ANTIPLASMID AND ANTIMICROBIAL ACTIVITIES OF SYNTHETIC AND NATURAL PRODUCTS FROM SELECTED MEDICINAL PLANTS

BLESSING MBAEBIE OYEDEMI

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THIS THESIS IS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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DR. PAUL STAPLETON

This thesis describes research conducted in the School of Pharmacy, University College London between 2010 and 2014 under the supervision of Prof Simon Gibbons and Dr Paul Stapleton. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication. This work has not been submitted at any other University in partial or entirety for the award of any degree.

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Signature Date

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

LIST OF ABBREVIATIONS

1D	One dimensional
2D	Two dimensional
δ	NMR chemical shift (ppm)
$CHCl_3$	Chloroform
COSY	Correlated Spectroscopy
d	Doublet
DEPT	Distortionless-Enhancement-by Polarisation-Transfer
DMSO	Dimethylsulphoxide
EI	Electron Ionisation
EtOAc	Ethyl acetate
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
ESI	Electrospray ionisation
Hz	Hertz
IR	Infrared
J	Spin-spin coupling constant (Hz)
m	Multiplet (NMR)
MeOH	Methanol
MDR	Multidrug resistant
MHB	Mueller-Hinton Broth
MHz	Mega Hertz
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MTT	3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
TLC	Preparative thin layer chromatography
SIC	Sub inhibitory concentration
MIC	Minimum inhibitory concentration
s	Singlet
sp.	Species
SPE	Solid phase extraction
HPLC	High performance liquid chromatography
UV	Ultraviolet
VLC	Vacuum liquid chromatography
VRE	Vancomycin-Resistant Enterococci

T4SS	Type-1V-secretion-system
RF	Retention factor
R-plasmid	Resistant plasmid
CA	*Capsicum annuum*
CS	*Cannabis sativa*
EV	*Evodia rutaecarpa*
Mat	*Mallotus phillipinensis*
Dtr	DNA transfer replication system
EDTA	Ethylenediaminetetraacetic acid
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
BHR	Broad host range
IncQ	Incompactibility group Q
IncP	Incompactibility group P
IncW	Incompactibility group W
IncX	Incompactibility group X
IncF11	Incompactibility group F
Inc12	Incompactibility group I
PBRT	PCR-based replicon typing
PCR	Polymerase chain reaction
EMSA	Electrophoretic gel mobility shift assay
LB	Luria Bertani broth
QBT	Equilibration buffer
QC	Wash buffer
QF	Elution buffer

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DEDICATION

Now unto the King eternal, Immortal, Invisible, the only wise God

Be glory, honour and power forever.

To my late dad for believing in me, to my hubby and sons, Josh & Dave

ABSTRACT

This PhD thesis is part of ongoing project to identify plant natural products and selected synthetic compounds that possess antimicrobial properties; and are able to promote plasmid loss or interfere with bacterial conjugation.

The conjugative broad host plasmids investigated include PKM101 (Inc N), TP114 (Inc I2), PUB307 (Inc P), and low- copy number plasmids: R6K (Inc X), R7K (Inc W) and R1-drd-19 (Inc F11). They represented the incompatibility plasmid groups that are currently associated with gross dissemination of antibiotic resistance in bacteria. A series of plant extracts evaluated at sub-inhibitory concentration of 100 mg/L, were shown to inhibit bacterial plasmid conjugation and their active constituents were isolated and characterised. *Mallotus philippinensis* yielded rottlerin and red compound, with good to moderate antibacterial activity against multidrug resistant *Staphylococcus aureus* strains, and had a broad range inhibition against the resistant plasmids. Investigation of extracts from the resin of *Cannabis sativa* L. identified tetrahydrocannabinolic acid (THCA) and cannabinolic acid (CBNA) which in addition to two synthetic cannabinoids: cannabigerol and olivetol inhibited the conjugal transfer of TP114 between *E. coli* strains. The antiplasmid activities of Δ9-THC, CBN, CBD, significantly reduced the transfer of amoxicillin–resistance conferring PKM 101. Methanolic extract from the dried fruits of *Evodia rutaecarpa* yielded evodiamine, rutaecarpine and naturally-isolated sucrose. Rutaecarpine was the most active alkaloid against NorA-expressing SA1199B and XU212 strain expressing TetK efflux mechanism. Evodiamine and sucrose had lesser antibacterial effect as well as low level of inhibition against the plasmids. Rutaecarpine and evocarpine remarkably reduced the transfer frequency of PKM 101, showing a high

level effect of inhibition by the compound. The bioassay-guided analysis of *Capsicum annuum* L. yielded capsaicin and dihydrocapsaicin (DHC) which demonstrated moderate antibacterial activities but inhibited conjugal transfer of R-plasmids actively. Capsaicin exhibited a broad range antiplasmid activity while DHC showed selective inhibition. The effect of synthetic compounds that were assessed: ferulenol, 6-gingerol and 6-shogoal were twice as effective against the transfer of PKM 101, TP114 and PUB307 compared to capsaicin, while nonivamide had no remarkable activity.

With the exception of promethazine, capsaicin and dihydrocapsaicin that showed some interaction with DNA due to decreased fluorescence which suggests binding, the rest of the compounds: rottlerin, red compound, ferulenol, evocarpine, rutaecarpine, 6-gingerol, 6-shogaol and nonivamide did not bind to DNA. This may indicate other probable mechanism of antiplasmid action of the compounds.

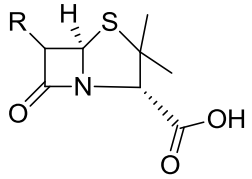
Together, some of these compounds were notable for their dual properties: robust antistaphylococcal activity and a broad host range antiplasmid effect, and are reported for the very first time. Such potentials are valuable in the discovery of next generation antimicrobial drugs.

CHAPTER ONE

INTRODUCTION

1.0 Antibiotic era

The discovery and development of antibiotics took a leap over 60 years ago, with credit to Alexander Fleming in 1928 for the discovery of the β-lactam penicillin, a natural product which came into clinical use in 1944 (Saga and Yamaguchi 2009). The initial use of penicillin (Benzylpenicillinic acid, **1**) led to the successful treatment and control of many infectious diseases; lethal wound infections, pneumonia, syphilis and with a positive impact on surgery and clinical practices that could not have been possible under the constant threat of infection (Bentley 2009). There was a significant decrease in morbidity and mortality rates with an overall increase in life expectancy.



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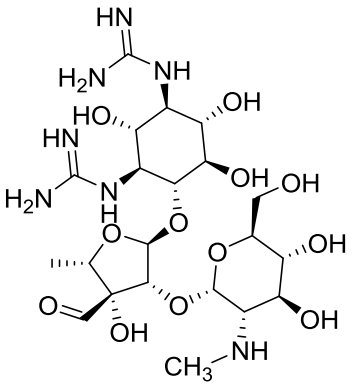

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Later on, streptomycin (**2**), an aminoglycoside antibiotic was discovered, and since then, various classes of antibiotics both from synthetic and natural sources, have been developed, which are now widely used for the treatment of microbial diseases (Saga and Yamaguchi 2009). Soon after antibiotic introduction, many bacteria became

resistant to the antibiotic treatment owing to either mutation or their acquisition of genes that conferred resistance to the antimicrobials (Sayyah *et al.,* 2004; Ye *et al.,* 2011). Up until recent times, the problem of bacterial resistance to antibiotics has reached huge proportions, followed by the appearance of resistant organisms up to a point at which the value of the antibiotics as a therapeutic agent have often been severely undermined.

1.1 Overview of current rise in bacterial resistance to antibiotics

Soon after the introduction of penicillin into widespread use, the resistance of *Staphylococcus aureus* to penicillin was observed, and nowadays, the value is about 80%. A similar pattern of resistance has been found with methicillin-resistant *S. aureus* (MRSA) and most antibiotics (Saga and Yamaguchi 2009). Resistance to classes of β-lactams were observed as well, examples included by *S. pneumoniae* to penicillin and *Haemophilus influenzae* to ampicillin. Ever since then, resistance to antibiotics continues to be a significant and growing medical problem across the globe, accompanied with the compromised option of treating infections effectively, prolonged hospital stay, a high risk of complications and death, and overall increased costs of healthcare (Woodford and Livermore 2009).

Current statistics of community outbreaks and hospital-acquired infections are underscored by multiresistant-MRSA and extremely- drug- resistant strains of *Mycobacterium tuberculosis* (XDR) or total-drug-resistant (TDR) strains of *Mycobacterium tuberculosis* (WHO 2013). Cases of *Neisseria gonorrhoea* resistance to ceftriaxone, as well as the rise of resistance of cefixime from 0% to 20% within the period 2005-2010 in the United Kingdom (Amabile-Cuevas 2013), have become

threatening while in the US, both cefixime or ceftriaxone at any single dose is no longer recommended as a first-line regimen treatment of gonococcal infections (Report 2012). Carbapenem-resistant Enterobacteriaceae (CRE), multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter, Clostridium difficile,* and *Klebsiella pneumoniae* continue to undermine available treatments due to either resistant or nearly resistant strains in many hospitals (Davies and Davies 2010). Cases were reported by the US Center for Disease Control that mortality rates rose from 1.2% to 4.2% over the decade from 2001 to 2011 associated with carbapenem-resistant infections, as well as *Klebsiella* compromised-treatments, which rose over the same time period from 1.6% to 10.4% (Prevention 2013) Unfortunately, carbapenems remain the only potent broad-spectrum β-lactam antibiotics traditionally reserved for the treatment of the most serious infections. The occurrences of such problematic types of resistance to carbapenems therefore require urgent attention. Other serious emerging threats include drug-resistant *Campylobacter*, vancomycin-resistant *Enterococcus* (VRE), drug-resistant *Salmonella typhi* and non-typhoidal *Salmonella*, and vancomycin-resistant *S. aureus* (VRSA) (Prevention 2013).

Given the above examples, antibiotic resistance is featured as a foremost public health concern and ranked as a big risk as an agent of bioterrorism by Dame Dr Sally Davies, the UK Chief Medical Officer. Numerous actions have been set out to slow the development and spread of antibiotic resistance, for example the UK antimicrobial resistance 5 year (2013-2018) strategy (Department of Health 2013), the US CDC document on antibiotic resistance threats, and the recent concluded two-day World Health Organisation (WHO) ministerial conference on a global call to take action on antimicrobial resistance, June 2014 (Organization 2014). These are

indicative of the magnitude of the situation, and advocate for a radical involvement of all levels of government and the community in the fight against bacterial drug resistance.

1.2 Mechanisms of antibiotic resistance

Resistance to all classes of antibiotics occurred after their use in the clinic or on the farm, by mutation or acquisition of resistance elements laterally from other microorganisms. Much evidence now supports that the source of resistance genes is the natural microbes which harbour the bacterial resistome; the collection of all resistance genes and their precursors in the both pathogenic and commensal bacterial populations (Wright 2012). The increased body of knowledge on the mechanisms of resistance to all classes of antibiotics (Table 1), has provided awareness and insights into the basis of antibiotic resistance both at the molecular and biochemical/cellular levels of the bacterial cell (Gale E F 1972; Alekshun and Levy 2007). This is a vital tool in the attempt to salvage compromised antibiotic treatments in the healthcare system. Basically, bacteria possess the ability to evolve these mechanisms that thwart antibiotic action via multiple or single elements of the following:

1. Alteration of drug target site.
2. Enzymatic detoxification of antibiotics.
3. Decreased drug accumulation.
4. Antibiotic efflux systems.

They can possess one, more or all of these mechanisms simultaneously which is evident in most cases of intrinsic- and acquired-multidrug resistance in bacterial communities (Ferber 2003 ; Abraham and Chain 1988; Martinez 2012).

Table 1: Modes of action and resistance mechanisms of commonly used antibiotics (Davies and Davies 2010; Morar and Wright 2010)

Antibiotic class	Example(s)	Target	Mode(s) of resistance mechanism
β-lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan synthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosporylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan synthesis	Reprogramming Peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monoxygenation, efflux, altered target
Macrolides	Erythromycin, azithromicin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase,(type B streptogramins, efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C_1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C_1 metabolism	Efflux, altered target
Rifamycin	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopepetides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target

1.2.1 Intrinsic resistance

Bacteria possess the inherent ability to become resistant to different classes of antibiotics, regardless of antibiotic selective pressure or the influence of horizontal gene transfer (Cox and Wright 2013a). A conventional example of intrinsic antibiotic resistance is the multi-drug resistant (MDR) phenotype exhibited by Gram-negative bacteria, which are insensitive to many classes of clinically effective antibiotics usually used in the treatment of Gram-positive bacteria. The molecular basis of this phenomenon is the presence of the Gram-negative outer membrane (OM), which is impermeable to many molecules, and the expression of numerous MDR efflux pumps that effectively reduce the intracellular concentration of the given drug (Nikaido 1994; Cox and Wright 2013b). Chromosomal mutation has triggered a number of resistant cases of organisms to antibiotics as seen in the rpoS mutation in *E. coli* to β-lactams (LeClerc *et al.,* 1996), ROS species and RecA mutations in *E. coli* and *S. aureus* to β-lactams, quinolone and aminoglycosides (Gutierrez *et al.,* 2013). However, this is a relatively rare event at a very low rate 10^{-5} to 10^{-10} per organism (Williams and Hergenrother 2008). The role of intrinsic resistance in Gram-negative bacteria is significantly becoming important to consider in the whole-targeted approach to overcoming antibiotic resistance in bacteria.

