Phytochemical Composition and Antimicrobial Properties of Four Plants Indicated for Traditional Medicine Use

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Abstract

The rapid development of seriously drug-resistant pathogen strains has created a dangerous problem to public health. The discovery of new effective antimicrobials remains an urgent task to control microbial resistance. Natural products can offer special stereochemistry and unlimited diversity of natural leads which are biologically active or ready for development and structure optimization strategies. Four medicinal plants *Cylicodiscus gabunensis* (CG), *Pogostemon cablin* (PC), *Perilla frutescens* (PF) and *Magnolia biondii* (MB) were selected for investigation of their phytochemical composition and antiplasmodial and/or antibacterial properties. A bioassay guided fractionation method has been followed to characterize the antiplasmodial and antibacterial constituents of CG. Flash column chromatography and preparative HPLC were used to obtain the bioactive compounds. GC-MS, LC-MS, and NMR have been used for chemical analysis purposes. For evaluation of the antibacterial activity, disk diffusion assay, alamar blue microplate assay, time kill kinetic and scanning electron microscopy (SEM) methods have been used. The work led to the isolation of the most potent antiplasmodial fraction of CG with IC_{50} of 4.7 µg/ml using Malaria Sybr Green I assay. Detailed chemical analysis suggested the presence of conjugates of oligosaccharide with gallic acid in this mixture. The active antibacterial compounds of CG were identified as benzoic acid derivatives such as ethyl gallate and gallic acid. Several gallate compounds were prepared *de novo* and the results showed that esterification of gallic acid increased both the antibacterial and antiplasmodial activities. Furthermore, the extracts of PC, PF and MB showed moderate antibacterial activities, whose mechanisms of action have been determined by SEM. In particular, patchouli alcohol, the major component of PC showed strong antibacterial activity and synergism with gallic acid against *Staphylococcus aureus*. These studies have provided evidences for the traditional use of these plants for the treatment of malaria and/or bacterial infections.

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Abbreviations

CC: Flash Column Chromatography

CFU/ml: Colony forming unit/ millilitre

CG: *Cylicodiscus gabunensis*

CGE: *Cylicodiscus gabunensis* ethanol extract

CGEAQ: *Cylicodiscus gabunensis* aqueous fraction of ethanol extract

CGEBU: *Cylicodiscus gabunensis* butanol fraction of ethanol extract

CGEEA: *Cylicodiscus gabunensis* ethyl acetate fraction of ethanol extract

CGH: *Cylicodiscus gabunensis* hexane extract

DCC: N,N'-Dicyclohexylcarbodiimide

DDA: Disk diffusion assay

DHFR: Dihydrofolate reductase (DHFR)

DHFS: Dihydrofolate synthetase

DHPS: Dihydropteroate synthase

DMSO: Dimethyl sulfoxide

EI: Electron ionization

EOs: Essential oils

ESI-MS: Electrospray ionization mass spectrometry

FIC: Fractional inhibitory concentration

FU: The fluorescence units

IZ: Inhibition zone

MB: *Magnolia biondii*

MDR: Multidrug-resistant

MIC: Minimum inhibitory concentration

MRSA: Methicillin resistance *Staphylococcus aureus*

MSSA: Methicillin sensitive *S. aureus*

ND: IZ not detected

NMR: Nuclear magnetic resonance spectroscopy

PBPs: Penicillin-binding proteins

PC: *Pogostemon cablin*

PCE: *Pogostemon cablin* ethanol extract

PCH: *Pogostemon cablin* hexane extract

PF: *Perilla frutescens*

PfCRT: The chloroquine resistance transporter

PFE: *Perilla frutescens* ethanol extract

PFH: *Perilla frutescens* hexane extract

PfMDR1: The multidrug resistance transporter

PfNHE1: The sodium/hydrogen exchanger 1

RI_s: Retention indices

RMAs: Resistance modifying agents

SD: Standard deviation

SEM: Scanning electron microscopy

TIC: Total ion chromatogram

WHO: World Health Organization

Publications

Journals:

Omar Aldulaimi, Wen-Wu Li. (2016) Fingerprint of Tiger Balm® By Thermal Desorption Gas Chromatography Mass Spectrometry. *Pharmacognosy Journal,* **8**(3), 230-233.

Omar Aldulaimi, Wen-Wu Li. Antibacterial effects and mechanism of the essential oil from Flower Buds of *Magnolia Biondii* Pamp. Submitted.

Omar Aldulaimi, Fidelia I Uche, Haddijatou Mbye, Imran Ullah, Hamza Hameed, Falko Drijfhout, Timothy D Claridge, Paul Horrocks, Wen-Wu Li. A characterization of the anti-malarial activity of the bark of *Cylicodiscus gabunensis* Harms. Submitted.

Omar Aldulaimi, Paul Horrocks, Wen-Wu Li. The antibacterial activity of *Pogostemon cablin* extracts and time kill kinetics of its main constituents. in preparation.

Conference Abstracts:

Omar Aldulaimi, Wen-Wu Li. (2013) Analysis of volatile compounds in phytomedicines using thermal desorption gas chromatography mass spectrometry. Planta Medica: natural products and medicinal plant research vol. 79, p. PK25. 61st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Münster, Germany, 1st – 5th September 2013 (Poster).

Omar Aldulaimi, Fidelia I Uche, Haddijatou Mbye, Paul Horrocks, Wen-Wu Li Antimicrobial and antimalarial compounds from *Cylicodiscus gabunensis* harms (2014) PharmSci 2014 Conference, 8th - 10th September 2014, University of Hertfordshire, Hatfield, UK (Poster).

Omar Aldulaimi, Paul Horrocks, Wen-Wu Li (2015) Antimicrobial activities and chemical compositions of *Pogostemon cablin* (Blanco) Benth. and *Perilla frutescens* (L.) Britt. in Huoxiang Zheng Qi formula. International symposium on phytochemicals in medicine and food 26th -29th June 2015 Shanghai China (ISPMF 2015) (Oral presentation).

Omar Aldulaimi, Fidelia I. Uche, Haddijatou Mbye, Paul Horrocks, Wen-Wu Li. (2015) Investigation of the antimalarial compounds from *Cylicodiscus gabunensis* harms. The 19th International congress of Phytopharm 21st -24th July 2015 Bonn, Germany (Poster).

Omar Aldulaimi, Paul Horrocks, Wen-Wu Li. (2015) Antibacterial effect of gallic acid and several analogues. PharmSci 2015 Conference, 7th - 9th September 2015, University of Nottingham, UK. 1st Poster prize award from The Academy of Pharmaceutical Sciences and GlaxoSmithKline (Poster).

Aldulaimi O, Horrocks P, Li WW. 2015. Antimicrobial activities and chemical compositions of *Pogostemon cablin* (Blanco) Benth. and *Perilla frutescens* (L.) Britt. in a traditional Chinese medicine formula. Nutrition and Medicine (vol. 204, pp. 497-498). ISSN: 2195-0083.

Omar Aldulaimi, Wen-Wu Li. (2016) Antibacterial effects of the essential oil and lignans from flower buds of *Magnolia biondii* Pamp. The 9th Joint Natural Products conference, 24th -27th July 2016, Copenhagen, Denmark (Poster).

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Chapter 1 : Introduction

1.1. Discovery of the antibacterial agents

The discovery of penicillin started the antibiotics era and for over seventy years provide huge victory over pathogens, however, the emergence and rapid development of seriously drug-resistant pathogens have created the greatest danger to public health and made the treatment of infectious diseases ineffective (Oluwatuyi, Kaatz et al. 2004).

Bacteria can resist antibiotic/s by several means: 1) the modification of protein targets to antibiotics; 2) the production of enzymes which can degrade or modify the antibiotic structure rendering it unsuccessful in infections treatment; 3) the changes of cell wall permeability due to mutations; 4) the use of the pumping system to expel the antibiotic molecules (Blair, Webber et al. 2015, Abreu, McBain et al. 2012).

Based on the various mechanisms of bacterial resistance listed above, resistance modifying agents (RMAs) which may address the resistance problem have been explored to modify the antimicrobial resistances. The details of resistance modification mechanisms and RMAs were reviewed in detail by Abreu (Abreu, McBain et al. 2012).

1.1.1. Antibacterial resistance

The emergence of resistant strains to antibacterials was determined by the level of exposure of the pathogen to the antibacterial, the biochemical and physiological effects of drugs on the microorganisms (pharmacodynamics) and the pharmacokinetic properties of the antibiotics (Andersson, Hughes 2010). The use of wrong dosage of antibacterials and the general abuse of the antibacterials were the contributing factors of the emergence and dissemination of resistance (Maisnier-Patin, Andersson 2004). The resistant microbes do not respond to the antibacterials due to either expression of resistance phenotype, or inherent resistant genes (Gilbert, Maira-Litran et al. 2002).

The multidrug-resistance is more serious than the resistance to a single antibiotic. Examples are the methicillin-resistant *Staphylococcus aureus* (MRSA) and the toughest type of *S. aureus* which also resists vancomycin (Abreu, McBain et al. 2012). These kinds of bacteria cause around 25000 deaths annually and cost the European economy more than one billion euros (Blair, Webber et al. 2015).

To control the antibiotic resistant microbes, the discovery of new effective antimicrobials with new mechanism of action against bacteria remains an urgent task to control the bacterial resistance (Theuretzbacher 2011). WHO adopted several strategies to address the resistance problems like using the antibiotics in the right dose and duration of treatment, enhancing healthy environment to control transmission of resistant strains and supporting the research for finding new antibiotics (WHO 2011). The use of synergism between antibiotics is also an important strategy to control drug resistance by targeting more than one site of action and increases the bioavailability or the modification of resistance mechanisms (Wagner, Ulrich-Merzenich 2009).

1.1.2. Plants as sources of antibacterials

Plant extracts have been utilized from ancient times to treat various ailments, particularly in Asia. A major proportion of the world's population is depending on traditional medicine for a healthy life (Prabuseenivasan, Jayakumar et al. 2006). In modern drug discovery and development processes, phytochemicals play a key role at the early stage of 'lead' discovery, i.e. discovery of the bioactive (determined by various bioassays) natural molecule, which could be an ideal drug candidate itself or as a lead compound to develop for its structural analogues (Sarker 2007, Cowan 1999).

Some of phytochemicals are utilized by the plants themselves as a defence against many invaders, therefore playing a distinctive role as antimicrobials (Simoes, Bennett et al. 2009).

The paucity of infections in wild plants supports the role of innate defence system of plants (Hemaiswarya, Kruthiventi et al. 2008). Many researchers have indicated that a broad range of plant extracts may act against bacterial resistance mechanisms, and the majority of them have now been focused on the combination of plant extracts and antibiotics in order to determine the existence and abundance of RMAs (Chlez, Hohmann et al. 2010, Sibanda, Okoh 2007).

Giant companies in the pharmaceutical sector have invested a huge amount of money to synthesize chemical compounds but without significant biological activities due to lack of special stereochemistry which natural products can offer, therefore an increase in drug discovery from nature in the future will be expected (Gibbons 2008). Another important motive to discover natural products and produce alternatives to the limited or failed therapeutic protocols is the unlimited diversity of natural leads which are biologically active or ready for development and structure optimization strategies. In addition to that, the toxicity and low margin of safety of some antimicrobials have promoted the interest in search for novel antimicrobial products from other sources, particularly plant secondary metabolites (phytochemicals) (Gibbons 2008).

There are different phytochemical approaches to follow when searching for natural antimicrobials. The plant species are selected randomly and then their phytochemicals are categorized to different chemical groups. The second approach is the random selection of plant species followed by antimicrobial assays; the third approach is to follow previous reports of researchers.

The most important approach is the follow-up of ethno-medical or traditional uses of plants against microbial infections (Fabricant, Farnsworth 2001). Traditional medicines like Ayurveda and Chinese medicine provide information about the medicinal use of plants. Information sources may include books, herbals, review articles, notes placed on voucher

herbarium specimens, field work and computer databases, such as NAPRALERT (Farnsworth 2015).

1.1.2.1. Extraction of antibacterial natural products

To extract the phytochemicals, researchers can use open extraction system or closed (continuous) system like refluxing with Soxhlet or Clevenger apparatus. Extraction of phytochemicals is carried out, using water or alcohol or other organic solvents like hexane, methanol or ethyl acetate under heat or at room temperature. Most of the antimicrobials are extracted by an aqueous alcoholic solution which is later dried by rotary evaporator under vacuum (Taylor, Edel et al. 1996).

1.1.2.2. Antibacterial assay methods

The assays of antibacterials from plants are conducted with crude plant extract (Silva, Duarte et al. 1996) and/or pure compounds (Afolayan, Meyer 1997, Batista, Duarte et al. 1994). Disk or agar well diffusion assay and broth micro or macro dilution assay are the most important assays used to evaluate the antibacterial activities (Ayafor, Tchuendem et al. 1994, Navarro, Villarreal et al. 1996). The bioautographic method (Rios, Recio et al. 1988) is another technique used to evaluate the antibacterial effects.

To detect the antibacterial action, the researchers can use the disk diffusion method, in which a paper disk impregnated with the natural extract or pure compound is laid on top of an inoculated agar plate. The volume or quantity of the natural material deposited on the paper disks, the thickness of the agar layer and whether a solvent is used can affect the results considerably between studies.

In the agar well test when a large number of extracts are to be screened or a large number of bacterial isolates need to be tested, the material assayed for antibacterial activity is introduced into wells cut into the agar plate (Deans, Simpson et al. 1993, Dorman, Deans 2000).

In broth dilution method, the antibacterial activity such as the minimal inhibitory concentrations (MIC) is quantified by measuring the optical density (OD) using an automated spectrophotometer, or by counting the viable colonies left after incubating the inoculum in the presence of the antibacterial agent for a defined period of time. Although this method is accurate, it is a tedious process and requires a longer period of time than OD measurement.

Resazurin (alamar blue) can be used in microplate assay method as a highly sensitive indicator, accurately reflect the activity of the bacterial cells (Sarker, Nahar et al. 2007). The alamar blue assay method is sensitive enough to be compared with OD measurement and viable colonies counting and it is more sensitive than the agar dilution method (Mann, Markham 1998).

The time-kill assay is used to define the time required to reach a bactericidal or bacteriostatic level. The time kill kinetic graph is presented by plotting the number of viable cells remaining in the broth after addition of the natural extract *versus* time. The destruction of the bacterial cell by antibacterial agent and loss of cellular contents can be studied by scanning electron microscopy (SEM) (Skandamis, Nychas 2001, Burt, Reinders 2003).

1.1.2.3. Volatile oils as antibacterial agents

Essential oils (EOs) are chemical compounds also called volatile or ethereal oils (Guenther 1948). EOs are aromatic and obtained from different plant parts, many methods are used to extract EOs; those methods are expression (cold pressing), enfleurage, or extraction by organic solvents, hexane for example. Steam distillation is a well-known method used to extract volatile oils in industry for the commercial production (Van de Braak, Leijten 1999). EOs may contain aromatic chemicals derived from shikimate pathway or mainly of terpenoids which are derived from methylerythritol phosphate or mevalonate pathways.

Some of the volatile oils are composed of the two types, aromatics and terpenoids, and one group of those two is predominated over the other (Paul 2009).

Terpenoids are composed of isoprene/s units, which are linked head to tail as in geraniol, farnesol, and geranyl geraniol. Isoprene units also linked tail to tail as in squalene. Terpenoids are classified into hemiterpenes composed of five carbon block, monoterpenes with C_{10}, sesquiterpenes with C_{15}, diterpenes C_{20}, sesterterpenes C_{25} and tetraterpenes C_{40} (Paul 2009). (Figure 1.1) shows the head to tail and tail to tail linked isoprene units.

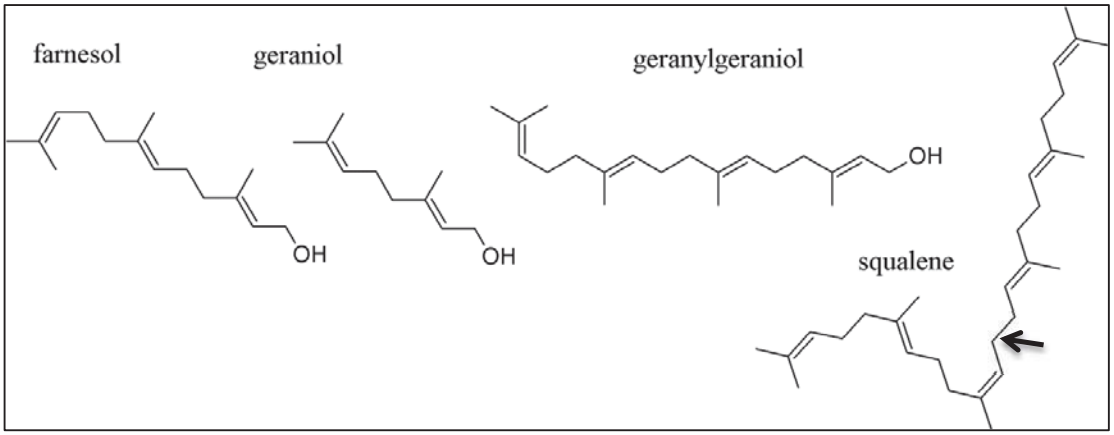


Figure 1.1: the head to tail and tail to tail linked isoprene units. The arrow shows the linkage point of tail to tail isoprene units in squalene.

1.1.2.3.1. Historical and current use of essential oils

However, the use of EOs was not common in Europe until the 16th century, where they were sold in London for different purposes such as perfumes, food additives and remedies for various ailments (Crosthwaite 1998). The environmental sustainability and encouragement for 'green' consumerism have generated scientific interests in these substances (Nychas 1995, Tuley de Silva 1996). EOs have been shown to have antibacterial actions (Deans, Ritchie 1987, Carson, Cookson et al. 1995a, Mourey, Canillac 2002), antiviral (Bishop 1995), anti-mycotic (Azzouz, Bullerman 1982, Akgul, Kivanc

1988, Jayashree, Subramanyam 1999, Mari, Bertolini et al. 2003) and insect killing properties (Konstantopoulou, Vassilopoulou et al. 1992).

EOs, in general, are a good source of antimicrobials (Burt 2004) when used alone or in combination with antibiotics to fight multidrug-resistant strains (Langeveld, Veldhuizen et al. 2014). The EOs extracted by hexane has been shown to exhibit better antimicrobial activity than the corresponding steam distilled EOs (Packiyasothy, Kyle 2002). The quantity and quality of the EO and hence its chemical compositions are varied substantially depending on the time of collection and geographical area (McGimpsey, Douglas et al. 1994, Faleiro, Miguel et al. 2002).

The strongest antimicrobial effects were observed from plants collected directly after their flowering (McGimpsey, Douglas et al. 1994). The chemical orientation or stereochemical orientation of the EOs components can affect the antimicrobial activity (Lis-Balchin, Ochoka et al. 1999). The MIC of EOs is calculated by μg/ml, however, some researchers reported EOs MIC as μl/ml instead of μg/ml (Tolossa, Asres et al. 2007).

Food scientists have found that terpenoids of EOs are useful in the control of *Listeria monocytogenes* (Aureli, Costantini et al. 1992). Basil oil is used to protect lettuce leaves from deterioration (Wan, Wilcock et al. 1998). The essential oil of Sweet basil *Ocimum basilicum* L. (*Lamiaceae*) showed antibacterial activity with MIC against *B. cereus* = 62.5 μg/ml, against *B. subtilis* = 125 μg/ml, against *E. coli* = 500 μg/ml and against *P. aeruginosa* > 1000 μg/ml (Hossain, Kabir et al. 2010).

A capsaicinoid alkaloid, capsaicin from chillies was bactericidal to *Helicobacter pylori* (Jones, Shabib et al. 1997) and trichorabdal A, a diterpene from a Japanese herb, could directly inhibit *H. pylori* (Kadota, Basnet et al. 1997). Another hot-tasting diterpene, aframodial, from a Cameroonian spice, is a broad-spectrum antifungal (Ayafor, Tchuendem et al. 1994).

The ethanol-soluble fraction of purple prairie clover yielded a terpenoid petalostemumol (Cowan 1999), which showed great activity with MICs of 0.78 μg/ml and 3.12 μg/ml against *B. subtilis* and *S. aureus*, respectively (Hufford, Jia et al. 1993).

Chrysothol and madreporanone, two terpenoids isolated from *Azorella cryptantha* (Clos) Reiche showed MIC of 50–100 μg/ml against *E. coli* and *Salmonella enteritidis* (Lima, Sanchez et al. 2015). Another terpenoid (dysidinoid A) isolated from China Sea sponge *Dysidea* sp. and showed a very potent activity with an MIC of 8 μg/ml against MRSA (Jiao, Li et al. 2014). (Figure 1.2) shows the structures of the terpenoids mentioned above.

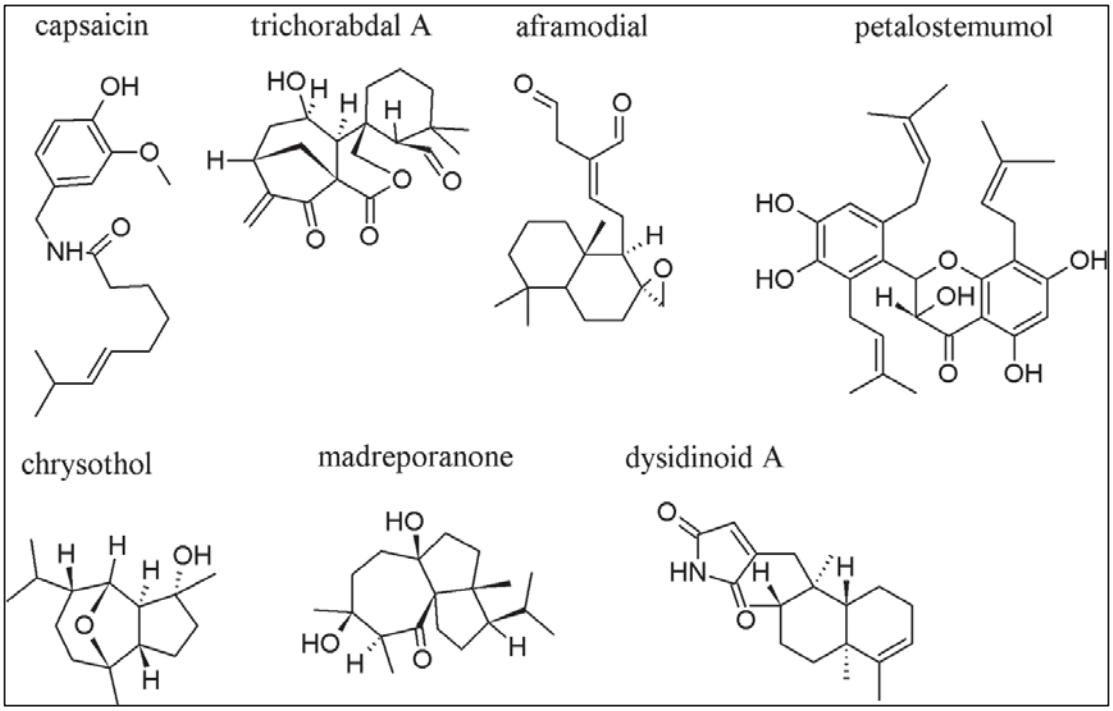


Figure 1.2: The structures of some terpenoids mentioned with antibacterial activities.

In a recent study (Maree, Kamatou et al. 2014), one hundred and fifty eight EOs were tested against the following microorganisms, *B. cereus*, *S. aureus*, *Enterococcus faecalis*, *E. coli*, *P. aeruginosa* and two yeasts, *Candida albicans* and *Cryptococcus neoformans*. The results revealed that the essential oils which contained eugenol demonstrated moderate activity against the studied cells, eighteen oils have MICs between 250 and 1000 µg/ml. *Cistus spp.*, *Eugenia caryophyllata*, and *Listea cubeba* have MIC of 4 µg/ml against *Cryptococcus neoformans*. EO which consists of α-pinene, limonene and sabinene showed weak antimicrobial activities (Maree, Kamatou et al. 2014). (Figure 1.3) shows the structure of eugenol and terpenes mentioned above.

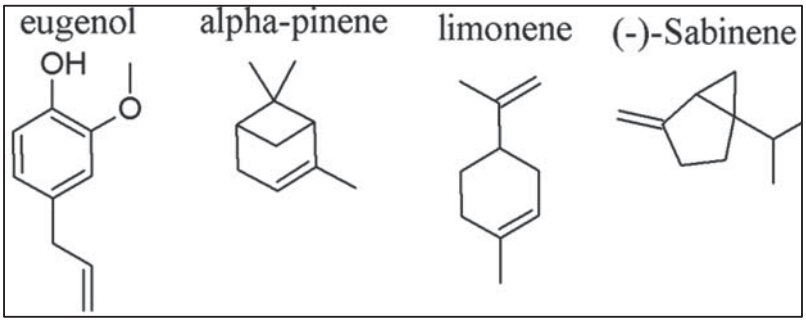


Figure 1.3: The structure of eugenol, α-pinene, limonene and sabinene.

The compositional analysis of the oil or its headspace is often accomplished by gas chromatography mass spectrometry (GC-MS) or thermal-desorption gas chromatography-mass spectrometry (TD GC- MS) (Salzer 1977, Scheffer, Svendsen 1981, Wilkins, Madsen 1991, Daferera, Ziogas et al. 2000, Juliano, Mattana et al. 2000).

There are numerous examples of essential oils studied for antibacterial activities and checked by GC-MS to define their metabolites profiles. The volatile oil of *Coriandrum sativum* L. was analyzed by GC-MS coupled with chemometric resolution methods to enhance the resolution of peak clusters (Zhou, Chen et al. 2011).

The EO of *Foeniculum vulgare* was analyzed by GC-MS, however, the antibacterial activity was found to be weak (Miguel, Cruz et al. 2010). The oil of *Piper arborescens* Roxb studied by GC-MS and showed activity against *S. aureus* MIC= 250 µg/ml (Salleh, Ahmad et al. 2016). *Artemisia stricta* Edgew. f. stricta Pamp showed MIC= 1.25 µg/ml against *S. aureus* and its fraction was analysed using GC-MS to detect its metabolic profile (Manika, Chanotiya et al. 2016). In summary, GC-MS is an important tool for essential oils characterization.

1.1.2.3.2. Mode of action and interactions between components of EOs

The hydrocarbons interact with the nonpolar parts of bacterial cells to exert their antibacterial action (Sikkema, De Bont et al. 1995). Bacterial cells contain many targets susceptible to the activity of volatile oil (Carson, Mee et al. 2002). The hydrophobicity of the oils enables them to partition into the lipids of the bacterial cell membrane, affecting their structures and leaving them more permeable (Sikkema, De Bont et al. 1994). Leakage of ions and cell materials is the second step (Zhang, Liu et al. 2016). The massive leakage of cell contents definitely leads to cell death (Shukla, Majumder et al. 2016).

Functional groups on the essential oil structures are vital for antibacterial activities; hydroxyl groups in carvacrol and thymol play a key role in antimicrobial behaviour (Ultee, Bennink et al. 2002). Thymol and carvacrol, were isolated from *Thymus vulgaris*, they increase the outer membrane permeability and assist the penetration of antibiotics in gram negative bacteria (Lambert, Skandamis et al. 2001). Acetate moiety increases the antibacterial activity of geraniol, where geranyl acetate was found to be more potent than geraniol against a range of gram-positive and negative species (Dorman, Deans 2000). Structures of carvacrol, thymol and geranyl acetate are shown in (Figure 1.4).

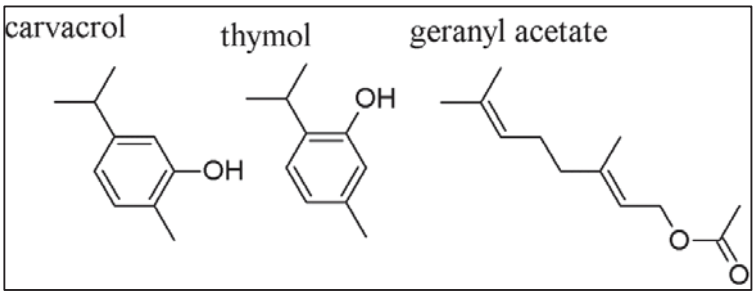


Figure 1.4: Structures of carvacrol, thymol and geranyl acetate.

The functional groups of the EO components may classify their antibacterial activities, phenolic materials are the most potent ones followed by alcohols, and esters are less active than the previously mentioned compounds while ethers rank among the least potent. Hydrocarbons are practically very weak antibacterials or inactive (Ait-Ouazzou, Cherrat et al. 2011). The hydrocarbon monoterpenes (α-pinene, camphene, myrcene, α -terpinene and para-cymene) with weak antimicrobial activities can potentiate the antimicrobial effects of carvacrol by interaction with the cell membrane to facilitate the penetration of carvacrol into the cell (de Azeredo, Stamford et al. 2011).

Additive effects were observed from a combination of 1,8-cineole (eucalyptol) and aromadendrene against MRSA and vancomycin-resistant *Enterococci* (*E. faecalis*) by using the checkerboard method (Mulyaningsih, Sporer et al. 2010). In conclusion the phenolic compounds in the EOs are more effective against bacteria than hydrocarbon molecules deprived of oxygen atoms. Structures of compounds from camphene to eucalyptol are shown in (Figure 1.5).

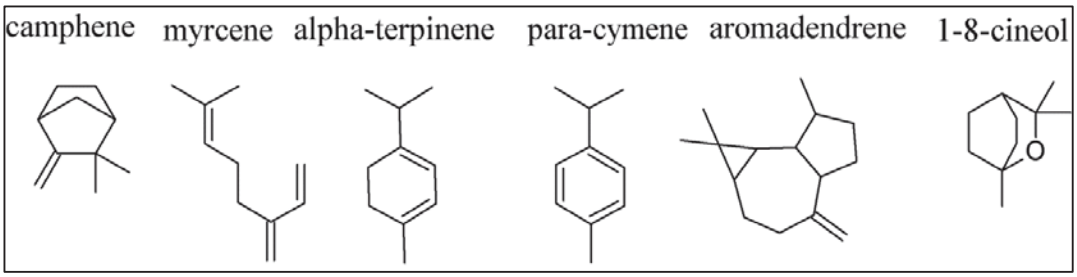


Figure 1.5: Structures of some of the hydrocarbon monoterpenes, with weak antibacterial activities, 1,8-cineole (eucalyptol) and aromadendrene showed additive effects against MRSA and vancomycin-resistant *Enterococci* (*E. faecalis*).

1.1.2.4. Phenols and phenolic acids

Phenols like catechol and pyrogallol showed activity against some microorganisms. For example, pyrogallol showed MIC= 2.4-2500 μg/ml and pyrocatechol MIC range was = 4.9-312.5 μg/ml against several microorganisms causing periodontitis (Shahzad, Millhouse et al. 2015). Taguri (2006) recorded the antibacterial activities of 22 polyphenolic structures against several gram positive and gram negative bacteria, the results showed that the pyrogallol-based compounds were more potent than others such as catechol or resorcinol (Taguri, Tanaka et al. 2006). There was evidence that increased hydroxylation of those phenols results in increased toxicity or antimicrobial activities (Geissman 1963). The mechanisms of action of simple phenols are probably proceed through interaction with sulfhydryl groups in microbial enzymes leading to inhibition of those enzymes or due to nonspecific proteins interactions (Mason, Wasserman 1987).

Some phenolic acids such as caffeic acid have antimicrobial activities. Caffeic acid showed weak antibacterial activities, MIC equal to 1600 μg/ml against *S. aureus* and *E. coli* compared to ampicillin which showed MIC= 0.1 and 3.2 μg/ml respectively (Lim, Subhan et al. 2016).

Gallic acid is a phenolic acid showing an effect against *E. faecalis* (Aires, Marques et al. 2013). It also showed the greatest effect, among several phenolic compounds, against a

group of bacterial strains including *P. aeruginosa*, *S. aureus*, *Moraxella catarrhalis*, *E. faecalis*, *S. agalactiae* and *S. pneumonia* (Cueva, Mingo et al. 2012). Gallic acid showed good activity against *Campylobacter* and produced bactericidal activity against two *Campylobacter coli* strains with MIC equal to 61.5-125 μg/ml (Sarjit, Wang et al. 2015).

In another study, the antibacterial activities of gallic acid and ferulic acid were detected against several bacterial isolates. Gallic acid showed MIC equal to 500 μg/ml against *P. aeruginosa*, 1500 μg/ml against *E. coli*, 1750 μg/ml against *S. aureus* and 2000 μg/ml against *L. monocytogenes*; MIC of ferulic acid was 100 μg/ml against *E. coli* and *P. aeruginosa*, 1100 μg/ml and 1250 μg/ml against *S. aureus* and *L. monocytogenes*, respectively. MBCs for both compounds against all microorganisms were 2500-5500 μg/ml (Borges, Ferreira et al. 2013).

Gallic acid and ferulic acid affect the bacterial cell wall of *S. aureus*, *E. coli* and *P. aeruginosa*, producing local destruction and leakage of cellular contents (Borges, Ferreira et al. 2013).

Wild Polish mushrooms which contained protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid, caffeic acids, p-coumaric and ferulic acids, showed intermediate antibacterial activities with MIC ranged from 156 to 5000 μg/ml, against a range of gram positive and negative bacteria with a stronger activity against gram-positive microorganisms (Nowacka, Nowak et al. 2015). (Figure 1.6) shows the structures of some phenols and phenolic acids like pyrogallol and gallic acid.

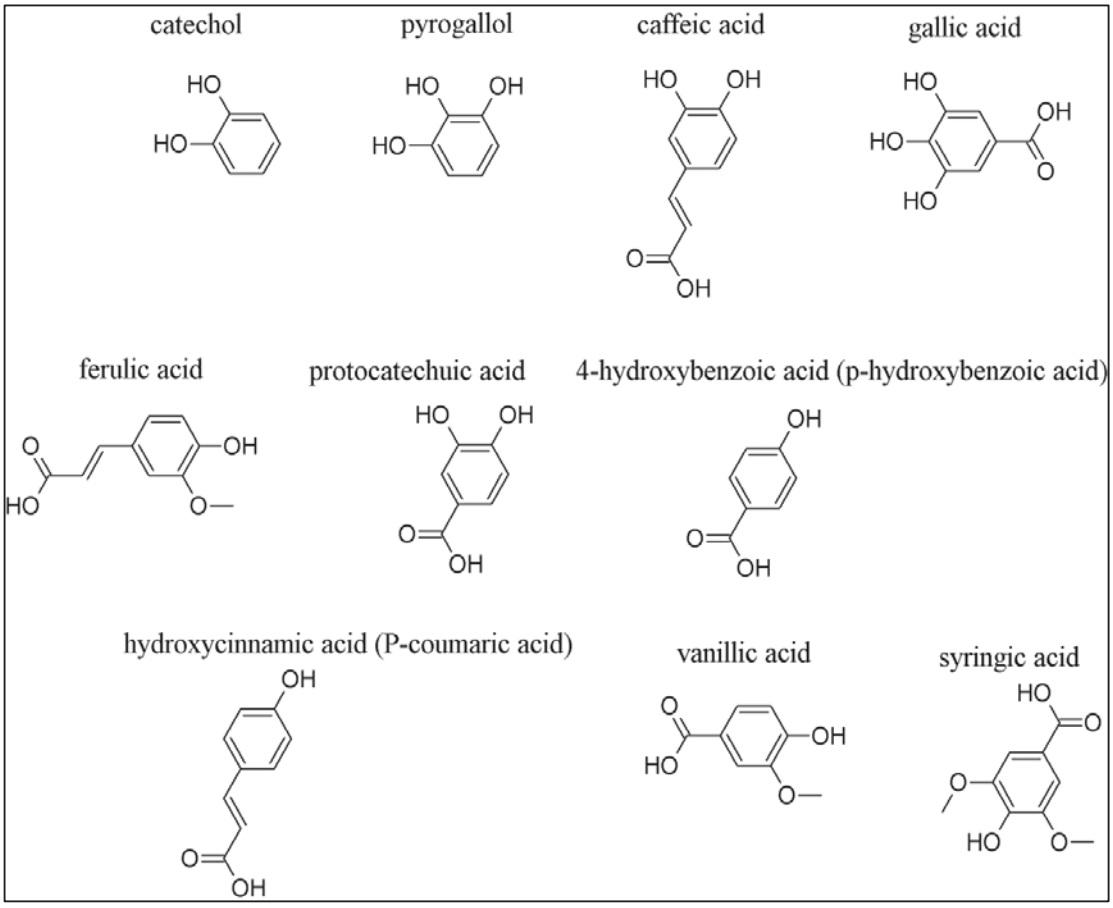


Figure 1.6: Structures of several phenols and phenolic acids.

Methyl gallate, a major metabolite from *Galla rhois* an Asian plant collected in Korea showed anti-*Salmonella* behavior against several *Salmonella* strains with MIC from 3.9 to 125 µg/ ml (Choi, Mun et al. 2014). The *in vivo* therapeutic activity of methyl gallate was well proved by the absence of lethargy and liver problems the two common consequences of *Salmonella* in treated mice (Choi, Mun et al. 2014). Methyl gallate demonstrated good antibacterial effect against *E. coli* and *Shigella flexneri* with MIC= 30 µg/ml (Madikizela, Aderogba et al. 2013).

Three *Potentilla* species were found to be rich in hyperoside, (+)-catechin, caffeic acid, ferulic acid, rutin and ellagic acid, which were tested for their antimicrobial activities. *P. fruticosa* had the highest effect against gram-positive bacteria, a gram negative bacterium

(*P. aeruginosa*) and a fungus *Candida albicans* with MIC values of 0.78-6.25 mg/ml (Wang, Wang et al. 2013). The structures of the methyl gallate and phenolic compounds detected in *Potentilla* species are shown in (Figure 1.7).

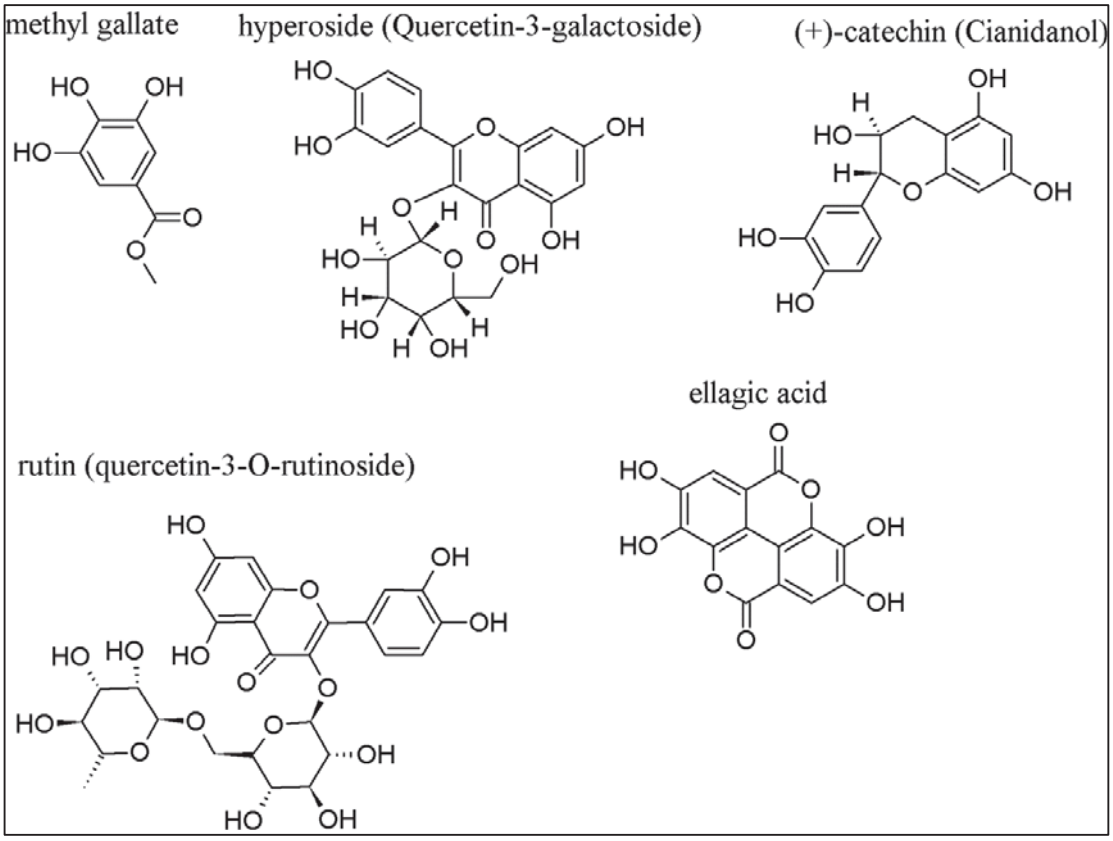


Figure 1.7: The structures of the methyl gallate and phenolic compounds detected in *Potentilla* species.

Plant polyphenols interact with each other to improve the antibacterial actions (Tomas-Menor, Barrajon-Catalan et al. 2015). Polyphenolic compounds present in a *Cistus salviifolius* extract showed synergism. Flavonoids in combination with ellagitannins in certain percentage showed inhibition against the growth of *S. aureus* (Tomas-Menor, Barrajon-Catalan et al. 2015).

The combination of isoquercitrin (10 μg/ml), (Figure 1.8) with gallic acid (10 μg/ml) was successful in inhibiting the growth of *S. aureus*, while their MICs separately were 10 times

more (Soberon, Sgariglia et al. 2014). The ethyl acetate fraction from the ethanol extract of *Searsia chirindensis* was the most active antibacterial fraction, contained methyl gallate, myricetin-3-O-arabinopyranoside, myricetin-3-O-rhamnoside, kaempferol-3-O-rhamnoside and quercetin-3-O-arabinofuranoside. The MICs of these compounds ranged from 30 to 250 µg/ml against *Campylobacter jejuni*, *E. coli*, *Shigella flexneri*, and *S. aureus* (Madikizela, Aderogba et al. 2013).

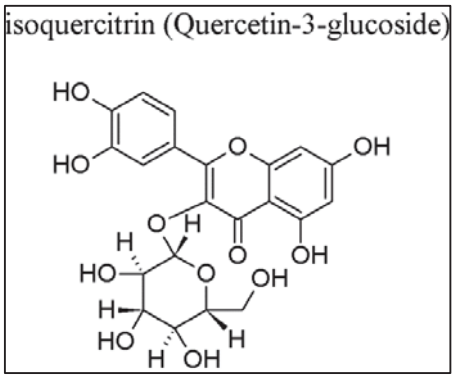


Figure 1.8: The structure of isoquercitrin, a compound showed synergism with gallic acid against *S. aureus*.

The seed kernels of *Mangifera induce* L. consisted of the following phenolic acids: gallic acid, methyl gallate and pentagalloyl glucopyranose. The extract inhibited the growth of MRSA, due to the major phenolic component, pentagalloylglucopyranose, which showed a synergism with penicillin G against MRSA (Jiamboonsri, Pithayanukul et al. 2011).

The structures of flavonoid glycosides, ellagitannin and pentagalloyl glucopyranose are shown in (Figure 1.9).

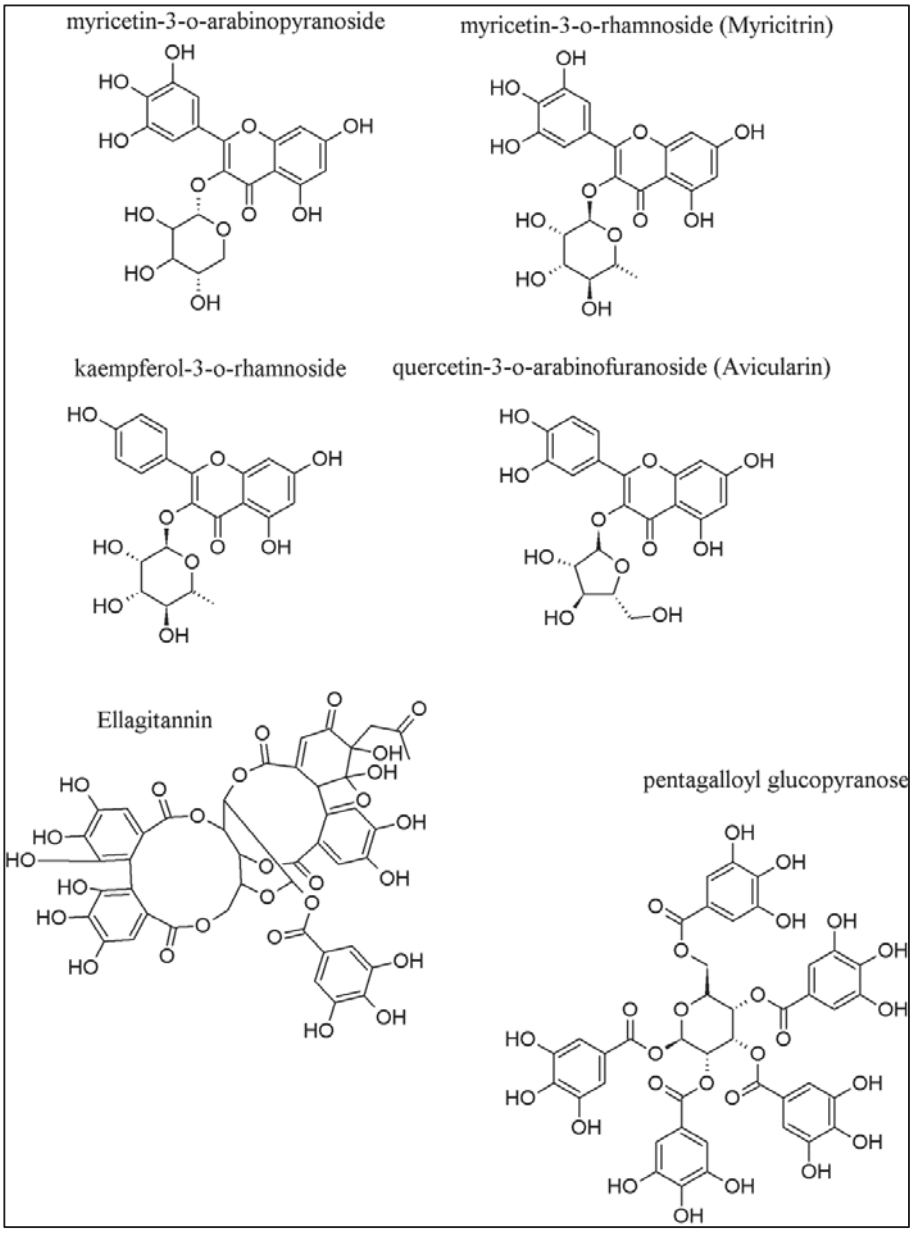


Figure 1.9: The structures of some flavonoid glycosides, ellagitannin and pentagalloyl glucopyranose.

Some phenolic compounds like hydroquinone, thymol, carvacrol, butylated hydroxyanisole, and octyl gallate were assayed against *S. aureus*. As a result, octyl gallate had MIC equal to 21µg/ml; hydroquinone had MIC= 103 µg/ml, and carvacrol and thymol showed MICs equal to 413 µg/ml (Rua, Fernandez-Alvarez et al. 2011).

Panduratin A a natural chalcone compound extracted from *Kaempferia pandurata* Roxb had potent activity against *S. aureus* with MIC equal to 0.06-2 µg/ml. An interesting study of synergism between phenolic materials and penicillins demonstrated the lowering MIC of oxacillin around 500 times by epicatechin gallate (Cushnie, Lamb 2011, Rukayadi, Lee et al. 2009). The structures of compounds have activity against *S. aureus* (hydroquinone to epicatechin gallate) are shown in (Figure 1.10).

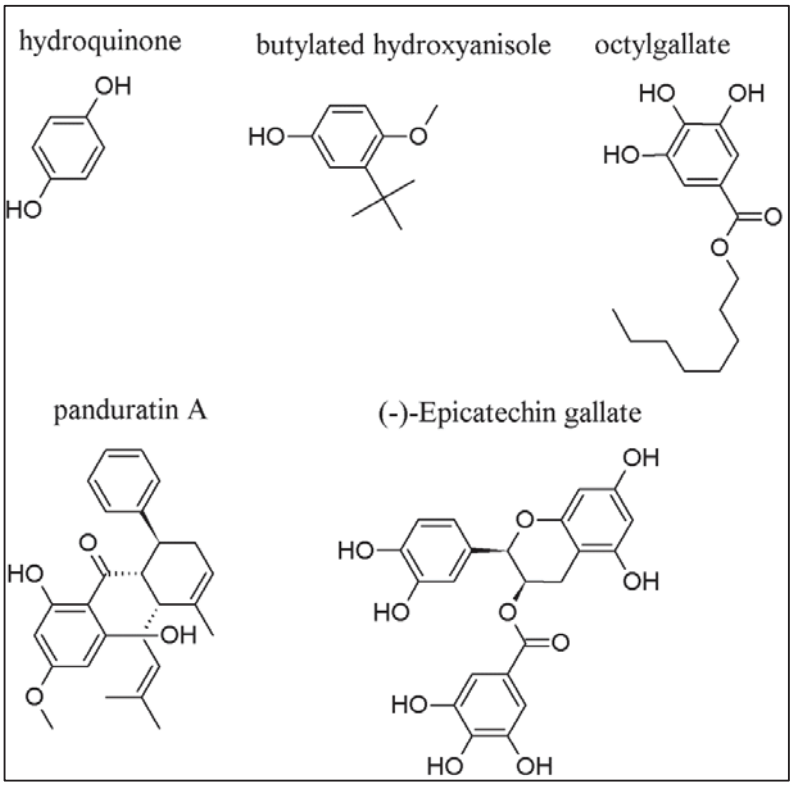


Figure 1.10: The structures of hydroquinone and other phenolic compounds recorded with anti-Staphylococcal activity.

Acylphloroglucinols (Olympicins A-E) isolated from the aerial parts of the plant *Hypericum olympicum* L. cf. uniflorum were evaluated as antibacterial agents against MRSA and multidrug-resistant *S. aureus*. They were found to be very active. Olympicin A had MIC of 0.5-1 µg/ml; others had MIC between 84 and 128 µg/ ml against several *S. aureus* isolates (Shiu, Rahman et al. 2012). Arzanol, a very strong anti-staphylococcal drug

from *Helichrysum italicum* subsp. *Microphyllum*, had MIC equal to 1-4 µg/ml against different *S. aureus* strains (Taglialatela-Scafati, Pollastro et al. 2013). Potent fraction isolated from *Hypericum beanie* contained two acylphloroglucinols, 1,5-dihydroxy-2-(2'-methylpropionyl)-3-methoxy-6-methylbenzene and 1,5-dihydroxy-2-(2'-methylbutanoyl)-3-methoxy-6-methylbenzene. This mixture showed MIC equal to 16-32 µg/ml against methicillin-resistant *S. aureus* (Po Shiu, Gibbons 2006). The structures of those acylphloroglucinols are shown in (Figure 1.11).

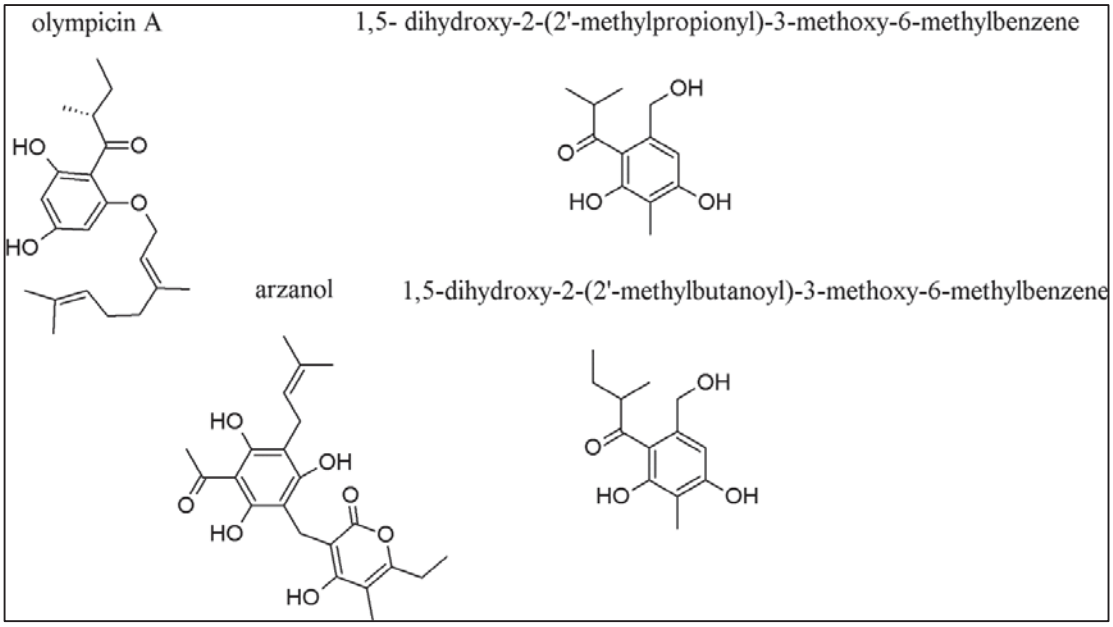


Figure 1.11: The structures of acylphloroglucinols.

Catechin gallate, epicatechin gallate and epigallocatechin gallate showed β-lactamase and PBP2a inhibition to potentiate the antibiotic activity against *S. aureus* and MRSA (Yam, Hamilton-Miller et al. 1998, Hu, Zhao et al. 2002, Sudano Roccaro, Blanco et al. 2004). Using epigallocatechin gallate (25 µg/ml) can restore MIC of imipenem to < 4 µg/ml against around 18 MRSA isolates out of 24 isolates (Hu, Zhao et al. 2002). Epigallocatechin gallate (50µg/ml) inhibited Tet (K) pump and increased intracellular concentration of tetracycline and ultimately its activity (Sudano Roccaro, Blanco et al.

2004). Epicatechin gallate decreased the MIC of norfloxacin four times against *S. aureus* with NorA (Gibbons, Moser et al. 2004). Catechins structures are shown in (Figure 1.12).

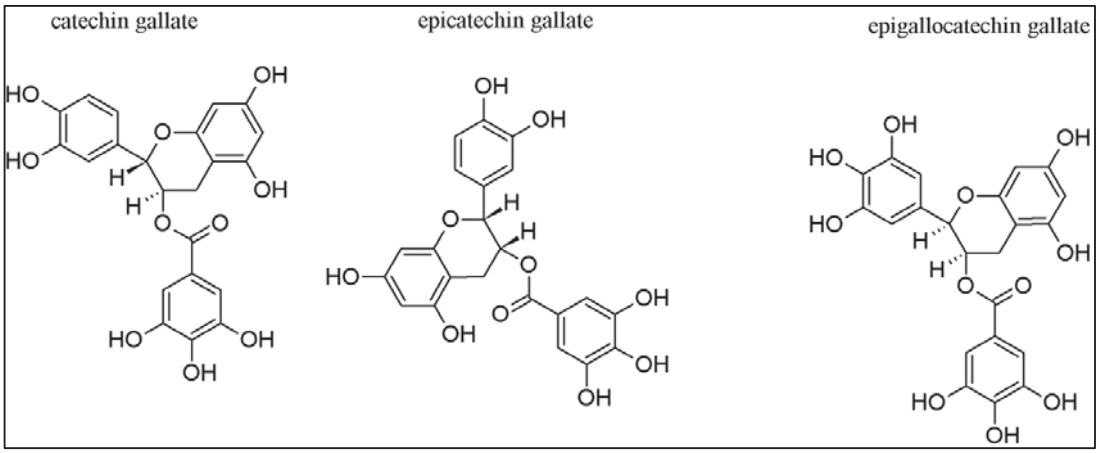


Figure 1.12: Structures of three catechins have resistance modification abilities.

Ethyl gallate and other alky gallate caused cell wall disruption of the methicillin sensitive *S. aureus* (MSSA) and MRSA, and potentiated the antibacterial activities of β-lactam antibiotics, probably by inhibition of PBP2a (Shibata, Kondo et al. 2005). Gallic acid showed synergism with sulfamethoxazole or tetracycline against several *P. aeruginosa* isolates (Jayaraman, Sakharkar et al. 2010) and with streptomycin against the same gram negative isolate (Saavedra, Borges et al. 2010) by disruptions of cell wall integrity.

1.1.2.5. Quinones

Quinones are responsible for the brown color formed on the fruit and vegetable cut pieces; they are also intermediates in the melanin synthesis pathway in human skin (Schmidt 1988). Quinones are electrophile and react readily with the nucleophile (amino acid) in proteins (Stern, Hagerman et al. 1996), leading to inactivation of the protein and loss of function. There is more than one mechanism which can illustrate the antimicrobial effects of quinones: they can bind to the cell wall polypeptide or membrane-bound enzymes. Murrayaquinone A had MIC equal to 50 μg/ml against *S. aureus* (Chakraborty,

Chakraborty et al. 2014). ω-Hydroxyemodin (Citreorosein) from *Cassia alata* showed an MIC equal to 4 µg/ml against *S. aureus* and 8 µg/ml against *B. cereus* (Promgool, Pancharoen et al. 2014). (Figure 1.13) shows the structures of quinones mentioned in this section.

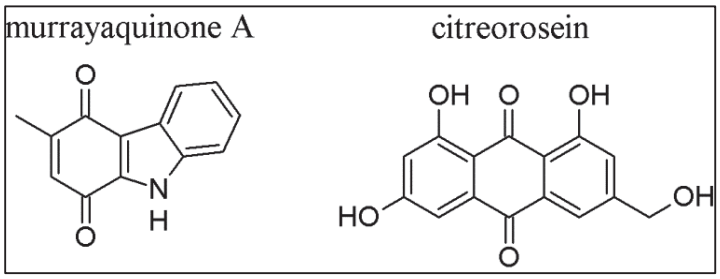


Figure 1.13: The structures of murrayaquinone A and ω-Hydroxyemodin.

1.1.2.6. Flavones and flavonols

Flavones are phenolic structures with one carbonyl group, the presence of a 3-hydroxyl group in flavones yields flavonols (Fessenden, Fessenden 1982). Galangin (3,5,7-trihydroxyflavone), derived from the perennial herb *Helichrysum aureonitens* showed activity against a wide range of gram-positive bacteria with MICs equal to 100 µg/ml against *B. cereus*, 500 µg/ml against *B. subtilis*, 500 µg/ml against *S. aureus* and 100 µg/ml against *Enterobacter cloacae*, but it was not active against *E. coli* (Afolayan, Meyer 1997).

The essential role of the hydroxyl groups on the (3-rings) of flavonoids is controversial, a research showed that the flavonoids lacking hydroxyl groups on their (3-rings) are more active against microorganisms than those with the hydroxyl groups since they are more lipophilic (Chabot, Bel-Rhlid et al. 1992). However, other research groups have reported the opposite effect, and have found that the more hydroxyl groups present on the flavonoids lead the greater antimicrobial activity (Sato, Fujiwara et al. 1996).

An isoflavone, 5,7-dihydroxy-2'-methoxy-3',4'-methylenedioxyisoflavanone was extracted from *Uraria picta* and showed MIC equal to 12.5 µg/ml against *S. aureus*, 50 µg/ml against *B. subtilis*, 100 µg/ml against *E. coli*, 25 µg/ml against *Proteus vulgaris*, 50 µg/ml against *Aspergillus niger* and100 µg/ml against *candida albicans* (Mukhlesur Rahman, Gibbons et al. 2007).

Hydroxygenkwanin-8-C-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside, isolated from the stem bark of *Margaritaria discoidea*, showed MICs of 500 µg/ml against *B. subtilis*, *S. aureus*, *Proteus vulgaris* and *P. aeruginosa* (Ekuadzi, Dickson et al. 2014). A flavonoid artonin I isolated from *Morus mesozygia* Stapf. showed an ability to block the bacterial efflux pump in multidrug-resistant (MDR) *S. aureus*, who's MIC was 20 µg/ml with possible application as a resistance modification agent (Farooq, Wahab et al. 2014). All the structures of the compounds mentioned here in this section are shown in (Figure 1.14).

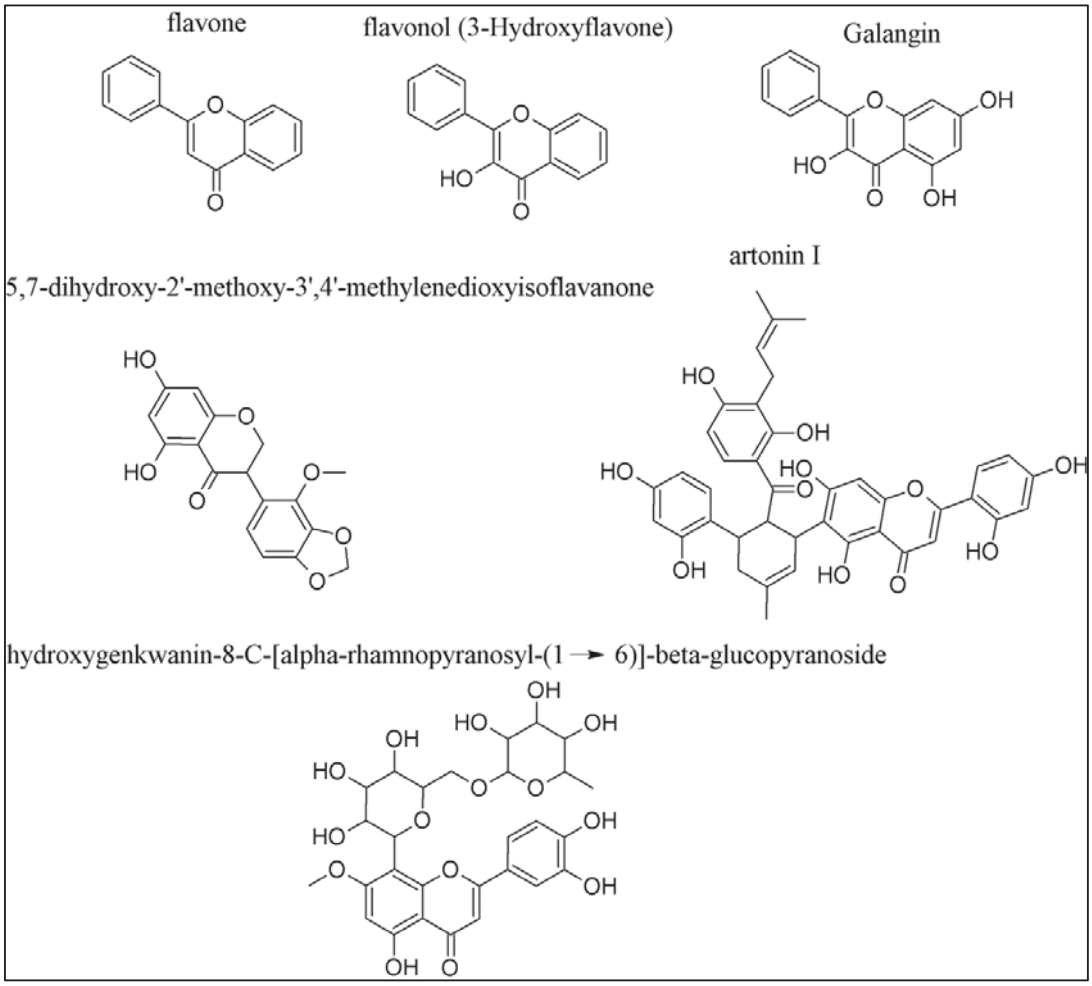


Figure 1.14: Structures of flavones and flavonols with some antibacterial activities.

1.1.2.7. Tannins

Tannins are derived from bark, wood, leaves, fruits and roots. More than one type of tannins is available in nature and the hydrolysable tannins (gallic acid ester with D-glucose) are less frequently found in nature than condensed tannin (proanthocyanidins) (Scalbert 1991). The antimicrobial action of tannins was attributed to their inhibition to microbial adhesions and complex formation with enzymes, cell envelope transport proteins and polysaccharide (Ya, Gaffney et al. 1988).

Condensed tannins were determined to bind cell walls of ruminal bacteria, and prevent growth and protease activity (Jones, McAllister et al. 1994).

Corilagin, a compound classified as ellagitannin, showed weak antibacterial activities with MICs equal to 1000-2000 μg/ml against *B. subtilis* and several gram-negative bacteria like *E. coli*. (Lipińska, Klewicka et al. 2014). Ellagitannins fraction from *Acalypha wilkesiana* var. *macafeana* hort showed moderate activity against *S. aureus* and its congener MRSA with inhibition zone of 11 and 8 mm respectively. The minimum bactericidal concentration against MRSA was 3000 μg/ml (Din, Jin et al. 2013).

The proanthocyanidins had stronger antibacterial abilities than catechins (Amarowicz, Pegg et al. 2000); they exerted their antibacterial activities by complexing with protein structures in the bacterial cells. For example, proanthocyanidins from green tea was shown to bind proteins and enzymes in *E. coli* (Amarowicz, Pegg et al. 2000). (Figure 1.15) shows the structure of corilagin and proanthocyanidins.

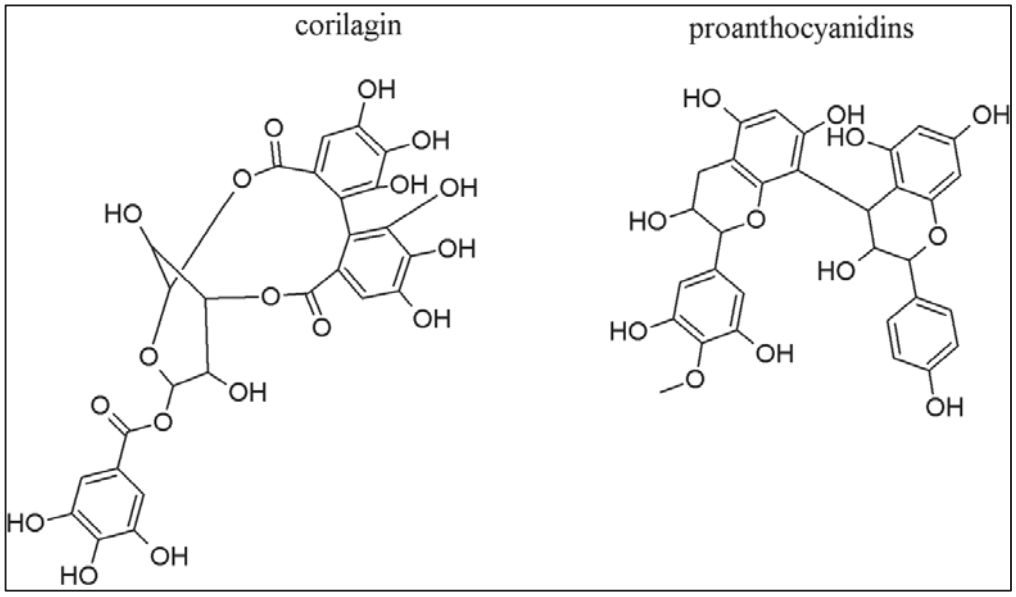


Figure 1.15: Structures of corilagin and proanthocyanidins

1.1.2.8. Coumarins

Coumarins are phenolic substances made of fused benzene and α-pyrone rings (O'Kennedy, Thornes 1997). They are responsible for the characteristic odor of hay and possess antithrombotic (Thastrup, Knudsen et al. 1985), anti-inflammatory (Piller 1975) and vasodilator (Namba, Morita et al. 1988) actions. Estrogenic effect of coumarin has also been detected (Soine 1964). Coumarins were found to stimulate macrophages (Casley-Smith, Casley-Smith 1997), which could have an indirect effect on infections.

The hydroxylated derivatives of coumarins which are produced in carrots were responsible for the antifungal activity (Hoult, Paya 1996). Isomammein from *Mammea africana* had strong antibacterial activities against *Campylobacter jejuni*, *Clostridium difficile*, *S. pneumoniae* with MIC of 0.25-0.5 μg/ml (Canning, Sun et al. 2013). Four coumarin derivatives isolated from *Arracacia tolucensis* var. *multifidi*: osthole, suberosin, 8-methoxypsoralen (methoxalen), and herniarin appeared to be active against the bacterium causing tuberculosis (*Mycobacterium tuberculosis*); the best activity was attributed to 8-methoxypsoralen with its MIC equal to 16μg/ml (Figueroa, Rivero-Cruz et al. 2007).

Licopyranocoumarin and glycyrin isolated from *Glycyrrhiza uralensis* showed MIC of 330 and 42 μM against *Enterococcus faecium,* respectively (Eerdunbayaer, Orabi et al. 2014). Coumarins structures are shown in (Figure 1.16).

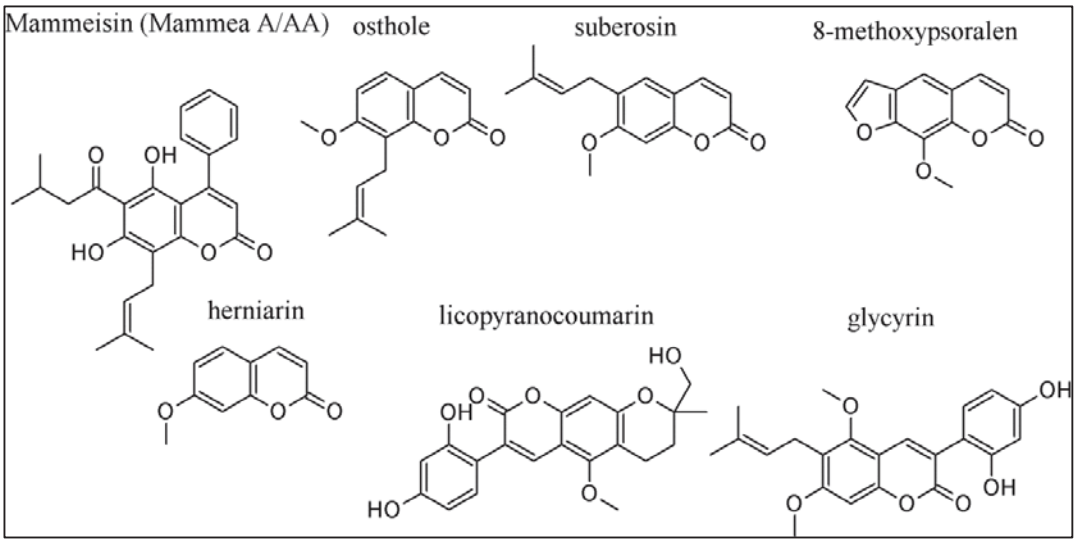


Figure 1.16: Structures of several coumarins recorded with good antibacterial activities.

1.1.2.9. Alkaloids

Alkaloids are nitrogen containing compounds with basic properties. Tetrandrine isolated from *Stephania tetrandra* S. Moore showed MIC equal to 128 μg/ml against MRSA (Zuo, Li et al. 2011). Two alkaloids from *Boophone disticha*, buphanidrine and distichamine showed antibacterial activities with MIC equal to 63 μg/ml against *S. aureus, E. coli* and *Klebsiella pneumoniae* (Cheesman, Nair et al. 2012). Oxyphylline B extracted from Zizyphus *oxyphylla* Edgew showed MIC equal to 25 μg/ml against *S. aureus* and 5 μg/ml against *E. coli* (Kaleem, Nisar et al. 2012). Tegerrardin A isolated from *Zanthoxylum zanthoxyloides* showed moderate antibacterial effect with MIC equal to 500 μg/ml against *S. aureus* and its MIC against *B. subtilis* was = 250 μg/ml (Wouatsa, Misra et al. 2013). Berberine an alkaloid isolated *Berberis microphylla* G. Forst had MIC equal to 167 μg/ml against *S. aureus* and *S. epidermidis*, but it was inactive against *B. cereus* and *B. subtilis* (Manosalva, Mutis et al. 2016). The structures of alkaloids mentioned in this section are shown in (Figure 1.17).

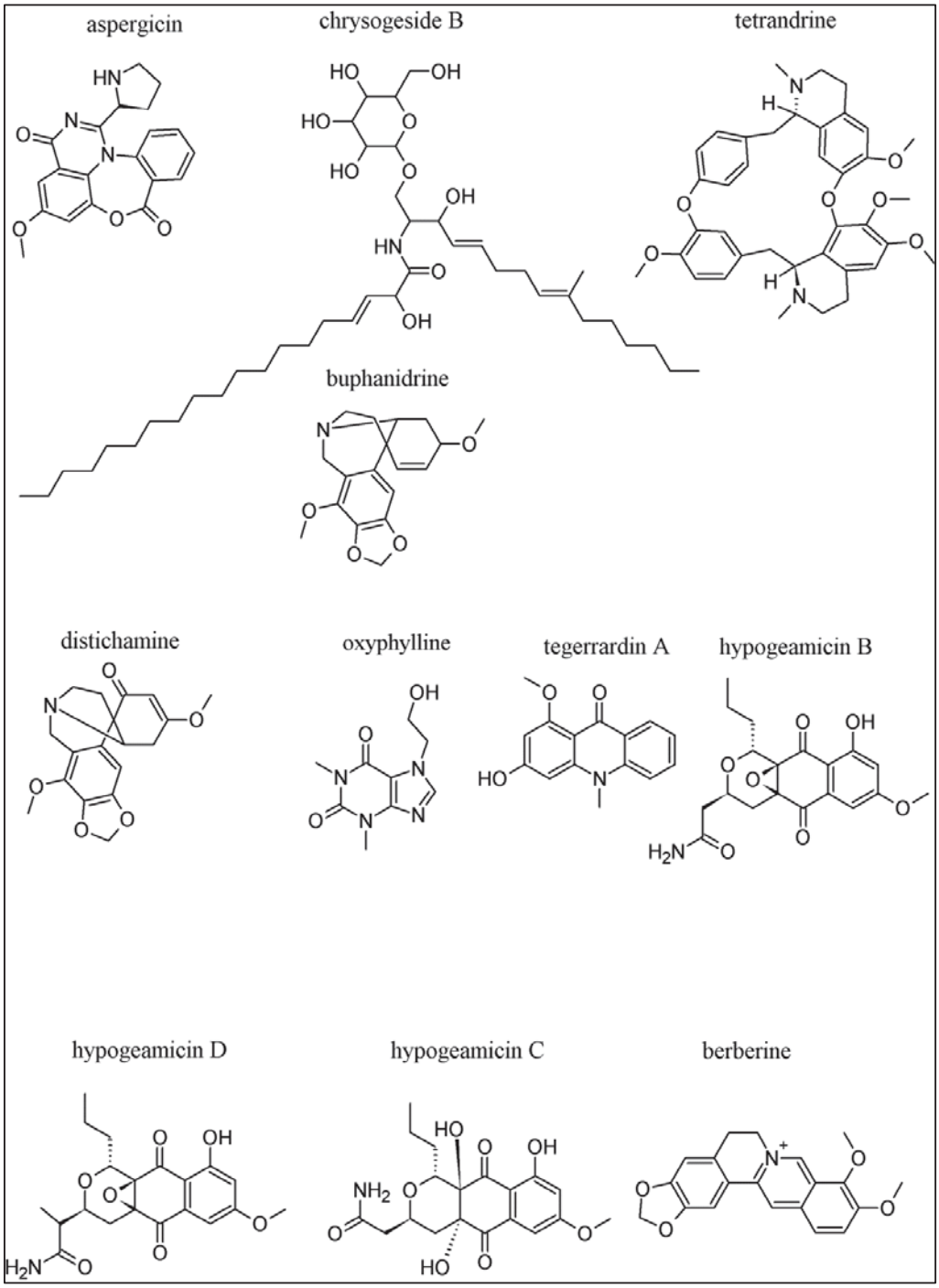


Figure 1.17: Structures of several alkaloids recorded with antibacterial activities.

1.1.2.10. Oligosaccharides

Oligosaccharides have been detected to have antibacterial activity. Tricolorin A isolated from *Ipomoea tricolor* the Mexican Morning Glory Species showed MICs equal to 16, 8, 8

and 4 μg/ml against *S. aureus* strains ATCC 25923, XU-212, SA-1199B, EMRSA-15, respectively. Orizabin VIII isolated from the same plant showed MIC equal to 4-32 μg/ml, against those strains (Pereda-Miranda, Kaatz et al. 2006). The structures of tricolorin A and orizabin VIII are shown in (Figure 1.18).

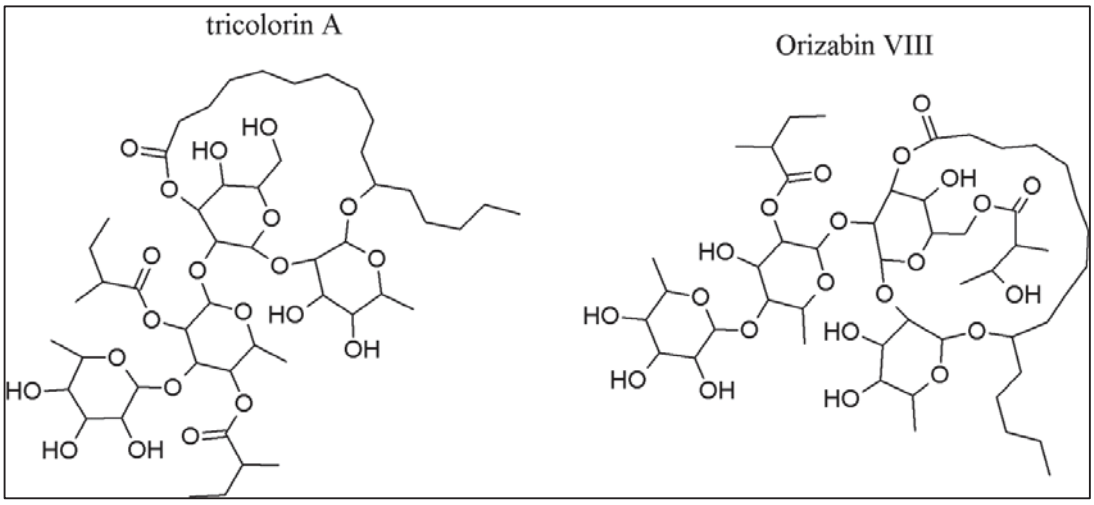


Figure 1.18: The structures of two oligosaccharides (tricolorin A and orizabin VIII) have antibacterial activities.

1.2. Discovery of the antiplasmodial agents

1.2.1. Malaria and antiplasmodial drugs resistance problem

Malaria is an infectious disease caused by the protozoa *Plasmodium*. It is widely spread in tropical countries, affecting 214 million persons in 2015, a twenty percentage less than the cases recorded in the year 2000 which were around 262 million, with 88% of the cases recorded in Africa (World Health Organization 2015). The causative agent of malaria is transmitted to the human biological compartments *via* a certain type of mosquito. There are about 3,500 mosquito species and those that transmit malaria all belong to a subset called the *Anopheles*. Approximately 40 *Anopheles* species are able to transmit malaria, causing serious human illness and death.

The four main parasites species causing malaria in human being are *Plasmodium falciparum, P. vivax, P. ovale, and P. malariae* (Grimberg, Mehlotra 2011). The most lethal specie is *P. falciparum* (Grimberg, Mehlotra 2011).

Phytotherapy plays an important role in the efficient treatment of malaria. The *Cinchona* species are well known for their antimalarial properties due to their bioactive alkaloid quinine (White 1985). For the treatment of malaria, quinine was widely used as the major antimalarial drug, it was originally isolated in 1820 by the French pharmacists Caventou and Pelletier from the bark of *Cinchona* species (e.g., *Cinchona officinalis*), which is used by Amazon people as antipyretic and in the early 1600s used in Europe for the treatment of malaria.

Quinine is an aryl amino alcohol and is usually kept as the last line in malaria therapy *via* intravenous injection. Doxycycline is usually used concurrently with quinine to increase its effect (Grimberg, Mehlotra 2011). The resistance to quinine started in South East Asia and South of America in the 1960s.

Quinine causes several adverse effects like arrhythmia and hypoglycemia (Schlitzer 2008). The mechanism of action of quinine is similar to chloroquine. The transporting proteins PfCRT (the chloroquine resistance transporter) and PfMDR1 (the multidrug resistance transporter) and PfNHE1 (sodium/hydrogen exchanger 1) are responsible for removal of quinine from the parasite and leads to a decrease in its activity (Cheruiyot, Ingasia et al. 2014).

In the 1930s, quinine was largely replaced by a series of synthetic drugs including 8-aminoquinolines (e.g. primaquine), 4-aminoquinolines (e.g. chloroquine, amodiaquine) and folic acid synthesis inhibitors (e.g. proguanil, pyrimethamine).

The resistant strains to chloroquine emerged in the 1950s, just ten years after its launch as a drug in the 1940s. The resistance problem began in South East Asia and South of America (Grimberg, Mehlotra 2011, Farooq, Mahajan 2004). Chloroquine inhibits the formation of haemozoin, the haem polymer. Haem in the un-polymerized form causes destruction of the parasite membrane and death (Combrinck, Mabotha et al. 2013).

Parasites sensitive to chloroquine accumulate chloroquine molecules in the digestive vacuole (Krogstad, Gluzman et al. 1987) and the resistance to this drug is attributed to change in this property. Transporting proteins PfCRT and PfMDR1 are responsible for chloroquine and multidrug resistance respectively (Ding, Beck et al. 2011, Eyasu 2015). Structures of the natural compound (quinine), chloroquine and other synthetic antiplasmodial drugs are shown in (Figure 1.19).

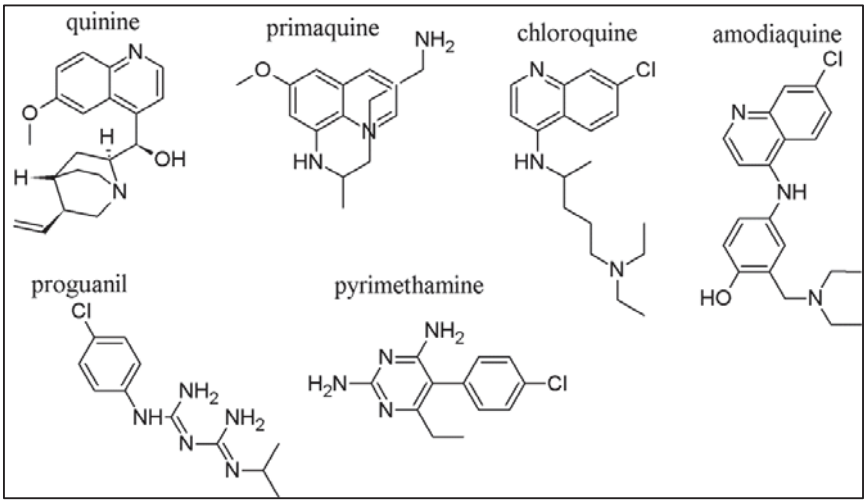


Figure 1.19: Structures of quinine and some synthetic antiplasmodial compounds.

Resistance emerged in both the vector and the parasite itself; the vector mosquito developed resistance to the potent insecticides such as dichlorodiphenyltrichloroethane (DDT) and by the early 1980s, several strains of *P. falciparum* had become multi-drug resistant. Decrease the accumulation of drugs in the parasite and affinity to the specific drug targets are the two major mechanisms of resistance (White 1999).

Mefloquine is a 4-aminoquinoline launched in the 1970s to treat chloroquine resistance (Schlitzer 2008) and works by prevention of haemozoin accumulation. Unfortunately the resistance to this drug was first observed ten years later in the Thai-Cambodian border where it was used intensively, in the Amazon and to a less extent in Africa (Farooq, Mahajan 2004). An amplified PfMDR1 gene is responsible for the resistance (Preechapornkul, Imwong et al. 2009). Artesunic acid in combination with mefloquine was used to overcome resistance (Price, Nosten et al. 1995).

The *Plasmodium* parasites biosynthesize folate using two enzymes: dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) (Dondorp, Fanello et al. 2010). Pyrimethamine and cycloguanil inhibit DHFR and interrupt folate synthesis. Sulfadoxine

and dapsone block the effect of DHPS (Dondorp, Fanello et al. 2010). Blocking the process of folate synthesis in the parasite, will lead to decrease the production of amino acids and parasite death (Sibley, Hyde et al. 2001). Fansidar® a drug contains sulphadoxine and pyrimethamine successively inhibits DHPS and DHFR (Petersen, Eastman et al. 2011, Sridaran, McClintock et al. 2010).