1.2.2 Horizontal Gene Transfer (HGT)

Horizontal gene transfer (HGT), also known as lateral gene transfer, refers to the movement of genetic information across normal mating barriers, between more or less distantly related organisms, thus differ from normal vertical transmission of genes from parent to offspring (Keeling and Palmer 2008). HGT, is the most frequent and responsible method for global and rapid dissemination of antibiotic resistance genes

among different bacterial species and genera, within the hospital and the community. The transfer can occur passively by transduction and transformation and actively by conjugation. Details of transfer of DNA to bacterial cells are represented in Figure 1, showing transduction (1), conjugation (2), and transposition (3) of mobile genetic cassettes.

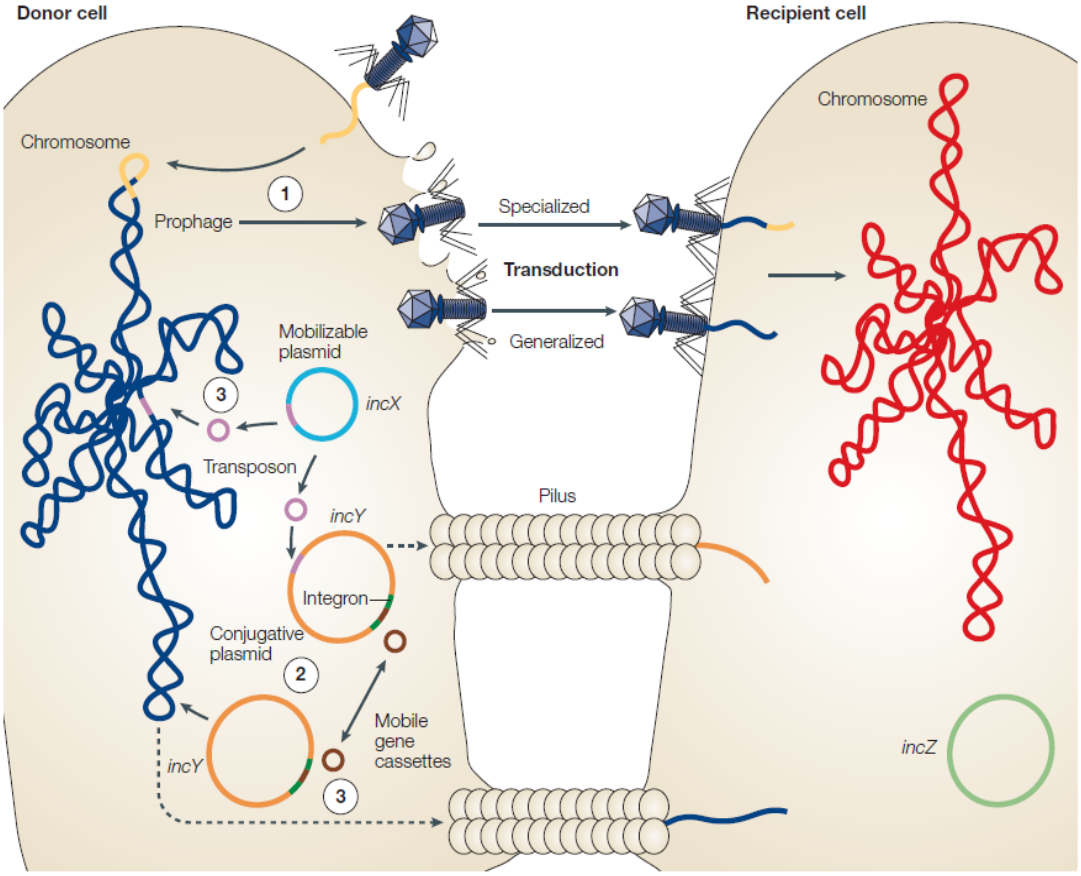


Figure 1: Transfer of DNA to bacterial cells. (Frost, Leplae *et al*., 2005)

Conjugation is generally considered to be an important mode of transfer of mobile genetic elements (plasmid, transposons and associated intergrons) compared to the other methods, and has heightened awareness of the great importance of horizontal gene transfer in genome evolution (Davies and Davies 2010).

1.3 Bacterial conjugation

Conjugation, involves the transfer of DNA between bacterial cells by a mechanism requiring cell to cell contact. This was first observed in *E. coli* (Ledeberg and Tatum 1946) and the first resistance genes spread from *Shigella* to *E. coli* via a conjugative plasmid (Hogan and Kolter 2002). Diverse scientific and experimental evidence has confirmed that the conjugation system is a highly successful method of transferring drug-resistance genetic determinants amongst bacterial populations. The significance of this promiscuous gene transfer is such that it provides a mechanism for the availability of a huge pool of genes for bacterial evolution. A dramatic example is the widespread development of resistance to antibiotics used in clinical medicine and agriculture.

1.3.1 Molecular Mechanism of bacterial conjugation

For the purpose of this thesis, discussion will be limited to conjugation systems of Gram-negative bacteria. In Gram-negative bacteria, plasmids widely employ type-IV secretion system (T4SS) (Figure 2), which is one of the three essential components of the conjugation, including the relaxosome and coupling proteins. Type-1V secretion system, T4SS, which is the transferome spans the cell envelope and is responsible for the synthesis of the conjugative pilus. The relaxasome is a complex of proteins that process the DNA at the origin of transfer (oriT), while the coupling protein, connects the two entities together. These relaxase proteins, which include nickases, transesterases, primases and RecA are involved with, and are in charge of, the binding and cleaving of DNA during plasmid transfer or replication processes (Christie and Vogel 2000; Data *et al.,* 2003). T4SS, therefore direct DNA and/or protein translocation into the recipient cell through the extracellular pili which are formed during mating pair formation.

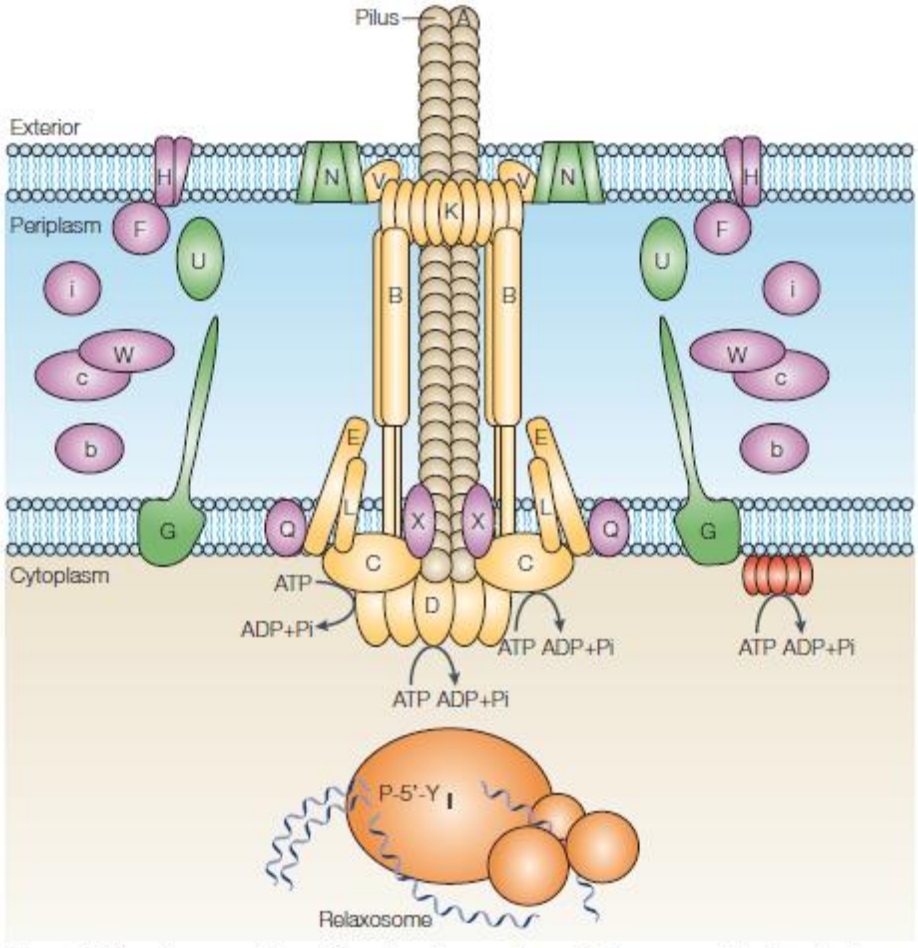


Figure 2: Signature proteins of conjugative systems in Gram-negative bacteria (Frost, Leplae *et al*., 2005). The F-type (F) conjugative system is shown located in the inner and outer membranes and extending through the periplasm. Tra proteins are labelled with capital letters and Trb proteins with lower-case letters. Shared proteins found in the T1 plasmid are yellow. For more details see the review by Frost *et al*., 2005

During conjugation, a unique single strand of DNA (ssDNA) called the transfer (T) strand undergoes 5' to 3' directional transfer from a donor bacterium to a recipient cell. This T-strand is cleaved in the donor by a specific nuclease at the nic site within the oriT (Origin of transfer). Cleavage is mediated by a plasmid-encoded relaxase enzyme, which establishes a covalent linkage with the 5' terminus of the T-strand, thereby producing a covalently bound protein-DNA transfer intermediate (Lanka and Wilkins

1995; Lang and Zechner 2012). For productive conjugation, a mating pair formation system facilitates cell-to-cell contact. Once contact is established, T-strand DNA is transferred from the donor to the recipient in a process that utilizes a type-IV secretion system (Fronzes *et al.*, 2009; De La Cruz *et al.*, 2010; Bhatty *et al.*, 2013; Christie *et al.*, 2014). The conjugative transposons and plasmids of Gram-positive bacteria do not use pili, but their transfer (*tra*) regions demonstrate some similarities with the Gram-negative bacteria (Christie *et al.*, 2014).

1.4. The role of plasmids in the origin and development of antibiotic resistance

1.4.1 Bacterial plasmids and the origin of plasmid-mediated antibiotic resistance

Resistant bacteria commonly harbour mobile genetic elements, for instance, conjugative plasmids that contain resistance genes to several classes of antibiotics. Simply defined, plasmids are small, extrachromosomal pieces of DNA in bacterial cells. They are classified as mobile genetic elements (MGE's), together with transposons and integrons and gene cassettes, all of which possess the ability to acquire and distribute an array of resistance genes in a single conjugation episode, among bacterial communities.

In particular, plasmids encode an enormous variety of functions which may not be essential for the host or plasmid survival. Such functions could include antibiotic resistance, heavy metal and toxin resistance, biofilm, and virulence factors (Bennett 2008). As noted earlier, many plasmids can capture, accumulate and disseminate resistance conferring genes among bacteria, not only from cell-to-cell of the same bacterial type, but also across genus and species, making them a primary passageway of antibiotic resistance and virulence genes spread. Thus, a plasmid is said to be resistant if it carries one or more antibiotic resistance genes. For example, it may be a metabolic plasmid, because it encodes a metabolic function, or a virulence plasmid, because it possesses one or more virulence genes. Carriage of one type of gene does not preclude carriage of other types that do not contribute towards maintenance and spread of the host plasmid (Bennett 2008).

Even though plasmids naturally occur in the environment, the occurrence of plasmids in pathogenic bacteria was very minimal before the introduction of antibiotic era in the 1940's (Hughes and Datta 1983). This goes to confirm that the prevalence of plasmid-

coded resistance is as a consequence of widespread use of antibiotics which exerts strong selective pressure on the original resistomes (Martinez 2012). Plasmid- encoded resistance genes originate during point mutations in the target genes of susceptible bacteria and also from genes that provide antibiotic-producing bacteria with protective mechanisms (Hughes and Datta 1983; Frost *et al.,* 2005). Once such a gene is introduced into a new host in which it lacks its original biochemical and genetic context, its function is limited to antibiotic resistance (Baquero *et al.,* 2009). Several reviews have established that the natural microbial pool contains a much larger number of resistance genes than those seen to be acquired by bacterial pathogens (Levy and Marshall 2004; Wright 2007; Davies and Davies 2010); and different ecosystems contain different resistance genes. By means of horizontal gene transfer especially by conjugation, plasmids therefore allow for a highly efficient spread of resistance independently or by means of bacteriophages upon its expression in a new host (Ojala *et al.,* 2013).