Mutation in genes responsible for DHPS and DHFR formation leads to decrease of their affinity to the drug and new resistance cases to Fansidar® have emerged (Gesase, Gosling et al. 2009). To address such resistance problem, a combination of chlorproguanil and dapsone was introduced to treat those resistance cases (Sridaran, McClintock et al. 2010). Structures of mefloquine, artesunic acid and inhibitors of DHFR and DHPS are shown in (Figure 1.20).

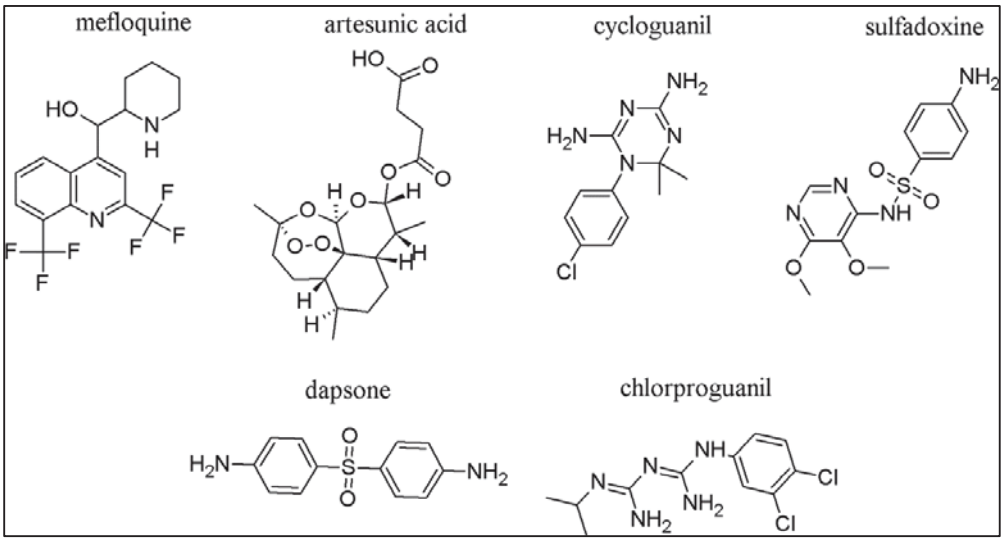


Figure 1.20: Structures of mefloquine, artesunic acid, and inhibitors of DHFR and DHPS.

Artemisinin, a sesquiterpene lactone from *Artemisia annua*, was a milestone in the treatment arsenal of malaria (Phillipson, O'Neill 1989, Wright 2005). It has an endoperoxide structure which is important for the antimalarial activities (Klayman 1985). Artemisinin is safer than chloroquine and it has a higher chemotherapeutic index. It is

effectively applied in the treatment of chloroquine-resistant strains of human malaria (Warhurst 1985). A reduction of the lactone ring in artemisinin gives its reduced form dihydroartemisinin which is more active than the original molecule (Wright 2005). (Figure 1.21) shows the structures of artemisinin and dihydroartemisinin.

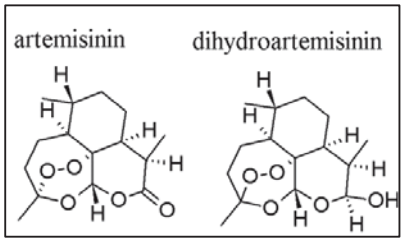


Figure 1.21: Structures of artemisinin and its reduced congener dihydroartemisinin

The drug, artemisinin (Grimberg, Mehlotra 2011) acts by alkylating the parasites protein's which leads to their damage (Meshnick, Taylor et al. 1996). The drug appeared to inhibit the uptake of radio-active hypoxanthine, indicating a decrease in nucleic acid synthesis (Saxena, Pant et al. 2003). The semisynthetic drugs of artemisinin such as artesunic acid were combined with mefloquine in order to increase the treatment efficacy and treat the condition within three days (Beeson, Boeuf et al. 2015). Despite the effectiveness of artemisinin, there have been reports of artemisinin treatment failure in South-East Asia (Burrows, Leroy et al. 2011, Grimberg, Mehlotra 2011). The proteins contributing to the resistance of artemisinin are PfCRT, PfMDR1 and PfATP6 (P. *falciparum* calcium-dependent ATPase) (Zakeri, Hemati et al. 2012).

1.2.2. Recent advances of antiplasmodial natural compounds from plants

Mortality rate has been increased due to the emergence and spread of multidrug-resistant *Plasmodium falciparum* parasite to the antiplasmodial compounds (Guantai, Chibale 2011, Ginsburg, Deharo 2011), therefore there was an urgent need to identify new drugs for the treatment of malaria (Sinha, Medhi et al. 2014). The search for new leads for the

development of treatments for malaria is a continuous process, which has drawn attention to drug discovery of new medicinal agents (McCracken, Kaiser et al. 2012).

WHO has established four stages to search for antiplasmodial drugs. The first stage is the primary screening of active molecules in order to find which one is active *in vitro* as antiplasmodial. The second step is further evaluation of the extent of activity, analysis of the quantity of the effect, and running the experiments required to determine the marginal safety of the tested molecule and comparative activities of analogues synthesised from the natural lead. The third stage is the study of various parasites (non-human and human) in biological systems. The fourth stage is the clinical bio-evaluation step (Mojab 2012).

Towards the search of new-antiplasmodial drugs, several recent reports have described the high throughput screening (HTS) of massive number of natural compounds and the identification of novel chemical scaffolds to be taken forward for development (Gamo, Sanz et al. 2010).

Prioritizing these compounds for further exploration of their pharmacokinetic and pharmacodynamics properties is essential and has been the focus of several recent developments in assay formats, and compounds extracted can be grouped into different ranks according to their properties and biological activities (Bahamontes-Rosa, Rodríguez-Alejandre et al. 2012, Sanz, Crespo et al. 2012).

Much effort has been made to explore the antimalarial abilities of plants; an important research published in (Lloydia) has been done by Spencer CF et al (1947) to reveal the antimalarial activities of 600 plants from 126 families, their extracts tested for activities, against *P. gallinaceum* in chickens, *P. chatemerium* and *P. lophurae* in ducklings. Thirty species yielded extracts which were active against these types of malaria affecting birds (Phillipson, O'Neill 1989).

The natural extracts with moderate activity *in vitro* or *in vivo* may have an important role in the treatment of malaria in mankind by modifying the disease course; shortening the period of illness by reducing anaemia, fever, and pain (Wright 2005). Finally, it is important to stress that plant extracts could also be effective against the parasite on hepatic stage. The results from *in vitro* experiments will enable researchers to decide whether the natural materials possess promising antiplasmodial activities should be followed by *in vivo* assays or not, however, most *in vivo* assays use parasites types unusual for the human being. The four-day suppressive test with *P. berghei* in rodents predicts antimalarial activity and toxicity in humans (Fandeur, Moretti et al. 1985, Phillipson, O'Neill 1989).

1.2.2.1. Antiplasmodial assay methods

Protozoa are microscopic and single-cell microorganisms that can be free-living or parasitic in nature and are able to multiply in humans.

The methods of *in vitro* culturing of the parasites causing malaria were developed within the last forty years. To test effects of the chemical compounds against *Plasmodium* species, researchers used a method called radiolabelled microorganisms technique where the erythrocyte infection was assayed with microtiter dishes (Francois, Ake Assi et al. 1996). In this case, [3H (G)]-hypoxanthine monohydrochloride was used to label the parasite *Plasmodium falciparum* (Desjardins, Canfield et al. 1979, Wright 2005).

A study developed by Makler in 1993 used lactate dehydrogenase (pLDH) assay as an alternative to the use of the radioactive material[3H(G)]-hypoxanthine monohydrochloride, the results produced were fast and accurate and provided an economic alternative to older methods (Makler, Ries et al. 1993, Wright 2005).

Defining the antiplasmodial mode of activity like the formation of toxic drug-haem complex to the parasite has been an achievable task now (Parapini, Basilico et al. 2000). For example, the mechanism of action of artemisinin which has an active site consisting of

a bridge between two oxygen atoms links to haem and produces free radicals to kill the parasites (Wright, Warhurst 2002).

SYBR Green I method is a reproducible, direct and economic method which can be defined as an alternative to the determination of parasite growth using a radioactive material. The parasitized erythrocytes are incubated with and without test substances, and then the *Plasmodium* organisms after certain period of incubation with SYBR dye are measured quantitatively. The fluorescence energy can be measured and dose- response curve to determine the IC_{50} of the drug can be sketch (Smilkstein, Sriwilaijaroen et al. 2004).

There have been different classifications with regard to the potency of the plant extracts and pure compounds in the literature. According to the potency of antiplasmodial activity *in vitro* Basco et al. (1994) specified that antiplasmodial compounds with IC_{50} < 10 μg/ml is good; IC_{50} of 10-50 μg/ml, is moderate in activity; IC_{50} of 50-100 μg/ml, is low in activity; and IC_{50} > 100 μg/ml, practically inactive (Basco, Mitaku et al. 1994, Dolabela, Oliveira et al. 2008).

On the other hand, Muriithi et al. (2002) classified compounds as inactive with IC_{50} > 100 μM; of limited activity, those with IC_{50} of 1–20 μM; and of low activity those displaying IC_{50} of 20–60 μM (Muriithi, Abraham et al. 2002).

Batista et al. (2009) have been established a new classification of antiplasmodial drugs according to *in vitro* activity, compounds with IC_{50} < 1 μM, excellent/potent activity; IC_{50} of 1-20 μM, good activity; IC_{50} of 20-100 μM, moderate activity; IC_{50} of 100-200 μM, low activity; and IC_{50} > 200 μM, inactive (Batista, Silva Ade et al. 2009).

Recently, plant extracts with antiplasmodial activities are classified on the basis of their IC_{50}s to be highly active ($\leq$ 5 μg/ml), promisingly active (5-10 μg/ml), have good activity

(10 -20 μg/ml), moderate activity (20-40 μg/ml), marginal potency (40-70 μg/ml), poor or inactive (> 70 μg/ml) (Singh, Kaushik et al. 2015).

Furthermore, two important indices are used to quantify the resistance and selectivity properties of the chemical compounds, the resistance index has been defined as the ratio between the IC_{50} values of a given compound against resistant strain and the sensitive strain, while the selectivity index is the ratio of the IC_{50} value of such substance against host normal cell line to that against the target strain.

Accordingly, a desirable chemotherapeutic lead structure should present both low resistance index and high selectivity index (Pedersen, Chukwujekwu et al. 2009).

In the following sections examples of plant natural compounds with antiplasmodial activity in different chemical classes will be discussed. The chloroquine sensitive plasmodium strains often used were *Plasmodium falciparum* MRC2; *P. falciparum* 3D7; *P. falciparum* D6; *P. falciparum* CDC1; *P. falciparum* NF54; *P. falciparum* D10. The chloroquine resistant strains used were *P. falciparum* RKL9; *P. falciparum* FCR3; *P. falciparum* W2; *P. falciparum* K1; *P. falciparum* FZR-8; and, *P. falciparum* Dd2.

1.2.2.2 Alkaloids

The nitrogenous compounds “alkaloids” form an important part of the natural compounds defending the human beings against malaria. Quinine is the first member of this family, which is discussed extensively above.

Orthidine F from the New Zealand ascidian *Aplidium orthium*, showed its IC_{50} of 0.89 μM against *P. falciparum* K1 strain (dual drug resistant strain). One of its analogues, 1,14-spermine-di-(2-hydroxyphenylacetamide) showed higher activities with IC_{50} of 8.6 nM against *P. falciparum* K1 strain. (Liew, Kaiser et al. 2013). Indolisoquinoline alkaloid, cryptolepine isolated from *Cryptolepis sanguinolenta* showed IC_{50} of 0.03 μg/ml (134 nM). This activity is higher than that of chloroquine against *P. falciparum* K1 *in vitro*;

however, it has no effect against *P. berghei* in an *in vivo* study conducted in mouse (Kirby, Paine et al. 1995). Later, a synthetic compound 2, 7-dibromocryptolepine was shown to be potent and less toxic than cryptolepine in mouse (Wright, Addae-Kyereme et al. 2001).

Bisbenzylisoquinoline alkaloids isolated from *Stephania erecta* were active against chloroquine-sensitive *P. falciparum*. (+)-2-N-methyltelobine showed IC_{50} of 0.25 µg/ml (444 nM) against *P. falciparum* W2 strain (Likhitwitayawuid, Angerhofer et al. 1993). These alkaloids have one or two N-methyl groups; however the presence of two N-methyl groups decreases the antiplasmodial activity against both sensitive and resistant parasites (Likhitwitayawuid, Angerhofer et al. 1993). Dehatrine, an alkaloid from *Beilschmiedia madang* exerted an antiplasmodial effect with an IC_{50} equal to 17 µM against *P. falciparum* (Kitagawa, Minagawa et al. 1993).

Tazopsine a novel morphinan alkaloid extracted from *Pseuduvaria setosa* showed IC_{50}s equal to 4.7 µM and 5.7 µM against *P. falciparum* 3D7 and *P. falciparum* FCR3, respectively (Carraz, Jossang et al. 2006). 8-Acetonyldihydronitidine from *Feroniella lucida* showed IC_{50} equal to 0.336 µg/ml (0.82 µM) against *P. falciparum* K1 (Sripisut, Cheenpracha et al. 2011). 2-Hydroxyathersperminine isolated from the stem bark of *Cryptocarya nigra*, showed IC_{50} of 0.75 µM against *P. falciparum* K1 (Nasrullah, Zahari et al. 2013). Olivacine isolated from *Aspidosperma ulei* Markgr, this alkaloid showed a weak antiplasmodial activities IC_{50} 4.5 mM against *P. falciparum* K1 (dos Santos Torres, Silveira et al. 2013).

Pseudopalmatine extracted from *Stephania rotunda* Lour a climber plant distributed in the high mountains of Cambodia showed an IC_{50} equal to 2.8 µM against *P. falciparum* W2 (Baghdikian, Mahiou-Leddet et al. 2013). N-methyl-tetrahydrolivacine isolated from *Aspidosperma olivaceum* showed an IC_{50} of 5.7 µg/ml (20.45 µM) against *P. falciparum* W2 (Chierrito, Aguiar et al. 2014).

(-)-O-O-dimethylgrisabine a bisbenzylisoquinoline alkaloid and (-)-milonine, a morphinandienones alkaloid both extracted from the bark of *Dehaasia longipedicellata* showed IC_{50} equal to 0.031 μM and 0.097 μM against *P. falciparum* K1, respectively. (Zahari, Cheah et al. 2014).

Normelicopidine isolated from *Zanthoxylum simullans* Hance (a common traditional Chinese medicine herb) showed IC_{50} equal to 21.1 μg/ml (70.5 μM) against *P. falciparum* Dd2 (Wang, Wan et al. 2014). Aspidoscarpine was extracted from *Aspidosperma olivaceum* a plant used locally in some regions of Brazil for alleviation of fever. Aspidoscarpine showed IC_{50} equal to 5.4 μg/ml (14.5 μM) against *P. falciparum* W2 (Chierrito, Aguiar et al. 2014). The structures of alkaloids mentioned in this section are shown in (Figure 1.22).

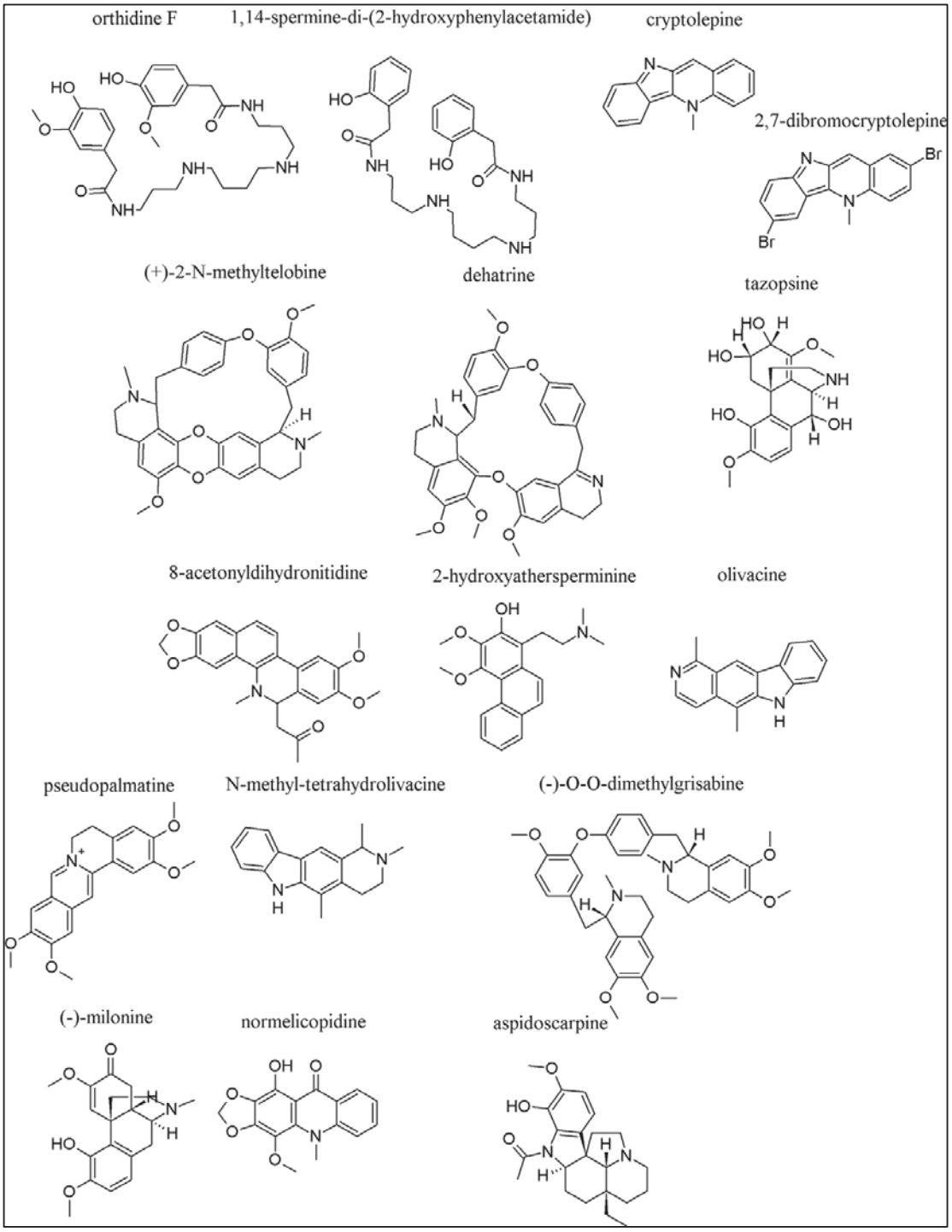


Figure 1.22: Structures of alkaloids with antiplasmodial activities.

1.2.2.3. Quassinoids

Quassinoids are degraded triterpenoids isolated from the plants of *Simaroubaceae* family. Usually their carbon skeletons are C-18, C-19, C-20, C-22 and C-25, with many oxygen bearing groups attached (Guo, Vangapandu et al. 2005). Quassinoids have many biological activities of which, antineoplastic and antiplasmodial are well reported. Bruceantin and bruceine A were isolated from *Brucea javanica*, and they showed very impressive IC_{50}s of 0.0008 μg/ml (0.0014 μM) and 0.011 μg/ml (0.021 μM) against *P. falciparum* K1 strain respectively (Anderson, O'Neill et al. 1991, Sharma, Agarwal 1993).

Orinocinolide and simalikalactone D isolated from *Simaba orinocensis* showed very low IC_{50}s of 3.27 and 8.53 ng/ml (0.0068 and 0.0177 μM) against *P. falciparum* D6, and 3.0 and 3.67 ng/ml (0.0062 and 0.0075 μM) against *P. falciparum* W2, respectively (Muhammad, Bedir et al. 2004). Cedronin is a quassinoid isolated from *Simaba cedron* and its IC_{50} against *P. falciparum* FZR-8 was 0.25 μg/ml (0.68μM) (Moretti, Deharo et al. 1994). A *Quassia indica* product called samaderine X showed good effect against *P. falciparum* K1 with a low IC_{50} of 0.015 μM (Kitagawa, Mahmud et al. 1996).

In general, the group of quassinoid compounds from plants showed excellent anti-plasmodial activities. Structures of several quassinoids mentioned above are shown in (Figure 1.23).

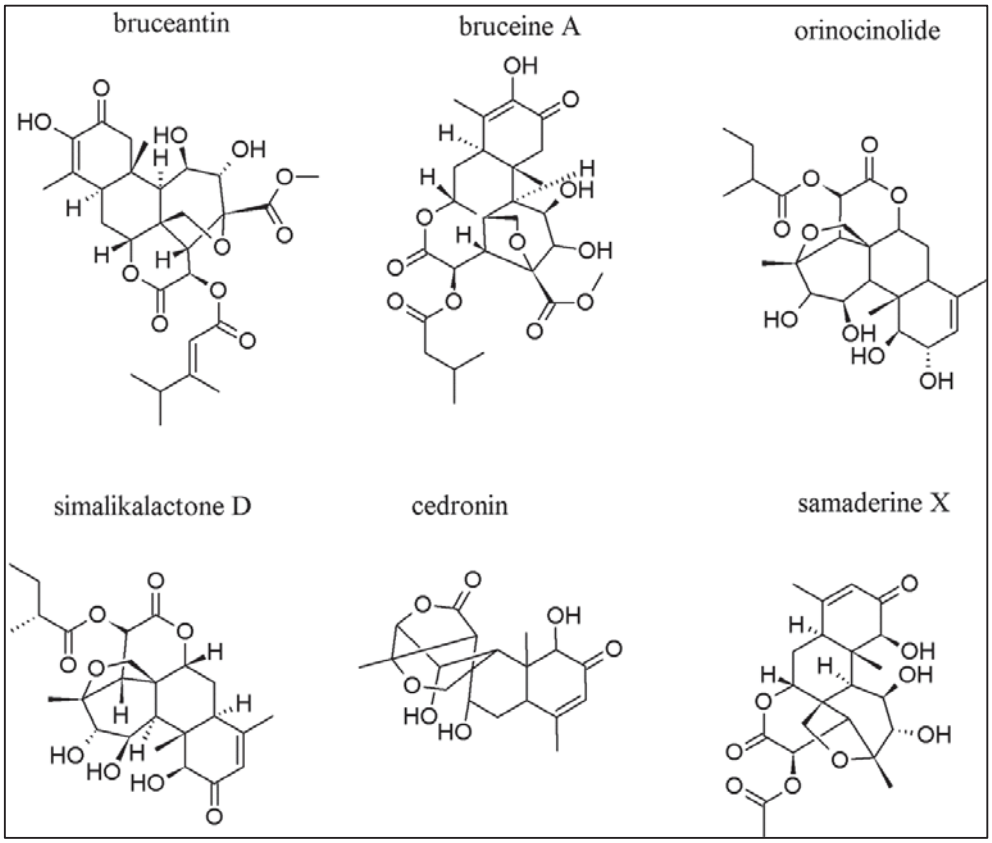


Figure 1.23: Structures of several quassinoids (potent antiplasmodial compounds)

1.2.2.4. Sesquiterpenes

The most famous example of sesquiterpene group is artemisinin, a strong antiplasmodial compound isolated from *Artemisia annua as discussed in details above.* 10,12-peroxycalamenene showed potent effect against *P. falciparum* K1 with IC_{50} equal to 2.33 μM (Thebtaranonth, Thebtaranonth et al. 1995). Neurolenin A isolated from *Neurolaena lobate* showed IC_{50} equal to 0.92 μM against *P. falciparum* NF-54 (Francois, Passreiter et al. 1996). Nardoperoxide a compound with Endoperoxide Bridge was extracted from *Nardostachys chinensis* and showed IC_{50} equal to 1.5 μM against *P. falciparum* NF-54 (Takaya, Kurumada et al. 1998).

The sesquiterpene lactone lipiferolide of *Liriodendron tulipifera* L. showed its IC_{50} equal to 1.8 µg/ml (5.8 µM) against *P. falciparum* D10 and 2.3 µg/ml (7.5 µM) against Dd2 a chloroquine resistant strain (Graziose, Rathinasabapathy et al. 2011). Urospermal A-15-O-acetate extracted from *Dicoma tomentosa* showed a low IC_{50} of <1 µg/ml (<3.1 µM) against *P. falciparum* 3D7 and W2 (Jansen, Tits et al. 2012). The structures of these sesquiterpenes are shown in (Figure 1.24).

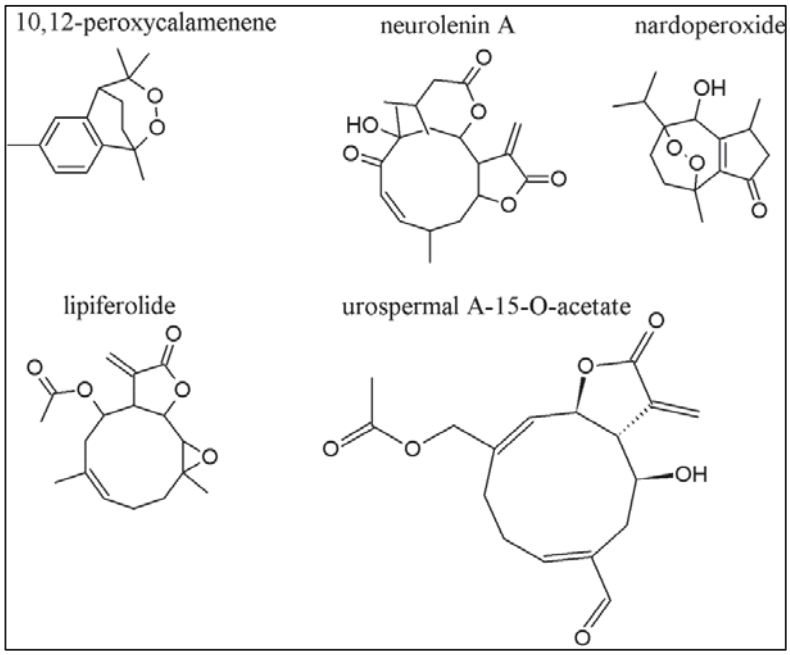


Figure 1.24: Structures of some sesquiterpenes with antiplasmodial activities.

1.2.2.5 Triterpenoids

There are many compounds with antiplasmodial activities reported in the triterpenoid group, gedunin is a limonoid closely related to quassinoids, which can produce a quassinoid like structure after treatment with base (Bray, Warhurst et al. 1990). Gedunin isolated from *Azadirachta indica* (L) showed IC_{50} of 0.72 µg/ml (1.5 µM) against *P. falciparum* K1 (Bray, Warhurst et al. 1990). The reduction of (α,β-unsaturated) ketone

produced dihydrogedunin which had less antiplasmodial activity (IC_{50} 2.62 µg/ml (5.4 µM)).

Betulinic acid extracted from *Triphyophyllum peltatum* showed an IC_{50} equal to 10.46 µg/ml (22.9 µM) against *P. falciparum* NF-54 (Bringmann, Saeb et al. 1997). Dihydrobruceajavanin A isolated from *Brucea javanica* had IC_{50} equal to 2.5 µg/ml (4.3 µM) against *P. falciparum* K1 (Sharma, Agarwal 1993). All of these triterpenes are shown in (Figure 1.25).

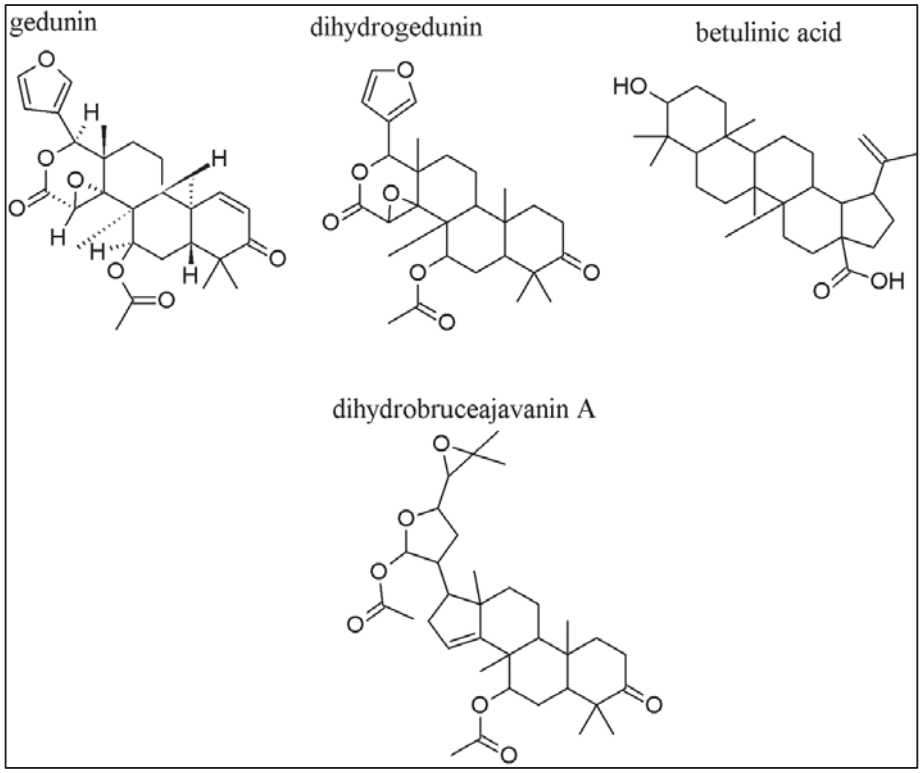


Figure 1.25: Structures of some triterpenes with antiplasmodial activities.

1.2.2.6. Flavonoids and their glycosides

3,7-dihydroxy-3′-(4-hydroxy-3-methylbutyl)-5,6,4′-trimethoxyflavone and 5,7-dihydroxy-3′-(2-hydroxy-3-methyl-3-butenyl)-3,6,4′-trimethoxyflavone isolated from *Duranta repens*, showed good antiplasmodial activity against *P. falciparum* D6 and W2 with IC_{50}s ranging between 6.4 µM and 7.0 µM, respectively (Ijaz, Ahmad et al. 2010).

3-O-methylquercetin extracted from *Tagetes minuta* L. showed IC_{50} of 4.37 µg/ml (13.8 µM) against *P. falciparum* D6 (Al-Musayeib, Mohamed et al. 2014).

Afzelin, quercitrin and myricitrin were extracted from the aerial parts of Euphorbia hirta Linn and showed IC_{50} of to 1.1 (2.5), 4.1 (9.1) and 5.4 (11.6) µg/ml (µM) against *P. falciparum* CDC1, respectively (Liu, Murakami et al. 2007). The structures of flavonoids and flavonoid glycosides are shown in (Figure 1.26).

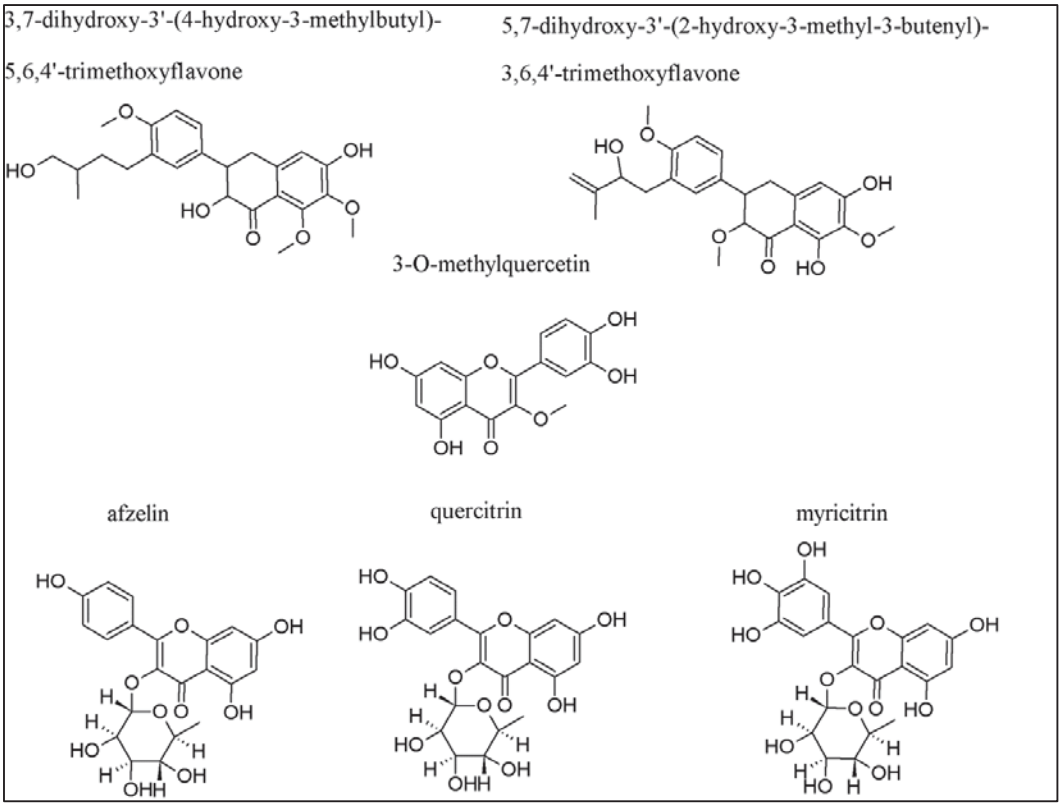


Figure 1.26: The structures of flavonoids and flavonoid glycosides with antiplasmodial activities.

1.2.2.7. Xanthones

Gerontoxanthone I, macluraxanthone and formoxanthone C were isolated from *Cratoxylum maingayi* and assayed against *P. falciparum* K1, which showed IC_{50} equal to 1.68 (4.2), 1.35 (3.4) and 1.19 (3) μg/ml (μM) respectively (Laphookhieo, Maneerat et al. 2009). Another xanthone, α-mangostin extracted from *Garcinia mangostana* and its IC_{50} was determined to be 2.2 μM against *P. falciparum* K1 (Al-Massarani, El Gamal et al. 2013). The structures of these xanthones with antiplasmodial activity are shown in (Figure 1.27).

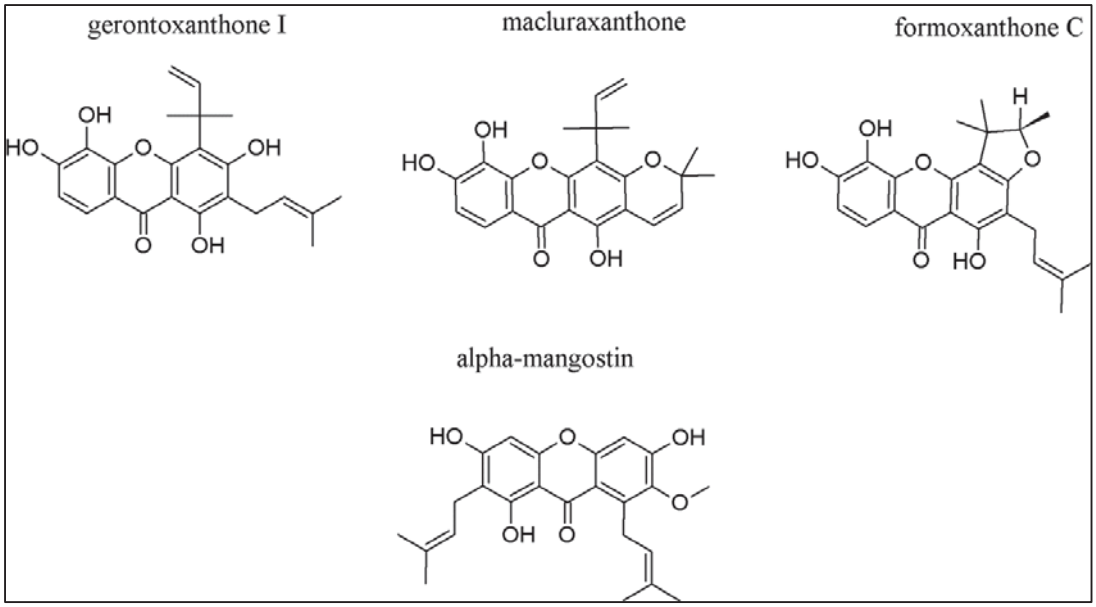


Figure 1.27: The structures of xanthones with antiplasmodial activities.

1.2.2.8 Quinones and anthracene

Quinones like digitolutein and damnacanthal were extracted from *Morinda lucida*, and their IC_{50} against *P. berghei* were determined to be 12.92 and 9.2 μg/ml (48.2 and 32.6 μM) respectively (Koumaglo, Gbeassor et al. 1992). Plumbagin was isolated from *Nepenthes thorelii* and its IC_{50} against *P. berghei* (in vitro) was 0.27 μM (Likhitwitayawuid, Kaewamatawong et al. 1998). Vismione B and vismione F were isolated from *Cratoxylum cochinchinense* and showed IC_{50} equal to 0.66 μg/ml and 2.02

μg/ml (1.68 and 4.9 μM) against *P. falciparum* K1, respectively (Laphookhieo, Maneerat et al. 2009). The quinones and anthracene structures are shown in (Figure 1.28).

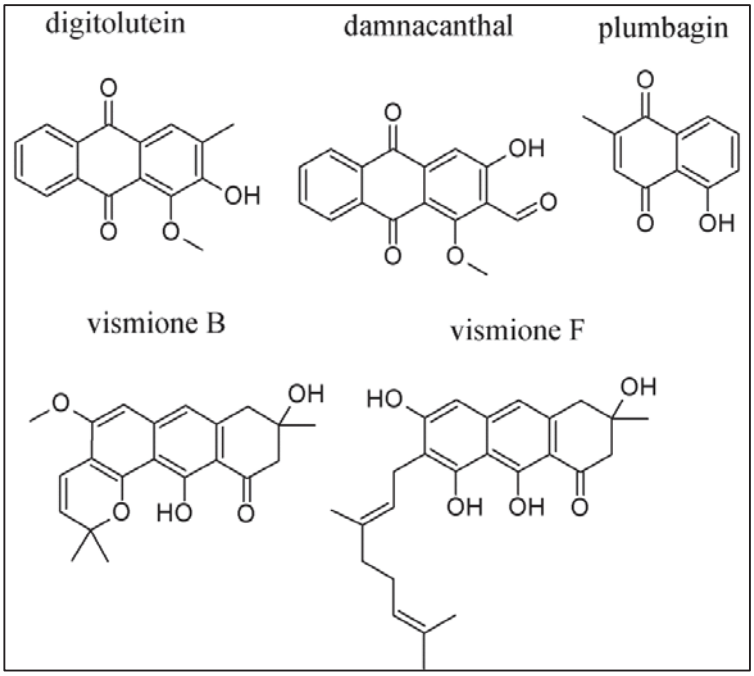


Figure 1.28: The structures of quinones and anthracene structures with antiplasmodial activities.

In summary, we are living in a crucial time to find new compounds able to fight multi-drug resistance pathogens. The plant kingdom is an indispensable source for both antimicrobials and resistance modifying agents. Natural products can offer special stereochemistry and unlimited diversity of natural compounds leads which are biologically active or ready for the development and structural optimization strategies.

1.3. Plants used in current research

1.3.1. Cylicodiscus gabunensis

Cylicodiscus gabunensis Harms, family *Mimosaceae* (CG) is a large tree which reaches 60 m in height, straight cylindrical and 11 m in girth, swollen at the base, natively distributed in the rainforests of West and Central Africa (Hutchinson, Daziel 1968).

The bark of CG used as an analgesic, antipyretic, anti-inflammatory, and for the treatment of jaundice and malaria by the Ibibio of Niger Delta region of Nigeria (Okokon, Ita et al. 2006). For the treatment of malaria, fever and internal abscess a decoction is prepared and used of two to three cups per day for 15 days (Ndah, Egbe et al. 2013).

The blood schizontocidal activities of the stem bark extract of CG was evaluated against *Plasmodium berghei* infection in mice. CG extracts administered orally, at 20–60 mg/kg/day exhibited a significant ($P < 0.05$) blood schizontocidal activity in 4-day early infection, repository evaluation and in established infection with a significant mean survival time comparable to that of the standard drug, chloroquine, 5 mg/kg/day (Okokon, Ita et al. 2006).

The ethyl acetate extract of *C. gabunensis* showed broad antibacterial activities against several clinical isolates (Kouitcheu Mabeku, Kouam et al. 2006). A study was conducted to define the toxicity level of CG extract against mice. The LD_{50} of the alcoholic bark extract administered intraperitoneally was 223.6 mg/kg while doses of 250 mg/kg and above were found to be lethal to mice (Okokon, Ita et al. 2006).

There have been a lot of chemical characterizations of *C. gabunensis*. Urs-12-ene-3,28-diol, β-amyrin-n-nonyl ether, 22α-hydroxyolean-12-en-3β-yl-β-D-galactopyranoside, and 24-hydroxyolean-12-en-3β-yl-β-D-glucopyranoside (structures are shown in Figure 1.29) were detected from the stem root of this plant (Mkounga, Tiabou et al. 2010).

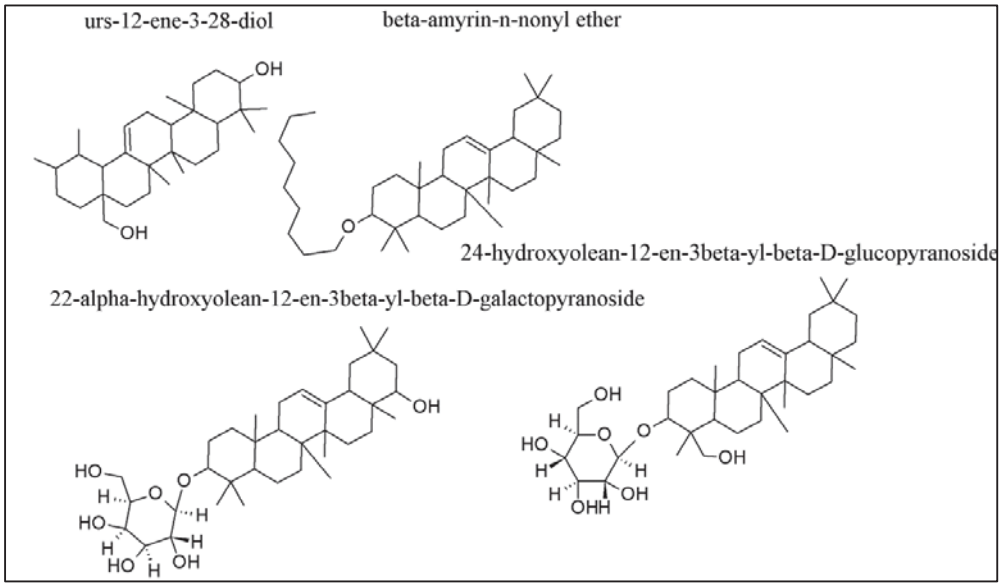


Figure 1.29: Structures of compounds isolated from the stem root of *Cylicodiscus gabunensis* by Mkounga et al. 2012.

Five triterpenoid saponins (Tchivounda, Koudogbo et al. 1991), cylicodiscic acid (Tchivounda, Koudogbo et al. 1990, Tane, Bergqust et al. 1995), lupeol, betulinic acid and cyclodione (Tane, Bergqust et al. 1995) were also isolated and characterized from the bark of CG. The structures of cylicodiscic acid, lupeol, betulinic acid and cyclodione are shown in (Figure 1.30).

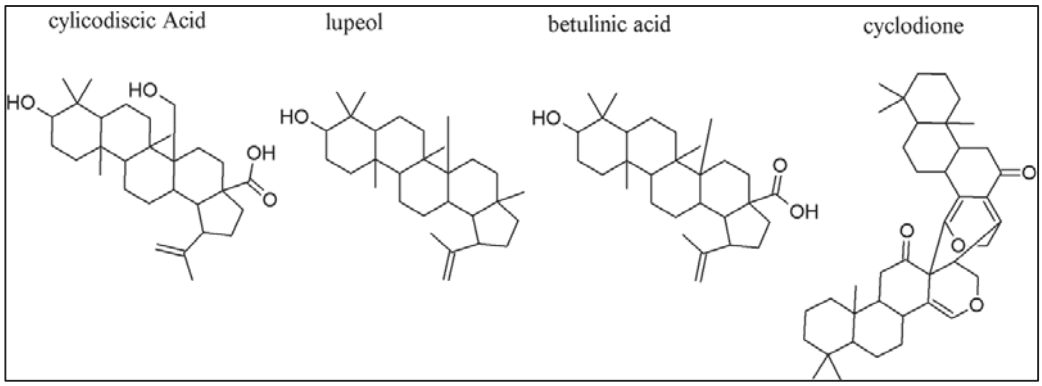


Figure 1.30: The structures of cylicodiscic acid, lupeol, betulinic acid and cyclodione isolated from the bark of *Cylicodiscus gabunensis*.

A triterpenoid glycosides (Gabunoside) or (3-O-[β-L-arabinopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranosyl] maslinic acid-28-α-L-rhamnopyranosyl ester), cylicodiscoside, and 3-O-[α-L-arabinopyranosyl-(1→3)-β-D-glucopyranosyl] cylicodiscic acid were isolated from the stem bark (Tene, Chabert et al. 2011). Despite the extensive phytochemical studies of CG, the exact active principles responsible for the antiplasmodial and antibacterial activities are yet to be confirmed. The structures of the chemical compounds isolated from the stem bark of *Cylicodiscus gabunensis* by Tene et al, 2011 are shown in (Figure 1.31).

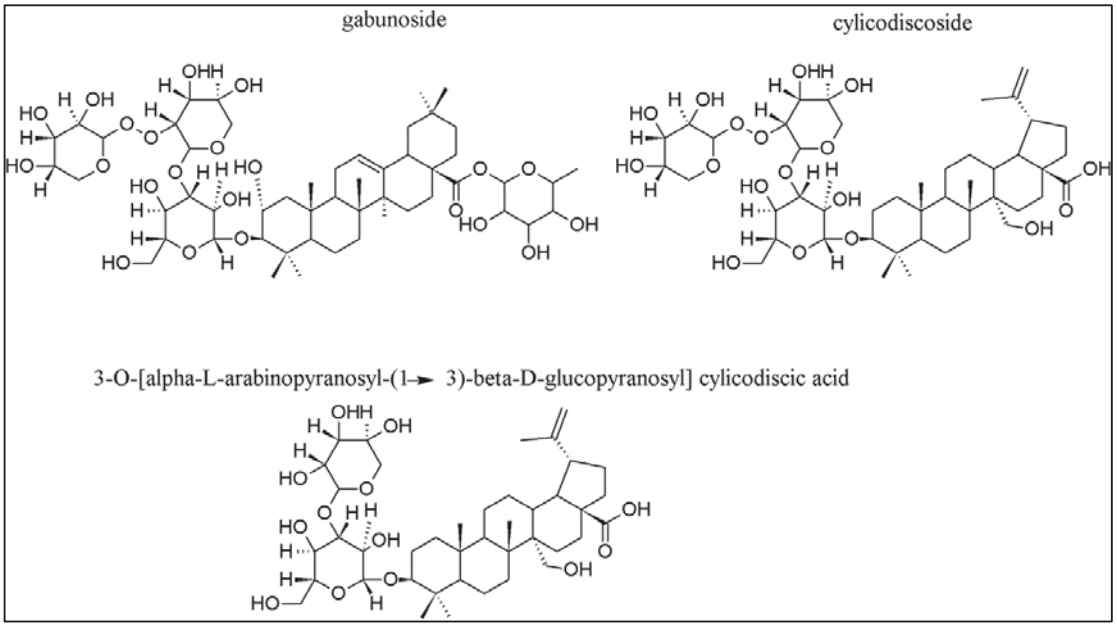


Figure 1.31: The structures of the chemical compounds isolated from the stem bark of *Cylicodiscus gabunensis* by Tene et al, 2011.

1.3.2. Pogostemon cablin

Pogostemon cablin (Blanco) Benth, family *Lamiaceae* (PC) is a traditional Chinese medicine used for the treatment of common cold, nausea, diarrhoea, headache and fever (Yang, Kinoshita et al. 1999). The chemical composition of the essential oil from *Pogostemon cablin* has been previously characterized using gas chromatography-mass spectrometry (GC-MS) (Hu, Li et al. 2006) and GC-MS-MS (Wu, Lu et al. 2004). Many terpenes such as patchouli alcohol and pogostone have been discovered in this plant (structures are shown in Figure 1.32).

The essential oil and its constituents showed antibacterial activity against periodontopathic bacteria (Osawa, Matsumoto et al. 1990), and antifungal activity against *Candida albicans* (Liu, Luten et al. 2012, Yi, He et al. 2013). Patchouli alcohol from *Pogostemon cablin* also shows dose-dependent activity against anti-influenza virus A/PR/8/34 (H1N1) with an IC_{50} value equal to 2.6 μM (Kiyohara, Ichino et al. 2012). The time kill kinetics of PC main constituents (patchouli alcohol and pogostone) against *S. aureus* and *E.coli* and SEM study of their effects on *S. aureus* are yet to be known.

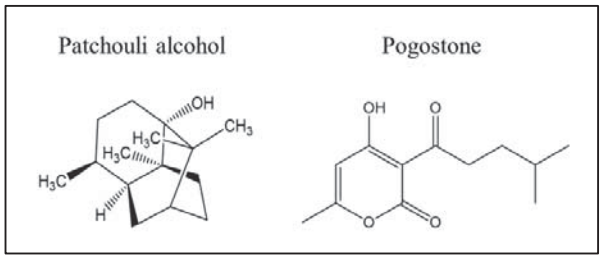


Figure 1.32: Structures of patchouli alcohol and pogostone, the main metabolites of *Pogostemon cablin*.

1.3.3. Perilla frutescens

Perilla frutescens (L.) Britt, family *Lamiaceae* (PF) is widely used as traditional Chinese medicine for the treatment of cold symptoms and as food additive (Qiu, Zhang et al. 2011). The volatile constituents of the steam distillate of the green leaves of *Perilla frutescens* showed broad antimicrobial activity (Kang, Helms et al. 1992).

The extract of *Perilla frutescens* seeds and a constituent, luteolin, also showed an antimicrobial effect against cariogenic *streptococci* and periodontopathic *Porphyromonas gingivalis* (Yamamoto, Ogawa 2002). *Perilla frutescens* oil inhibited the production of Staphylococcal enterotoxins which may be used as an additive to β-lactam antibiotics for the treatment of *S. aureus* infections (Qiu, Zhang et al. 2011).

Its essential oil and perilla ketone also showed significant anti-*Bacillus* and anti-*Shigella* activity (Lim, Shin 2014). The chemical compositions of the essential oil extracted from *Perilla frutescens* from different countries has been investigated, in which perillaldehyde; perilla ketone; limonene and β-caryophylline were all found to be the main volatile components (Huang, Lei et al. 2011, Liu, Wan et al. 2013, Seo, Baek 2009). (Figure 1.33) shows the structures of several metabolites detected in *Perilla frutescens*. Assaying the antimicrobial activity of both the non-polar and polar extracts of *Perilla frutescens* with a broader spectrum of microorganisms and study the effect of PF hexane extract using SEM have yet to be carried out.

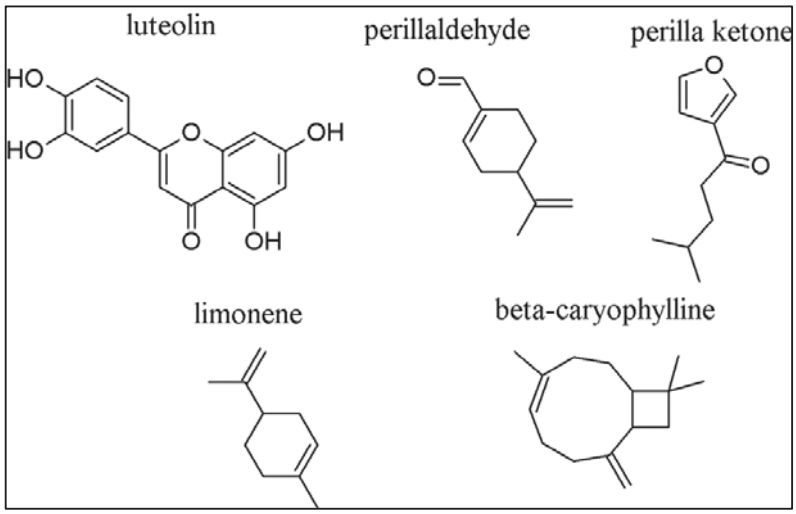


Figure 1.33: The structures of several compounds detected in *Perilla frutescens*.

1.3.4. Magnolia biondii

The flower buds of *Magnolia biondii* Pamp, family *Magnoliaceae* are known as Xin-yi in the Chinese Pharmacopoeia and are widely used for the treatment of allergic rhinitis, sinusitis, nasal congestion and headache (Shen, Li et al. 2008). Extracts and phytochemicals of Flos *Magnolia biondii* showed anti-inflammatory, anti-histamine, and anti-hypertensive activities (Shen, Li et al. 2008, Lee, Song et al. 2014). Bacterial infection caused by *S. aureus* in the nasal-sinus mucosa is one of key factors which could cause rhinosinusitis (Redinbo 2014), thus it is important to evaluate the antibacterial effect of the extracts from *Magnolia biondii*, which may provide scientific evidence of using Xin-Yi for the treatment of rhinosinusitis.

The essential oil from *Magnolia biondii* was analysed by gas chromatography-mass spectrometry (GC-MS) indicating the presence of eucalyptol, beta-pinene and alpha-terpineol as the major compounds (Lu, Xu et al. 2008, Zeng, Xie et al. 2011).

Furthermore, the tetrahydrofurofuran lignans such as fargesin, aschantin, and magnolin, the sesquiterpene lactone parthenolide, and the sesquiterpene alcohol oplodiol were isolated

and characterized from this plant (Schuhly, Skarbina et al. 2009). (Figure 1.34) shows the structure of these lignans.

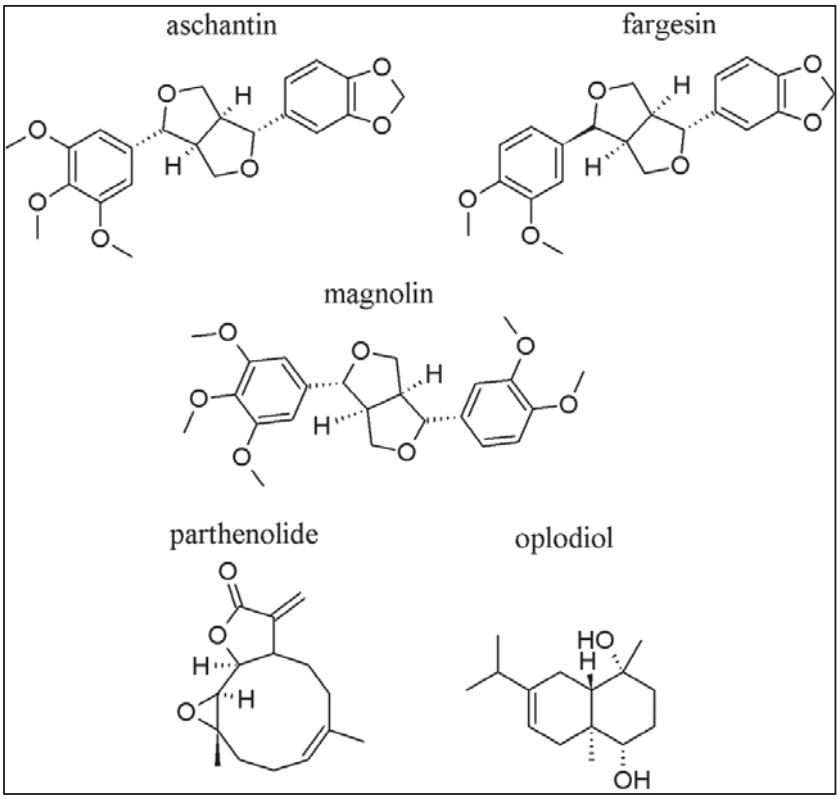


Figure 1.34: Structures of lignans, parthenolide and oplodiol isolated from *Magnolia biondii*.

1.4. The aim and objectives of this work are

The aim of this research is to evaluate the *in vitro* antiplasmodial and antibacterial activities of a native African medicinal plant (*Cylicodiscus gabunensis*) and identify the bioactive compounds, and to evaluate the antibacterial activity of three medicinal plants used in Chinese medicine (*Pogostemon cablin*, *Perilla frutescens* and *Magnolia biondii*). The knowledge gained here can provide scientific basis for using these plants for traditional uses.

The objectives are

1. To evaluate the antibacterial activities of *Cylicodiscus gabunensis* by disk diffusion assay and alamar blue microplate assay; to isolate the active compounds using a bioassay-guided extraction technique; to identify the active compounds by Nuclear Magnetic Resonance (NMR), Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography-Mass Spectrometry (GC-MS); to synthesize the derivatives of the bioactive molecule (gallic acid); to study the synergism between gallic acid and patchouli alcohol against *S. aureus* using checkerboard; to study the effects of gallic acids and esters of gallic acids on *S. aureus* using Scanning Electron Microscopy (SEM), to study gallic acid esters as possible food preservatives and to find the relationship between the structures of benzoic acid derivatives and their bio-activities (antibacterial and antiplasmodial).
2. To evaluate the *in vitro* antiplasmodial activities of *Cylicodiscus gabunensis* against a chloroquine resistant *Plasmodium falciparum* Dd2 using Malaria Sybr Green I fluorescence assay method; to isolate the active compounds using a bioassay-guided extraction technique; to identify the active compounds by NMR, LC-MS, and GC-MS.
3. To evaluate the antibacterial activities of the nonpolar and polar extracts and pure components of *Pogostemon cablin* using disk diffusion assay, alamar blue microplate assay; to characterize their chemical composition by GC-MS; to study the mechanism of action of active compounds using time-kill assay and SEM.
4. To evaluate the antibacterial activities of the nonpolar and polar extracts and pure components of *Perilla frutescens* using disk diffusion assay, alamar blue microplate assay; to characterize their chemical composition by GC-MS; to study the mechanism of action of active extract using SEM.

5. To evaluate the antibacterial activity of the essential oil and lignans of *Magnolia biondii*, by determination of MIC, SEM and time-kill kinetics. To characterize the chemical composition of oil and lignans using GC-MS and NMR techniques.

Chapter 2 : Materials and Methods

2.1. Plants collection and authentication

The bark of *Cylicodiscus gabunensis* Harms, family *Mimosaceae* was collected from the South East zone of Nigeria, Imo state, in September 2011. The plant was identified by H. Donyeachusim and a voucher specimen (UPH1028) was kept in the herbarium at the University of Port Harcourt, Nigeria.

The stem parts of *Pogostemon cablin* (Blanco) Benth, family *Lamiaceae*, and the aerial parts of *Perilla frutescens* (L.) Britt, family *Lamiaceae* were purchased from a registered pharmacy in Chongqing, China. The specimen (each 10 g) of *Pogostemon cablin* stems (KUISTM00**2**) and aerial parts of *Perilla frutescens* (KUISTM00**3**) were kept in the Institute of Science and Technology in Medicine, Keele University.

Flower buds of *Magnolia biondii* Pamp, family *Magnoliaceae* were harvested in Guangxi province. They were purchased from Hunan Jinshitang Traditional Chinese Medicine Co Ltd., China (2013120101). A voucher specimen (KUISTM00**4**) has been deposited in the Institute of Science and Technology in Medicine, Keele University. All the plant specimens were kept in dark glass jars until the time of the study.

2.2. Grinding of the plant materials

The plants were cleaned and pulverized separately by mechanical mills for the subsequent extraction procedure.

2.3. Solvents, standards, and other materials

All the chemicals used in this research are enumerated in (Table 2.1).

Table 2.1: The chemical compounds, standards and solvents used in the current study.

Sigma Aldrich
Material name
(−)-Borneol
(−)-Caryophyllene oxide
2,4-Di-tert-butylphenol
2,6-Di-tert-butylphenol
2-Methyl-1-butanol
2-Methyl-1-propanol
3,4-Dihydroxybenzoic acid
3-Methyl-1-butanol
3-Methyl-2-buten-1-ol
Acetone-d6
Bis-(2-ethylhexyl) phthalate
BSTFA + TMCS, 99:1
Camphene
Caryophylline
Chloroform-d
D-(−)-Ribose
D-(+)-Mannose
D-(+)-Xylose
Ethanol
Ethyl gallate
Eucalyptol
Eugenol
Gallic acid
Gallic acid trimethyl ether
L-(+)-Arabinose
Lauryl gallate
Limonene
Linalool
L-menthol
L-menthone
L-Rhamnose
N,N′-Dicyclohexylcarbodiimide
n-alkanes
o-cymene
Patchouli oil (density 0.963 g/ml)
Preparative TLC plates with indicator
Propyl gallate
Racemic-menthol
Trifluoroacetic acid-d
TWEEN® 20
Vanillic acid
α-D-Glucose
α-phellandrene
α-pinene
α-terpineol
β-phellandrene
β-pinene
γ-terpinene
Fischer Scientific
Material name
Acetone
Alamar Blue Cell Viability Assay Reagent
Ampicillin sodium salt
Chloroform
Ethyl acetate
Hexane
Isopropanol
Methanol
Methanol-d4
Nutrient agar powder Oxoid
Nutrient broth powder Oxoid
Anhydrous pyridine
Tetrahydrofuran
Carbosynth Limited
Material name
Patchouli alcohol
Pogostone

2.4. Extraction of the active constituents

2.4.1. Extraction of Cylicodiscus gabunensis, Pogostemon cablin and Perilla frutescens

The powder of CG bark (120 g) was macerated in two liters of hexane in Duran™ Borosilicate Glass Bottle twice and each for two days. The hexane filtrates were combined and evaporated at 30°C under vacuum (Buchi Rotavapor RII) to yield the hexane extract of *Cylicodiscus gabunensis* (CGH, 0.9 g, 0.75% w/w). The residue was extracted again by maceration using two liters of 70% ethanol two times and each for two days, the ethanol filtrates were combined and dried at 45°C under vacuum to give the ethanol extract of *Cylicodiscus gabunensis* (CGE, 11.3 g, 9.4 % w/w).

50 g of each of PC and PF powders were macerated (twice and each for two days) in 500 ml of hexane in Duran™ Borosilicate Glass Bottle. The hexane extracts were filtered and the solvent was removed by evaporation at a temperature not exceeding 30°C under vacuum to yield the hexane extracts of *Pogostemon cablin* (PCH) and *Perilla frutescens* (PFH), respectively. The plant residues were further macerated (twice and each for two days) in 500 ml of 70% ethanol in water and the ethanol extracts were filtered and evaporated at 45 ºC under vacuum to give the ethanol extracts of *Pogostemon cablin* (PCE) and *Perilla frutescens* (PFE), respectively. The yields of PCH, PCE, PFH, and PFE were 3.9 %, 5.5 %, 3.3% and 1.7 % w/w, respectively. Density of PCH was 1.1 g/ml after removal of higher alkanes by freezing.

2.4.2. Extraction of Magnolia biondii

2.4.2.1. Extraction of the essential oil

Powdered flower buds of MB (200 g) was immersed in 500 ml of deionized water (deionised by water distillator, PURELAB Option-Q, UK) and subjected to steam distillation for 6 hours, using a reflux-type apparatus; extraction flask was fed from a side inlet by water when required. The oil droplets were extracted from the distillate using 600 ml of hexane, two times

and the hexane extracts were combined and dried over sodium sulphate and carefully removing the hexane solvent at 30 °C under vacuum, the essential oil (1 ml) of *Magnolia biondii* was obtained and kept in a sealed dark glass vial at 4 °C for further chemical analysis and antibacterial assays.

2.4.2.2. Extraction of lignans

Flower buds of MB (2 g) were crushed and sonicated twice with 100 ml of chloroform for 50 min. The combined extracts were filtered and the solvent was evaporated under vacuum to obtain the chloroform lignan-rich extract of *Magnolia biondii* (120 mg, 6 % w/w yield).

2.5. Fractionation of Cylicodiscus gabunensis ethanol extract (CGE)

Each 5.6 g were dispersed in 350 ml of deionized water and partitioned with 350 ml × 2 of ethyl acetate and butanol successively, the ethyl acetate, butanol and aqueous extracts were combined separately and evaporated as required yielding the ethyl acetate fraction (CGEEA) 0.9 g, butanol fraction (CGEBU) 8.3 g and aqueous fraction (CGEAQ) 1.6 g which were kept in dark bottles at 4°C for future work. The key aspects of the extraction processes are summarized in (Figure 2.1).

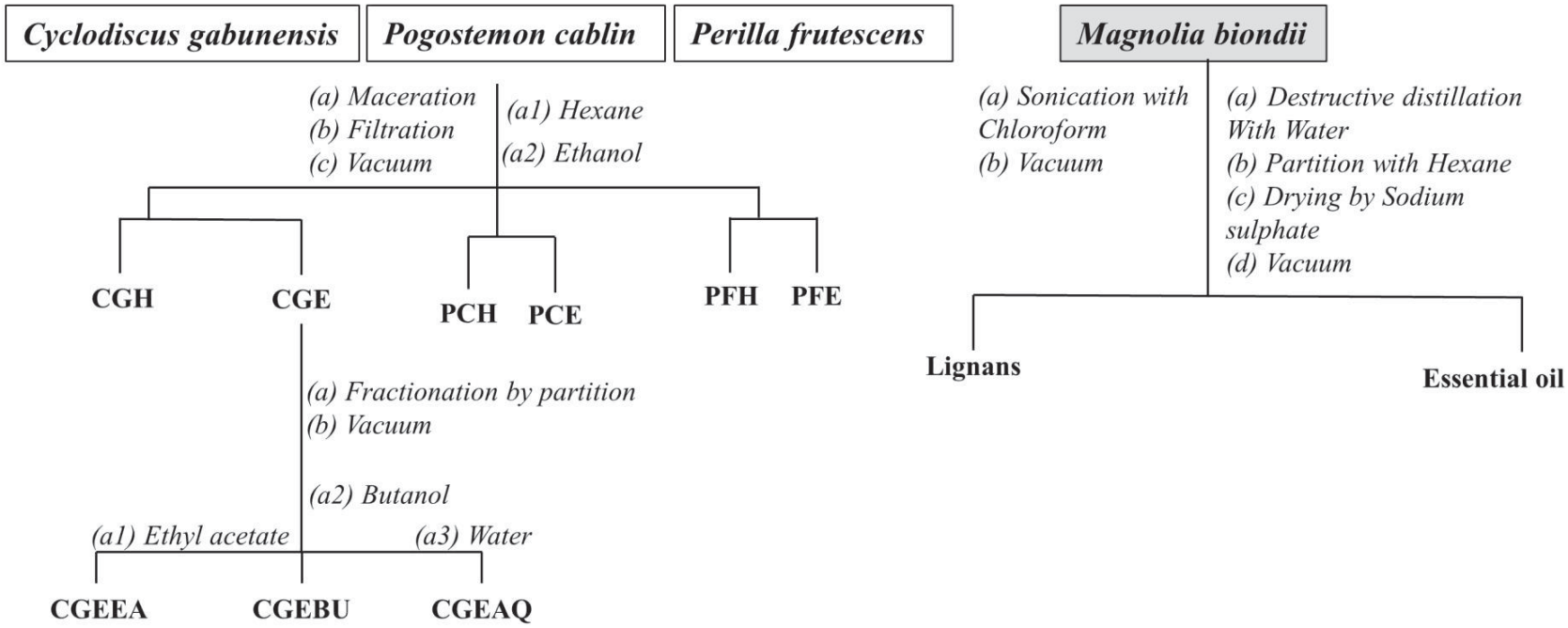


Figure 2.1: Schematic diagram illustrating key aspects of the extraction of *Cylicodiscus gabunensis*, *Pogostemon cablin*, *Perilla frutescens* and *Magnolia biondii*.The solvents used in the separation steps are indicated using italics. CGH and CGE are hexane and ethanol extracts of *C. gabunensis*. CGEEA, CGEBU and CGEAQ are the ethyl acetate, butanol and aqueous fractions of *C. gabunensis* ethanol extract respectively. PCH and PCE are the hexane and ethanol extracts of *P. cablin* respectively. PFH and PFE are the hexane and ethanol extracts of *Perilla frutescens* respectively.

2.6. Fractionation by Flash Column Chromatography (CC)

2.6.1. Fractionation of CGEEA

0.85 g of CGEEA was ran on silica gel (35-75 μm; 2.5×50 cm), hexane: ethyl acetate 4:1 with increased polarity until washing the column by methanol, 3 ml/ min; RT of each fraction was 100 ml and 18 fractions were collected; detection of eluates by TLC (SiO_2, hexane: ethyl acetate 1.5: 1, UVGL-25 compact UV lamp 254/365 nm UV/4- Watt, Iodine vapour). Some of fractions were combined and dried using vacuum, yielding 8 fractions. F1, 29 mg; F2, 9.1 mg; F3, 8.8 mg; F4, 8.9 mg; F5, 450 mg; F6, 130 mg; F7, 72.3 mg and F8, 97.2 mg with around 94% of recovery.

2.6.2. Fractionation of CGEBU

7 g of CGEBU were ran on silica gel (35-75 μm; 4×50 cm), $CHCl_3$: MeOH: H_2O 10: 1: 0.1 ml with increased polarity, 3 ml/min; RT of fractions 1-18 was 100 ml and for fraction 19 was 200 ml; detection of eluates by TLC (SiO_2, $CHCl_3$: MeOH 7:3, UVGL-25 compact UV lamp 254/365 nm UV/4- Watt, Iodine vapour). Some of fractions were combined and dried using vacuum, yielding 10 fractions. F1= 100mg, F2= 95mg, F3= 118mg, F4= 75mg, F5= 90mg, F6= 650, F7= 900 mg, F8= 2100, F9= 1750mg, F10= 350mg with ≈ 89% of recovery.

2.7. Preparative High Performance Liquid Chromatogarphy (HPLC)

Agilent 1200 series, preparative system consists of, G2258A DLA Dual Loop Auto-sampler, G1361A Preparative pump, X bridge Prep C-18, 5 μm OBD 19 × 100 mm column from Waters, G1315D DAD Diode Array Detector at 254 nm and G1364B Prep FC Fraction Collector. Flow rate of solvents (A: 0.1% glacial acetic acid in water, B: 0.1% glacial acetic acid in methanol) was 16 ml/ min and max pressure 400 bar. (Table 2.2) shows the % of methanol in the solvent system throughout the experiment.

Table 2.2: The percentage concentration (v/v) of solvent methanol (solvent B) during the preparative HPLC method.

Time in min	0	5	25	25.1	31	31.1	32	32.1
B %	0 %	5 %	60 %	100 %	100 %	5 %	5 %	0 %

2.7.1. Fractionation of CGEEA-F5 by preparative HPLC

EtOAc fraction 5 or as abbreviated CGEEA-F5 (0.42 g) was fractionated by preparative HPLC; twelve fractions were collected according to time intervals and peaks selection. The fractions were subjected to decrease their volume by evaporation at 45 °C under vacuum evaporator then completely dry by lyophilisation using Modulyo freeze dryer from Edwards. F1= 12mg, F2= 2mg, F3= 2mg, F4= 2mg, F5= 2mg, F6= 4mg, F7 16mg, F8= 21 mg, F9= 83mg, F10= 223 mg, F11=13mg, F12= 8mg with ≈ 92% of recovery.

2.7.2. Fractionation of CGEBU-F10 by preparative HPLC

n-BuOH fraction 10 or as abbreviated CGEBUF10 (0.350 g) was fractionated by preparative HPLC; eight fractions were collected according to time intervals and peaks selection. The fractions were subjected to decrease their volume by evaporation at 45 °C using vacuum evaporator then completely dry by lyophilisation. F1= 20mg, F2=3.5mg, F3= 1.5mg, F4= 5.5mg, F5= 86mg, F6= 185mg, F7= 16mg, F8= 9mg with ≈ 93% of recovery.

2.8. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GC-MS system consisted of an Agilent 7890 A gas chromatography system with HP-5ms column 5 % phenyl-methylpolysiloxane, 30 m × 0.25 mm× 0.25 μm (Agilent Technologies, USA), coupled to an Agilent MS model 5975C MSD with triple axis detector (Agilent Technologies, USA). Front SS Inlet He mode is split and the split ratio is 5:1, thermal Aux 2 (MSD transfer line) temperature 280 °C for 0 min.

2.8.1. Program or method A

Initial temperature 60°C for 2 min, was elevated to 300°C at 10°C/min, and held at 300°C for 4 min, (total time 30 min) under a constant pressure (10 psi). Spectral data were picked up at *m/z* range 16-1050.

2.8.2. Program or method B

Initial temperature 160°C for 0 min, was elevated to 300°C at 10°C/min, and held at 300°C for 6 min, (total time 20 min) under a constant pressure (10 psi). Spectral data were picked up at *m/z* range 16-1050. This method was used in association of method A to run the benzoates derivatives synthesized *de novo* for this research.

2.8.3. Program or method C

Initial temperature 60°C for 2 min, was elevated to 300°C at 6°C/min, and held at 300°C for 18 min, (total time 60 min) under a constant pressure (10 psi). Spectral data were picked up at *m/z* range 16-1050.

2.8.4. Identification and proportional quantification of the plant metabolites

The compounds were identified by matching their EI-MS spectra with those in the NIST 2011 Mass Spectral Library using MSD Chem-Station (Agilent Technologies, USA) with a high matching quality (greater than 95 %) and with the retention times of standard compounds where available. retention indices (RI_s) were calculated utilizing alkanes C_8-C_{20} assayed under the same conditions. The relative amount of individual compounds was expressed as a percentage of abundance ± standard deviation (SD) of three measurements, n=3.

2.8.5. Sample preparation

2.8.5.1. Preparation and injection of hexane extract and alkane standards

Hexane extract was prepared as 1mg/ml of anhydrous hexane and directly analyzed, 1 µl was injected into the GC-MS injection port. 1 µl of n-alkane standard mixture was injected directly without dilution into the injection port.

2.8.5.2. Preparation of polar extract and compounds

Polar extracts or pure compounds were derivatized to their corresponding trimethyl silyl derivatives to increase their volatility. Polar materials are including but not limited to alcohols, alkaloids, amines, carboxylic acids, phenols, and steroids were silylated by N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) in the presence of trimethylchlorosilane (TMCS) as a silylation catalyst, small quantity of pyridine is essential to dissolve the sample (Li, Barz 2006).

The reaction is simple and direct and it was modified from (Li, Barz 2006), 1mg of extract or pure compounds was mixed with 20 µl of anhydrous pyridine and 50 µl of BSTFA + TMCS, 99:1 from Sigma Aldrich, UK. The mixture was incubated in a hybridization oven from Hybrid for 30 min at 60 °C to prepare the corresponding trimethyl silyl (TMSi) derivatives and 1 µl was injected into the GC-MS injection port.

2.8.6. Calculation of Retention indices (RI_s)

The serial alkanes were separated into distinct peaks and the following equation was used for (RI) calculation. (RI) = 100 × n a1 + 100 × (RT c – RT a1) / (RT a2 – RT a1)

n a1: carbon number of alkane eluted before the tested compound (a1)

RT c: retention time of the tested compound (c)

RT a1: retention time of the alkane eluted before the tested compound

RT a2: retention time of the alkane eluted after the tested compound (a2)

2.9. Detection method of the compounds developed on silica gel layer (TLC)

Application of UV light using UVGL-25 compact UV lamp 254/365 nm UV/4- Watt was used to detect the compounds separated by TLC method on TLC plate ALUGRAM 40mm x 80mm SIL G/ UV254 thickness of silica 0.20mm from Fischer Scientific, UK. Another technique was used for the detection of chemical compounds which were separated on TLC, was the non-destructive visualization technique using Iodine vapour.

2.10. Analysis of CGEEA-F5 by HPLC

CGEEA-F5 was analyzed along with three standards; gallic acid, ethyl gallate and protocatechuic acid, using an analytical C-8 column from thermoscientific (5 µm; 250×4.6 mm), methanol/water as the solvent system. 1 ml/min, UV-detector 218 nm, (Table 2.3) shows the % of methanol in the solvent system throughout the experiment.

Table 2.3: The percentage concentration (v/v) of solvent methanol (solvent B) during the analytical HPLC of CGEEA-F5.

Time in min	0	5	25	25.1	31	31.1	32	32.1
B %	0 %	5 %	60 %	100 %	100 %	5 %	5 %	0 %

2.11. Analysis of CGEEA-F5-8 by HPLC

CGEEA-F5-8 was ran on C18 analytical column from Thermo Scientific (5 µm; 250× 4.6 mm), methanol/water. 1 ml/min, UV-detector 218 nm, along with ethyl gallate and then we spiked the fraction by few crystals of ethyl gallate to notice the change in magnitude of ethyl gallate peak. **(**Table 2.4) shows the % of methanol in the solvent system throughout the experiment.

Table 2.4: The percentage concentration (v/v) of solvent methanol (solvent B) during the analytical HPLC of CGEEA-F5-8.

Time in min	0	25	25.1	35	35.1
B %	10 %	70 %	100 %	100 %	10 %

2.12. Accurate mass Liquid Chromatography-Mass Spectrometry TOF (LC-MS)

Infinity 1260 series system consists of G4225A Hip Degasser, G1312B Binary Pump, G1329B ALS Auto sampler, G1316A TCC Thermostated Column Compartment, Column 50x2.1mm 5 µm HyPURITY C8 from Thermo Fisher Scientific, UK and 6530 Accurate Mass Q-TOF as detector, qualitative analysis mass hunter work station software, ion Source Dual ESI, Ion Polarity positive, Mass range 100-1700 m/z Detector. Flow rate of solvent A: 0.1% Formic acid in water, B: acetonitrile, flow rate 0.5 ml/ min and max pressure 600 bar, the concentration of sample prepared was 100 µg/ml. (Table 2.5) shows the % of acetonitrile in the solvent system throughout the experiment. Analysis of the samples was done by Dr. Falko Drijfhout, Chemical Sciences Research Centre, Keele University.

Table 2.5: The percentage concentration (v/v) of solvent acetonitrile (solvent B) during the Accurate mass TOF LC-MS.

Time in min	0	10	18	18.1
B %	5 %	95 %	95 %	5 %

2.13. Acidic hydrolysis and anomerisation of sugars

2.13.1. Acidic hydrolysis

CGEBU-F10-7, was hydrolysed as described in (Aires, Marques et al. 2013, Tene, Chabert et al. 2011) with modifications, 2 mg of the extract were mixed with 2 ml of 2 M HCl in 50 % methanol and heated in ultrasonic bath at 50 °C for 4 hours and incubated overnight in oven at 90 °C. After centrifugation at 10000 rpm for 10 min using Eppendorf Centrifuge 5418R, the supernatant was dried and1 mg was mixed by BSTFA and pyridine to prepare the TMSi derivatives and checked by GC-MS.

2.13.2. Anomerisation of sugars

Standard sugars were supplied by Sigma Aldrich UK, aldohexoses like α-D-glucose, α-D-galactose and D-(+)-mannose, in addition to some aldopentoses like L-(+)-arabinose, (+)-xylose and (−)-ribose and one deoxy sugar, L-rhamnose, 1 mg of each standard sugar was dissolved in 150 µl of deionized water and kept overnight in an oven at 100 °C, water was removed by rota-vapour at 45 °C and the residue was derived into its trimethyl silyl derivatives according to the method mentioned in section **2.8.5.2.**, then 1 µl was injected into the GC system after dilution with sufficient quantity of chloroform.

2.14. Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H NMR Spectroscopy of the extracts and pure materials was recorded on a Bruker DPX 300 MHz spectrometer and ^{13}C NMR spectroscopy of the extracts and pure materials was recorded on a Bruker DPX 101 MHz spectrometer, 10 mg of material was dissolved using 0.6 ml of d-chloroform or d4-methanol as solvent. ^{1}H NMR spectrum of CGEBU-F10-7 was recorded on a Bruker 700 MHz instrument, 4 mg was dissolved in 0.6 ml of (CD3COCD3:D2O (1:1)) . NMR data were analysed using ACD/Labs 12 software.

2.15. Bacterial isolates

The bacterial colonies were supplied by the National Collection of Industrial and Marine Bacteria NCIMB Ltd, Aberdeen, Scotland. *Bacillus cereus* NCIMB 9945, *Bacillus subtilis* NCIMB 3610, *Staphylococcus aureus* NCIMB 11832, *Staphylococcus epidermidis* NCIMB 8853, *Enterobacter cloacae* NCIMB 10101, *Escherichia coli* NCIMB8277, *Alcaligenes faecalis* NCIMB 8156, *Pseudomonas aeruginosa* NCIMB 10848 and *Enterococcus faecalis* NCIMB 2707.

2.15.1. Preparation of bacterial inoculum

2.15.1.1. Preparation of the broth media

Nutrient broth from Thermo Scientific, UK was prepared according to the manufacturer directions, by the addition of 13g to 1 litre of distilled water. Then we mixed the suspension well using a magnetic bar until complete solubility. The resulted solution was distributed into volumes of 5ml and 20 ml in glass bottles with cap, those bottles were sterilised by autoclaving at 121°C for 15 minutes, then kept at 4 ºC for further study.

2.15.1.2. Preparation of the primary inoculum

Three to five single colonies were selected from the agar plate using a sterile poly styrene inoculating loop from Fischer Scientific, UK and transferred into 20 ml sterilized glass tube with cap contained 5 ml of broth media, vortexed for one min using Fisons Whirlimixer vortex stirrer. Cultures were incubated at 37 °C in a Thermo / Lab-Line / Barnstead MAX Q 4000 Benchtop Shaker from Thermo Scientific, USA at 225 rpm for 2-6 hr.

2.15.1.3. Calibration of growth density

One ml of the bacterial culture was transferred into a cuvette macro disposable polystyrene 4.0ml capacity 10mm light path from Fischer Scientific, UK and the absorbance was read against the absorbance of broth media as a blank, using Genesys 10 S UV-VIS

Spectrophotometer at 625nm. A visible turbidity that is equal to the turbidity of a McFarland Standard 0.5 should be optimised and this can be verified by recording absorbance of 0.08–0.13 which indicates the presence of approximately $1\text{-}2\times10^{8}$ Colony Forming Unit/ml (CFU/ml).

2.15.2. Preparation of the agar plates

Nutrient agar from Thermo Scientific, UK was prepared according to the manufacturer directions, 28 g were suspended in 1 litre of distilled water, heated until boiling to ensure complete solubility with the aid of stirring using a magnetic bar, and then we sterilised the solution by autoclaving at 121°C for 15 minutes. The agar was cooled down to about 50 °C and distributed into cell culture dishes diameter 100 mm × 20 mm height, 60.8 cm^2 from Thermo Scientific, UK, about 15 ml of agar was distributed into each plate, agar plates let to cool down completely and kept at 4 °C in a cold room until future work.

2.15.3. Preparation of the purity plate and control plate

Using the streak method, both the control plate and purity plate were prepared to ensure the standardisation of the assay. Control plate was necessary to check the sensitivity of the bacterial isolate to the drug used as a control which was Ampicillin here; the control plate should be prepared concurrently with each test set. An important step in the bioassay is to guarantee the purity of the culture cells and this has been done by the addition of 10µl of a bacterial inoculum in to an agar plate, a serial streaking of the inoculum into an agar plate in order to obtain individual colony forming units, after the procedure was completed the agar plate was incubated at 37 °C for the appropriate amount of time (usually 20-24 h) and colonies were inspected for uniformity.

2.16. Disk diffusion assay (DDA)

Antimicrobial susceptibility testing (Bukvicki, Gottardi et al. 2012, Matuschek, Brown et al. 2013) disk diffusion method was initially used to assess the antimicrobial activities of the plant extracts. 100 µl of 0.5 McFarland inoculum suspensions were uniformly spread on the agar plate. The plant extracts were prepared in four concentrations of 12.5, 25, 50 and 100 mg/ml in DiMethyl SulfOxide (DMSO) and 20 µl dispensed to Whatman sterile filter paper disks with diameter of 6 mm from Fischer Scientific, UK, two controls were used in the study one for the solvent DMSO and the second one was Ampicillin in a concentration of 10 or 2 µg per disk, the disks were applied firmly to the surface of the inoculated agar plate. The plates were inverted and incubated at 37 °C using LMS series 4 cooled incubators.

Inhibition zone (IZ) is an indication of the antibacterial activities and it is increased as the potency of the compound increased. The diameters of the IZs were measured to the nearest millimetre with a ruler and the inhibitory zone sizes of three plates were prepared for each isolate on three different days, the standard deviation (SD) of the readings were calculated.

In case of detection of colonies growth throughout the IZ, the infiltrate colonies were checked on different plate for purity if the colonies were pure then the zone recorded as not detected or (ND), if the infiltrate colonies were contaminated then redoing the test was essential.

2.17. Determination of MICs using microplate alamar blue assay

2.17.1. Technical steps

MIC of the plant extracts and pure materials were determined using microplate alamar blue assay (Collins, Franzblau 1997, Kumar, Khan et al. 2005, Leonard, Coronel et al. 2008). alamar blue is ready to use reagent gave rapid assay results and gave us a real quantification of microbial cell viability, it is usually added as 10 % of the growth medium, for example 20 µl into 200 µl well volume.

The recommended final inoculum size for broth dilution is 5×10^5 CFU/ml. The bacterial isolates were adjusted to McFarland standard 0.5 ($1\text{-}2 \times 10^8$ CFU/ml), and diluted by a factor of 1:100 by addition of 0.2 ml bacterial suspension to 19.8 ml sterile broth medium to give a bacterial concentration of 1×10^6 CFU/ml. The plant extracts and pure compounds were dissolved in 3% DMSO, or 0.5% Tween 20 in broth media for use. Black Microplate FluoroNunc 96 well flat bottom polystyrene not treated from Fischer Scientific, UK was used for the assay.

100 µl of nutrient broth were distributed into all wells except wells 10, to which 100 µl of 3% DMSO or 0.5% Tween 20 were added. 100 µl of the extracts or pure compounds were distributed into wells 1A to 1H and mixed well with the broth, to made a serial dilution (two folds dilution) until wells 8, then 100 µl of bacterial broth containing 1×10^6 CFU/ ml were distributed into wells 1-10. Wells 9 were the growth control which contained broth media and bacteria and wells 10 were the solvent control which contained either 3% DMSO or 0.5% Tween 20 and bacteria, the growth in wells nine and ten must not much different, and they form the growth control average. Wells 11 were used as the sterility control and wells 12 were contained a serial dilution of Ampicillin as drug control from 12A- 12H as 128-0.5 µg/ml. After incubation at 37 °C for 18-24 hours, 20 µl of Alamar blue from Fischer Scientific, UK were added to each well followed by further incubation for 1-4 hours. The fluorescence units (FU) were read in GloMax-plus® Multi Detection System, using filter at excitation wavelength of 525 nm and emission wavelength of 580-640nm.

2.17.2. Calculation

The following equations were used to calculate the percentage of inhibition for each drug used. To ensure the reproducibility of the assay the experiment has been done four times on two days, duplicate on each day. The background effect must be excluded by subtraction from

each reading, percent inhibition was defined as [1 – (FU test well – FU SC well) / (FU GC well – FU SC well)] × 100.

FU test well: Mean of fluorescence in test wells

FUSC well: Mean of fluorescence in sterility control

FUGC well: Mean of fluorescence in growth controls

The lowest extracts/pure compounds concentration effecting an inhibition growth of 90% was considered as the MIC (Collins, Franzblau 1997). MICs could be further verified visually as the lowest concentration of extracts/pure compounds that prevented a blue-to-pink alamar blue colour change.

2.18. Time Kill assay

Time-kill assay was performed according to guideline M26-A of the National Committee for Clinical Laboratory standards (Karkare, Adou et al. 2007, McKay, Beaulieu et al. 2009), with the following modifications: polypropylene 96-well plates were used for time-kill assays with *S. aureus* and *E. coli*.

Exponential-phase bacteria were diluted to 5×10^5 - 1×10^6 CFU/ml and exposed to the drug over a 24 h period. Eight wells were dedicated for each plant extract or pure compound used in the study. The drug used was distributed into three different concentrations ½ MIC of the drug used, MIC and 2 MIC levels of the plant extract or pure compound and 0 (control) when no drug was used. 100 µl Specimens were withdrawn and diluted up to 10^{-7} in some heavy growth wells and 100 µl cell cultures were seeded on agar growth media at different time slots, 0, 3, 6 and 24 hr in duplicates. The average of cell density (CFU/ml) was calculated based on the viable cell counting and the killing kinetics of the drug was determined. The

bactericidal effect was achieved when the drug caused a 3-Log reduction in bacterial counts which equates to a 99.9% reduction in microorganisms.

2.19. Scanning Electron Microscopy (SEM)

Eppendorf tubes each containing 3 ml of *S. aureus* NCIMB 11832 at a cellular density of (10^8 CFU/ml) were prepared for this experiment (Monson, Stringham et al. 2010). Tube A was kept as a control where 3 ml of 6% DMSO in broth media were added. 3 ml of plant extract or pure compound were prepared in 6% DMSO at their 2 MIC level and were added to tube B. After one hour the tubes were centrifuged at 16000 rpm using an Eppendorf centrifuge 5418R for 10 min. The supernatant liquid was removed carefully to avoid disturbing the cellular pellets to which 2.5% glutaraldehyde in a buffer containing 0.1 M sodium cacodylate and 2mM calcium chloride was added and left standing for 2 h. This was the primary fixation step followed by buffer removal and addition of 1% osmium tetroxide in (buffer), which kept for one hour. After centrifugation, the sample pellets were cleaned with the buffer three times and dried by ethanol with increased concentration (70 to 100 %) and anhydrous 100% ethanol. The cells were distributed into 10 µl of hexamethyldisilazane, and spread onto a glass coverslip attached to carbon tab 12 mm in diameter PK100 from Agar Scientific, Hitachi, which was placed on the surface of aluminum stubs 15mm × 6 mm from Agar Scientific, Hitachi. The samples were left to dry in air overnight, and then coated by gold using Emscope FD 500 sputter coater. The examination was performed using scanning electron microscope (Model S-4500, Hitachi, Japan). Scanning of the specimens by SEM was performed by Ms. Karen Walker, Electron Microscope Unit/ Keele University.

2.20. Study the synergism between gallic acid and patchouli alcohol

Checker board was used to evaluate the synergism between gallic acid and patchouli alcohol; checker board is a two dimensions assay where two agents were mixed in each well at different dilutions prepared previously for the study (Eliopoulos, Eliopoulos 1988). A1 well in

the plate was used as growth control where just the bacterial cells were added without the drug and H12 used as sterility control where just the broth solution has been added.

Column one was used to determine the MIC of drug B, double dilution concentrations starting from H1 to B1 wells 100 µl each, for determination of drug A MIC we were used row A starting from A12 to A2 wells 100 µl each. The wells used to mix the two drugs together were containing 50 µl from each drug in a concentration corresponding to the concentration in the column or row used to determine the MIC of the drug as shown in (Table 2.6). To calculate the fractional inhibitory concentration and define the type of interaction whether it is synergism or not we need to do the following calculations. Fractional inhibitory concentration (FIC) A= MIC A in combination/ MIC A alone; FIC B= MIC B in combination/ MIC B alone; $X= \sum FIC= FIC\ A + FIC\ B$; Synergism $X \leq 0.5$; Indifference $0.5 < X \leq 1$; Antagonism $X > 4$.

Table 2.6: The mixing strategy for two compounds **A** in rows and **B** in columns

	1	2	3	4	5	6	7	8	9	10	11	12
A	GC	1.95	3.9	7.8	15.63	31.25	62.5	125	250	500	1000	2000
B	31.25	1.95+ 31.25	3.9+ 31.25	7.8+ 31.25	15.63+ 31.25	31.25+ 31.25	62.5+ 31.25	125+ 31.25	250+ 31.25	500+ 31.25	1000+ 31.25	2000+ 31.25
C	62.5	1.95+ 62.5	3.9+ 62.5	7.8+ 62.5	15.63+ 62.5	31.25+ 62.5	62.5+ 62.5	125+ 62.5	250+ 62.5	500+ 62.5	1000+ 62.5	2000+ 62.5
D	125	1.95+ 125	3.9+ 125	7.8+ 125	15.63+ 125	31.25+ 125	62.5+ 125	125+ 125	250+ 125	500+ 125	1000+ 125	2000+ 125
E	250	1.95+ 250	3.9+ 250	7.8+ 250	15.63+ 250	31.25+ 250	62.5+ 250	125+ 250	250+ 250	500+ 250	1000+ 250	2000+ 250
F	500	1.95+ 500	3.9+ 500	7.8+ 500	15.63+ 500	31.25+ 500	62.5+ 500	125+ 500	250+ 500	500+ 500	1000+ 500	2000+ 500
G	1000	1.95+ 1000	3.9+ 1000	7.8+ 1000	15.63+ 1000	31.25+ 1000	62.5+ 1000	125+ 1000	250+ 1000	500+ 1000	1000+ 1000	2000+ 1000
H	2000	1.95+ 2000	3.9+ 2000	7.8+ 2000	15.63+ 2000	31.25+ 2000	62.5+ 2000	125+ 2000	250+ 2000	500+ 2000	1000+ 2000	SC

2.21. Malaria Sybr Green I fluorescence assay method

Professor Paul's group of molecular parasitology, Huxley building, Keele University did all the laboratory experimental evaluation of the anti-malarial activities. To evaluate the anti-malarial activities of the plant extracts at three different concentrations and to determine the IC_{50} of the plant extracts or pure compounds against *P. falciparum* Dd2, the group used a fluorescent dye called Sybr Green l Fluorescence dye (5000X concentration) from Sigma Aldrich, UK. The stain was utilised here to stain double-stranded DNA in parasite.

Sybr Green-conjugated DNA emits a fluorescent light which can be quantified using a blue fluorescent filter (excitation 490 nm, emission 510-570 nm) and a GloMax-plus® Multi Detection System.

Stock solution was prepared at 1 mg/ml in DMSO for plant extract and 10 mM in DMSO for pure compounds. The drug solutions prepared were used immediately or stored at 4°C for not longer than one month before use. The stock solutions were diluted to the desired solutions in μg/ml for extracts and μM for the pure compounds using Roswell Park Memorial Institute (RPMI) 1640 Medium from Fischer Scientific, UK.

100 μl of re-suspended parasite culture were transferred into a Black Microplate FluoroNunc 96 well flat bottom polystyrene not treated from Fischer Scientific, UK. Wells containing no drug but the parasite culture were included on each plate as control. Chloroquine was used as a control and the IC_{50} of Chloroquine against *P. falciparum* Dd2 was 0.171 μM.

The plate was placed in a modular incubator chamber and gassed (gas contains 92.5% N_2, 5.5% CO_2, 2% O_2). The chamber was placed in an incubator set at 37ºC for 72 hours.

Laboratory reference clones, *P. falciparum* Dd2, regarded as chloroquine resistance were assayed periodically as internal control.

For the outcomes measurements, 100 µl of MSF lysis buffer (200mM Tris HCl PH 7.5, 50mM EDTA, 0.08% Saponin and 0.8% Triton X100) supplemented with 1X Sybr Green I dye was added and mixed by pipetting. The plate was covered with aluminium foil and incubated at room temperature in the dark for at least two hours. After the incubation, the fluorescence of the samples was measured (Smilkstein, Sriwilaijaroen et al. 2004). Determination of 50% inhibitory concentration (IC_{50}) were carried out using a serial two folds dilution method to provide a log-concentration response curve analysed using graph prism software (v6.0).

2.22. Synthesis of alkyl and alkenyl 3,4,5-trihydroxybenzoates

To a solution of gallic acid (3.00 mmol; MW 170.12) amount used ≈ 510 mg and anhydrous alcohol (4.00 mmol) in THF (6 ml) were mixed in 50 ml dry flask and cooled in ice bath at 0 °C, we added a solution of N,N'-dicyclohexylcarbodiimide (DCC) (3.50 mmol; MW 206.33) amount used ≈ 720 mg in THF (6 ml). The mixture was stirred for 24 h; the reaction vessel was connected to a vacuum evaporator to evaporate the solvent, yielded dark yellow residue. The product extracted by cold ethyl acetate three times and filtered. The filtrate combined and evaporated to dryness.

List of alcohols used

1- 2-methyl-1-propanol; MW 74.122 g mol^{-1}; density 0.803 g ml^{-1}, amount used was ≈ 370 µl.

2- 2-methyl-1-butanol; MW 88.15 g mol^{-1}; density 0.809 g ml^{-1}, amount used was ≈436 µl.

3- 3-methyl-2-buten-1-ol; MW 86.13 g mol^{-1}; density 0.848 g ml^{-1}, amount used was ≈ 406 µl.

4- 3-methyl-1-butanol; MW 88.15 g mol^{-1}; density 0.809 g ml^{-1}, amount used was 436 µl.

5- Isopropanol; MW 60.10 g mol^{-1}; density 0.785 g ml^{-1}, amount used was 306 µl.

2.23. Purification of the synthesised esters

The synthesised esters were purified by column chromatography, the dried product was ran on silica gel (35-75 µm; 4×50 cm), hexane: ethyl acetate 7: 3, isocratic, 3 ml/min, detection of eluates by TLC (SiO_2, hexane: ethyl acetate 7: 3, UVGL-25 compact UV lamp 254/365 nm UV/4- Watt, Iodine vapour).

2.24. Analysis of Alkyl and alkenyl 3,4,5-trihydroxybenzoate by HPLC

Compounds synthesized in the laboratory were run on C18 analytical column from Thermo Scientific (5 µm; 250× 4.6 mm), methanol/water, UV-detector 254 nm. (Table 2.7) shows the % of methanol in the solvent system throughout the experiment.

Table 2.7: The percentage concentration (v/v) of solvent methanol (solvent B) during the analytical HPLC of synthesised esters.

Time in min	0	25	25.1	35	35.1
B %	10 %	70 %	100 %	100 %	10 %

Chapter 3 : Results and discussion of antibacterial and antiplasmodial activities of CG

3.1. Results

3.1.1. Antibacterial effects of Cylicodiscus gabunensis extracts

3.1.1.1. Disk diffusion assay

IZs in mm of CGH and CGE against a panel of bacteria were recorded three times, on three different plates according to section **2.16** and the results are shown in (Table 3.1), the effect of CGE against *S. epidermidis* is displayed in (Figure 3.1). (Table 3.2) shows the IZ in mm and MIC in µg/ml of ampicillin against several bacterial isolates recorded according to sections **2.16** and **2.17** respectively.

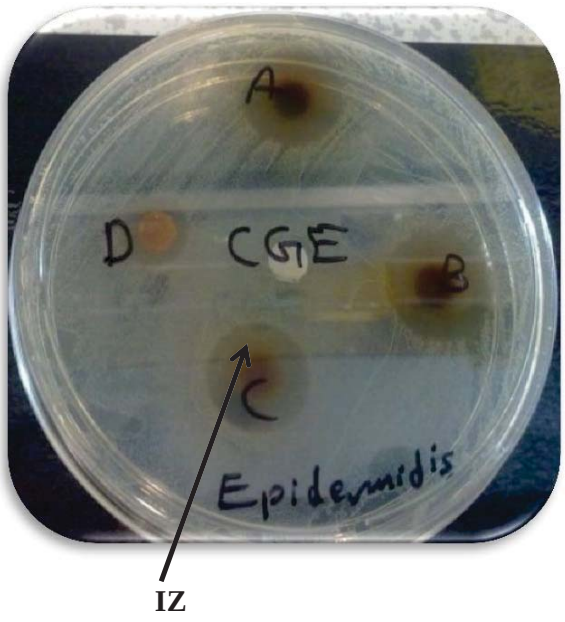


Figure 3.1: The effect of CGE against *Staphylococcus epidermidis* (8853) growing at 37 °C, A: concentration of CGE 2mg/disk, B: 1mg/disk, C: 0.5 mg/disk, and D: 0.25 mg/disk. The test has been done in triplicate, n=3 according to section 2.16.

Table 3.1: Antibacterial activity of *Cylicodiscus gabunensis*, hexane extract (HE) and ethanol extract (EE). The test has been done in triplicate, n=3 according to section 2.16.

Bacteria strain	**Extracts**	**Mean diameter of IZ mm ± SD (n =3)**			
(gram stain)		2 mg/disk	1 mg/disk	0.5 mg/disk	0.25 mg/disk
***Staphylococcus aureus* (+)**	HE	7.5 ± 0.5	7.0 ± 0.4	NC	NC
	EE	16.5 ± 0.5	12.0 ± 1.0	10.5±0.5	7.5±0.6
***Staphylococcus epidermidis* (+)**	HE	Neg.	Neg.	Neg.	Neg.
	EE	19.5 ±1.0	14.5±0.5	13.5±0.5	10.0±1.0
***Bacillus cereus* (+)**	HE	Neg.	Neg.	Neg.	Neg.
	EE	12.5±0.6	11.0±1.0	10.0±0.5	8.0±1.0
***Bacillus subtilis* (+)**	HE	Neg.	Neg.	Neg.	Neg.
	EE	10.6±0.5	8.5±0.5	7.0±0.5	Neg.
***Streptococcus (Enterococcus) faecalis* (+)**	HE	Neg.	Neg.	Neg.	Neg.
	EE	10.5±0.5	8.0±0.5	7.0±0.5	Neg.
***Escherichia coli* (-)**	HE	Neg.	Neg.	Neg.	Neg.
	EE	Neg.	Neg.	Neg.	Neg.
***Enterobacter cloacae* (-)**	HE	Neg.	Neg.	Neg.	Neg.
	EE	Neg.	Neg.	Neg.	Neg.
***Pseudomonas aeruginosa* (-)**	HE	Neg.	Neg.	Neg.	Neg.
	EE	Neg.	Neg.	Neg.	Neg.
***Alcaligenes faecalis* (-)**	HE	9.5 ± 0.5	7.5 ± 0.5	Neg.	Neg.
	EE	17.0 ± 2.0	15.6 ± 1.4	15.0 ± 1.0	11.0 ±2.0

Neg.: negative result, NC: IZ not calculated due to the growth of some colonies throughout the IZ.

Table 3.2: Antibacterial activity of ampicillin using disk diffusion assay in triplicate n=3, and alamar blue microplate assay in quadruplicate, n=4. The test has been done according to section 2.16 and 2.17 respectively.

Bacteria strains (gram stain)	Mean diameter of IZ mm ± SD (n =3)		
	Ampicillin 10 µg/disk	Ampicillin 2 µg/disk	MIC µg/ml
Staphylococcus aureus (+)	na	32±3.0	≤ 0.5
Staphylococcus epidermidis (+)	na	36±2.5	≤ 0.5
Bacillus cereus (+)	Neg.	na	>64
Bacillus subtilis (+)	na	28±1.0	≤ 0.5
Streptococcus faecalis (+)	na	24±2.0	≤ 0.5
Escherichia coli (-)	22±2.0	16±1.0	≤ 0.5
Enterobacter cloacae (-)	24±1.0	17±1.5	≤ 0.5
Pseudomonas aeruginosa (-)	na	na	na
Alcaligenes faecalis (-)	10±1.0	na	16

na: not assayed

3.1.1.2. Partition of CG ethanol extract

Previous results showed that CGE is more active than CGH against most of the sensitive bacterial isolates used. CGE was fractionated according to section **2.5**. Three fractions were separated, butanol fraction CGEBU 77% w/w, ethyl acetate fraction 8% w/w and aqueous fraction CGEAQ 15% w/w.

3.1.1.3. Disk diffusion assay of ethanol fractions

IZ in mm of CGEEA, CGEBU, and CGEAQ against *S. aureus, S. apidermidis, B. cereus* and *Alcaligenes faecalis* were calculated according to section **2.16**, and the results are

shown in (Table 3.3). The selected bacterial isolates were showed sensitivity to CGE as it is clear from (Table 3.1).

Table 3.3: Antibacterial activity of *Cylicodiscus gabunensis*, ethanol fractions, the test has been done in triplicate, n=3 according to section 2.16.

Bacterial strain	0.5 mg/disk	IZ ± SD (n =3)
Staphylococcus aureus	CGEEA	14.0±0.4
	CGEBU	11.5±1.5
	CGEAQ	7.5±0.4
Staphylococcus epidermidis	CGEEA	15.0±1.0
	CGEBU	10.5±0.6
	CGEAQ	11.5±0.6
Bacillus cereus	CGEEA	11.0±0.4
	CHEBU	8.0±0.5
	CGEAQ	8.5±0.5
Alcaligenes faecalis	CGEEA	16.0±1.0
	CGEBU	13.0±0.5
	CGEAQ	11.5±1.0

CGEEA: CG ethanol ethyl acetate; CGEBU: CG ethanol butanol; CGEAQ: CG ethanol aqueous, IZ by mm, SD: Standard deviation, n= number of assay replication

3.1.1.4. CGEEA fractionation

CGEEA was separated into eight fractions using column chromatography according to section **2.6.1**. The fractions were prepared for alamar blue microplate assay according to section **2.17**.

3.1.1.5. MICs of CGEEA fractions

Eight fractions, CGEEA-F1-8 were prepared in eight different concentrations 8-1024 µg/ml in 3% DMSO according to section **2.17**. The results are shown in (Table 3.4). Fraction five (CGEEA-F5) showed higher activities than the other fractions with the best effect against *S. epidermidis* with MIC of 128 µg/ml, *S. aureus* with MIC of 256 µg/ml and *Alcaligenes faecalis* with MIC of 256 µg/ml, CGEEA-F6 also showed some activities against the three isolates mentioned above, other fractions showed MIC > 1024 µg/ml against all bacterial isolates.

Table 3.4: MIC results in μg/ml of CG ethanol ethyl acetate fractions CGEEA-F5, CGEEA-F6, and Ampicillin. The test has been done in quadruplicate, n= 4 according to section 2.17.

Name of Bacteria	CGEEA-F5	CGEEA-F6	Ampicillin
Bacillus cereus	1024	> 1024	>64
Bacillus subtilis	> 1024	> 1024	≤0.5
Escherichia coli	1024	> 1024	≤0.5
Enterobacter cloacae	1024	> 1024	≤0.5
Enterococcus faecalis	1024	> 1024	≤0.5
Staphylococcus aureus	256	512	≤0.5
Staphylococcus epidermidis	128	256	≤0.5
Pseudomonas aeruginosa	1024	> 1024	Not assayed
Alcaligenes faecalis	256	1024	16

3.1.1.6. Preparative HPLC of CGEEA-F5

CGEEA-F5 was fractionated by preparative HPLC according to section **2.7.1**, twelve fractions were collected according to time intervals, and (Figure 3.2) shows the HPLC chromatogram.

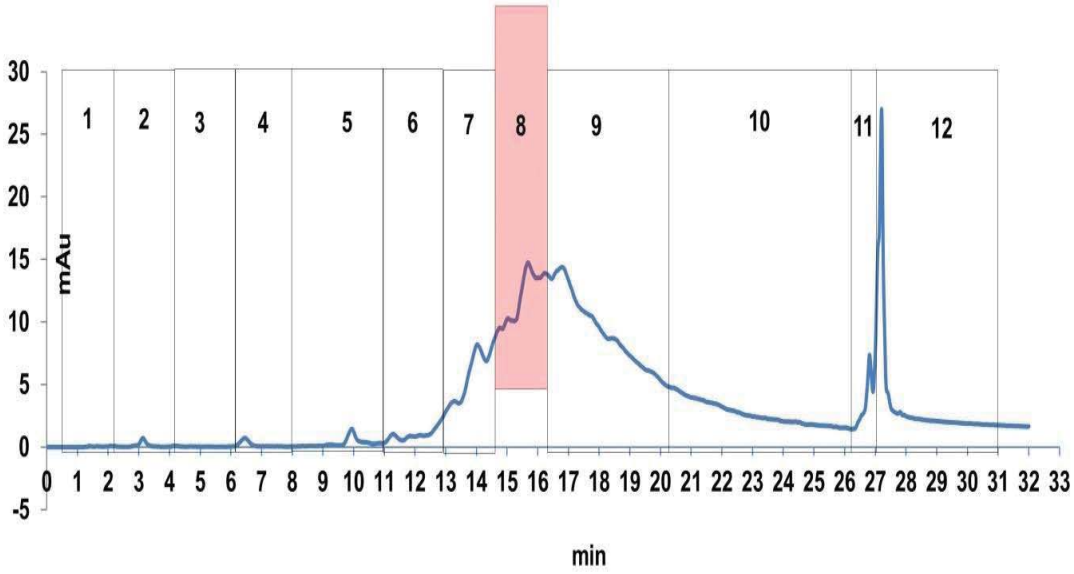


Figure 3.2: Preparative HPLC of CGEEA-F5. ethyl acetate fraction five, fractions were collected according to defined time intervals. The separation has been done according to section 2.7.1.

3.1.1.7. MICs CGEEA-F5 fractions

Twelve fractions, CGEEA-F5 (1-12) were prepared in eight different concentrations 4-512 µg/ml in 3% DMSO according to section **2.17** to calculate their MIC against three bacterial isolates. Small fractions were checked only against *S. epidermidis*, the results are shown in (Table 3.5). The results revealed that fraction 8, CGEEA-F5-8 was the most potent fraction and its MIC was 64 µg/ml against *S. epidermidis* and the MIC against each of *S. aureus* and *Alcaligenes faecalis* was 128µg/ml, however it showed moderate activity against *E. coli*.

Table 3.5: MIC (µg/ml) of fractions CGEEA-F5 (1-12) and Ampicillin. The test has been done in quadruplicate, n= 4 according to section 2.17.

Fraction	Bacterial isolate			
	S. aureus	*S. epidermidis*	*E. coli*	*A. faecalis*
CGEEA-F5-1	>512	> 512	>512	>512
CGEEA-F5-2	na	>512	na	na
CGEEA-F5-3	na	>512	na	na
CGEEA-F5-4	na	>512	na	na
CGEEA-F5-5	na	>512	na	na
CGEEA-F5-6	na	>512	na	na
CGEEA-F5-7	256	256	>512	512
CGEEA-F5-8	128	64	512	128
CGEEA-F5-9	256	128	>512	512
CGEEA-F5-10	>512	512	512	>512
CGEEA-F5-11	>512	>512	512	>512
CGEEA-F5-12	>512	>512	>512	>512
Ampicillin	≤0.5	≤0.5	≤0.5	16

n: Number of assay replication, n=4; na= Not assayed.

3.1.1.8. The effects on S. aureus cell wall integrity

Scanning electron microscopy was used to study the effect of gallic acid and ethyl gallate on *S. aureus* cell wall integrity according to the method mentioned in section **2.19**. Several images of cells from the control and after exposure to gallic acid and ethyl gallate were taken and here we present the results of both side by side for comparison purposes, (Figure 3.3) shows the SEM results of gallic acid and ethyl gallate against *S. aureus*.

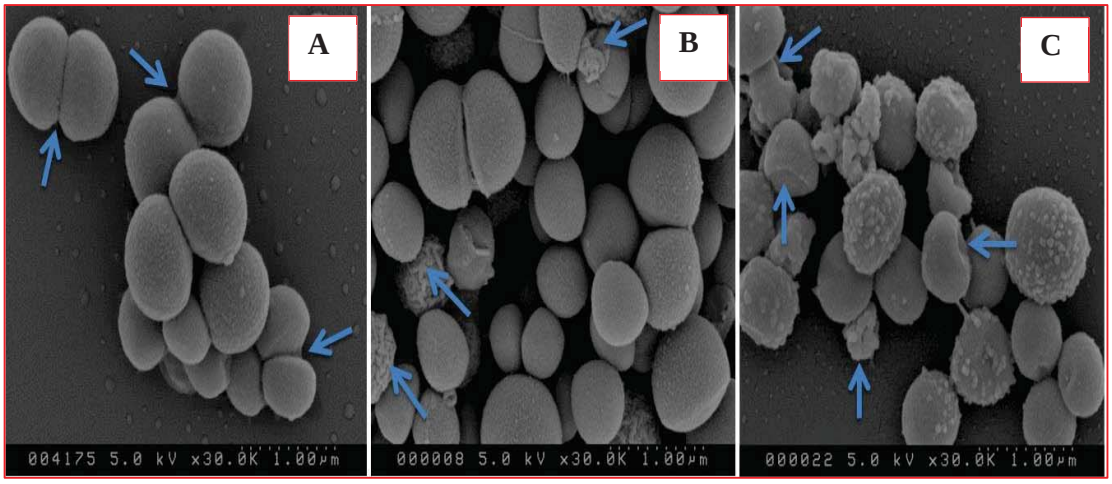


Figure 3.3: Electron scanning micrographs (SEM) of *S. aureus* NCIMB 11832 exposed to ethyl gallate and gallic acid. A: SEM of *S. aureus* shows the electron micrograph of control after 1 hr, arrows pointing to the division of cells. B: SEM of *S. aureus* after exposure to 2MIC level of ethyl gallate for 1 hr, arrows pointing to the leakage of cell contents in addition to the collapse of some cells. C: SEM of *S. aureus* after exposure to 2MIC level of gallic acid for 1 h, arrows pointing to doughnut shape cells and lysed cells. The test has been done according to section 2.19.

To summarize the bioassay-guided separation process, (Figure 3.4) shows all the steps of separation and bioactivity evaluation of the antibacterial activities.

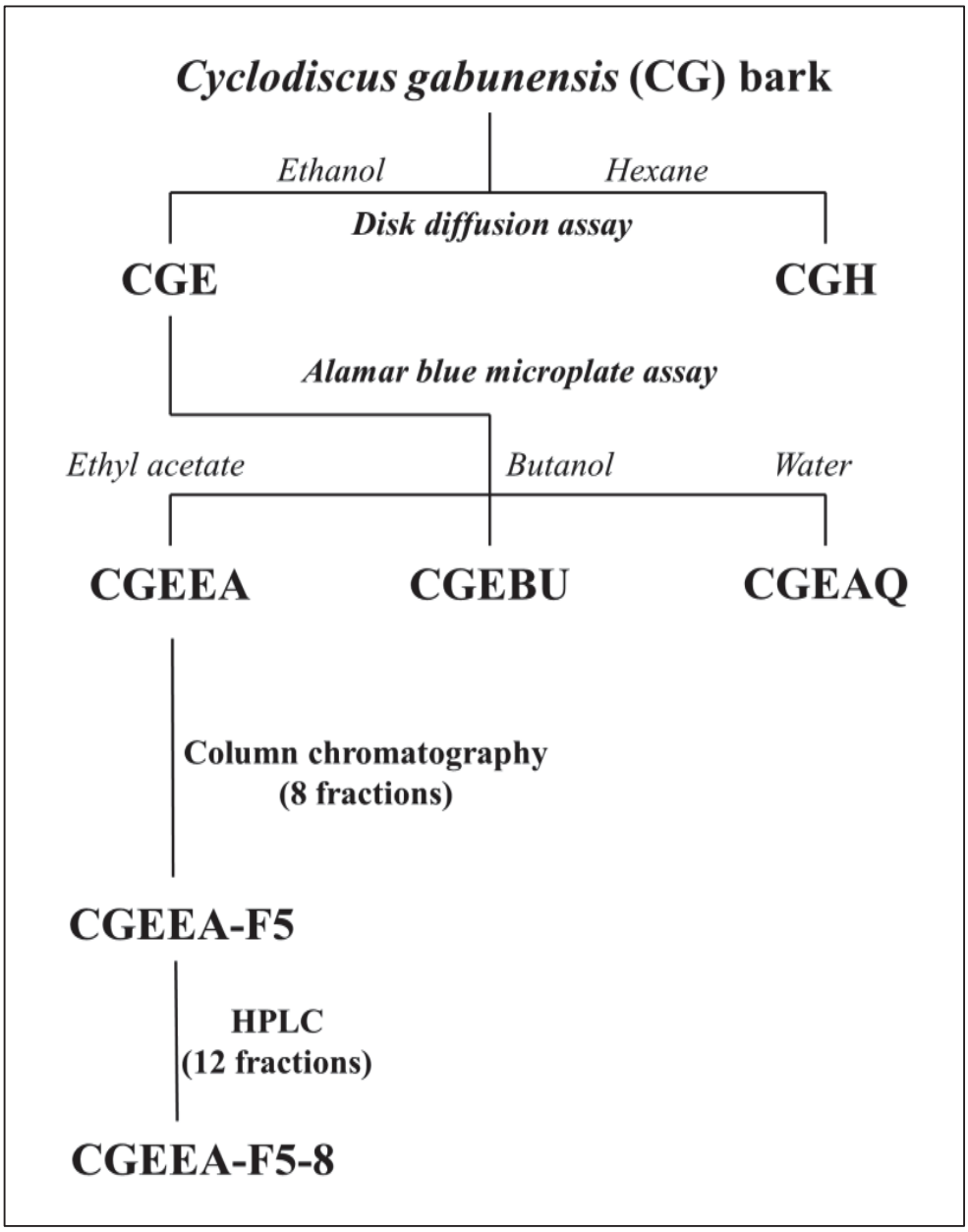


Figure 3.4: Schematic diagram illustrating key aspects of the bioassay guided fractionation of *Cylicodiscus gabunensis*. The solvents used in the initial separation steps are indicated using *italics*, biological replicates of disk diffusion assay =3 and alamar blue microplate assay =4 to detect the IZ in mm and MIC in µg/ml respectively. Different kinds of chromatography have been used for separation purposes. CGEEA: CG ethanol ethyl acetate; CGEBU: CG ethanol butanol; CGEAQ: CG ethanol aqueous.

3.1.2. Chemical analysis of the active antibacterial fractions

3.1.2.1. Analysis of CGEEA using GC-MS

3.1.2.1.1. Calculation of RI_s

Retention indices are important as an assistant of the compounds identity confirmation; n-alkanes were used for calculation of RI, according to section **2.8**. (Table 3.6) mentions the RT of alkanes' standard C_{8-20} ran in two different conditions (method A section **2.8.1)** and (method C section **2.8.3)**, (Figure 3.5) shows a graph represents the linear relationship between RT of alkanes and their carbon number.

Table 3.6: Alkanes' standard C8-C20 ran in two different conditions, RT in min. The analysis has been done in triplicate, n=3 according to sections 2.8.1 and 2.8.3.

Alkane	Carbon number	RT Method A	RT Method C
Octane	8	3.14±0.002	2.96±0.121
Nonane	9	4.59±0.120	4.54±0.054
Decane	10	6.31±0.021	6.75±0.001
Undecane	11	8.03±0.067	9.26±0.301
Dodecane	12	9.68±0.214	11.84±0.023
Tridecane	13	11.22±0.170	14.39±0.143
Tetradecane	14	12.67±0.310	16.66±0.005
Pentadecane	15	14.03±0.009	18.83±0.080
Hexadecane	16	15.31±0.101	20.89±0.090
Hepatdecane	17	16.54±0.210	22.86±0.124
Octadecane	18	17.68±0.167	24.73±0.134
Nonadecane	19	18.79±0.223	26.53±0.020
Eicosane	20	19.83±0.243	28.25±0.090

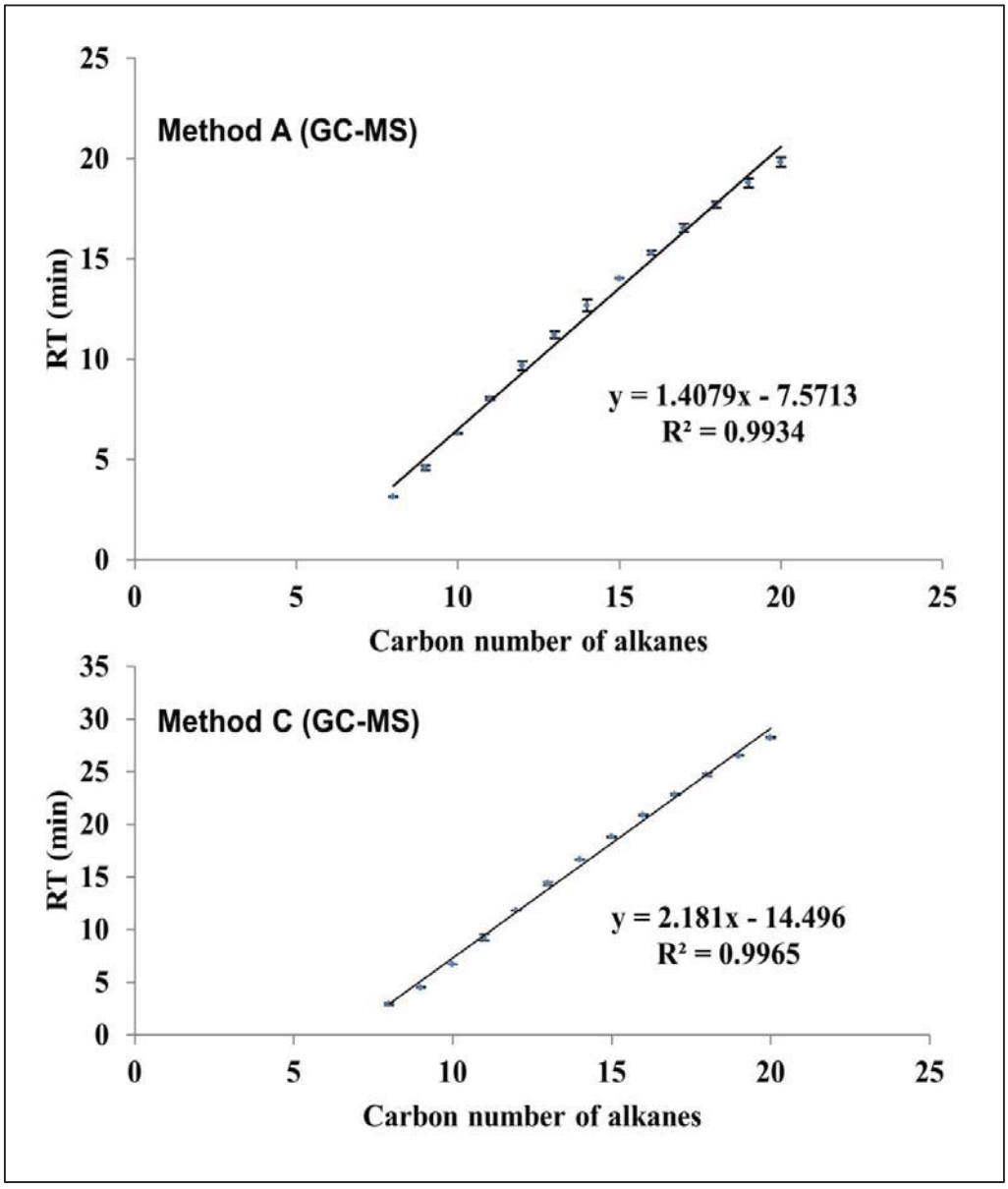


Figure 3.5: The linear relationship between alkane's carbon number and RT.Alkanes were run under different temperatures in a GC-MS system. The analysis has been done in triplicate, n=3 according to sections 2.8.1 and 2.8.3.

3.1.2.1.2. Chemical composition of CGEEA

After derivatisation of CGEEA to its corresponding silylated derivatives according to the procedure mentioned in section **2.8.5.2**, the derived fraction was run by method A section **2.8.1**. (Figure 3.6) presents the GC-MS chromatogram and (Table 3.7) shows the compounds detected in CGEEA.

Several benzoic acid derivatives were detected in CGEEA, those benzoic acid derivatives were represented a clear characteristic of this fraction, carboxylic acids and some fatty acids were also detected in the ethyl acetate fraction.

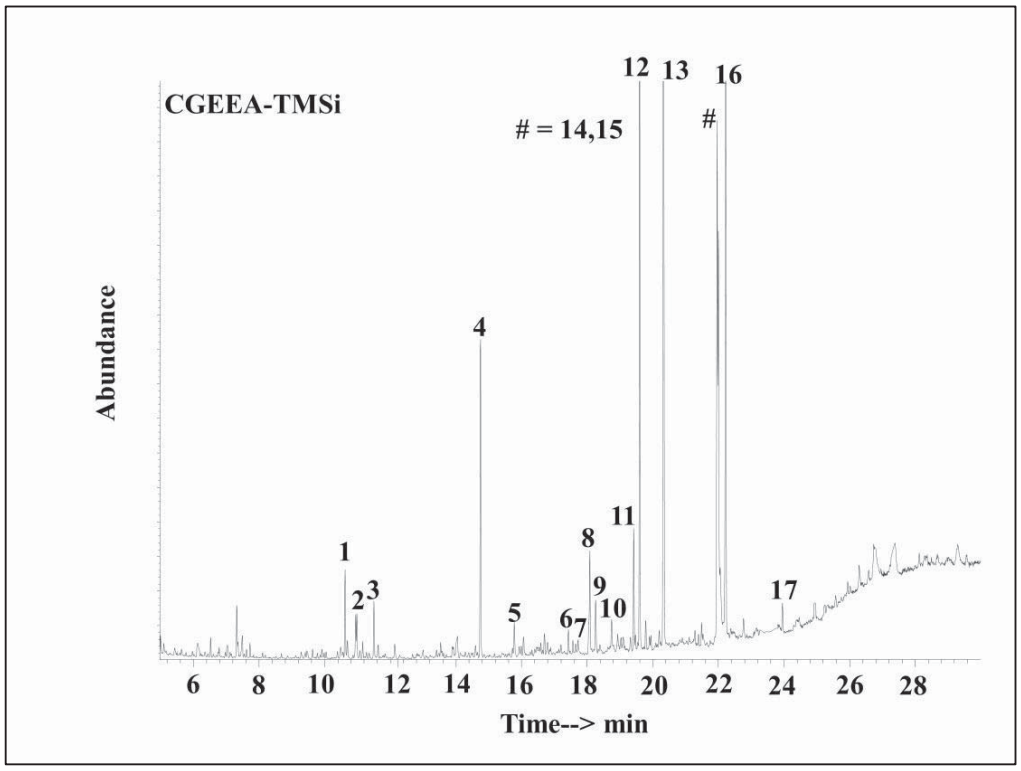


Figure 3.6: GC-MS chromatogram of *Cylicodiscus gabunensis* CGEEA. The analytes are derived according to section 2.8.5.2 and analysed according to section 2.8.1 in triplicate, n=3.

Table 3.7: Compounds (after TMSi derivation, section 2.8.5.2) detected from CGEEA by GC-MS, section 2.8.1. RT = Retention time, SD= Standard deviation, n= Number of experiment replication, RI= Retention index.

no.	RT (min)	Compound directly detected by GC-MS	The name of the compound before derivatisation	percentages±SD (n=3)	RI
1	10.60	1,3-bis(1,1-dimethylethyl)- benzene	1,3-bis(1,1-dimethylethyl)- Benzene	1.42±0.14	1261
2	10.98	Glycerol, tris(trimethylsilyl) ether	Glycerine	0.54±0.01	1285
3	11.50	Butanedioic acid, bis(trimethylsilyl) ester	Succinic acid	0.88±0.07	1319
4	14.75	Trimethyl(2,6 ditert.-butylphenoxy)silane	2,6-ditert-butylphenol	5.40±0.60	1556
5	15.73	Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	4-hydroxy-benzoic acid	0.42±0.01	1634
6	17.43	Benzoic acid, 3-methoxy-4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	3-methoxy-4-hydroxy-benzoic acid or vanillic acid	0.44±0.06	1778
7	17.70	Azelaic acid, bis(trimethylsilyl) ester	Azelaic acid	0.38±0.01	1804
8	18.08	Benzoic acid, 3,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	3,4-dihydroxy-benzoic acid or protocatechuic acid	2.18±0.07	1836
9	18.26	Tetradecanoic acid, trimethylsilyl ester	Tetradecanoic acid	1.05±0.13	1852
10	18.90	Trimethylsilyl 3,5-dimethoxy-4-(trimethylsilyloxy)benzoate	3,5-dimethoxy-4-hydroxy-benzoic acid or syringic acid	0.47±0.04	1914
11	19.40	3,4,5-Trihydroxybenzoic acid ethyl ester, tris(O-trimethylsilyl)-	3,4,5-trihydroxybenzoic acid ethyl ester or ethyl gallate)	1.88±0.10	1960
12	19.60	Benzoic acid, 3,4,5-tris(trimethylsilyloxy)-, trimethylsilyl ester	3,4,5-trihydroxy benzoic acid	13.24±0.10	1973
13	20.30	Hexadecanoic acid, trimethylsilyl ester	Hexadecanoic acid	31.14±2.50	-
14	21.96	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	(Z,Z)-9,12-octadecadienoic acid	8.52±0.45	-
15	21.99	11-trans-Octadecenoic acid, trimethylsilyl ester	11-trans-octadecenoic acid	8.95±0.43	-
16	22.22	Octadecanoic acid, trimethylsilyl ester	Octadecanoic acid	15.58±0.93	-
17	23.96	Eicosanoic acid, trimethylsilyl ester	Eicosanoic acid	0.36±0.01	-

3.1.2.2. Chemical composition of CGEEA-F5

This fraction was derived to its TMSi-derivative according to section **2.8.5.2** and was run by method A section **2.8.1**. (Figure 3.7) shows the GC-MS chromatogram and (Table 3.8) shows the compounds detected in this fraction.

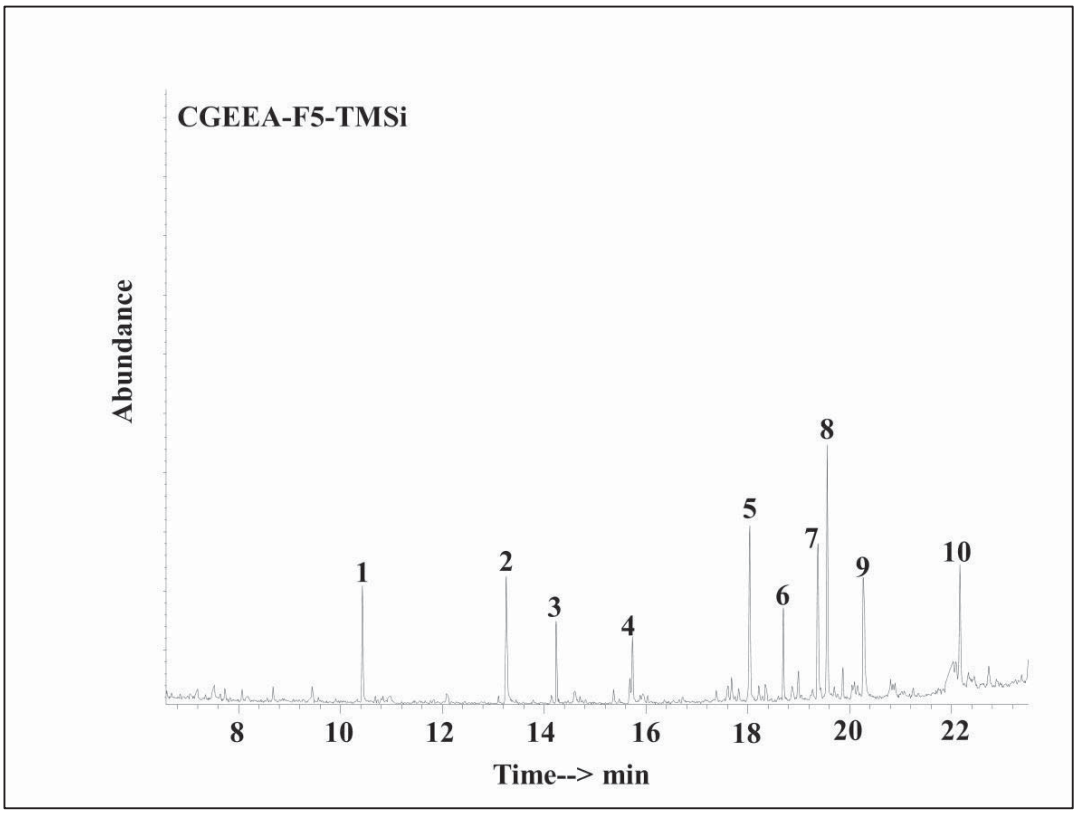


Figure 3.7: GC-MS chromatogram of *Cylicodiscus gabunensis* CGEEA-F5. The analytes are derived according to section 2.8.5.2 and analysed according to section 2.8.1 in triplicate, n=3.

Ten compounds were detected. Several benzoic acid derivatives represented about 68% of the detected compounds like 4-hydroxy benzoic acid, protocatechuic acid; syringic acid, gallic acid and ethyl gallate were detected from CGEEA-F5. Fatty acids abundance is 17% compared to more than 60% in CGEEA.

Table 3.8: Compounds (after TMSi derivation, section 2.8.5.2) detected from CGEEA-F5 by GC-MS, section 2.8.1, n=3.

no.	RT (min)	Compound directly detected by GC-MS	The name of the compound before derivatisation	percentages ±SD (n=3)	RI
1	10.57	1,3-bis(1,1-dimethylethyl)- Benzene	1,3-bis(1,1-dimethylethyl)- Benzene	8.96 ±0.45	1262
2	13.24	Benzene, 1-(trimethylsilyloxy)-2-(trimethylsilyloxymethyl)-	2-(Hydroxymethyl)phenol or salicylic alcohol	9.60 ±0.34	1442
3	14.45	Benzoic acid, 4-ethoxy-, ethyl ester	Benzoic acid, 4-ethoxy-, ethyl ester	6.99 ±1.01	1533
4	15.72	Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	4-hydroxy-benzoic acid	3.97 ±0.01	1634
5	18.03	Benzoic acid, 3,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	3,4-dihydroxy-benzoic acid or protocatechuic acid	10.45 ±1.20	1836
6	18.69	Trimethylsilyl 3,5-dimethoxy-4-(trimethylsilyloxy)benzoate	3,5-dimethoxy-4-hydroxy-benzoic acid or syringic acid	5.73 ±0.70	1892
7	19.37	3,4,5-Trihydroxybenzoic acid ethyl ester, tris(O-trimethylsilyl)-	3,4,5-trihydroxybenzoic acid ethyl ester or ethyl gallate	8.97 ±0.80	1960
8	19.55	Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester	3,4,5-trihydroxy benzoic acid or gallic acid	22.30 ±3.56	1973
9	20.27	Hexadecanoic acid, trimethylsilyl ester	Hexadecanoic acid	7.56 ±0.35	-
10	22.16	Octadecanoic acid, trimethylsilyl ester	Octadecanoic acid	9.98 ±1.23	-

RT = Retention time, SD= Standard deviation, n= Number of experiment replication, RI= Retention index

3.1.2.3. Analysis of CGEEA-F5 by HPLC

To confirm the presence of gallic acid, protocatechuic acid and ethyl gallate, the three standards were run on analytical C-8 column, according to section **2.10**, (Figure 3.8) shows the HPLC chromatogram.

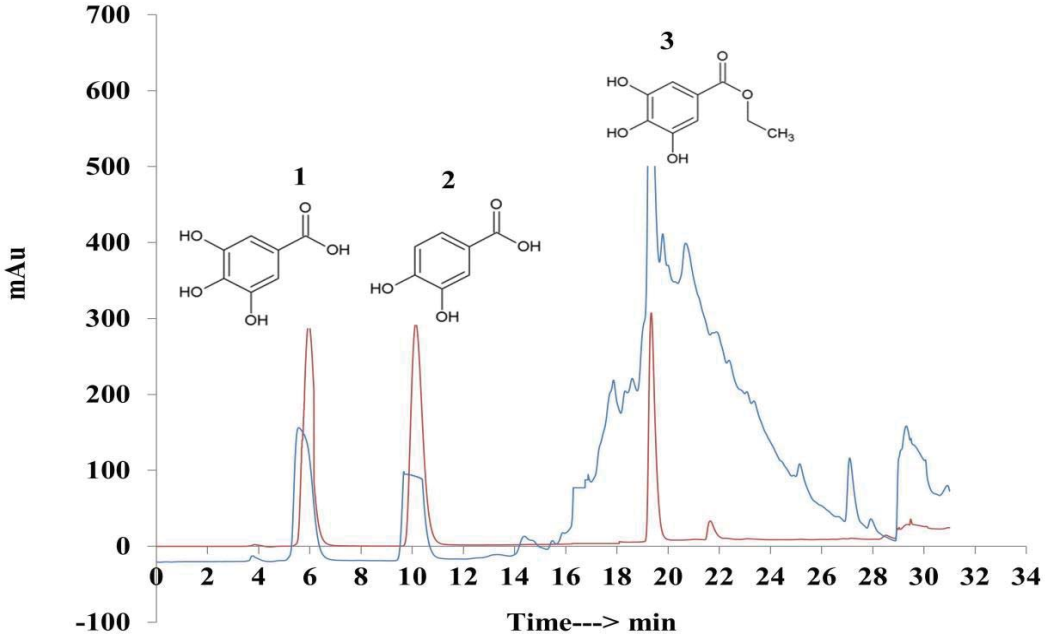


Figure 3.8: Analytical HPLC of CGEEA-F5 (blue line).Standards from left to right (red line) **1**: gallic acid, RT 5.8 min; **2**: protocatechuic acid, RT 10.5 min; **3**: ethyl gallate, RT 19.5 min. The analysis has been done according to section 2.10, in triplicate, n=3.

3.1.2.4. Chemical composition of CGEEA-F5-8

3.1.2.4.1. GC-MS

Checking the most active fraction from CGEEA-F5 by GC-MS using method A section **2.8.1**, showed three benzoic acid derivatives; syringic acid, gallic acid and ethyl gallate (Figure 3.9 and Figure 3.10).

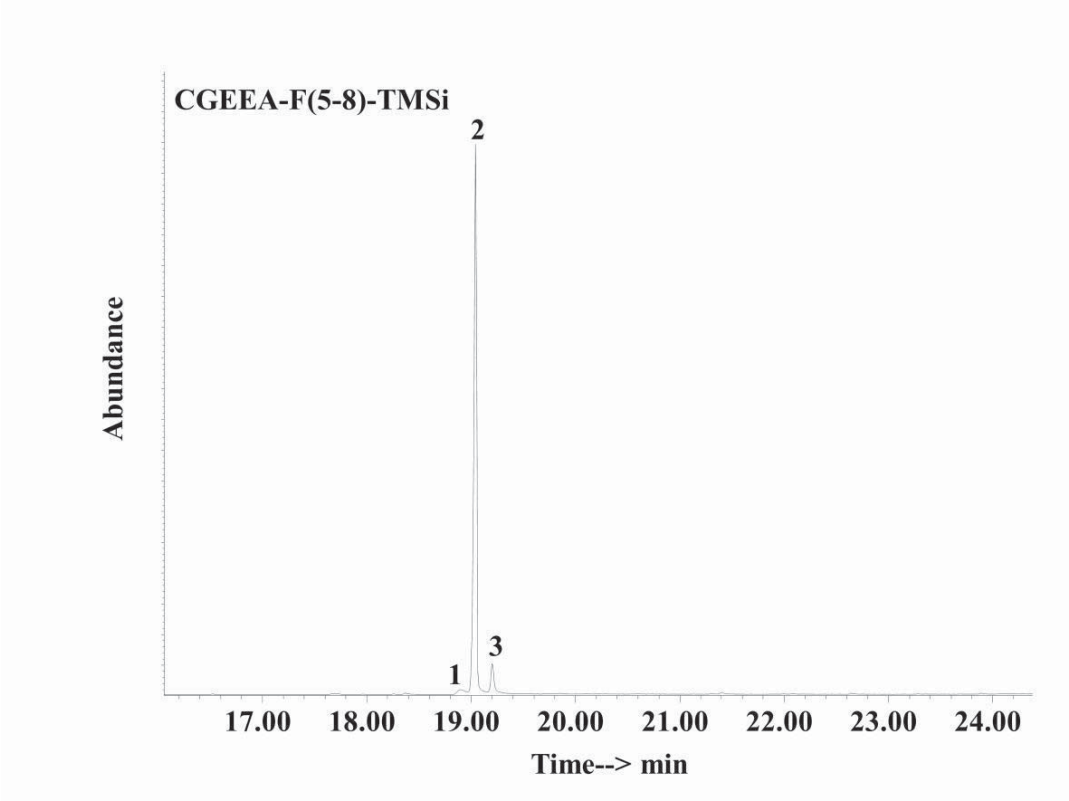


Figure 3.9: GC-MS chromatogram of CGEEA-F5-8.1: syringic acid-TMSi; 2: ethyl gallate-TMSi; 3: gallic acid-TMSi. The fraction was derived according to section 2.8.5.2 and analyse according to section 2.8.1 in triplicate, n=3.

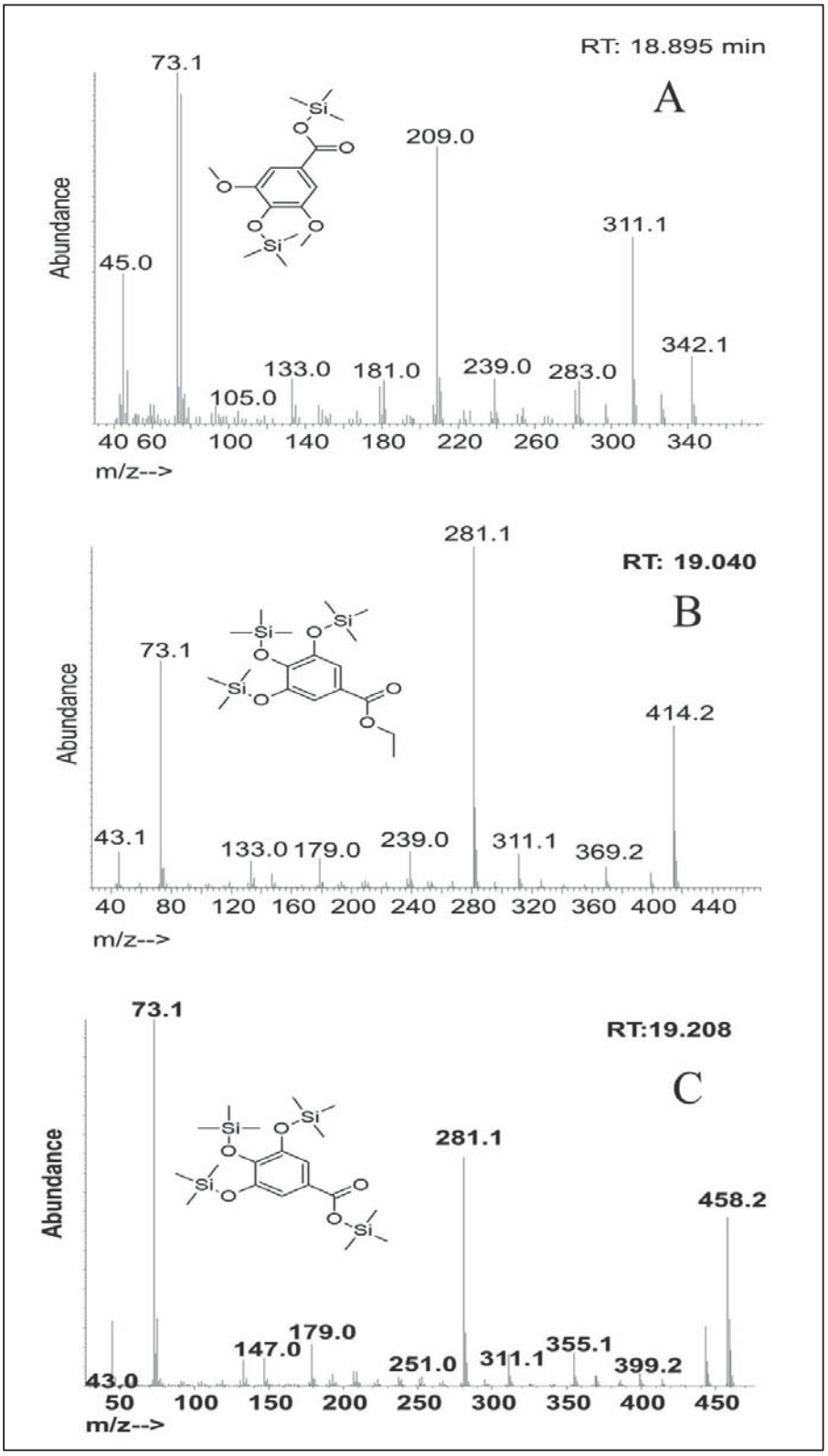


Figure 3.10: EI-Mass spectra of compounds detected in CGEEA-F5-8. A: syringic acid-TMSi; B: ethyl gallate- TMSi; C: gallic acid-TMSi.

3.1.2.4.2. HPLC

CGEEA-F5-8 ran according to section **2.11** on C18 analytical column and then we spiked the fraction by few crystals of ethyl gallate to notice the change in the magnitude of ethyl gallate peak (Figure 3.11).

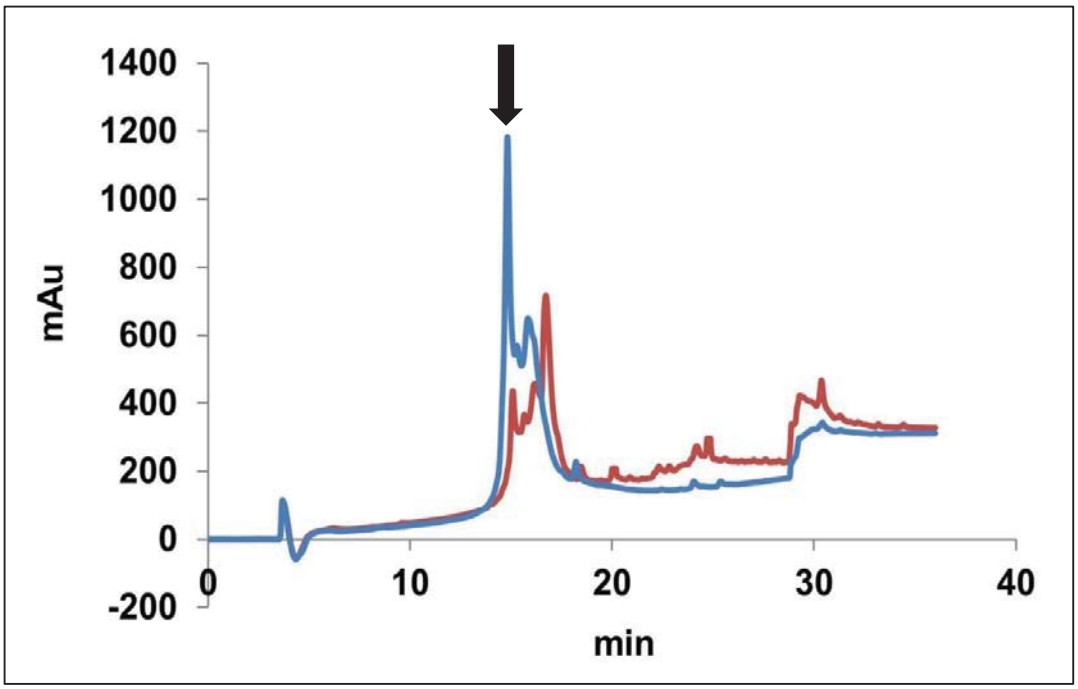


Figure 3.11: Analytical HPLC of CGEEA-F5-8.*Cylicodiscus gabunensis* ethanol ethyl acetate fraction 5-8 (red), the blue coloured chromatogram is representing CGEEA-F5-8 spiked with ethyl gallate. The black arrow refers to ethyl gallate. The analysis has been done according to section 2.11 in triplicate, n=3.

3.1.2.4.3. TOF LC-MS

CGEEA-F5-8 ran by LC-MS as discussed in section **2.12**., (Figure 3.12) shows the extraction of ethyl gallate peak which constitutes about 63% of CGEEA-F5-8 and the electrospray ionization mass spectrometry (ESI-MS). (Table 3.9) shows all the masses detected from this active fraction.

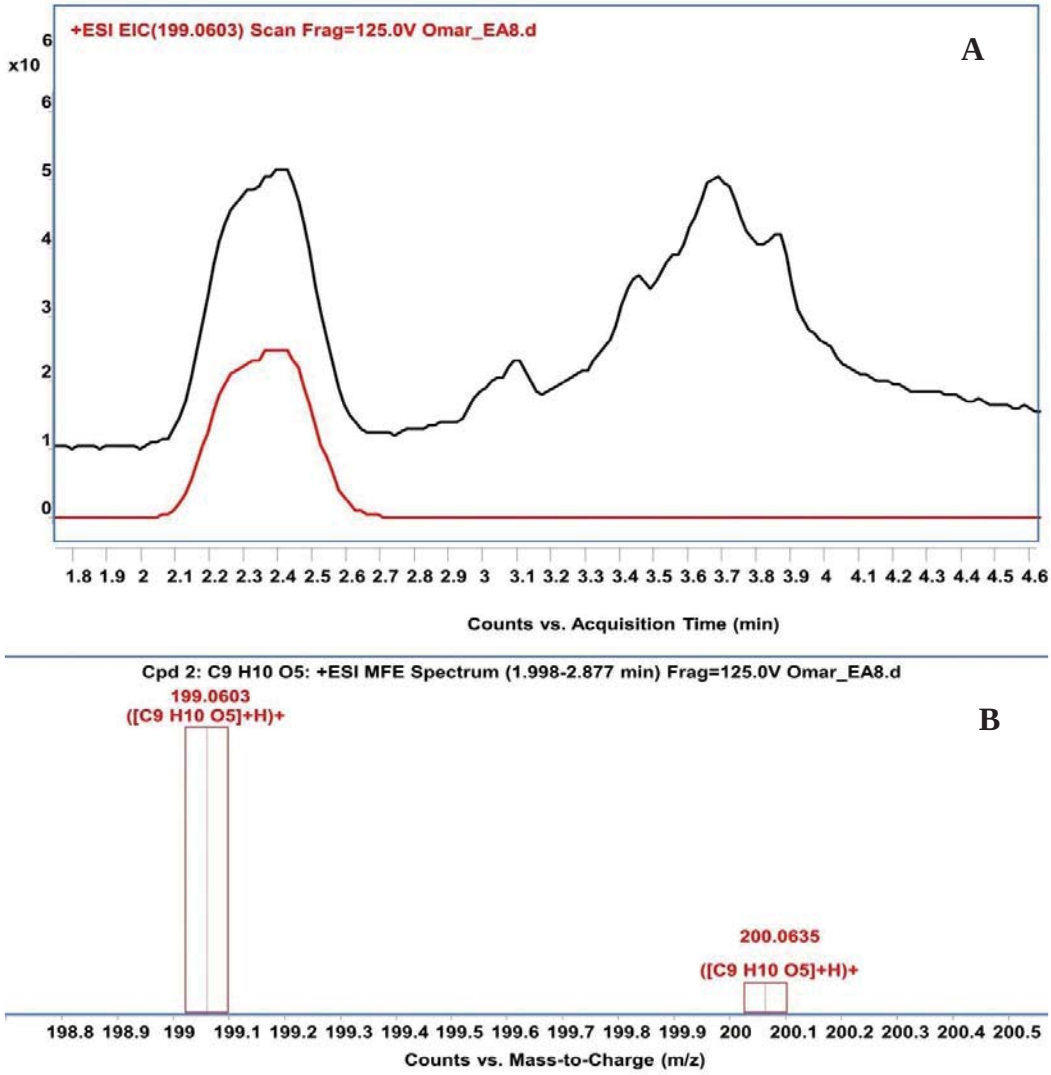


Figure 3.12: A: LC-MS chromatogram of CGEEA-F5-8. B: positive ESI-MS of Ethyl gallate MW: 198.0603 g/mol. Both acquired from qualitative analysis mass hunter work station software. The analysis has been done according to section 2.12, n=3.

Table 3.9: Molecular masses of compounds pooled in CGEEA-F5-8 detected using LC-MS.

Formula	Score	Mass	*m/z*	RT	Vol %
C7 H4 O4	99.81	152.011	153.0183	2.356	1.91
C7 H6 O5	99.91	170.0214	171.0287	2.363	3.01
C9 H10 O5	99.84	198.0528	199.0603	2.364	63.23
C15 H12 O6	97.5	288.0635	289.0706	2.926	1.45
C22 H18 O8	99.88	410.1001	411.1074	3.448	2.45
C20 H22 O9	99.83	406.1265	407.1338	3.072	4.78
C22 H26 O13	99.81	498.1373	499.1446	3.438	1.31
C37 H30 O14	99.42	698.1639	699.1713	3.677	6.83
C37 H30 O15	99.85	714.1585	715.1658	3.719	2.26
-	-	834.2151	835.2223	3.862	3.61
-	-	850.2104	851.2177	3.673	6.22
-	-	1002.2222	1003.2294	3.864	2.62

The positive ESI-MS of the twelve compounds masses detected are shown in appendix A as (Figure A-1).

3.1.3. Antiplasmodial effects of Cylicodiscus gabunensis extracts

3.1.3.1. Antiplasmodial activity of CGH and CGE

CGH and CGE were prepared in DMSO as 100, 33 and 11 µg/ml and checked for antiplasmodial activity according to section **2.21**. (Figure 3.13) shows the % of untreated control (live cells) versus drug concentration. The IC_{50} of CGE and CGH are shown in (Figure 3.14).

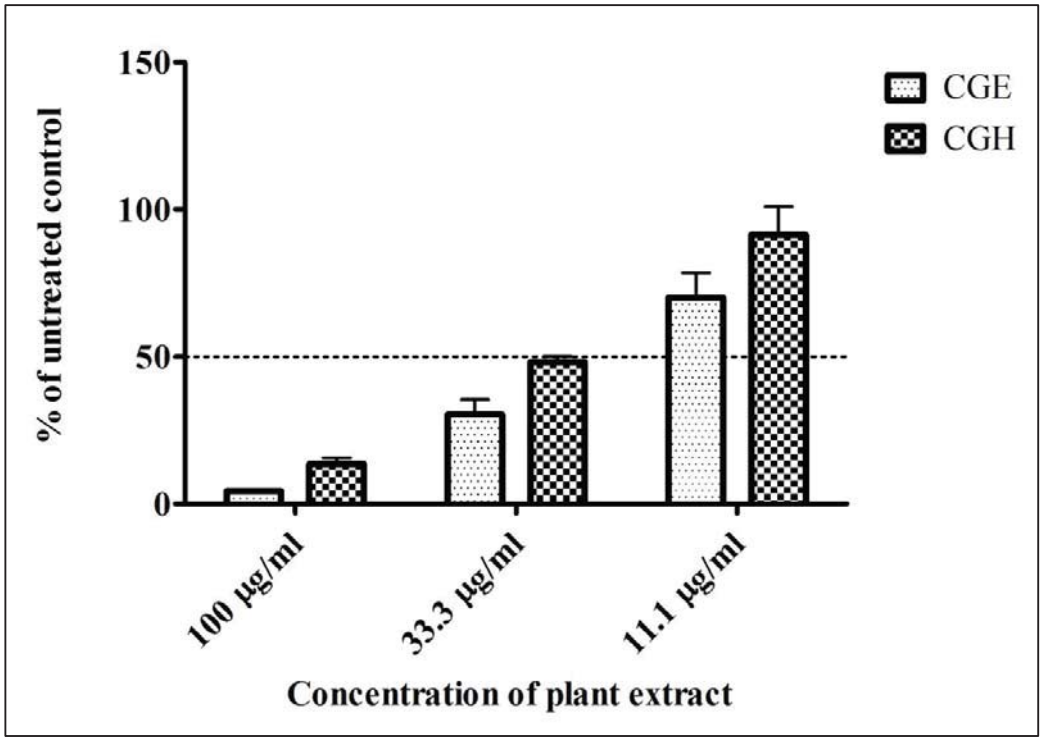


Figure 3.13: The % of untreated *P. falciparum* Dd2 cells versus three concentrations of CG plant extracts (100, 33.3, and 11.1 µg/ml). Extracts used are *Cylicodiscus gabunensis* ethanol (CGE) and hexane (CGH), n=3. The assay has been done according to section 2.21.

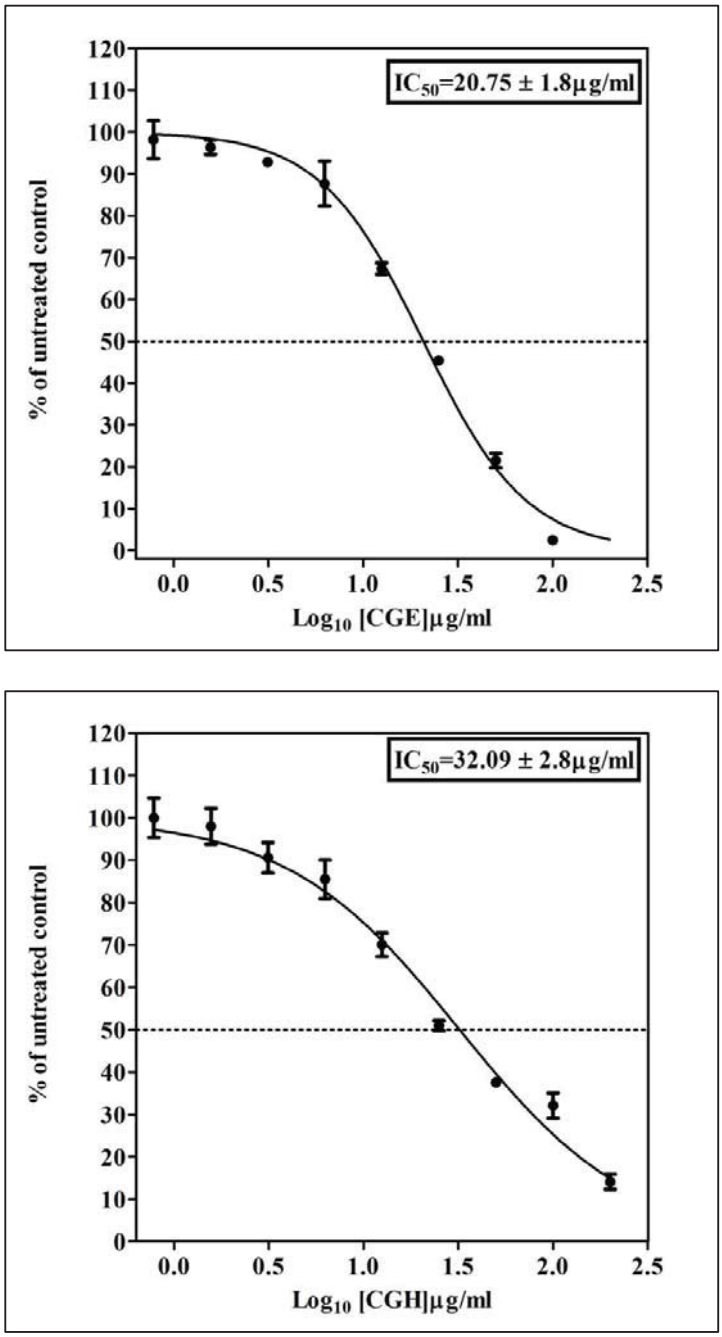


Figure 3.14: IC_{50} of *Cylicodiscus gabunensis* ethanol (CGE) and hexane extract (CGH) against *P. falciparum* Dd2, n=3. The IC_{50} of Chloroquine against *P. falciparum* Dd2 was 0.171 µM. The assay has been done according to section 2.21.

3.1.3.2. Antiplasmodial activity of CGE fractions

CGEEA, CGEBU and CGEAQ were prepared in DMSO, a dilution of 100, 33 and 11 µg/ml were checked for antiplasmodial activity according to section **2.21**, (Figure 3.15) describes the antiplasmodial potentials of CGE fractions and (Figure 3.16) shows their (IC_{50})s. The results revealed that CGEBU fraction was more active than CGEEA and CGEAQ.

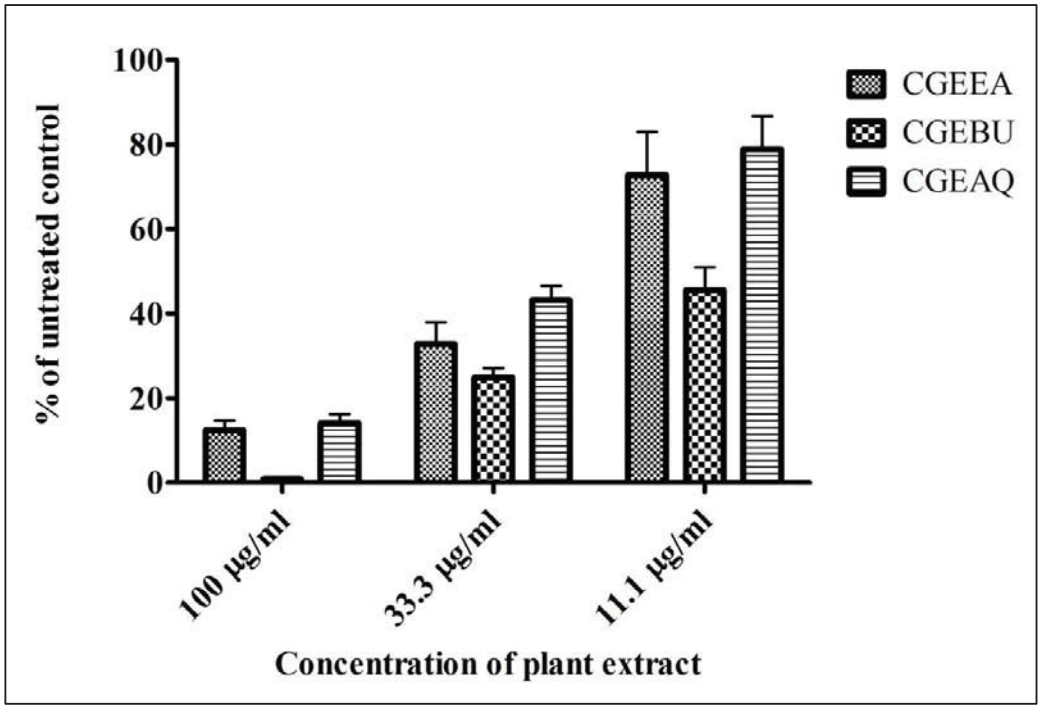


Figure 3.15: The % of untreated *P. falciparum* Dd2 cells versus three concentrations of CGEEA, CGEBU and CGEAQ plant extracts (100, 33.3, and 11.1 µg/ml). Extracts used are *C. gabunensis* ethanol ethyl acetate fraction (CGEEA), ethanol butanol fraction (CGEBU), ethanol aqueous fraction (CGEAQ), n=3. The assay has been done according to section 2.21.

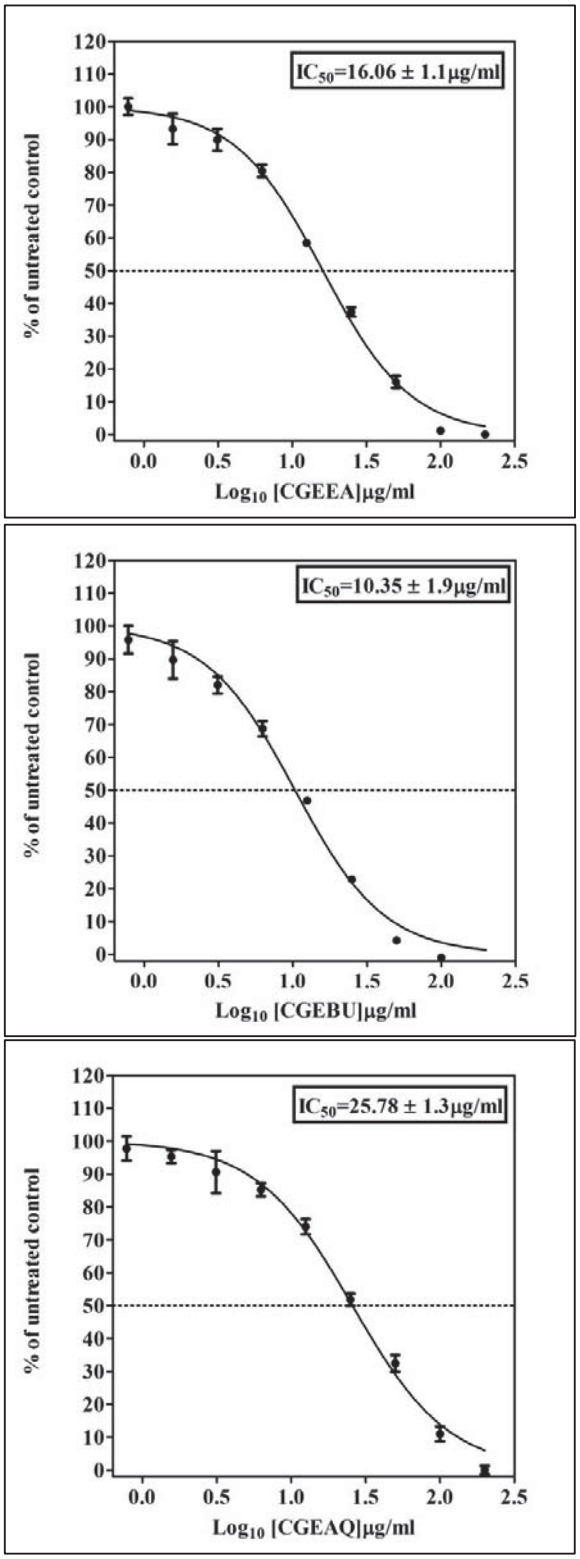


Figure 3.16: IC_{50} of *Cylicodiscus gabunensis* CGEBU, CGEEA and CGEAQ against *P. falciparum* Dd2, n=3.The IC_{50} of Chloroquine against *P. falciparum* Dd2 was 0.171 µM. The assay has been done according to section 2.21.

3.1.3.3. Fractionation of CGEBU by Column Chromatography

CGEBU was separated into ten fractions over silica gel column according to the method mentioned in section **2.6.2**.

3.1.3.4. Antiplasmodial activity of CGEBU-F1-10

Butanol fractions CGEBU-F (1-10) were prepared in DMSO as 100, 33, and 11 µg/ml and checked for antiplasmodial activity according to section **2.21**. (Figure 3.17) shows the antiplasmodial effect of the ten fractions. (Figure 3.18) shows the IC_{50} 6.5 µg/ml of the more potent fraction CGEBU-F10.

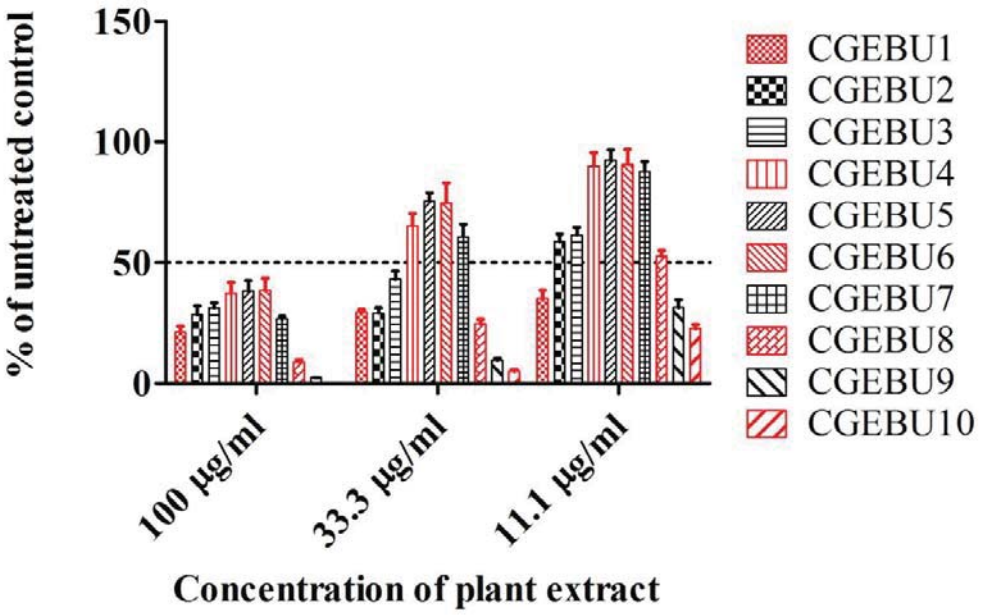


Figure 3.17: The % of untreated *P. falciparum* Dd2 cells versus three doses of CGEBU fractions (100, 33.3, and 11.1 µg/ml). Extracts used are *Cylicodiscus gabunensis* ethanol butanol fractions (CGEBU-F1-10), n=3. The assay has been done according to section 2.21.