1.5 Plasmids are crucial to multidrug antibiotic resistance and pathogen virulence

Plasmid–mediated antibiotic resistance continue to impact on the overall burden of antibiotic resistance. Evident cases are present in Gram-positive and Gram-negative bacteria that boost the difficulties in maintaining effective antibiotic activity against what might now be referred to as "the superbugs"" like *E. coli*, *M tuberculosis*, *K. pneumonia*e and *Salmonella enterica* serovar *Typhimurium*. Plasmid resistance genes are present for many classes of antimicrobials and pathogens, namely, plasmid-mediated quinolone resistance (PMQR), aminoglycoside resistance methylases (ARM), plasmid-mediated 16S rRNA methylase (RMT) and sulphonamide resistance (Carattoli 2009; García-Fernández *et al.,* 2011), varying phenotypes of qacA/B in methicillin-

resistant *Staphylococcus aureus* (MRSA) (Zheng *et al.,* 2009; Cheng-Mao Ho *et al.,* 2012), MDR IncX1-plasmid in *E. coli* (Norman *et al.,* 2008) and blaCMY-2-like allele in an IncI plasmid expressing β-lactams resistance in *Salmonella enterica* serovar *Typhimurium* (Cordeiro *et al.,* 2013). In particular, plasmid-coded resistance genes have been associated with the five major groups of extended-spectrum β-lactamases (ESBLs), which dominate world widely, namely KPC's-carbapenamase, OXA-48 non-metallo-enzymes and IMP, blaNDM-1, and VIM metallo-carbapenemases extended-spectrum β-lactamases (ESBLs) (Nordmann *et al.,* 2012; Gopi and Robert 2013). Unlike KPCs and NDM-1 which have been associated with a variety of plasmids, the overall rise of single 62kb self-conjugative IncL/M-type plasmids has supported greatly the distribution of blaOXA−48 in Europe (Poirel *et al.,* 2012).

The prevalent virulence of *Bacillus anthracis* is attributed to the presence of pXO1 plasmids, the loss of which leads to a loss of pathogenicity. A plethora of plasmid-encoded pathogenic islands PAI IV536, PAI ICFT073, and PAI IICFT073, in addition to IncF plasmid (blaCTX-M-15, and aac(6')-lb-cr) resistance genes were found in *E. coli* ST131 strains, a combination of factors that renders β-lactam antibiotics inactive (Calhau *et al.,* 2013). Likewise, plasmid-encoded and multiple chromosomal genes in *Chlamydia trachomatis*, a causative organism of chronic inflammatory diseases of the eye and genital tract, was associated with chlamydial virulence, as plasmid-deficient organisms are highly susceptible to antibiotic treatment (Song *et al.,* 2013).

Not surprisingly, plasmid-mediated transfer of resistance has been a major focus of recent research and in particular, how it can be reversed in order to enhance bacteria sensitivity to antibiotics.

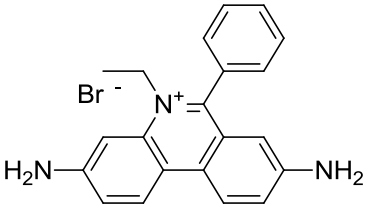
The term "superbugs" refers to microbes with enhanced morbidity and mortality due to multiple mutations endowing high levels of resistance to the antibiotic classes specifically recommended for their treatment (Davies and Davies 2010).

1.6 Various approaches to curb plasmid-mediated antibiotic resistance

The act of displacement of a resident plasmid, through changes in environmental conditions or by genetic or chemical means, from its natural host to establish a plasmid-free cell, is commonly referred to as "plasmid curing". Naturally, plasmids seem to be unstable and may be easily lost from the host, but many are extremely stable and would require exposure to conditions or agents in order to induce elimination. Therefore during the curing process, a plasmid is lost from a cell due to an inability of the plasmid to replicate, unsuccessful partitioning of the plasmid into daughter cells upon cell division, or induced death of plasmid-containing cells through activation of plasmid-kill mechanisms ultimately resulting in the loss of specific phenotypes such as drug-resistance.

One of the earliest attempts of plasmid curing was the addition of nickel and cobalt to the growth medium to promote the loss of the F plasmid from *E. coli* (Adepoju and Adebanjo 2011). Since then, various procedures have been used including growth of plasmids at elevated temperature (Sonstein and Baldwin 1972b; (Jain *et al.,* 2011), thymine starvation (Sat, Reches *et al.,* 2003; Williams and Hergenrother 2008) and protoplast regeneration (Abdalla-Galal *et al.,* 2000). The use of intercalating drugs such as ethidium bromide (**3**) (Koffi-Nevry *et al.,* 2011; Santos *et al.,* 2012), acriflavine (**4**), acridine (**5**), and quinacrine (**6**), have been studied on different plasmid types and their responses respectively (Sonstein and Baldwin 1972a; Guiney 1993; Novick *et al.,*

2008), (Molnar *et al.,* 1979; Chopra 2000), (Koffi-Nevry *et al.,* 2011; Santos *et al.,* 2012). The curing potential of sodium dodecyl sulphate (**7**) and other surface active alkyl sulphates (SDS) has been applied to plasmid found in *S. aureus* (Sonstein and Baldwin 1972b) and *E. coli* K12 (Grindley *et al.,* 1972; Noumedem *et al.,* 2013). Under the various conditions, different plasmids responded quite differently to the same agents or applied conditions, and could not achieve 100% plasmid loss from the host bacterial population (Guiney 1993). These intercalating agents are also associated with high toxicity that makes them unsuitable for human treatment.



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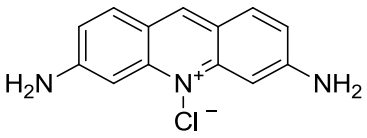

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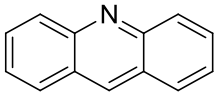

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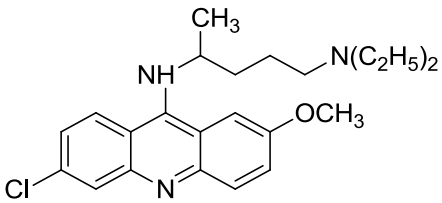

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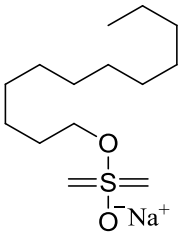

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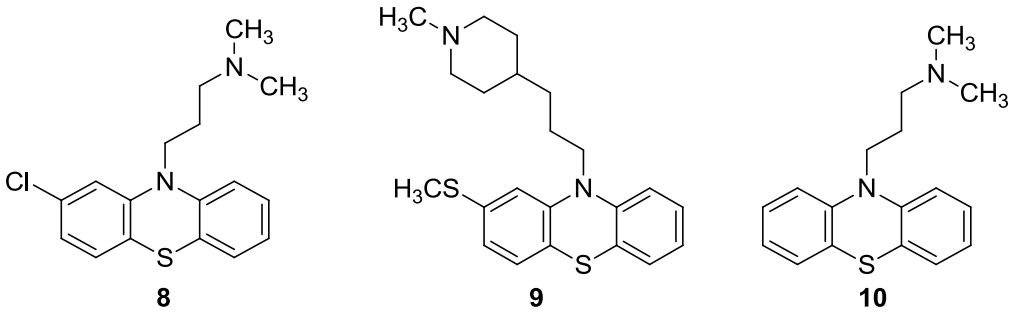
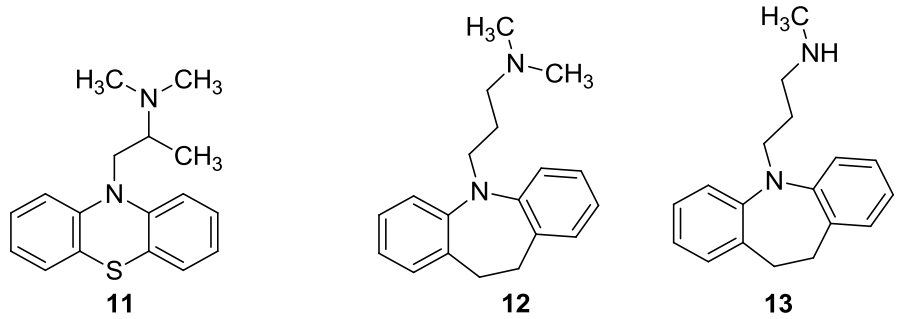
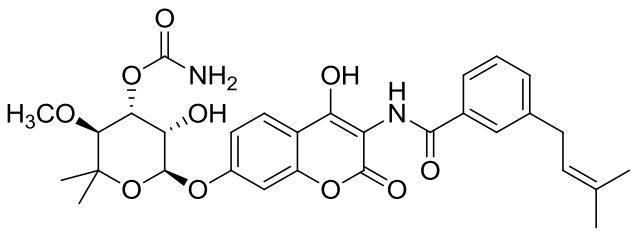
Tricyclic neuroleptics and the anti-depressant phenothiazines like chlorpromazine (**8**), thioridazine (**9**), promazine (**10**), promethazine (**11**), imiprazine (**12**) and desipramine (**13**), which possessed strong *in vitro* antibacterial activity triggered by their plasmid elimination properties attributable to inhibition of plasmid replication, partition and conjugal transfer processes (Kawase *et al.,* 2001; Spengler *et al.,* 2003; Molnar *et al.,* 2004; Takacs *et al.,* 2011).





In the last decades, various coumarins and quinolones have shown a variety of plasmid curing efficiencies (Michel-Briand *et al.,* 1986; Weisser and Wiedemann 1986). Novobiocin (25 mg/L) showed significant antibacterial activity as well as loss of the IncF plasmids R446b and R386 from multidrug-resistant *E. coli* (Michel-Briand *et al.,* 1986), in addition to pBR322, which conferred tetracycline-resistance in both *E. coli* W3110 and *E. coli* C600 strains.

Though novobiocin (**14**) has a track record of remarkable anti-plasmid and antimicrobial properties compared to other quinolone agents, its use has been seriously hampered by toxicity issues and as a result withdrawn from human use (Federal Register 2011).



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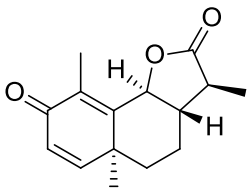

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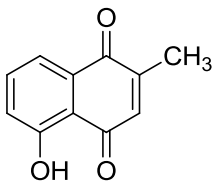
Due to failed antibiotic activity and resistance limitations, several studies have focused on naturally derived compounds as an alternative source of active compounds for total elimination of plasmids from their resistant bacteria host. Such works are valuable and continue to lend credence to nature as reservoirs of biologically active agents as well as extend our understanding of the outcome of natural compound as anti-plasmid agents. Examples include α-santonin (**15**), a sesquiterpene lactone isolated from *Artemisia maritima* flowers which induced the loss of the ColE1 group plasmids, pBR322 and pBR329, from *E. coli* (Hamisy *et al.,* 2000). *Coptis chinensis*, a traditional Chinese medicinal plant known for a high concentration of berberine and used as an immune booster, as in gastrointestinal and respiratory health, recorded a 23% plasmid loss from *E. coli* under elevated temperature (Perdue *et al.,* 1986). The crude extract from *Chinese pulsatilla* (Sims 1981) induced the loss of a 20kbp plasmid band which encoded streptomycin-resistance after treatment with 0.125 mg/L of the extract.

Plumbagin (**16**) is a naphthoquinone (5-hydroxy-2-methyl-1, 4-naphthaquinone), and is a characteristic yellow pigment common to the plants of the Plumbaginaceae family. It is also found in the Droseraceae, Ancestrocladaceae and Dioncophyllaceae plant families (Sandur *et al.,* 2006). Plumbagin has been in use traditionally since ancient times and was first isolated from the root of *Plumbago zeylanica* with a wide variety of biological activities including anti-carcinogenic, anti-atherosclerotic effects. It is an

inhibitor of superoxide production in nox-4 cells (Ding *et al.,* 2005). The antimicrobial and antiplasmid properties of plumbagin (**16**) have been reported on various plasmids such as R6K, TP154, TP181 and R162 (Lakshmi *et al.,* 1987). Loss of pUK651 from *E. coli* X^+ was observed in plumbagin extract-treated cells which gave rise to a 14% loss at a sub-inhibitory concentration of 7 mg/L of the crude drug.



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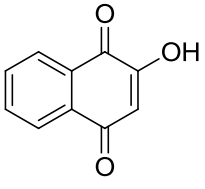
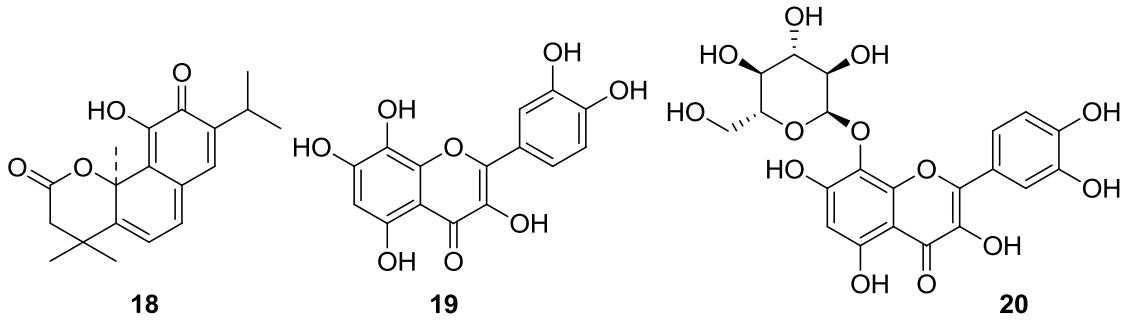


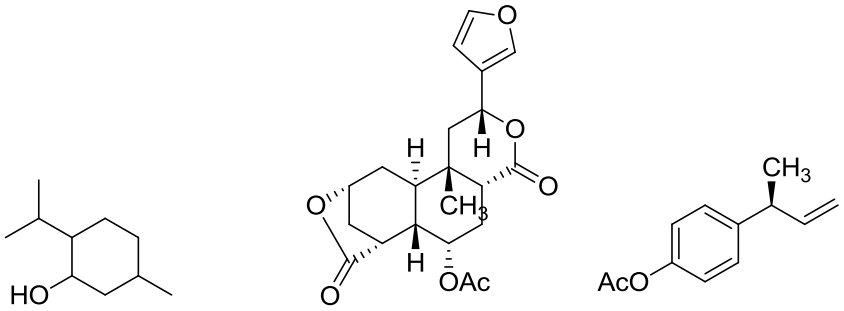
17

Lawsone (**17**), a structurally-related naturally occurring naphthoquinone and plumbagin exhibited antibacterial activity against vancomycin-resistant *Staphylococcus aureus* (VRSA) and was correlated to loss of encoding plasmids (Jahagirdar *et al.,* 2008). The mode of anti-plasmid action of plumbagin is said to be based on the intercalation of DNA and induction of topoisomerase-II-mediated DNA cleavage *in-vitro,* but this has not been completely elucidated (Fuji *et al.,* 1992). The effect of phenolic compounds namely bharangin (**18**) isolated from *Pygmacopremna herbacea* (Roxb), together with gossypetin (**19**) and gossypin (**20**) have been implicated in the loss of a penicillinase-conferring plasmid in *E. coli* 46R41 (Marie-Magdeleine *et al.,* 2010). Results showed that bharangin and gossypetin eliminated 30% and 21% of the TP181 plasmid respectively but remained inactive to ColE1 plasmid, pBR322 and pBR329 plasmids. Bharangin at a higher SIC value of 400 mg/L demonstrated 70% elimination of the plasmids P181, R162, and TP154 (Marie-Magdeleine *et al.,* 2010).