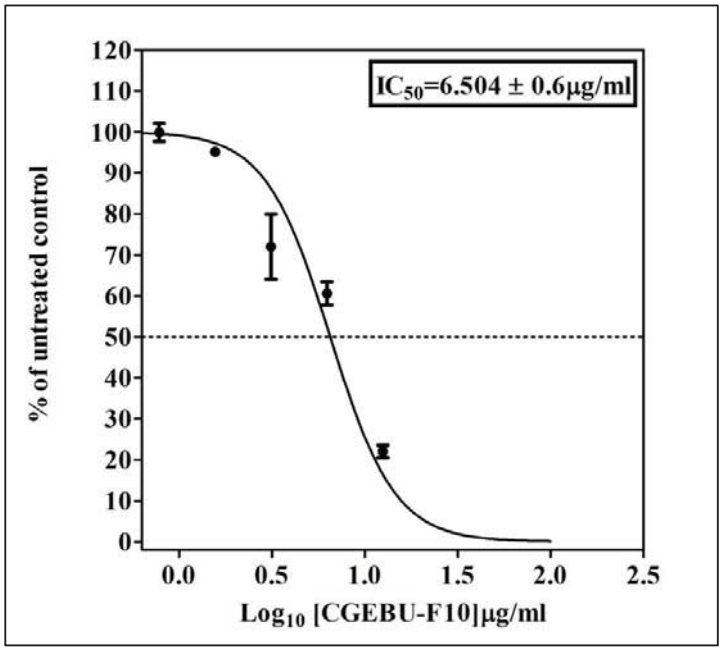


Figure 3.18: The dose-response curve shows the IC_{50} of the most active fraction from butanol extract (CGEBU-F10).The IC_{50} of Chloroquine against *P. falciparum* Dd2 was 0.171 µM. The assay has been done according to section 2.21, n=3.

3.1.3.5. Preparative HPLC of CGEBU-F10

The most active fraction from butanol extract (CGEBU-F10) was separated into eight fractions using preparative HPLC column according to section **2.7.2**, The HPLC chromatogram is shown in (Figure 3.19).

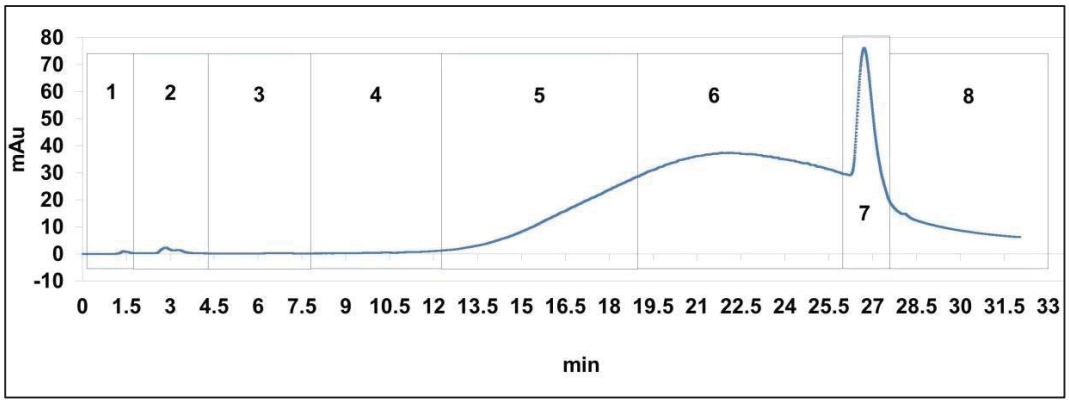


Figure 3.19: Preparative HPLC of CGEBU-F10. The fractionation has been done according to section 2.7.2.

3.1.3.6. Antiplasmodial activity of CGEBU-F10 (1-8)

Butanol fractions (CGEBU-F10 (1-8)) were prepared in DMSO as 50, 10, and 2 µg/ml according to the method mentioned in section **2.21**, for the evaluation of their antiplasmodial activities. (Figure 3.20) shows the effect of the butanol fractions (CGEBU-F10 (1-8)) against *P. falciparum* Dd2. (Figure 3.21) shows the dose-response curve of the most active fraction (CGEBU-F10-7), IC_{50} 4.694 µg/ml.

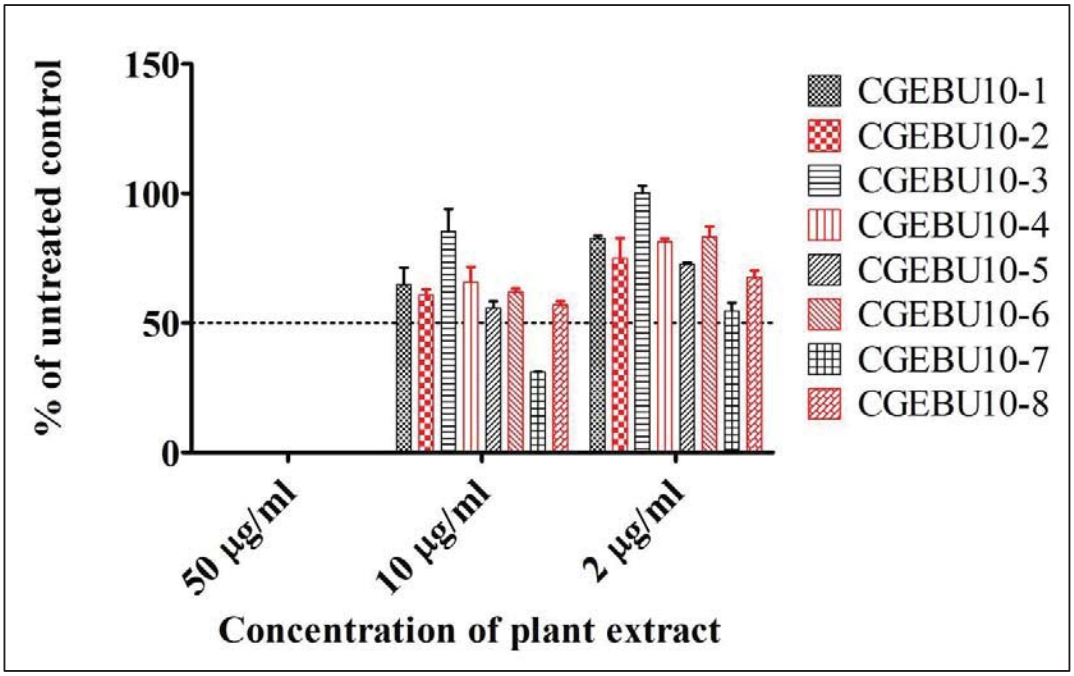


Figure 3.20: The % of untreated *P. falciparum* Dd2 cells versus three doses of CGEBU-F10 subfractions (50, 10, and 2 µg/ml). Extracts used are *Cylicodiscus gabunensis* CGEBU-F10 fractions (1-8), n=3. All the fractions causing complete eradication of the parasites at concentration 50 µg/ml. The assay has been done according to section 2.21.

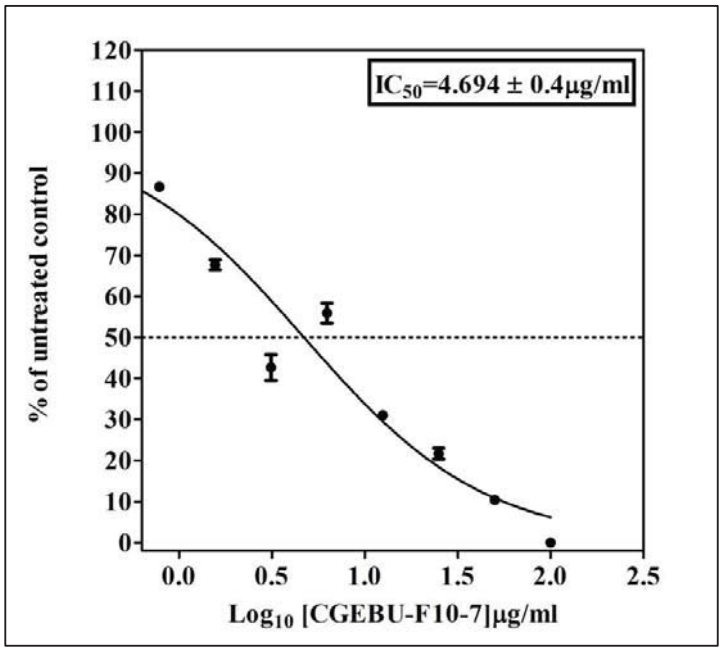


Figure 3.21: The Dose- response curve shows the IC_{50} of the most active fraction from butanol fraction 10 (CGEBU-F10-7). The IC_{50} of Chloroquine against *P. falciparum* Dd2 was 0.171 µM. The assay has been done according to section 2.21, in triplicate n=3.

3.1.4. Chemical analysis of the active antiplasmodial compounds

3.1.4.1. Analysis of CGEBU, CGEAQ and CGH

The trimethyl silyl derivatives of CGEBU and CGEAQ were prepared according to section **2.8.5.2** and CGH dissolved in hexane according to section **2.8.5.1** to check by GC-MS according to method A described in section **2.8.1**. (Table 3.10) shows the metabolites detected from CGEBU and (Figure 3.22) shows the GC-MS total ion chromatogram TIC. (Table B-1) and (Figure B-1) show the compounds detected and TIC of CGEAQ respectively. (Table B-2) and (Figure B-2) show the compounds detected and TIC of CGH respectively. (Figure B-3) shows the EI-Mass spectrometry of Lup-20(29)-en-3-one, a compound detected from CGH (Appendix-B).

Table 3.10: Compounds (after TMSi derivation, section 2.8.5.2) detected in CGEBU by GC-MS, section 2.8.1, n=3.

no.	RT (min)	Compound directly detected by GC-MS	The name of the compound before derivatisation	percentages (n=3)	±SD	RI
1	10.63	Benzene, 1,3-bis(1,1-dimethylethyl)-	Benzene, 1,3-bis(1,1-dimethylethyl)-	2.70±0.07		1262
2	10.99	Glycerol, tris(trimethylsilyl) ether	Glycerine	4.51±0.06		1285
3	11.51	Butanedioic acid, bis(trimethylsilyl) ester	Butanedioic acid	1.44±0.06		1320
4	14.75	Trimethyl(2,6 ditert.-butylphenoxy)silane	2,6-ditert-butylphenol	9.70±0.29		1556
5	18.26	Tetradecanoic acid, trimethylsilyl ester	Tetradecanoic acid	2.58±0.10		1852
6	19.60	Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester	3,4,5-trihydroxy benzoic acid or gallic acid	12.06±0.90		1978
7	20.33	Hexadecanoic acid, trimethylsilyl ester	Hexadecanoic acid	32.33±1.01		-
8	22.23	Octadecanoic acid, trimethylsilyl ester	Octadecanoic acid	24.21±0.57		-
9	23.75	cis-11,14-Eicosadienoic acid, trimethylsilyl ester	cis-11,14-eicosadienoic acid	1.69±0.06		-
10	23.96	Eicosanoic acid, trimethylsilyl ester	Eicosanoic acid	6.79±0.16		-

RT = Retention time, SD= Standard deviation, n= Number of experiment replication, RI= Retention index

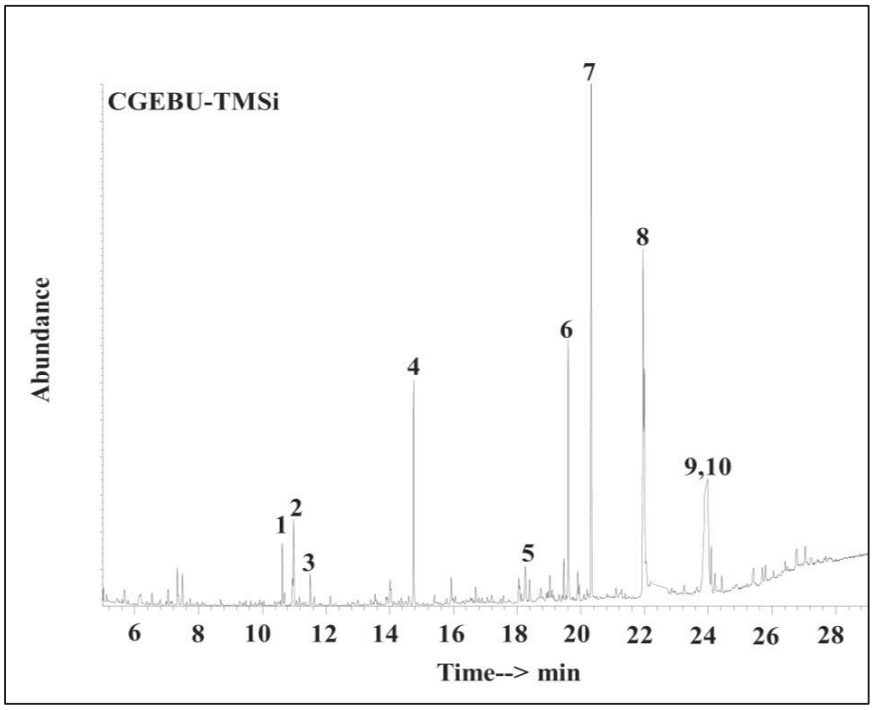


Figure 3.22: GC-MS chromatogram of *Cylicodiscus gabunensis* butanol fraction, CGEBU. According to section 2.8.1 in triplicate, n=3.

3.1.4.2. ^{1}H NMR of CGEBU-F10-7

Four mg of CGEBU-F10-7 was prepared for ^{1}H NMR according to section **2.14**; (Figure 3.23) shows the NMR spectrum of CGEBU-F10-7.

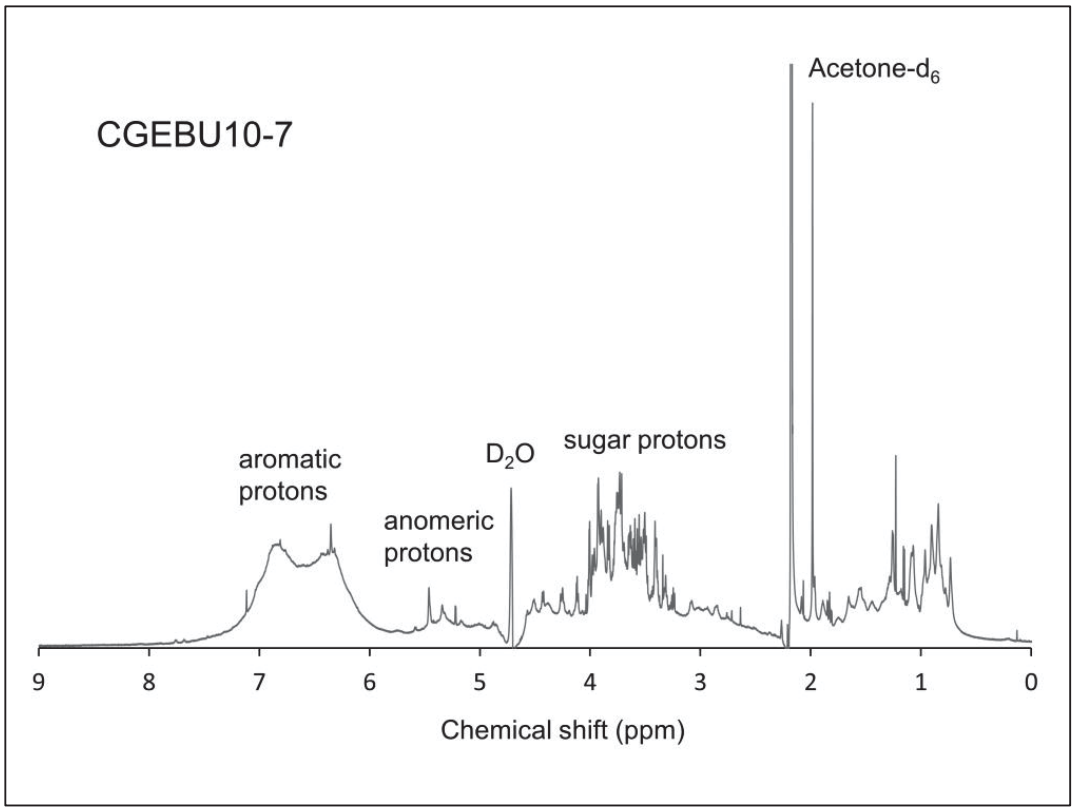


Figure 3.23: ^{1}H NMR spectrum of CGEBU-F10-7 in CD_3COCD_3:D_2O (1:1), section 2.14.

3.1.4.3. Acid hydrolysis and GC-MS assay of CGEBU- F10-7

Two mg of CGEBU-F10-7 hydrolysed according to the procedure specified in section **2.13**. (Figure 3.24) shows the GC-MS chromatogram of the hydrolysed fraction CGEBU-F 10-7.

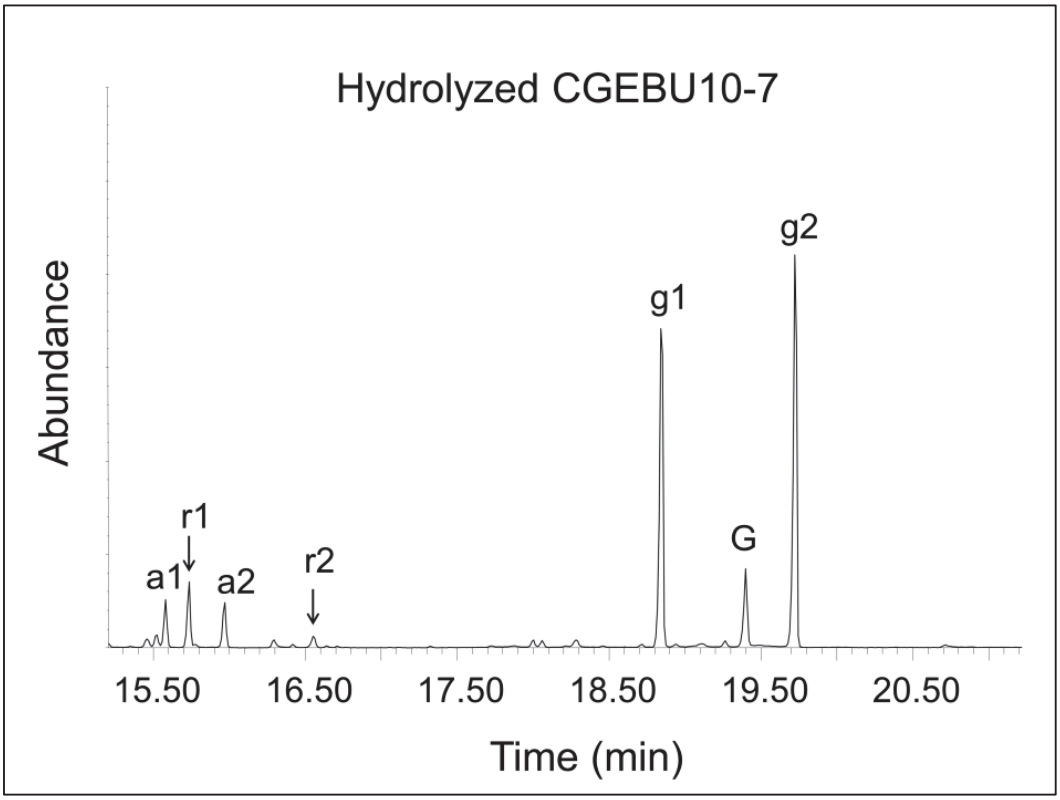


Figure 3.24: GC-MS chromatogram of hydrolysed CGEBU-F10-7 after TMSi derivation. Peaks from left to right are a1: α-L-arabinose-TMSi; r1: α-L-rhamnose-TMSi; a2: β-L-arabinose-TMSi; r2: β-L-rhamnose-TMSi; g1: α-D-glucose-TMSi; g2: β-D-glucose-TMSi; G: gallic acid-TMSi. The assignment of these peaks was based on the NIST library and comparison with standard compounds. The derivatives were prepared according to section 2.8.5.2 and analysed according to section 2.8.1, in triplicate n=3.

CGEBU-F10-7 apparently composed of oligosaccharides units attached to either gallic acid or other molecules as confirmed from the results of LC-MS which showed high molecular weights of the compounds detected from this fraction. (Figure A-2) in appendix A shows the positive ESI-MS of five compounds picked as masses from CGEBU-F10-7 by qualitative analysis mass hunter work station software. Masses detected were **1**: 1260.5497; **2**: 1436.6116 and 1406.6074; **3**: 1044.5522; **4**: 1244.5487.

3.1.4.4. Mass spectrometry of the anomerised sugars

Sugar molecules were identified by running standard sugars and do anomerisation of sugars according to section **2.13.2**, then confirm the identity of the sugar molecules detected from the hydrolysed CGEBU-F10-7 fraction with those standards through matching of EI-MS spectrum and RT. (Figures 3.25, 3.26, and 3.27) are show the TIC, EI-MS of the monosaccharides detected from the hydrolysed CGEBU-F10-7 fraction.

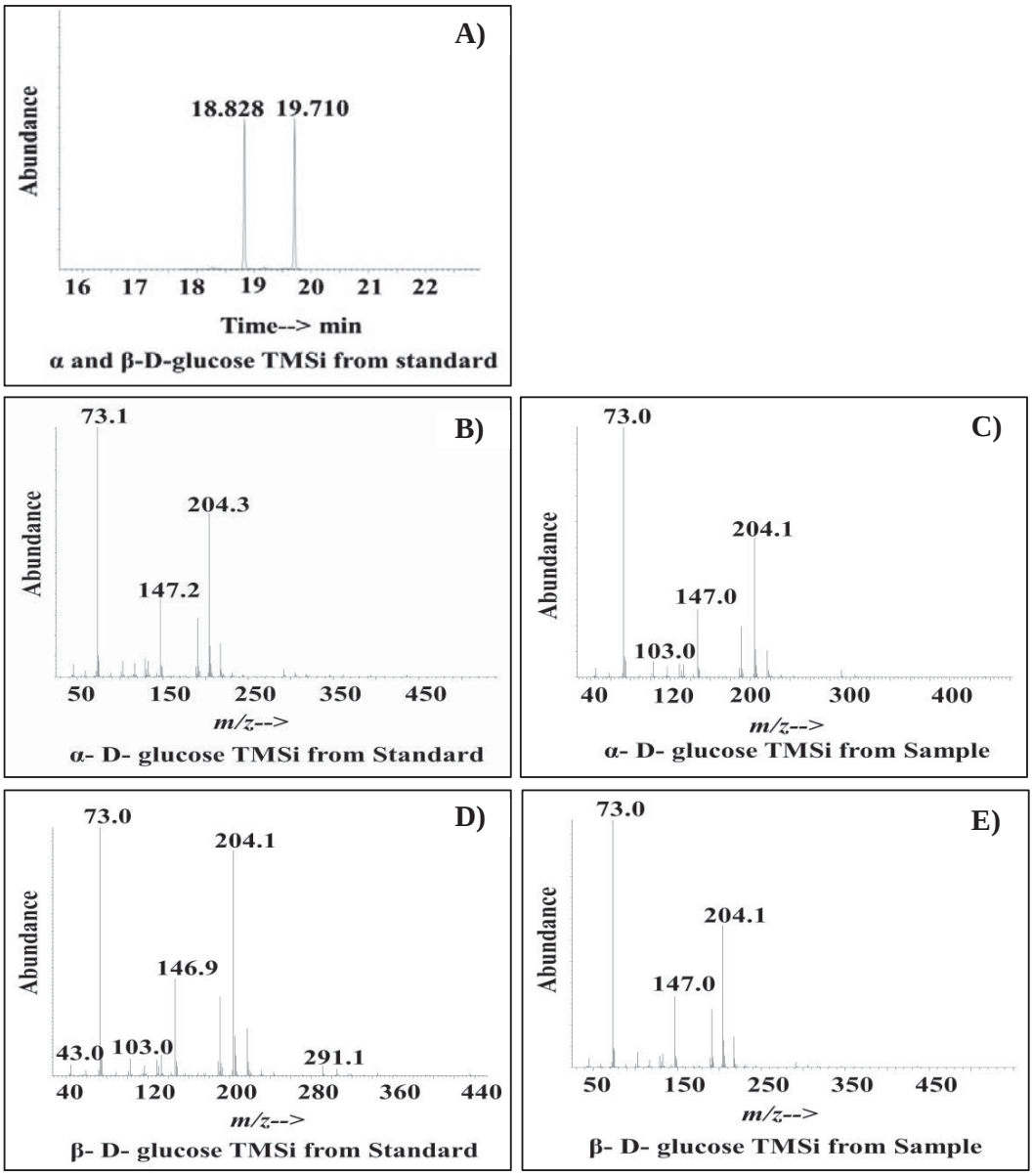


Figure 3.25: GC-MS chromatogram (TIC) and EI-MS of the anomerised glucose. A: TIC of glucose shows two peaks of alpha and beta-glucose. B and C: EI-MS of α-glucose from the standard and the anomerised sample respectively. D and E: EI-MS of beta-glucose from the standard and the anomerised sample respectively.

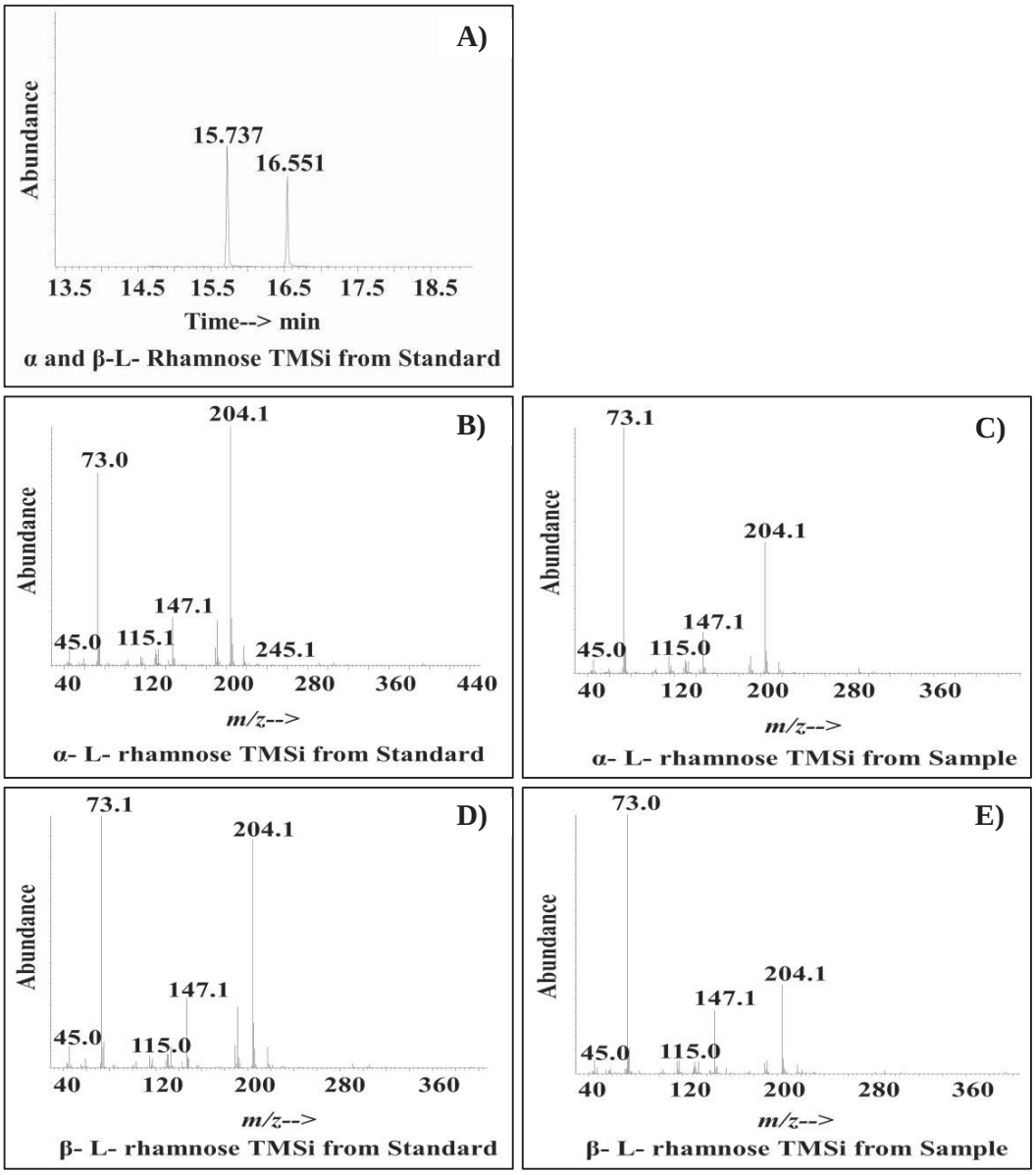


Figure 3.26: GC-MS chromatogram (TIC) and EI-MS of anomerised rhamnose. A: TIC of rhamnose shows two peaks of alpha and beta-rhamnose. B and C: EI-MS of alpha-rhamnose from the standard and the anomerised sample respectively. D and E: EI-MS of beta-rhamnose from the standard and the anomerised sample respectively.

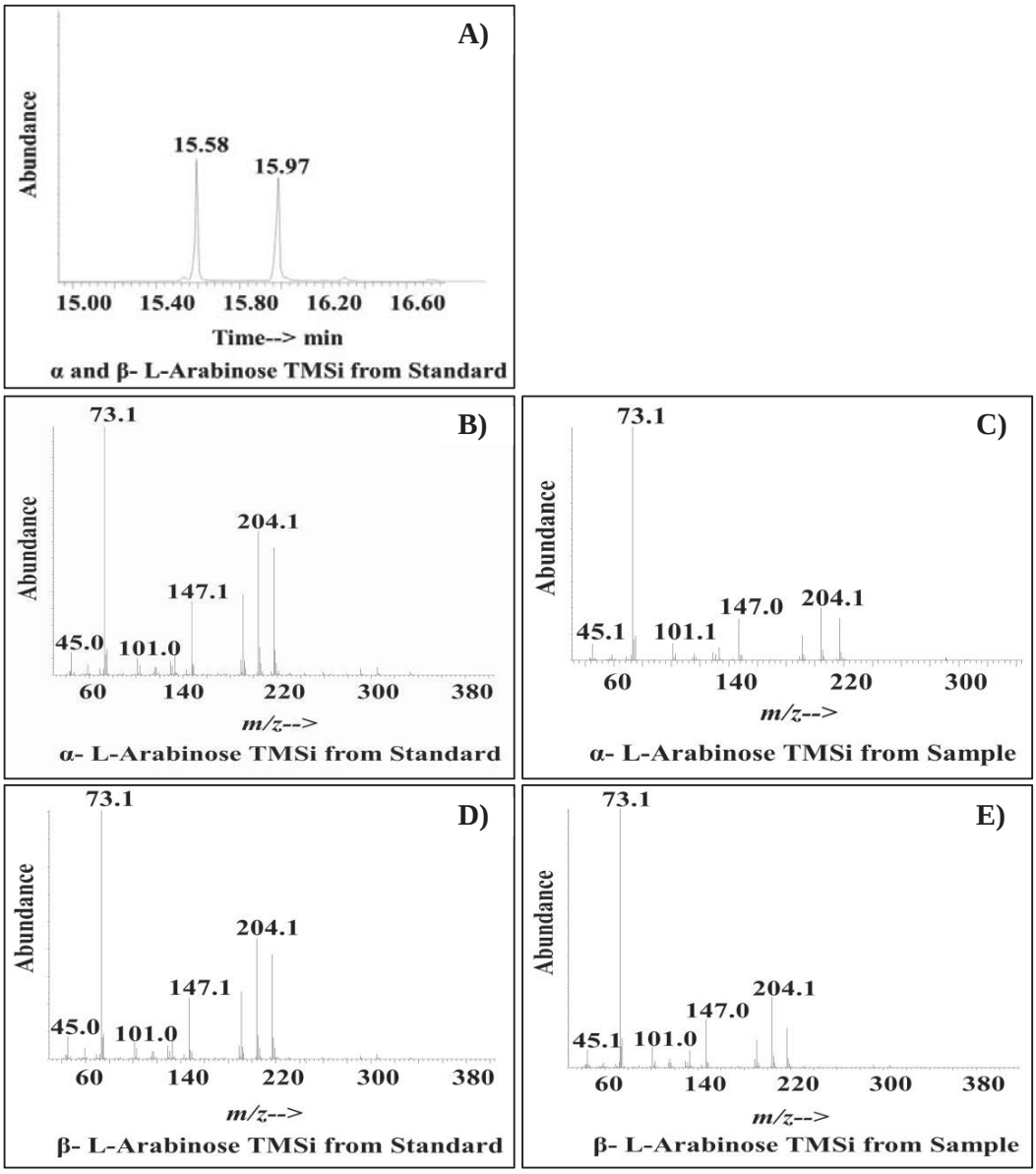


Figure 3.27: GC-MS chromatogram (TIC) and EI-MS of anomerised arabinose. A: TIC of arabinose shows two peaks of alpha and beta-arabinose. B and C: EI-MS of alpha-arabinose from the standard and the anomerised sample respectively. D and E: EI-MS of beta-arabinose from the standard and the anomerised sample respectively.

(Figure 3.28) mentions the bioguided extraction scheme of *Cylicodiscus gabunensis* to identify its antiplasmodial compounds.

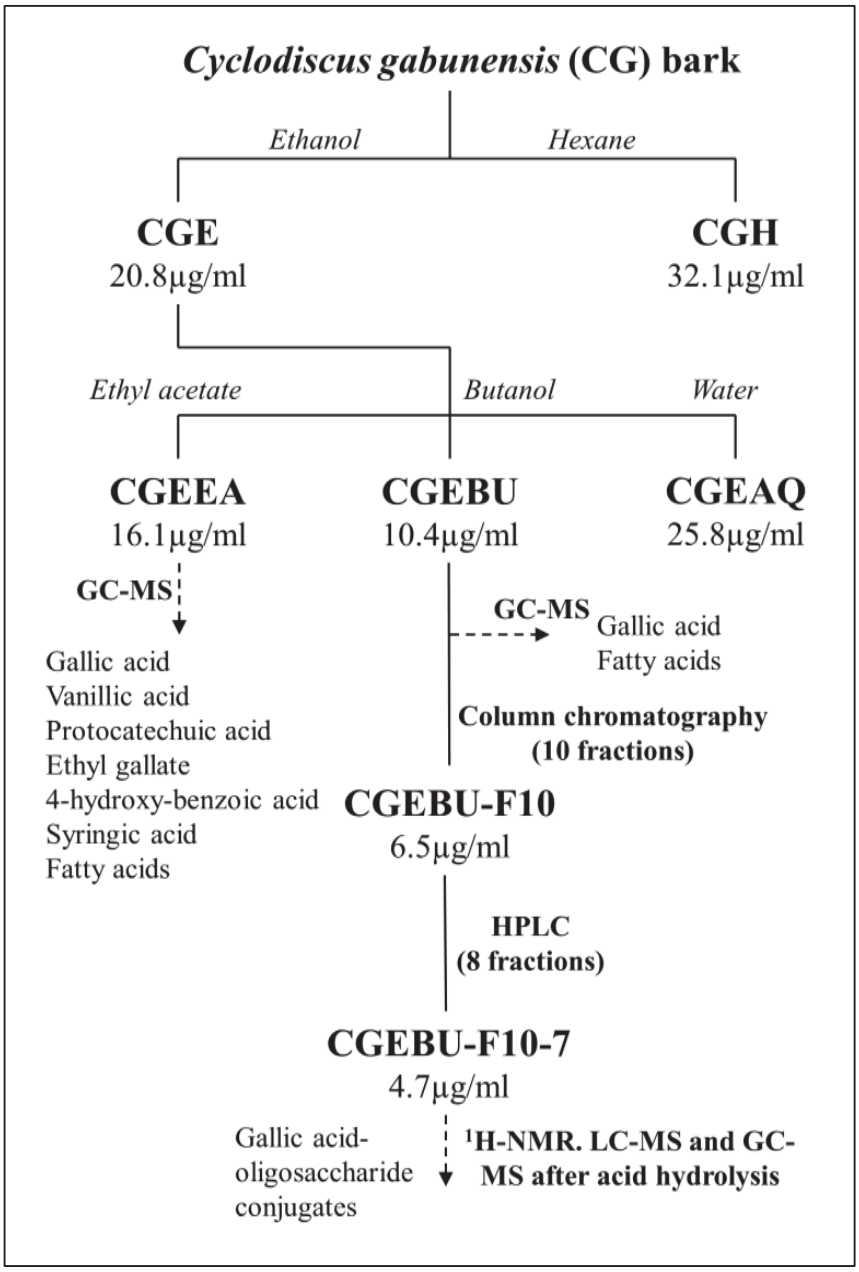


Figure 3.28: Schematic diagram illustrating key aspects of the bioassay guided fractionation of *Cylicodiscus gabunensis* bark. Indicating key fractionation methods and the phytochemical identified. The solvents used in the initial separation steps are indicated using italics, with the IC_{50} in µg/ml of derived fractions indicated below each (mean from n=3 biological replicates).

3.2. Discussion

3.2.1. Antibacterial activities of Cylicodiscus gabunensis

The choice of the appropriate solvent for extraction of compounds from natural sources is essential for obtaining fractions with high antibacterial activity (Feng, Xu 2014). To extract the non-polar and polar compounds of CG, we used hexane and aqueous ethanol respectively. Phenolics were extracted efficiently with polar solvents, as aqueous ethanol was ranked in the best solvents set for extraction of phenolic compounds and hexane the least (Feng, Xu 2014). More phenolic materials were detected in ethanol than other solvents of extraction like chloroform and ethyl acetate (Al-Dabbas, Suganuma et al. 2006); CG bark was rich in phenolic materials which were extracted efficiently by aqueous ethanol.

3.2.1.1. Disk diffusion assay

The agar diffusion method or disk diffusion assay is not a quantitative but a qualitative technique (Rios, Recio et al. 1988). The extracts showing IZ above 9 mm were selected as good antibacterial agents (Michielin, Salvador et al. 2009).

CGE showed potent activity against gram positive bacterial isolates used in this study compared to the weak antibacterial activity of CGH. On some plates the IZs were not recorded due to the growth of bacterial colonies throughout the IZ.

The highest effect of CGE recorded against *S. epidermidis* IZ= 19.5 ±1.0 mm, and against *S. aureus* IZ= 16.5± 0.5 mm. *S. aureus* can cause skin abscess, sinusitis, and food intoxication. *S. aureus* is extremely prevalent in atopic dermatitis patients (NHS Choices 2015). *S. epidermidis* is one of causes of the biofilms formation, because the bacterial cells can stack all over to make a multilayer biofilm (Otto 2009). CGE showed moderate effect against *B. cereus*, which contaminates the food and causes food poisoning, nausea, vomiting and diarrhea (Asaeda, Caicedow et al. 2005, Ehling-Schulz, Fricker et al. 2004).

Ethyl acetate extract of CG showed moderate activities against several isolates of bacteria, however, the active compounds have not been revealed yet (Kouitcheu Mabeku, Kouam et al. 2006). The ethyl acetate extract prepared and assayed against *S. aureus*. 2.5 mg/disk showed IZ 8-20 mm, those results were in agreement with our results (Kouitcheu Mabeku, Kouam et al. 2006).

3.2.1.2. Partition of CGE

The compounds which were extracted using aqueous ethanol were semi-polar or polar, hence the partition of these compounds was dependent on their solubility behaviour in ethyl acetate, butanol and deionized water. The sum of butanol and aqueous fractions was about 92% w/w of the total ethanol extract, indicating the high polarity properties of the chemical compounds isolated from the ethanol extract.

CGEEA, CGEBU and CGEAQ were showed good antibacterial activities against the gram positive isolates used in this study. CGEEA was more potent than the other fractions.

3.2.1.3. Antibacterial effect of CGEEA fractions

There is no clear cut to compare the antibacterial activities of the plant extracts with those of standard antibiotics or pure compounds, however there was a classification system of natural antibacterials based on their MICs which generally classified natural extracts as strong when MIC are less than 500 µg/ml, moderate 600-1500 µg/ml and weak with MIC above 1600 µg/ml (Duarte, Leme et al. 2007, Michielin, Salvador et al. 2009).

Disk diffusion assay revealed that CGEEA was more active than CGEBU and CGEAQ, therefore the active fraction CGEEA was separated further into eight fractions over silica gel column. The most potent fraction was CGEEA-F5 collected near the end of chromatography indicating the semi polar to polar nature of active materials. The fraction was rich in benzoic acid derivatives.

Gallic acid, protocatechuic acid and ethyl gallate in CGEEA-F5 were eluted together and detected using analytical HPLC, which were in agreement with GC-MS results. Preparative HPLC was used to separate the CGEEA-F5 into 12 fractions. Fractions 7, 8 and 9 were eluted in the middle of chromatography. Microdilution assay defined CGEEA-F5-8 as the most potent fraction against *S. epidermidis*, *S. aureus*, *Alcaligenes faecalis* and *E. coli*; but this fraction was not separated further due to a low quantity and very similar elution properties of its individual components.

The most active fraction CGEEA-F5-8 was derivatized to its corresponding trimethyl silyl derivatives and checked by GC-MS; as a result, three benzoic acid derivatives were detected. Analysis of CGEEA-F5-8 by TOF LC-MS showed the presence of twelve molecules with higher molecular weight than 500 Dalton.

Ethyl gallate formed about 63% of this fraction, and the antibacterial activities are due to synergistic interaction between the components present in this fraction as roughly estimated from the higher MIC of each of these compounds when used alone against the bacterial isolates compared to the MIC of the total fraction (results will be presented in Chapter IV).

Succinic acid detected in CGEEA is used as an acidity regulator in some pharmaceutical preparation. Fatty acids represent a major percentage of the extract; hexadecanoic acid (Palmitic acid), (Z,Z)-9,12-octadecadienoic acid (linoleic acid), octadecanoic acid (stearic acid), eicosanoic acid (arachidic acid), and 11-trans-octadecenoic acid (vaccenic acid) are used in the production of soaps and detergents.

Free fatty acids have general antimicrobial activity and can control the fungal cells and bacterial isolates effectively. The petroleum ether extract of *Pleurotus eous* containing fatty acids esters such as 7,10-octadecadienoic acid, methyl ester and 16-methyl-heptadecanoic acid methyl ester, showed good antibacterial effects with MIC of 4.4 μg/ml

against *S. aureus*, 3.1 µg/ml against *B. subtilis*, 4.2 µg/ml against *B. cereus*, 8.8 µg/ml against *P. aeruginosa*, 3.1 µg/ml against *E. coli* and 4.4 µg/ml against *K. pneumoniae* (Suseem, Saral 2013). Fatty acids can inhibit the biofilms formation and cease the production of toxins and harmful enzymes from the bacterial cells; they can be used as antibacterial agents where antibiotic are undesirable or forbidden (Desbois 2012).

CGEEA contains several benzoic acid derivatives like 4-hydroxy benzoic acid, 3-methoxy-4-hydroxybenzoic acid or vanillic acid, 3,4-dihydroxybenzoic acid or protocatechuic acid, 3,5-dimethoxy-4-hydroxybenzoic acid or syringic acid, 3,4,5-trihydroxybenzoic acid ethyl ester or ethyl gallate, and 3,4,5-trihydoxy benzoic acid or gallic acid, which were reported previously with antibacterial activity.

Wild mushrooms contain several phenolic acids which have a moderate antibacterial activity against methicillin sensitive and resistant *S. aureus* (MSSA and MRSA), *S. epidermidis* and *Enterococcus faecalis*. 4-hydroxybenzoic acid had MIC >1000 µg/ml against all of the microorganisms used in the study, while its 3-methoxy derivative (vanillic acid) performed a better antibacterial role with MIC of 500 µg/ml against MRSA and >1000 µg/ml against other bacterial isolates, 3,4-dihydroxybenzoic acid or protocatechuic acid showed MIC 1000 µg/ml against both MRSA and MSSA and >1000 µg/ml against the other two types of bacteria. 3,5-dimethoxy-4-hydroxybenzoic acid or syringic acid showed MIC of 500 µg/ml against MRSA and >1000 against the other three isolates, and gallic acid showed MIC >1000 µg/ml against all of the four bacteria (Alves, Ferreira et al. 2013).

Caffeic acid, protocatechuic acid and chlorogenic acid are three phenolic compounds isolated from *Coffea arabica*. They showed MIC of 2000 µg/ml against *Streptococcus mutans* a gram positive bacterial isolate responsible for tooth decay (Almeida, Naghetini et al. 2012). Other study showed that caffeic, vanillic and protocatechuic acids showed MIC

of 300 µg/ml against two gram negative bacteria *E. coli* and *K. pneumoniae* (Aziz, Farag et al. 1998).

Several phenolic acids from wine, like gallic acid, ethyl gallate, and caffeic acid showed the greatest antimicrobial activity against *S. aureus* ATCC25923 with IC_{50} 18.9, 257, 319 µg/ml, respectively. Their IC_{50} against *Enterococcus faecalis* V583 were 440, 484 and 975 µg/ml respectively. *E. faecalis*, a gram positive lactobacillus, can cause dangerous infections to patients in hospital wards (Cueva, Mingo et al. 2012).

Gallic acid found naturally abundant in beer at a concentration of 0.6 mg/kg and 35 mg/ kg of wine is used in food industry as food additive or flavoring agent from 1- 500 mg/kg and maximum 5-2000 mg/kg because of its ability to inhibit the growth of *S. aureus* ([EFSA] European Food Safety Authority 2010).

3.2.1.4. SEM results

Gallic acid and ethyl gallate were tested for their effects on *S. aureus* cell wall. Double concentration of MIC of these two compounds was used here and the fixation process started within the first hour of incubation with the antibacterial agents, the reason for this is the quick and complete destruction of cells within 3 hours of incubation, indicating the necessity for fast fixation to follow the effect of antibacterials against *S. aureus*.

Gallic acid and ethyl gallate increase the permeability of the cell wall and enhance the leakage of cell contents to end with collapsed cells; gallic acid activity was more prominent than ethyl gallate ability within the first hour of incubation.

Previous study on mechanism of action of gallic acid showed that gallic acid increased the cell wall permeability, decreased the negative surface charge and formed pores in the cell wall of *S. aureus*, indicating that gallic acid might link several targets in the bacterial cell wall (Borges, Ferreira et al. 2013). Those results were agreed with our results, as we have observed the roughness on *S. aureus* cell wall and leakage of cell contents.

3.2.2. Antiplasmodial activities of Cylicodiscus gabunensis

In order to identify the antiplasmodial compounds from CG bark we used hexane and 70 % ethanol to extract the non-polar materials and polar components, respectively, butanol fraction had most of the materials from the ethanol extract around 77% w/w, except benzoic acid derivatives which were extracted efficiently by ethyl acetate. Hexa- and octadecanoic acids represented about 40% of this fraction. Plants with possible anti-malarial activities are usually extracted by ethanol (Okokon, Ita et al. 2006, Vale, Vilhena et al. 2015). The results indicated that the ethanol extract has more potent activity over the hexane extract, which comes in agreement with the previous research which used an ethanolic extract of CG in an *in vivo* study in mice infected with *P. berghei* (Okokon, Ita et al. 2006).

Several phenolic acids in fractions of CGEBU and CGEEA detected by GC-MS analysis were assayed for their antiplasmodial activities, and the results indicated that they contributed to the antiplasmodial activity of these fractions, those results will be discussed in details in Chapter IV.

Bioassay guided fractionation gave an HPLC fraction (CGBU-F10-7) which was the most active and ranked as a highly active extract. Plant extracts with possible anti-malarial activities were classified to highly active with $IC_{50} \leq 5$ µg/ml, promisingly active 5.1-10 µg/ml, good activity 10.1-20 µg/ml, moderate activity 20.1-40 µg/ml, marginal potency 40.1-70 µg/ml and poor or inactive 70.1 to > 100 µg/ml (Singh, Kaushik et al. 2015).

Although different columns such as C8 and amino-derived columns have been tried to isolate single pure components for structural elucidation and evaluation its biological activity, unfortunately this was not successful probably because those components may have very close physiochemical properties. Therefore we had to characterize the most active fraction CGEBU-F10-7 as a mixture.

LC-MS of CGEBU-F10-7 indicated the presence of higher molecular weight compounds between 1000 and 1500 Da. After acid hydrolysis and TMSi derivation, GC-MS analysis indeed revealed that the presence of gallic acid and several monosaccharides (D-glucose, L-arabinose, and L-rhamnose), where the peaks for glucose is apparently higher than other sugars peaks.

High resolution ^{1}H NMR indicated the presence of sugar and aromatic protons as well as aliphatic protons. These results suggested that conjugates of oligosaccharide with gallic acid or terpenoids might be present in this mixture. However, the exact linkage between the monosaccharides and gallic acid is unknown. Previously an oligosaccharide derivative (a resin glycoside, crypthophilic acid C) from *Scrophularia cryptophila* showed potent antiplasmodial activity against *P. falciparum* with IC_{50} (4.2 μg/ml) (Tasdemir, Brun et al. 2008).

Ellagic acid (a dimer of gallic acid) and a galloyl glycoside from *Tristaniopsis calobuxus* bark extract had very potent antiplasmodial activities against *P. falciparum* IC_{50} values of 0.5 and 3.2 μM, respectively (Verotta, Dell'Agli et al. 2001). 1-O-galloyl-6-O-luteoyl-α-D-glucose from *Phyllanthus niruri* also showed very low IC_{50} (1.4 μg/ml) against *P. falciparum* (Subeki, Matsuura et al. 2005). All these indicate that galloyl moiety plays an important role in the inhibition of *P. falciparum*. The structures of crypthophilic acid C and 1-O-galloyl-6-O-luteoyl-α-D-glucose are shown in (Figure 3.29).

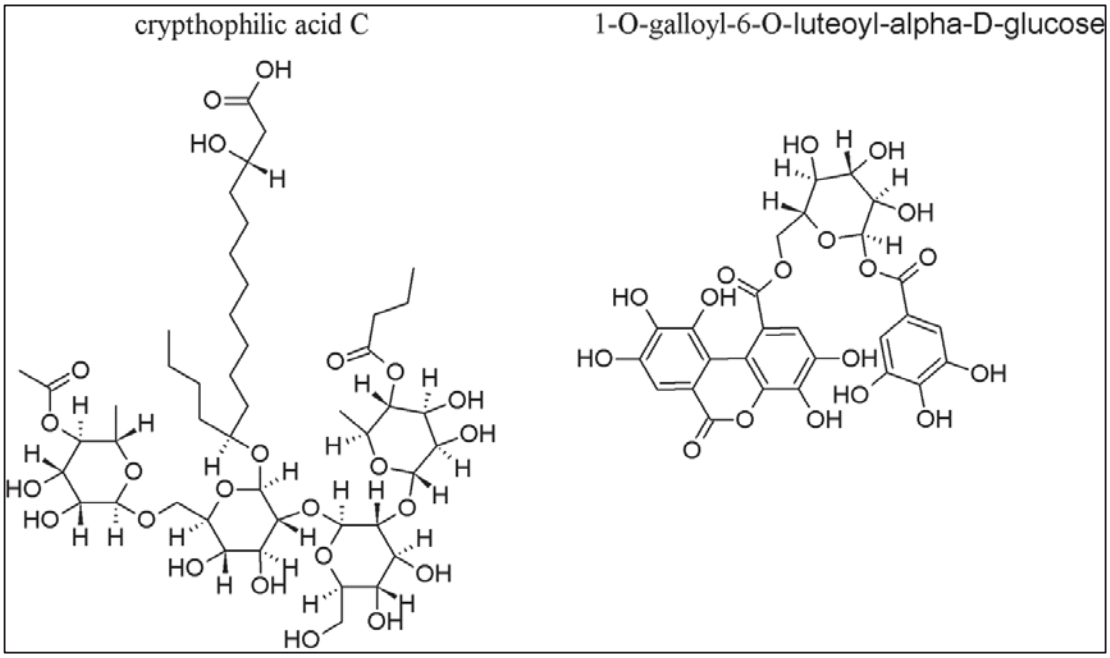


Figure 3.29: The structures of crypthophilic acid C and 1-O-galloyl-6-O-luteoyl-α-D-glucose.

Furthermore, HR LC-MS showed presence of a mass of 1044.5522 which matched to the molecular mass (calcd. 1044.5505) of a known gabunoside, 3-O-[β-L-arabinopyranosyl-(1-2)-α-L-arabinopyranosyl-(1-3)-β-D-glucospyranosyl] maslinic acid-28-α-L-rhamnosyl ester from CG (Tene, Chabert et al. 2011). However, further confirmation using the standard sample is needed.

Chapter 4 : Results and discussion of the antibacterial and antiplasmodial activities of several benzoic acid derivatives.

4.1. Results

4.1.1. Synthesis of alkyl and alkenyl 3,4,5-trihydroxybenzoates

Initially, chemical screening using GC-MS was applied to both CGEEA and CGEBU fractions (Chapter III, **3.1.2.1.2** and **3.3.2.1.**) in order to quickly identify the possible antibacterial and antiplasmodial compounds. As a result, 3,4,5-trihydroxybenzoic acid (gallic acid) and several fatty acids were detected in both CGEEA and CGEBU. In addition, 4-hydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid (vanillic acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), syringic acid, and 3,4,5-trihydroxybenzoic ethyl ester (ethyl gallate) were detected from CGEEA.

Benzoic acid derivatives may play an important role in the antibacterial and antiplasmodial activities of *Cylicodiscus gabunensis*, here we prepared *de novo* several 3,4,5-trihydroxybenzoates for this study and several benzoic acid derivatives were available from commercial sources to test for antibacterial and antiplasmodial activities. The possible application of those materials as food preservatives and antibiotic resistance modification agents will be discussed.

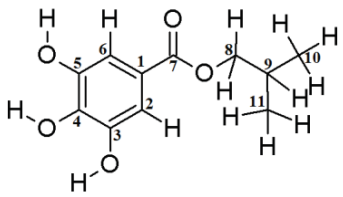
Five alkyl or alkenyl 3,4,5-trihydroxybenzoates were synthesized according to section **2.22** and purified according to section **2.23**. White crystals were collected for all compounds with yields exceeding 80%. The yields were as follows 583 mg (85%) of 2-methylpropyl 3,4,5-trihydroxybenzoate, **1;** 597 mg (82%) of 2- methylbutyl 3,4, 5-trihydroxybenzoate, **2;** 606 mg (84%) of 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate, **3;** 604 mg (83%) of

3-methylbutyl 3,4,5-trihydroxybenzoate,**4;** 534 mg (83%) of propan-2-yl 3,4,5-trihydroxybenzoate, **5**. The molecular weights of the synthesised esters were 226, 240, 238, 240, and 212 g/mol respectively.

4.1.2. NMR, positive-ion TOF LC-MS and GC-MS of the synthesised benzoate derivatives

The aromatic type structures of the synthesised 3.4.5-trihydroxybenzoates (**1-5**) were established by spectroscopic analysis. NMR experiments were conducted according to section 2.14. The compounds were prepared in d_4-methanol according to section **2.14**.

1

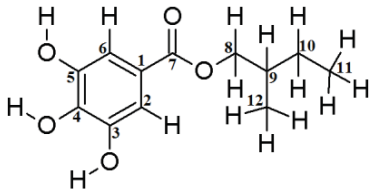


1: 2-methylpropyl 3,4,5-trihydroxybenzoate

^{1}H NMR (300MHz, METHANOL-d_4) δ = 7.08 (2H, s, H-2, 6), 4.02 (2H, d, *J* = 6.6 Hz, H-8, 8), 2.04 (1H, m, H-9), 1.03 (6H, d, *J* = 6.6 Hz, H-10, 11).

^{13}C NMR (101MHz, METHANOL-d_4) δ = 167.2 (C-7), 145.1 (C-3, 5), 138.3 (C-4), 120.3 (C-1), 108.6 (C-2, 6), 70.3 (C-8), 27.8 (C-9), 18.1 (C-10, 11).

2

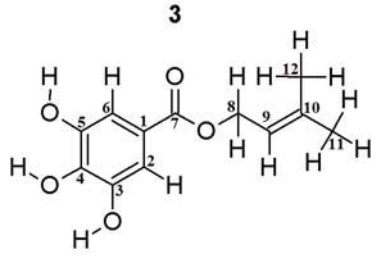


2: 2- methylbutyl 3,4,5-trihydroxybenzoate

^{1}H NMR (300MHz, METHANOL-d_4) δ = 7.07 (2H, s, H-2, 6), 4.02 - 4.16 (2H, m, H-8, 8), 1.79 - 1.87 (1H, m, H-9), 1.45-1.55 (1H, m, H-10), 1.26 - 1.34 (1H, m, H-10), 1.02 (3H, d, *J* = 6.8 Hz, H-12), 0.95 (3H, t, *J*=7.45 Hz, H-11).

^{13}C NMR (101MHz, METHANOL-d_4) δ = 167.2 (C-7), 145.1 (C-3, 5), 138.3 (C-4), 120.3 (C-1), 108.6 (C-2, 6), 68.8 (C-8), 34.3 (C-9), 25.8 (C-10), 15.5 (C-12), 10.3 (C-11).

3

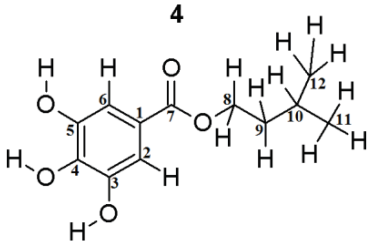


3: 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate

^{1}H NMR (300MHz, METHANOL-d_4) δ = 7.05 (2H, s, H-2, 6), 5.45 (1H, m, H-9), 4.74 (2H, d, *J* = 7.2 Hz, H-8, 8), 1.79 (3H, s, H-11), 1.82 (3H, s, H-12).

^{13}C NMR (101MHz, METHANOL-d_4) δ = 167.1 (C-7), 145.1 (C-3, 5), 138.6 (C-10), 138.3 (C-4), 120.4 (C-1), 118.7 (C-9), 108.6 (C-2, 6), 61.0 (C-8), 24.5 (C-12), 16.7 (C-11).

4

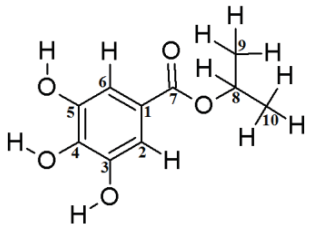


4: 3-methylbutyl 3,4,5-trihydroxybenzoate

^{1}H NMR (300MHz, METHANOL-d4) δ = 7.06 (2H, s, H-2, 6), 4.27 (2H, t, *J* = 6.6 Hz, H-8,8), 1.64 (2H, q, *J* = 6.7 Hz, H-9), 1.25 (1H, m, H-10), 0.99 (6H, d, *J* = 6.6 Hz, H-11, 12).

^{13}C NMR (101MHz, METHANOL-d4) δ = 167.2 (C-7), 145.1 (C-3, 5), 138.3 (C-4), 120.3 (C-1), 108.6 (C-2, 6), 62.8 (C-8), 37.3 (C-9), 25.0 (C-10), 21.5 (C-11, 12).

5



5: propan-2-yl 3,4,5-trihydroxybenzoate

^{1}H NMR (300MHz, METHANOL-d_4) δ = 7.06 (2H, s, H-2, 6), 5.09 - 5.17 (1H, m, H- 8), 1.34 (6H, d, *J* = 6.4 Hz, H-9, 10).

^{13}C NMR (101MHz, METHANOL-d_4) δ = 166.7 (C-7), 145.0 (C-3, 5), 138.2 (C-4), 120.8 (C-1), 108.6 (C-2,6), 67.8 (C-8), 20.8 (C-9,10).

The positive-ion TOF LC-MS was done according to section **2.12**. The positive-ion ESI-MS of 3.4.5-trihydroxybenzoates (1-5) generated the following pseudo-molecular $[M+H]^+$ ions at *m/z*: 227.0917, **1**; 241.1073, **2**; 239.0922, **3**; 241.1071, **4**, $[M+Na]^+$ ion at *m/z*: 235.0576, **5**.

Using GC-MS, samples were prepared according to section **2.8.5.2** and analyzed according to section **2.8.1**. The molecular peaks of the trimethylsilyl derivatives of the synthesized

compounds were detected at *m/z*: 442.3, **1-TMSi**; 456.3, **2-TMSi**; 454.2, **3-TMSi**; 456.3, **4-TMSi**; and 428.2, **5-TMSi**.

(Figures C1-C10) in Appendix C show the ^{1}H and ^{13}C NMR data with detailed structures of compounds (1-5). (Figure A-3) in appendix A shows the positive mode ESI-MS of the synthesized compounds. (Figure A-4) in appendix A shows the EI-MS of compounds (1-5) trimethylsilyl derivatives. The esters synthesized were run on C18 column according to section **2.24** and RT recorded. (Table 4.1) shows the RT of those compounds in three different systems, TOF LC-MS, HPLC, and GC-MS.

Table 4.1: The retention time in min of the five synthetic benzoate esters in three different columns using three different techniques, LC-MS, HPLC and GC-MS. Analysis has been done in triplicate, n=3 according to sections 2.12, 2.24, and 2.8.1 respectively.

Compound	RT using LC-MS	RT using HPLC	RT using GC-MS
1	4.245	22.409	20.592
2	4.980	25.368	21.440
3	4.523	23.353	21.798
4	5.007	25.346	21.396
5	5.720	18.107	19.141

4.1.3. Antibacterial activity of gallic acid and other structurally related molecules

To study the effects of carboxylic acid esterification, O-methylation and dehydroxylation of gallic acid on the antibacterial activities of this phenolic acid detected in CGEEA, section **3.1.2.1.2.,** five phenolic esters **(**2-methylpropyl 3,4,5-trihydroxybenzoate, **1**; 2-methylbutyl 3,4,5-trihydroxybenzoate, **2**; 3-methylbut-2-en-1-yl 3,4, 5-trihydroxybenzoate, **3**; 3-methylbutyl 3, 4, 5-trihydroxybenzoate, **4**; propan-2-yl 3,4,5-trihydroxybenzoate, **5)**,

in addition to seven phenolic acids and esters of phenolic acids available from commercial sources **(**3,4,5-trihydroxybenzoic acid, **6**; 3,4-dihydroxybenzoic acid, **7**; 4-hydroxy-3-methoxybenzoic acid, **8**; 3,4,5- trimethoxybenzoic acid, **9**; Ethyl 3,4,5-trihydroxybenzoate, **10**; Propyl 3,4,5-trihydroxybenzoate, **11**; and Dodecyl 3,4,5-trihydroxybenzoate, **12)** were prepared in 3% DMSO, dodecyl 3,4,5-trihydroxybenzoate was prepared in 0.5 % Tween 20, at eight different double diluted concentrations 1000-8 μg/ml and MICs of those compounds against several bacterial isolates were determined according to section **2.17**, **(**Table 4.2) shows the MIC of several phenolic compounds. The structures and IUPAC names of the synthesized compounds (1-5) and of compounds (6-12) available from commercial sources are shown in (Figure 4.1).

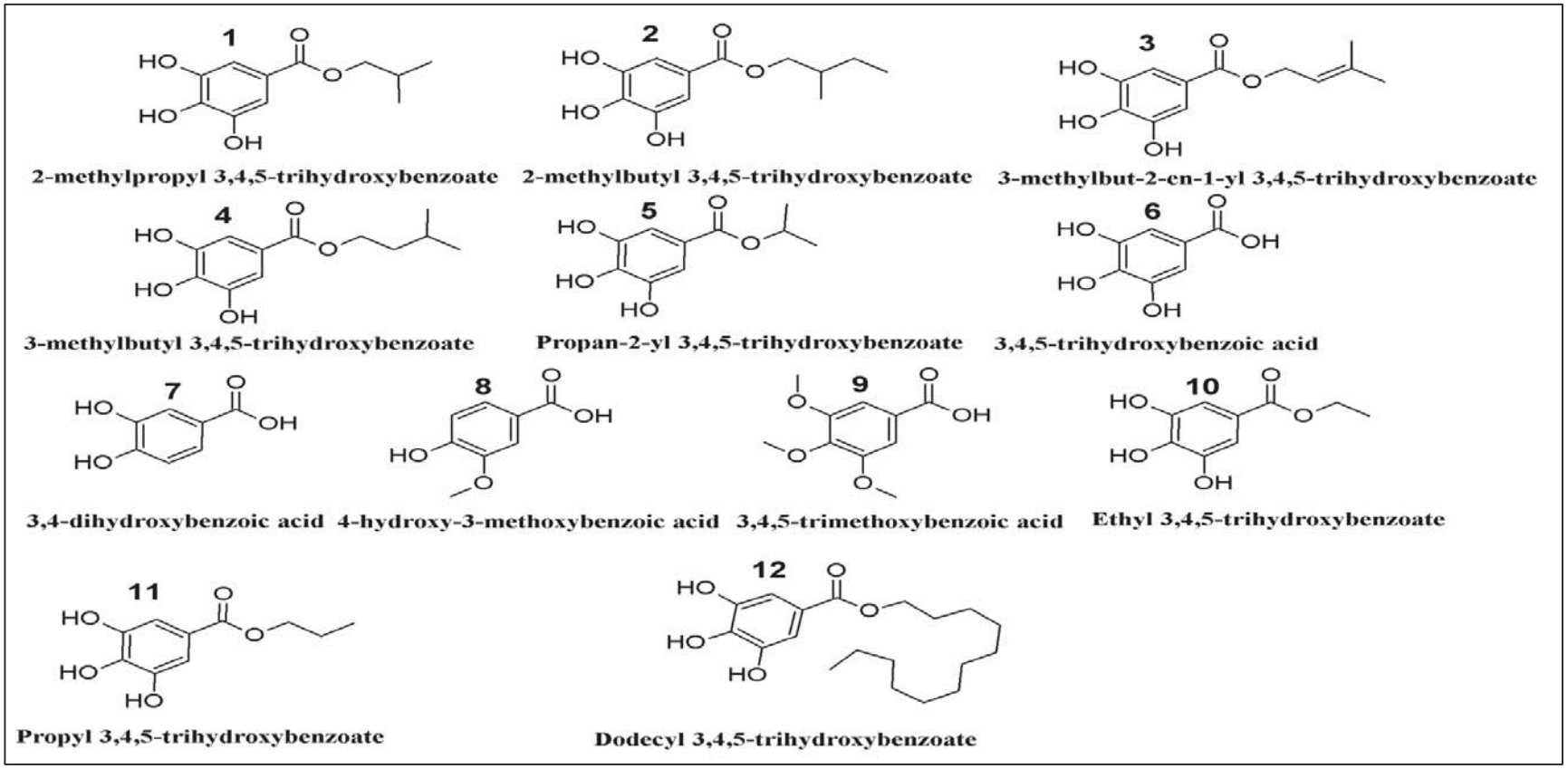


Figure 4.1: structures of several phenolic acids and phenolic acid esters, compounds (**1-12**).

Table 4.2: Gallic acid and other structurally related molecules (MICs) assayed according to section 2.17, against several gram-positive and negative bacterial isolates. Commercially sourced compounds or those synthesized for this study (marked with*). The calculated Log Partition coefficient (Log P) using ChemBioDraw software and antibacterial activities MIC in µg/ml (mean ± SD from n=4 biological replicates) are shown in the table.

Compound	*S. aureus*	*S. epidermidis*	*E. coli*	*B. subtilis*	*B. cereus*	*A. faecalis*	Log *P*
2-methylpropyl 3,4,5-trihydroxybenzoate (**1**)*	500	250	500	1000	1000	500	0.42
2- methylbutyl 3,4,5-trihydroxybenzoate (**2**)*	250	250	250	250	500	250	0.81
3-methylbut-2-en-1-yl 3,4, 5-trihydroxybenzoate (**3**)*	250	250	500	500	1000	500	1.08
3-methylbutyl 3, 4, 5-trihydroxybenzoate (**4**)*	250	250	250	250	500	500	1.21
propan-2-yl 3,4,5-trihydroxybenzoate (**5**)*	1000	250	62.5	>1000	>1000	250	1.02
3,4,5-trihydroxybenzoic acid (**6**)	2000	500	1000	1000	1000	1000	1.51
3,4-dihydroxybenzoic acid (**7**)	1000	500	1000	1000	1000	500	1.34
4-hydroxy-3-methoxybenzoic acid (**8**)	1000	1000	1000	1000	1000	500	1.91
3,4,5- trimethoxybenzoic acid (**9**)	1000	1000	1000	1000	1000	500	2.33
Ethyl 3,4,5-trihydroxybenzoate (**10**)	1000	250	62.5	1000	1000	250	1.92
Propyl 3,4,5-trihydroxybenzoate (**11**)	500	250	125	1000	1000	250	2.26
Dodecyl 3,4,5-trihydroxybenzoate (**12**)	8	8	>1000	31.25	31.25	1000	5.27
Ampicillin	≤0.5	≤0.5	≤0.5	≤0.5	>64	16	1.35

4.1.4. Effects of 2-methylpropyl 3, 4, 5-trihydroxybenzoate and propan-2-yl 3,4,5-trihydroxybenzoate on S. aureus cell wall

Compounds 2-methylpropyl 3,4,5-trihydroxybenzoate, (**1)** and propan-2-yl 3,4,5-trihydroxybenzoate, (**5)** were selected as the least active compounds of the synthesised esters against *S. aureus*. They were tested for their effects on *S. aureus* cell wall and their effects studied using SEM according to the method mentioned in section **2.19**. Compounds 2-methylpropyl 3,4,5-trihydroxybenzoate, (**1)** and propan-2-yl 3,4,5-trihydroxybenzoate, (**5)** have prominent effects on *S. aureus* cell wall integrity as displayed in (Figure 4.2).

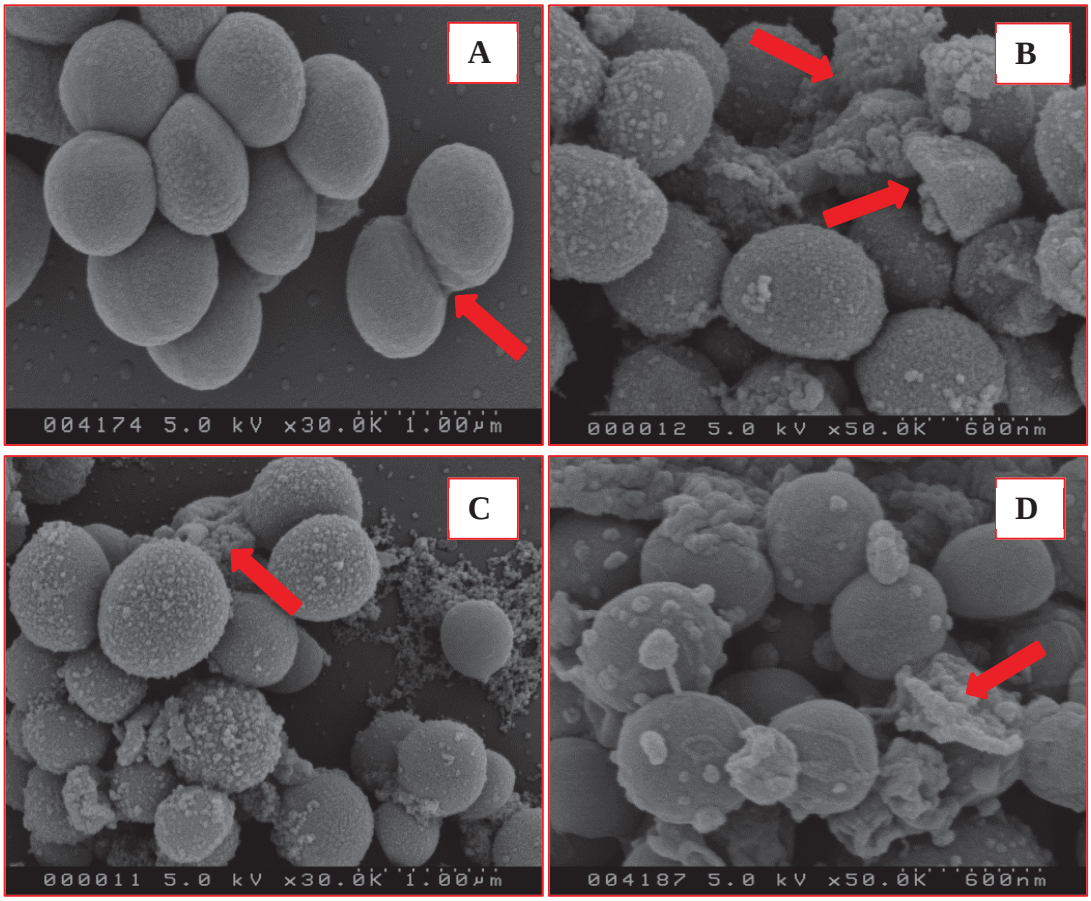


Figure 4.2: The effects of 2-methylpropyl 3,4,5-trihydroxybenzoate, **(1)** and propan-2-yl 3,4,5-trihydroxybenzoate , (**5**) against *S. aureus*. A: *S. aureus* control, arrows pointing to the cell divisions; B and C: micrography shows the effects of **1** on *S. aureus* cell wall, arrows pointing to the collapsed cells and leakage of cellular contents; D: micrography shows the effects of **5** against *S. aureus*, arrows pointing to the exudates from the bacterial cell walls. The assay has been done according to section 2.19.

4.1.5. Antiplasmodial activities of gallic acid and other structurally related molecules

Several benzoic acid derivatives (**1-12**) which were mentioned in section **4.1.2** were tested for their antiplasmodial activity according to method mentioned in section **2.21**, the dose-response curves were sketched using Graph pad prism 6 and IC_{50} s were calculated in µM and µg/ml. (Table 4.3) shows the antiplasmodial potentials of several benzoic acid derivatives and (Figures 4.3-4.4) show the dose-response curve of several benzoic acid derivatives against *P. falciparum* Dd2.

Table 4.3: The antiplasmodial activities of several benzoic acid derivatives. IC_{50} (mean ± SD from n=3 biological replicates. Commercially sourced compounds or those synthesized for this study (marked with*). The IC_{50} of Chloroquine against *P. falciparum* Dd2 was 0.171 µM. The assay has been done according to section 2.21.

Compound name	**IC_{50} ± SD in µg/ml (µM)**
2-methylpropyl 3,4,5-trihydroxybenzoate (1)*	3.8 ± 0.5 (16.9 ± 2.3)
2- methylbutyl 3,4,5-trihydroxybenzoate (2)*	2.8 ± 0.3 (11.8 ± 1.2)
3-methylbut-2-en-1-yl 3,4, 5-trihydroxybenzoate (3)*	4.1 ± 0.5 (17.5 ± 2.2)
3-methylbutyl 3, 4, 5-trihydroxybenzoate (4)*	2.1 ± 0.4 (8.8 ± 1.5)
propan-2-yl 3,4,5-trihydroxybenzoate (5)*	3.0 ± 0.4 (13.9 ± 1.8)
3,4,5-trihydroxybenzoic acid (6)	4.5 ± 0.7 (26.8 ± 4.4)
3,4-dihydroxybenzoic acid (7)	12.9 ± 1.5 (83.4 ± 10.0)
4-hydroxy-3-methoxybenzoic acid (8)	11.97 ± 1.8 (71.2 ± 11.0)
3,4,5- trimethoxybenzoic acid (9)	23.8 ± 3.2 (112.3 ± 15.2)
Ethyl 3,4,5-trihydroxybenzoate (10)	1.8 ± 0.6 (9.3 ± 3.1)
Propyl 3,4,5-trihydroxybenzoate (11)	5.2 ± 0.6 (24.5 ± 2.9)
Dodecyl 3,4,5-trihydroxybenzoate (12)	0.7 ± 0.1 (2.2 ± 0.2)

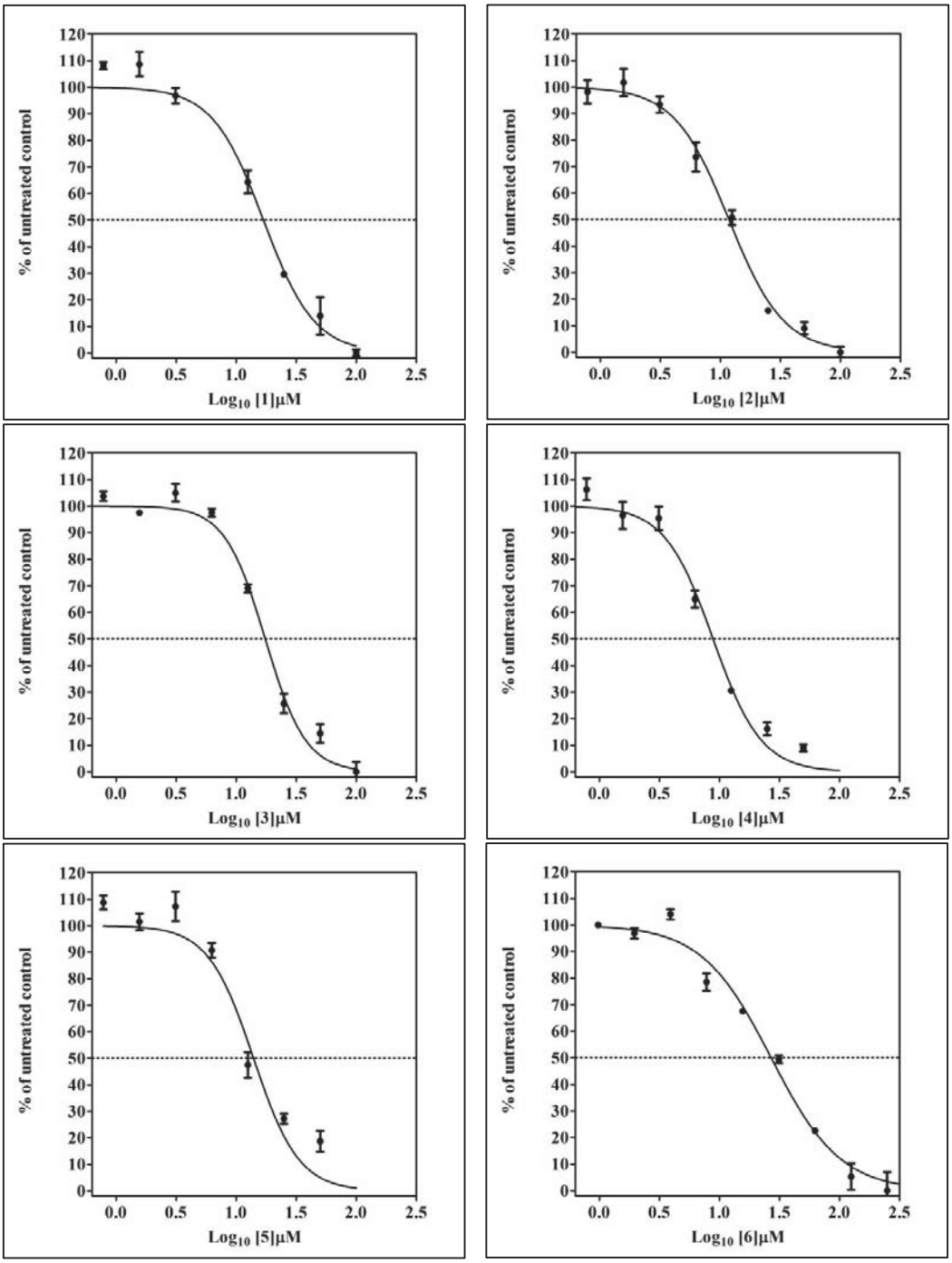


Figure 4.3: Dose-response curve of antiplasmodial activity against *P. falciparum* Dd2, compounds (**1-6**). 2-methylpropyl 3,4,5-trihydroxybenzoate, **1**; 2- methylbutyl 3,4,5-trihydroxybenzoate, **2**; 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate, **3**; 3-methylbutyl 3,4,5-trihydroxybenzoate,**4**;propan-2-yl 3,4,5-trihydroxybenzoate,**5**;3,4,5 trihydroxybenzoic acid, **6**. The assay has been done according to section 2.21, in triplicate n=3. Chloroquine IC_{50}= 0.171 µM.

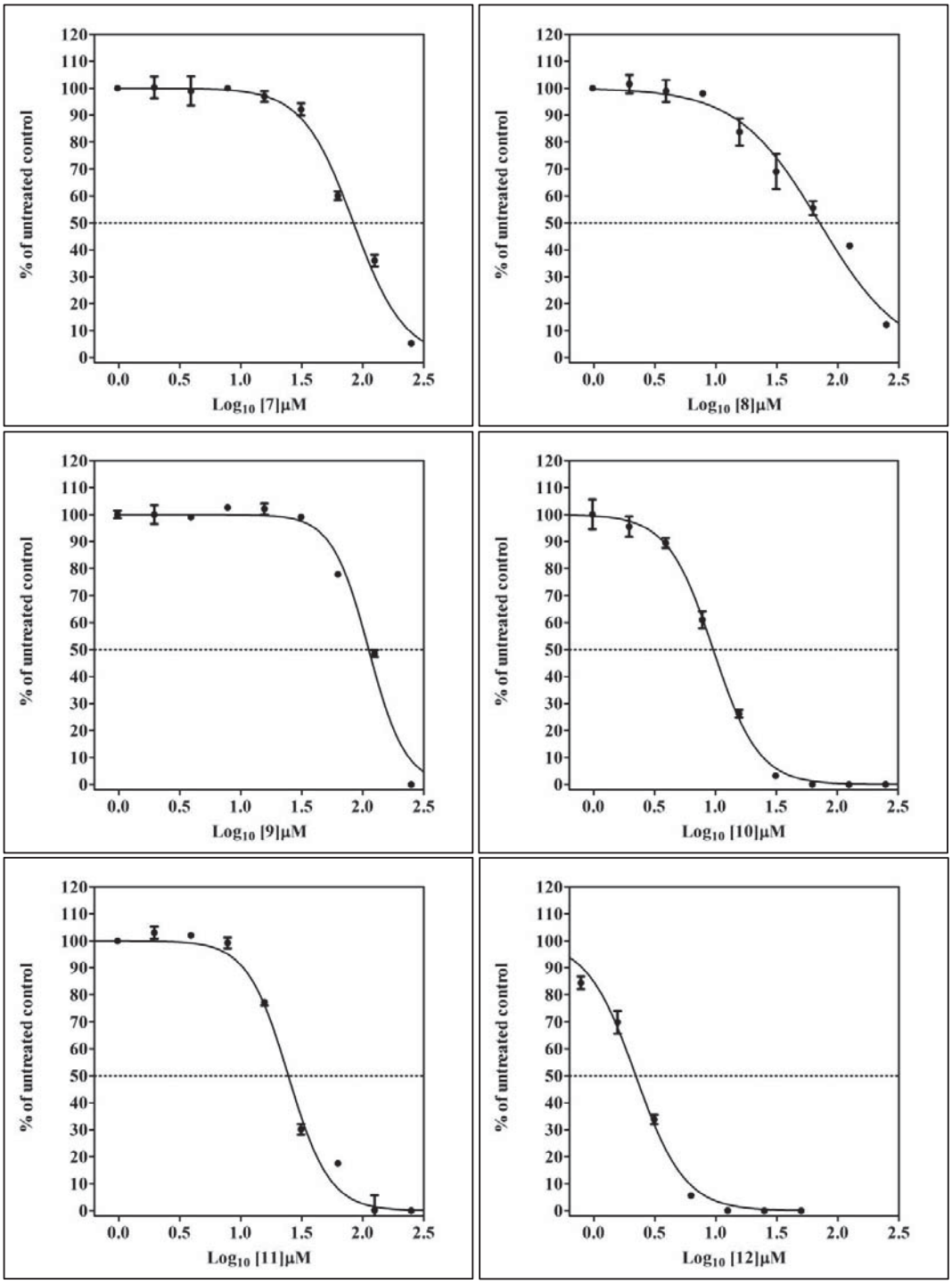


Figure 4.4: Dose-response curve of anti-malarial activity against *P. falciparum* Dd2, compounds (**7-12**). 3,4-dihydroxybenzoic acid,**7**; 4-hydroxy-3-methoxybenzoic acid, **8**; 3,4,5-trimethoxybenzoic acid,**9**; ethyl 3,4,5-trihydroxybenzoate, **10**; propyl 3,4,5-trihydroxybenzoate, **11**; and dodecyl 3,4,5-trihydroxybenzoate, **12**. The assay has been done according to section 2.21, in triplicate n=3. Chloroquine IC_{50}= 0.171 µM.

4.2. Discussion

4.2.1. Synthesis of alkyl and alkenyl 3,4,5-trihydroxybenzoates

Benzoates can be synthesised from 3,4,5-trihydroxybenzoyl chloride, the procedure was utilized excess of thionyl chloride. Production yields for esters from primary and secondary alcohols were 72% and 55%, respectively (Khatkar, Nanda et al. 2013). The other method is by heating gallic acid with nine times excess of alkanol with sulphuric acid, and the yield was 77% (Khatkar, Nanda et al. 2013). Esters can be synthesised using a coupling reagents like N,N'-dicyclohexylcarbodiimide (DCC), and this reaction needs some base as a catalyst (Gilles, Vieira et al. 2015).