18 **19** **20**

The anti-plasmid activities of 10 essential oils (with promethazine as a control) were assayed against *E. coli* harbouring F□*lac*. Peppermint, *Eucalyptus* and Rosemary oils eliminated F□*lac* determinant with various levels of responses (Schelz *et al.,* 2006). Menthol (**21**), the main constituent of peppermint oil promoted near total plasmid elimination from the host independently, and in combination with promethazine. A *nor*-diterpene compound, 8-epidiosbulbin-E-acetate (EEA) (**22**), was isolated from the bulbs of *Dioscorea bulbifera* with plasmid-curing activities against pUB110, RP1; RM163, RIP164, from *E. coli* and *P. aeruginosa*, respectively (Shriram *et al.,* 2008).



21 **22** **23**

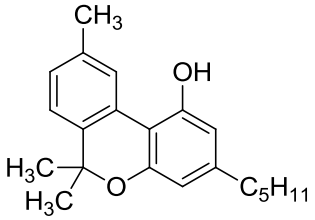
The compound, 1-acetoxychavicol acetate, (**23**) isolated from *Alpinia galanga* was identified as the responsible agent for the loss of the broad host pUB110 occurring in *S. typhi, P. aeruginosa, E. coli* and *Enterococcus* as well as RP4 in *B. cereus* and *E. coli*. Comparatively, the crude extract of *Alpinia galanga*, demonstrated higher plasmid curing activity than the isolated single entity, 1'-acetoxychavicol acetate. However, this

is not unusual as crude drug extracts often exhibit synergistic activities (Latha *et al.*, 2009).

Within the last decade, marine organisms are increasingly gaining attention as sources of new antimicrobial drugs, and the search for anti plasmid agents are not excluded from this source. Crude drugs of two sea weeds from the genera *Halimeda* and *Sarconema* were investigated for their anti-plasmid activity against a penicillinase-bearing plasmid in *E. coli* and both active fractions cured plasmids from *E. coli*. This was validated by an agarose gel profile which showed the physical loss of the plasmids within the cured cells (Samy 2012). Further research is needed in this area to uncover possible marine metabolites that could have clinical potential as anti-plasmid agents.

1.6.1 Cannabinoids as antiplasmid agents

The antibacterial activities and plasmid curing efficiency of active constituents of *Cannabis* and its phenanthryl derivatives have been reported (Spengler *et al.*, 2006). Cannabinol (**24**), Δ8-tetrahydrocannabinol and Δ9-tetrahydrocannabinol (**25**) recorded MIC values of 3, 3 and 10 mg/L respectively against *E. coli*; whereas poor elimination of the F□*lac* plasmid in *E. coli* was observed (<0.5% plasmid loss).



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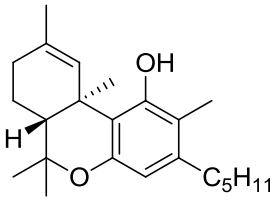

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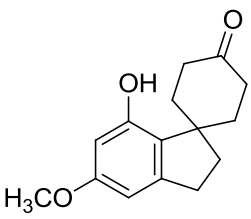
F'*lac* plasmid in *E. coli* K12 was sensitive to tetrahydrocannabidiolic acid which caused 30% loss of the plasmid, whilst acetylcannabispirol, cannabispirone (**26**) and cannabispirenone (**27**) at a sub-inhibitory concentration of 0.15 mg/L were less active

(Molnár *et al.,* 1986; Spengler *et al.,* 2006). Each compound, except for acetylcannabispirol, inhibited R144 plasmid conjugal transfer between *E. coli* isolates.



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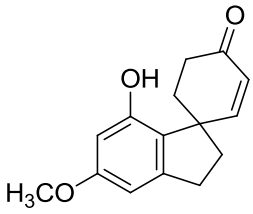

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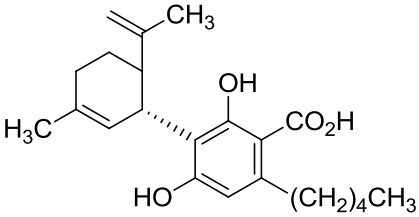

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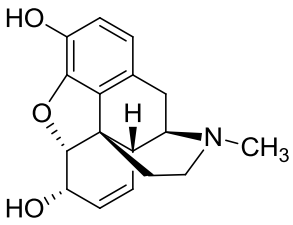

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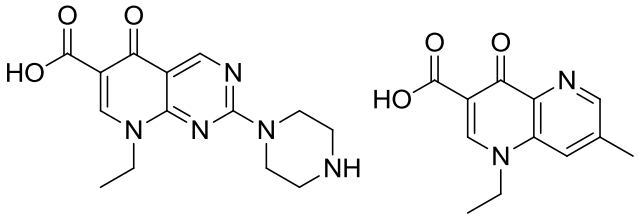
Other compounds with similar activity reported by the same authors included cannabidiolic acid (**28**), morphine (**29**) and 1-dimethylamino-3-(9-phenanthryl)-3-propanol. The mechanism by which cannabinoids interfere with the bacterial plasmid system is yet to be elucidated, but could be associated with binding to the transconjugal DNA, intercellular inhibition of the mating pair formation, replication or plasmid partition processes (Weisser and Wiedemann 1985). The beneficial antimicrobial activities of cannabinoids against multidrug-resistant *S. aureus* have been highlighted by several authors (Katz and Weaver 2003; Appendino. *et al.,* 2008). Further investigation into their chemistry to harness possible anti-plasmid potentials is an interesting niche of research. Major cannabinoids like tetrahydrocannabinol and cannabidiol are already existing medicinal agents used to ameliorate the symptoms associated with multiple sclerosis, and are undergoing clinical development for cancer pain (Barnes 2006).

The above evidence shows that the development of naturally sourced anti-conjugation or anti plasmid agents is possible. While, these studies indicated the activity of the compounds studied to cure plasmids in bacteria, many potential natural agents remain unexploited in antimicrobial discovery. It cannot be overemphasized that plants remain a natural reservoir of biologically active metabolites with unique chemistry; heavily utilized in traditional medicines for primary healthcare by 75-90% of the world population (WHO 2014)

1.7 Inhibition of the conjugal DNA transfer system

Whereas most studies have highlighted several techniques to induce plasmid elimination, only a few have focused on the process of interfering with bacterial conjugation. The inhibition of plasmid conjugation has been proposed as a potential way of combating the spread of plasmid-mediated antibiotic resistance (Fernandez-Lopez *et al.,* 2005; Williams and Hergenrother 2008). Some conjugative plasmids from various incompatibility groups have been studied and the effect of inhibitors on their transferability of resistance genes into a number of species. For example, the effect of pipemidic acid (**30**) and related compounds, nalidixic acid (**31**) and nitrofurans were studied on the transfer of F plasmids harboured by enterobacterial strains (Nakamura *et al.,* 1976; Barrero *et al.,* 1998; Yang *et al.,* 2008). Nitrofurans are known to act on DNA and to disrupt cell metabolism, so they may not necessarily be considered specific anti-conjugal agents, even though the anti-plasmidic activity observed was attributed to the reduction of the -NO_2 group of nifurzide (**32**) (Rajwar *et al.,* 2011). Sulbactamox, a current ESBL antibiotic, reportedly acted as an inhibitor of plasmid transfer in *E. coli.* However, the recorded antiplasmid activity of the antibiotic was achieved at the MIC

value of 0.25 mg/L (Chaudhary and Arygu 2012) as opposed to the sub-inhibitory concentration. The purported antiplasmid activity by Chaudhary's group is the effect of the bacterial growth inhibition while the antiplasmid activity of sulbactamox merits further investigation.



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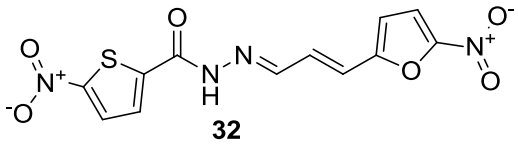

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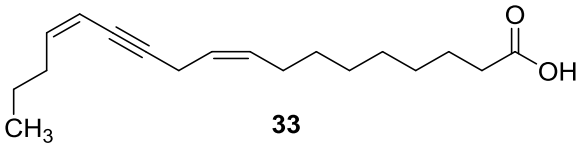

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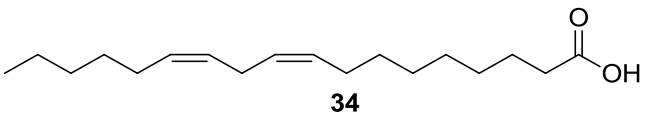

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One of the few attempts made to identify naturally-derived inhibitors of conjugal DNA transfer was a high-throughput conjugation screen of the NatChem library of 12,000 compounds against R388 and other plasmid transfers between *E. coli* isolates (Fernandez-Lopez *et al.,* 2005). The results identified two unsaturated fatty acids; dehydrocrepenynic acid (DHCA) (**33**) from *Sistotrema semanderi*-FX21 and linoleic acid (**34**) from *Mollisia ventosa*-FX14 which showed transfer inhibitory activity against plasmids R388 and R1, but not against RP4, RK6 and PKM101. The features of these acids, their carboxylic group, chain length and the double bond positions were

suggested to have an impact on their selective inhibitory action on the plasmid DNA transfer replication (Dtr) system (Fernandez-Lopez *et al.*, 2005).

1.8 Description of some Broad Host Range (BHR) conjugative plasmids used in our study

As earlier mentioned, resistance genes are horizontally transferred by conjugative plasmids which requires type IV secretion systems (T4SS). Genes encoding T4SSs occur in plasmids or occasionally within transposons and enable these mobile genetic elements (MGE's) to not only determine their own transfer but also to mobilise the transfer of a non-conjugative plasmid that is present in the bacterial cell (Frost *et al.*, 2005; Jain and Srivastava 2013). Conjugative plasmids can either be broad or narrow host range. Narrow host plasmids are limited in transfer and move within closely related species of the bacterial population while BHR, indeed, are very promiscuous and can transfer between bacteria from different species and are maintained stably within them. The transfer often exceeds the range of species in which they can be stably maintained (Guiney 1993; Jain and Srivastava 2013).

The initial classification of plasmids into these two ranges was done by (Datta and Hedges 1972) based on the ability of a plasmid to transfer among Enterobacteriaceae and *Pseudomonas spp*. This is a phenomenon known as 'incompatibility' (Inc) in plasmids, such that plasmids with the same replication mechanism cannot co-habit the same bacterial cell. Incompatibility groups have been defined for plasmids of the Enterobacteriaceae (26 groups), the Pseudomonads (14 groups), and for the Gram-positive staphylococci (~18 groups) (Frost *et al.*, 2005).

Plasmids of the IncP group (RP1, RP4, RK2, R18, R68), IncW (R388, pSa, R7K, pIE321, and pIE522, IncQ and IncN are classic examples of BHR and are stably

maintained in almost all Gram-negative bacteria (Revilla, Garcillán-Barcia *et al*., 2008). The efficient transfer of these large BHR plasmids is due to their antirestriction functions which enable them to overcome host restriction and maintain themselves in the host (Belogurov *et al.,* 1992).

1.9 Selected examples of plasmids belonging to different Incompactibility groups

Selected plasmids that were used in the study include: PKM 101 (IncN), TP114 (IncI2), pUB 307(IncP), R7K (IncW), R6K (IncX), and R1-drd-19 (IncF11). These plasmids exemplify plasmid groups that are currently associated with gross spread of antibiotic resistance, especially in Enterobacteriaceae.

1.9.1 PKM 101 (IncN)

The PKM 101 plasmid belongs to IncN, and is a 35.4kb derivative from the parent R46 (Langer and Walker 1981). It possesses an interesting property that make *E. coli* and *S. typhimurium* resistant to the lethal effects of UV radiation and mutagenesis. An *in vivo* deletion of a single DNA region of R46 removed the gene coding for resistance to sulphonoamides, streptomycin, and spectinomycin but earmarked the ability of PKM 101 to enhance UV–resistance as well as the presence of ampicillin resistance and the F factor to *E. coli* (Langer and Walker 1981). Generally, IncN plasmids are classically implicated in the resistance types among Enterobacteriaceae of human and animal origin (Carattoli 2009). They are broad host range and self-conjugative plasmids. These plasmids have been associated with genes conferring resistance to many antimicrobial classes, in particular genes encoding extended-spectrum β-lactamases (ESBLs). IncN plasmids are linked with the spread of several *bla* CTX-M variants, which are currently a major cause of infectious outbreaks worldwide, carbapenemases of the Ambler class A and class B, such as *Klebsiella pneumoniae* carbapenemase (KPC) and Verona integron-encoded metallo-β-lactamase (VIM) respectively (Nordmann *et al.,* 2012;

Poirel *et al.*, 2012; Gopi and Robert 2013), and the IncN–VIM-1 plasmids detected in *E. coli*, *Klebsiella pneumoniae*, *K. oxytoca*, *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, *Providencia stuartii* and *Proteus mirabilis* (Nordmann *et al.*, 2012).

1.9.2 TP114 (Inc I2)

The TP114, 62.1kb of molecular size, is a self-transmissible plasmid belonging to $IncI_2$, originally isolated from *E. coli* (Carattoli *et al.*, 2005). It encodes genes for aminoglycoside resistance such as the kanamycin determinant. TP114 is compatible with the plasmids of the I_1 group, and all support multiplication of the I-specific phage Ifl (Grindley *et al.*, 1972). Both Inc I1 and I2 groups possess thick conjugative pili which are morphological distinct from other incompatibility groups of I5, B, K and Z (Bradley 1984). Using the PCR-based replicon typing (PBRT) approach that revealed the major plasmid families involved in the current spread of resistance in Enterobacteriaceae, the IncI1 group was associated with a high prevalence of AmpC, ESBLs, 16S rRNA methylases, Qnr, and MBL drug resistance in *E. coli*, *K. pneumoniae*, *Samonella enterica* and *S. sonnei* that were isolated from both human and animal sources (Carattoli 2009).

1.9.3 PUB 307 (IncP)

The PUB 307 plasmid belongs to IncP group, a conjugative broad host range derivative of RP1 that carries tetracycline and kanamycin resistance markers. PUB 307 is known for its ability to mobilise gonococcal resistance plasmids from *E. coli* to *Neisseria gonorrhoea* (Piffaretti *et al.*, 1988).

From a phylogenetic and amino acid sequence analyses, IncP plasmids are grouped into six known sub-groups namely -α, -β, -γ, -ε, -δ and –ζ, encoding various levels of drug

resistance and widely dispersed in Gram-negative bacteria for eaxample, in *Pseudomonas sp, Klebsiella aerogenes, E. coli* and *Sphingomonas* (Thomas 2000; Shintani *et al.,* 2010; Popowska and Krawczyk-Balska 2013). These genes often code for resistances to a broad spectrum of antibiotics, heavy metals, and quaternary ammonium compounds used as disinfectants (Popowska and Krawczyk-Balska 2013). Due to their highly stable backbone which utilizes horizontal gene transfer, their transfer system (*tra*) is increasingly used in the genetic analysis and manipulation of most Gram-negative bacteria (Yakobson and Guiney 1983). To illustrate their BHR and stable nature, RP4, R68.45, RP1::TnSOl, and pUB307 were transferred to acidophilic, obligately chemolithotrophic *Thiobacillus ferrooxidans* from *E. coli* successfully via conjugation (Fürste *et al.*, 1989; Peng *et al.*, 1994).

1.9.4 R7K (IncW)

The R7K plasmid is atypical of the IncW group which took its name from T. Watanabe, including classical members: the pSa and R388 plasmids. R7K was originally isolated from *Providencia rettgeri* and could transfer the factor to *E. coli* K12 (Coetzee *et al.,* 1972). It encodes ampicillin, spectinomycin and streptomycin antibiotic resistance determinants and the genetic backbone is comprised of 42 genes of 39.7kb in molecular weight (Revilla *et al.,* 2008).

The IncW plasmid has gained much interest from researchers because of its small size, a wide spectrum of antibiotic resistance and broad host range nature, which are transferred by conjugation to species in the genera *Escherichia, Salmonella, Shigella*, *Pseudomonas* and *Proteobacteria.* IncW plasmids transfer efficiently in solid but not in liquid media, and possess thick w-pili, usually 10-12nm width and 450nm length found in pSa, R388 and R7K (Bradley and Cohen 1976).

1.9.5 R6K (IncX)

The plasmid R6K belongs to the Inc group X, a recombinant transmissible plasmid of 39.9kb molecular weight. It is distributed in *E. coli* K12 J53 and carries ampicillin and spectinomycin resistance determinants. R6K was thought to be a narrow host range plasmid (Espinosa *et al.*, 2000), until it was shown that R6K and its variants possess robust replicons in a variety of enteric and non-enteric bacterial populations (Wild *et al.,* 2004). The result is a broader host range and the implications of R6K and its multiple origins of replication has been documented (Rakowski and Filutowicz 2013).

1.9.6 R1-drd-19 (IncF11)

The conjugative R plasmid, R1-drd-19 belongs to the IncF11 group and a derepressed mutant of R1 (Meynell and Datta 1967). Plasmid R1 represses conjugal pilus synthesis by the FinO and FinP of TraJ protein, a repressor of the *tra* operon. On the other hand, R1-drd-19 synthesizes these pili constitutively, and has a molecular size of ca 33μm and weight of 62.5 Md. It carries the gene for β-lactamases (*bla*+), adenylytransferase (*aadA*+), acetyltransferase (cat+), phosphotransferase (*aphA*+) and *Su*+ gene specified for dihydropteroate synthetase, that determines the resistance to ampicillin, streptomycin, chloramphenicol, kanamycin and sulphonamides respectively (Blohm and Goebel 1978). All these five resistance genes are located in 2 different transposon, *Tn3* and *Tn4* shuttles. The restriction map of R1-drd-19 has been studied by the same authors using four different restriction enzymes namely, *Bam*H1, *Hind*111, *Eco*R1 and *Sal*1.

1.10 Natural products as potential source of antimicrobial agents

Plants remain a natural reservoir of biologically active metabolites with unique chemistry that may hold a solution to plasmid elimination. The focus of recent antimicrobial drug discovery has centred on synthetic and combinatorial chemistry of existing drugs, though with a good level of success but not without bacteria evolving resistance upon resistance against the drug outcomes. Thus, research into natural products cannot be overlooked even though it has its own challenges of high cost of research and development from crude to the market shelf and an average of 12 years to achieve the goal. Despite that, natural products contain more potential drug sources than compounds synthesized by combinatorial chemistry given that 50% of new drugs derived directly or indirectly from natural sources (Zhang *et al.,* 2010). Evolutionary evidence suggests that a certain number of human genes should have orthologs in plants and even microbes, and therefore, some secondary metabolites produced by plants and microbes to modulate their own metabolism could hit the targets implicated in microbial diseases (Zhang *et al.,* 2010). Furthermore, the strong potential of drug development of natural products can be explained in terms of the co-interaction of the microbes with their biological environment, which may create various natural agents that are therefore of medicinal interest to humans. For example, these natural compounds produced by plants to combat microbial pathogens could act as antimicrobial drugs, while some of the compounds involved in defense against herbivores could possess pharmacological activities as laxatives, emetics, cardio-tonics and muscle relaxants. The medicinal properties exhibited by the compounds are often due to similar physiological functions either in competing microorganisms, plants, and animals or mimic the action of vital body metabolites, ligands and hormones (Briskin 2000). In addition, structures of secondary products which have evolved to interact with

molecular targets affecting the cells, tissues in human thereby making them medicinally effective on humans or bacteria due to similarities in their potential target sites (Kaufman PB *et al.,* 1999; Briskin 2000).

It is necessary that new drug leads with newer mechanisms be harnessed from natural products to combat antibiotic resistance, in particular, the problematic dissemination of plasmid-mediated resistance in bacteria. Not much attention has been given to the search of naturally derived plasmid-inhibitor compounds as equally revealed from the paucity of literature available from databases such as PubMed, SciFinder and Scidirect.

1.11 Selected medicinal plants for screening for conjugal plasmid DNA transfer inhibition

Based on the ethnomedicinal evidence and literature search, the following plant species have been shown to be of long traditional usage and possess biologically active principles. To the best of our knowledge, none of the selected plants has been studied on their ability to effect inhibition of plasmid DNA conjugal transfer in *E. coli* strains. Hence, they are chosen for further investigation to verify their antibacterial activity and potential anti-plasmid effects.

1.11.1 *Mallotus philippinensis* (Lam.) Mull. Arg

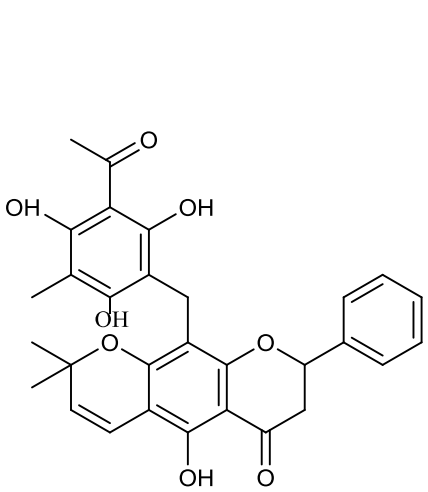
Mallotus philippinensis (Lam.) Mull. Arg. (Figure 3) is a well-known medicinal plant from Asia and Australia. It is known as Monkey-face tree (English), Kamala (Hindu), Kampillaka, while other minor names are Raini, Sindur, Sinduri, kumala, Kamal, Kumila. It is an evergreen shrub or small tree, belonging to the plant family

Euphorbiaceae, with different parts used in folk medicine.

Figure 3: *Mallotus philippinensis* (Lam.) Mull. Arg. Picture taken by Kakalou, 2010.

The characteristic red powder, kamala, from the glandular hairs of the plant capsule is used as a dye for silk and possesses anthelmintic and cathartic properties (Satyavati *et*

al., 1987). Distinct groups of compounds have been isolated from different parts of the plant. Flavonoids such as kamalachalcones A and B, 4′-hydroxy isorottlerin (**35**) , and two new chalcone derivatives, kamalachalcones C (**36**) and D, isorottlerin , rottlerin and 5, 7-dihydroxy-8-methyl-6-prenylflavanone have been isolated from the plant (Tanaka *et al.,* 1998; Furusawa *et al.,* 2005).



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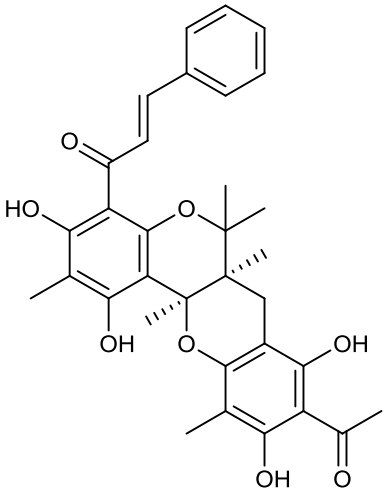

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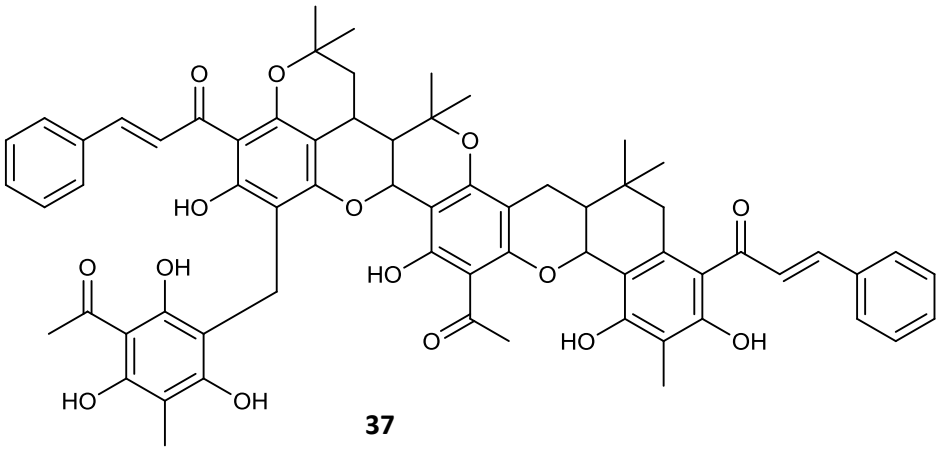
A new kamalachalcone E (**37**) along with known rottlerin (5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene) also called mallotoxin,1-(5,7-dihydroxy-2,2,6-trimethyl-2H-1-benzopyran-8-yl)-3-phenyl-2-propen-1-one, and 4'-hydroxyrottlerin was recently characterised and showed to possessed anti-inflammatory activity (Kulkarni *et al.,* 2014b).