Kubo et al. (2002) synthesized esters of gallic acid utilizing 4 mM of DCC as a coupling agent and 1 mM of each gallic acid and alcohol, however, the purification steps were very tedious and contained many step of washing with citric acid, alkali and water before using the column chromatography (Kubo, Xiao et al. 2002).

The synthetic approach we adapted here was a modified sequence of steps, which used excess of alcohol (4 mmol), 3 mmol of gallic acid and 3.5 mmol of DCC. This combination enabled us to avoid the unnecessary excess of coupling reagent which complicated the purification steps as we noticed from our synthesis.

Our procedure did not use several washing steps. Column chromatography using an isocratic solvent system of a mixture of ethyl acetate and hexane was used for the purification of products; the yields were high and exceeded 80% for all derivatives even for propan-2-yl 3,4,5-trihydroxybenzoate compared to the 55% yield when using 3,4,5-trihydroxybenzoyl chloride (Khatkar, Nanda et al. 2013).

Using a coupling agent in the presence of a base was reported; the proposed mechanism is the formation of acid anhydride by the effect of coupling reagent and base (Gilles, Vieira et al. 2015). (Figure 4.5) shows the mechanism of ester formation via acid anhydride,

modified from (Gilles, Vieira et al. 2015). In our synthesis scheme we did not use a base; we speculate that the conversion of two moles of gallic acid into one mole of gallic acid anhydride is spontaneous in the presence of DCC and excess amount of alcohol, however, detailed study of all the products and intermediates is essential to confirm this.

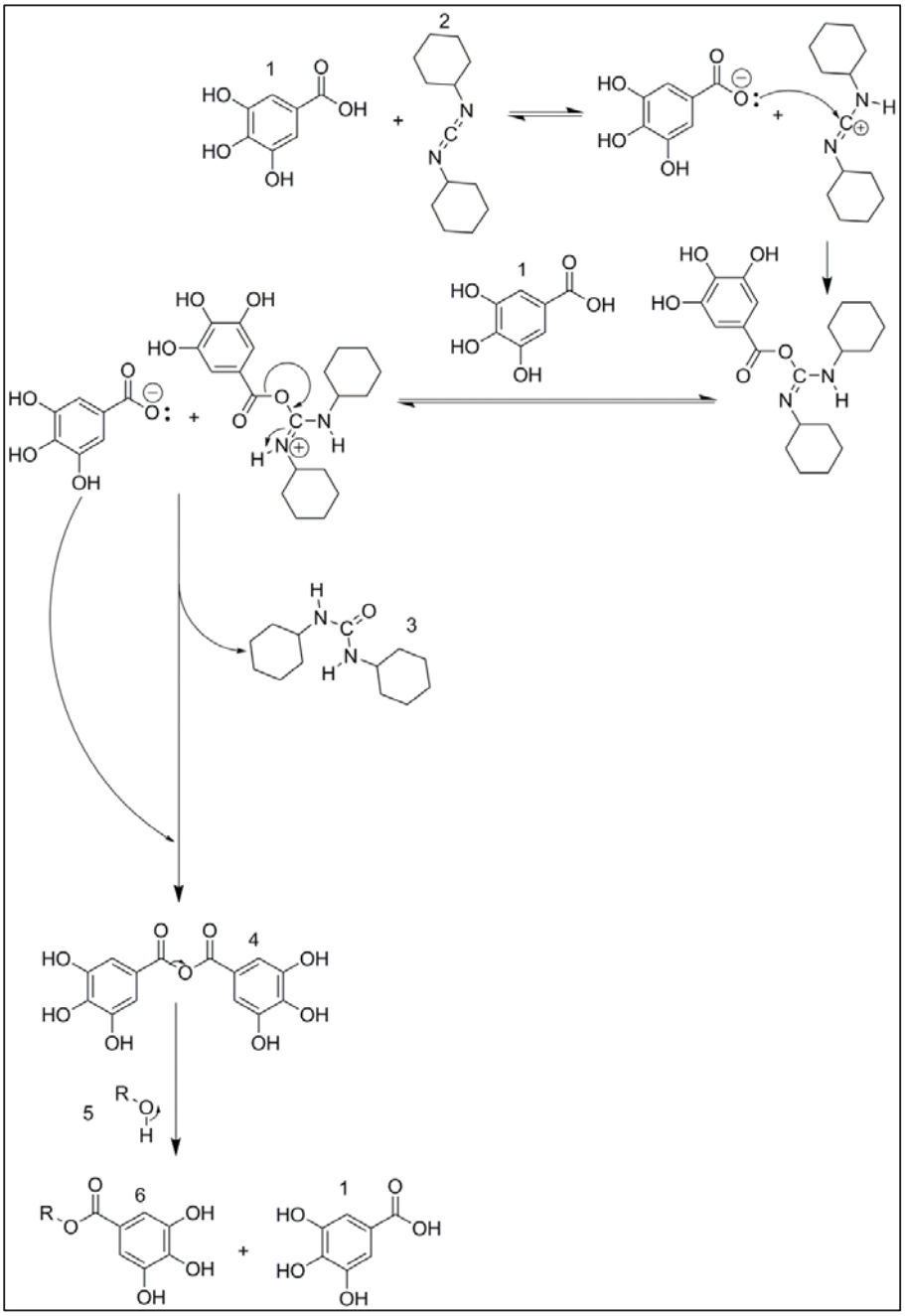


Figure 4.5: Mechanism of benzoate ester formation. Modified from (Gilles, Vieira et al. 2015). Compounds are gallic acid, **1**; DCC, **2**; dicyclohexylurea, **3**; gallic anhydride, **4**; alcohol, **5**; benzoate esters, **6**.

4.2.2. NMR

n-heptyl gallate, n-nonyl gallate, n-decyl gallate, and n-undecyl gallate were synthesised before and their ^{1}H NMR showed a chemical shift = 7.07 ppm for the two aryl protons (H-2,6) (Shibata, Kondo et al. 2005) which was in agreement with our results. The ^{1}H NMR spectra of 2-methylpropyl 3,4,5-trihydroxybenzoate were in good agreement with the ^{1}H NMR of isobutyl benzoate, recorded on Varian T-60 spectrometer and CCl_4 as a solvent. Isobutyl benzoate was showed proton chemical shifts as follows δ 0.97 (d, *J* = 6.7 Hz, 6 H), 2.02 (m, 1 H), 4.10 (d, *J* = 6.7 Hz.2 H), 7.4 (m, 3 H), 8.0 (m, 2 H) (Rossi, De Rossi 1974), supported the characterization of 2-methylpropyl 3,4,5-trihydroxybenzoate. The structures of isobutyl benzoate and 2-methylpropyl 3,4,5-trihydroxybenzoate are shown in (Figure 4.6).

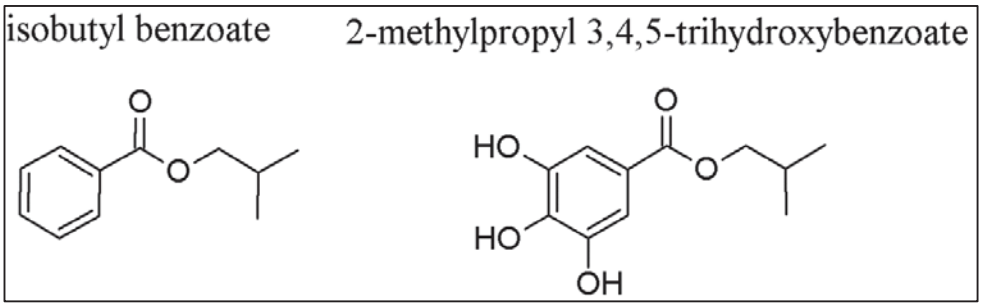


Figure 4.6: The structures of isobutyl benzoate and 2-methylpropyl 3,4,5-trihydroxybenzoate.

For 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate (**3**), the proton (H-9) attached to C-9 appeared at δ = 5.45 ppm (m, H-9) instead of doublet, due to two types of coupling; a coupling with the two geminal protons (CH_2-8), and a long range coupling with the six protons of the two methyl groups (CH_3-11/12). In return, the six protons attached to carbon number 11 and 12 appeared as two adjacent singlets δ = 1.79 (3H, s, H-11), δ = 1.82 (3H, s, H-12) instead of one singlet, reflecting the different chemical environments of these two

methyl groups in the molecule. The structure of 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate is shown in (Figure 4.7).

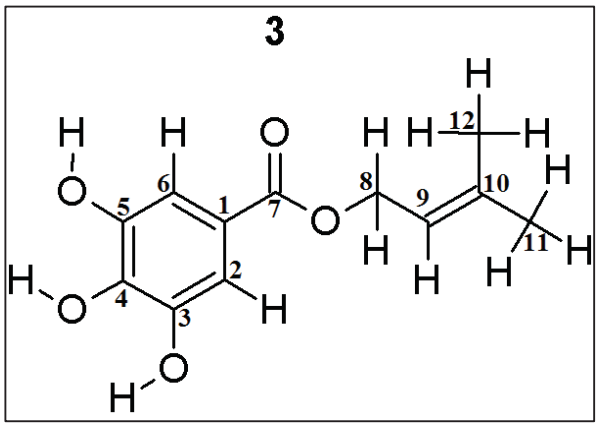


Figure 4.7: The structure of 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate.

Interestingly, ^{13}C NMR of 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate (**3**) shows the different chemical shifts for CH_3-12 (24.5 ppm) and CH_3-11 (16.7 ppm) probably due to their different chemical environments resulted from the alkene preventing the free rotation around the bond.

4.2.3. Antibacterial activity of several benzoic acid derivatives

Foodstuffs are targets to many pathogenic bacteria like *S. aureus*, *E. coli*, *B. cereus* and *Pseudomonas spp*. which are the major causes of food poisoning. *S. aureus* gastroenteritis is one of the major food hygiene problems (Seo, Boach 2007); dairy products inoculated with Shiga toxin producers (*E. coli*) present another problem in food preservation and probably cause an outbreak of infection between people (Nataro, Bopp et al. 2007). Food

packaging companies are seeking new kinds of preservatives to improve the products safety and phenolics are kinds of promising food preservatives.

Phenolic compounds have antibacterial activities with more effectiveness against gram positive than gram negative bacteria (López-Malo Vigil, Palou et al. 2005). One of those phenolic acids detected in CGEEA and CGEBU (Chapter III, **3.1.2.1.2** and **3.3.2.1.**) is gallic acid which has MIC equal to 100-1600 μg/ml against different *S. aureus* strains (Gutiérrez-Larraínzar, Rúa et al. 2012).

Phenolics are antioxidants and their hydroxyl number, arrangement and structural conjugation determine antioxidant abilities (Rice-Evans, Miller et al. 1996), those effects give them advantage as possible food additives. Previous studies showed that antibacterial activity increases with degree of hydroxylation on the ring, however, this assumption is not always true (Cowan 1999). Several benzoates are approved to use in Europe as food additives due to their antioxidant activities, like propyl gallate, octyl gallate, and dodecyl gallate which were coded as E310, E311, and E312 respectively (Gov.UK 2016).

The results showed that esterification of gallic acid with alkyl or alkenyl group with four carbons or more increased the antibacterial activity against *B. cereus* and *B. subtilis* compared to gallic acid (MIC 1000 μg/ml). The most prominent anti-bacillus agent was lauryl gallate (MIC 31.25 μg/ml). The effect of the esterified gallic acid was more pronounced on both *Staphylococcus* species used in this research. *S. epidermidis* was more liable to the compounds used, the order of activity was as following gallic acid, protocatechuic acid, vanillic acid, and 3,4,5-trimethoxybenzoic acid< ethyl gallate, propyl and isopropyl gallate < lauryl gallate and other esters used. Lauryl gallate showed potent effect against *S. aureus* and *S. epidermidis* (MIC ≤ 8 μg/ml).

The results showed that the phenolic hydroxyls are essential for antibacterial activities as we can see from the decrease in activity against *S. aureus* and *S. epidermidis* by the

methylation of phenolic hydroxyls. Saturation of the double bond in 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate, **3**; compared to 3-methylbutyl 3,4,5-trihydroxybenzoate, **4**, leads to a two-fold increase of the antibacterial effect against *E. coli, B. cereus and B. subtilis* .

The results indicated that the esterification of carboxyl moiety increased the activity against *E. coli*, however this increment in antibacterial activity is limited to a short chain of alkyl group attached to the carboxyl group, the most potent compounds against *E. coli* were propan-2-yl 3,4,5-trihydroxybenzoate and ethyl gallate (MIC 62.5 μg/ml), both of these compounds were more active than their corresponding acidic molecule gallic acid which showed MIC of 1000 μg/ml being half of MIC of propyl gallate.

Propan-2-yl 3,4,5-trihydroxybenzoate (isopropyl gallate, **5)** characterized by the three carbon chain unit attached to the carbonyl moiety as isopropyl group, showed a more potent anti *E. coli* effect with MIC of 62.5 μg/ml than that (125 μg/ml) of propyl 3,4,5-trihydroxybenzoate (propyl gallate). The increase in such activity may be owed to the geometry of the side chain in the molecule. Interestingly, compound **5** showed less inhibition against *S. aureus* with its MIC 1000 μg/ml than propyl gallate with MIC of 500 μg/ml.

Dodecyl gallate lost completely the activity against *E. coli*; this could be attributed to the nature of *E. coli* cell wall composed of an outer membrane containing lipopolysaccharides, a periplasmic space with a peptidoglycan layer which may prevent penetration of the highly non-polar molecules.

Gram negative bacteria have impermeable outer cell wall which prohibits many molecules to go through and the membrane bound efflux proteins which expels many intruders outside the cells. These make the finding of strong anti-gram negative agent a big challenge and needs great attention (Gibbons 2008). Gallic acid and lauryl gallate showed

weak activity against *A. faecalis* (MIC 1000 μg/ml) while other compounds were more potent with MIC of 250-500 μg/ml.

Alkyl gallates at concentrations lower than MIC effectively reduced the MIC of oxacillin against MRSA (Shibata, Kondo et al. 2005), because the amphiphilic properties of alkyl gallates enable them to interact with *S. aureus* cell wall (Shibata, Kondo et al. 2005). The galloyl moiety plays a key role in beta lactam activity intensification rather than the alkyl group which increases the anti-staphylococcal activity of benzoic acid derivatives (Shibata, Kondo et al. 2005). The structure of the alkyl gallates enables them to interact with many targets in the bacterial cell wall, which disrupt the structure of the cell wall and render the process of cell wall synthesis inefficient (Shibata, Kondo et al. 2005).

3,4,5-trihydroxybenzoic acid showed weak antifungal activity against *Saccharomyces cerevisiae* ATCC 7754, MIC > 3200 μg/ml. The esterification of gallic acid increased the antifungal effects with MIC of 12.5 μg/ml for dodecyl gallate, which showed that both the alkyl group and catechol or pyrogallol groups are essential for the antifungal activity (Kubo, Xiao et al. 2002).

The SEM micrography pictures indicate that alkyl gallates synthesised here are important antibacterial candidates which effectively disrupt the cell wall integrity of *S. aureus* and could offer a synergistic activity with some antibiotics against resistant bacterial cells with impermeable cell wall or efflux pump proteins.

4.2.4. Antiplasmodial activity of several benzoic acid derivatives

Evaluation of the antiplasmodial activity of several phenolic acids indicated that the free hydroxyl groups on the aromatic ring for gallic acid and gallate esters are essential for antiplasmodial effect.

Gallic acid has been detected in the active fraction of butanol CG extract. Gallic acid and protocatechuic acid from *Chrozophora senegalensis* aqueous extract showed moderate

antiplasmodial activities against chloroquine-resistant *P. falciparum* strain W2-Indochina (Garcia-Alvarez, Moussa et al. 2013). Ethyl gallate from *Talisia nervosa* showed a weak antiplasmodial effect *in vitro* against *P. falciparum* W2 Indochina with IC_{50} of 9 - 35 μM (Calderón, Romero et al. 2006, Ramanandraibe, Grellier et al. 2008).

These results were in agreement with our findings. Furthermore, alky gallates, in particular lauryl gallate, are more potent antiplasmodial compounds than gallic acid itself. Esterification of the acidic group in gallic acid increases the antiplasmodial activity.

Addition of one methyl group to the alkyl side chain of 2-methylpropyl increased the effect and decreased the IC_{50} from 3.8 to 2.8 μg/ml; the attachment of methyl group to position 3 instead of position 2 increased the antiplasmodial effect more, while unsaturation of the side chain decreased the effect. The results suggest that there is an increase in antiplasmodial activity of benzoic acid derivatives by esterification of the carboxylic acid group and the free hydroxyl groups on the aromatic ring are essential for anti-malarial effect.

Chapter 5 : Results and discussion of antibacterial activity of *Pogostemon* cablin

5.1. Results

5.1.1. Antibacterial effects of PC

5.1.1.1. Disk diffusion assay

The hexane (PCH) and ethanol (PCE) extracts of *Pogostemon cablin* (PC) were tested for their antibacterial activities according to section **2.16**. (Table 5.1) shows the DDA results.

Table 5.1: Antimicrobial activity of the hexane and ethanol extracts PC in disk diffusion assay. +/-: indicates Gram positive and negative staining; NC: Not calculated due to the growth of some colonies throughout the IZ; Neg: negative. Section 2.16, n=3.

Microorganism strain	Extracts	Mean diameter of inhibition zone (IZ) ± SD (n =3) (mm)			
		2 mg	1 mg	0.5 mg	0.25 mg
S. aureus (+)	PCH	11.5 ± 0.6	8.5 ± 0.5	8.0 ± 0.6	NC
	PCE	10.5 ± 0.5	8.0 ± 0.5	NC	NC
S. epidermidis (+)	PCH	12.5 ± 0.5	10.5 ± 0.4	8.5 ± 0.6	NC
	PCE	10.5 ± 0.6	Neg.	Neg.	Neg.
B. cereus (+)	PCH	8.5 ± 0.5	7.0 ± 0.6	Neg.	Neg.
	PCE	NC	Neg.	Neg.	Neg.
B. subtilis (+)	PCH	8.0 ± 0.5	6.5 ± 0.4	Neg.	Neg.
	PCE	NC	Neg.	Neg.	Neg.
E. faecalis (+)	PCH	8.5 ± 0.5	7.0 ± 0.4	Neg.	Neg.
	PCE	Neg.	Neg.	Neg.	Neg.
E. coli (-)	PCH	11.0 ± 0.4	10.0 ± 0.6	8.5 ± 0.5	8 ± 0.5
	PCE	7.0 ± 0.5	6.5 ± 0.4	6.0 ± 0.5	Neg.
E. cloacae (-)	PCH	Neg.	Neg.	Neg.	Neg.
	PCE	NC	NC	NC	NC
P. aeruginosa (-)	PCH	Neg.	Neg.	Neg.	Neg.
	PCE	Neg.	Neg.	Neg.	Neg.
A. faecalis (-)	PCH	14.5 ± 0.5	10.0 ± 0.5	7.5 ± 0.5	7.0 ± 0.4
	PCE	11.5 ± 0.5	9.0 ± 0.4	7.0 ± 0.5	Neg.

5.1.1.2. MIC of the extracts

Given the broad antibacterial activity of the PCH, the MIC was determined, along with an ampicillin control, against a panel of bacteria shown to be inhibited in the previous disk diffusion assay using the alamar blue microdilution method according to section **2.17**, the MIC of PCH, and the essential oil of PC (Sigma Aldrich, UK) are listed in (Table 5.2). PCH generally are more potent than PC oil against most of the bacteria tested.

Table 5.2: MICs in µg/ml and µl/ml of the hexane extract of PC and PC oil against the growth of eight bacteria.na: not assayed. Assay according to section 2.17, n=4.

Microorganism strain	PCH		*P. cablin* oil		Ampicillin
	µg/ml	µl/ml	µg/ml	µl/ml	µg/ml
S. aureus (+)	256	0.23	1024	1.06	≤0.5
S. epidermidis (+)	128	0.12	512	0.53	≤0.5
B. cereus (+)	16	0.01	256	0.27	>64
B. subtilis (+)	16	0.01	128	0.13	≤0.5
E. faecalis (+)	1024	0.93	>2048	>2.12	≤0.5
E. coli (-)	512	0.47	2048	2.12	≤0.5
E. cloacae (-)	2048	1.86	>2048	>2.12	≤0.5
P. aeruginosa (-)	na	na	na	na	na
A. faecalis (-)	512	0.47	2048	2.12	16

In order to identify which compounds in PCH are likely to contribute to the antibacterial activities of the whole extracts against these bacterial strains, the principal components identified by GC-MS and components reported in the literature to be antibacterials were selected for testing. The MICs of those components against several isolates of bacteria were determined and are shown in (Table 5.3).

Table 5.3: MIC in µg/ml of several metabolites detected in PCH and PC oil (na: not assayed). Assay according to section 2.17, n=4.

Bacteria	Patchouli alcohol	pogostone	α-pinene	β- pinene	β- caryophylline	2,4-Bis (1,1-dimethy lethyl)-phenol	Ampicillin
S. aureus	512	256	>2048	>2048	>2048	1024	≤0.5
S. epidermidis	256	128	>2048	>2048	>2048	512	≤0.5
E. faecalis	512	1024	>2048	>2048	>2048	1024	≤0.5
E. coli	256	512	256	2048	>2048	1024	≤0.5
E. cloacae	>2048	512	>2048	>2048	>2048	>2048	≤0.5
A. faecalis	>2048	>2048	64	256	>2048	>2048	16
B. subtilis	64	128	>2048	>2048	>2048	>2048	≤0.5
B. cereus	256	512	>2048	>2048	>2048	>2048	>64

5.1.1.3. Time Kill assay

PCH showed promising antibacterial activity. Patchouli alcohol and pogostone, the two key compounds of PCH were prepared for the killing kinetics and assayed according to section **2.18** against *S. aureus* and *E. coli*; the results are shown in (Figure 5.1) and (Figure 5.2).

Patchouli alcohol against *S. aureus* achieved bactericidal level at 2 MIC concentration with in the first 3 hours of experiment, the drug at concentration of MIC failed to kill 99.9 % of *S. aureus* cells, ½ MIC produced inhibition of cells with in the first 3 hours then the division of cells reactivated again. On *E. coli* the story showed some differences in the antibacterial ability of the drug at ½ MIC the microorganism achieved a high population

number reaching 370 times the initial population compared to 41 times of *S. aureus* initial population, at MIC level the drug killed about 90 % of the initial cells number within 3 hours and 98% at the end of experiment course. Patchouli alcohol at 2 MIC level kill all the *E. coli* cells within 3 hours (Figure 5.1).

Pogostone at ½ MIC concentrations showed a delay in growth for three hours, followed by rapid growth of the bacterial population to 496 times the initial population of *E. coli* and 103 times the initial population of *S. aureus*. 2 MIC of pogostone achieved bactericidal level after 3 hours on *E. coli* and 6 hours on *S. aureus*, indicating a slow onset of action of pogostone against *S. aureus* (Figure 5.2).

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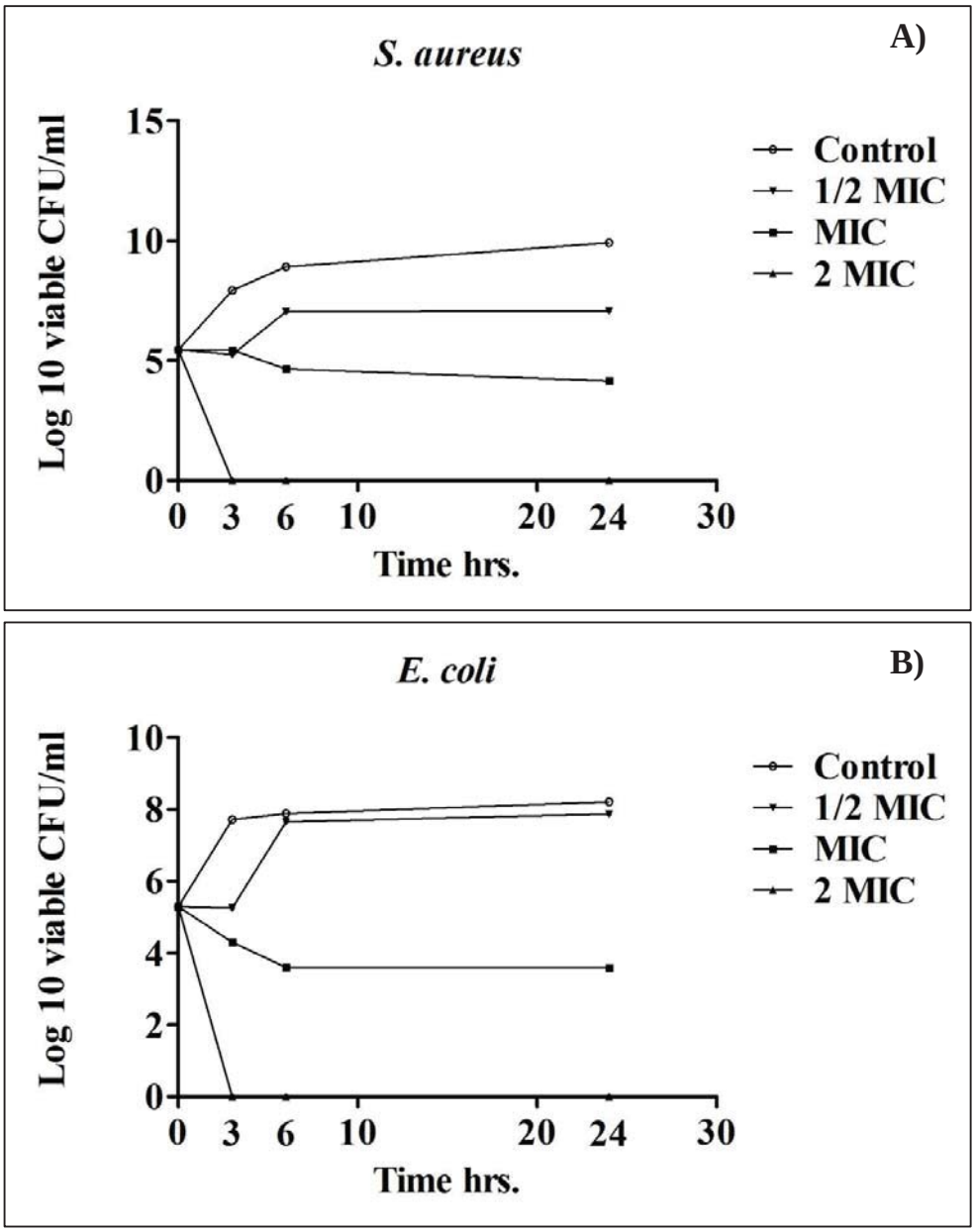


Figure 5.1: Time-kill kinetics of patchouli alcohol against *S. aureus* (A) and *E. coli* (B) , MIC of patchouli alcohol against *S. aureus* and *E. coli* are 512 and 256 µg/ml respectively. The assay has been done in duplicate, n=2 according to section 2.18.

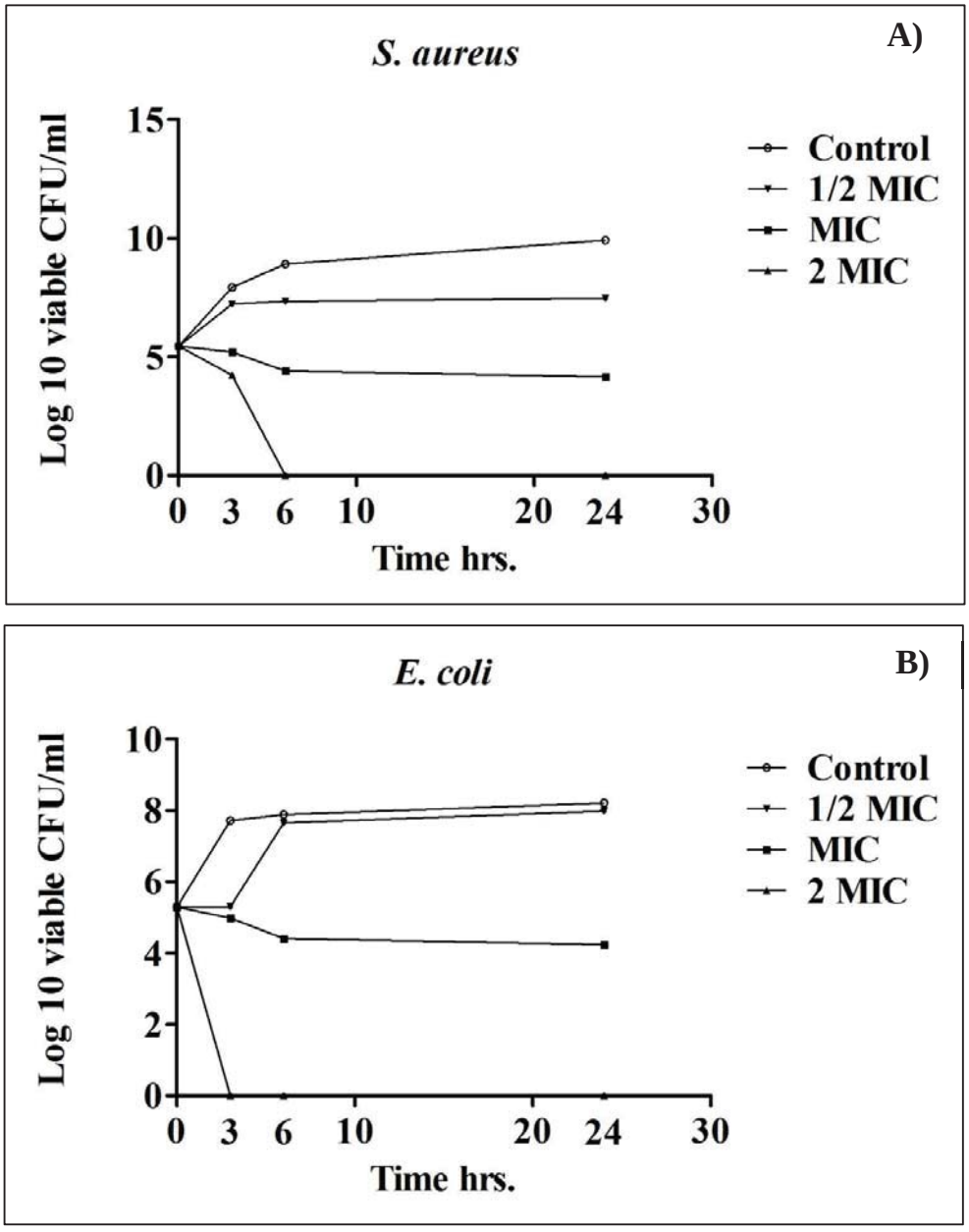


Figure 5.2: Time-kill kinetics of pogostone against *S. aureus* (A) and *E. coli* (B) , MIC of pogostone against *S. aureus* and *E. coli* are 256 and 512 µg/ml respectively. The assay has been done in duplicate, n=2 according to section 2.18.

5.1.1.4. Scanning Electron Microscopy (SEM)

(Figure 5.3 (A-E)) shows the SEM images of *S. aureus* after exposure to medium (control) and 2MIC of PCH, patchouli alcohol, and pogostone for 1h according to section **2.19**.

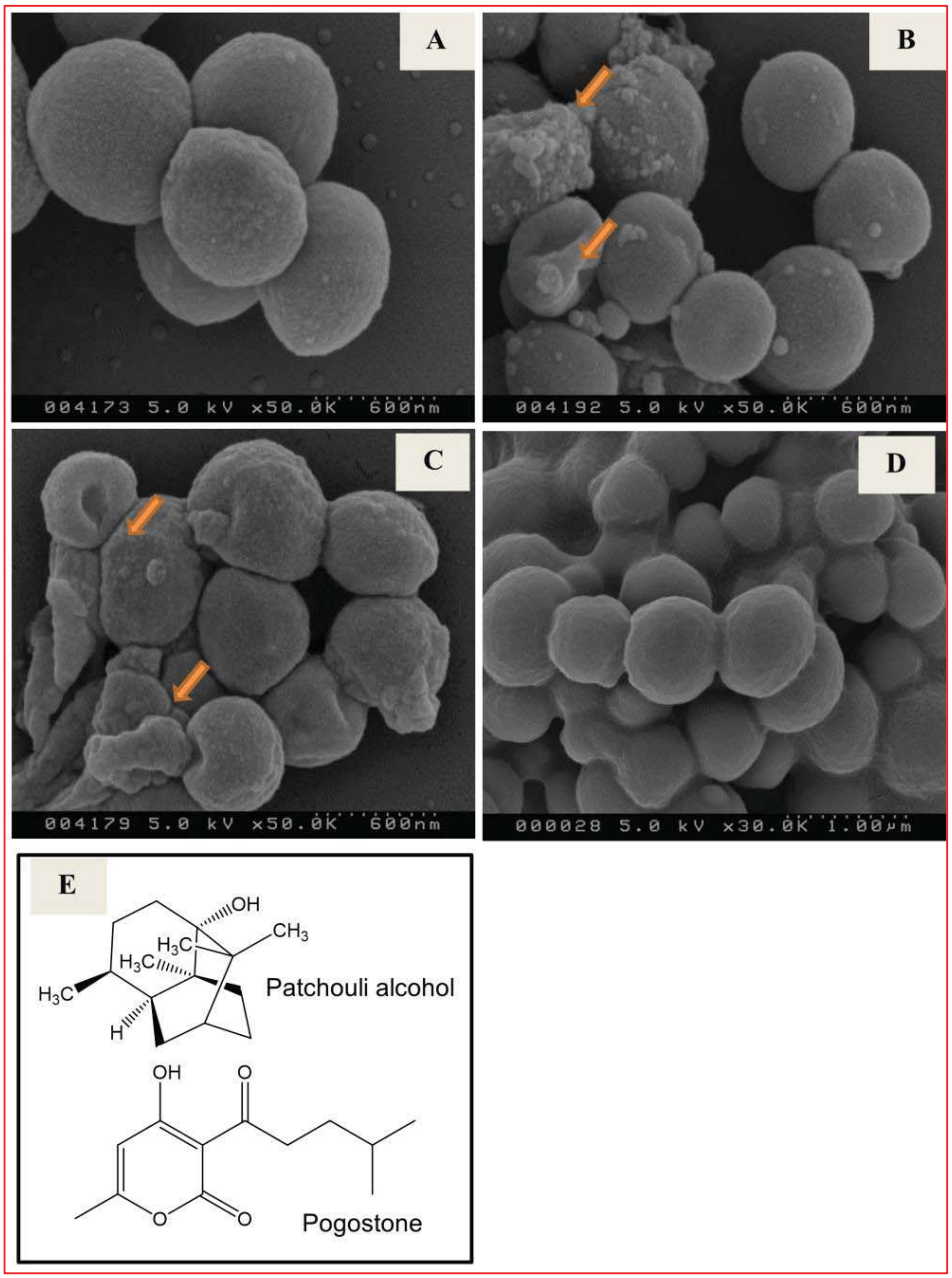


Figure 5.3: SEM images of *S. aureus* after exposure to medium as control (A), PCH (B), patchouli alcohol (C), and pogostone (D) for 1h at a concentration of 2 MIC level; Structures of patchouli alcohol and pogostone (E). The assay is according to section 2.19.

5.1.1.5. Synergism studies

Previous studies showed that gallic acid exerted its antibacterial action by increasing the bacterial cell wall permeability and pores formation, section **3.2.4.** (Borges, Ferreira et al. 2013). The main sesquiterpene metabolite of PC, patchouli alcohol showed great affinity for the two key enzymes of bacterial biosynthesis pathways; PBPs and dihydrofolate synthase (DHFS) using the molecular docking technology (Yang, Zhang et al. 2013). In this section we tried to design a suitable combination of two molecules detected as main active constituents from two different medicinal plants and targeting two different sites of action in *S. aureus*.

Patchouli alcohol as drug A and gallic acid as drug B were prepared in double dilution sets in µg/ml. Evaluation of the interaction between them against *S. aureus* has been done according to procedure mentioned in section **2.20**. (Figure 5.4) shows blue wells in which the bacteria have been inhibited and red wells in which bacterial growth has occurred. (Table 5.4) shows the interpretation of interaction between patchouli alcohol and gallic acid against *S. aureus*.

Patchouli alcohol and gallic acid are probably working on different targets in bacterial cells and they showed good synergism versus *S. aureus* over variable concentrations range. The best synergism between patchouli alcohol and gallic acid, was at $\sum FIC = 0.265$ when 125 µg/ml or one quarter the MIC of patchouli alcohol interact with 31.25 µg/ml of gallic acid which is just 1.5 % of its MIC.

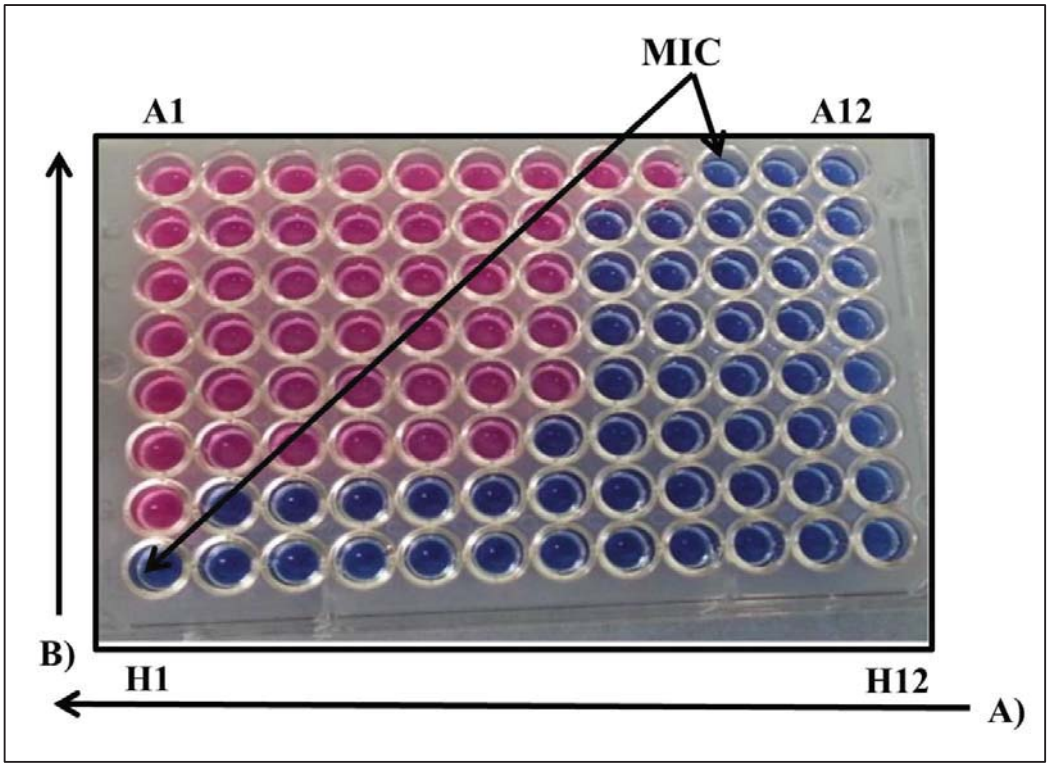


Figure 5.4: Plate to assay synergism between patchouli alcohol and gallic acid against *S. aureus*. Patchouli alcohol (Drug A) concentrations set from 2000 µg/ml (A12) to 1.95 µg/ml (A2). Gallic acid (Drug B) concentrations set from 2000 µg/ml (H1) to 3.9 µg/ml (B1). Two black arrows refer to MIC of patchouli alcohol and gallic acid against *S. aureus*, n=3. Assay has been done according to section 2.20.

Table 5.4: Interpretation of the interaction between patchouli alcohol and gallic acid against *S. aureus* , data evaluated, IND as Indifference, PSYN as partial synergism and SYN as synergism.

Well With no growth	Patchouli alcohol		Gallic acid		∑FIC= FIC A+FIC B	Interpretation
	Con. µg/ ml	FIC of A	Con. µg/ml	FIC of B		
A10	500	MIC of A	0	MIC of A	MIC of A	MIC of A
B10	500	1	31.25	0.015	1.015	IND
B9	250	0.5	31.25	0.015	0.515	PSYN or IND
B8	125	0.25	31.25	0.015	0.265	SYN
C8	125	0.25	62.5	0.03	0.28	SYN
D8	125	0.25	125	0.06	0.31	SYN
E8	125	0.25	250	0.125	0.375	SYN
F7	62.5	0.125	500	0.25	0.375	SYN
G6	31.25	0.06	1000	0.5	0.56	PSYN or IND
G5	15.62	0.03	1000	0.5	0.53	PSYN or IND
G4	7.8	0.015	1000	0.5	0.515	PSYN or IND
G3	3.9	0.0078	1000	0.5	0.507	PSYN or IND
G2	1.95	0.0039	1000	0.5	0.503	PSYN or IND
H1	0	MIC of B	2000	MIC of B	MIC of B	MIC of B

5.1.2. GC-MS

PCH and PC oil were prepared according to section **2.8.5.1**. PCE was prepared according to section **2.8.5.2**. Samples were injected according to method mentioned in section **2.8.1** method A. (Table 5.5) mentions the compounds detected in PCH and (Figure 5.5) shows the GC-MS chromatogram. (Table 5.6) and (Figure 5.6) shows the compounds detected in PC oil and GC-MS chromatogram respectively.

Table 5.5: Compounds detected in PCH using GC-MS, according to section 2.8.1, n=3.

no.	RT (min)	Compound name	percentages ±SD (n=3)	RI
1	5.25	α-pinene	0.15±0.02	939
2	6.01	β-pinene	0.39±0.06	982
3	9.29	Endo-borneol	0.17±0.03	1176
4	10.62	1,3-Bis(1,1-dimethylethyl)-benzene	2.73±0.60	1359
5	12.67	β-patchoulene	1.29±0.15	1400
6	13.20	β-caryophyllene	0.54±0.06	1439
7	13.40	α-guaiene	5.43±0.51	1454
8	13.58	Seychellene	4.76±0.42	1467
9	13.76	α-Patchoulene	2.81±0.37	1480
10	14.04	Pentadecane	1.74±0.09	1501
11	14.12	α-Selinene	0.49±0.05	1508
12	14.28	2,4-Bis(1,1-dimethylethyl)-phenol	5.41±1.43	1520
13	14.33	α-Bulnesene	1.76±0.21	1524
14	15.16	Cashmeran	2.26±0.09	1588
15	15.41	Caryophyllene oxide	2.21±0.19	1608
16	15.80	(-)-Spathulenol	1.52±0.09	1640
17	16.46	Patchouli alcohol	27.74±0.80	1694
18	16.96	Pogostone	35.95±4.85	1738

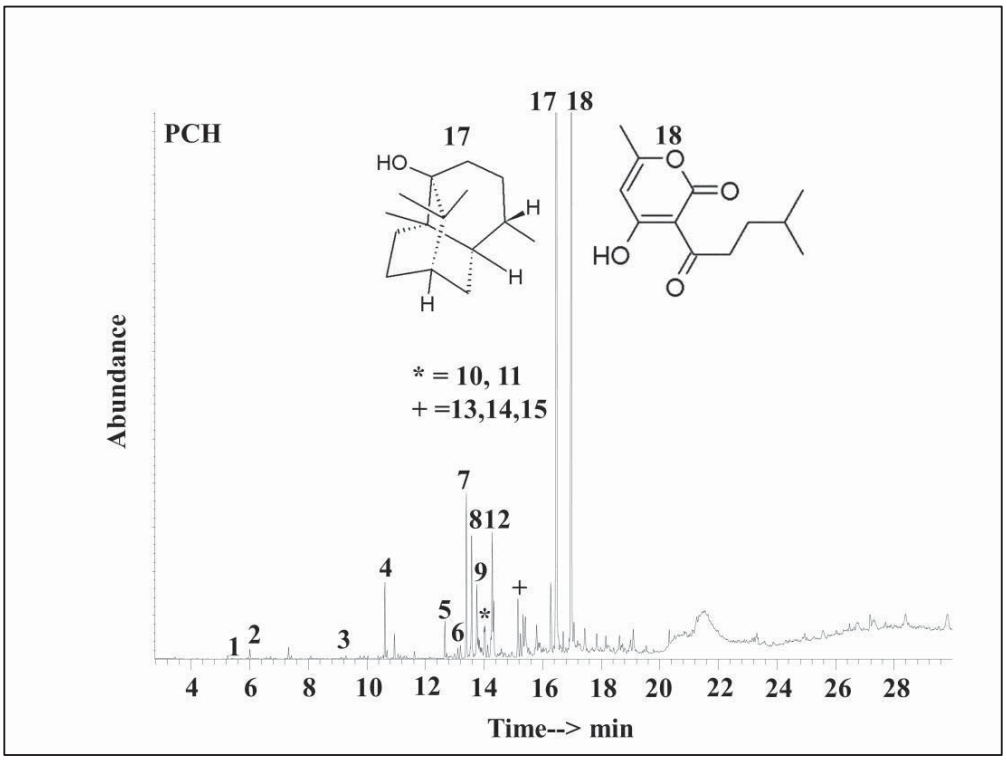


Figure 5.5: GC-MS chromatogram of compounds of the PCH. Two principal components (patchouli alcohol (17) and pogostone (18)) are shown in the inset and number bearing of peaks refers to (Table 5.5). The analysis has been done according to section 2.8.1, n=3.

Table 5.6: Compounds detected in PC oil using GC-MS, section 2.8.1, n=3.

no.	RT (min)	Compound name	Averages percentages± SD (n=3)	RI
1	12.50	α-Copaene	0.09±0.01	1390
2	12.60	β-patchoulene	2.65±0.05	1400
3	12.70	β-elemene	1.11±0.20	1401
4	13.17	β-caryophylline	4.96±0.56	1439
5	13.40	α-Guaiene	15.06±2.20	1454
6	13.60	Seychellene	7.07±0.70	1467
7	13.70	α-caryophyllene (Humulene)	0.81±0.03	1475
8	13.80	α-patchoulene	5.40±0.27	1480
9	13.81	α-Elemene	1.83±0.31	1481
10	14.10	D-Germacrene	0.26±0.07	1506
11	14.12	γ-Gurjunene	0.58±0.01	1507
12	14.20	Cedrene-V6	3.99±0.05	1513
13	14.30	α-Bulnesene	17.48±3.23	1524
14	14.50	(+)-epi-Bicyclosesquiphellandrene	0.40±0.08	1537
15	15.10	Cashmeran	0.62±0.10	1588
16	15.40	Caryophyllene oxide	0.78±0.15	1608
17	15.50	2-methyl-3-methylene-Cyclopentanecarboxaldehyde	0.05±0.01	1615
18	16.30	epi-α-Selinene	1.82±0.48	1680
19	16.50	Patchouli alcohol	31.50±5.29	1694
20	16.90	pogostone	1.07±0.34	1737

RT = Retention time in min, SD= Standard deviation, n= Number of experiment replication, RI= Retention index.

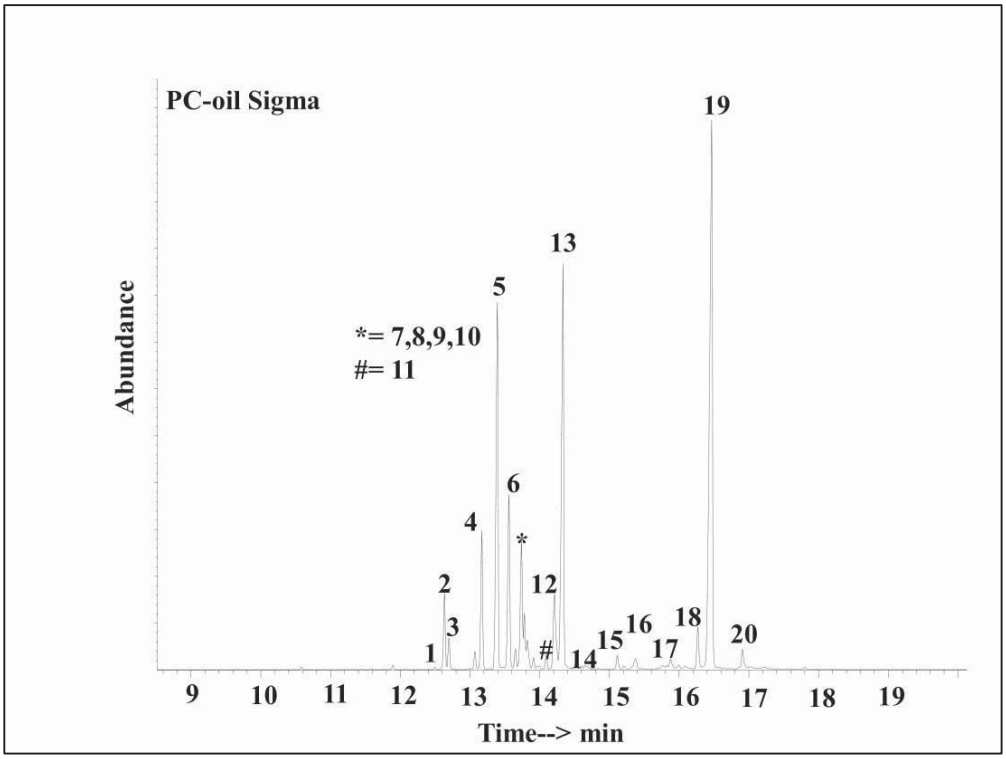


Figure 5.6: GC-MS chromatogram of compounds of the PC oil. The analysis has been done according to section 2.8.1, n=3.

Nine compounds were detected in both PCH and PC oil, the percentage of those compounds were different in extracts, (Figure 5.7) shows the difference in compounds abundance in PCH and PC oil. It is clear that pogostone quantity, one of the main compounds of PC is very low in PC oil compared to its quantity in PCH.

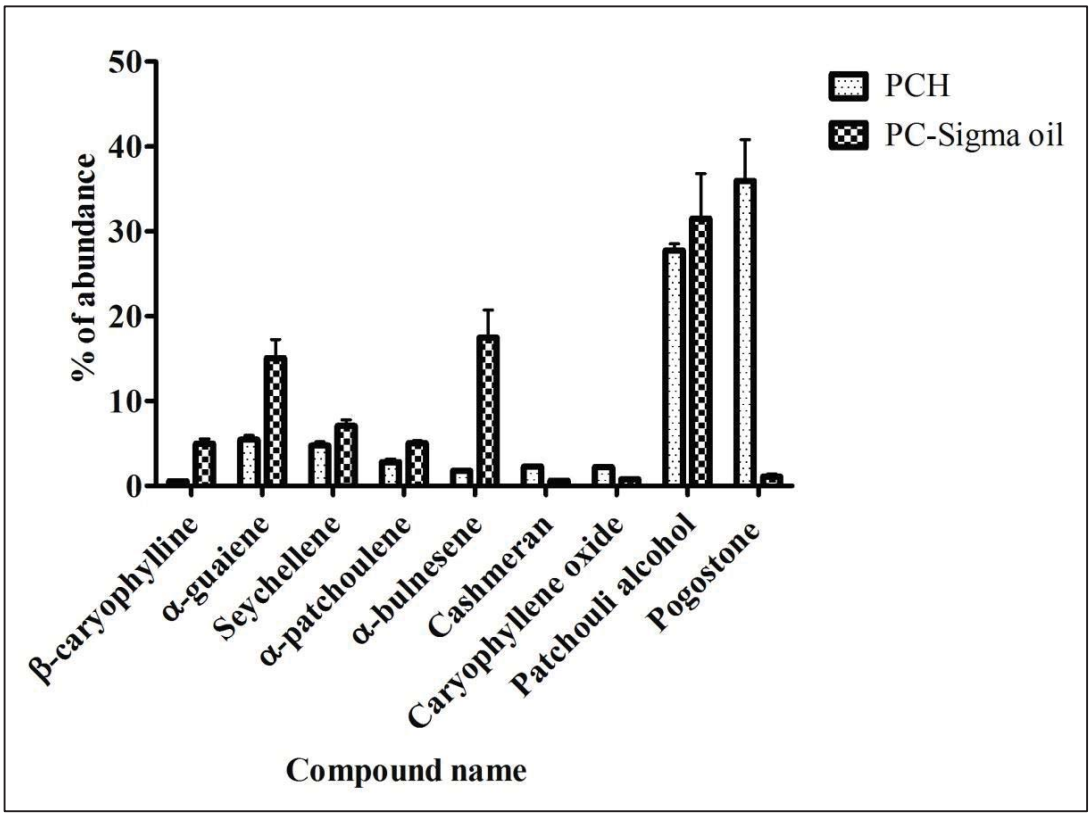


Figure 5.7: Comparison of the relative quantity of nine compounds detected in PCH and PC oil.

Appendix D shows the GC-MS data obtained from PCE, (Table-D1) shows the compounds detected in PCE. (Figure-D1) shows the GC-MS chromatograms of PCE.

5.2. Discussion

5.2.1. Disk diffusion assay

A general outlook about the results of DDA revealed that PCH is a promising antibacterial extract and hexane was more efficient than ethanol in extraction of the active antimicrobial compounds from *Pogostemon cablin*.

In all experiments, as expected, decreasing the quantity of the extracts tested resulted in the reduction of diameter of IZ. The extracts showed no or little inhibitory effect against *E. facecalis* and the two gram negative bacteria *P. aeruginosa* and *E. cloacae*.

5.2.2. MIC of PCH and Patchouli oil

The MICs of PCH, and patchouli oil along with ampicillin as a control, were recorded against the bacteria, which were shown to be inhibited by DDA. *Pseudomonas aeruginosa* was not tested here because it showed no sensitivity to PCH or PCE. The essential oil of patchouli extracted by destructive distillation was included in this experiment to study the effect of the extraction method on the antibacterial activity.

The MICs of PCH against *S. aureus* and *S. epidermidis* were 256 and 128μg/ml respectively which were in agreement or in good correlation with the IZ values showed in the DDA (Table 5.1), however, this correlation between MIC and IZ is not true for other microorganisms such as *B. cereus* and *B. subtilis*.

B. cereus and *B. subtilis* showed very high sensitivity to PCH from alamar blue microplate assay (MICs = 16 μg/ml) compared to only moderate antibacterial effects shown by DDA.

Such results without significant correlations were recognized before, for example, when *Wedelia chinensis* methanolic extract showed IZ against *B. cereus, B. subtilis* and *S. aureus* equal to 14, 12 and 15 mm respectively and MIC equal to 3.13, 6.25 and 6.25 mg/ml respectively (Darah, Lim et al. 2013). However, no explanation for this has been mentioned.

B. cereus and *B. subtilis* have the ability to form endospores when nutrients levels are decreased. When the microorganisms are subjected to various stress conditions, these endospores can reactivate again when the conditions are appropriate. The reactivation process occurs, and endospores can germinate again into vegetative cells when the nutrients, organic metabolites and moisture become available (Wijnands, Dufrenne et al. 2006, von Stetten, Mayr et al. 1999), the early destructed bacterial cells on the agar plate offer an enrich source of decomposable organic metabolites essentials for spores reactivation.

The uncorrelated results could be attributed to the slow release of the oil active constituents into the agar plate and possible reactivation of some of *Bacillus* spores leading to decrease the IZ compared to the direct contact with the active constituents of *Pogostemon cablin* in broth microdilution method which may kill both the endospores and vegetative cells. The other explanation of this result deduced from the fact that *B. cereus* grew very fast in the disk diffusion assay on agar plate (Chang, Lee et al. 1998) which may lead to increasing the bacterial population facing the limited quantity of the antibacterial extract, however, more experiments need to be conducted to confirm these explanation.

Alcaligenes faecalis showed moderate sensitivity to PCH using alamar blue microplate assay (MIC 512 µg/ml) compared to the strong inhibition of growth recorded by DDA (IZ = 15 mm) indicating that the DDA could provide enough information about the boundary of antibacterial activity/inactivity of nonpolar extracts. However, the determination of MIC using microplate assay is crucial to evaluate the extent of the antibacterial activities.

The antibacterial activity of PCH against *B. cereus* was strong compared to ampicillin and it showed moderate effect against *E. coli* (MIC 512 µg/ml).

Patchouli oil in general showed less antibacterial activities compared to PCH against all of the bacterial isolates used in this study, which could be attributed to the difference in

metabolites types and quantity. Pogostone abundance in patchouli oil is very low compared to that in PCH, which could be a major reason for the decrease in antibacterial activities of the oil. Similarly, the hexane extract of nonpolar compounds showed better antibacterial activities than its corresponding essential oil extracted by fractional distillation (Packiyasothy, Kyle 2002).

It is well-accepted that the MIC of oil against microorganism could be recorded by µl/ml unit in addition to the µg/ml unit that is why we included MICs in µl/ml as a second column in (Table 5.3), (Abu-Darwish, Cabral et al. 2016, Knezevic, Aleksic et al. 2016, Bouzabata, Bazzali et al. 2013).

Patchouli essential oil from leaves of *Pogostemon cablin* showed weak antibacterial activities MIC 5 µl/ml against *Candida albicans*, 1.2 µl/ml against *Enterococcus facecalis*, 2.5 µl/ml against *S. aureus* and >20 µl/ml against *E. coli*, *K. pneumoniae* and *P. aeruginosa* (Hammer, Carson et al. 1999). Patchouli oil from Guangdong, China showed weak effects with MIC >4 mg/ml against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus proteus* and *Shigella dysenteriae* (Yang, Zhang et al. 2013). The main constituent patchouli alcohol showed MIC equal to 1mg/ml against *E. coli*, 2 mg/ml against *S. aureus* while pogostone showed MIC equal to 0.45 mg/ml against *E. coli* and 1 mg/ml against *S. aureus* (Yang, Zhang et al. 2013), which are much higher than the MICs obtained in this study.

In the current study, the composition of PCH was characterized by GC-MS and it was characterised to contain patchouli alcohol and pogostone as major compounds together with a number of other monoterpenes and sesquiterpenes, which were also found in the previous reports (Hu, Li et al. 2006, Wu, Lu et al. 2004).

Terpenes such as, α-pinene, β-pinene and β-caryophyllene in PCH all showed weak antibacterial activity with MICs > 2048 µg/ml against *S. aureus, S. epidermidis, E. coli* and

E. cloacae, however, α-pinene showed strong growth inhibition against *E. coli* and *A. faecalis* with MIC values of 256 and 64 μg/ml respectively. The positive enantiomers of the α- and β-isomers of pinene were active compared to the negatives against MRSA with MICs being 4150 and 6250 μg/ml respectively (Rivas, Lopes et al. 2012).

The bicyclic sesquiterpene β-caryophyllene showed some antimicrobial activity against *E. coli, S. aureus, K. pneumoniae, P. aeruginosa and C. albicans* (Goren, Piozzi et al. 2011). MICs of α-caryophyllene and β-caryophyllene against specific *P. acnes* were found to be 15.6 and 7.8 μg/ml, respectively. MIC of β-caryophyllene against *E. faecalis* was found to be 6 ppm (~6 μg/ml) (Schmidt, Bail et al. 2010). The weak antimicrobial activity of β-caryophyllene (MICs > 1000 μg/ml) against *S. aureus* and *E. coli* (Kang, Helms et al. 1992) were consistent with our results (MICs > 2048 μg/ml).

Interestingly, patchouli alcohol showed MICs equal to 256, 64 and 256 μg/ml against *S. epidermidis, B. subtilis* and *E. coli*, respectively. 2,4-bis(1,1-dimethylethyl)-phenol which constituted about 5 % of PCH, showed weak to moderate antibacterial activities with the best effect recorded against *S. epidermidis* with MIC equal to 512 μg/ml.

Previously the extract of *Pogostemon cablin* and its components (patchouli alcohol and pogostone) showed antibacterial activity against periodontopathic bacteria, including *Actinobacillus*, *Capnocytophaga*, *Fusobacterium*, *Eikenella* and *Bacteroides* species (Osawa, Matsumoto et al. 1990).

The results demonstrated the moderate antibacterial properties of pogostone against *E. coli*, MIC 512 μg/ml and moderate to strong antibacterial action against *S. aureus* and *S. epidermidis* with MIC equal to 256 and 128 μg/ml respectively. Pogostone showed inhibition against a fungus *Candida albicans* with MICs ranging from 12 to 97 μg/ml against all strains and moderate inhibition against bacteria including *Shigella flexneri, Pneumococcus* and *S. aureus* (MIC 250 μg/ml) but weak effect against *E. coli*, *Salmonella*

typhi, *S. enteritidis*, *P. aeruginosa*, and *Pneumococcus* (MICs > 2000 μg/ml) (Yi, He et al. 2013). Pogostone was recorded with rapid absorption from the gut of the mice after oral administration (Li, Liang et al. 2012). Recently pogostone was also reported to show strong antibacterial activity against *Corynebacterium xerosis* and *Chryseobacterium indologenes* (MIC < 0.098 μg/ml) and moderate activities (MIC 200-800 μg/ ml) against different *E. coli* isolates (Peng, Wan et al. 2014). Patchouli alcohol has been shown to possess selective antibacterial activity against *Helicobacter pylori* (Yu, Xie et al. 2015), anti-influenza virus A/PR/8/34 (H1N1) (Kiyohara, Ichino et al. 2012) and also exhibited cytotoxic activity in human colorectal cancer cells (Jeong, Choi et al. 2013).

5.2.3. Time-kill assay

Time-kill kinetic study of the *S. aureus* and *E. coli* after treatment by patchouli alcohol and pogostone also demonstrated a rapid killing of these bacteria. To the best of current knowledge this is the first time to show the kinetics of those bacteria after exposure to the pure components from *Pogostone cablin*.

5.2.4. SEM analysis

PCH caused collapse of cells, a "doughnut" shape, and the leakage of cell contents as compared to the control sample which showed well defined *S. aureus* cells. PBPs, DHFS, dihydrofolate reductase (DHFR), RNA polymerase and DNA gyrase, are essential enzymes for bacterial biosynthesis; the two main constituents of Patchouli: patchouli alcohol and pogostone may show multi-target activity on these enzymes. Patchouli alcohol and pogostone were documented to interact with PBPs and DHFS, respectively (Yang, Zhang et al. 2013).

Patchouli alcohol produced "doughnut"-shaped cells with massive destruction to the cells but without the change in permeability, which is probably due to the interaction of patchouli alcohol with bacterial enzymes in accordance with Yang's results (Yang, Zhang

et al. 2013). The bacterial cells subjected to pogostone looked well defined with continuous division; however, pogostone produced cell wall roughness which comes in agreement with the time-kill assay results, i.e. pogostone at its 2 MIC level approaches bactericidal level only after 6 h which indicates a slow onset of action.

Chapter 6 : Results and discussion of antibacterial activity of *Perilla frutescens* (PF)

6.1. Results

6.1.1 Antibacterial effects of PF

6.1.1.1. Disk diffusion assay (DDA)

The hexane and ethanol extracts of PF antibacterial activities were assayed according to section **2.16**, using DDA. The mean diameters of (IZ)s against five gram-positive and four gram-negative microorganisms at four concentrations of PF hexane extract (PFH), and PF ethanol extract (PFE) were measured (Table 6.1**)**. In general, increasing the quantity of extracts increased the IZs for the susceptible bacteria strains tested and PFH showed stronger antibacterial activities than PFE. PFH also showed moderate antibacterial activity against both gram positive and negative bacteria.

6.1.1.2. Determination of MIC of the extracts and pure components

The MICs of PFH along with an ampicillin control, against seven bacterial isolates which showed positive results to PFH by DDA were determined (Table 6.2**)** according to section **2.17**.

Furthermore, the MICs of pure components of PFH including limonene (both isomers R and L), eucalyptol and linalool (Table 6.2) were determined. Linalool showed only weak effect against *E. cloacae* with MIC equal to 1024 µg/ml, against *E. coli* and *Alcaligenes faecalis* with MICs equal to 2048 µg/ml, against all other bacterial cells tested with MIC > 2048 µg/ml. MICs of limonene and eucalyptol were > 2048 µg/ml against all bacterial

isolates tested. Perilla ketone and several fatty acids detected in PF extracts are important to check for their antibacterial activities.

Table 6.1: Antimicrobial activity of the hexane (PFH) and ethanol (PFE) extracts of *Perilla frutescens* by disk diffusion assay. +/-: indicates gram positive and negative staining; NC: Not calculated due to the growth of some colonies throughout the IZ; Neg: negative, assay has been done according to section 2.16, in triplicate n=3.

Microorganism strain	Extracts	Mean diameter of inhibition zone (IZ) ± SD (n =3) (mm)			
		2 mg	1 mg	0.5 mg	0.25 mg
S. aureus (+)	PFH	9.5 ± 0.5	7.0 ± 0.5	NC	NC
	PFE	7.0 ± 0.5	Neg.	Neg.	Neg.
S. epidermidis (+)	PFH	10.5 ± 0.6	9.5 ± 0.5	8.0 ± 0.5	7.0 ± 0.6
	PFE	8.0 ± 0.6	7.0 ± 0.5	7.0 ± 0.4	6.5 ± 0.4
B. cereus (+)	PFH	8.5 ± 0.5	8.0 ± 0.4	7.0 ± 0.5	Neg.
	PFE	NC	Neg.	Neg.	Neg.
B. subtilis (+)	PFH	8.5 ± 0.5	8.0 ± 0.4	7.5 ± 0.5	Neg.
	PFE	NC	Neg.	Neg.	Neg.
E. faecalis (+)	PFH	Neg.	Neg.	Neg.	Neg.
	PFE	Neg.	Neg.	Neg.	Neg.
E. coli (-)	PFH	7.5 ± 0.5	7.0 ± 0.4	6.5 ± 0.4	6.4 ± 0.4
	PFE	7.0 ± 0.5	7.5 ± 0.5	6.5 ± 0.4	6.5 ± 0.4
E. cloacae (-)	PFH	8.5 ± 0.5	8.0 ± 0.4	7.5 ± 0.6	7.0 ± 0.6
	PFE	Neg.	Neg.	Neg.	Neg.
P. aeruginosa (-)	PFH	Neg.	Neg.	Neg.	Neg.
	PFE	Neg.	Neg.	Neg.	Neg.
A. faecalis (-)	PFH	10.5 ± 0.5	9.5 ± 0.4	8.0 ± 0.4	7.5 ± 0.5
	PFE	15.0 ± 1.0	13.5 ± 1.5	11.5 ± 1.0	9.0 ± 1.5

Table 6.2: MICs in µg/ml of PFH and linalool against seven bacterial isolates, shown to be sensitive in the previous DDA, section 2.17, assay has been done in quadruplicate n=4.

Microorganism strain	PFH	Linalool	Ampicillin
S. aureus (+)	1024	> 2048	≤0.5
S. epidermidis (+)	256	> 2048	≤0.5
B. cereus (+)	64	> 2048	>64
B. subtilis (+)	128	> 2048	≤0.5
E. coli (-)	>2048	2048	≤0.5
E. cloacae (-)	1024	1024	≤0.5
A. faecalis (-)	1024	2048	16

6.1.1.3. Scanning Electron Microscopy (SEM)

(Figure 6.1) shows the SEM images of *S. aureus* after exposure to medium (control) and 2MIC of PFH for 1hour according to section **2.19**. There is significant change of their morphology such as the damage of cell wall of *S. aureus* by PFH.

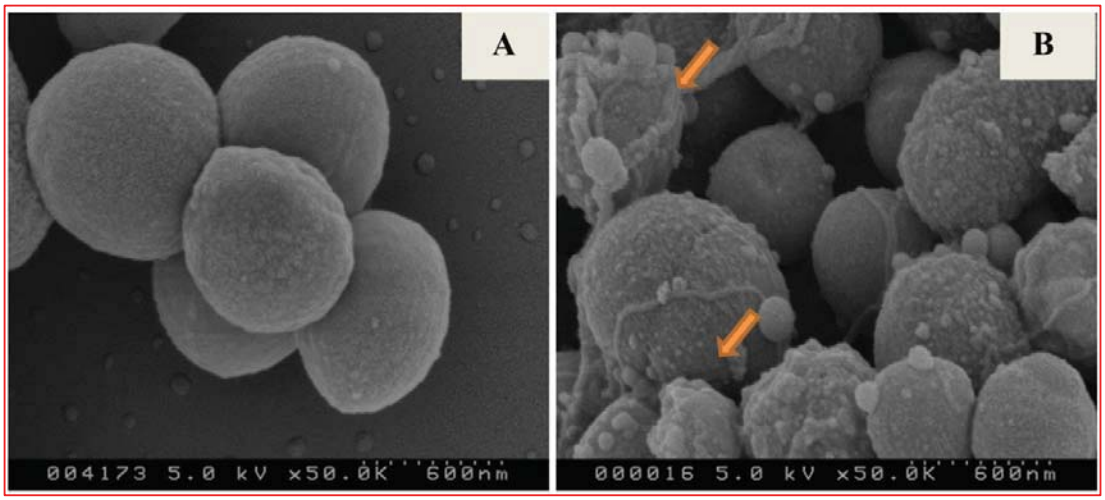


Figure 6.1: SEM images of *S. aureus* after exposure to medium as control (A) and PFH (B) for 1h at a concentration of 2 MIC level. The study has been done according to section 2.19.

6.1.2. Chemical analysis of PFH and PFE using GC-MS

PFH was prepared according to section **2.8.5.1**. PFE was prepared according to section **2.8.5.2**. Samples were injected according to method mentioned in section **2.8.1** method A. (Table 6.3 and 6.4) list the compounds detected in PFH and PFE respectively. (Figure 6.2 and 6.3) show the TIC of the PFH and PFE using GC-MS, respectively.

Table 6.3: Compounds detected in the hexane extract of *Perilla frutescens* (PFH) by GC-MS. The analysis has been done according to section 2.8.1, n=3.

no.	RT (min)	Compound	percentages ±SD (n=3)	RI
1	6.88	D-Limonene	0.12±0.004	1033
2	6.94	Eucalyptol	0.04±0.01	1037
3	8.08	Linalool	0.42±0.08	1103
4	9.14	Isoborneol	0.09±0.01	1167
5	9.29	Endo-Borneol	0.16±0.04	1176
6	10.54	1-(3-Furyl)-4-methyl-1-pentanone (Perilla ketone)	6.86±1.53	1256
7	10.68	2-Isopropyl-5-methyl-3-cyclohexen-1-one	0.7±0.18	1265
8	12.53	Copaene	0.09±0.02	1391
9	13.20	β-Caryophyllene	1.15±0.25	1439
10	13.40	α-Guaiene	0.21±0.02	1454
11	13.58	Seychellene	0.18±0.003	1467
12	13.68	Humulene	0.14±0.01	1474
13	14.02	(*Z*,*E*)-α-Farnesene	0.58±0.1	1499
14	14.11	1,2-Dimethoxy-4-(1-propenyl)-benzene	0.41±0.04	1506
15	14.29	2,4-Bis(1,1-dimethylethyl)-phenol	0.25±0.09	1520
16	14.50	Myristicin	1.37±0.19	1536
17	14.84	Elemicin	1.85±0.29	1563
18	15.43	Caryophyllene oxide	1.18±0.09	1610
19	16.06	Asarone (isoelemicin)	0.38±0.02	1661
20	19.09	Hexadecanoic acid methyl ester	0.29±0.14	1929
21	19.60	*E*-11-Hexadecenoic acid ethyl ester	0.58±0.24	1978
22	19.81	Hexadecanoic acid ethyl ester	11.7±1.3	1997
23	21.51	Linoleic acid ethyl ester	15.7±0.95	-
24	21.59	Linolenic acid ethyl ester	38.2±0.62	-

RT = Retention time, SD= Standard deviation, n= Number of experiment replication, RI= Retention index.

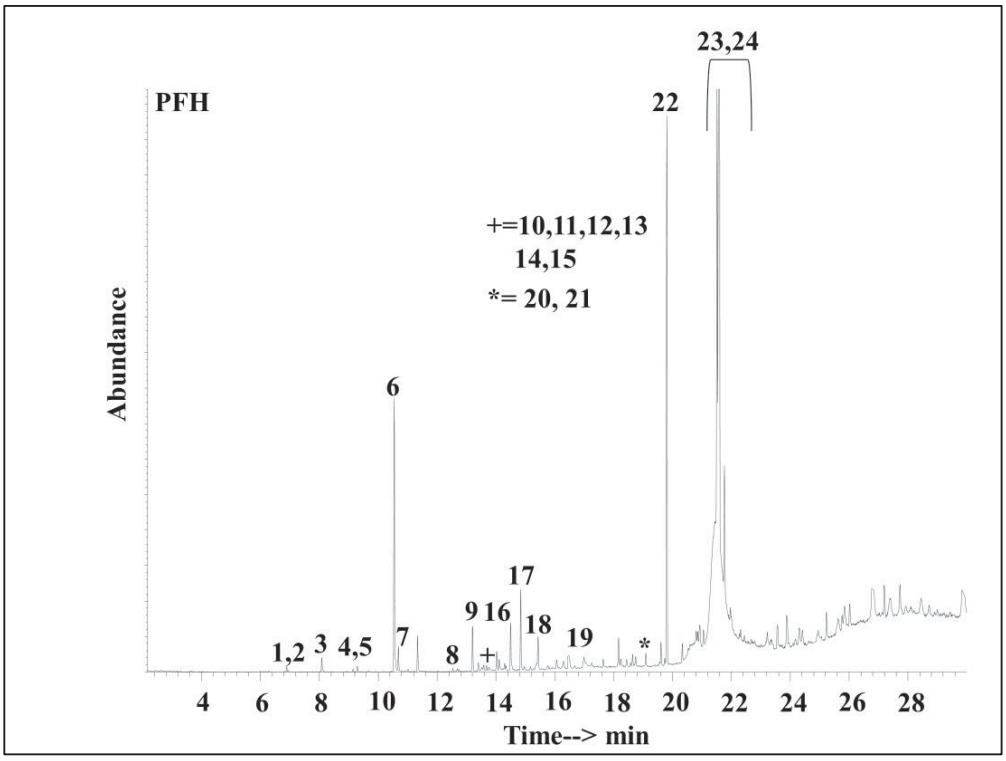


Figure 6.2: GC-MS chromatogram of compounds of the hexane extract of *Perilla frutescens* (PFH). The analysis has been done according to section 2.8.1, n=3.

Table 6.4: Compounds (after TMSi derivation, section 2.8.5.2) in PFE detected using GC-MS, section 2.8.1, n=3.

no.	RT (min)	Compound directly detected by GC-MS	The name of the compound before derivatisation	Percentages ± SD (n=3)
1	9.90	Propanedioic acid, bis(trimethylsilyl) ester	Propanedioic acid	0.79±0.02
2	11.50	Butanedioic acid, bis(trimethylsilyl) ester	Butanedioic acid	1.17±0.20
3	11.90	2,3-Dihydroxypropanoic acid tris(trimethylsilyl)	2,3-Dihydroxypropanoic acid	2.24±0.30
4	11.98	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	E-2-butenedioic acid	0.49±0.07
5	14.05	2-Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	2-hydroxybutanedioic acid	5.2±0.60
6	14.26	L-Threitol, tetrakis(trimethylsilyl) ether	L-threitol-TMSi	3.68±0.23
7	14.50	(2S)-1-(Trimethylsilyl)-5-oxopyrrolidine-2-carboxylic acid	pyroglutamic acid	1.02±0.15
8	14.90	Trimethylsilyl 2,3,4-tris[(trimethylsilyl)oxy]butanoate	2,3,4-trihydroxybutyric acid	1.16±0.10
9	15.06	L-Threonic acid, tris(trimethylsilyl) ether	L-threonic acid	0.4±0.03
10	16.05	L-(+)-Tartaric acid, bis(trimethylsilyl) ether, bis(trimethylsilyl) ester	tartaric acid	5.96±0.28
11	17.08	Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	Xylitol	8.67±0.3
12	18.06	D-Fructose, pentakis(trimethylsilyl) ether	D-fructose	4.9±0.08
13	18.16	Propane-1,2,3-tricarboxylic acid, 1-hydroxy, tetrakis-TMS	2-hydroxy-1,2,3-propanetricarboxylic acid	5.97±0.16
14	18.28	2-(Trimethylsilyl)ethyl beta-D-glucopyranoside	ethyl-D-glucopyranoside	12.95±0.48
15	18.67	D-(+)-Talose, pentakis(trimethylsilyl) ether	alpha-D-talose	1.68±0.65
16	18.93	D-Gluconic acid, 2,3,4,6-tetrakis-O-(trimethylsilyl)-, δ-lactone	D-Gulonic acid, 1,4-lactone	1.70±0.26
17	19.15	α-D-Galactopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	alpha-D-galactopyranose	3.75±0.98
18	19.60	Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester	3,4,5-trihydroxybenzoic acid	0.34±0.03
19	19.74	scyllo-Inositol, hexakis-TMS	scyllo-inositol	0.17±0.01
20	19.92	β-D-Allopyranose, pentakis(trimethylsilyl) ether	beta-D-allopyranose	8.29±0.23
21	20.25	D-Gluconic acid, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, trimethylsilyl ester	D-gluconic acid	9.30±0.21

no.	RT (min)	Compound directly detected by GC-MS	The name of the compound before derivatisation	Percentages ± SD (n=3)
22	20.34	Hexadecanoic acid, trimethylsilyl ester	hexadecanoic acid	4.14±0.35
23	20.40	Mucic acid, TMS	Mucic acid	0.31±0.01
24	21.11	myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	myo-inositol	3.27±0.12
25	21.35	Trimethylsilyl 3,4-bis(trimethylsiloxy)cinnamate	3,4-dihydroxycinnamic acid = caffeic acid	0.3±0.10
26	21.62	1-hydroxy-3,7,11,15-tetramethyl-2-hexadecene	1-hydroxy-3,7,11,15-tetramethyl-2-hexadecene	0.37±0.01
27	21.97	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	(Z,Z)-9,12- octadecadienoic acid)	2.78±2.50
28	22.22	Octadecanoic acid, trimethylsilyl ester	octadecanoic acid	2.19±0.13
29	22.38	D-Mannitol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	D-mannitol	0.5±0.07

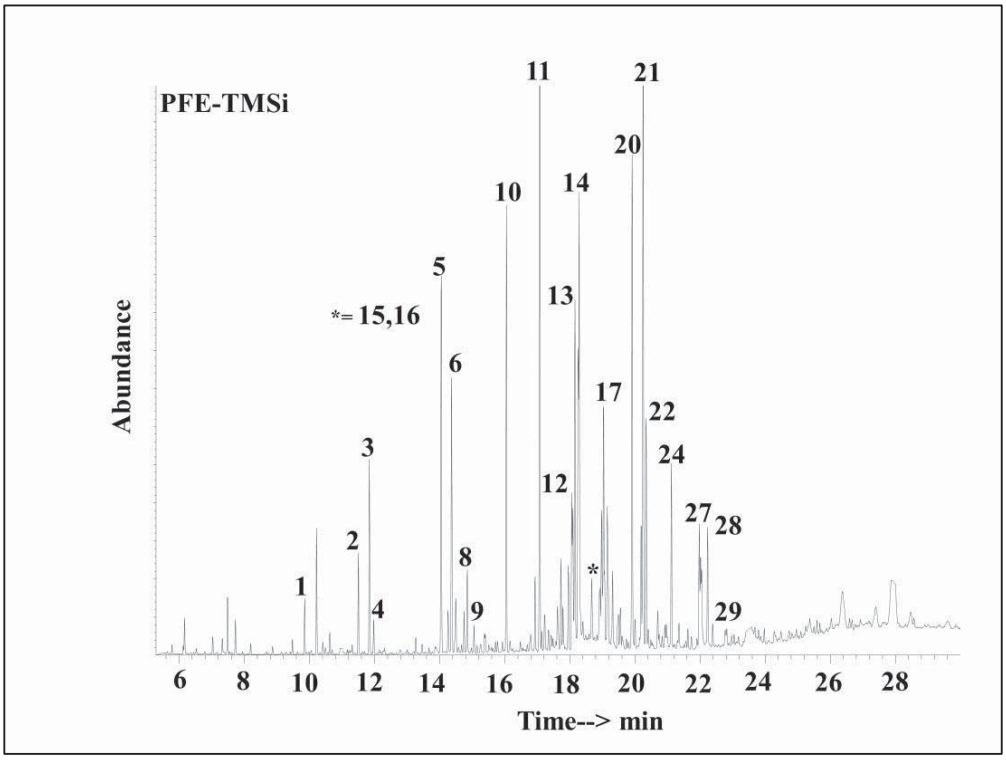


Figure 6.3: GC-MS chromatogram of compounds of PFE. The assay has been done according to section 2.8.1, n=3.

6.2. Discussion

6.2.1. Disk diffusion assay

PFH showed stronger antibacterial activity than PFE by using disk diffusion assay, which provided a fast and cheap method to determine the bacterial susceptibility of plant extracts before the more expensive and time-consuming determination of their MICs. PFE showed weak activity against most bacterial strains, however, the PFE showed interesting and good antibacterial activities against *Alcaligenes faecalis* with an IZ of 15 mm. Investigation of this extract by GC-MS showed the existence of gallic acid and caffeic acid in PFE which showed moderate to good antibacterial effects (Chapter III and IV). Previous report also

showed that the aqueous leave extract of PF contained gallic acid and caffeic acid (Asif 2012).

Caffeic acid showed good anti-staphylococcal activity against MSSA and MRSA with MICs equal to 62 and 250 µg/ml, respectively (Luís, Silva et al. 2014). In another study caffeic acid showed an MIC equal to 625 µg/ml against *S. aureus* and *S. epidermidis*, while it showed weak effect against *K. pneumonia* with MIC equal to 5000 µg/ml (Pinho, Ferreira et al. 2014). These phenolics or other unknown compounds might contribute to the inhibition of *A. faecalis*, which need to be investigated in the future.

6.2.2. MIC of PFH and metabolites profile

The MICs of PFH extract along with ampicillin as positive control were recorded against seven bacterial isolates which were recorded moderately sensitive to PFH by DDA (Table 6.1 and 6.2). *E. faecalis* and *P. aeruginosa* were not tested for MIC determination, as there was no inhibition of growth for these two screened by DDA.

PFH showed stronger activity against *B. cereus* with MIC of 64 µg/ml compared to the MIC of 128 µg/ml against *B. subtilis*. PFH generally recorded better activity against gram positive isolates used compared to the negative ones used in this study. PFH showed moderate antibacterial activity against *S. aureus* and *S. epidermidis* and weak activity against *E. coli*.

Limonene, eucalyptol and β-caryophyllene in PFH all showed weak antibacterial activity with MICs > 2048 µg/ml against *S. aureus, S. epidermidis, E. coli* and *E. cloacae.* Limonene showed a strong inhibition of *S. aureus* with MIC of 125 µg/ml (Kang, Helms et al. 1992), which contradicted the results of current research. The results indicated that both L-limonene and D-limonene showed very weak activity against *S. aureus* as also reported (Van Vuuren, Viljoen 2007).

Linalool a terpene alcohol detected from PFH showed an MIC equal to 1024 µg/ml against *E. cloacae*, which has been previously shown to exhibit a strong antibacterial activity against periodontopathic and cariogenic bacteria (Park, Lim et al. 2012). The MICs against *Porphylomonas gingivalis* ATCC 49417 and *Prevotella intermedia* ATCC 49046 were determined to be 100 µg/ml and 1600 µg/ml, respectively (Park, Lim et al. 2012). Linalool had MIC > 1000 µg/ml against *S. aureus*, *S. mutans* and *E. coli* (Kang, Helms et al. 1992). The antimicrobial activity of the methanol and steam distillate leaves extracts of *Perilla frutescens* and its components against some bacterial strains including *Propionibacterium acnes* and *S. aureus*, two of fungus *Mucor mucedo*, and *Penicillium chrysogenum* and a yeast, *Saccharomyces cerevisiae* showed that the MICs were > 500 µg/ml and < 250 µg/ml respectively against all mentioned isolates (Kang, Helms et al. 1992).

Caffeic acid and gallic acid were two of the phenolic compounds present in the defatted ethanol extract of *Perilla* seeds, they showed MIC 100-200 µg/ml and 50-800 µg/ml respectively against several *Porphyromonas gingivalis* strains (Yamamoto, Ogawa 2002). The results also indicated that PFH had antibacterial activity against *S. aureus* as well as other species such as *B. subtilis*, *E. cloacae* and *A. facecalis*, which are reported here for the first time.

Furthermore, the chemical composition of essential oils obtained by steam distillation/extraction has been well characterized as volatile compounds such as perilla ketone, linalool and β-caryophyllene (Kang, Helms et al. 1992, Seo, Baek 2009, Huang, Lei et al. 2011, Liu, Wan et al. 2013). Leaves of Korean *Perilla frutescens* were extracted using solvent-assisted flavour evaporation, liquid-liquid continuous and hydrodistillation extraction yielding perilla ketone as a major metabolite (>80%) compared with its low percentage (6%) present in the sample assayed in current research.