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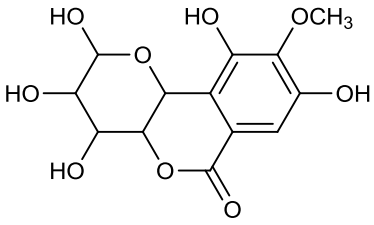
Other compounds that have been derived from *M. philippinensis* include the red compound, β-sitosterol, stigmasterol, bergenin (**38**), alpha–amyrin, 3′-prenylrubranine, atypical phloroglucinol derivatives like mallotophilippen A (**39**) and B, mallotophilippen C, D and E, (Daikonya, 2002).



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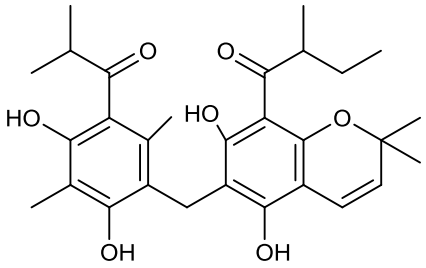

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A novel, 8-cinnamoyl-5,7-dihydroxy-2,2-dimethyl-6-geranylchromene, named mallotophilippen F and 8-cinnamoyl-2,2-dimethyl-7-hydroxy-5-methoxychromene was isolated including rottlerin, isoallorottlerin (isorottlerin) and the red compound (8-cinnamoyl-5,7-dihydroxy-2,2,6-trimethylchromene) (Hong *et al.,* 2010). The various compounds from different aerial parts of the plant, including kamala and rottlerin have exhibited anti-tumor and cytotoxic effects (Arisawa *et al.,* 1990; Tanaka *et al.,* 2008), anti-allergic (Daikonya *et al.,* 2002), antiviral (Kumar *et al.,* 2006; Kulkarni *et al.,* 2014a), antibacterial activity against resistant *Helicobacter pylori* strains (Zaidi *et al.,*

2009), anti-leukemic (Khan *et al.,* 2013) and anti-tubercular activities (Hong *et al.,* 2010b; Gangwar *et al.,* 2014). The ethanol extract from *Mallotus philippinensis* stem bark improved on mesenchymal stem cell proliferation, their migration, and wound healing *in vitro* and *in vivo* (Furumoto *et al.,* 2014). A present study showed that *M. philippinensis* crude drug was active against leukaemic cells and it is proposed that some potent anti-carcinogenic compounds exist in *M. philippinensis* that call for their identification (Khan *et al.,* 2013). Despite the several studies on the biological and pharmacological potentials of *M. philippinensis*, no report shows that its plasmid inhibition activities have been investigated.

1.11.2 *Cannabis sativa* L.

Cannabis sativa L. (Figure 4) belongs to the family of Cannabinaceae, and is one of the oldest known medicinal plants likely originating from Asia (Hazekamp *et al.,* 2005). *C. sativa* is known by various names worldwide, but most popularly marijuana and

hashish.

Figure 4: *Cannabis sativa* L. Picture taken by me (20/08/2011) in UCL School of Pharmacy green house.

The folk medicine is used traditionally for the treatment of rabies, cholera, rheumatism, epilepsy, tetanus, allergies, burns, cuts, wounds, inflammation, scabies, smallpox, and sexually transmitted diseases (Ali *et al.,* 2012). *Cannabis* has an ancient tradition of usage as in obstetrics and gynaecology. *Cannabis* extracts have been shown to be safe in the treatment of conditions in women including dysmenorrhea, dysuria, and *Hyperemesis gravidarum* and menopausal symptoms. Traditionally, the plant was used in ancient China as an anaesthetic during surgery. Furthermore, in Europe and America, it is traditionally used for the treatment of severe headaches and migraine attacks. C*annabis* is currently undergoing development for pain management (Hazekamp *et al.,* 2005)

Numerous phytochemicals are present in *Cannabis* and generally grouped into classical and non-classical cannabinoids. The term cannabinoids refers to a unique group of secondary metabolites found in the *Cannabis* plant, responsible for its pharmacological effects (Ali *et al.,* 2012). The naturally occurring cannabinoids are grouped as phytocannabinoids and synthetic cannabinoids (those synthesized in the laboratory). Endocannabinoids are those produced in the body of humans and animals. In confirmation of age-long standing antimicrobial abilities of the *Cannabis* plant and the cannabinoids, Appendino's team reported the antibacterial effect of five major cannabinoids; cannabidiol, cannabichromene, cannabigerol, Δ9-tetrahydrocannabinol and cannabinol with MIC values ranged between 0.5-2 µg/mL against methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Appendino *et al.,* 2008).

Antibacterial, anti-leishmanial, and antifungal effects of *Cannabis* on a variety of resistant pathogenic organisms were also reported (Novak *et al.,* 2001; Lone *et al.,* 2012). In addition to their antibacterial potentials, several classical and non-classical cannabinoids have been studied for their effect on F' *lac* plasmid elimination from *E. coli,* with tetrahydrocannabidiolic acid (THCA) causing 30% loss (Spengler *et al.,* 2006). Although, this is the only report regarding the effect of cannabinoids on R-plasmid transfer inhibition, it indicates that compounds from *Cannabis* plant may have effect on bacterial plasmid elimination. The various compounds tested for antiplasmid effects were in structural and chemical analogy, which made it possible to study their structure-activity–relationship (SAR) and to correlate their antiplasmid effect and supermolecular complexes of these plasmid-curing compounds. The heterocyclic and potent nature of cannabinoids, both natural and synthetic, opens up useful opportunities for the development or modification of antibacterial and antiplasmid agents from natural sources.

1.11.3 *Capsicum annuum* L.

The species *Capsicum annuum* L. (Figure 5) belongs to the large genus *Capsicum* in the

family Solanaceae, native to South America, and represents one of the leading vegetables grown globally with a worldwide production of approximately 26 million tonnes per year (Krajewska and Powers 1987).

Figure 5: *Capsicum annuum* L. Source: *http://www.virginiatobaccoseeds.com*

C. annuum contains capsaicinoids, a group of compounds that give them the characteristic pungent taste, and the following five naturally occurring capsaicinoids noted are capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin. These capsaicinoids are the major components of most *Capsicum* species, constituting approximately 95% or more of the total capsaicinoid content (Krajewska and Powers 1987), while capsaicin and dihydrocapsaicin are responsible for up to 90% of the total pungency of the pepper (Garces-Claver, A. *et al.*, 2007). Capsaicinoid compounds have been widely studied for their medicinal purposes, as pharmaceuticals, and as food additives and industrial sprays (Garces-Claverr *et al*., 2006). *Capsicum* species and capsaicin have been shown by several studies to possess antimicrobial activity against metronidazole-resistant *Helicobacter pylori* (Zeyrek Yildiz and Oguz 2005) and various multidrug-resistant Gram-positive and Gram-negative bacteria such as *E. coli*, *P. solanacearum*, and *B. subtilis* cultures (Wei *et al.*, 2006); (Koffi-Nevry *et al.*, 2011; Santos *et al.*, 2012), (Noumedem *et al.*, 2013). Recently, capsaicin demonstrated efflux pump inhibition against NorA-conferring resistance on notorious *S. aureus* to norfloxacin (Kalia *et al.*, 2012). Further study into potential antiplasmid action of capsaicinoids could be useful for developing new drug leads; modifying or enhancing the potency of valuable antimicrobial therapeutic agents present in this plant.

1.11.4 *Evodia rutaecarpa* (Juss) Benth

Evodia rutaecarpa (Juss.) Benth, (Figure 6) commonly known as Wuzhuyu, is an ancient traditional medicinal plant used in Chinese medicine. It is been used as a stimulant, and a remedy for illnesses such as headache, leg edema, dysmenorrhea, vomiting, diarrhoea, abdominal pain and colic, weakness, *postpartum* haemorrhage, migraines, nausea (Pharmacopoeia, 2005).

Figure 6: *Evodia rutaecarpa* (Juss.) Benth. Source: *http://kampo.ca/herbs-formulas/herbs/goshuyu*

Several phytochemical studies have revealed a rich variety of indole and quinolone-type alkaloids, including indoloquinazoline-type dehydroevodiamine, evodiamine, rutaecarpine, and evocarpines, which are the major active compounds (Liu *et al.,* 2005; Ko *et al.,* 2007; Huang *et al.,* 2012; Zhang *et al.,* 2014). The quinolone alkaloids from the fruits of *E. rutaecarpa* have shown some pharmacological activity on human

granulocytes (Adams *et al.,* 2005; Pei-ting Zhang *et al.,* 2013) and display highly selective antibacterial activity against *Helicobacter pylori* (Hamasaki *et al.,* 2000). The major quinolone alkaloids had exhibited anti-mycobaterial activities against *Mycobacterium fortuitum*, *M. smegmatis*, and *M. phlei* at MIC value range of 32-2 mg/L (Adams *et al.,* 2005). Extracts from different parts of the plant, the leaves, stem wood, root and fruit of *Evodia* have continued to demonstrate antibacterial activity among 300 herbal remedies, screened for anti-hepatitis B surface antigen capability (Zheng and Zhang 1990), antibacterial activity with MIC ranges between 0.25 mg/L and 1 mg/L against some Gram–positive bacteria, *P. aeruginosa*, and *Candida* yeast at 0.5 mg/L (Thuille *et al.,* 2003). Several researches have also highlighted the anti-inflammatory activity of the quinolone alkaloids; dehydroevodiamine, evodiamine, rutaecarpine and synephrine against influenza A virus (H1N1)-induced chemokines production and on differentiated neutrophils and microglial cell *in vitro* (Ko *et al.,* 2007; Chiou, W.-F. *et al.,* 2011; Liao, J.-F. *et al.,* 2011).

1.12 Problem statement

The possibility of treating infections caused by either mono- or multidrug-resistant bacteria is becoming increasingly difficult to manage with the existing antibiotics. Several reviews have proposed different approaches to curb antibiotic resistance over these past years: such as the need to control the fitness cost of resistant bacteria by reducing prescription of antibiotic drugs (Andersson and Hughes 2011), the development of new antibiotics with superior mechanisms over the existing ones and to develop resistance-modifying efflux inhibitors (Stavri *et al.,* 2007) or plasmid-curing compounds (Spengler *et al.,* 2006). Reported examples include the process of phage-killing of conjugative plasmids that bear antibiotic resistance (Ojala *et al.,* 2013), and conjugational delivery of antimicrobial agents via bacterial plasmid (Filutowicz *et al.,* 2008). The fact remains that only a few new antibiotics are coming onto the market or in clinical trials, and none is based on anti-plasmid mechanism; certainly not enough to address the resistance issue over the long term. For major pharmaceutical companies, their level of interest and involvement in antimicrobial drug discovery and market is presently low with negligible interest, owing to the low financial rewards compared to new medicines for other more lucrative areas such as hyperlipidaemia, cancer, arthritis and depression, greatly outweigh the returns associated with new anti-infectives (Gibbons 2008).

However, the challenge remains that the research into new compounds with alternative mechanisms to outsmart resistance mechanisms in bacteria is much needed at a time like this. Given that plasmids are an integral part of antibiotic resistance development and spread, one obvious strategy would be to identify therapeutic compounds that are

present in medicinal plants that can either promote plasmid loss or interfere with bacterial conjugation. In combination with existing antibiotics, compounds that inhibit plasmids by either directly binding to render them inert, or inhibiting their transport systems and coupling proteins involved in the T4SS mechanism, hold the potential to reverse an organism that was originally resistant to become sensitive (Spengler, Molnar *et al.*, 2006). Compounds that could interfere with the T4SS bio-target and in turn inhibit plasmid transfer are promising as T4SS inhibitors and are applicable against other organisms such as *Helicobacter pylori* that utilise such systems to transport effector molecules into host cells.

1.13 Aims and Objectives of the study

The overall aim of this project is to explore potential inhibitors of plasmid DNA conjugal transfer in *E. coli* using both natural and synthetic compounds. In line with the aim, the objectives set to be achieved during this study are broadly grouped in to four:

1. Bacterial conjugation inhibition screening

    i. This involves the screening of crude extracts of *Mallotus philippinensis*, *Cannabis sativa*, *Evodia rutaecarpa*, *Capsicum annuum* and subsequent step-wise screening for active fractions and or pure compounds for their ability to inhibit bacterial plasmid conjugal transfer among *E. coli*.

    ii. Successfully isolated compounds including selected synthetic analogues that were commercially purchased will be assayed against a

panel of clinically relevant plasmids PKM 101, TP114, PUB307, R6K and R7K to determine their anti-plasmid activity.

iii. The effect of time on the rate of inhibition of conjugal transfer of PKM 101, TP114, and PUB307 by the compounds.

2. Bioassay-guided isolation/phytochemical analysis of the active principles

iv. This involved the bioassay-guided isolation of the active fractions from (1) above, identification, characterisation and structure elucidation of the active chemical principles responsible for their bioactivity, using chromatographic and spectrophotometric methods.

3. Determination of the antimicrobial activities (MIC) of the isolated/synthetic compounds against a variety of multidrug resistant Gram–positive and Gram–negative strains.

v. The MIC of the compounds or semi-pure fractions were determined on model *E. coli* NCTC 10418 to determine their sub-inhibitory concentrations (SIC) required for (1) above

4. The DNA binding studies of the compounds using electrophoretic DNA mobility shift assay (EMSA), to determine if the compounds act by interfering with the plasmid DNA

CHAPTER TWO

2.0 MATERIAL AND METHODS

2.1 Phytochemical methods

All plants materials were pre-screened for biological activity by carrying out small-scale extractions (hot and cold) prior to testing the resulting extract.

2.1.1 Extraction

Dried and powdered parts (fruit, resin or fruit powder) depending on the individual plant were exhaustively extracted by cold agitation with solvents in order of increasing polarity (hexane, chloroform and methanol). The solutions were placed over an ultra-sonication bath for 48h In the case of *E. rutaecarpa*; the methanol extract was further subjected to acid-base partitioning in order to obtain the basified extract rich in alkaloids (section 3.0.3). The resulting extracts were dried under vacuum in a rotary evaporator, weighed (Appendix 14) and stored in a refrigerator for further analysis.

2.1.2 Vacuum Liquid chromatography

In natural product chemistry, there is a constant need to separate small and large quantities of complex mixtures efficiently and rapidly using the most cost–effective method. Unfortunately, classical chromatographic techniques cannot meet these requirements and efforts to improve on these techniques include the use of multi-bore columns, flash chromatography, automated systems and dry column chromatography. Thus, Vacuum Liquid Chromatography (VLC) allows for the effective fractionation of a large quantity of material by gradient elution and the flow of which is switched on by a vacuum (Pelletier *et al.,* 1986) The technique employs the use of sintered glass column (60 mm x 120 mm) with a fritted disk and a quick-fit joint which fits

into a 24/29 or 24/40 T-piece adapter with a side arm connected to a vacuum line. The column was packed up to two-third of the total volume with adsorbent (preparative layer chromatography silica kiesegel 60 $PF_{254 + 366}$) and allowed to settle by gentle tapping. The whole experimental setup was under the influence of gravity. The column was conditioned with the first eluent (*n*-hexane), and next, a weighed amount of the plant sample which was mixed with the silica (TLC standard grade, Merck 7749), was transferred onto the surface of the column before further elution with solvents: from 100% hexane to 100% ethyl acetate and methanol solvent in order of increasing polarity. Round bottomed flasks were fitted at the end of the column to collect the fractions; and each of these was dried under vacuum on a rotary evaporator. VLC differs from flash chromatography in that the column is allowed to run dry after each fraction is collected.

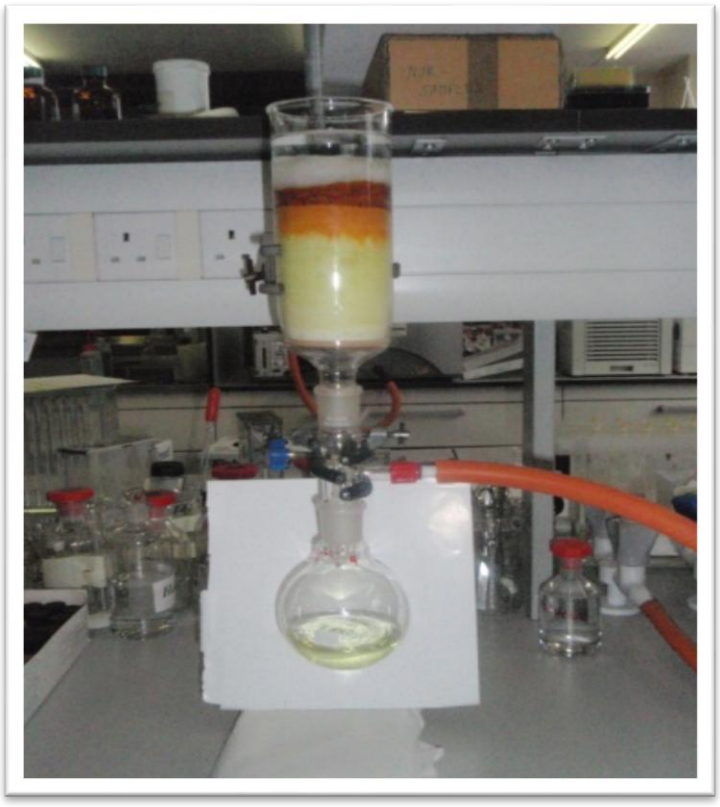


Figure 7: Vacuum liquid chromatography setup

Table 2: Plant materials and sources

Plant material	Family	Area of collection/Source
Mallotus philippinensis (Lam.) Mull. Arg	Euphorbiaceae	Collected by Vaibhav Shinde and Kamlesh Shinde from the premises of Poona College of Pharmacy, Pune, India. The voucher specimen SOPMa001 is deposited at the SOP UCL herbarium.
Cannabis sativa L	Cannabinaceae	Collected from the SOP UCL herbarium storage of *Cannabis* collections (SOPCaS002)
Capsicum annuum L	Solanaceae	Supplied by Herbs in a bottle Ltd, UK
Evodia rutaecarpa (Juss) Benth	Rutaceae	Supplied by Herbs in a bottle Ltd, UK

Table 3: Antibacterial agents/chemicals

Antibacterial agents/chemicals	Source
Tetracycline	Sigma chemicals, UK
Erythromycin	Sigma chemicals, UK
Norfloxacin	Sigma chemicals, UK
Oxacillin	Sigma chemicals, UK
Kanamycin	Sigma chemicals, UK
Streptomycin	Sigma chemicals, UK
Amoxicillin	Sigma chemicals, UK
Nalidixic acid	Sigma chemicals, UK
Actinomycin-D	Sigma chemicals, UK
Ciprofloxacin	Sigma chemicals, UK
DMSO	Sigma chemicals, UK
MTT (Thiazolyl blue tetrazolium)	Sigma chemicals, UK

Antibacterial agents/chemicals continued	Source
Silica (pore size 60 Å, 5-25 μm particle size)	Merck Chemicals, UK
Reverse phase C18 plates	Merck Chemicals, UK
Solid phase extraction cartridges (silica, C18)	Strata Phenomenex products
Solvents (HPLC grade)	Fisher Scientific UK Ltd / BDH Chemicals, UK
Sodium Chloride	Sigma Chemicals, UK
bijou containers (6 mL, 20 mL)	SLS Ltd
96-well plate (flat bottom)	Fisher Scientific UK Ltd
Micropipettes (1mL, 100uL and multipipette)	Gilson Ltd.
Nutrient agar	Oxoid UK Ltd
MacConkey agar	Oxoid UK Ltd
Luria-Bertani Broth (LB)	Oxoid UK Ltd
Muller Hinton Broth (MHB)	Oxoid UK Ltd

2.1.3 Thin Layer chromatography

Thin Layer Chromatography is a technique that can be used to analyse and separate plant extracts qualitatively and quantitatively. It reveals the characteristic constituents of a crude mixture and also the purity of an isolated compound. It operates on the principle of different migration properties of compounds in different solvent systems. The stationary phase is a thin layer of adsorbent material (e.g. silica gel) and is immobilised on an aluminium plate. The mobile phase is a mixture of solvents into which one end of the plate is immersed. Because of capillary action the solvent mixture migrates to the top of the plate. The composition of the solvent mixture and the affinity of compounds for the solvents determine which kind of compounds will travel onto the top of the plate and their rate of ascent. For polar compounds, the solvent mixture needs to have polar properties and *vice versa*. The fluorescent coating on the plates allows visualisation of UV active compounds once viewed under long (λ = 365 nm) and short (λ = 254 nm) wave UV light while colourless compounds are visualized by application of staining reagents. To quantify the migration of the compounds on a particular plate and the solvent system, the R_f value was used and defined as

$$\frac{\text{compounds distance from the origin}}{\text{solvent front distance from the origin}} = \leq 1$$

The R_f values are always ratios and are never greater than 1, the greater affinity the compound has for the adsorbent the smaller the R_f value and *vice versa.*

TLC was performed on all of the extracts. A small amount of sample was dissolved in 50 µl of an appropriate solvent and the sample applied 2 cm from the bottom of a silica gel 60-F_{254} pre-coated TLC plate. The plate was then placed in a tank saturated

with an appropriate mobile phase (v/v). After the separation was complete, the plate was air dried and viewed under UV 254 nm and 365 nm to determine the absorbance and fluorescence properties of the extracts. Substances that quench fluorescence appear dark short wavelength (λ = 254 nm) and those that fluoresce under long wavelength (λ = 356 nm) appear as either bright blue or yellow bands. The plates were sprayed with different detection reagents to visualize the characteristic compounds. The reagent-sprays used in the study were 1% vanillin-sulphuric acid reagent (VS), on *M philippinensis*, *C. sativa* and *C. annuum* to visualise flavonoids, phenolics and Dragendorff's reagent (DRG) on *E. rutaecarpa*, to visualize alkaloids and quaternary amine compounds.

2.1.4 Solid phase extraction

Solid phase extraction (SPE) is a separation technique by which compounds can be separated according to their physical and chemical properties. SPE is an alternative to liquid/liquid extraction, since it is more efficient, rapid and easy to perform. It employs the principle of liquid-solid extraction (de Fátima Alpendurada 2000) and utilises adsorbents that are available commercially in the form of pre-packed cartridges, which are disposable. Separation efficiency is largely a function of sample application flow rate and sample/adsorbent volume ratio. Lower flow rates improve separation but increase run time and if the flow rate is too high, the sample may not have sufficient contact time with the adsorbent. When used for sample clean up, the cartridge either retains the components of interest while the undesired components pass through depending on the mobile and stationary phases. In cases of fractionation, the extract or mixture is adsorbed onto the adsorbent matrix and fractionated. Therefore, it has become the method of choice for the separation of a wide range of compounds.

50 mL cartridges were used in the case of normal phase SPE, while 75 mL C_{18} cartridges were used during the reverse phase SPE.

Figure 8: A solid-phase extraction setup

2.1.5 Silica column chromatography

Various sizes of chromatography columns are used for this simple absorption chromatography technique for routine purification of organic compounds. It is very convenient for large scale separation as well as small scale separation depending on the size of column used. Traditionally, large scale preparative separations are carried out in long column chromatography, although the results are satisfactory, it is time consuming. In recent years, various preparative systems have evolved such as flash column chromatography which reduce separation times to 1-3 h and allow for good resolution of the components on the TLC plate.

The apparatus required consists of a chromatography column and a flow controller valve. The column is a flattened, bottom packed silica mixed in the appropriate solvent to form slurry. A tap is opened so that the solvent can flow through the column and is collected in a beaker or flask at the bottom. The column is never allowed to dry out and fresh solvent is added in such a way to minimise disturbance to the packing material and the sample adsorbed onto the silica.

1g of sample was adsorbed onto silica and packed into the column after packing slurry had settled. The column was equilibrated by washing with 100% of the appropriate solvent (hexane, toluene or chloroform). Separation was achieved by eluting solvents in order of increasing polarity (100% hexane-chloroform- methanol in 1% to 5% increments) under gravitational flow. Eluents were collected in test tubes for further analysis.

2.1.6 High Performance Liquid chromatography (HPLC)

HPLC is a versatile, robust and widely used form of chromatography. Although mainly used quantitatively, it can also yield qualitative information and is commonly used in the isolation of natural products. The sample is applied through an injection port to the top of a tightly packed column (diameter usually around 3-10 μm) through which a predetermined mobile phase is flowing continuously. Separation of sample on a reverse phase is achieved due to differences in the relative affinity for the stationary and mobile phases. Eluting substances were monitored with a suitable detector and the output plotted as a graph with a series of peaks, which correspond to the separated components.

HPLC equipment and conditions used	
HPLC system	Waters 996 photodiode array detector (DAD)
Column	Waters 5 μm Novapak C 18 RP x10 cm
Mobile phase	100% water- 100% methanol (gradient mode)
Flow rate	1 mL/min
Run time	20 min
Injection volume	20 μL
Detection wavelength	280 nm

In this project, HPLC was used only on some fractions of *Evodia* and *Capsicum*. 2 mg/mL solution of the SPE/VLC fractions were analysed by HPLC after filtering through Nalgene 4 mm syringe filters, 0.45 μm, nylon. The same system was used on preparative HPLC at a concentration of 50 mg/mL (injection volume of 1mL) to isolate individual compounds.

2.2 Spectroscopic methods

The full identification of all pure compounds was carried out using various forms of spectroscopy such as: one (^{1}H, ^{13}C, C-DEPT-135) and two dimensional (HSQC, HMQC, HMBC, COSY, NOESY) Nuclear Magnetic Resonance (NMR), and Mass Spectrometry (MS)-fast bombardment (FAB), -electron ionisation (EI), and -electron-spray ionisation (ESI). NMR spectra were recorded on Bruker AVANCE 500 MHz NMR spectrometers. Chemical shifts (δ) were reported in parts per million (ppm) relative to appropriate internal standard and coupling constant (*J* values) are given in Hertz.

2.2.1 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance is a powerful and versatile tool which is employed routinely by chemists in the structure elucidation of organic molecules or to give information on the type of compounds present in an extract (Soininen 2008). The importance in chemistry is attributed largely to the detailed information that can be obtained from it; spectroscopic features correlate with individual atoms rather than as with other techniques such as UV and IR spectroscopy. It is now routinely possible to determine the structures of organic compounds with a molecular mass of less than 1000 Daltons and above.

The phenomenon of nuclear magnetic resonance occurs because the nuclei of certain atoms possess spin and this spin is characterised by the nuclear spin quantum number *I* which may take integer or half-integer values. The most commonly used nuclei are that of ^{1}H and ^{13}C. ^{1}H nucleus has almost 100% natural abundance and is one of most sensitive nuclei in NMR.