6.2.3. SEM analysis

PFH also caused dramatic change of cellular shape, the peeling of the cell wall and leakage of cell contents shown by SEM (Figure 6.1) for the first time. *Perilla* oil effectively decreased the production of α-toxin, enterotoxin A and B, and toxic shock syndrome toxin 1, in MRSA and MSSA (Qiu, Zhang et al. 2011). This encouraged the use of *Perilla* oil in combination with β-lactam to increase the antibacterial activities and to incorporate this oil to many food products for their protection (Qiu, Zhang et al. 2011).

Chapter 7 : Results and discussion of Antibacterial activities of the essential oil and lignans from flower buds of *Magnolia biondii* Pamp.

7.1. Results

Flower buds of *Magnolia biondii* Pamp (MB), family *Magnoliaceae* were recorded in Chinese Pharmacopoeia to treat various ailments like allergy, inflammation, rhinosinusitis, and accumulation of pus in the respiratory system. This study was designed to evaluate the antibacterial activity and to characterize the metabolites of MB.

In this study, the essential oil and lignans from the flower buds of MB were analysed using GC-MS and nuclear magnetic resonance (NMR) spectroscopy. The antibacterial activities against *S. aureus* and other bacteria on the basis of the MIC were evaluated. Furthermore, time-kill kinetics of *S. aureus* and *E. coli* in the presence of the *Magnolia* essential oil was also investigated. The micrography of *Magnolia* oil effects against *S. aureus* was studied using SEM.

7.1.1. Antibacterial activity

7.1.1.1. Determination of MIC using microplate method

MICs were determined according to the method detailed in section **2.17**; the results are shown in (Table 7.1) where the IC_{50}s were calculated using GraphPad prism software (v6.0).

Table 7.1: MICs of the essential oil and lignan-rich chloroform extract from flower buds of *Magnolia biondii* Pamp. The assay has been done according to section 2.17, n=4.

Bacterium	MIC		MIC (µg/ml)	IC_{50} (µg/ml)	MIC (µg/ml)
	essential oil		chloroform extract	essential oil	Ampicillin
	µg/ml	µl/ml			
B. cereus	500	0.5	>1000	260	>64
B. subtilis	250	0.25	>1000	210	≤0.5
S. aureus	500	0.5	>1000	320	≤0.5
S. epidermidis	500	0.5	>1000	280	≤0.5
E. coli	500	0.5	>1000	360	≤0.5

7.1.1.2. Time-kill assay

Magnolia essential oil killing kinetics against *S. aureus* and *E. coli* was determined using the method mentioned in section **2.18**; (Figure 7.1) shows the time kill assay results. Four concentrations (1000 µg/ml (2 MIC), 500 µg/ml (1 MIC), 250 µg/ml (1/2 MIC) and 0 (control) µg/ml) of the *Magnolia* oil were used for the time-kill kinetics assay in both *S. aureus* and *E. coli*. The oil at 1 MIC and ½ MIC level was not efficient to achieve the bactericidal effect after 24 hours. At 3-h exposure using 1000 µg/ml of the oil 5% and 9 % of live cells for *S. aureus* and *E. coli* were observed, respectively. After 24 hours less than 0.1% of viable cells (99.9 % or 3 log reduction in viable cells) for both of bacteria were observed, which indicated bactericidal effects.

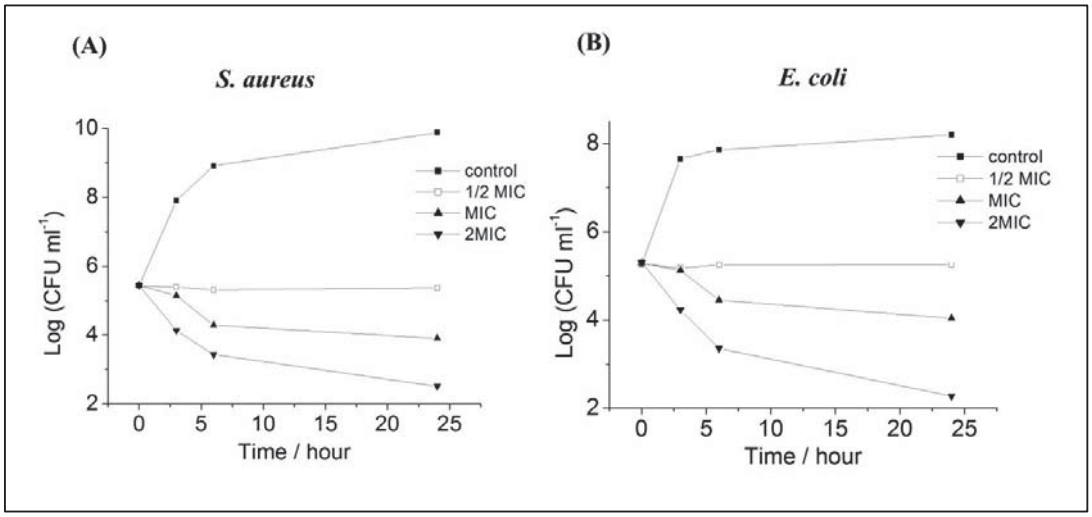


Figure 7.1: Time-kill kinetics of the *Magnolia biondii* Pamp essential oil at four different concentrations. (0 (control), 250 (½ MIC), 500 (MIC) and 1000 µg/ml (2 MIC)) against *S. aureus* (A) and *E. coli* (B), the assay has been done according to section 2.18, in duplicate.

7.1.1.3. Effects of MB oil on S. aureus cell wall

Effects of *Magnolia* oil against *S. aureus* cell wall were studied using SEM and the images are shown in (Figures 7.2 and 7.3). The cells fixed within one hour after exposure to the oil showed cell wall damage, leakage of contents and some cells were completely empty and appeared like empty shells, while the 24 hours sample showed complete destruction of the cells.

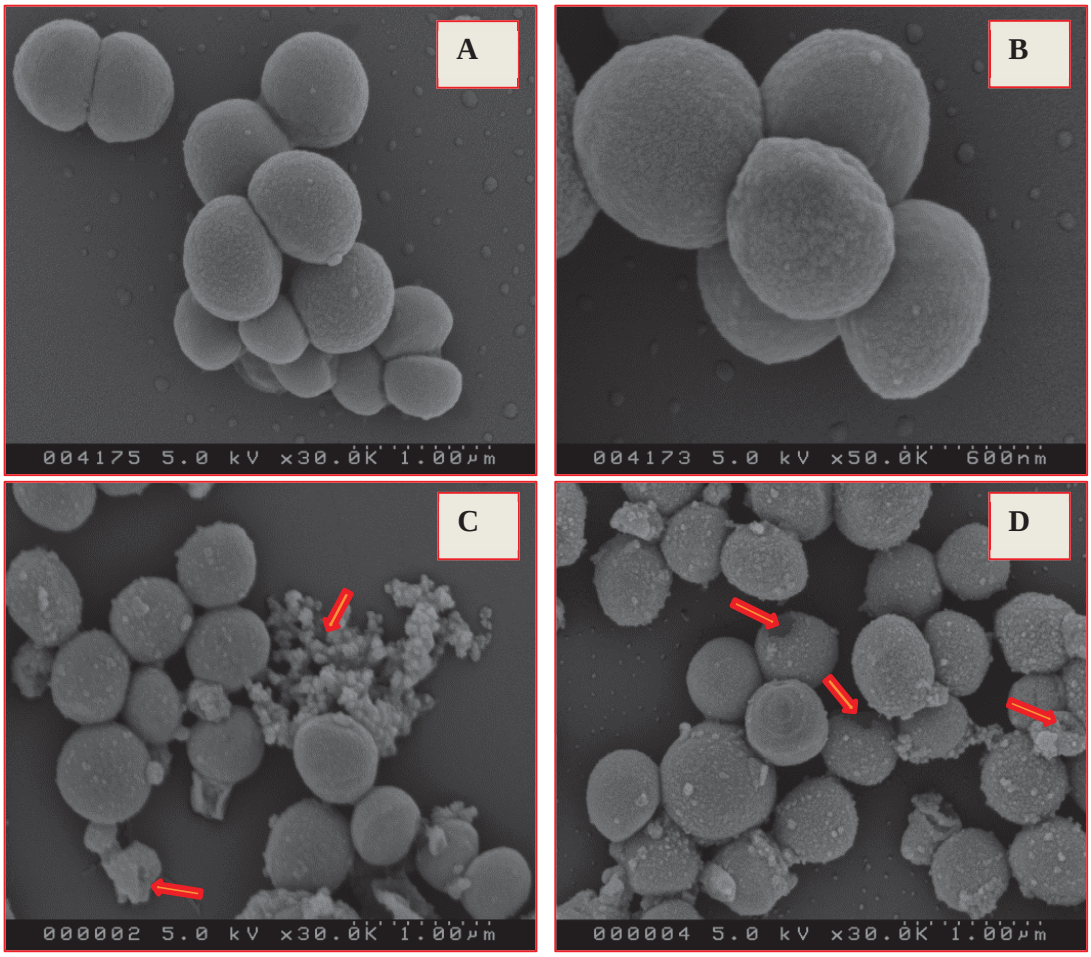


Figure 7.2: *Magnolia* oil against *S. aureus* cell wall (exposure time to the oil is 1 hr). A and B: SEM micrograph of *S. aureus* control after 1 hour of incubation at 37°C. C and D: Micrographs show the effects of *Magnolia* oil against *S. aureus* cell wall within 1 hr of exposure to the oil, arrows pointing the sites of cellular contents leakage and collapsed cells. The study has been done according to section 2.19.

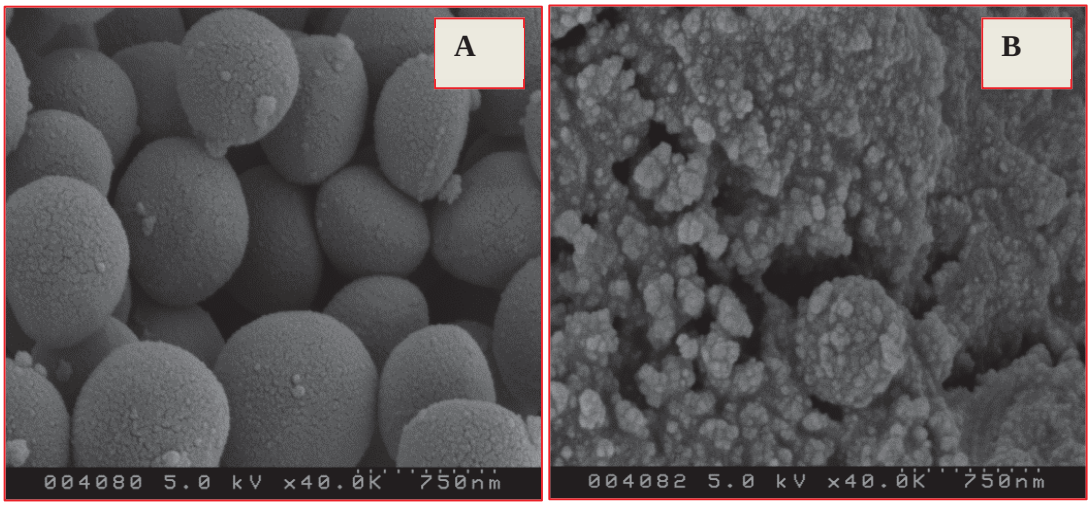


Figure 7.3: *Magnolia* oil against *S. aureus* cell wall (exposure time to the oil is 24 hr). A. Micrograph of *S. aureus* control after 24 hours of incubation at 37°C. Micrograph B shows the effects of Magnolia oil against *S. aureus* cells. We can see the destruction of cells and complete death of the culture after 24 hr of contact with oil. The study has been done according to section 2.19.

7.1.2. Spectroscopical analysis

7.1.2.1. GC-MS

The essential oil and lignan fractions of MB were prepared according to section **2.8.5.1** and injected into the system to identify their metabolites, using GC-MS method C mentioned in section **2.8.3**. The compounds detected are shown in (Table 7.2) and (Table 7.3) for the materials detected in MB oil and lignan fraction respectively. (Figure 7.4) and (Figure 7.5), show the GC-MS chromatograms of the oil and chloroform extract respectively.

Table 7.2: Compounds identified in the essential oil of *Magnolia biondii* Pamp. by GC-MS. *Trace, less than 0.1 %. References: (Qu, Qi et al. 2009, Zeng, Xie et al. 2011) which represent (a, b) respectively, samples prepared according to section 2.8.5.1 and analysed according to section 2.8.3 in triplicate n=3.

No.	RT (min)	Compound name	percentages ±SD (n=3)	Reference
1	5.39	α-Pinene	trace*	[a]
2	5.73	Camphene	trace	[a,b]
3	6.27	Sabinene	trace	
4	6.36	β-Pinene	trace	
5	6.66	β-Myrcene	trace	
6	6.84	Decane	trace	
7	7.01	α-Phellandrene	trace	[a]
8	7.30	(+)-3-carene	trace	[a]
9	7.59	D-limonene	0.27 ± 0.02	[b]
10	7.76	Eucalyptol (1,8-cineol)	25.00 ± 1.60	
11	8.34	γ-terpinene	trace	[b]
12	8.71	Linalool oxide	trace	[b]
13	8.74	Ethyl-2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	trace	
14	9.12	*Trans*-2-menthen-1-ol	trace	
15	9.13	Fenchone	trace	[a]
16	9.31	Undecane	trace	
17	9.47	3,7-dimethyl-1,6-Octadien-3-ol (linalool)	5.80 ± 0.30	[a,b]
18	9.67	Tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-2H-Pyran	0.17 ± 0.02	[a]
19	10.01	*Cis*-2-menthenol	0.50 ± 0.02	
20	10.49	*Cis*-1-methyl-4-(1-methylethyl)-2-cyclohexen-1-ol	trace	
21	10.64	Camphor ((+)-2-Bornanone)	10.60 ± 0.20	[a,b]
22	10.71	Fenchol	0.87 ± 0.03	[a]
23	11.49	Terpinen-4-ol	8.40 ± 0.30	[a,b]
24	11.91	α-terpineol	19.80 ± 1.80	[a]
25	12.70	Citronellol	2.90 ± 0.10	[a]
26	13.34	Geraniol	2.30 ± 0.10	[b]
27	14.05	Bornyl acetate	trace	
28	15.55	(3R-trans)-4-Ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, cyclohexene	trace	
29	15.89	1,3,3-trimethyl- 2-oxabicyclo[2.2.2]octan-6-ol acetate	trace	[a]
30	16.20	α-Copaene	trace	[b]
32	17.23	Caryophyllene	1.20 ± 0.05	[a,b]
33	17.92	(E)-β-farnesene	0.38 ± 0.16	
34	17.98	Humulene	0.15 ± 0.02	
35	18.14	Alloaromadendrene	trace	
36	18.38	1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-(1.alpha.,4a.beta.,8a.alpha)- naphthalene	trace	[a]
37	18.46	1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	0.20 ± 0.03	[b]
38	18.59	β-Copaene	0.54 ± 0.20	
39	18.75	(Z,Z)-alpha-Farnesene	trace	[a]
40	18.82	α-Cubebene	trace	[a,b]
41	18.97	Ioledene	0.57 ± 0.02	[a]
42	19.29	γ-Muurolene	0.58 ± 0.02	[b]
43	19.47	1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-(1S-*cis*)-naphthalene	2.40 ± 0.20	[a]
44	19.67	1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene	trace	[a]

45	19.77	1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-[1S-(1.alpha.,4a.beta.,8a.alpha.)]- naphthalene	trace	[a]
46	20.26	3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	0.25 ± 0.03	[a]
47	20.69	(-)-Spathulenol	trace	[a,b]
48	20.80	Caryophyllene oxide	trace	[a]
49	21.98	tau-Muurolol	2.30 ± 0.20	[b]
50	22.05	1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-[1R-(1.alpha.,4.beta.,4a.beta.,8a.beta.)]-1-naphthalenol	1.70 ± 0.90	[a]
51	22.26	α-Cadinol	3.30 ± 0.40	[a,b]
52	23.49	*trans*-Farnesol	8.70 ± 1.20	[a,b]
53	23.93	7-Acetyl-2-hydroxy-2-methyl-5-isopropylbicyclo[4.3.0]nonane (= oplopanone)	0.64 ± 0.09	[a]
54	24.25	*trans*-Geranylgeraniol	trace	
55	25.52	*trans*-Longipinocarveol	trace	[b]
56	25.57	3,7,11-Trimethyl-[S-(Z)]-1,6,10-dodecatrien-3-ol (Nerolidol)	trace	

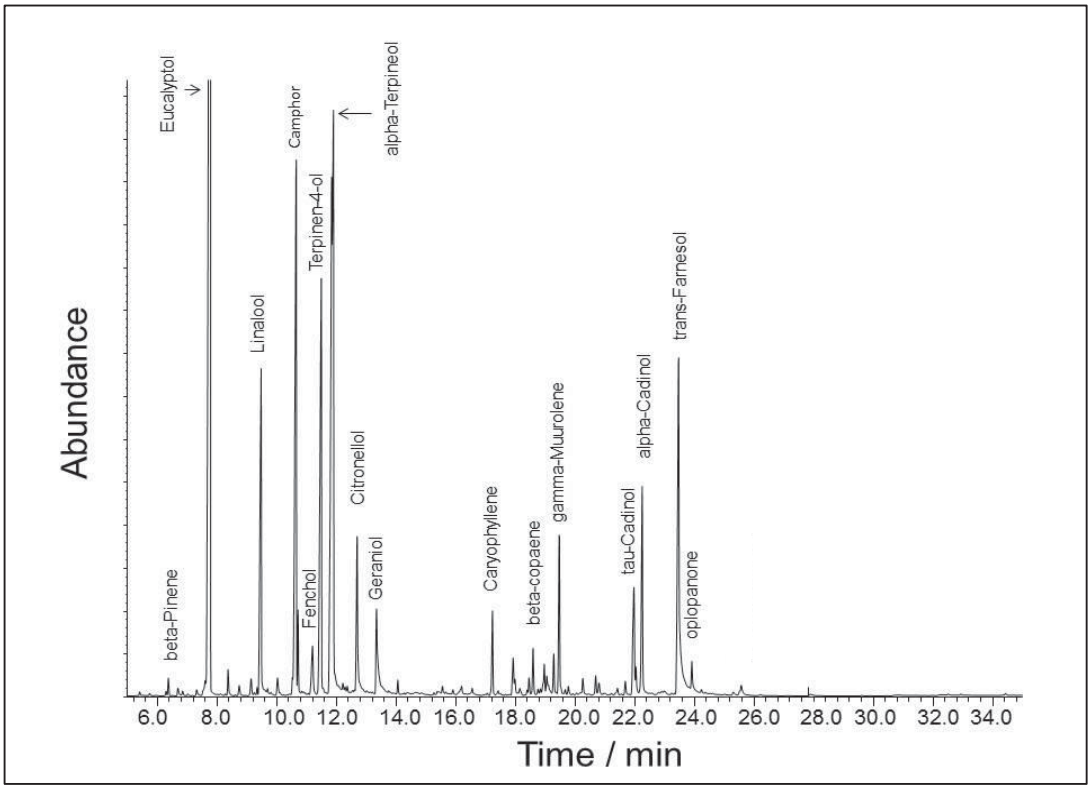


Figure 7.4: GC-MS chromatogram of the essential oil from flower buds of *Magnolia biondii* Pamp. 56 compounds (99.1 % of the total oil composition) were identified. Sections 2.8.5.1 and 2.8.3 in triplicate n=3.

Table 7.3: Lignans identified in the chloroform extract of MB Pamp. by GC-MS. Samples prepared according to section 2.8.5.1 and analysed according to section 2.8.3 in triplicate n=3

no.	RT (min)	Lignans	% of abundance ± SD (n=3)
1	25.08	demethoxyaschantin	2.7 ± 0.2
2	25.26	fargesin	2.0 ± 0.1
3	25.73	epieudesmin	1.0 ± 0.1
4	25.97	eudesmin	21.2 ± 0.3
5	27.33	aschantin	< 0.1
6	28.30	magnolin	55.9 ± 2.9
7	30.71	yangambin	5.5 ± 0.1

RT = Retention time in min, SD= Standard deviation, n= Number of the experiment replication, RI= Retention index.

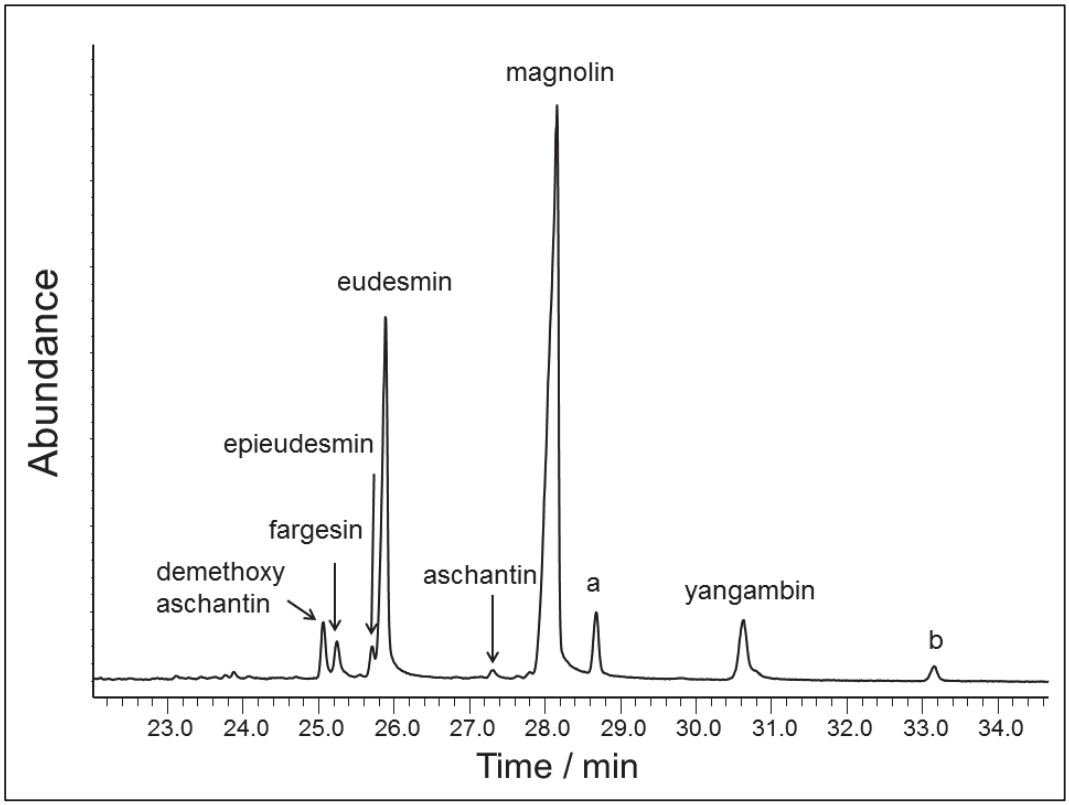


Figure 7.5: GC-MS chromatogram of the lignans from the chloroform extract of flower buds of *Magnolia biondii* Pamp. Two unknown compound a (MW = 646.5) and b (MW = 662.5) were also detected. Assay according to 2.8.3 in triplicate n=3.

7.1.2.2. NMR results

^{1}H NMR and ^{13}C NMR spectrum of the chloroform extract was done according to section 2.14. (Figure 7.6) shows the ^{1}H NMR spectrums of MB chloroform extract.

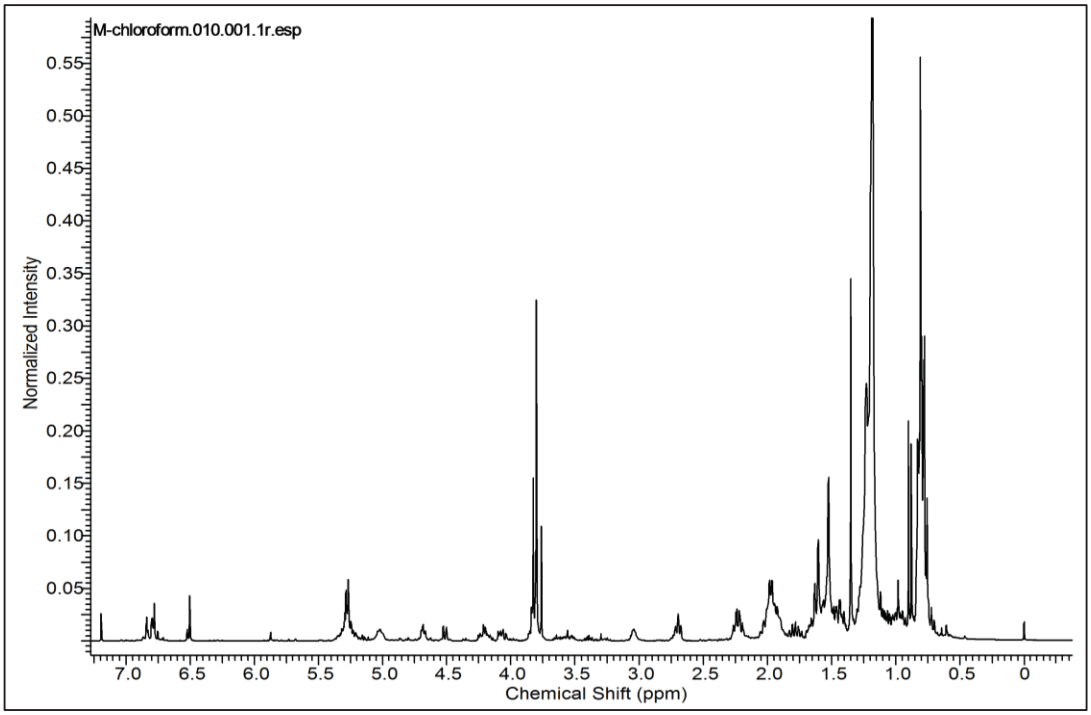


Figure 7.6: ^{1}H NMR of the chloroform extract of flower buds of *Magnolia biondii* Pamp. The analysis has been done according to section 2.14.

The ^{1}H NMR literature data of four lignans magnolin, eudesmin, fargesin and yangambin (Figure 7.7) are shown in (Table 7.4), they were in agreement with current research results as shown in the proton NMR chromatogram magnified at the areas corresponding to the proton shifts detailed in (Table 7.4), the magnified areas are shown in Appendix E, (Figures E-(1-6)). (Figure 7.8) shows the ^{13}C NMR spectra with assignment of yangambin carbon chemical shifts, which was identical with literature data.^{13}C NMR data of yangambin was as follows 153.4 (C-3,5,3′,5′), 136.7 (C-1,1′), 137.5 (C-4,4′), 102.8 (C-2,6,2′,6′), 85.9 (C-7,7′), 72.0 (C-9,9′), 60.8 (4,4′-OCH3), 56.2 (3,5,3′,5′-OCH3), 54.3 (C-8,8′).

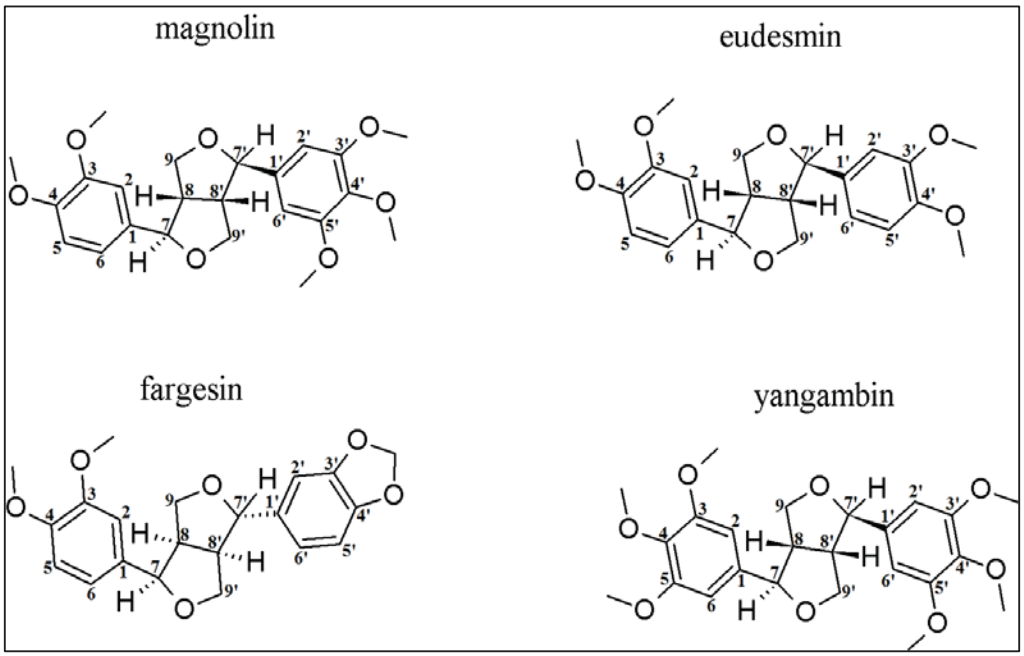


Figure 7.7: The chemical structures of four lignans magnolin, eudesmin, fargesin and yangambin detected from *Magnolia Biondii*.

Table 7.4: ^{1}H-NMR data of several lignans detected in MB. Magnolin, eudesmin and fargesin (Kakisawa, Kusumi et al. 1970) and of yangambin (Tulake, Jiang et al. 2012). Structures of the lignans are shown above in (Figure 7.7).

protons	magnolin	eudesmin	fargesin	yangambin
8-H	3.15 m (2H)	3.15 m (2H)	3.35 m	3.11 m (2H) 8,8′
8′-H			2.90 m	
7-H	4.8 d	4.75 d	4.88 d	4.76 d (2H) 7,7′
7′-H			4.45 d	
9-H	4.2-4.5 m (2H)	3.8-4.0 m (2H)	3.25-3.45 m	4.32 dd (2H) Hα-9,9′
9′-H	3.8-4.0 m (2H)	4.2-4.4 m (2H)	3.7-4.0 m (2H)	3.94 dd (2H) Hβ-9,9′
			4.1-4.25 m	
OCH3	3.85,3.90	3.86,3.88	3.90	3.88 (12H) 3,3′,5,5′
				3.85 (6H) 4,4′
-OCH2O-	-	-	5.95	
Aromatic	6.61, 6.90	6.8-7.0	6.8-6.95	6.58 (4H) 2,6,2′,6′

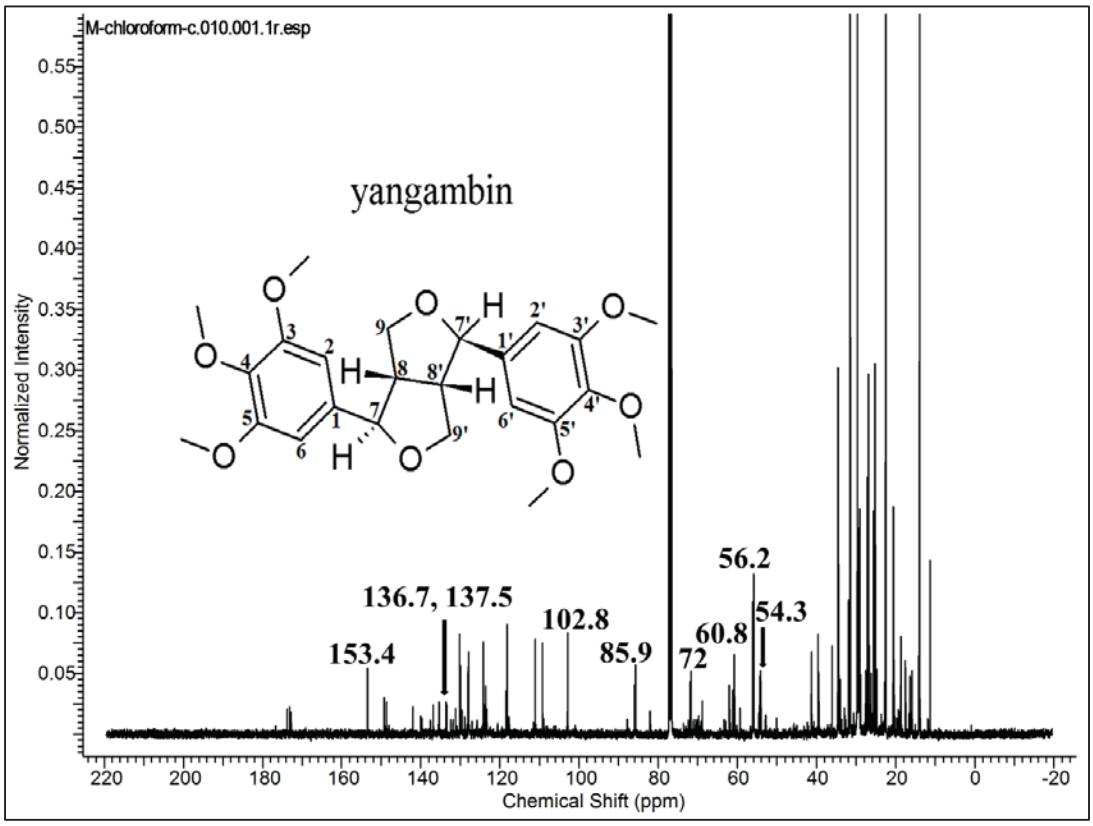


Figure 7.8: ^{13}C-NMR chart of *Magnolia biondii* chloroform extract , the chemical structure of magnolin shows the numbering system, chemical shifts are recorded in ppm and they are as follows. 153.4 (C-3,5,3′,5′), 136.7 (C-1,1′), 137.5 (C-4,4′), 102.8 (C-2,6,2′,6′), 85.9 (C-7,7′), 72.0 (C-9,9′), 60.8 (4,4′-OCH3), 56.2 (3,5,3′,5′-OCH3), 54.3 (C-8,8′). The analysis has been done according to section 2.14.

7.2. Discussion

7.2.1. Antibacterial activities

The *Magnolia* oil and lignan-rich chloroform extract were tested for their antibacterial activities against *B. cereus, B. subtilis, S. aureus, S. epidermidis* and *E. coli* by determination of their MICs using microplate alamar blue assay. The oil showed moderate antibacterial activity with MICs ranging from 250-500 µg/ml (0.25-0.5 µl/ml) against the

bacterial isolates used in this study with the most prominent effect against *B. subtilis* with MIC of 250 μg/ml (0.25 μl/ml).

The IC_{50}s of the essential oil against the five bacteria were also determined using Graph Pad software for more detailed comparison. The EO of *Magnolia* was more effective against *B. cereus* and *B. subtilis* (IC_{50} 260 and 210 μg/ml respectively) than its effect against *S. aureus* and *S. apidermidis* with the least effect on *E. coli* (IC_{50} 360 μg/ml), which is attributed to the nature of non-polar compounds from the oil. However, the lignan-rich extract showed weaker antibacterial activity than the essential oil, which suggested that the lignans played a less important antibacterial role than those terpenes and terpenoids in the essential oil.

The antibacterial activity of the essential oil observed above is probably due to the monoterpenoids such as linalool, alpha-terpineol, citronellol, geraniol, and trans-farnesol (Cerca, Gomes et al. 2013, Togashi, Inoue et al. 2008) which are some of the main parts of the essential oil. Caryophyllene oxide, borneol, camphor and 1,8-cineole revealed no antibacterial activities against several plant pathogenic bacteria (Dadasoglu, Kotan et al. 2015). *Lippia multiflora* Moldenke oil which contains 1,8-cineole (60.5%) shows weak antibacterial activities 1250 μg/ml against *S. aureus*, *B. cereus* and *E. coli* (Owolabi, Ogundajo et al. 2009).

In addition, geraniol and geranyl geraniol (both present in the *Magnolia* oil) could enhance the antibacterial activity of farnesol (Togashi, Inoue et al. 2008). Farnesol was found to induce the *S. epidermidis* biofilm detachment (Cerca, Gomes et al. 2013). In addition, some minor compounds such as bornyl acetate, β-carophyllene, β-copaene, γ-murrolene and α-murrolene could exert antibacterial effects in synergy with the major bioactive compounds (Bajpai, Rahman et al. 2008).

The essential oil antibacterial activities of another plant species of the genus *Magnolia* (*Magnolia liliiflora* Desr.) have been evaluated against several food borne pathogenic bacteria to show MIC values equal to 125 µg/ml against *S. aureus* , *B. subtilis* and *P. aeruginosa* and weaker effects against *E. coli* (MIC 500-1000 µg/ml) (Bajpai, Rahman et al. 2008).

The lignans detected still have important biological activities: magnolin from *Magnolia fargesii* showed inhibition of cancerous cell metastasis (Cheol-Jung Lee, Mee-Hyun Lee et al. 2015), yangambin showed antihypertensive activities (Albuquerque Araújo, Silva et al. 2014) and eudesmin showed anticonvulsant and sedative activities (Liu, Song et al. 2015). Eudesmin, magnolin and yangambin previously showed inhibitory effects on TNF-alpha production in LPS-stimulated murine macrophage cell line, and eudesmin showed the strongest activity (Chae, Kim et al. 1998). They also inhibited the NO production in LPS-activated microglia, suggesting a role in the treatment of neurodegenerative diseases (Kim, Lim et al. 2009).

Functional groups on the essential oil structure are essential for antibacterial activities, hydroxyl groups on the structure of carvacrol and thymol have key role in antimicrobial behavior (Knobloch, Weigand et al. 1986, Ultee, Bennink et al. 2002). Bacterial cell contains many targets susceptible for activity of volatile oil (Skandamis, Koutsoumanis et al. 2001, Skandamis, Nychas 2001, Carson, Mee et al. 2002). The hydrophobicity of the oils enables them to partition into the lipids of the bacterial cell membrane, affecting their structures and leaving them more permeable (Knobloch, Weigand et al. 1986, Sikkema, De Bont et al. 1994). Leakage of ions and cell materials is the second step (Oosterhaven, Poolman et al. 1995, Cox, Mann et al. 2000). The massive leakage of cell contents definitely leads to cell death (Denyer, Hugo 1991a).

Citronellol could show significant inhibition against *S. aureus* and *E. coli* by acting on the cell surface and causing the disruption of cell membrane (Lopez-Romero, Gonzalez-Rios et al. 2015). Farnesol may induce leakage of potassium ion by acting on membrane of *S. aureus* (Inoue, Shiraishi et al. 2004).

Many lipophilic compounds have been reported to increase membrane permeability (Trombetta, Castelli et al. 2005) and therefore potentiate the antibacterial potential of antibiotics that interact with intracellular targets.

7.2.2. GC-MS results

GC-MS revealed the presence of 56 volatile and semi-volatile compounds (99.1% of the total oil composition) from MB essential oil. The main composition of this oil is similar to the oil of MB reported previously (Qu, Qi et al. 2009, Zeng, Xie et al. 2011), however, some metabolites were detected here in this species for the first time which could be attributed to the difference in the collection time or place of collection. GC-MS analysis of the chloroform extract of MB indicated the presence of an abundance of lignans.

Four peaks were deduced for two pairs of stereoisomeric (epimeric) tetrahydrofurofuran lignans fargesin and demethoxyaschantin; eudesmin ((+)-pinoresinol dimethyl ether) and epieudesmin. The peaks were assigned based on the assumption that the *trans*-diaryl (*epi*-) isomers were eluted earlier than their *cis*-counterparts on the given stationary phase (5 % phenyl-methylpolysiloxane) (Yamamoto, Otto et al. 2004, Radulovic, Mladenovic et al. 2012).

The other three lignans were aschantin, magnolin, and yangambin (lirioresinol-B dimethyl ether) (Schuhly, Skarbina et al. 2009, Zhao, Zhou et al. 2007, Ma, Han 1995). These compounds were initially identified based on the NIST library comparison and previous phytochemical studies on this plant (Ma, Han 1995, Schuhly, Skarbina et al. 2009, Zhao, Zhou et al. 2007).

Further ^{1}H NMR (Figure 7.6) analysis of the total chloroform extract confirmed the presence of magnolin, eudesmin, fargesin (Kakisawa, Kusumi et al. 1970, Tulake, Jiang et al. 2012) and yangambin (Tulake, Jiang et al. 2012). The presence of yangambin was also confirmed by ^{13}C NMR in comparison with their reported data (Tulake, Jiang et al. 2012). Two unknown compounds detected with molecule weights of 646.5 and 662.5 (Figure 7.10) were not confirmed by NIST library indicating the probability to be novel, however, their separation and identification is needed to confirm their chemical identities.

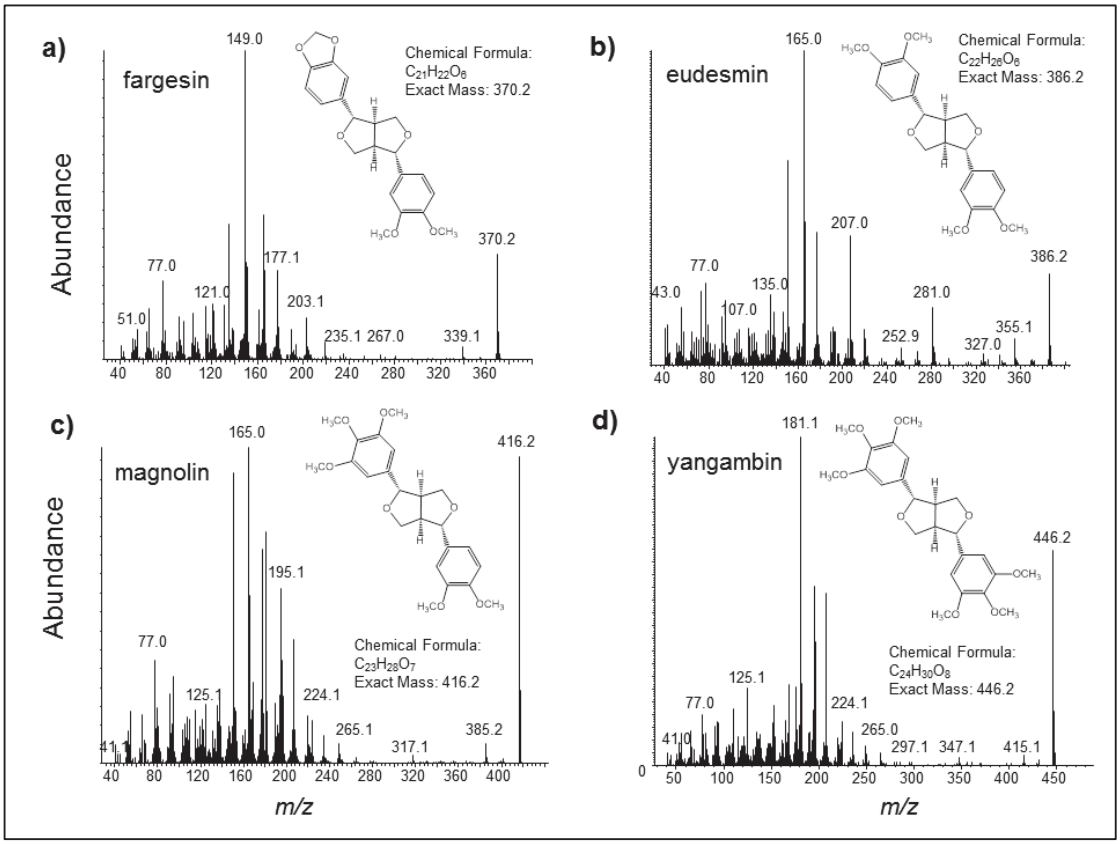


Figure 7.9: EI-MS of fargesin (a), eudesmin (b), magnolin (c), and yangambin (d) from the chloroform extract of flower buds of *Magnolia biondii* Pamp. Their chemical structures, chemical formula, and exact masses are shown in inset.

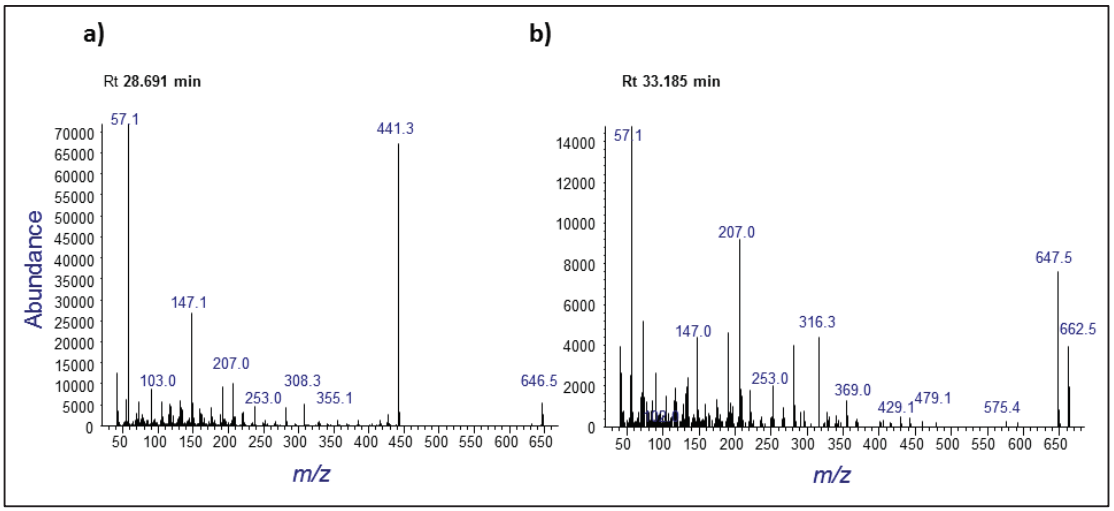


Figure 7.10: EI-MS of two unknown compounds (a) with RT, 28.691 min (a), and compound b with RT, 33.185 min (b).

Chapter 8 : General discussion, conclusion and future studies

8.1. General discussion

8.1.1. Microbial resistance

Bacteria, parasites, viruses and fungi have the abilities to develop resistance to antimicrobials. The encoding for the resistance can be transferred between microorganisms aggravating the problem; some microorganisms are multidrug resistance and called superbugs, prescribing the antibiotics using uncontrolled system contributes negatively to the resistance tragedy (Maisnier-Patin, Andersson 2004). Infection by resistant strains may lead to death and it is increasing the cost of treatment. The mortality number of resistant bacterial infections is around 25000 per year and the cost of treatment of the causative agents in Europe is exceeding one billion euros (Blair, Webber et al. 2015).

In 2015 the World Health Organization (WHO) estimated, that there were some 214 million new cases of malaria globally, resulting in approximately 438,000 deaths. Of these deaths, some 90% occur within Sub-Saharan Africa, over the last decade, the mortality rates are falling significantly, but the development and spread of resistance was the opponent of this success (Guantai, Chibale 2011, Ginsburg, Deharo 2011, World Health Organization 2015).

Bacterial resistance in general is developed when the bacterial cell wall permeability is changed, the antibacterial compounds are pumped out of the cells, bacterial enzymes destroy the antibacterial compounds and the receptor of the antibacterial material has been changed and becomes not accessible anymore for the antibacterial compound (Abreu, McBain et al. 2012). On the other hand, decrease the accumulation of drugs in the parasite

and affinity to some of the specific drug targets are the two major mechanisms of resistance to anti-malarial drugs (White 1999). In Introduction, brief explanations of resistance mechanisms and the role of natural compounds in controlling bacteria and parasites were presented.

Plant extracts, fractions and pure compounds prove themselves as important resistance modification agents, effectively modify the resistance mechanisms of the microbe and synergise the antimicrobials activities (Chlez, Hohmann et al. 2010, Sibanda, Okoh 2007). The inhibition of β-lactamase and PBP2a by gallate derivatives, to potentiate the drug activity against MRSA is an important example, epigallocatechin gallate decreases the concentration of imipenem required to inhibit the growth of several MRSA strains to 16% of its original concentration (Hu et al., 2002). Epigallocatechin gallate potentiates the effect of tetracycline by inhibition of Tet (K) pump (Sudano Roccaro et al., 2004). In the same direction, several esters of gallic acid synergise the antibacterial activities of β-lactam antibiotics against MRSA by inactivation of PBP2a (Shibata et al., 2005). Targeting more than one site of action in the microorganism is one of the important strategies to control microbial resistance (Wagner, Ulrich-Merzenich 2009).

8.1.2. The medicinal role of natural antimicrobials and challenges facing the bioguided-extraction procedure.

Natural products from plants play a role in controlling diseases particularly in Asia. Many peoples are dependent on natural therapy for wellbeing and controlling of various illnesses (Prabuseenivasan, Jayakumar et al. 2006). An organized system of medicinal plants recording dated back 2600 BC in Mesopotamia (Atanasov, Waltenberger et al. 2015).

The best way to find new antibacterial molecules with new mode of action is the following of an organized bioguided extraction method.

The starting of the bioguided extraction studies faced many challenges. The limited quantities of some active metabolites in the plant species in addition to limitation of the collected plant quantity due to protection rights of some plant, is one of these challenges (David, Wolfender et al. 2015).

The problem of shortage in medicinal plants is serious and must be put in the circle of care, therefore the European Medicines Agency launched the controlled agricultural and collection code of practice to ensure a sustainable plants collection (Atanasov, Waltenberger et al. 2015). In addition to the previously mentioned challenges, only very few numbers of natural products are successful to reach experiments circle beyond the *in vitro* assays.

Drug discovery of compounds from natural sources is hindered by the complexity of the chemical structures of natural molecules which have complex stereochemistry and functional groups. This makes their synthesis in laboratories a tedious task compared to pharmaceutical compounds produced by medicinal chemistry (Henrich, Beutler 2013), however, the complex stereochemistry of natural compounds could be advantageous from another side of view. Synthetic compounds have less chiral centres, more flexible bonds, and small molecule weights which produce low biological activities and less specific pharmacological action (Feher, Schmidt 2003), while the natural products have more complex structures and chirality and specifically bind proteins on the target cells (Koehn, Carter 2005). This brings the attention of pharmaceutical companies, back to the natural medicines. Pharmaceutical sector recognises that the synthesised molecules lack the special stereochemistry which natural products can offer and an increase in investments in natural products sector is expected (Gibbons 2008).

8.1.3. Selection of the plant species for the phytochemical study

There are several selection methods of plants for phytochemical studies. The random screening of plant extracts, fractions and pure compounds may lead to discovery of unexpected pharmacological activities, however, this strategy is suffering from low hit rate due to the limitation of pharmacological assays can be applied (Henrich, Beutler 2013).

Ethnopharmacology is a collection of treatment reports raised from local communities, botanical descriptions, plants pharmacopeia and other printed materials. Plants can be selected according to ethnopharmacological reports with a high possibility to find pure chemicals with pharmacological activities identical to the activities reported for the plants with ethnopharmacological background. Selection of the plant utilizing the chemotaxnomical information is another approach which phytochemist can use, certain plant species contains certain metabolites form its chemotaxnomical character (Atanasov, Waltenberger et al. 2015).

8.1.4. The selection of plants under research

The selection of *Cylicodiscus gabunensis*, *Pogostemon cablin*, *Perilla frutescens*, and *Magnolia biondii* was depending on ethnopharmacological reports. About 80% of 122 natural molecules used as medicines were extracted from plants with ethnopharmacological indication identical to the indication of the purified drugs (Farnsworth, Akerele et al. 1985).

8.1.5. Extraction of the active principles from plants under research

In most phytochemical studies, the active metabolites are unknown and the scientist needs a solvent able to extract most of the active constituents, 70% aqueous methanol or ethanol, is the solvent of choice in this case (Atanasov, Waltenberger et al. 2015). In this study we used an alternative method by extraction of active constituents using more than one solvent

of increasing polarity, which gives us extracts with small number of metabolites and almost uniform in polarity (Liu 2008).

The active plant extract is subjected to cascade of bioguided fractionation steps starting with liquid-liquid chromatography (in current study we used ethyl acetate and butanol) to get several fractions of different polarities (Liu 2008). Different kinds of chromatography were used for separation of active metabolites and purification of chemicals. The benzoate derivatives were identified by different kinds of chromatography and spectroscopy.

8.1.6. Evaluation of the antibacterial activities

To evaluate the antibacterial activities, we utilized the disk diffusion assay which is a routine technique in microbiology laboratories. To ensure reproducibility microbiologist needs to do up to the standards of official protocols (Matuschek, Brown et al. 2013). Disk diffusion assay is usually conducted to evaluate the antibacterial activities before using other techniques like broth microdilution, time kill kinetics and SEM (Burt 2004, Boire, Zhang et al. 2016, Jr.Valle, Cabrera et al. 2016).

In broth microdilution using alamar blue as an indicator, active cells have the reducing system able to convert resazurin (safe compound, enter cells freely) from blue colour to a red coloured material called resorufin. Resorufin is a fluorescent material and its emitted fluorescent energy can be quantified using spectrofluorometric apparatus such as GloMax-plus® Multi Detection System used in Keele University laboratories. This method is very sensitive and can quantify cells down to 50 cells (Léguillier, Lecsö-Bornet et al. 2015, Westhrin, Xie et al. 2015).

The time kill assay or time kill kinetics is used to determine whether the antibacterial of the extract, fraction, or pure compound is bactericidal or bacteriostatic (Igbinosa, Idemudia 2016, El-Azizi 2016). The bactericidal concentration reduces the initial number of the live colonies 99.9% or 3 log10 cfu/ml (Olajuyigbe, Afolayan 2015). The drug is bactericidal,

when the minimum bactericidal concentration (MBC) is no more than double of the MIC and the drug is bacteriostatic if the MBC is eight times more than the MIC (Coqueiro, Regasini et al. 2014). In the current research we developed the study in 96 well plates to conserve the active compounds and to ensure the required replications of experiment.

8.1.7. Assessment of the detection facilities used in the study

The detection systems of plant secondary products changed radically during the previous years, many characters like detection limit, analysis time and separation effectiveness have been changed dramatically. Gas chromatography is sensitive and has ability to detect many compounds in the complex plant extracts and using of mass spectrometry as a detector increases its efficacy compared to LC-MS which does not contain the required compounds data bases like the National Institute of Standards and Technology/Gaithersburg, MD, USA (Rohloff 2015).

The silylation step in this research was essential to detect polar and high boiling points metabolites (Lisec, Schauer et al. 2015), and the compounds detected in this study were not limited to sugars (monosaccharides), fatty acids, phenolics, and terpenoids. LC-MS is suitable for identification of more polar materials and high molecule weight materials of up to 2000 Da (Halket, Waterman et al. 2005). The glycosylated polyphenols and hydrolysable tannins have high molecular weight and need LC-MS for detection (Rohloff 2015) as used for the analysis of antiplasmodial and antibacterial compounds from CG plant in this study.

8.1.8. The antibacterial potential of the plants under study

Analysing the results revealed from *C. gabunensis* study showed that the gram positive isolates used in this study were more sensitive than the gram negative bacteria except *Alcaligenes faecalis* which is a non-pathogenic bacterium.

Several benzoic acid derivatives have been detected in the most active fraction of *C. gabunensis* which are probably the compounds responsible for the antibacterial activities. Gallic acid, one of the active metabolite detected in CG showed weak antibacterial activity against *S. aureus* and *S. epidermidis*, however, the esterification of carboxylic moiety in gallic acid increases the antibacterial activity against those bacteria drastically. In particular, lauryl gallate showed MIC of (8 µg/ml) against *S. aureus* and *S. epidermidis*, indicating that the carboxylic acid moiety may hinder the penetration of benzoic acid derivatives into the gram positive bacterial cells.

The EO of *Pogostemon cablin* showed less antibacterial activities compared to its hexane extract which was attributed to the less quantity of pogostone in the oil. The compounds extracted by hexane have been shown to exhibit better antimicrobial activity than the corresponding EOs obtained by steam distillation (Packiyasothy, Kyle 2002).

S. aureus is a microorganism with abilities to cause serious infections for patients in hospitals and vulnerable persons of community. *S. aureus* infects the skin producing boils and skin abscess. Other infections caused by *S. aureus* are endocarditis and pneumonia (Smith, Gould et al. 2010, Waryah, Gogoi-Tiwari et al. 2016). *S. aureus* causes food poisoning and the control of its activity is one of the important challenges to food suppliers and consumers (Tong, Chen et al. 2012, Aires, Marrinhas et al. 2016).

S. epidermidis is causing hospital-acquired infection, by lodging the medical devices like vascular catheter (Otto 2009, National Nosocomial Infections Surveillance System 2004). Treatment of these kinds of infection presents a challenge for the medical sector in hospitals since *S. epidermidis* cells are forming biofilms and may express resistance genes to certain treatment protocols (Carvalhais, Cerveira et al. 2015). *S. epidermidis* ranked the second after *S. aureus* in infecting prosthetic valve which leads to prosthetic valve endocarditis (Chu, Miro et al. 2009).

B. cereus causes food adulteration and food poisoning among consumers (Eneroth, Svensson et al. 2001, Ghelardi, Celandroni et al. 2002). *B. cereus* produces β-lactamases and can resists third generation cephalosporins (Cormican, Jones 1995). Ampicillin, oxacillin, and cephalotin showed MIC ≥ 32 μg/ml against *B. cereus* (Schlegelova, Brychta et al. 2003). Food stuffs of plant origin and products of milk especially become rotten by *B. cereus* (Zhang, Feng et al. 2016, Zhang, Tao et al. 2016). A serious consequences caused by *B. cereus* after seeding the catheters is the biofilm formation which is usually difficult to treat by conventional antibiotics (Kuroki, Kawakami et al. 2009, Celandroni, Salvetti et al. 2016).

PCH showed strong antibacterial activities against *B. cereus* with MIC of (16 μg/ml); however its main components showed less activity indicating a possible synergism between individual compounds.

B. subtilis used in biotechnology to produce different proteins (proteases and α-amylases), vitamins, and antibiotics. This kind of bacteria is safe to human and free of any toxin (van Dijl, Hecker 2013, Ploss, Reilman et al. 2016). *E. coli* cell wall is impermeable to some materials and it has pumps to expel unwanted compounds. The exposure to β-lactam antibiotics promotes selection of resistant strains (Gibbons 2008, Sanchez, Master et al. 2012). *E. coli* has polar properties (Borges, Ferreira et al. 2013), which prevents compounds like lauryl gallate from exerting its antibacterial activities.

After contact angle measurement, gallic acid increases the hydrophilicity of *E. coli* and decreases the hydrophilicity of *S. aureus*, this effect in addition to several physicochemical effects of gallic acid on *E. coli* and *S. aureus* studied by Borges et al. 2013 indicated that gallic acid can interact with the cell membrane of both types of bacteria (Borges, Ferreira et al. 2013). Gallic acid increases the permeability of *S. aureus* cell wall and induces the leakage of contents (Borges, Ferreira et al. 2013).

Simple phenols inactivate some bacterial enzymes by nonspecific interaction (Mason, Wasserman 1987). Ethyl gallate and other alkyl gallate synergise the β-lactam antibiotics against both MRSA and MSSA. The structure of the benzoates enable them to dwelling the external part of the bacterial cell membrane, disrupt its fluidity and decrease binding to PBP2a (Shibata, Kondo et al. 2005). Gallic acid potentiates the effects of tetracyclines against *P.aerognosa* (Jayaraman, Sakharkar et al. 2010) and with streptomycin against *E. coli* and *P.aerognosa* (Saavedra, Borges et al. 2010).

Essential oil from *Enteromorpha linza* L. exerts its activity by increasing the cell wall permeability and leakage of K^+, resulting in cell destruction and death (Patra, Baek 2016). Patchouli alcohol and pogostone showed multi-target antibacterial activities on penicillin binding proteins (PBPs), dihydrofolate synthetase (DHFS), dihydrofolate reductase (DHFR), RNA polymerase and DNA gyrase. The best effect of patchouli alcohol and pogostone was recorded by interaction with PBPs and DHFS respectively (Yang, Zhang et al. 2013).

Plant extracts contain more than one compound which can work synergistically by targeting more than one site of action (Wagner, Ulrich-Merzenich 2009). Sesquiterpenoid lactones are working by disrupting the bacterial cell wall integrity (Chadwick, Trewin et al. 2013). Other research reported that the nonpolar properties of terpenoids enable them to host the bacterial cell wall and increase the wall permeability (Gershenzon, Dudareva 2007). As we mentioned previously, patchouli alcohol targets PBPs to a high degree among the four proteins used (Yang, Zhang et al. 2013) which gives terpenoids more than single site of action.

Pencillin binding protein has a crucial role in peptidoglycan biosynthesis, and initiates several reactions (transpeptidation, transglucosylation and carboxypeptidation) essential for cell wall synthesis. β-lactam antibiotic interrupt all these steps by binding PBPs (Alves,

Froufe et al. 2014). The molecular docking system showed that the fitting score of patchouli alcohol is comparable to benzyl penicillin on PBPs (Yang, Zhang et al. 2013).

The antibacterial combination we used in this study consists of patchouli alcohol and gallic acid which could work on two targets in the bacterial cells and showed a synergism between the two components in most combinations.

8.1.9. The anti-malarial potential of the plants under the study.

The other important issue we need to cover in current research is Malaria, which is one of the most dangerous diseases in the world and it is one of the major health problems in the developing countries (Garcia-Alvarez, Moussa et al. 2013). The need for new antiplasmodials is raised up again and nature is the place where we need to search for the new antiplasmodial candidates. Phenolic acids and gallic acid oligosaccharides conjugates could play a vital role in the antiplasmodial activities of CG. Gallic acid and protocatechuic acid were isolated from *Sebastiania chamaelea* which showed $IC_{50} > 10$ μg/ml against the chloroquine resistant strain, *P. falciparum* W2 (Garcia-Alvarez, Moussa et al. 2013).

Ellagic, gentisic, and gallic acids were isolated from *Anogeissus leiocarpus*, and showed moderate to potent *in vitro* antiplasmodial activities against *P. falciparum* 3D7 with IC_{50} of 2.88-11.74 μM, in which the most potent compound was ellagic acid (IC_{50} 2.88 μM) (Ndjonka, Bergmann et al. 2012). Gallic acid and β-glucogallin were isolated from *Phyllanthus niruri* and their IC_{50} were (14.8 μg/ml and 4.6 μg/ml) respectively, indicating that attachment of glucose molecule to gallic acid increases the anti-malarial activities (Subeki, Matsuura et al. 2005).

8.1.10. The benzoates compounds used in this study

Current results showed that alkyl or alkenyl benzoates are more potent than gallic acid against *Plasmodium parasite*. Previous report on *Sorindeia juglandifolia* showed that

esterification of the carboxylic functional group in benzoic acid derivatives increased its antiplasmodial activities. 2,3,6-Trihydroxy benzoic acid showed IC_{50} of 16.5 μM against *P. falciparum* W2 while its methyl ester derivative 2,3,6-trihydroxy methyl benzoate showed IC_{50} of 13 μM (Kamkumo, Ngoutane et al. 2012).

8.2. Conclusions

The results of this research support the ethnobotanical use of *Cylicodiscus gabunensis* as a treatment of malaria, the most active fraction CGEBUF-10-7 showed most potent antiplasmodial activities (IC_{50} 4.7 μg/ml) against *P. falciparum* Dd2. The active constituents were benzoic acid derivatives which act as antibacterial and antiplasmodial agents.

The improved method for 3,4,5-trihydroxy benzoates synthesis offer a fast, clean and single step of purification and it was successful to synthesise esters from primary alcohols and secondary alcohols. Those compounds have a better activity than gallic acid against *S. aureus*, *S. epidermidis*, *E. coli*, *B. subtilis* and *B. cereus*. The products synthesised here are promising for further studies to prove their role as antibacterials with probability to use as food additives.

The hexane and ethanol extracts of both *Pogostemon cablin* and *Perilla frutescens* (the two key compounds of the traditional medicine Huo-Xiang-Zhen-Qi-Wan), have been characterized by GC-MS, and they both showed antibacterial activities against a range of gram-positive and negative strains using DDA and alamar blue microplate assay. In particular, patchouli alcohol in *Pogostemon cablin* shows promising antibacterial activity against *S. epidermidis* and *E. coli*. The anti-bacillus activity of those two extracts present a chance for further developments of them as possible food additives or as resistant modifying agents as it is supported by the SEM results.

The tetrahydrofurofuran lignans in *Magnolia biondii* were readily detected by GC-MS for the first time without prior trimethylsilylation as usually required for the analysis of lignans (Li, Barz 2006), which could offer a simple method for the quantification of these lignans from MB, alternative to the HPLC method (Zhao, Zhou et al. 2007).

The combination usage of GC-MS and NMR techniques allowed the identification of 56 volatile compounds and 7 non-volatile lignans present in the extracts of MB. The *Magnolia* essential oil showed antibacterial and bactericidal activity, which could provide a scientific basis for the treatment of rhinosinusitis caused by *S. aureus* infection (Redinbo 2014) and suggested its potential use as antibacterial agents. *Magnolia* oil showed activity toward *S. aureus, E. coli* and *B. cereus* which are the major causes of food poisoning, therefore providing a chance of using this oil in food preservation.

8.3. Future work

The future work is suggested to investigate the antibacterial activities of the benzoate derivatives against antibiotic resistant bacterial strains alone and in combination with antibiotics. LC-MS-MS study of the most antiplasmodial fraction from *Cylicodiscus gabunensis* (CGEBU-F10-7) is highly recommended to elucidate their detailed chemical structure including the linkages between the sugars and gallic acid. It would be interesting to check any synergism between this active antiplasmodial fraction of *Cylicodiscus gabunensis* and chloroquine against chloroquine resistant *Plasmodium* strains.

References

[EFSA] EUROPEAN FOOD SAFETY AUTHORITY, 2010. Scientific opinion on flavouring group evaluation 20, revision 2 (FGE.20Rev2): benzyl alcohols, benzaldehydes, a related acetal, benzoic acids, and related esters from chemical groups 23 and 30. *EFSA Journal,* **8**(7), pp. 1405-1-136.

ABREU, A.C., MCBAIN, A.J. and SIMOES, M., 2012. Plants as sources of new antimicrobials and resistance-modifying agents. *Natural product reports,* **29**(9), pp. 1007-1021.

ABU-DARWISH, M.S., CABRAL, C., GONCALVES, M.J., CAVALEIRO, C., CRUZ, M.T., ZULFIQAR, A., KHAN, I.A., EFFERTH, T. and SALGUEIRO, L., 2016. Chemical Composition and Biological Activities of Artemisia judaica Essential Oil from Southern Desert of Jordan. *Journal of ethnopharmacology,* **191**, pp. 161-168.

AFOLAYAN, A. and MEYER, J., 1997. The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of Helichrysum aureonitens. *Journal of ethnopharmacology,* **57**, pp. 177-181.

AIRES, A., MARRINHAS, E., CARVALHO, R., DIAS, C. and SAAVEDRA, M.J., 2016. Phytochemical Composition and Antibacterial Activity of Hydroalcoholic Extracts of Pterospartum tridentatum and Mentha pulegium against *Staphylococcus aureus* Isolates. *BioMed research international,* **2016**, pp. 5201879.

AIRES, A., MARQUES, E., CARVALHO, R., ROSA, E.A.S. and SAAVEDRA, M.J., 2013. Evaluation of biological value and appraisal of polyphenols and glucosinolates from organic baby-leaf salads as antioxidants and antimicrobials against important human pathogenic bacteria. *Molecules (Basel, Switzerland),* **18**(4), pp. 4651-4668.

AIT-OUAZZOU, A., CHERRAT, L., ESPINA, L., LORÁN, S., ROTA, C. and PAGÁN, R., 2011. The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innov. Food Sci. Emerg.,* **12**, pp. 320-329.

AKGUL, A. and KIVANC, M., 1988. Inhibitory effects of selected Turkish spices and oregano components on some foodborne fungi. *International journal of food microbiology,* **6**, pp. 263-268.

ALBUQUERQUE ARAÚJO, I.G., SILVA, D.F., DO CARMO, D.A., GONDIM DIAS, K.L., MARQUES CAVALCANTE, K.V., CAVALCANTE VERAS, R., BARBOSA-FILHO, J., DOS, A.N., BENDHACK, L.M., DE, A.C. and DE MEDEIROS, I.A., 2014. Calcium Influx Inhibition is Involved in the Hypotensive and Vasorelaxant Effects Induced by Yangambin. *Molecules,* **19**(5), pp. 6863-6876.

AL-DABBAS, M.M., SUGANUMA, T., KITAHARA, K., HOU, D.X. and FUJII, M., 2006. Cytotoxic, antioxidant and antibacterial activities of Varthemia iphionoides Boiss. extracts. *Journal of ethnopharmacology,* **108**(2), pp. 287-293 7p.

AL-MASSARANI, S.M., EL GAMAL, A.A., AL-MUSAYEIB, N.M., MOTHANA, R.A., BASUDAN, O.A., AL-REHAILY, A.J., FARAG, M., ASSAF, M.H., EL TAHIR, K.H. and MAES, L., 2013. Phytochemical, antimicrobial and antiprotozoal evaluation of Garcinia mangostana pericarp and alpha-mangostin, its major xanthone derivative. *Molecules (Basel, Switzerland),* **18**(9), pp. 10599-10608.

ALMEIDA, A.A.P., NAGHETINI, C.C., SANTOS, V.R., ANTONIO, A.G., FARAH, A. and GLÓRIA, M.B.A., 2012. Influence of natural coffee compounds, coffee extracts and increased levels of caffeine on the inhibition of Streptococcus mutans. *Food Research International,* **49**(1), pp. 459-461.

AL-MUSAYEIB, N.M., MOHAMED, G.A., IBRAHIM, S.R. and ROSS, S.A., 2014. New thiophene and flavonoid from Tagetes minuta leaves growing in Saudi Arabia. *Molecules (Basel, Switzerland),* **19**(3), pp. 2819-2828.

ALVES, M.J., FERREIRA, I.C.F.R., FROUFE, H.J.C., ABREU, R.M.V., MARTINS, A. and PINTADO, M., 2013. Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *Journal of applied microbiology,* **115**(2), pp. 346-357.

ALVES, M.J., FROUFE, H.J.C., COSTA, A.F.T., SANTOS, A.F., OLIVEIRA, L.G., OSÓRIO, S.R.M., ABREU, R.M.V., PINTADO, M. and FERREIRA, I.C.F.R., 2014. Docking Studies in Target Proteins Involved in Antibacterial Action Mechanisms: Extending the Knowledge on Standard Antibiotics to Antimicrobial Mushroom Compounds. *Molecules,* **19**(2), pp. 1672-1684.

AMAROWICZ, R., PEGG, R.B. and BAUTISTA, D.A., 2000. Antibacterial activity of green tea polyphenols against *Escherichia coli* K 12. *Die Nahrung,* **44**(1), pp. 60-62.

ANDERSON, M.M., O'NEILL, M.J., PHILLIPSON, J.D. and WARHURST, D.C., 1991. In vitro cytotoxicity of a series of quassinoids from Brucea javanica fruits against KB cells. *Planta Medica,* **57**(1), pp. 62-64.

ANDERSSON, D.I. and HUGHES, D., 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature reviews.Microbiology,* **8**(4), pp. 260-271.

ASAEDA, G., CAICEDOW, G. and SWANSON, C., 2005. Fried rice syndrome. *JEMS : a journal of emergency medical services,* **30**(12), pp. 30-32.

ASIF, M., 2012. Phytochemical study of polyphenols in Perilla Frutescens as an antioxidant. *Avicenna journal of phytomedicine,* **2**(4), pp. 169-178.

ATANASOV, A.G., WALTENBERGER, B., PFERSCHY-WENZIG, E.M., LINDER, T., WAWROSCH, C., UHRIN, P., TEMML, V., WANG, L., SCHWAIGER, S., HEISS, E.H., ROLLINGER, J.M., SCHUSTER, D., BREUSS, J.M., BOCHKOV, V., MIHOVILOVIC, M.D., KOPP, B., BAUER, R., DIRSCH, V.M. and STUPPNER, H., 2015. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances,* **33**(8), pp. 1582-1614.

AURELI, P., COSTANTINI, A. and ZOLEA, S., 1992. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. *J. Food Prot.,* **55**, pp. 344-348.

AYAFOR, J., TCHUENDEM, M. and NYASSE, B., 1994. Novel bioactive diterpenoids from *Aframomum aulacocarpos*. *J. Nat. Prod.,* **57**, pp. 917-923.

AZIZ, N.H., FARAG, S.E., MOUSA, L.A. and ABO-ZAID, M., 1998. Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios,* **93**(374), pp. 43-54.

AZZOUZ, M. and BULLERMAN, L., 1982. Comparative antimycotic effects of selected herbs, spices, plant components and commercial antifungal agents . *Journal of Food Protection,* **45**(14), pp. 1298-1301.

BAGHDIKIAN, B., MAHIOU-LEDDET, V., BORY, S., BUN, S.S., DUMETRE, A., MABROUKI, F., HUTTER, S., AZAS, N. and OLLIVIER, E., 2013. New antiplasmodial alkaloids from Stephania rotunda. *Journal of ethnopharmacology,* **145**(1), pp. 381-385.

BAHAMONTES-ROSA, N., RODRÍGUEZ-ALEJANDRE, A., GONZÁLEZ-DEL-RIO, R., GARCÍA-BUSTOS, J.F. and MENDOZA-LOSANA, A., 2012. A new molecular approach for cidal vs static antimalarial determination by quantifying mRNA levels. *Molecular and biochemical parasitology,* **181**(2), pp. 171-177.

BAJPAI, V.K., RAHMAN, A., DUNG, N.T., HUH, M.K. and KANG, S.C., 2008. In vitro inhibition of food spoilage and foodborne pathogenic bacteria by essential oil and leaf extracts of Magnolia liliflora Desr. *Journal of Food Science,* **73**(6), pp. M314-20.

BASCO, L.K., MITAKU, S., SKALTSOUNIS, A.L., RAVELOMANANTSOA, N., TILLEQUIN, F., KOCH, M. and LE BRAS, J., 1994. In vitro activities of furoquinoline and acridone alkaloids against Plasmodium falciparum. *Antimicrobial Agents and Chemotherapy,* **38**(5), pp. 1169-1171.

BATISTA, O., DUARTE, A., NASCIMENTO, J. and SIMONES, M., 1994. Structure and antimicrobial activity of diterpenes from the roots of *Plectranthus hereroensis*. *J. Nat. Prod.,* **57**, pp. 858-861.

BATISTA, R., SILVA ADE, J.,Jr and DE OLIVEIRA, A.B., 2009. Plant-derived antimalarial agents: new leads and efficient phytomedicines. Part II. Non-alkaloidal natural products. *Molecules (Basel, Switzerland),* **14**(8), pp. 3037-3072.

BEESON, J.G., BOEUF, P. and FOWKES, F.J., 2015. Maximizing antimalarial efficacy and the importance of dosing strategies. *BMC medicine,* **13**, pp. 110-015-0349-9.

BISHOP, C., 1995. Antiviral activity of the essential oil of *Melaleuca alternifolia* (Maiden and Betche) Cheel (tea tree) against *tobacco* mosaic virus. *Journal of Essential Oil Research,* **7**, pp. 641-644.

BLAIR, J.M., WEBBER, M.A., BAYLAY, A.J., OGBOLU, D.O. and PIDDOCK, L.J., 2015. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol,* **13**(1), pp. 42-51.

BOIRE, N., ZHANG, S., KHUVIS, J., LEE, R., RIVERS, J., CRANDALL, P., KEEL, M.K. and PARRISH, N., 2016. Potent Inhibition of Pseudogymnoascus destructans, the Causative Agent of White-Nose Syndrome in Bats, by Cold-Pressed, Terpeneless, Valencia Orange Oil. *PLoS ONE,* **11**(2), pp. 1-10.

BORGES, A., FERREIRA, C., SAAVEDRA, M.J. and SIMOES, M., 2013. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microbial drug resistance (Larchmont, N.Y.),* **19**(4), pp. 256-265.

BOUZABATA, A., BAZZALI, O., CABRAL, C., GONÇALVES, M.J., CRUZ, M.T., BIGHELLI, A., CAVALEIRO, C., CASANOVA, J., SALGUEIRO, L. and TOMI, F., 2013. New compounds, chemical composition, antifungal activity and cytotoxicity of the essential oil from Myrtus nivellei Batt. & Trab., an endemic species of Central Sahara. *Journal of ethnopharmacology,* **149**(3), pp. 613-620 8p.

BRAY, D.H., WARHURST, D.C., CONNOLLY, J.D., O'NEILL, M.J. and PHILLIPSON, J.D., 1990. Plants as sources of antimalarial drugs. Part 7. Activity of some species of Meliaceae and their constituent limonoids. *Phytother Res.,* **4**, pp. 29-35.

BRINGMANN, G., SAEB, W., ASSI, L.A., FRANCOIS, G., SANKARA NARAYANAN, A.S., PETERS, K. and PETERS, E.M., 1997. Betulinic acid: isolation from Triphyophyllum peltatum and Ancistrocladus heyneanus, antimalarial activity, and crystal structure of the benzyl ester. *Planta Medica,* **63**(3), pp. 255-257.

BUKVICKI, D., GOTTARDI, D., VELJIC, M., MARIN, P., VANNINI, L. and GUERZONI, M., 2012. Identification of Volatile Components of Liverwort (Porella cordaeana) Extracts Using GC/MS-SPME and Their Antimicrobial Activity. *Molecules,* **17**, pp. 6982-6995.

BURROWS, J.N., LEROY, D., LOTHARIUS, J. and WATERSON, D., 2011. Challenges in antimalarial drug discovery. *Future Medicinal Chemistry,* **3**(11), pp. 1401-1412.

BURT, S. and REINDERS, R., 2003. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. *Letters in Applied Microbiology,* **36**(3), pp. 162-167.

BURT, S., 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. *International journal of food microbiology,* **94**(3), pp. 223-253.

CALDERÓN, ÁI., ROMERO, L.I., ORTEGA-BARRÍA, E., BRUN, R., CORREA A., M.D. and GUPTA, M.P., 2006. Evaluation of Larvicidal and in Vitro Antimalarial Activities of Plants in a Biodiversity Plot in the Altos de Campana National Park, Panama. *Pharmaceutical Biology,* **44**(7), pp. 487-498.

CANNING, C., SUN, S., JI, X., GUPTA, S. and ZHOU, K., 2013. Antibacterial and cytotoxic activity of isoprenylated coumarin mammea A/AA isolated from Mammea africana. *Journal of ethnopharmacology,* **147**(1), pp. 259.

CARRAZ, M., JOSSANG, A., FRANETICH, J.F., SIAU, A., CICERON, L., HANNOUN, L., SAUERWEIN, R., FRAPPIER, F., RASOANAIVO, P., SNOUNOU, G. and MAZIER,

D., 2006. A plant-derived morphinan as a novel lead compound active against malaria liver stages. *PLoS medicine,* **3**(12), pp. e513.

CARSON, C., COOKSON, B., FARRELLY, H. and RILEY, T., 1995a. Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia*. *Journal of Antimicrobial Chemotherapy,* **35**, pp. 421-424.

CARSON, C., MEE, B. and RILEY, T., 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time kill, lysis, leakage and salt tolerance assays and electron microscopy. *Antimicrobial Agents and Chemotherapy,* **46**(6), pp. 1914-1920.

CARVALHAIS, V., CERVEIRA, F., VILANOVA, M., CERCA, N. and VITORINO, R., 2015. An immunoproteomic approach for characterization of dormancy within *Staphylococcus epidermidis* biofilms. *Molecular immunology,* **65**(2), pp. 429-435.

CASLEY-SMITH, J. and CASLEY-SMITH, J., 1997. Coumarin in the treatment of lymphoedema and other high-protein oedemas. In: R. O'KENNEDY and R. THORNES, eds, *Coumarins: biology, applications and mode of action.* New York, N.Y.: John Wiley & Sons, Inc., pp. 348.

CELANDRONI, F., SALVETTI, S., GUEYE, S.A., MAZZANTINI, D., LUPETTI, A., SENESI, S. and GHELARDI, E., 2016. Identification and Pathogenic Potential of Clinical Bacillus and Paenibacillus Isolates. *PLoS ONE,* **11**(3), pp. 1-13.

CERCA, N., GOMES, F., BENTO, J.C., FRANCA, A., ROLO, J., MIRAGAIA, M., TEIXEIRA, P. and OLIVEIRA, R., 2013. Farnesol induces cell detachment from established S. epidermidis biofilms. *The Journal of antibiotics,* **66**(5), pp. 255-258.

CHABOT, S., BEL-RHLID, R., CHENEVERT, R. and PICHE, Y., 1992. Hyphal growth promotion in vitro of the *VA mycorrhizal fungus, Gigaspora margaritaBecker and Hall*, by the activity of structurally specific flavonoid compounds under CO2-enriched conditions. *New Phytol.*, **122**, pp. 461-467.

CHADWICK, M., TREWIN, H., GAWTHROP, F. and WAGSTAFF, C., 2013. Sesquiterpenoids Lactones: Benefits to Plants and People. *International Journal of Molecular Sciences,* **14**(6), pp. 12780-12805.

CHAE, S.H., KIM, P.S., CHO, J.Y., PARK, J.S., LEE, J.H., YOO, E.S., BAIK, K.U., LEE, J.S. and PARK, M.H., 1998. Isolation and identification of inhibitory compounds on TNF-alpha production from Magnolia fargesii. *Archives of Pharmacal Research,* **21**(1), pp. 67-69.

CHAKRABORTY, B., CHAKRABORTY, S. and SAHA, C., 2014. Antibacterial Activity of Murrayaquinone A and 6-Methoxy-3,7-dimethyl-2,3-dihydro-1H-carbazole-1,4(9H)-dione. *International Journal of Microbiology,* , pp. 1-8.

CHANG, B., LEE, Y., KU, Y., BAE, K. and CHUNG, C., 1998. Antimicrobial activity of magnolol and honokiol against periodontopathic microorganisms. *Planta Medica,* **64**(4), pp. 367-369.

CHEESMAN, L., NAIR, J.J. and VAN STADEN, J., 2012. Antibacterial activity of crinane alkaloids from Boophone disticha (Amaryllidaceae). *Journal of ethnopharmacology*, **140**(2), pp. 405-408.

CHEOL-JUNG LEE, MEE-HYUN LEE, SUN-MI YOO, KYUNG-IL CHOI, JI-HONG SONG, JEONG-HOON JANG, SEI-RYANG OH, HYUNG-WON RYU, HYE-SUK LEE, YOUNG-JOON SURH and YONG-YEON CHO, 2015. Magnolin inhibits cell migration and invasion by targeting the ERKs/RSK2 signaling pathway. *BMC Cancer*, **15**(1), pp. 1-12.

CHERUIYOT, J., INGASIA, L.A., OMONDI, A.A., JUMA, D.W., OPOT, B.H., NDEGWA, J.M., MATIVO, J., CHERUIYOT, A.C., YEDA, R., OKUDO, C., MUIRURI, P., BIDII, N.S., CHEBON, L.J., ANGIENDA, P.O., EYASE, F.L., JOHNSON, J.D., BULIMO, W.D., ANDAGALU, B., AKALA, H.M. and KAMAU, E., 2014. Polymorphisms in Pfmdr1, Pfcrt, and Pfnhe1 genes are associated with reduced in vitro activities of quinine in Plasmodium falciparum isolates from western Kenya. *Antimicrobial Agents and Chemotherapy*, **58**(7), pp. 3737-3743.

CHIERRITO, T.P.C., AGUIAR, A.C.C., DE ANDRADE, I.M., CERAVOLO, I.P., GONÇALVES, R.A.C., DE OLIVEIRA, ARILDO J. B. and KRETTLI, A.U., 2014. Antimalarial activity of indole alkaloids isolated from Aspidosperma olivaceum. *Malaria Journal*, **13**(1), pp. 1-22.

CHLEZ, Z., HOHMANN, J. and MOLNAR, J., 2010. A Source of Complementary Therapeutics. In: D. CHATTOPADHYAY, ed, *Ethnomedicine.* 1 edn. india: Research. Signpost, pp. 179-201.

CHOI, J.G., MUN, S.H., CHAHAR, H.S., BHARAJ, P., KANG, O.H., KIM, S.G., SHIN, D.W. and KWON, D.Y., 2014. Methyl gallate from Galla rhois successfully controls clinical isolates of Salmonella infection in both in vitro and in vivo systems. *PloS one*, **9**(7), pp. e102697.

CHU, V.H., MIRO, J.M., HOEN, B., CABELL, C.H., PAPPAS, P.A., JONES, P., STRYJEWSKI, M.E., ANGUERA, I., BRAUN, S., MUNOZ, P., COMMERFORD, P., TORNOS, P., FRANCIS, J., OYONARTE, M., SELTON-SUTY, C., MORRIS, A.J., HABIB, G., ALMIRANTE, B., SEXTON, D.J., COREY, G.R., FOWLER, V.G.,Jr and INTERNATIONAL COLLABORATION ON ENDOCARDITIS-PROSPECTIVE COHORT STUDY GROUP, 2009. Coagulase-negative staphylococcal prosthetic valve endocarditis--a contemporary update based on the International Collaboration on Endocarditis: prospective cohort study. *Heart (British Cardiac Society)*, **95**(7), pp. 570-576.

COLLINS, L. and FRANZBLAU, S.G., 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. *Antimicrobial Agents and Chemotherapy*, **41**(5), pp. 1004-1009.

COMBRINCK, J.M., MABOTHA, T.E., NCOKAZI, K.K., AMBELE, M.A., TAYLOR, D., SMITH, P.J., HOPPE, H.C. and EGAN, T.J., 2013. Insights into the role of heme in the mechanism of action of antimalarial s. *ACS chemical biology*, **8**(1), pp. 133-137.

COQUEIRO, A., REGASINI, L.O., STAPLETON, P., DA, S.B. and GIBBONS, S., 2014. In Vitro Antibacterial Activity of Prenylated Guanidine Alkaloids from Pterogyne nitens and Synthetic Analogues. *Journal of natural products*, **77**(8), pp. 1972-1975 4p.

CORMICAN, M.G. and JONES, R.N., 1995. Antimicrobial activity of cefotaxime tested against infrequently isolated pathogenic species (unusual pathogens). *Diagnostic microbiology and infectious disease*, **22**(1-2), pp. 43-48.

COWAN, M.M., 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews*, **12**(4), pp. 564-582.

COX, S., MANN, C., MARKHAM, J., BELL, H., GUSTAFSON, J., WARMINGTON, J. and WYLLIE, S., 2000. The mode of antimicrobial action of essential oil of *Melaleuca alternifola* (tea tree oil). *Journal of Applied Microbiology*, **88**, pp. 170-175.

CROSTHWAITE, D., 1998. *UK trade within the flavour and fragrance industry. International Federation of Essential Oils and Aroma Trades.* 21st International Conference on Essential Oils and Aroma's edn. London: IFEAT.

CUEVA, C., MINGO, S., MUÑOZ-GONZÁLEZ, I., BUSTOS, I., REQUENA, T., DEL CAMPO, R., MARTÍN-ÁLVAREZ, P.J., BARTOLOMÉ, B. and MORENO-ARRIBAS, M., 2012. Antibacterial activity of wine phenolic compounds and oenological extracts against potential respiratory pathogens. *Letters in applied microbiology*, **54**(6), pp. 557-563.

CUSHNIE, T.P.T. and LAMB, A.J., 2011. Recent advances in understanding the antibacterial properties of flavonoids. *International journal of antimicrobial agents*, **38**(2), pp. 99-107.

DADASOGLU, F., KOTAN, R., CAKIR, A., CAKMAKCI, R., KORDALI, S., OZER, H., KARAGOZ, K. and DIKBAS, N., 2015. ANTIBACTERIAL ACTIVITIES OF ESSENTIAL OILS, EXTRACTS AND SOME OF THEIR MAJOR COMPONENTS OF Artemisia spp. L. AGAINST SEED-BORNE PLANT PATHOGENIC BACTERIA. *Fresenius Environmental Bulletin*, **24**(9), pp. 2715-2724.

DAFERERA, D., ZIOGAS, B. and POLISSIOU, M., 2000. GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. *Journal of Agricultural and Food Chemistry*, **48**(6), pp. 2576-2581.

DARAH, I., LIM,S.H. and NITHIANANTHAM, K., 2013. Effects of Methanol Extract of Wedelia chinensis Osbeck (Asteraceae) Leaves against Pathogenic Bacteria with Emphasise on Bacillus cereus. *Indian Journal of Pharmaceutical Sciences*, **75**(5), pp. 533-539.

DAVID, B., WOLFENDER, J. and DIAS, D.A., 2015. The pharmaceutical industry and natural products: historical status and new trends. *Phytochemistry Reviews*, **14**(2), pp. 299-315.

DE AZEREDO, G.A., STAMFORD, T.L.M., NUNES, P.C., GOMES NETO, N.J., DE OLIVEIRA, M.,Elieidy Gomes and DE SOUZA, E.L., 2011. Combined application of

essential oils from Origanum vulgare L. and Rosmarinus officinalis L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables. *Food Research International,* **44**(5), pp. 1541-1548.

DEANS, S. and RITCHIE, G., 1987. Antibacterial properties of plant essential oils. *International Journal of Food Microbiology,* **5**, pp. 165-180.

DEANS, S., SIMPSON, E., NOBLE, R., MACPHERSON, A. and PENZES, L., 1993. Natural antioxidants from *Thymus vulgaris* (thyme) volatile oil: the beneficial effects upon mammalian lipid metabolism. *Acta Horticulturae,* **332**, pp. 177-182.

DENYER, S. and HUGO, W., 1991a. Biocide-induced damage to the bacterial cytoplasmic membrane . In: S. DENYER and W. HUGO, eds, *Mechanisms of Action of Chemical Biocides. The Society for Applied Bacteriology, Technical Series* . 27 edn. Oxford: Oxford Blackwell Scientific Publication, pp. 177-188.

DESBOIS, A.P., 2012. Potential applications of antimicrobial fatty acids in medicine, agriculture and other industries. *Recent Patents On Anti-Infective Drug Discovery,* **7**(2), pp. 111-122.

DESJARDINS, R.E., CANFIELD, C.J., HAYNES, J.D. and CHULAY, J.D., 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrobial Agents and Chemotherapy,* **16**(6), pp. 710-718.

DIN, W.M., JIN, K.T., RAMLI, R., KHAITHIR, T.M.N. and WIART, C., 2013. Antibacterial Effects of Ellagitannins from Acalypha wilkesiana var. macafeana hort.: Surface Morphology Analysis with Environmental Scanning Electron Microcopy and Synergy with Antibiotics. *Phytotherapy Research,* **27**(9), pp. 1313-1320 8p.

DING, X.C., BECK, H.P. and RASO, G., 2011. Plasmodium sensitivity to artemisinins: magic bullets hit elusive targets. *Trends in parasitology,* **27**(2), pp. 73-81.

DOLABELA, M.F., OLIVEIRA, S.G., NASCIMENTO, J.M., PERES, J.M., WAGNER, H., PÓVOA, M.M. and DE OLIVEIRA, A.B., 2008. In vitro antiplasmodial activity of extract and constituents from Esenbeckia febrifuga, a plant traditionally used to treat malaria in the Brazilian Amazon. *Phytomedicine,* **15**(5), pp. 367-372 6p.

DONDORP, A.M., FANELLO, C.I., HENDRIKSEN, I.C., GOMES, E., SENI, A., CHHAGANLAL, K.D., BOJANG, K., OLAOSEBIKAN, R., ANUNOBI, N., MAITLAND, K., KIVAYA, E., AGBENYEGA, T., NGUAH, S.B., EVANS, J., GESASE, S., KAHABUKA, C., MTOVE, G., NADJM, B., DEEN, J., MWANGA-AMUMPAIRE, J., NANSUMBA, M., KAREMA, C., UMULISA, N., UWIMANA, A., MOKUOLU, O.A., ADEDOYIN, O.T., JOHNSON, W.B., TSHEFU, A.K., ONYAMBOKO, M.A., SAKULTHAEW, T., NGUM, W.P., SILAMUT, K., STEPNIEWSKA, K., WOODROW, C.J., BETHELL, D., WILLS, B., ONEKO, M., PETO, T.E., VON SEIDLEIN, L., DAY, N.P., WHITE, N.J. and AQUAMAT GROUP, 2010. Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. *Lancet (London, England),* **376**(9753), pp. 1647-1657.

DORMAN, H. and DEANS, S., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils . *Journal of Applied Microbiology,* **88**, pp. 308-316.

DOS SANTOS TORRES, Z.E., SILVEIRA, E.R., ROCHA E SILVA, L.F., LIMA, E.S., DE VASCONCELLOS, M.C., DE ANDRADE UCHOA, D.E., FILHO, R.B. and POHLIT, A.M., 2013. Chemical composition of Aspidosperma ulei Markgr. and antiplasmodial activity of selected indole alkaloids. *Molecules (Basel, Switzerland),* **18**(6), pp. 6281-6297.

DUARTE, M.C., LEME, E.E., DELARMELINA, C., SOARES, A.A., FIGUEIRA, G.M. and SARTORATTO, A., 2007. Activity of essential oils from Brazilian medicinal plants on *Escherichia coli*. *Journal of ethnopharmacology,* **111**(2), pp. 197-201.

EERDUNBAYAER, ORABI, M.A.A., AOYAMA, H., KURODA, T. and HATANO, T., 2014. Structures of New Phenolics Isolated from Licorice, and the Effectiveness of Licorice Phenolics on Vancomycin-Resistant Enterococci. *Molecules,* **19**(9), pp. 13027-13041.

EHLING-SCHULZ, M., FRICKER, M. and SCHERER, S., 2004. Bacillus cereus, the causative agent of an emetic type of food-borne illness. *Molecular nutrition & food research,* **48**(7), pp. 479-487.

EKUADZI, E., DICKSON, R., FLEISCHER, T., ANNAN, K., PISTORIUS, D., OBERER, L. and GIBBONS, S., 2014. Flavonoid glycosides from the stem bark of Margaritaria discoidea demonstrate antibacterial and free radical scavenging activities. *Phytotherapy Research,* **28**(5), pp. 784-787 4p.

EL-AZIZI, M., 2016. Novel Microdilution Method to Assess Double and Triple Antibiotic Combination Therapy In Vitro. *International Journal of Microbiology,* , pp. 1-10.

ELIOPOULOS, G.M. and ELIOPOULOS, C.T., 1988. Antibiotic combinations: should they be tested? *Clinical microbiology reviews,* **1**(2), pp. 139-156.

ENEROTH, A., SVENSSON, B., MOLIN, G. and CHRISTIANSSON, A., 2001. Contamination of pasteurized milk by Bacillus cereus in the filling machine. *The Journal of dairy research,* **68**(2), pp. 189-196.

EYASU, M., 2015. Antimalarial Drug Resistance: In the Past, Current Status and Future Perspectives. *British Journal of Pharmacology and Toxicology,* **6**(1), pp. 1-15.

FABRICANT, D.S. and FARNSWORTH, N.R., 2001. The value of plants used in traditional medicine for drug discovery. *Environmental health perspectives,* **109 Suppl 1**, pp. 69-75.

FALEIRO, M., MIGUEL, M., LADEIRO, F., VENANCIO, F., TAVARES, R., BRITO, J., FIGUEIREDO, A., BARROSO, J. and PEDRO, L., 2002. Antimicrobial activity of essential oils isolated from Portuguese endemic species of Thymus. *Letters in Applied Microbiology,* **36**, pp. 35-40.

FANDEUR, T., MORETTI, C. and POLONSKY, J., 1985. In vitro and in vivo assessement of the antimalarial activity of sergeolide. *Planta Medica*, **51**(1), pp. 20-23.

FARNSWORTH, N., 2015-last update, Natural Products Alert (NAPRALERT) [Homepage of University of Illinois at Chicago, Chicago, U.S.A.], [Online]. Available: https://www.napralert.org/ [March/09, 2016].

FARNSWORTH, N.R., AKERELE, O., BINGEL, A.S., SOEJARTO, D.D. and GUO, Z., 1985. Medicinal plants in therapy. *Bulletin of the World Health Organization*, **63**(6), pp. 965-981.

FAROOQ, S., WAHAB, A.T., FOZING, C.D.A., RAHMAN, A.U. and CHOUDHARY, M.I., 2014. Artonin I inhibits multidrug resistance in *Staphylococcus aureus* and potentiates the action of inactive antibiotics in vitro. *Journal of applied microbiology*, **117**(4), pp. 996-1011.

FAROOQ, U. and MAHAJAN, R.C., 2004. Drug resistance in malaria. *Journal of vector borne diseases*, **41**(3-4), pp. 45-53.

FEHER, M. and SCHMIDT, J.M., 2003. Property distributions: differences between drugs, natural products, and molecules from combinatorial chemistry. *Journal of chemical information and computer sciences*, **43**(1), pp. 218-227.

FENG, S. and XU, J., 2014. Profile of antioxidant and antibacterial activities of different solvent extracts from Rabdosia rubescens. *International Journal of Food Science & Technology*, **49**(11), pp. 2506-2513.

FESSENDEN, R. and FESSENDEN, J., 1982. *Organic chemistry*. 2nd edn. Boston, Mass.: Willard Grant Press.

FIGUEROA, M., RIVERO-CRUZ, I., RIVERO-CRUZ, B., BYE, R., NAVARRETE, A. and MATA, R., 2007. Constituents, biological activities and quality control parameters of the crude extract and essential oil from Arracacia tolucensis var. multifida. *Journal of ethnopharmacology*, **113**(1), pp. 125-131 7p.

FRANCOIS, G., AKE ASSI, L., HOLENZ, J. and BRINGMANN, G., 1996. Constituents of *Picralima nitida* display pronounced inhibitory activities against asexual erythrocyticforms of *Plasmodium falciparum* in vitro. *J. Ethnopharmacol.*, **54**, pp. 113-117.

FRANCOIS, G., PASSREITER, C.M., WOERDENBAG, H.J. and VAN LOOVEREN, M., 1996. Antiplasmodial activities and cytotoxic effects of aqueous extracts and sesquiterpene lactones from Neurolaena lobata. *Planta Medica*, **62**(2), pp. 126-129.

GAMO, F., SANZ, L.M., VIDAL, J., DE COZAR, C., ALVAREZ, E., LAVANDERA, J., VANDERWALL, D.E., GREEN, D.V.S., KUMAR, V., HASAN, S., BROWN, J.R., PEISHOFF, C.E., CARDON, L.R. and GARCIA-BUSTOS, J., 2010. Thousands of chemical starting points for antimalarial lead identification. *Nature*, **465**(7296), pp. 305-310.

GARCIA-ALVAREZ, M., MOUSSA, I., NJOMNANG SOH, P., NONGONIERMA, R., ABDOULAYE, A., NICOLAU-TRAVERS, M., FABRE, A., WDZIECZAK-BAKALA, J., AHOND, A., POUPAT, C., IKHIRI, K. and BENOIT-VICAL, F., 2013. Both plants Sebastiania chamaelea from Niger and Chrozophora senegalensis from Senegal used in African traditional medicine in malaria treatment share a same active principle. *Journal of ethnopharmacology*, **149**(3), pp. 676-684.

GEISSMAN, T., 1963. Flavonoid compounds, tannins, lignins and related compounds. In: M. FLORKIN and E. STOTZ, eds, *pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9.* New York, N.Y.: Elsevier, pp. 265.

GERSHENZON, J. and DUDAREVA, N., 2007. The function of terpene natural products in the natural world. *Nature chemical biology*, **3**(7), pp. 408-414.

GESASE, S., GOSLING, R.D., HASHIM, R., ORD, R., NAIDOO, I., MADEBE, R., MOSHA, J.F., JOHO, A., MANDIA, V., MREMA, H., MAPUNDA, E., SAVAEL, Z., LEMNGE, M., MOSHA, F.W., GREENWOOD, B., ROPER, C. and CHANDRAMOHAN, D., 2009. High resistance of Plasmodium falciparum to sulphadoxine/pyrimethamine in northern Tanzania and the emergence of dhps resistance mutation at Codon 581. *PloS one*, **4**(2), pp. e4569.

GHELARDI, E., CELANDRONI, F., SALVETTI, S., BARSOTTI, C., BAGGIANI, A. and SENESI, S., 2002. Identification and characterization of toxigenic Bacillus cereus isolates responsible for two food-poisoning outbreaks. *FEMS microbiology letters*, **208**(1), pp. 129-134.

GIBBONS, S., 2008. Phytochemicals for Bacterial Resistance - Strengths, Weaknesses and Opportunities. *Planta Medica*, **74**(6), pp. 594-602.

GIBBONS, S., MOSER, E. and KAATZ, G.W., 2004. Catechin gallates inhibit multidrug resistance (MDR) in *Staphylococcus aureus*. *Planta Medica*, **70**(12), pp. 1240-1242.

GILBERT, P., MAIRA-LITRAN, T., MCBAIN, A.J., RICKARD, A.H. and WHYTE, F.W., 2002. The physiology and collective recalcitrance of microbial biofilm communities. *Advances in Microbial Physiology*, **46**, pp. 202-256.

GILLES, V., VIEIRA, M.A., JR., L.V., CASTRO, E.V.R., SANTOS, R.B., ORESTES, E., CARNEIRO, J.W.A. and GRECO, S.J., 2015. A New, Simple and Efficient Method of Steglich Esterification of Juglone with Long-Chain Fatty Acids: Synthesis of a New Class of Non-Polymeric Wax Deposition Inhibitors for Crude Oil. *J. Braz. Chem. Soc.*, **26**(1), pp. 74-81.

GINSBURG, H. and DEHARO, E., 2011. A call for using natural compounds in the development of new antimalarial treatments - an introduction. *Malaria journal*, **10 Suppl 1**, pp. S1-2875-10-S1-S1.

GOREN, A., PIOZZI, F., AKCICEK, E., KILIÇ, T., ÇARIKÇI, S., MOZIOĞLU, E. and SETZER, W., 2011. Essential oil composition of twenty-two Stachys species (mountain tea) and their biological activities. *Phytochem. Lett.*, **4**, pp. 448-453.

GOV.UK, 21/07/2016, 2016-last update, Current EU approved additives and their E Numbers[Homepage,of,Gov.UK],[Online].Available:http://www.food.gov.uk/science/additives/enumberlist [09/10, 2016].

GRAZIOSE, R., RATHINASABAPATHY, T., LATEGAN, C., POULEV, A., SMITH, P.J., GRACE, M., LILA, M.A. and RASKIN, I., 2011. Antiplasmodial activity of aporphine alkaloids and sesquiterpene lactones from Liriodendron tulipifera L. *Journal of ethnopharmacology,* **133**(1), pp. 26-30 5p.

GRIMBERG, B.T. and MEHLOTRA, R.K., 2011. Expanding the Antimalarial Drug Arsenal-Now, But How? *Pharmaceuticals (Basel, Switzerland),* **4**(5), pp. 681-712.

GUANTAI, E. and CHIBALE, K., 2011. How can natural products serve as a viable source of lead compounds for the development of new/novel antimalarials? *Malaria journal,* **10 Suppl 1**, pp. S2-2875-10-S1-S2.

GUENTHER, E., 1948. *The Essential Oils.* New York: D. Van Nostrand.

GUO, Z., VANGAPANDU, S., SINDELAR, R.W., WALKER, L.A. and SINDELAR, R.D., 2005. Biologically active quassinoids and their chemistry: potential leads for drug design. *Current medicinal chemistry,* **12**(2), pp. 173-190.

GUTIÉRREZ-LARRAÍNZAR, M., RÚA, J., CARO, I., DE CASTRO, C., DE ARRIAGA, D., GARCÍA-ARMESTO, M.R. and DEL VALLE, P., 2012. Evaluation of antimicrobial and antioxidant activities of natural phenolic compounds against foodborne pathogens and spoilage bacteria. *Food Control,* **26**(2), pp. 555-563.

HALKET, J.M., WATERMAN, D., PRZYBOROWSKA, A.M., PATEL, R.K., FRASER, P.D. and BRAMLEY, P.M., 2005. Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. *Journal of experimental botany,* **56**(410), pp. 219-243.

HAMMER, K.A., CARSON, C.F. and RILEY, T.V., 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal of applied microbiology,* **86**(6), pp. 985-990.

HEMAISWARYA, S., KRUTHIVENTI, A.K. and DOBLE, M., 2008. Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology,* **15**(8), pp. 639-652.

HENRICH, C.J. and BEUTLER, J.A., 2013. Matching the power of high throughput screening to the chemical diversity of natural products. *Natural product reports,* **30**(10), pp. 1284-1298.

HOSSAIN, M.A., KABIR, M.J., SALEHUDDIN, S.M., RAHMAN, S.M.M., DAS, A.K., SINGHA, S.K., ALAM, M.K. and RAHMAN, A., 2010. Antibacterial properties of essential oils and methanol extracts of sweet basil Ocimum basilicum occurring in Bangladesh. *Pharmaceutical Biology,* **48**(5), pp. 504-511.

HOULT, J. and PAYA, M., 1996. Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential . *Gen. Pharmacol.,* **27**, pp. 713-722.

HU, L.F., LI, S.P., CAO, H., LIU, J.J., GAO, J.L., YANG, F.Q. and WANG, Y.T., 2006. GC-MS fingerprint of Pogostemon cablin in China. *Journal of pharmaceutical and biomedical analysis,* **42**(2), pp. 200-206.

HU, Z.Q., ZHAO, W.H., ASANO, N., YODA, Y., HARA, Y. and SHIMAMURA, T., 2002. Epigallocatechin gallate synergistically enhances the activity of carbapenems against methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* **46**(2), pp. 558-560.

HUANG, B., LEI, Y., TANG, Y., ZHANG, J., QIN, L. and LIU, J., 2011. Comparison of HS-SPME with hydrodistillation and SFE for the analysis of the volatile compounds of Zisu and Baisu, two varietal species of Perilla frutescens of Chinese origin. *Food Chemistry,* **125**(1), pp. 268-275.

HUFFORD, C., JIA, Y., CROOM, E., MUHAMMED, J., OKUNADE, A., CLARK, A. and ROGERS, R., 1993. Antimicrobial compounds from *Petalostemum purpureum*. *J. Nat. Prod.,* **56**, pp. 1878-1889.

HUTCHINSON, J. and DAZIEL, J., 1968. *Flora of West Tropical Africa.* London: Agent for overseas Government.

IGBINOSA, E.O. and IDEMUDIA, O.G., 2016. Anti-vibrio potentials of acetone and aqueous leaf extracts of Ocimum gratissimum (Linn). *Tropical Journal of Pharmaceutical Research,* **15**(4), pp. 743-750.

IJAZ, F., AHMAD, N., AHMAD, I., UL HAQ, A. and WANG, F., 2010. Two new anti-plasmodial flavonoid glycosides from Duranta repens. *Journal of enzyme inhibition and medicinal chemistry,* **25**(6), pp. 773-778.

INOUE, Y., SHIRAISHI, A., HADA, T., HIROSE, K., HAMASHIMA, H. and SHIMADA, J., 2004. The antibacterial effects of terpene alcohols on *Staphylococcus aureus* and their mode of action. *FEMS microbiology letters,* **237**(2), pp. 325-331.

JANSEN, O., TITS, M., ANGENOT, L., NICOLAS, J., DE MOL, P., NIKIEMA, J. and FRÉDÉRICH, M., 2012. Anti-plasmodial activity of Dicoma tomentosa (Asteraceae) and identification of urospermal A-15-O-acetate as the main active compound. *Malaria Journal,* **11**, pp. 289-289.

JAYARAMAN, P., SAKHARKAR, M.K., LIM, C.S., TANG, T.H. and SAKHARKAR, K.R., 2010. Activity and interactions of antibiotic and phytochemical combinations against Pseudomonas aeruginosa in vitro. *International journal of biological sciences,* **6**(6), pp. 556-568.

JAYASHREE, T. and SUBRAMANYAM, C., 1999. Antiaflatoxigenic activity of eugenol is due to inhibition of lipid peroxidation. *Letters in Applied Microbiology,* **28**, pp. 179-183.

JEONG, J.B., CHOI, J., LOU, Z., JIANG, X. and LEE, S.H., 2013. Patchouli alcohol, an essential oil of Pogostemon cablin, exhibits anti-tumorigenic activity in human colorectal cancer cells. *International immunopharmacology*, **16**(2), pp. 184-190.

JIAMBOONSRI, P., PITHAYANUKUL, P., BAVOVADA, R. and CHOMNAWANG, M.T., 2011. The inhibitory potential of Thai mango seed kernel extract against methicillin-resistant *Staphylococcus aureus*. *Molecules (Basel, Switzerland)*, **16**(8), pp. 6255-6270.

JIAO, W., LI, J., LIU, Q., XU, T., SHI, G., YU, H., YANG, F., HAN, B., LI, M. and LIN, H., 2014. Dysidinoid A, an unusual meroterpenoid with anti-MRSA activity from the South China Sea sponge Dysidea sp. *Molecules (Basel, Switzerland)*, **19**(11), pp. 18025-18032.

JONES, G., MCALLISTER, T., MUIR, A. and CHENG, K., 1994. Effects of sainfoin (*Onobrychis viciifolia scop.*) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. *Appl. Environ. Microbiol.*, **60**, pp. 1374-1378.

JONES, N., SHABIB, S. and SHERMAN, P., 1997. Capsaicin as an inhibitor of the growth of the gastric pathogen *Helicobacter pylori*. *FEMS Microbiol. Lett.*, **146**, pp. 223-227.

JR.VALLE, D.L., CABRERA, E.C., PUZON, J.J.M. and RIVERA, W.L., 2016. Antimicrobial Activities of Methanol, Ethanol and Supercritical CO2 Extracts of Philippine Piper betle L. on Clinical Isolates of Gram Positive and Gram Negative Bacteria with Transferable Multiple Drug Resistance. *PLoS ONE*, **11**(1), pp. 1-14.

JULIANO, C., MATTANA, A. and USAI, M., 2000. Composition and in vitro antimicrobial activity of the essential oil of *Thymus herbabarona Loisel* growing wild in Sardinia. *Journal of Essential Oil Research*, **12**, pp. 516-522.

KADOTA, S., BASNET, P., ISHII, E., TAMURA, T. and NAMBA, T., 1997. Antibacterial activity of trichorabdal from *Rabdosia trichocarpa* against *Helicobacter pylori*. *Zentbl. Bakteriol.*, **286**, pp. 63-67.

KAKISAWA, H., KUSUMI, T., HSU, H. and CHEN, Y., 1970. structures of lignans of magnolia fargesii. *B CHEM SOC JPN*, **43**(11), pp. 3631-3631.

KALEEM, W.A., NISAR, M., QAYUM, M., ZIA-UL-HAQ, M., ADHIKARI, A. and DE FEO, V., 2012. New 14-membered cyclopeptide alkaloids from Zizyphus oxyphylla Edgew. *International journal of molecular sciences*, **13**(9), pp. 11520-11529.

KAMKUMO, R.G., NGOUTANE, A.M., TCHOKOUAHA, L.R.Y., FOKOU, P.V.T., MADIESSE, E.A.K., LEGAC, J., KEZETAS, J.J.B., LENTA, B.N., BOYOM, F.F., DIMO, T., MBACHAM, W.F., GUT, J. and ROSENTHAL, P.J., 2012. Compounds from Sorindeia juglandifolia (Anacardiaceae) exhibit potent anti-plasmodial activities in vitro and in vivo. *Malaria Journal*, **11**(1), pp. 382-388.

KANG, R., HELMS, R., STOUT, M., JABER, H., CHEN, Z. and NAKATSU, T., 1992. Antimicrobial activity of the volatile constituents of Perilla frutescens and its synergistic effects with polygodial. *J. Agric. Food Chem.*, **40**(11), pp. 2328-2330.

KARKARE, S., ADOU, E., CAO, S., BRODIE, P., MILLER, J.S., ANDRIANJAFY, N.M., RAZAFITSALAMA, J., ANDRIANTSIFERANA, R., RASAMISON, V.E. and KINGSTON, D.G., 2007. Cytotoxic cardenolide glycosides of Roupellina (Strophanthus) boivinii from the Madagascar rainforest. *Journal of natural products*, **70**(11), pp. 1766-1770.

KHATKAR, K., NANDA, A., KUMAR, P. and NARASIMHAN, B., 2013. Synthesis, antimicrobial evaluation and QSAR studies of gallic acid derivatives. *Arabian Journal of Chemistry*, **doi: http://dx.doi.org/10.1016/ j.arabjc.2013.11.014**.

KIM, J.Y., LIM, H.J., LEE DA, Y., KIM, J.S., KIM, D.H., LEE, H.J., KIM, H.D., JEON, R. and RYU, J.H., 2009. In vitro anti-inflammatory activity of lignans isolated from Magnolia fargesii. *Bioorganic & medicinal chemistry letters*, **19**(3), pp. 937-940.

KIRBY, G.C., PAINE, A., WARHURST, D.C., NOAMESE, B.K. and PHILLIPSON, J.D., 1995. **In vitro** and **in vivo** antimalarial activity of cryptolepine, a plant-derived indoloquinoline. *Phytother. Res.*, **9**(5), pp. 359-363.

KITAGAWA, I., MINAGAWA, K., ZHANG, R., HORI, K., DOI, M., INOUE, M., ISHIDA, T., KIMURA, M., UJI, T. and SHIBUYA, H., 1993. Dehatrine, an antimalarial bisbenzylisoquinoline alkaloid from the Indonesian medicinal plant Beilschmiediamadang, isolated as a mixture of two rotational isomers. *Chem Pharm Bull*, **41**(5), pp. 997-999.

KITAGAWA, I., MAHMUD, T., YOKOTA, K., NAKAGAWA, S., MAYUMI, T., KOBAYASHI, M. and SHIBUYA, H., 1996. Indonesian medicinal plants. XVII. Characterization of quassinoids from the stems of Quassia indica. *Chemical & pharmaceutical bulletin*, **44**(11), pp. 2009-2014.

KIYOHARA, H., ICHINO, C., KAWAMURA, Y., NAGAI, T., SATO, N. and YAMADA, H., 2012. Patchouli alcohol: in vitro direct anti-influenza virus sesquiterpene in Pogostemon cablin Benth. *Journal of natural medicines*, **66**(1), pp. 55-61.

KLAYMAN, D.L., 1985. Qinghaosu (artemisinin): an antimalarial drug from China. *Science (New York, N.Y.)*, **228**(4703), pp. 1049-1055.

KNEZEVIC, P., ALEKSIC, V., SIMIN, N., SVIRCEV, E., PETROVIC, A. and MIMICA-DUKIC, N., 2016. Antimicrobial activity of Eucalyptus camaldulensis essential oils and their interactions with conventional antimicrobial agents against multi-drug resistant Acinetobacter baumannii. *Journal of ethnopharmacology*, **178**, pp. 125-136.

KNOBLOCH, K., WEIGAND, H., WEIS, N., SCHWARM, H. and VIGEN- SCHOW, H., 1986. Action of terpenoids on energy metabolism. In: E. BRUNKE, ed, *Progress in Essential Oil Research: 16th International Symposium on Essential Oils.* Berlin: De Gruyter, pp. 429-445.

KOEHN, F.E. and CARTER, G.T., 2005. The evolving role of natural products in drug discovery. *Nature reviews.Drug discovery*, **4**(3), pp. 206-220.

KONSTANTOPOULOU, I., VASSILOPOULOU, L., MAVRAGANI-TSIPIDOU, P. and SCOURAS, Z., 1992. Insecticidal effects of essential oils. A study of the effects of essential oils extracted from eleven Greek aromatic plants on *Drosophila auraria*. *Experientia,* **48**(6), pp. 616-619.

KOUITCHEU MABEKU, L.B., KOUAM, J., PENLAP, B.V., NGADJUI, B.T., FOMUM, Z.T. and ETOA, F.X., 2006. Evaluation of antimicrobial activity of the stem bark of *Cylicodiscus gabunensis* (Mimosaceae). *African journal of traditional, complementary, and alternative medicines : AJTCAM / African Networks on Ethnomedicines,* **4**(1), pp. 87-93.

KOUMAGLO, K., GBEASSOR, M., NIKABU, O., DE SOUZA, C. and WERNER, W., 1992. Effects of three compounds extracted from Morinda lucida on Plasmodium falciparum. *Planta Medica,* **58**(6), pp. 533-534.

KROGSTAD, D.J., GLUZMAN, I.Y., KYLE, D.E., ODUOLA, A.M., MARTIN, S.K., MILHOUS, W.K. and SCHLESINGER, P.H., 1987. Efflux of chloroquine from Plasmodium falciparum: mechanism of chloroquine resistance. *Science (New York, N.Y.),* **238**(4831), pp. 1283-1285.

KUBO, I., XIAO, P., NIHEI, K., FUJITA, K., YAMAGIWA, Y. and KAMIKAWA, T., 2002. Molecular design of antifungal agents. *Journal of Agricultural and Food Chemistry,* **50**(14), pp. 3992-3998.

KUMAR, M., KHAN, I.A., VERMA, V. and QAZI, G.N., 2005. Microplate nitrate reductase assay versus Alamar Blue assay for MIC determination of Mycobacterium tuberculosis. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease,* **9**(8), pp. 939-941.

KUROKI, R., KAWAKAMI, K., QIN, L., KAJI, C., WATANABE, K., KIMURA, Y., ISHIGURO, C., TANIMURA, S., TSUCHIYA, Y., HAMAGUCHI, I., SAKAKURA, M., SAKABE, S., TSUJI, K., INOUE, M. and WATANABE, H., 2009. Nosocomial bacteremia caused by biofilm-forming Bacillus cereus and Bacillus thuringiensis. *Internal medicine (Tokyo, Japan),* **48**(10), pp. 791-796.

LAMBERT, R.J., SKANDAMIS, P.N., COOTE, P.J. and NYCHAS, G.J., 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of applied microbiology,* **91**(3), pp. 453-462.

LANGEVELD, W.T., VELDHUIZEN, E.J. and BURT, S.A., 2014. Synergy between essential oil components and antibiotics: a review. *Critical reviews in microbiology,* **40**(1), pp. 76-94.

LAPHOOKHIEO, S., MANEERAT, W. and KOYSOMBOON, S., 2009. Antimalarial and cytotoxic phenolic compounds from Cratoxylum maingayi and Cratoxylum cochinchinense. *Molecules (Basel, Switzerland),* **14**(4), pp. 1389-1395.

LEE, H.K., SONG, H.E., LEE, H.B., KIM, C.S., KOKETSU, M., NGAN, L.T. and AHN, Y.J., 2014. Growth inhibitory, bactericidal, and morphostructural effects of dehydrocostus

lactone from Magnolia sieboldii Leaves on antibiotic-susceptible and -resistant strains of Helicobacter pylori. *PloS one,* **9**(4), pp. e95530.

LEE, S.Y., SO, Y., SHIN, M.S., CHO, J.Y. and LEE, J., 2014. Antibacterial effects of afzelin isolated from Cornus macrophylla on Pseudomonas aeruginosa, a leading cause of illness in immunocompromised individuals. *Molecules (Basel, Switzerland),* **19**(3), pp. 3173-3180.

LÉGUILLIER, T., LECSÖ-BORNET, M., LÉMUS, C., ROUSSEAU-RALLIARD, D., LEBOUVIER, N., HNAWIA, E., NOUR, M., AALBERSBERG, W., GHAZI, K., RAHARIVELOMANANA, P. and RAT, P., 2015. The Wound Healing and Antibacterial Activity of Five Ethnomedical Calophyllum inophyllum Oils: An Alternative Therapeutic Strategy to Treat Infected Wounds. *PLoS ONE,* **10**(9), pp. 1-20.

LEONARD, B., CORONEL, J., SIEDNER, M., GRANDJEAN, L., CAVIEDES, L., NAVARRO, P., GILMAN, R.H. and MOORE, D.A., 2008. Inter- and intra-assay reproducibility of microplate Alamar blue assay results for isoniazid, rifampicin, ethambutol, streptomycin, ciprofloxacin, and capreomycin drug susceptibility testing of Mycobacterium tuberculosis. *Journal of clinical microbiology,* **46**(10), pp. 3526-3529.

LI, W.W. and BARZ, W., 2006. Structure and accumulation of phenolics in elicited Echinacea purpurea cell cultures. *Planta Medica,* **72**(3), pp. 248-254.

LI, Y.C., LIANG, H.C., CHEN, H.M., TAN, L.R., YI, Y.Y., QIN, Z., ZHANG, W.M., WU, D.W., LI, C.W., LIN, R.F., SU, Z.R. and LAI, X.P., 2012. Anti-Candida albicans activity and pharmacokinetics of pogostone isolated from Pogostemonis Herba. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology,* **20**(1), pp. 77-83.

LIEW, L.P.P., KAISER, M. and COPP, B.R., 2013. Discovery and preliminary structure–activity relationship analysis of 1,14-sperminediphenylacetamides as potent and selective antimalarial lead compounds. *Bioorganic & medicinal chemistry letters,* **23**(2), pp. 452-454.

LIKHITWITAYAWUID, K., ANGERHOFER, C.K., CORDELL, G.A., PEZZUTO, J.M. and RUANGRUNGSI, N., 1993. Cytotoxic and antimalarial bisbenzylisoquinoline alkaloids from Stephania erecta. *Journal of natural products,* **56**(1), pp. 30-38.

LIKHITWITAYAWUID, K., KAEWAMATAWONG, R., RUANGRUNGSI, N. and KRUNGKRAI, J., 1998. Antimalarial naphthoquinones from Nepenthes thorelii. *Planta Medica,* **64**(3), pp. 237-241.

LIM, H. and SHIN, S., 2014. Anti-Bacillus and Anti-Shigella Activities of the Essential Oil from Perilla fruescens var. japonica Hara. *Journal of essential oil bearing plants,* **17**(2), pp. 309-316.

LIM, A., SUBHAN, N., JAZAYERI, J.A., JOHN, G., VANNIASINKAM, T. and OBIED, H.K., 2016. Plant Phenols as Antibiotic Boosters: In Vitro Interaction of Olive Leaf Phenols with Ampicillin. *Phytotherapy Research: PTR,* **30**(3), pp. 503-509.

LIMA, B., SANCHEZ, M., AGÜERO, M.B., TAPIA, A., PALERMO, J.A. and FERESIN, G.E., 2015. Antibacterial activity of extracts and compounds isolated from the Andean medicinal plant Azorella cryptantha (Clos) Reiche, Apiaceae. *Industrial Crops & Products,* **64**, pp. 152-157.

LIPIŃSKA, L., KLEWICKA, E. and SÓJKA, M., 2014. Structure, Occurrence and Biological Activity of Ellagitannins: a General Review. *Acta Scientiarum Polonorum.Technologia Alimentaria,* **13**(3), pp. 289-299.

LIS-BALCHIN, M., OCHOKA, R., DEANS, S., ASZTEMBORSKA, M. and HART, S., 1999. Differences in bioactivity between the enantiomers of alpha-pinene . *Journal of Essential Oil Research,* **11**, pp. 393-397.

LISEC, J., SCHAUER, N., KOPKA, J., WILLMITZER, L. and FERNIE, A.R., 2015. Corrigendum: Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature protocols,* **10**(9), pp. 1457-1457a. Epub 2015 Aug 27.

LIU, Q., LUTEN, W., PELLENS, K., WANG, Y., WANG, W., THEVISSEN, K., LIANG, Q., CAMMUE, B., SCHOOFS, L. and LUO, G., 2012. Antifungal activity in plants from Chinese traditional and folk medicine. *Journal of ethnopharmacology,* **143**(3), pp. 772-778.

LIU, H., SONG, Z., LIAO, D., ZHANG, T., LIU, F., ZHUANG, K., LUO, K., YANG, L., HE, J. and LEI, J., 2015. Anticonvulsant and Sedative Effects of Eudesmin isolated from Acorus tatarinowii on mice and rats. *Phytotherapy Research,* **29**(7), pp. 996-1003 8p.

LIU, J., WAN, Y., ZHAO, Z. and CHEN, H., 2013. Determination of the content of rosmarinic acid by HPLC and analytical comparison of volatile constituents by GC-MS in different parts of Perilla frutescens (L.) Britt. *Chemistry Central journal,* **7**(1), pp. 61-153X-7-61.

LIU, Y., MURAKAMI, N., JI, H., ABREU, P. and ZHANG, V., 2007. Antimalarial Flavonol Glycosides from Euphorbia hirta. *Pharmaceutical Biology,* **45**(4), pp. 278-281.

LIU, Z., 2008. Preparation of botanical samples for biomedical research. *Endocrine, metabolic & immune disorders drug targets,* **8**(2), pp. 112-121.

LÓPEZ-MALO VIGIL, A., PALOU, E. and ALZAMORA, S.M., 2005. Naturally occurring compounds. Plant sources. In: D. P. M., S. J. N. and B. A. L., eds, *Antimicrobials in food.* 3 edn. Boca Raton, Florida: CRC Press, Taylor and Francis Group, pp. 429-452.

LOPEZ-ROMERO, J.C., GONZALEZ-RIOS, H., BORGES, A. and SIMOES, M., 2015. Antibacterial Effects and Mode of Action of Selected Essential Oils Components against *Escherichia coli* and *Staphylococcus aureus*. *Evidence-based complementary and alternative medicine : eCAM,* **2015**, pp. 795435.

LU, J.Q., XU, Y.T., ZHAN, X.L., WAN, W., HE, X.F., WU, D.H., CAO, R.B., HU, X.Y. and YANG, X.H., 2008. Analysis of the chemical constituents of essential oil from

Magnolia biondii by GC-MS. *Zhong yao cai = Zhongyaocai = Journal of Chinese medicinal materials,* **31**(11), pp. 1649-1651.

LUÍS, Â, SILVA, F., SOUSA, S., DUARTE, A.P. and DOMINGUES, F., 2014. Antistaphylococcal and biofilm inhibitory activities of gallic, caffeic, and chlorogenic acids. *Biofouling,* **30**(1), pp. 69-79.

MA, Y. and HAN, G., 1995. Biologically active lignins from Magnolia biondii Pamp. *Zhongguo zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica,* **20**(2), pp. 102-4, 127.

MADIKIZELA, B., ADEROGBA, M.A. and VAN STADEN, J., 2013. Isolation and characterization of antimicrobial constituents of Searsia chirindensis L. (Anacardiaceae) leaf extracts. *Journal of ethnopharmacology,* **150**(2), pp. 609-613.

MAISNIER-PATIN, S. and ANDERSSON, D., 2004. Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Res Microbiol.,* **155**(5), pp. 360-369.

MAKLER, M.T., RIES, J.M., WILLIAMS, J.A., BANCROFT, J.E., PIPER, R.C., GIBBINS, B.L. and HINRICHS, D.J., 1993. Parasite lactate dehydrogenase as an assay for Plasmodium falciparum drug sensitivity. *The American Journal of Tropical Medicine and Hygiene,* **48**(6), pp. 739-741.

MANIKA, N., CHANOTIYA, C.S., DAROKAR, M., SINGH, S. and BAGCHI, G.D., 2016. Compositional Characters and Antimicrobial Potential of Artemisia stricta Edgew. f. stricta Pamp. Essential Oil. *Records of Natural Products,* **10**(2), pp. 40-46.

MANN, C. and MARKHAM, J., 1998. A new method for determining the minimum inhibitory concentration of essential oils . *Journal of Applied Microbiology,* **84**, pp. 538-544.

MANOSALVA, L., MUTIS, A., URZUA, A., FAJARDO, V. and QUIROZ, A., 2016. Antibacterial Activity of Alkaloid Fractions from Berberis microphylla G. Forst and Study of Synergism with Ampicillin and Cephalothin. *Molecules (Basel, Switzerland),* **21**(1), pp. 10.3390/molecules21010076.

MAREE, J., KAMATOU, G., GIBBONS, S., VILJOEN, A. and VAN VUUREN, S., 2014. The application of GC–MS combined with chemometrics for the identification of antimicrobial compounds from selected commercial essential oils. *Chemometrics & Intelligent Laboratory Systems,* **130**, pp. 172-181.

MARI, M., BERTOLINI, P. and PRATELLA, G., 2003. Non-conventional methods for the control of post-harvest pear diseases. *Journal of Applied Microbiology,* **94**, pp. 761-766.

MASON, T. and WASSERMAN, B., 1987. Inactivation of red beet betaglucan synthase by native and oxidized phenolic compounds. *Phytochemistry,* **26**, pp. 2197-2202.

MATUSCHEK, E., BROWN, D.F.J. and KAHLMETER, G., 2013. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin Microbiol Infect,* **20**, pp. O255-O266.

MCCRACKEN, S.T., KAISER, M., BOSHOFF, H.I., BOYD, P.D.W. and COPP, B.R., 2012. Synthesis and antimalarial and antituberculosis activities of a series of natural and unnatural 4-methoxy-6-styryl-pyran-2-ones, dihydro analogues and photo-dimers. *Bioorganic & medicinal chemistry,* **20**(4), pp. 1482-1493.

MCGIMPSEY, J., DOUGLAS, M., VAN KLINK, J., BEAUREGARD, D. and PERRY, N., 1994. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris L.* in New Zealand. *Flavour and Fragrance Journal,* **9**, pp. 347-352.

MCKAY, G.A., BEAULIEU, S., ARHIN, F.F., BELLEY, A., SARMIENTO, I., PARR, T.,Jr and MOECK, G., 2009. Time-kill kinetics of oritavancin and comparator agents against *Staphylococcus aureus*, Enterococcus faecalis and Enterococcus faecium. *The Journal of antimicrobial chemotherapy,* **63**(6), pp. 1191-1199.

MESHNICK, S.R., TAYLOR, T.E. and KAMCHONWONGPAISAN, S., 1996. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiological reviews,* **60**(2), pp. 301-315.

MICHIELIN, E.M.Z., SALVADOR, A.A., RIEHL, C.A.S., SMÂNIA, A., SMÂNIA, E.F.A. and FERREIRA, S.R.S., 2009. Chemical composition and antibacterial activity of Cordia verbenacea extracts obtained by different methods. *Bioresource technology,* **100**(24), pp. 6615-6623.

MIGUEL, M., CRUZ, C., FALEIRO, L., SIMÕES, M., FIGUEIREDO, A., BARROSO, J. and PEDRO, L., 2010. Foeniculum vulgare essential oils: chemical composition, antioxidant and antimicrobial activities . *Nat Prod Commun,* **5**(2), pp. 319-328.

MKOUNGA, P., TIABOU, A.T. and KOUAM, J., 2010. Triterpenoid derivatives from Cylicodiscus gabunensis. *Chemical & pharmaceutical bulletin,* **58**(8), pp. 1100-1102.

MOJAB, F., 2012. Antimalarial Natural Products: A Review. . *Avicenna Journal of Phytomedicine,* **2**(2), pp. 52-62.

MONSON, B.K., STRINGHAM, J., JONES, B.B., ABDEL-AZIZ, S., CUTLER PECK, C.M. and OLSON, R.J., 2010. Scanning electron microscopy visualization of methicillin-resistant *Staphylococcus aureus* after contact with gatifloxacin with and without preservative. *Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics,* **26**(2), pp. 133-136.

MORETTI, C., DEHARO, E., SAUVAIN, M., JARDEL, C., DAVID, P.T. and GASQUET, M., 1994. Antimalarial activity of cedronin. *Journal of ethnopharmacology,* **43**(1), pp. 57-61.

MOUREY, A. and CANILLAC, N., 2002. Anti-*Listeria monocytogenes* activity of essential oils components of conifers. *Food Control,* **13**, pp. 289-292.

MUHAMMAD, I., BEDIR, E., KHAN, S.I., TEKWANI, B.L., KHAN, I.A., TAKAMATSU, S., PELLETIER, J. and WALKER, L.A., 2004. A new antimalarial quassinoid from Simaba orinocensis. *Journal of natural products,* **67**(5), pp. 772.

MUKHLESUR RAHMAN, M., GIBBONS, S. and GRAY, A.I., 2007. Isoflavanones from Uraria picta and their antimicrobial activity. *Phytochemistry,* **68**(12), pp. 1692-1697.

MULYANINGSIH, S., SPORER, F., ZIMMERMANN, S., REICHLING, J. and WINK, M., 2010. Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of Eucalyptus globulus against antibiotic-susceptible and antibiotic-resistant pathogens. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology,* **17**(13), pp. 1061-1066.

MURIITHI, M.W., ABRAHAM, W., ADDAE-KYEREME, J., SCOWEN, I., CROFT, S.L., GITU, P.M., KENDRICK, H., NJAGI, E.N.M. and WRIGHT, C.W., 2002. Isolation and in vitro antiplasmodial activities of alkaloids from Teclea trichocarpa: in vivo antimalarial activity and X-ray crystal structure of normelicopicine. *Journal of natural products,* **65**(7), pp. 956-959.

NAMBA, T., MORITA, O., HUANG, S., GOSHIMA, K., HATTORI, M. and KAKIUCHI, N., 1988. Studies on cardio-active crude drugs. I. Effect of coumarins on cultured myocardial cells. *Planta Med.,* **54**, pp. 277-282.

NASRULLAH, A.A., ZAHARI, A., MOHAMAD, J. and AWANG, K., 2013. Antiplasmodial alkaloids from the bark of Cryptocarya nigra (Lauraceae). *Molecules (Basel, Switzerland),* **18**(7), pp. 8009-8017.

NATARO, J.P., BOPP, C.A., FIELDS, P.I., KAPER, J.B., STROCKBINE and N. A., 2007. Escherichia, Shigella, and Salmonella. In: M. P. R., ed, *Manual of clinical microbiology, Vol. 1.* 9th ed. edn. Washington, D.C.: ASM Press., pp. 670-687.

NATIONAL NOSOCOMIAL INFECTIONS SURVEILLANCE SYSTEM, 2004. National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004. *American Journal of Infection Control,* **32**(8), pp. 470-485.

NAVARRO, V., VILLARREAL, M., ROJAS, G. and LOZOYA, X., 1996. Antimicrobial evaluation of some plants used in Mexican traditional medicine for the treatment of infectious diseases. *J. Ethnopharmacol.,* **53**, pp. 143-147.

NDAH, N., EGBE, A., BECHEM, E., ASAHA, S., YENGO, T., CHIA, E. and AND EYENIEH, N., 2013. Ethnobotanical study of commonly used medicinal plants of the Takamanda Rainforest South West, Cameroon African. *Journal of Plant Science,* **7**(1), pp. 21-34.

NDJONKA, D., BERGMANN, B., AGYARE, C., ZIMBRES, F., LÜERSEN, K., HENSEL, A., WRENGER, C. and LIEBAU, E., 2012. In vitro activity of extracts and isolated polyphenols from West African medicinal plants against Plasmodium falciparum. *Parasitology research,* **111**(2), pp. 827-834.

NHS CHOICES, 15/04/2015, 2015-last update, Staphylococcal-infections [Homepage of NHS.UK], [Online]. Available: http://www.nhs.uk/conditions/Staphylococcal-infections/pages/introduction.aspx [April/22, 2016].

NOWACKA, N., NOWAK, R., DROZD, M., OLECH, M., LOS, R. and MALM, A., 2015. Antibacterial, Antiradical Potential and Phenolic Compounds of Thirty-One Polish Mushrooms. *PLoS ONE,* **10**(10), pp. 1-13.

NYCHAS, G., 1995. Natural antimicrobials from plants. In: G. GOULD, ed, *New Methods of Food Preservation.* London: Blackie Academic and Professional, pp. 58-89.

O'KENNEDY, R. and THORNES, R., 1997. *Coumarins: biology, applications and mode of action.* New York, N.Y.: John Wiley & Sons, Inc.

OKOKON, J.E., ITA, B.N. and UDOKPOH, A.E., 2006. Antiplasmodial activity of Cylicodiscus gabunensis. *Journal of ethnopharmacology,* **107**(2), pp. 175-178.

OLAJUYIGBE, O.O. and AFOLAYAN, A.J., 2015. In vitro Synergy and Time-kill Assessment of Interaction between Kanamycin and Metronidazole against Resistant Bacteria. *Tropical Journal of Pharmaceutical Research,* **14**(5), pp. 837-843.

OLUWATUYI, M., KAATZ, G. and GIBBONS, S., 2004. Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. *Phytochemistry,* **65**(24), pp. 3249-3254.

OOSTERHAVEN, K., POOLMAN, B. and SMID, E., 1995. S-carvone as a natural potato sprout inhibiting, fungistatic and bacteristatic compound. *Industrial Crops and Products,* **4**, pp. 23-31.

OSAWA, K., MATSUMOTO, T., MARUYAMA, T., TAKIGUCHI, T., OKUDA, K. and TAKAZOE, I., 1990. Studies of the antibacterial activity of plant extracts and their constituents against periodontopathic bacteria. *The Bulletin of Tokyo Dental College,* **31**(1), pp. 17-21.

OTTO, M., 2009. *Staphylococcus epidermidis*-the 'accidental' pathogen. *Nature reviews.Microbiology,* **7**(8), pp. 555-567.

OWOLABI, M.S., OGUNDAJO, A., LAJIDE, L., OLADIMEJI, M.O., SETZER, W.N. and PALAZZO, M.C., 2009. Chemical Composition and Antibacterial Activity of the Essential Oil of Lippia multiflora Moldenke from Nigeria. *Records of Natural Products,* **3**(4), pp. 170-177.

PACKIYASOTHY, E. and KYLE, S., 2002. Antimicrobial properties of some herb essential oils. *Food Australia,* **54**(9), pp. 384-387.

PARAPINI, S., BASILICO, N., PASINI, E., EGAN, T.J., OLLIARO, P., TARAMELLI, D. and MONTI, D., 2000. Standardization of the physicochemical parameters to assess in vitro the beta-hematin inhibitory activity of antimalarial drugs. *Experimental parasitology,* **96**(4), pp. 249-256.

PARK, S.N., LIM, Y.K., FREIRE, M.O., CHO, E., JIN, D. and KOOK, J.K., 2012. Antimicrobial effect of linalool and alpha-terpineol against periodontopathic and cariogenic bacteria. *Anaerobe,* **18**(3), pp. 369-372.

PATRA, J.K. and BAEK, K., 2016. Antibacterial Activity and Action Mechanism of the Essential Oil from Enteromorpha linza L. against Foodborne Pathogenic Bacteria. *Molecules,* **21**(3), pp. 388-398.

PAUL, M.D., 2009. *Medicinal Natural Products: A Biosynthetic Approach.* 3 edn. USA: John Wiley & Sons, Ltd.

PEDERSEN, M.M., CHUKWUJEKWU, J.C., LATEGAN, C.A., STADEN, J.V., SMITH, P.J. and STAERK, D., 2009. Antimalarial sesquiterpene lactones from Distephanus angulifolius. *Phytochemistry,* **70**(5), pp. 601-607.

PENG, F., WAN, F., XIONG, L., PENG, C., DAI, M. and CHEN, J., 2014. In vitro and in vivo antibacterial activity of Pogostone. *Chinese medical journal,* **127**(23), pp. 4001-4005.

PEREDA-MIRANDA, R., KAATZ, G.W. and GIBBONS, S., 2006. Polyacylated oligosaccharides from medicinal Mexican morning glory species as antibacterials and inhibitors of multidrug resistance in *Staphylococcus aureus*. *Journal of natural products,* **69**(3), pp. 406-409.

PETERSEN, I., EASTMAN, R. and LANZER, M., 2011. Drug-resistant malaria: molecular mechanisms and implications for public health. *FEBS letters,* **585**(11), pp. 1551-1562.

PHILLIPSON, J.D. and O'NEILL, M.J., 1989. New leads to the treatment of protozoal infections based on natural product molecules . *Acta Pharm. Nord.*, **1** (3), pp. 131-144.

PILLER, N., 1975. A comparison of the effectiveness of some anti-inflammatory drugs on thermal oedema. *Br. J. Exp. Pathol.,* **56**(554), pp. 559.

PINHO, E., FERREIRA, I.C.F.R., BARROS, L., CARVALHO, A.M., SOARES, G. and HENRIQUES, M., 2014. Antibacterial potential of northeastern Portugal wild plant extracts and respective phenolic compounds. *Biomed Research International,* **2014**, pp. 814590-814590.

PLOSS, T.N., REILMAN, E., MONTEFERRANTE, C.G., DENHAM, E.L., PIERSMA, S., LINGNER, A., VEHMAANPERÄ, J., LORENZ, P. and VAN DIJL, J.M., 2016. Homogeneity and heterogeneity in amylase production by Bacillus subtilis under different growth conditions. *Microbial Cell Factories,* **15**, pp. 1-16.

PO SHIU, W.K. and GIBBONS, S., 2006. Anti-staphylococcal acylphloroglucinols from Hypericum beanii. *Phytochemistry,* **67**(23), pp. 2568-2572.

PRABUSEENIVASAN, S., JAYAKUMAR, M. and IGNACIMUTHU, S., 2006. In vitro antibacterial activity of some plant essential oils. *BMC complementary and alternative medicine,* **6**, pp. 39.

PREECHAPORNKUL,P.,IMWONG, M., CHOTIVANICH, K., PONGTAVORNPINYO, W., DONDORP, A.M., DAY, N.P., WHITE, N.J. and PUKRITTAYAKAMEE, S., 2009. Plasmodium falciparum pfmdr1 amplification, mefloquine resistance, and parasite fitness. *Antimicrobial Agents and Chemotherapy,* **53**(4), pp. 1509-1515.

PRICE, R.N., NOSTEN, F., LUXEMBURGER, C., KHAM, A., BROCKMAN, A., CHONGSUPHAJAISIDDHI, T. and WHITE, N.J., 1995. Artesunate versus artemether in combination with mefloquine for the treatment of multidrug-resistant falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **89**(5), pp. 523-527.

PROMGOOL, T., PANCHAROEN, O. and DEACHATHAI, S., 2014. Antibacterial and antioxidative compounds from Cassia alata Linn. *Songklanakarin Journal of Science & Technology,* **36**(4), pp. 459-463.

QIU, J., ZHANG, X., LUO, M., LI, H., DONG, J., WANG, J., LENG, B., WANG, X., FENG, H., REN, W. and DENG, X., 2011. Subinhibitory concentrations of perilla oil affect the expression of secreted virulence factor genes in *Staphylococcus aureus*. *PloS one,* **6**(1), pp. e16160.

QU, L., QI, Y., FAN, G. and WU, Y., 2009. Determination of the volatile oil of Manolia biodii Pamp by GC-MS combined with chemometric techniques. *Chromatographia,* **70**, pp. 905-913.

RADULOVIC, N.S., MLADENOVIC, M.Z. and ETHORDEVIC, N.D., 2012. Chemotypification of Astrantia major L. (Apiaceae): essential-oil and lignan profiles of fruits. *Chemistry & biodiversity,* **9**(7), pp. 1320-1337.

RAMANANDRAIBE, V., GRELLIER, P., MARTIN, M.T., DEVILLE, A., JOYEAU, R., RAMANITRAHASIMBOLA, D., MOURAY, E., RASOANAIVO, P. and MAMBU, L., 2008. Antiplasmodial Phenolic Compounds from Piptadenia pervillei. *Planta Medica,* **74**(4), pp. 417.

REDINBO, M.R., 2014. The microbiota, chemical symbiosis, and human disease. *Journal of Molecular Biology,* **426**(23), pp. 3877-3891.

RICE-EVANS, C.A., MILLER, N.J. and PAGANGA, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free radical biology & medicine,* **20**(7), pp. 933-956.

RIOS, J., RECIO, M. and VILLAR, A., 1988. Screening methods for natural antimicrobial products with antimicrobial activity. *Journal of Ethnopharmacology,* **23**, pp. 127-149.

RIVAS, D.S., LOPES, P.M., BARROS, D.A., COSTA, D.C.M., ALVIANO, C.S. and ALVIANO, D.S., 2012. Biological activities of a-pinene and ß-pinene enantiomers. *Molecules (Basel, Switzerland),* **17**(6), pp. 6305-6316.

ROHLOFF, J., 2015. Analysis of Phenolic and Cyclic Compounds in Plants Using Derivatization Techniques in Combination with GC-MS-Based Metabolite Profiling. *Molecules,* **20**(2), pp. 3431-3462.

ROSSI, R.A. and DE ROSSI, R.H., 1974. Preparation of benzoate esters of tertiary alcohols by transesterification. *J. Org. Chem.*, **39**(6), pp. 855-865.

RUA, J., FERNANDEZ-ALVAREZ, L., DE CASTRO, C., DEL VALLE, P., DE ARRIAGA, D. and GARCIA-ARMESTO, M.R., 2011. Antibacterial activity against foodborne *Staphylococcus aureus* and antioxidant capacity of various pure phenolic compounds. *Foodborne pathogens and disease*, **8**(1), pp. 149-157.

RUKAYADI, Y., LEE, K., HAN, S., YONG, D. and HWANG, J.K., 2009. In vitro activities of panduratin A against clinical *Staphylococcus* strains. *Antimicrobial Agents and Chemotherapy*, **53**(10), pp. 4529-4532.

SAAVEDRA, M.J., BORGES, A., DIAS, C., AIRES, A., BENNETT, R.N., ROSA, E.S. and SIMOES, M., 2010. Antimicrobial activity of phenolics and glucosinolate hydrolysis products and their synergy with streptomycin against pathogenic bacteria. *Medicinal chemistry (Shariqah (United Arab Emirates))*, **6**(3), pp. 174-183.

SALLEH, W., AHMAD, F. and KHONG, H., 2016. Essential Oil Compositions and Antimicrobial Activity of Piper arborescens Roxb. *Marmara Pharmaceutical Journal*, **20**(2), pp. 111-115.

SALZER, U., 1977. The analysis of essential oils and extracts (oleoresins) from seasonings—a critical review. *Critical Reviews in Food Science and Nutrition*, **9**(4), pp. 345-373.

SANCHEZ, G.V., MASTER, R.N., KARLOWSKY, J.A. and BORDON, J.M., 2012. In vitro antimicrobial resistance of urinary *Escherichia coli* isolates among U.S. outpatients from 2000 to 2010. *Antimicrobial Agents and Chemotherapy*, **56**(4), pp. 2181-2183.

SANZ, L.M., CRESPO, B., DE-CÓZAR, C., DING, X.C., LLERGO, J.L., BURROWS, J.N., GARCÍA-BUSTOS, J.,F. and GAMO, F., 2012. P. falciparum in vitro killing rates allow to discriminate between different antimalarial mode-of-action. *Plos One*, **7**(2), pp. e30949-e30949.

SARJIT, A., WANG, Y. and DYKES, G.A., 2015. Antimicrobial activity of gallic acid against thermophilic Campylobacter is strain specific and associated with a loss of calcium ions. *Food Microbiology*, **46**, pp. 227-233.

SARKER, S.D., 2007. *Chemistry for pharmacy students : general, organic and natural product chemistry*. Hoboken, N.J.; Chichester: Wiley; John Wiley distributor.

SARKER, S.D., NAHAR, L. and KUMARASAMY, Y., 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods (San Diego, Calif.)*, **42**(4), pp. 321-324.

SATO, M., FUJIWARA, S., TSUCHIYA, H., FUJII, T., IINUMA, M., TOSA, H. and OHKAWA, Y., 1996. Flavones with antibacterial activity against cariogenic bacteria. *J. Ethnopharmacol.*, **54**, pp. 171-176.

SAXENA, S., PANT, N., JAIN, D.C. and BHAKUNI, R.S., 2003. Antimalarial agents from plant sources. *Current Science,* **85**(9), pp. 1314-1329.

SCALBERT, A., 1991. Antimicrobial properties of tannins. *Phytochemistry,* **30**, pp. 3875-3883.

SCHEFFER, J. and SVENDSEN, A., 1981. Zur Analytik ätherische Öle: Zehn Jahre Ātherische-Öle-Forschung 1969 bis 1979. *Riechstoffe, Aromen, Kosmetica,* **31**(2), pp. 39-41.

SCHLEGELOVA, J., BRYCHTA, J., KLIMOVA, E., NAPRAVNIKOVA, E. and BABAK, V., 2003. The prevalence of and resistance to antimicrobial agents of Bacillus cereus isolates from foodstuffs. *Vet. Med. – Czech,* **48**(11), pp. 331-338.

SCHLITZER, M., 2008. Antimalarial drugs - what is in use and what is in the pipeline. *Archiv der Pharmazie,* **341**(3), pp. 149-163.

SCHMIDT, H., ed, 1988. *Phenol oxidase (E.I.14.18.1), a marker enzyme for defense cells. Progress in histochemistry and cytochemistry, vol. 17.*. New York, N.Y.: Gustav Fischer.

SCHMIDT, E., BAIL, S., FRIEDL, S.M., JIROVETZ, L., BUCHBAUER, G., WANNER, J., DENKOVA, Z., SLAVCHEV, A., STOYANOVA, A. and GEISSLER, M., 2010. Antimicrobial activities of single aroma compounds. *Natural product communications,* **5**(9), pp. 1365-1368.

SCHUHLY, W., SKARBINA, J., KUNERT, O., NANDI, O.I. and BAUER, R., 2009. Chemical characterization of Magnolia biondii (Flos Magnoliae, Xin Yi). *Natural product communications,* **4**(2), pp. 231-234.

SEO, K.S. and BOACH, G.A., 2007. *Staphylococcus aureus*. In: M.P. DOYLE and L.R. BEUCHAT, eds, *Food microbiology: Fundamentals and frontiers.* 3 edn. Washington, D.C.: ASM Press., pp. 493-518.

SEO, W.H. and BAEK, H.H., 2009. Characteristic aroma-active compounds of Korean perilla (Perilla frutescens Britton) leaf. *Journal of Agricultural and Food Chemistry,* **57**(24), pp. 11537-11542.

SHAHZAD, M., MILLHOUSE, E., CULSHAW, S., EDWARDS, C.A., RAMAGE, G. and COMBET, E., 2015. Selected dietary (poly)phenols inhibit periodontal pathogen growth and biofilm formation. *Food & Function,* **6**(3), pp. 719-729.

SHARMA, S.C. and AGARWAL, V.K., 1993. Brucea javanica (Linn.) Merr.: A potent anticancer and antimalarial plant – a review. *Indian J. Pharm. Sci.,* **55**, pp. 77-85.

SHARMA, R., KISHORE, N., HUSSEIN, A. and LALL, N., 2013. Antibacterial and anti-inflammatory effects of Syzygium jambos L. (Alston) and isolated compounds on acne vulgaris. *BMC complementary and alternative medicine,* **13**, pp. 292-6882-13-292.

SHEN, Y., LI, C.G., ZHOU, S.F., PANG, E.C., STORY, D.F. and XUE, C.C., 2008. Chemistry and bioactivity of Flos Magnoliae, a Chinese herb for rhinitis and sinusitis. *Current medicinal chemistry,* **15**(16), pp. 1616-1627.

SHIBATA, H., KONDO, K., KATSUYAMA, R., KAWAZOE, K., SATO, Y., MURAKAMI, K., TAKAISHI, Y., ARAKAKI, N. and HIGUTI, T., 2005. Alkyl gallates, intensifiers of beta-lactam susceptibility in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* **49**(2), pp. 549-555.

SHIU, W.K.P., RAHMAN, M.M., CURRY, J., STAPLETON, P., ZLOH, M., MALKINSON, J.P. and GIBBONS, S., 2012. Antibacterial acylphloroglucinols from Hypericum olympicum. *Journal of natural products,* **75**(3), pp. 336-343.

SHUKLA, S., MAJUMDER, R., AHIRWAL, L. and MEHTA, A., 2016. Proposed mechanism of antibacterial mode of action of Caesalpinia bonducella seed oil against food-borne pathogens. *Bangladesh Journal of Pharmacology,* **11**(1), pp. 257-263.

SIBANDA, T. and OKOH, A.I., 2007. The challenges of overcoming antibiotic resistance: Plant extracts as potential sources of antimicrobial and resistance modifying agents. *Afr J Biotechnol.,* **6**(25), pp. 2886-2896.

SIBLEY, C.H., HYDE, J.E., SIMS, P.F., PLOWE, C.V., KUBLIN, J.G., MBERU, E.K., COWMAN, A.F., WINSTANLEY, P.A., WATKINS, W.M. and NZILA, A.M., 2001. Pyrimethamine-sulfadoxine resistance in Plasmodium falciparum: what next? *Trends in parasitology,* **17**(12), pp. 582-588.

SIKKEMA, J., DE BONT, J. and POOLMAN, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews,* **59**(2), pp. 201-222.

SIKKEMA, J., DE BONT, J. and POOLMAN, B., 1994. Interactions of cyclic hydrocarbons with biological membranes. *Journal of Biological Chemistry,* **269**(11), pp. 8022-8028.

SILVA, O., DUARTE, A., CABRITA, J., PIMENTEL, M., DINIZ, A. and GOMES, E., 1996. Antimicrobial activity of Guinea-Bissau traditional remedies. *J. Ethnopharmacol.,* **50**, pp. 55-59.

SIMOES, M., BENNETT, R.N. and ROSA, E.A., 2009. Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Natural product reports,* **26**(6), pp. 746-757.

SINGH, N., KAUSHIK, N.K., MOHANAKRISHNAN, D., TIWARI, S.K. and SAHAL, D., 2015. Antiplasmodial activity of medicinal plants from Chhotanagpur plateau, Jharkhand, India. *Journal of ethnopharmacology,* **165**, pp. 152-162.

SINHA, S., MEDHI, B. and SEHGAL, R., 2014. Challenges of drug-resistant malaria. *Parasite (Paris, France),* **21**, pp. 61.

SKANDAMIS, P., KOUTSOUMANIS, K., FASSEAS, K. and NYCHAS, G., 2001. Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. *Italian Journal of Food Science,* **13**(1), pp. 65-75.

SKANDAMIS, P. and NYCHAS, G., 2001. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. *Journal of Applied Microbiology,* **91**, pp. 1011-1022.

SMILKSTEIN, M., SRIWILAIJAROEN, N., KELLY, J.X., WILAIRAT, P. and RISCOE, M., 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrobial Agents and Chemotherapy,* **48**(5), pp. 1803-1806.

SMITH, K., GOULD, K.A., RAMAGE, G., GEMMELL, C.G., HINDS, J. and LANG, S., 2010. Influence of tigecycline on expression of virulence factors in biofilm-associated cells of methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* **54**(1), pp. 380-387.

SOBERON, J.R., SGARIGLIA, M.A., DIP MADERUELO, M.R., ANDINA, M.L., SAMPIETRO, D.A. and VATTUONE, M.A., 2014. Antibacterial activities of Ligaria cuneifolia and Jodina rhombifolia leaf extracts against phytopathogenic and clinical bacteria. *Journal of bioscience and bioengineering,* **118**(5), pp. 599-605.

SOINE, T., 1964. Naturally occurring coumarins and related physiological activities. *J. Pharm. Sci.,* **53**, pp. 231-264.

SRIDARAN, S., MCCLINTOCK, S.K., SYPHARD, L.M., HERMAN, K.M., BARNWELL, J.W. and UDHAYAKUMAR, V., 2010. Anti-folate drug resistance in Africa: meta-analysis of reported dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) mutant genotype frequencies in African Plasmodium falciparum parasite populations. *Malaria journal,* **9**, pp. 247-2875-9-247.

SRIPISUT, T., CHEENPRACHA, S. and LAPHOOKHIEO, S., 2011. Chemical constituents from the roots of Feroniella lucida. *Journal of Asian natural products research,* **13**(6), pp. 556-560.

STERN, J., HAGERMAN, A., STEINBERG, P. and MASON, P., 1996. Phlorotannin-protein interactions. *J. Chem. Ecol.,* **22**, pp. 1887-1899.

SUBEKI, S., MATSUURA, H., TAKAHASHI, K., YAMASAKI, M., YAMATO, O., MAEDE, Y., KATAKURA, K., KOBAYASHI, S., TRIMURNINGSIH, T., CHAIRUL, C. and YOSHIHARA, T., 2005. Anti-babesial and anti-plasmodial compounds from Phyllanthus niruri. *Journal of natural products,* **68**(4), pp. 537-539.

SUDANO ROCCARO, A., BLANCO, A.R., GIULIANO, F., RUSCIANO, D. and ENEA, V., 2004. Epigallocatechin-gallate enhances the activity of tetracycline in staphylococci by inhibiting its efflux from bacterial cells. *Antimicrobial Agents and Chemotherapy,* **48**(6), pp. 1968-1973.

SUSEEM, S.R. and SARAL, A.M., 2013. Analysis on Essential Fatty Acid Esters of Mushroom Pleurotus Eous and its Antibacterial Activity. *Asian Journal of Pharmaceutical & Clinical Research,* , pp. 188-191.

TAGLIALATELA-SCAFATI, O., POLLASTRO, F., CHIANESE, G., MINASSI, A., GIBBONS, S., ARUNOTAYANUN, W., MABEBIE, B., BALLERO, M. and APPENDINO, G., 2013. Antimicrobial phenolics and unusual glycerides from Helichrysum italicum subsp. microphyllum. *Journal of natural products,* **76**(3), pp. 346-353.

TAGURI, T., TANAKA, T. and KOUNO, I., 2006. Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. *Biological & pharmaceutical bulletin,* **29**(11), pp. 2226-2235.

TAKAYA, Y., KURUMADA, K., TAKEUJI, Y., KIM, H., SHIBATA, Y., IKEMOTO, N., WATAYA, Y. and OSHIMA, Y., 1998. Novel antimalarial guaiane-type sesquiterpenoids from Nardostachys chinensisroots. *Tehtrahedron Lett.,* **39**(11), pp. 1361-1364.

TANE, P., BERGQUST, K., TENE, M., NGADJUI, B., AYAFOR, J. and STERNER, O., 1995. Cyclodione, an unsymmetrical dimeric diterpene from *Cylicodiscus gabunensis*. *Tetrahedron,* **51**(42), pp. 11595-11600.

TASDEMIR, D., BRUN, R., FRANZBLAU, S.G., SEZGIN, Y. and CALIS, I., 2008. Evaluation of antiprotozoal and antimycobacterial activities of the resin glycosides and the other metabolites of Scrophularia cryptophila. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology,* **15**(3), pp. 209-215.

TAYLOR, R., EDEL, F., MANANDHAR, N. and TOWERS, G., 1996. Antimicrobial activities of southern Nepalese medicinal plants. *J. Ethno- pharmacol.*, **50**, pp. 97-102.

TCHIVOUNDA, H., KOUDOGBO, B., BESACE, Y. and CASADEVALL, E., 1990. Cylicodiscic acid, a dihydroxy pentacyclic triterpene carboxylic acid from *Cylicodiscus gabunensis*. *Phytochemistry,* **29**, pp. 3255-3258.

TCHIVOUNDA, H.P., KOUDOGBO, B., BESACE, Y. and CASADEVALL, E., 1991. Triterpene saponins from *Cylicodiscus gabunensis*. *Phytochemistry,* **30**(8), pp. 2711-2716.

TENE, M., CHABERT, P., NOTE, O., KENLA, T. and LOBSTEIN, P., 2011. Triterpenoid saponins from *Cylicodiscus gabunensis*. *phytochemistry letters,* **4**, pp. 89-92.

THASTRUP, O., KNUDSEN, J., LEMMICH, J. and WINTHER, K., 1985. Inhibitions of human platelet aggregation by dihydropyrano- and dihydrofurano- coumarins, a new class of cAMP phosphodiesterase inhibitors. *Biochem. Pharmacol.,* **34**, pp. 2137-2140.

THEBTARANONTH, C., THEBTARANONTH, Y., WANAUPPATHAMKUL, S. and YUTHAVONG, Y., 1995. Antimalarial sesquiterpenes from tubers of Cyperus rotundus: structure of 10,12-peroxycalamenene, a sesquiterpene endoperoxide. *Phytochemistry,* **40**(1), pp. 125-128.

THEURETZBACHER, U., 2011. Resistance drives antibacterial drug development. *Current opinion in pharmacology,* **11**(5), pp. 433-438.

TOGASHI, N., INOUE, Y., HAMASHIMA, H. and TAKANO, A., 2008. Effects of two terpene alcohols on the antibacterial activity and the mode of action of farnesol against *Staphylococcus aureus*. *Molecules,* **13**, pp. 3069-3076.

TOLOSSA, K., ASRES, K., EL-FIKY, F., SINGAB, A.N.B. and BUCAR, F., 2007. Composition of the Essential Oils of Satureja abyssinica ssp. abyssinica and Satureja paradoxa: Their Antimicrobial and Radical Scavenging Activities. *Journal of Essential Oil Research,* **19**(3), pp. 295-300.

TOMAS-MENOR, L., BARRAJON-CATALAN, E., SEGURA-CARRETERO, A., MARTI, N., SAURA, D., MENENDEZ, J.A., JOVEN, J. and MICOL, V., 2015. The promiscuous and synergic molecular interaction of polyphenols in bactericidal activity: an opportunity to improve the performance of antibiotics? *Phytotherapy Research : PTR,* **29**(3), pp. 466-473.

TONG, S.Y., CHEN, L.F. and FOWLER, V.G.,Jr, 2012. Colonization, pathogenicity, host susceptibility, and therapeutics for *Staphylococcus aureus*: what is the clinical relevance? *Seminars in immunopathology,* **34**(2), pp. 185-200.

TROMBETTA, D., CASTELLI, F., SARPIETRO, M.G., VENUTI, V., CRISTANI, M., DANIELE, C., SAIJA, A., MAZZANTI, G. and BISIGNANO, G., 2005. Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotherapy,* **49**(6), pp. 2474-2478.

TULAKE, A., JIANG, Y. and TU, P., 2012. Nine lignans from Artemisia absinthium L. *Journal of Chinese Pharmaceutical Sciences,* **21**, pp. 360-364.

TULEY DE SILVA, K., 1996. *A Manual on the Essential Oil Industry.* Vienna: United Nations Industrial Development Organization.

ULTEE, A., BENNINK, M. and MOEZELAAR, R., 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology,* **68**(4), pp. 1561-1568.

VALE, V.V., VILHENA, T.C., SANTOS TRINDADE, R.C., FERREIRA, M.R.C., PERCÁRIO, S., SOARES, L.F., PEREIRA, W.L.A., BRANDÃO, G.C., OLIVEIRA, A.B., DOLABELA, M.F. and DE VASCONCELOS, F., 2015. Antimalarial activity and toxicity assessment of Himatanthus articulatus, a plant used to treat malaria in the Brazilian Amazon. *Malaria Journal,* **14**(1), pp. 1-10.

VAN DE BRAAK, S. and LEIJTEN, G., 1999. *Essential Oils and Oleoresins: A Survey in the Netherlands and other Major Markets in the European Union.* Rotterdam: Centre for the Promotion of Imports from Developing Countries.

VAN DIJL, J.M. and HECKER, M., 2013. Bacillus subtilis: from soil bacterium to super-secreting cell factory. *Microbial cell factories,* **12**, pp. 3-2859-12-3.

VAN VUUREN, S. and VILJOEN, A.M., 2007. Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination. *Flavour and Fragrance Journal,* **22**(6), pp. 540-544.

VEROTTA, L., DELL'AGLI, M., GIOLITO, A., GUERRINI, M., CABALION, P. and BOSISIO, E., 2001. In vitro antiplasmodial activity of extracts of Tristaniopsis species and identification of the active constituents: ellagic acid and 3,4,5-trimethoxyphenyl-(6'-O-galloyl)-O-beta-D-glucopyranoside. *Journal of natural products,* **64**(5), pp. 603-607.

VON STETTEN, F., MAYR, R. and SCHERER, S., 1999. Climatic influence on mesophilic Bacillus cereus and psychrotolerant Bacillus weihenstephanensis populations in tropical, temperate and alpine soil. *Environmental microbiology,* **1**(6), pp. 503-515.

WAGNER, H. and ULRICH-MERZENICH, G., 2009. Synergy research: approaching a new generation of phytopharmaceuticals. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology,* **16**(2-3), pp. 97-110.

WAN, J., WILCOCK, A. and COVENTRY, M., 1998. The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. *J. Appl. Microbiol.,* **84**, pp. 152-158.

WANG, C., WAN, J., MEI, Z. and YANG, X., 2014. Acridone alkaloids with cytotoxic and antimalarial activities from Zanthoxylum simullans Hance. *Pharmacognosy magazine,* **10**(37), pp. 73-76.

WANG, S.S., WANG, D.M., PU, W.J. and LI, D.W., 2013. Phytochemical profiles, antioxidant and antimicrobial activities of three Potentilla species. *BMC complementary and alternative medicine,* **13**, pp. 321-6882-13-321.

WARHURST, D.C., 1985. New drugs and their potential use against drug-resistant malaria. *Annali dell'Istituto Superiore di Sanita,* **21**(3), pp. 327-336.

WARYAH, C.B., GOGOI-TIWARI, J., WELLS, K., ETO, K.Y., MASOUMI, E., COSTANTINO, P., KOTIW, M. and MUKKUR, T., 2016. Diversity of Virulence Factors Associated with West Australian Methicillin-Sensitive *Staphylococcus aureus* Isolates of Human Origin. *BioMed Research International,* , pp. 1-10 10p.

WESTHRIN, M., XIE, M., OLDERØY, M.Ø, SIKORSKI, P., STRAND, B.L. and STANDAL, T., 2015. Osteogenic Differentiation of Human Mesenchymal Stem Cells in Mineralized Alginate Matrices. *PLoS ONE,* **10**(3), pp. 1-16.

WHITE, N.J., 1985. Clinical pharmacokinetics of antimalarial drugs. *Clinical pharmacokinetics,* **10**(3), pp. 187-215.

WHITE, N., 1999. Antimalarial drug resistance and combination chemotherapy. *Philosophical transactions of the Royal Society of London.Series B, Biological sciences,* **354**(1384), pp. 739-749.

WHO, 2011. *European Strategic Action Plan on Antibiotic Resistance.* Copenhagen, Denmark: World Health Organisation.

WIJNANDS, L.M., DUFRENNE, J.B., ZWIETERING, M.H. and VAN LEUSDEN, F.M., 2006. Spores from mesophilic Bacillus cereus strains germinate better and grow faster in simulated gastro-intestinal conditions than spores from psychrotrophic strains. *International journal of food microbiology,* **112**(2), pp. 120-128.

WILKINS, C. and MADSEN, J., 1991. Oregano headspace constituents. *Zeitschrift für Lebensmittel Untersuchung und Forschung,* **192**, pp. 214-219.

WORLD HEALTH ORGANIZATION, 2015. *World Malaria Report 2015.* ISBN: 978 92 4 156515 8. Switzerland: WHO Press.

WOUATSA, V.N., MISRA, L., KUMAR, S., PRAKASH, O., KHAN, F., TCHOUMBOUGNANG, F. and VENKATESH, R.K., 2013. Aromatase and glycosyl transferase inhibiting acridone alkaloids from fruits of Cameroonian Zanthoxylum species. *Chemistry Central journal,* **7**, pp. 125-153X-7-125. eCollection 2013.

WRIGHT, C.W. and WARHURST, D.C., 2002. The mode of action of artemisinin and its derivatives. In: C.W. WRIGHT, ed, *Artemisia.* Taylor and Francis, London, pp. 249-288.

WRIGHT, C.W., 2005. Traditional antimalarial s and the development of novel antimalarial drugs. *Journal of ethnopharmacology,* **100**(1-2), pp. 67-71.

WRIGHT, C.W., ADDAE-KYEREME, J., BREEN, A.G., BROWN, J.E., COX, M.F., CROFT, S.L., GOKCEK, Y., KENDRICK, H., PHILLIPS, R.M. and POLLET, P.L., 2001. Synthesis and evaluation of cryptolepine analogues for their potential as new antimalarial agents. *Journal of medicinal chemistry,* **44**(19), pp. 3187-3194.

WU, J., LU, X., TANG, W., KONG, H., ZHOU, S. and XU, G., 2004. Application of comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry in the analysis of volatile oil of traditional Chinese medicines. *Journal of chromatography.A,* **1034**(1-2), pp. 199-205.

YA, C., GAFFNEY, S., LILLEY, T. and HASLAM, E., 1988. Carbohydrate polyphenol complexation. In: R. HEMINGWAY and J. KARCHESY, eds, *Chemistry and significance of condensed tannins.* New York, N.Y.: Plenum Press, pp. 553.

YAM, T.S., HAMILTON-MILLER, J.M. and SHAH, S., 1998. The effect of a component of tea (Camellia sinensis) on methicillin resistance, PBP2' synthesis, and beta-lactamase production in *Staphylococcus aureus*. *The Journal of antimicrobial chemotherapy,* **42**(2), pp. 211-216.

YAMAMOTO, H. and OGAWA, T., 2002. Antimicrobial activity of *perilla* seed polyphenols against oral pathogenic bacteria. *Bioscience, biotechnology, and biochemistry,* **66**(4), pp. 921-924.

YAMAMOTO, S., OTTO, A. and SIMONEIT, B.R., 2004. Lignans in resin of Araucaria angustifolia by gas chromatography/mass spectrometry. *Journal of mass spectrometry : JMS,* **39**(11), pp. 1337-1347.

YANG, X., ZHANG, X., SHUI-PING YANG and WEI-QI LIU, 2013. Evaluation of the Antibacterial Activity of Patchouli Oil. *Iranian Journal of Pharmaceutical Research,* **12**(3), pp. 307-316.

YANG, Y., KINOSHITA, K., KOYAMA, K., TAKAHASHI, K., TAI, T., NUNOURA, Y. and WATANABE, K., 1999. Anti-emetic principles of Pogostemon cablin (Blanco) Benth. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology,* **6**(2), pp. 89-93.

YI, Y., HE, J., SU, J., KONG, S., SU, J., LI, Y., HUANG, S., LI, C., LAI, X. and SU, Z., 2013. Synthesis and antimicrobial evaluation of pogostone and its analogues. *Fitoterapia,* **84**(1), pp. 135-139.

YU, X.D., XIE, J.H., WANG, Y.H., LI, Y.C., MO, Z.Z., ZHENG, Y.F., SU, J.Y., LIANG, Y.E., LIANG, J.Z., SU, Z.R. and HUANG, P., 2015. Selective antibacterial activity of patchouli alcohol against Helicobacter pylori based on inhibition of urease. *Phytotherapy Research : PTR,* **29**(1), pp. 67-72.

ZAHARI, A., CHEAH, F.K., MOHAMAD, J., SULAIMAN, S.N., LITAUDON, M., LEONG, K.H. and AWANG, K., 2014. Antiplasmodial and antioxidant isoquinoline alkaloids from Dehaasia longipedicellata. *Planta Medica,* **80**(7), pp. 599-603.

ZAKERI, S., HEMATI, S., PIRAHMADI, S., AFSHARPAD, M., RAEISI, A. and DJADID, N.D., 2012. Molecular assessment of atpase6 mutations associated with artemisinin resistance among unexposed and exposed Plasmodium falciparum clinical isolates to artemisinin-based combination therapy. *Malaria journal,* **11**, pp. 373-2875-11-373.

ZENG, Z., XIE, R., ZHANG, T., ZHANG, H. and CHEN, J.Y., 2011. Analysis of volatile compositions of Magnolia biondii pamp by steam distillation and headspace solid phase micro-extraction. *Journal of oleo science,* **60**(12), pp. 591-596.

ZHANG, Y., LIU, X., WANG, Y., JIANG, P. and QUEK, S., 2016. Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. *Food Control,* **59**, pp. 282-289.

ZHANG, Z., FENG, L., XU, H., LIU, C., SHAH, N.P. and WEI, H., 2016. Detection of viable enterotoxin-producing Bacillus cereus and analysis of toxigenicity from ready-to-eat foods and infant formula milk powder by multiplex PCR. *Journal of dairy science,* **99**(2), pp. 1047-1055.

ZHANG, Z., TAO, X., SHAH, N.P. and WEI, H., 2016. Antagonistics against pathogenic Bacillus cereus in milk fermentation by Lactobacillus plantarum ZDY2013 and its anti-adhesion effect on Caco-2 cells against pathogens. *Journal of dairy science,* **99**(4), pp. 2666-2674.

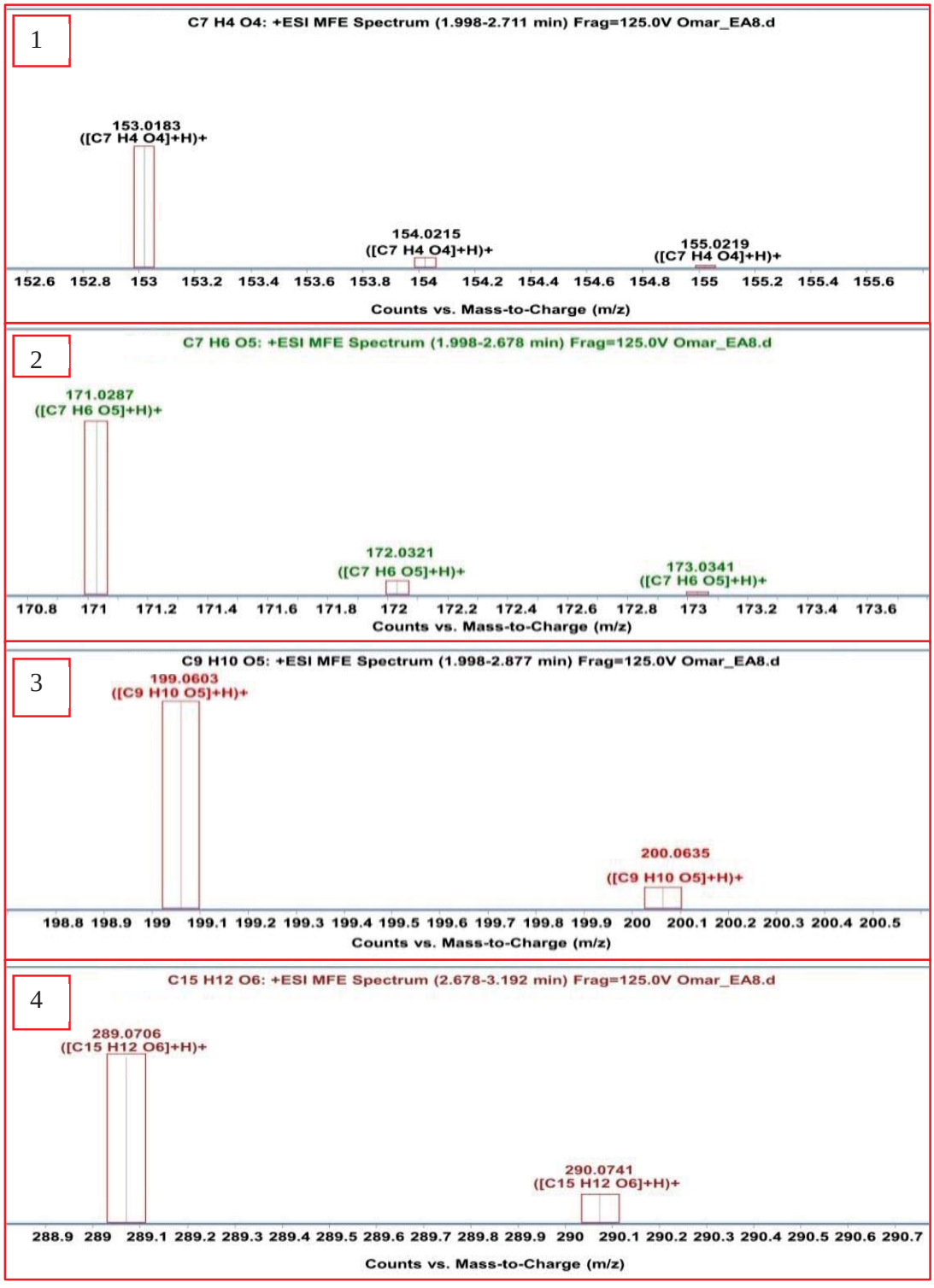
ZHAO, W., ZHOU, T., FAN, G., CHAI, Y. and WU, Y., 2007. Isolation and purification of lignans from Magnolia biondii Pamp by isocratic reversed-phase two-dimensional liquid chromatography following microwave-assisted extraction. *Journal of separation science,* **30**(15), pp. 2370-2381.

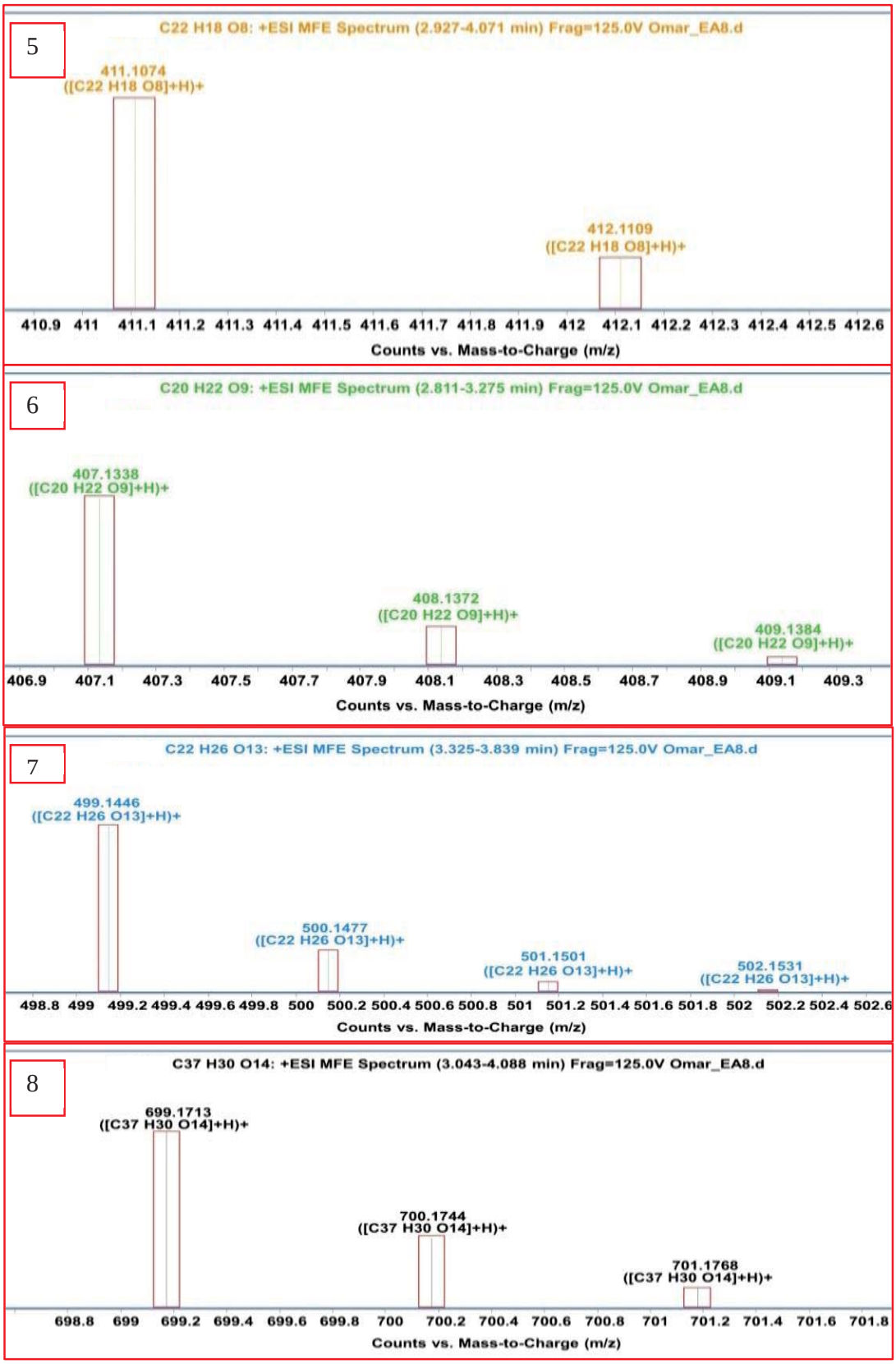
ZHOU, Z., CHEN, L., SHEN, M., MA, A., YANG, X. and ZOU, F., 2011. Analysis of the essential oils of *Coriandrum sativum* Using GC-MS coupled with chemometric resolution methods. *Chem Pharm Bull,* **59**(1), pp. 28-34.

ZUO, G.Y., LI, Y., WANG, T., HAN, J., WANG, G.C., ZHANG, Y.L. and PAN, W.D., 2011. Synergistic antibacterial and antibiotic effects of bisbenzylisoquinoline alkaloids on clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). *Molecules (Basel, Switzerland),* **16**(12), pp. 9819-9826.

Appendices

Appendix A




5
C22 H18 O8: +ESI MFE Spectrum (2.927-4.071 min) Frag=125.0V Omar_EA8.d
411.1074
([C22 H18 O8]+H)+
412.1109
([C22 H18 O8]+H)+
410.9 411 411.1 411.2 411.3 411.4 411.5 411.6 411.7 411.8 411.9 412 412.1 412.2 412.3 412.4 412.5 412.6
Counts vs. Mass-to-Charge (m/z)
6
C20 H22 O9: +ESI MFE Spectrum (2.811-3.275 min) Frag=125.0V Omar_EA8.d
407.1338
([C20 H22 O9]+H)+
408.1372
([C20 H22 O9]+H)+
409.1384
([C20 H22 O9]+H)+
406.9 407.1 407.3 407.5 407.7 407.9 408.1 408.3 408.5 408.7 408.9 409.1 409.3
Counts vs. Mass-to-Charge (m/z)
7
C22 H26 O13: +ESI MFE Spectrum (3.325-3.839 min) Frag=125.0V Omar_EA8.d
499.1446
([C22 H26 O13]+H)+
500.1477
([C22 H26 O13]+H)+
501.1501
([C22 H26 O13]+H)+
502.1531
([C22 H26 O13]+H)+
498.8 499 499.2 499.4 499.6 499.8 500 500.2 500.4 500.6 500.8 501 501.2 501.4 501.6 501.8 502 502.2 502.4 502.6
Counts vs. Mass-to-Charge (m/z)
8
C37 H30 O14: +ESI MFE Spectrum (3.043-4.088 min) Frag=125.0V Omar_EA8.d
699.1713
([C37 H30 O14]+H)+
700.1744
([C37 H30 O14]+H)+
701.1768
([C37 H30 O14]+H)+
698.8 699 699.2 699.4 699.6 699.8 700 700.2 700.4 700.6 700.8 701 701.2 701.4 701.6 701.8
Counts vs. Mass-to-Charge (m/z)

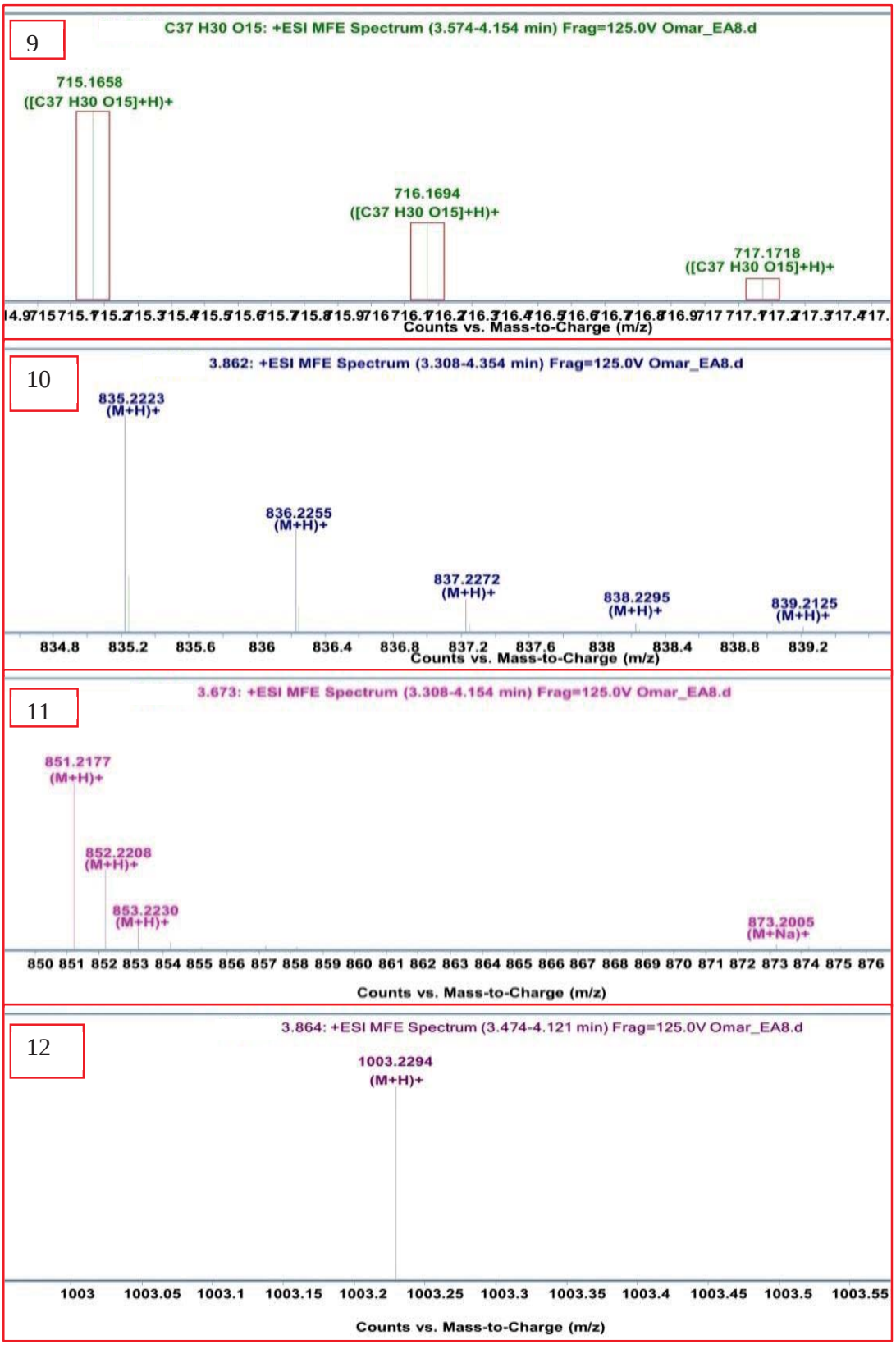


Figure A-1: The positive mode ESI-MS of CGEEA-F5-8 by qualitative analysis mass hunter work station software.

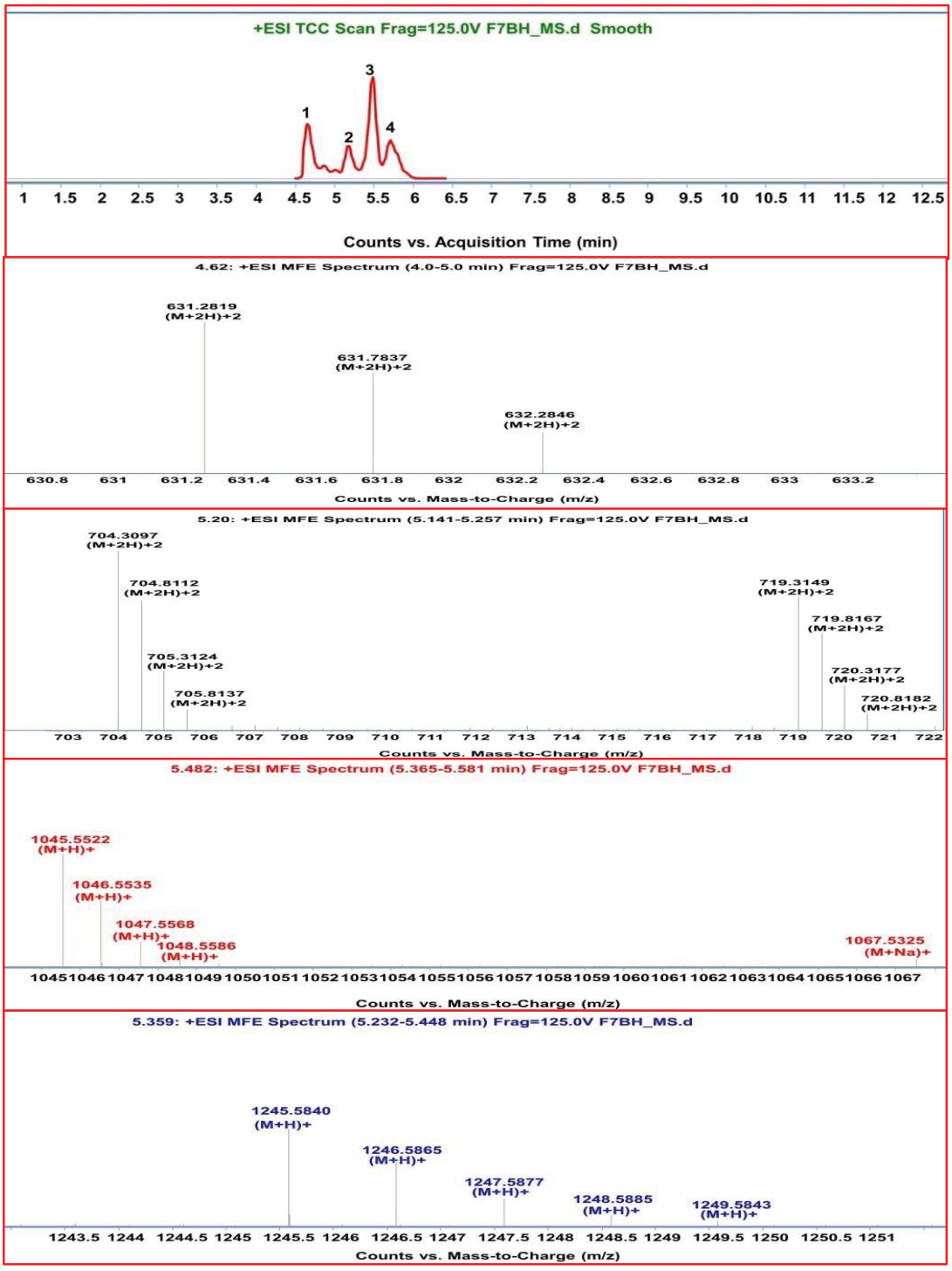


Figure A-2: the positive ESI-MS of five compounds picked as masses from CGEBU-F10-7 by qualitative analysis mass hunter work station software. Masses detected are **1**: 1260.5497; **2**: 1436.6116 and 1406.6074; **3**: 1044.5522; **4**: 1244.5487.

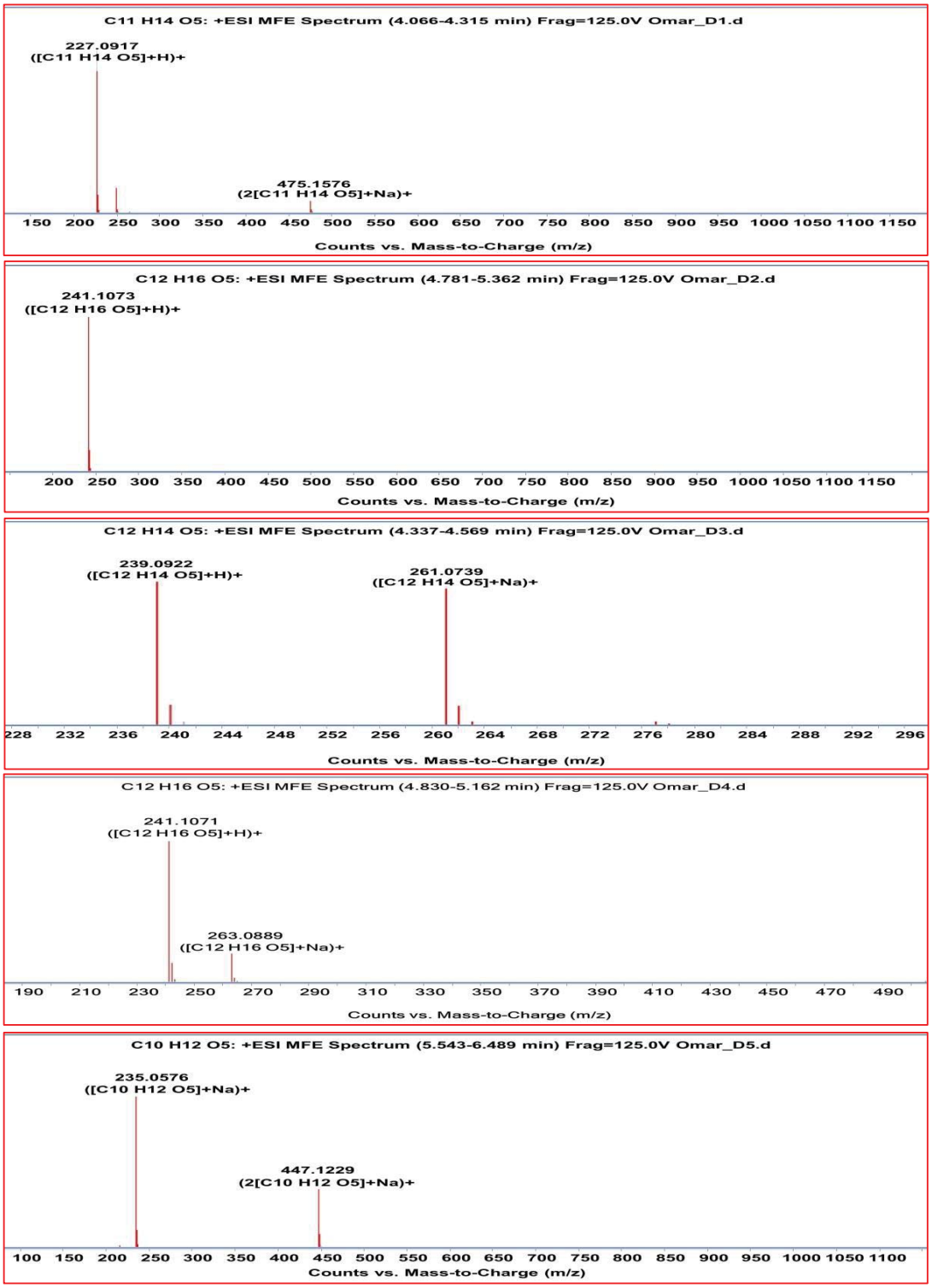


Figure A-3: Positive ion ESI-MS $[M+H]^+$ for **1**, **2**, **3**, **4**, and $[M+Na]^+$ for **5**. 2-methylpropyl 3,4,5-trihydroxybenzoate, **1**; 2- methylbutyl 3,4,5-trihydroxybenzoate, **2**; 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate, **3**; 3-methylbutyl 3,4,5-trihydroxybenzoate, **4**; propan-2-yl 3,4,5-trihydroxybenzoate, **5**.

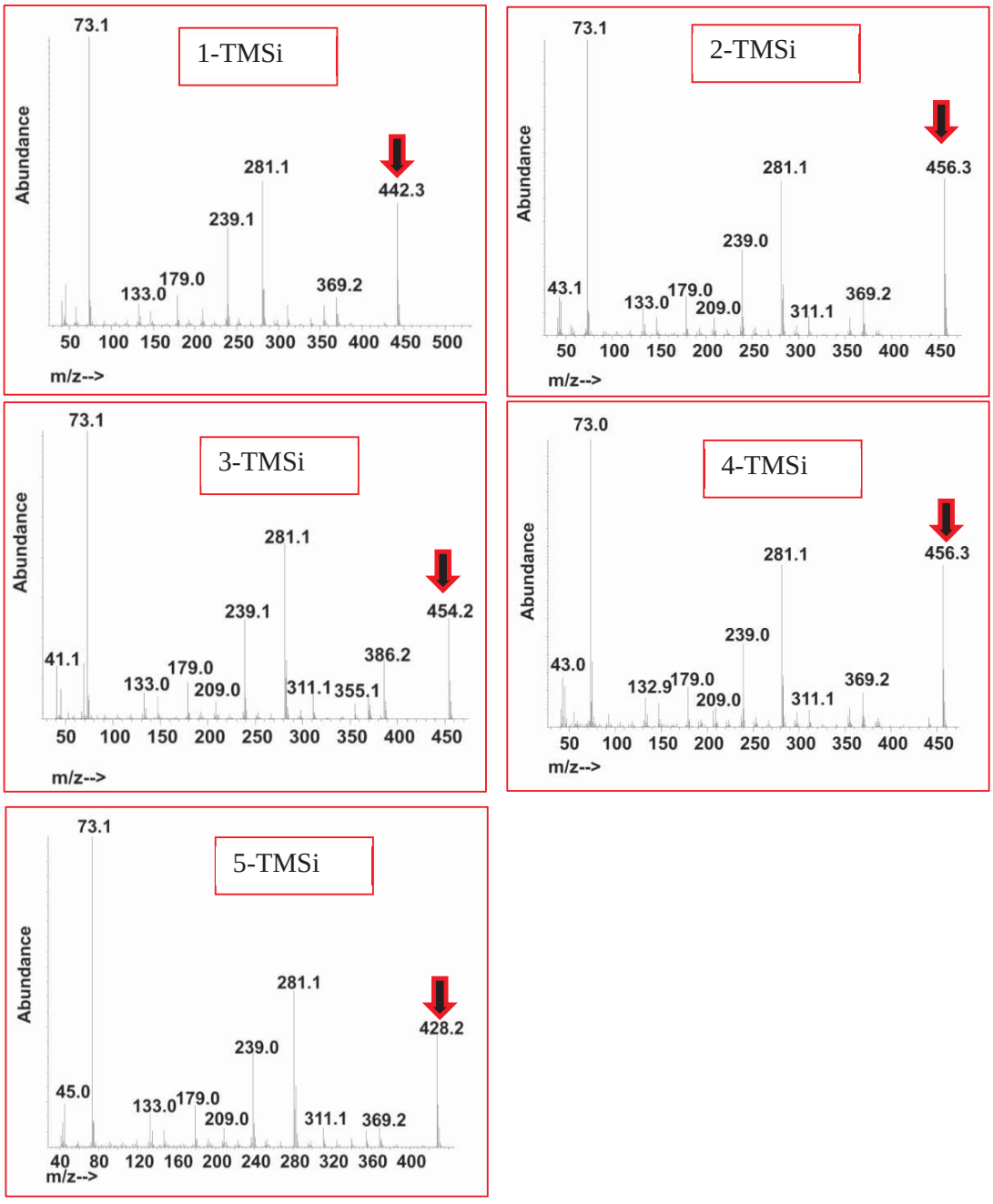


Figure A-4: EI-MS of trimethylsilyl derivatives of the synthesised esters, arrows pointing to the molecular ions, **1**-TMSi= 442.3; **2**-TMSi= 456.3; **3**-TMSi= 454.2; **4**-TMSi= 456.3 and **5**-TMSi= 428.2.

Appendix B

Table B-1: Compounds detected (after TMS derivation) in CGEAQ by GC-MS

no.	RT (min)	Compound directly detected by GC-MS	The name of the compound before derivatisation	percentages ±SD (n=3)	RI
1	10.63	Benzene, 1,3-bis(1,1-dimethylethyl)-	Benzene, 1,3-bis(1,1-dimethylethyl)-	3.23±0.20	1262
2	11.50	Butanedioic acid, bis(trimethylsilyl) ester	Butanedioic acid or succinic acid	1.9±0.10	1320
3	14.75	Trimethyl(2,6 ditert.-butylphenoxy)silane	2,6-ditert-butylphenol	11.31±0.46	1556
4	18.25	Tetradecanoic acid, trimethylsilyl ester	Tetradecanoic acid or myristic acid	3.8±0.01	1852
5	19.60	Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester	3,4,5-trihydroxy benzoic acid or gallic acid	2.84±0.07	1978
6	20.33	Hexadecanoic acid, trimethylsilyl ester	Hexadecanoic acid or palmitic acid	48.15±1.70	-
7	21.10	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	Myoinositol	1.62±0.39	-
8	21.96	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	(Z,Z)-9-12-octadecadienoic acid	7.40±0.90	-
9	21.99	Oleic acid, trimethylsilyl ester	Oleic acid or cis-9-octadecenoic acid	10.88±0.09	-
10	23.95	Eicosanoic acid, trimethylsilyl ester	Eicosanoic acid or arachidic acid	5.64±1.130	-

RT = Retention time in min, SD= Standard deviation, n= Number of experiment replication, RI= Retention index

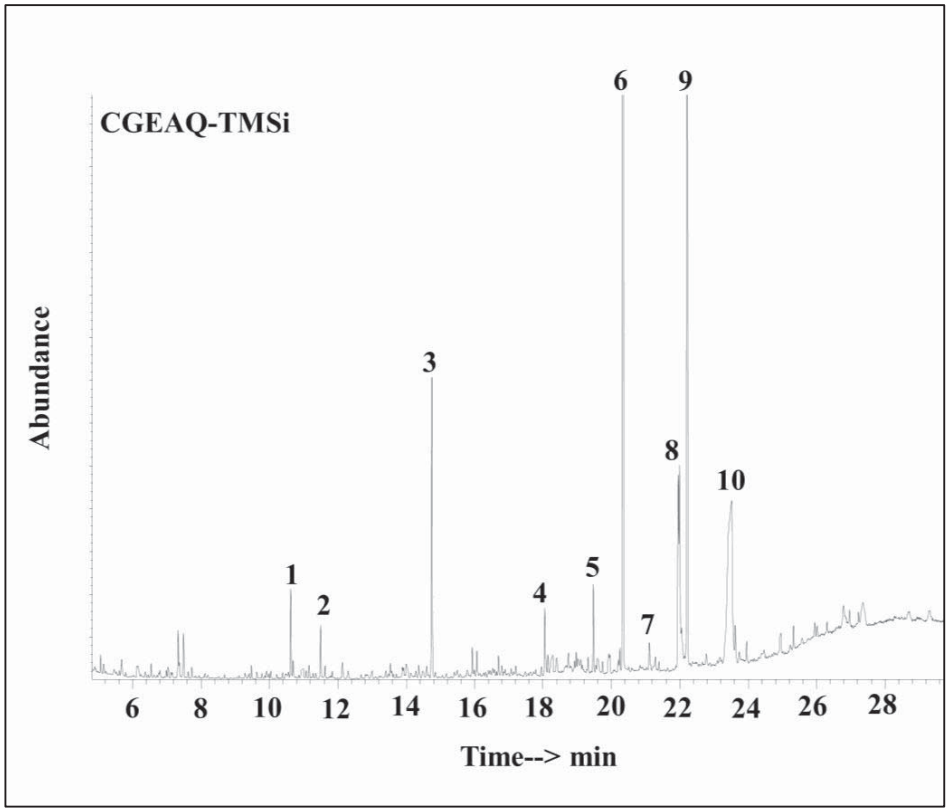


Figure B-1: GC-MS chromatogram of *Cylicodiscus gabunensis* harms ethanol aqueous fraction (CGEAQ)

Table B-2: Compounds detected in CGH by GC-MS

no.	RT (min)	Compound name	Percentage ±SD (n=3)
1	10.62	Benzene, 1,3-bis(1,1-dimethylethyl)-	11.66± 1.31
2	14.28	Phenol, 2,4-bis(1,1-dimethylethyl)-	25.91± 1.74
3	28.11	Lup-20(29)-en-3-one†	36.00± 1.39
4	29.88	Stigmast-4-en-3-one	26.42± 3.38

RT = Retention time, SD= Standard deviation, n= Number of experiment replication

†Previous research showed that, the methanolic extract of the *Cylicodiscus gabunensis* stem bark and after fractionation by chloroform yielded, lupeol, the reduced form of Lup-20(29)-en-3-one (Tane, Bergqust et al. 1995). The EI-Mass chromatogram (Figure B-3) of Lup-20(29)-en-3-one deprived from the peak of trimethyl silyl (Si $(CH_3)_3^{\cdot}$), which has molecular weight equal to (73.1); the detection of the Lup-20(29)-en-3-one molecular peak of 424.4 was clear.

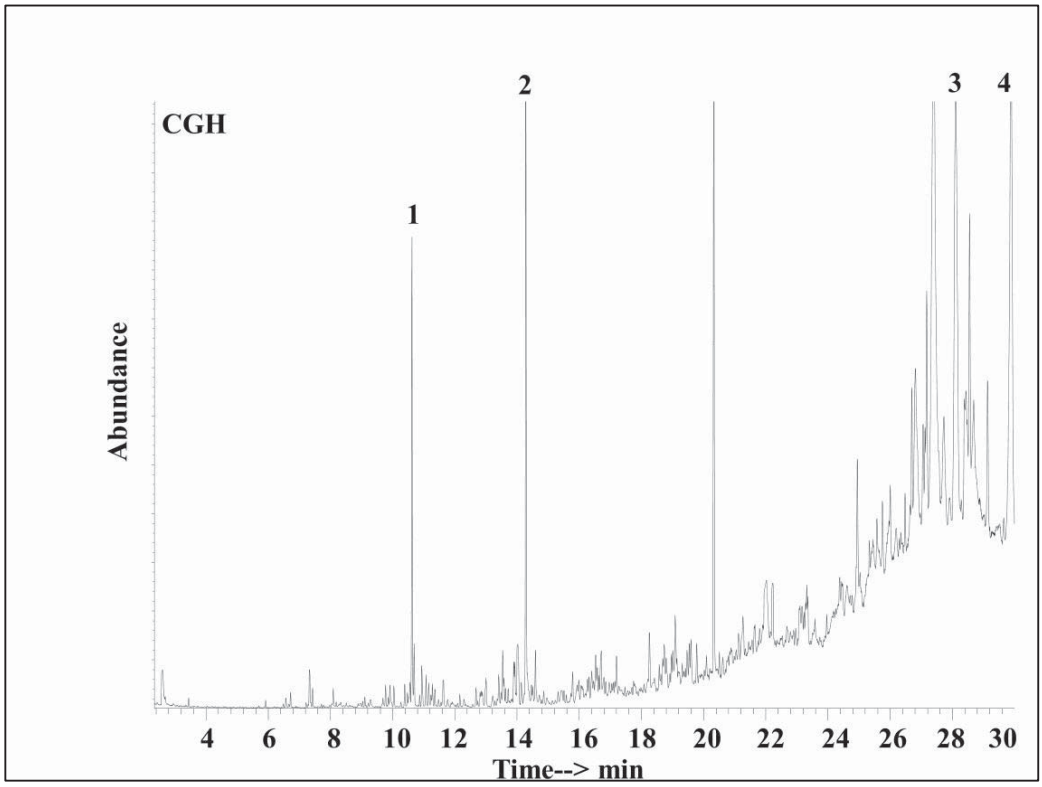


Figure B-2: GC-MS chromatogram of *Cylicodiscus gabunensis* harms hexane extract, CGH.

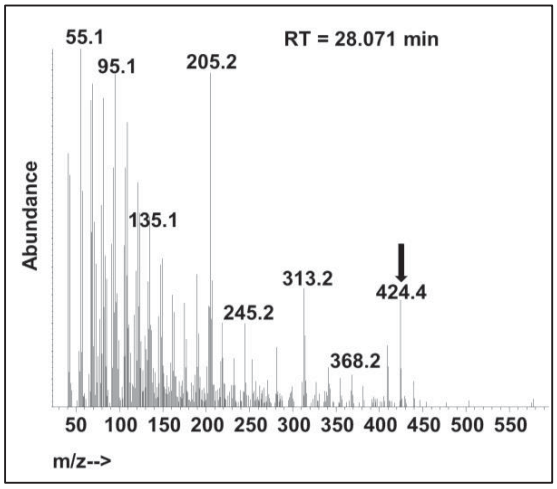


Figure B-3: EI- Mass spectrum of Lup-20(29)-en-3-one

Appendix C

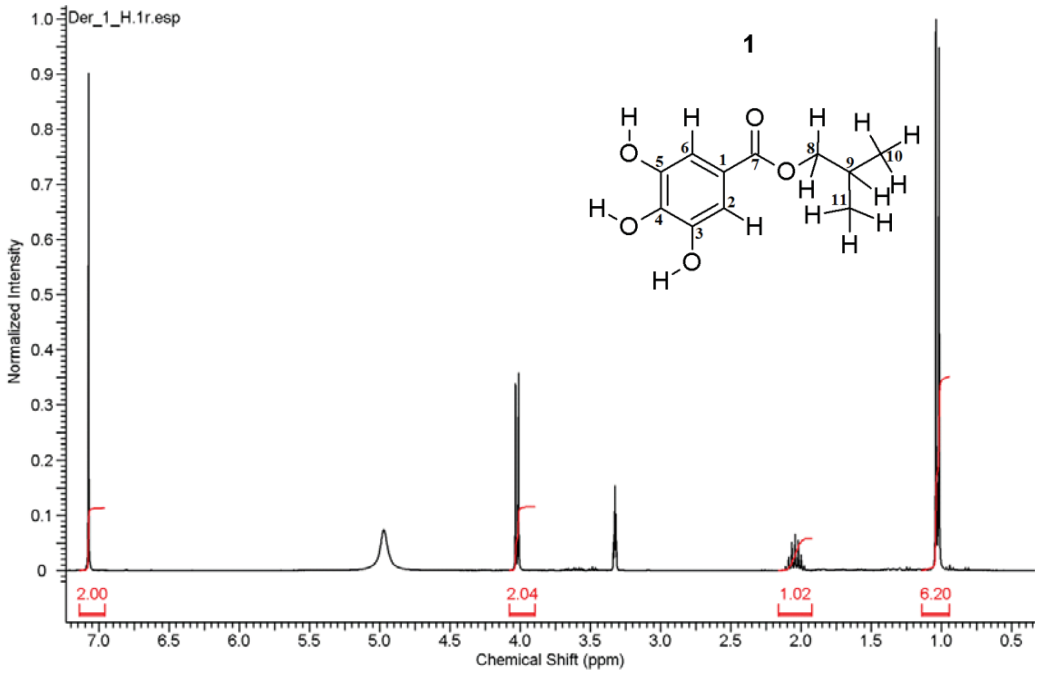


Figure C-1: ^{1}H NMR (300MHz, METHANOL-d_4) δ = 7.08 (2H, s, H-2, 6), 4.02 (2H, d, *J* = 6.6 Hz, H-8, 8), 2.04 (1H, m, H-9), 1.03 (6H, d, *J* = 6.6 Hz, H-10, 11).

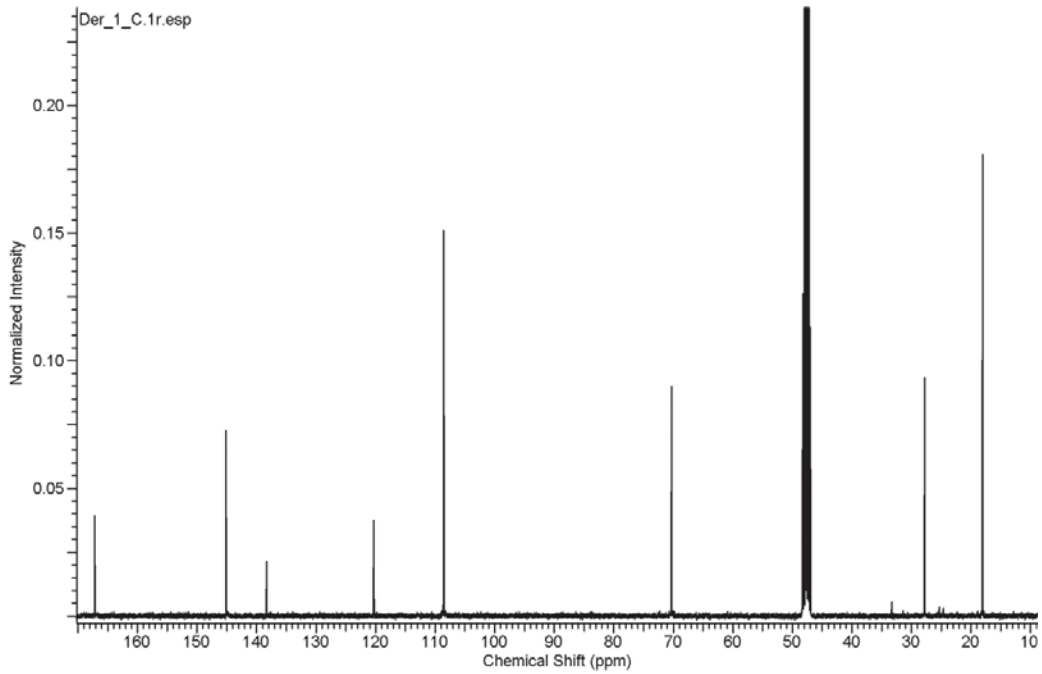


Figure C-2: ^{13}C NMR of 2-methylpropyl 3, 4, 5-trihydroxybenzoate; ^{13}C NMR (101MHz, METHANOL-d4) δ = 167.2 (C-7), 145.1 (C-3, 5), 138.3 (C-4),120.3 (C-1), 108.6 (C-2, 6), 70.3 (C-8), 27.8 (C-9), 18.1 (C-10, 11).

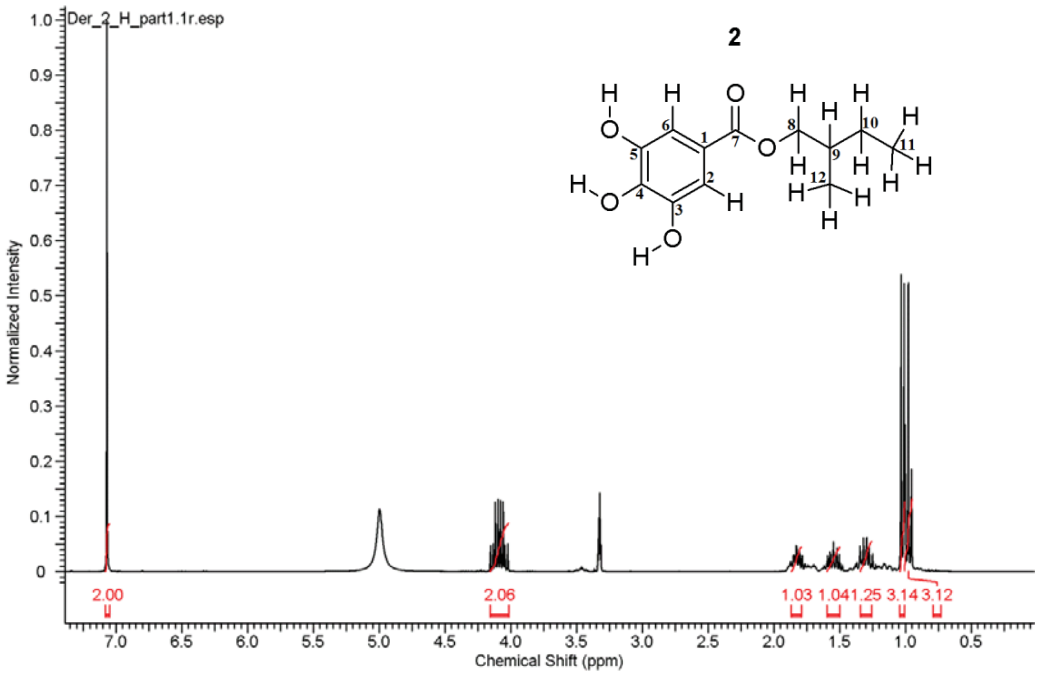


Figure C-3: [1]H NMR (300MHz, METHANOL-d4) δ = 7.07 (2H, s, H-2, 6), 4.02 - 4.16 (2H, m, H-8, 8), 1.79 - 1.87 (1H, m, H-9), 1.45-1.55 (1H, m, H-10), 1.26 - 1.34 (1H, m, H-10), 1.02 (3H, d, *J* = 6.8 Hz, H-12), 0.95 (3H, t, *J*=7.45 Hz, H-11).

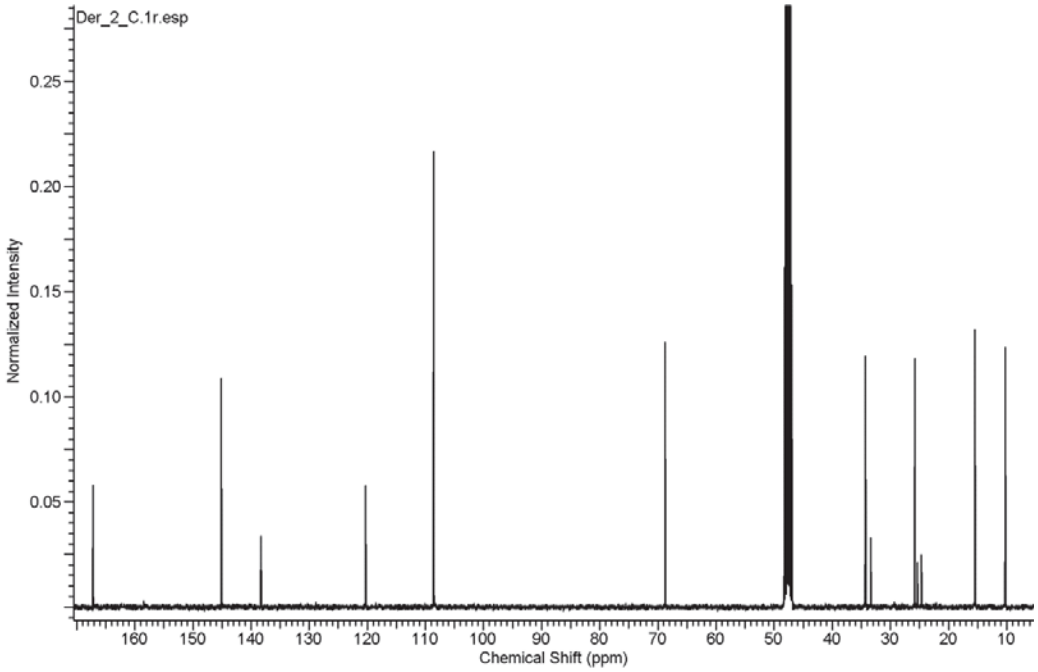


Figure C-4: [13]C NMR of 2- methylbutyl 3, 4, 5-trihydroxybenzoate;[13]C NMR (101MHz, METHANOL-d4) δ = 167.2 (C-7), 145.1 (C-3, 5), 138.3 (C-4),120.3 (C-1), 108.6 (C-2, 6), 68.8 (C-8), 34.3 (C-9), 25.8 (C-10), 15.5 (C-12), 10.3 (C-11).

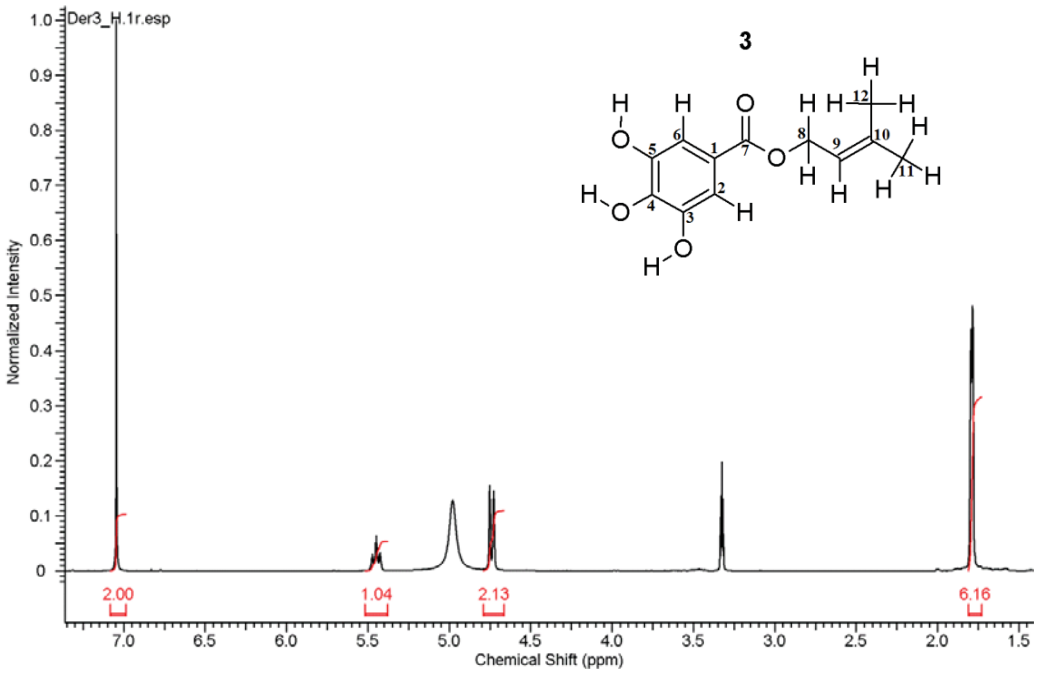


Figure C-5: ^{1}H NMR (300MHz, METHANOL-d_4) δ = 7.05 (2H, s, H-2, 6), 5.45 (1H, m, H-9), 4.74 (2H, d, *J* = 7.2 Hz, H-8, 8), 1.79 (3H, s, H-11), 1.82 (3H, s, H-12).

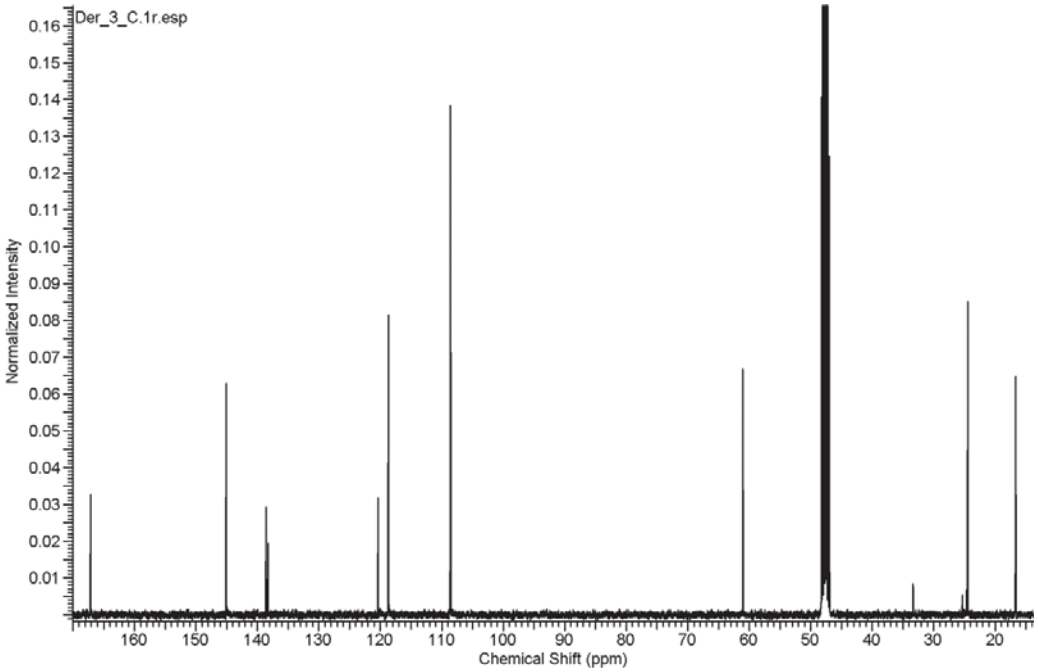


Figure C-6: ^{13}C NMR of 3-methylbut-2-en-1-yl 3,4,5-trihydroxybenzoate; ^{13}C NMR (101MHz, METHANOL-d_4) δ = 167.1 (C-7), 145.1 (C-3, 5), 138.6 (C-10), 138.3 (C-4), 120.4 (C-1), 118.7 (C-9), 108.6 (C-2, 6), 61.0 (C-8), 24.5 (C-11), 16.7 (C-12).

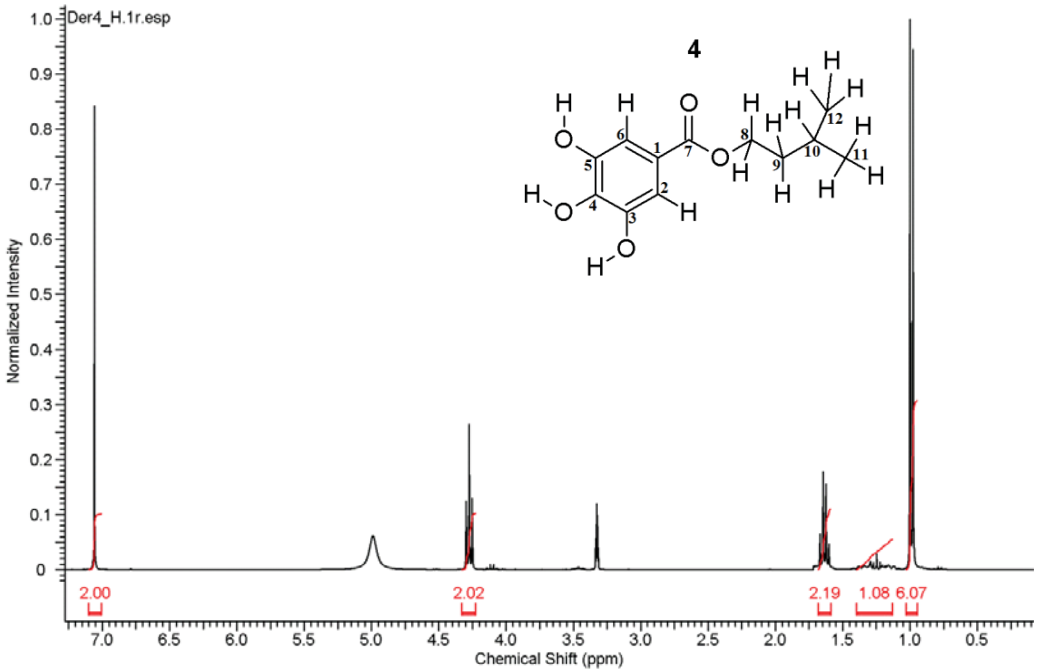


Figure C-7: ^{1}H NMR of 3-methylbutyl 3,4,5-trihydroxybenzoate; ^{1}H NMR (300MHz, METHANOL-d4) δ = 7.06 (2H, s, H-2, 6), 4.27 (2H, t, *J* = 6.6 Hz, H-8), 1.64 (2H, q, *J* = 6.7 Hz, H-9), 1.25 (1H, m, H-10), 0.99 (6H, d, *J* = 6.6 Hz, H-11, 12).

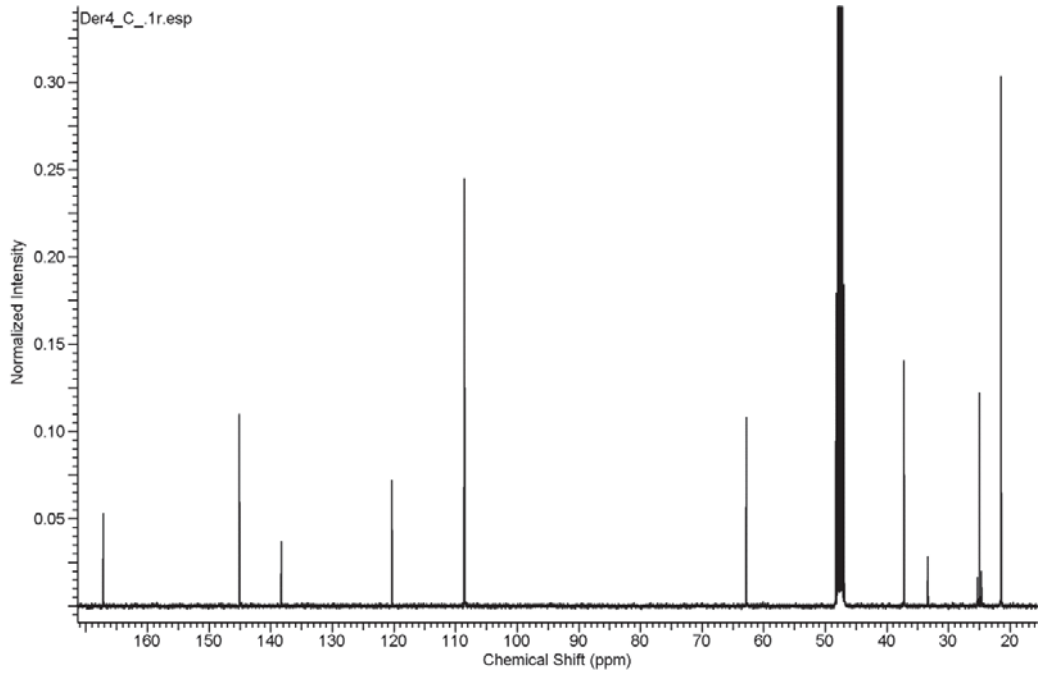


Figure C-8: ^{13}C NMR of 3-methylbutyl 3,4,5-trihydroxybenzoate; ^{13}C NMR (101MHz, METHANOL-d4) δ = 167.2 (C-7), 145.1 (C-3, 5), 138.3 (C-4), 120.3 (C-1), 108.6 (C-2, 6), 62.8 (C-8), 37.3 (C-9), 25.0 (C-10), 21.5 (C-11, 12).

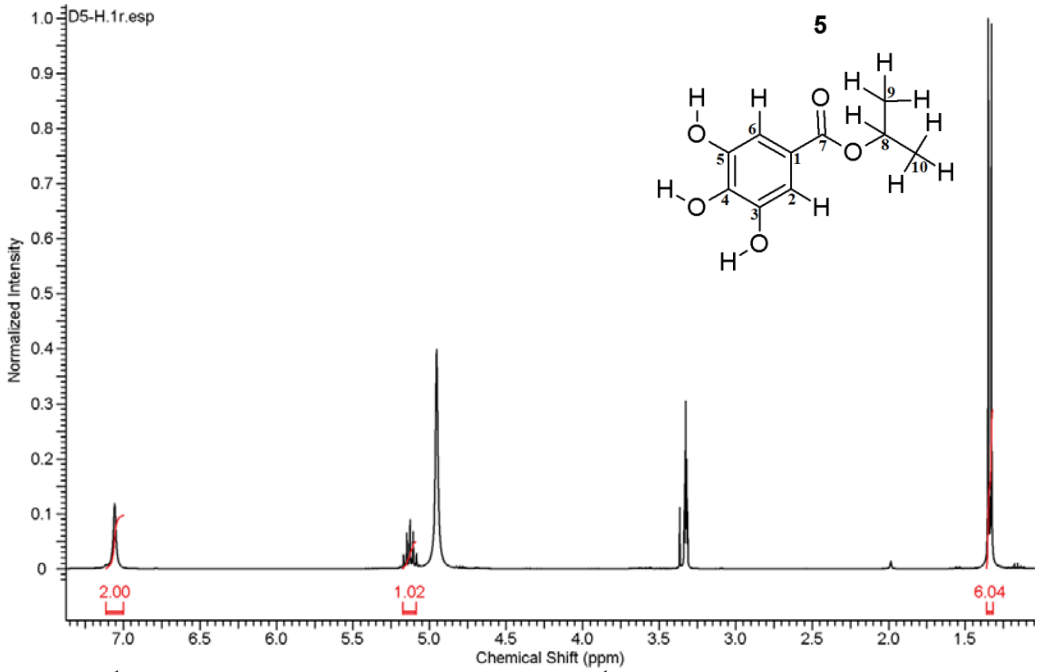


Figure C-9: [1]C NMR propan-2-yl 3,4,5-trihydroxybenzoate; [1]H NMR (300MHz, METHANOL-d4) δ = 7.06 (2H, s, H-2, 6), 5.09 - 5.17 (1H, m, H-8), 1.34 (6H, d, *J* = 6.4 Hz, H-10, 11).

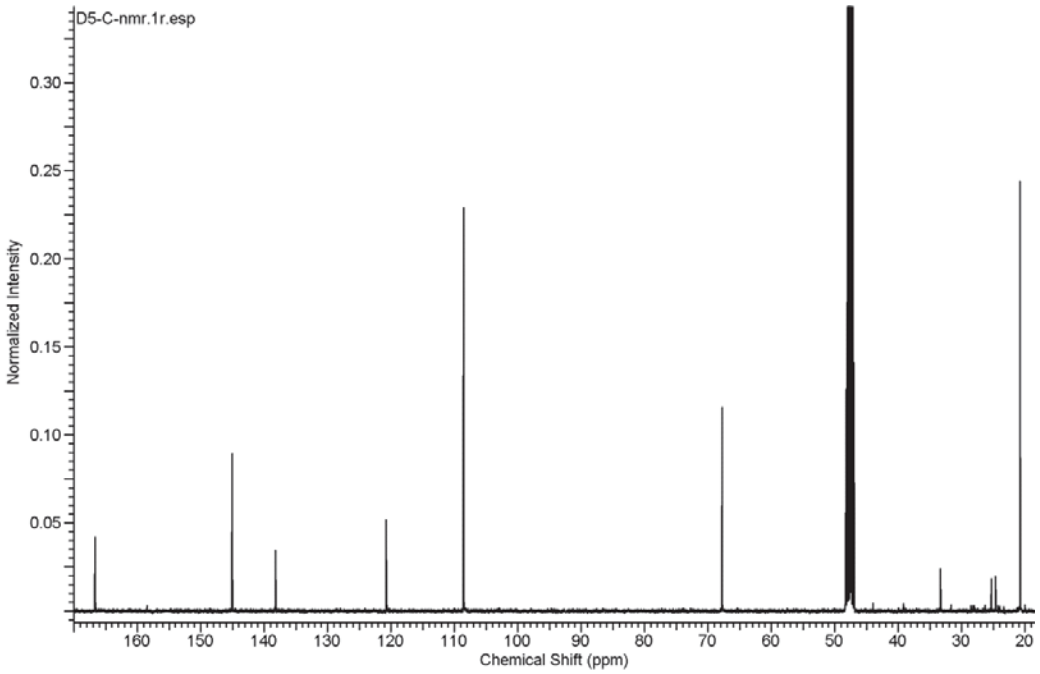


Figure C-10: [13]C NMR propan-2-yl 3,4,5-trihydroxybenzoate; [13]C NMR (101MHz, METHANOL-d4) δ = 166.7 (C-7), 145.0 (C-3, 5), 138.2 (C-4), 120.8 (C-1), 108.6 (C-2,6), 67.8 (C-8), 20.8 (C-9,10).

Appendix D

Table D-1: Compounds detected (after TMS derivation) in PCE by GC-MS

no.	RT (min)	Compound directly detected by GC-MS	The name of the compound before derivatisation	percentages ±SD (n=3)
1	8.19	L-alanine- trimethylsilyl ester	L-alanine	0.08±0.01
2	10.62	1,3-bis(1,1-dimethylethyl)- Benzene	1,3-bis(1,1-dimethylethyl)- Benzene	0.16±0.005
3	11.51	Butanedioic acid, bis(trimethylsilyl) ester	Butanedioic acid	0.59±0.029
4	11.84	2,3-Dihydroxypropanoic acid tris(trimethylsilyl)	2,3-Dihydroxy-propanoic acid	0.57±0.03
5	11.97	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	E-2-butenedioic acid	0.14±0.004
6	14.05	Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	Hydroxy-butanedioic acid	5.95±0.32
7	14.50	(2S)-1-(Trimethylsilyl)-5-oxopyrrolidine-2-carboxylic acid	5-oxopyrrolidine-2-carboxylic acid	0.21±0.08
8	14.60	Butanoic acid, 4-[bis(trimethylsilyl)amino]-, trimethylsilyl ester	4-amino-butanoic acid	0.16±0.06
9	14.80	Trimethylsilyl 2,3,4-tris[(trimethylsilyl)oxy]butanoate	2,3,4-Trihydroxybutyric acid	0.42±0.02
10	15.06	L-Threonic acid, tris(trimethylsilyl) ether, trimethylsilyl ester	L-threonic acid	0.13±0.006
11	15.50	Pentanedioic acid, 3-methyl-3-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	3-hydroxy-3-methyl pentanedioic acid	0.21±0.01
12	15.90	6-Deoxy-1,2,3,4-tetrakis-O-(trimethylsilyl)-α-L-mannopyranose	L- (6-Deoxy mannopyranose)	0.16±0.002
13	16.17	D-arabinopyranose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-	D-arabionpyranose	0.16±0.006
14	17.10	Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	Xylitol	5.96±0.22
15	18.10	1,2,3,4,6-Pentakis-O-(trimethylsilyl)-α-D-psicofuranose	D-psicofuranose	6.4±0.2
16	18.16	D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether	D-fructofuranose	5.50±0.32
17	18.29	2-(Trimethylsilyl)ethyl beta-D-glucopyranoside	Ethyl-D-glucopyranoside	14.96±0.07
18	18.68	D-(+)-Talose, pentakis(trimethylsilyl) ether	D-Talose	4.3±0.868
19	18.93	d-Gluconic acid, 2,3,4,6-tetrakis-O-(trimethylsilyl)-, δ-lactone	D-(+)-Gluconic acid δ-lactone	0.89±0.2
20	19.05	D-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	D-glucopyranose	14.14±0.3
21	19.17	β-D-(+)-Talopyranose, pentakis(trimethylsilyl) ether	β-D-(+)-D-talopyranose	7.52±0.27
22	19.93	β-D-Allopyranose, pentakis(trimethylsilyl) ether	β-D-Allopyranose	21.33±0.63

no.	RT (min)	Compound directly detected by GC-MS	The name of the compound before derivatisation	percentages ±SD (n=3)
23	20.26	D-Gluconic acid, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, trimethylsilyl ester	D-gluconic acid-TMSi	2.95±0.14
24	20.30	Hexadecanoic acid, trimethylsilyl ester	hexadecanoic acid	1.83±0.14
25	21.10	myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	Myo-Inositol	2.60±0.05
26	21.97	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	(Z,Z)-9,12-octadecadienoic acid	0.94±0.15
27	22.89	Glycerol, 2-O-galactopyranoside, TMS	glyceryl glycoside	0.17±0.07
28	26.90	Trimethylsilyl-D-(+)-trehalose	D(+)-trehalose	0.96±0.28
29	27.90	Lactose, octakis(trimethylsilyl)-	Lactose	2.25±0.049

RT = Retention time in min, SD= Standard deviation, n= Number of experiment replication, RI= Retention index

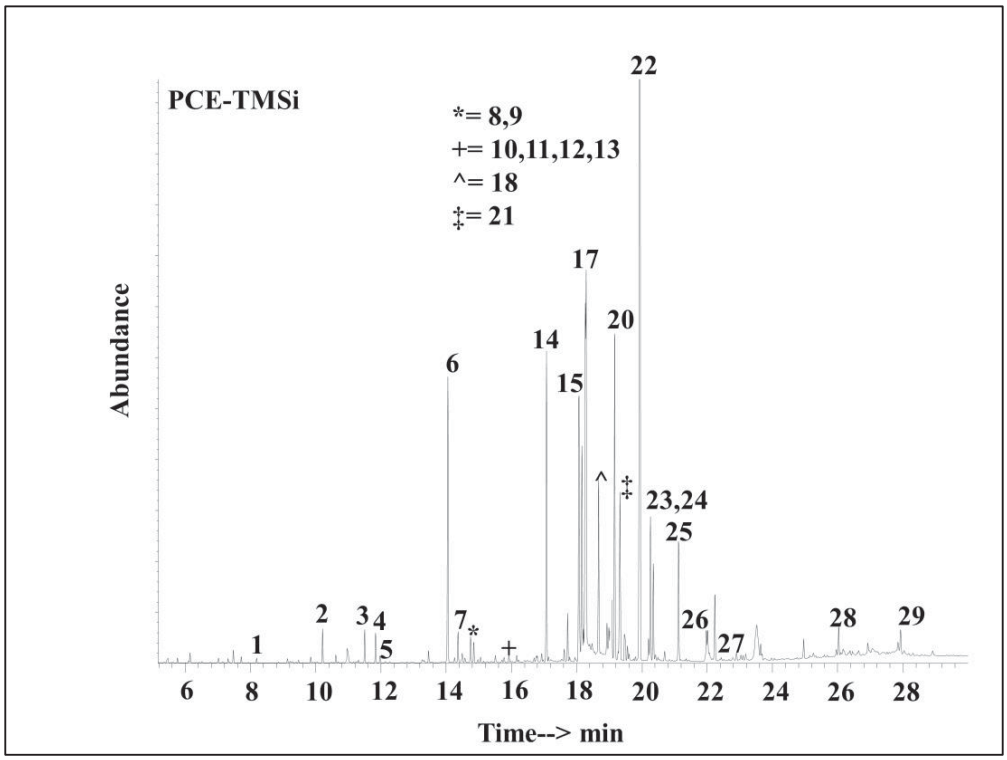


Figure D-1: GC-MS chromatogram of compounds of PCE

Appendix E

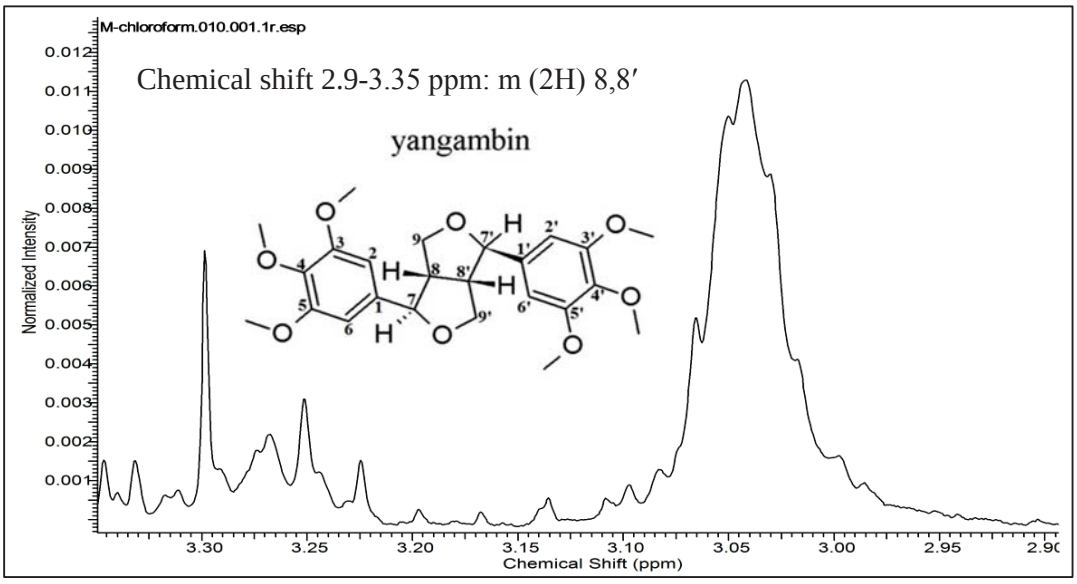


Figure 1-E: The proton NMR of lignans mixture from *Magnolia Biondii*, the area shown is related to the two protons of the Tetrahydrofuran Bridge. Yangambin structure is included here as an example of lignans, the numbering system is clear on the structure for correlation purposes.

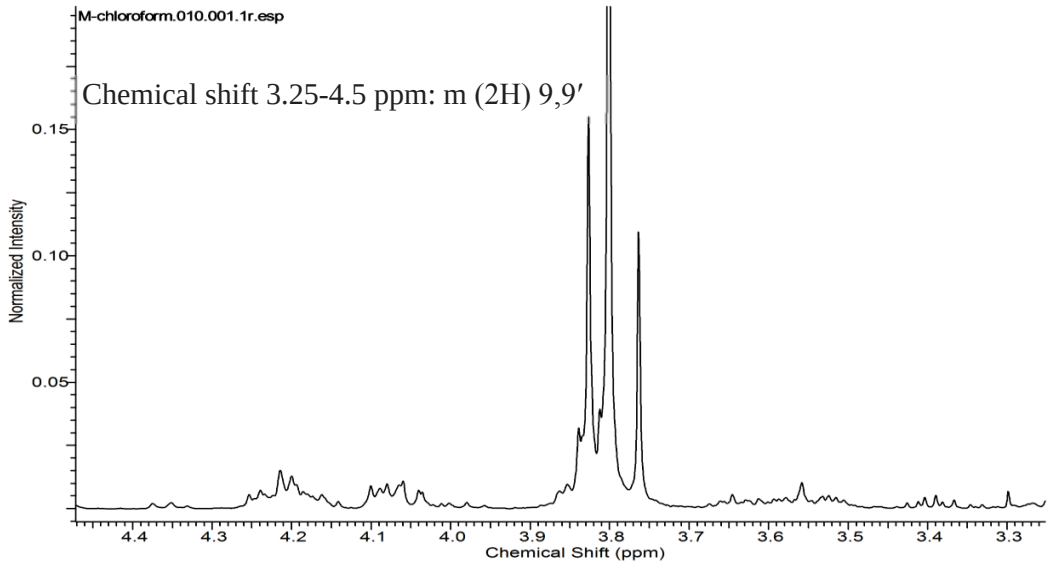


Figure 2-E: The proton NMR of lignans mixture from *Magnolia Biondii*, the area shown is related to the two aliphatic protons 9 and 9′.

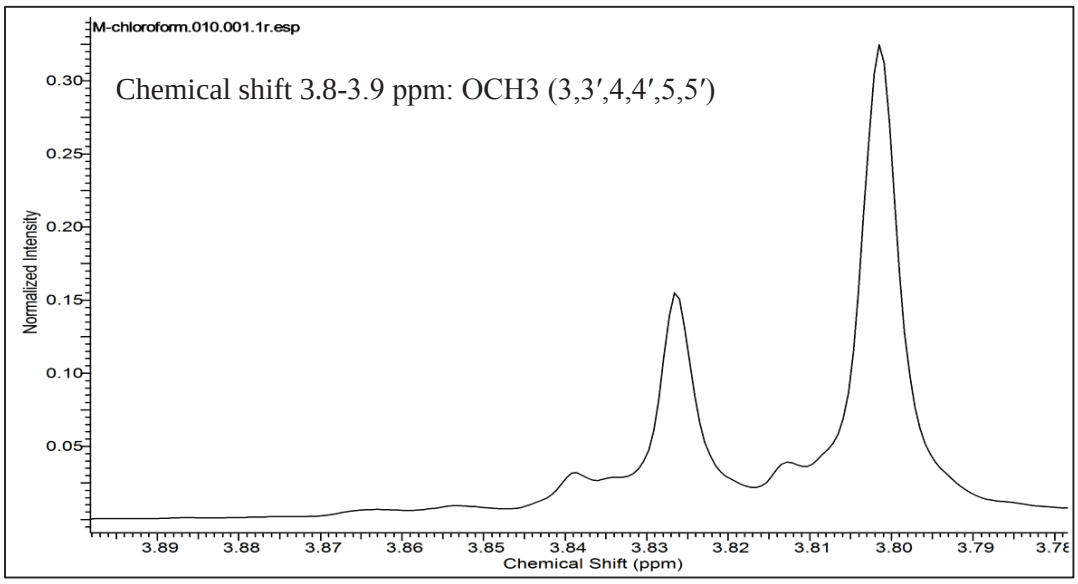


Figure 3-E: The proton NMR of lignans mixture from *Magnolia Biondii*, the area shown is related to the methoxy groups protons at carbons 3,3′,4,4′,5,5′.

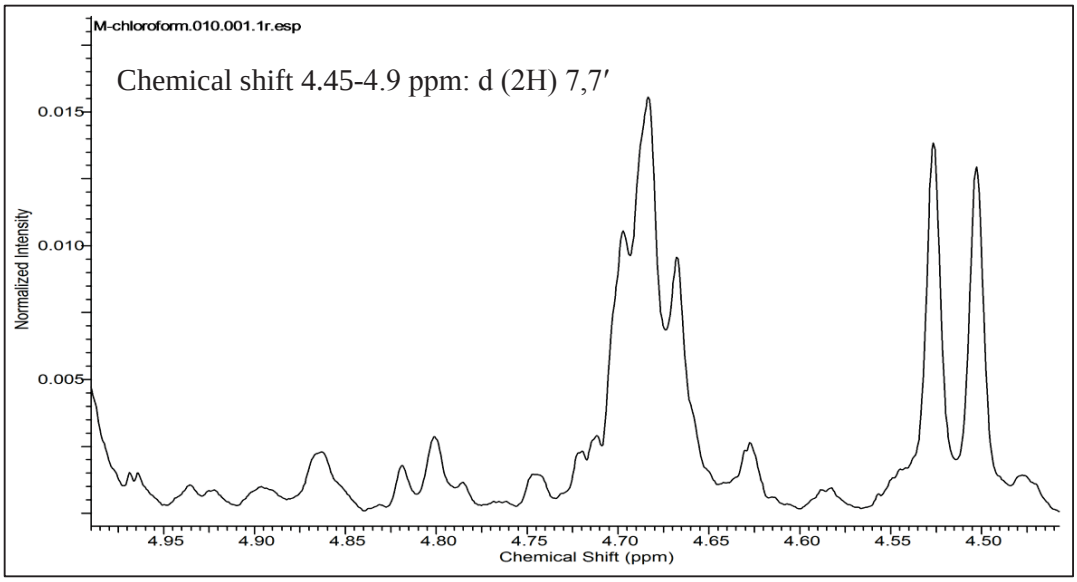


Figure 4-E: The proton NMR of lignans mixture from *Magnolia Biondii*, the area shown is related to the two aliphatic protons 7 and 7′.

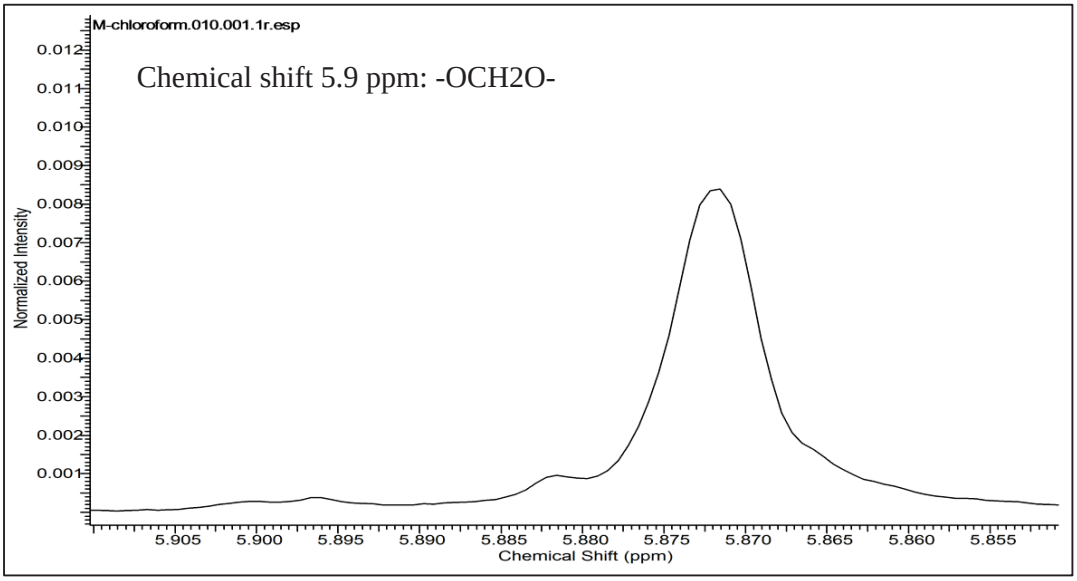


Figure 5-E: The proton NMR of lignans mixture from *Magnolia Biondii*, the area shown is related to the two protons of -OCH2O-.

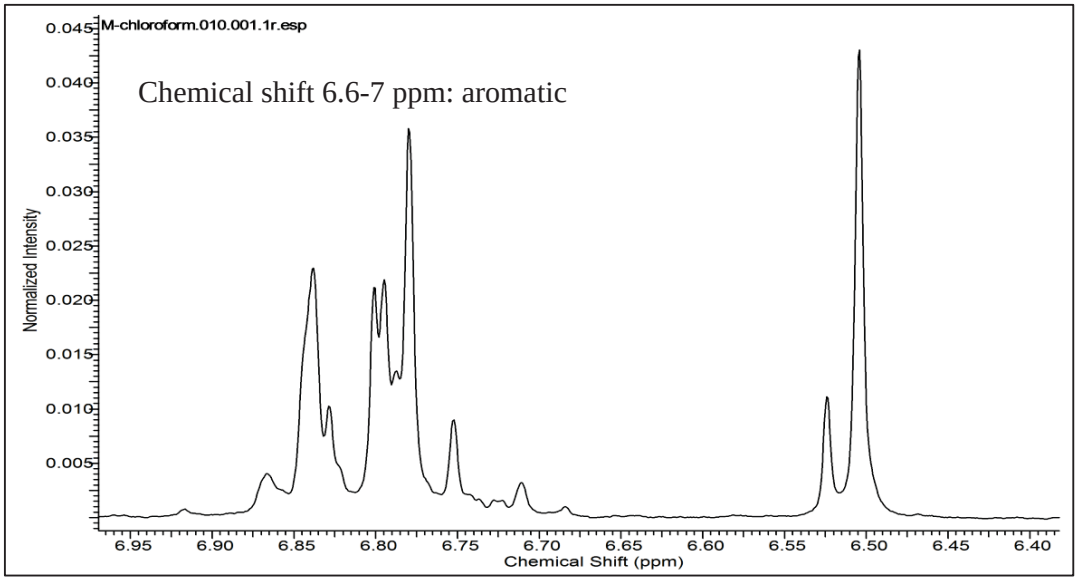


Figure 6-E: The proton NMR of lignans mixture from *Magnolia Biondii*, the area shown is related to aromatic protons.