NMR spectra were recorded on Bruker AVANCE 400 and 500 MHz spectrometers, and samples dissolved in deuterated chloroform, methanol or water. The spectra were calibrated accordingly to the solvent peaks and the spectra processed using TopSpin Bruker software.

Solvent	^{1}H peak(s) ppm	^{13}C peak ppm
Chloroform	7.26	77.2
Methanol	4.87, 3.31	49.1
Water	4.8	

2.2.1.1 Proton NMR (^{1}H)

The ^{1}H NMR spectrum gives information on the number of protons present in a compound and their chemical environment. It will also give an indication of the purity of a sample. Integration of the spectrum using the area under the peaks reveals the relative number of protons associated with each peak. The resonance of each proton is given a chemical shift value δ which is reported in parts per million (ppm). When the nucleus is in a shielded (electron donating) environment, a higher field strength is required to cause resonances resulting in low δ values. Therefore, the ppm indicates whether the protons are in a deshielded (high ppm) or shielded (low ppm) environment. The proton spectrum reveals the types of functional groups likely to be present, for example, the proton of an aldehyde group gives a characteristic signal at 9.5-10.5 ppm and aromatic protons resonate between 6-8ppm. Methyl groups in a shielded environment give signals around 0.17- 1.5 ppm. Coupling constants known as *J* values can reveal the position of protons in respect to each other, for example whether they are in *ortho* or *meta* position in an aromatic ring or in the case of a double bond, whether they are *cis* or *trans* to each other.

The proton spectrum can reveal considerable information about a sample and can be used as a dereplication tool, for example, to identify samples containing undesired chemicals groups or types of compounds, i.e impure compounds. Where the proton spectrum indicated a pure compound, full NMR spectra, both one- and the two-dimensional were acquired to determine the structure.

2.2.1.2 Carbon-13 NMR (^{13}C)

Carbon NMR uses the comparatively rare ^{13}C isotope as ^{12}C is not magnetic. Two types of carbon spectra were acquired. The broadband decoupled carbon spectrum gives a peak for each carbon atom present in a compound. The peak is a singlet, since

the protons are decoupled during the experiment. The ^{13}C DEPT-135 (Distortionless Enhancement by Polarisation Transfer) spectrum only shows a signal for carbons with a proton attached which makes for easy identification of methane, methylene and methyl groups attached to the carbons. Comparison of the two spectra will reveal quaternary carbons, which do not show in up in the DEPT experiment.

2.2.1.3 Two-dimensional spectra

The ^{1}H and ^{13}C experiments are referred to as one-dimensional techniques displaying the data along the x and y axes. A two-dimensional spectrum shows experimental data additionally on the z axis, which is the intensity of the signal. The data may be from the same experiment, or from the separate experiments, for example proton data along the x axis and carbon data on the y axis.

2.2.1.4 Correlation Spectroscopy (COSY)

The COSY technique provides information on proton coupling. It is described as homonuclear as it acquires only data from hydrogen nuclei. However, the data is displayed along three axes on the spectrum, and it is therefore a two-dimensional technique. The chemical shift data is plotted on the diagonal and proton coupling is shown by cross peaks which lie off the diagonal axis, indicating protons which are only two-to four bonds from each other.

2.2.1.5 Heteronuclear Single Quantum Coherence (HSQC) spectroscopy

This technique is heteronuclear as it uses data acquired from both proton and carbon nuclei. An HSQC spectrum reveals which protons are attached to which carbons.

Quaternary carbons will not give a signal since they do not have any protons attached.

2.2.1.6 Heteronuclear Multibond Quantum Coherence (HMQC) spectroscopy

The HMQC technique provides long-range correlation information on protons and carbons which are separated by two or three bonds. From the information given in the HMBC spectrum, the structure of the compound can begin to be assembled. This is done by assembly of partial fragments of the compounds.

2.2.1.7 Nuclear Overhauser Effect Spectroscopy (NOESY)

The NOESY spectrum is another homonuclear technique involving proton data only. It gives information on the spatial proximity of protons; therefore, protons which are not close together in terms of bond distance, but are close together in space may give a signal. The NOESY technique can therefore facilitate in assigning the relative stereochemistry of a compound.

2.3 Biological Methods

All bacterial strains (Table 4) were obtained from Dr Paul Stapleton; cultured on nutrient agar slopes and incubated for 24 hours at 37^{o}C prior to MIC determination. An inoculum turbidity equivalent to tube a 0.5 McFarland standard (1 x10^{8} cfu/mL) was prepared in normal saline for each test organism, and then diluted 1:100 in Mueller-Hinton broth just before inoculation of the plates.

2.3.1 Minimum inhibitory concentration (MIC)

100µL of sterile Mueller-Hinton broth (MHB; Oxoid) containing 20 mg/L and 10mg/L of Ca^{2+} and Mg^{2+} respectively, was dispensed into 11 of the wells of a 96-

well microtitre plate (Nunc, 0.3 mL volume per well). All antibacterial agents apart from norfloxacin were dissolved in dimethylsulphoxide (DMSO) and diluted in MHB to give a stock solution of 2048 mg/L. 100µL of stock was serially diluted into each well and then 100µL of the bacterial inoculum was added to each well to give a final concentration range of 512 - 1 mg/L in the wells. All procedures were performed in duplicate and the plates incubated for 18 hours at 37^{o}C. 20µL of a 5 mg/L methanolic solution of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well and incubated for 30 minutes. A dark purple colouration indicated bacterial growth. The MIC was recorded as the lowest concentration at which no colouration was observed.

Table 4: Bacterial strains/materials

Bacterial strain	Resistance determinants
SA1199B	Over-expresses the NorA multidrug resistance efflux pump
RN4220	Over-expresses MsrA protein pump
XU212	Possess the TetK tetracycline pump
EMRSA-15, EMRSA-16,	Possess mec genes
ATCC 259232	Standard strain
MRSA 346724	Multidrug resistant *S. aureus*
MRSA 774812	Multidrug resistant *S. aureus*
MRSA 274829	Multidrug resistant *S. aureus*
MRSA 12981	Multidrug resistant *S. aureus*
SA13373	Multidrug resistant *S. aureus*
Enterococcus faecalis 13379	Gram-positive
Enterococcus faecalis 12697	Gram-positive
Bacillus subtilis BsSOP01	Gram-positive

Bacterial strain continued	
E. coli NCTC 10418	Gram-negative,
Pseudomonas aeruginosa 10662	Gram-negative
Klebsiella pneumoniae 342	Gram-negative
Proteus sp. P10830	Gram-negative

2.3.2 Plasmid Conjugation Inhibition Assay

The plasmid conjugation inhibition assay was performed by the broth mating method described by (Rice and Bonomo 2005) with some modifications. The plasmids used for the study, their resistance markers, and respective hosts are listed in Table 5. Mating between the plasmid-containing donor strain *E. coli* K12 J53 and the recipients *E. coli* ER1793 and *E. coli* JM109 was performed in LB broth. The independent overnight cultures of the plasmids were inoculated into 5 mL fresh Luria broth and incubated overnight with shaking at 37^{o}C. Donor and recipient cultures were mixed 1:1 in 100 μL LB with 100 μg/mL of each of the compounds to a final volume of 200 μL and incubated overnight at 37^{o}C. For time course experiments, overnight cultures were mixed and mated in the presence of the drug and incubated for the time periods stated. Only the bacteria that have successfully taken up the resistance gene become resistant and will grow despite the effect of compounds.

The overnight mating cultures were serially diluted from well 10^{-1} to10^{-7} and transconjugants were identified by plating wells 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} bacterial mixtures onto selective media containing the appropriate antibiotics; wells 4 and 5 mixtures onto streptomycin (to select for the recipient) plus either amoxicillin (to detect transfer of PKM101 or PUB307) or kanamycin (to detect transfer of TP114); and plate wells 6 and 7 serial diluted mixtures onto single antibiotic plates of either kanamycin or amoxicillin. Concentrations of the antibiotics used were 30 mg/L (amoxicillin) and 10 mg/L (kanamycin). Transfer frequency is calculated as number of transconjugants (cfu/mL)/ total number of donor (cfu/mL) equals to a transposition frequency as a fraction of 1. Inoculum counts for each strain were plated from wells 6 and 7 to determine the cfu/mL(colony forming unit/mL) of starting cultures.

Statistical analysis

In order to determine the overall significance level and measure of variability that may exist within the sample populations. The MIC determination was repeated twice with replicates on different days, while the plasmid broth mating assay was repeated three times with replicates. Each replicate was used as a data point n=6 and an average mean of the results were determined. The data were expressed as mean $\pm$ standard deviation (SD). Differences between two mean values were calculated by the Student's t-test. The chosen level of significance for all statistical tests was $P>0.05$. Overall data were represented in tables, bar and line graphs.

The summary of plasmids that were assayed, the resistance markers and their respective hosts are listed in Table 5.

Table 5: Plasmid strains used, host and resistance markers

Plasmid	Molecular weight	Incompatibility group	Host	Resistance marker(^{r})
TP114	62.1Kb	IncI2	*E. coli* K12 J53	Km^r
PKM 101	35.4kb	IncN	*E. coli* WP2 uvrA	Ap^r
PUB 307:RP1	56.4Kb	IncP	*E. coli* K12 J53	Ap^r, Km^r, Tet^r
R6K	39.4Kb	IncX	*E. coli* K12 J53	Ap^r, Sm^r
R7K	30.3 Kb	IncW	*E. coli* K12 J53-2	Ap^r, Sm^r, Sp^r
R1-drd-19	93.9 Kb	IncF11	*E. coli* K12 J53	Ap^r, Cm^r, Km^r, Sm^r, Sp^r, Su^r
E. coli ER1793	Recipient			Sm^r
JM 109	Recipient			Nal^r

Km^r = kanamycin, Ap^r =ampicillin, Tet^r = tetracycline, Sm^r = streptomycin, Sp^r = spectinomycin, Cm^r = chloramphenicol, Su^r = sulphonamide, Nal^r = nalidixic acid

Table 6: Materials needed for DNA electrophoresis

pAKlux3, PKM 101	Collected from Dr Paul Stapleton
HpaI	Sigma, UK
SphI	Sigma, UK
Lamba DNA 111	New England Biolabs,UK
Agarose gel	Sigma UK
TAE (Tris-acetate-EDTA)	Sigma, UK
TBE (Tris-borate-EDTA)	Sigma, UK
Electrophoresis gel box with power pac	Bio-Rad
Ethidium bromide	VWR International
Phenol blue dye	Sigma UK
Loading/elution buffer	Qiagen, UK
G:BOX transilluminator with syngene software	Syngene LTD
QIAprep Spin Mini and midi prep Kit	Qiagen, UK

2.3.3 DNA mechanistic studies

2.3.4 Electrophoretic Gel Mobility Shift Assay (EMSA)

In free solution, DNA moves under the influence of an electric field in a way that is independent of its shape and molecular mass but dependent on charge. When this movement takes place in a gel, the speed of migration becomes dependent on size and shape as well as charge of DNA. The pH of the gel is important and is maintained at a relatively constant value of 7.5 - 8.0 by the presence of buffers. The role of buffers is to conduct electricity and ensure that the DNA molecules at the pH become ionized and negatively charged and will migrate as desired. This permits a high resolution and good separation of the DNA sample. When a power supply is connected, the negatively charged DNA fragments that are loaded into the sample well at the cathode end of a gel move through the gel towards the anode. The location of the DNA within the gel can be determined by staining with low concentration of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1-10 ng of DNA can be detected by direct examination of the gel under ultraviolet light. Electrophoresis is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation (Sidorova *et al.,* 2005)

The materials that were used for the agarose gel electrophoresis assay are found in Table 6. Agarose gel electrophoresis for plasmid DNA was performed by mixing 1g of dry agarose powder in 110 mL of Tris-acetate-EDTA (TAE) aqueous buffer and heating in a microwave oven for about 2 mins at P-80 until a clear solution is obtained. After cooling to about 50^{o}C, the gel was poured into a casting tray; the sample comb inserted and allowed to solidify at a room temperature. The staining of

the gel is done after the gel is run in order to avoid ethidium bromide binding to the DNA, i.e. the ethidium might block binding of the compounds to DNA. Prior to the running of the gel, 2-4 μL of plasmid DNA was first incubated with 8 μL of the test compounds in their various concentrations (10 or 100 μg/mL), 1 μL of elution loading buffer and 1uL of bromo-phenol blue. A mixture of a total volume of 10 -12 μL was usually loaded slowly into the wells, while the reference ladder DNA was loaded at both extreme well of the gel submerged in 1% TBE buffer. The ladder is a set of DNA fragments of known size that is used to estimate the sizes of the unknown fragments of the plasmid DNA that were separated. The electrophoresis gel box (Pharmacia Biotech GNA-200 submarine unit with Power Pac 300 power supply, Bio-Rad), was then switched on and allowed to run at a constant voltage of 40 - 65V for 2 hours or more until the dye was approximately 75-80% of the way down the gel. The gel was subsequently stained in a container of ethidium bromide for 30 min to visualise the location of the DNA fragments. Notably, ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels. The visualisation was done with G: BOX gel documentation system (Syngene), and images were inverted (black to white) with GeneSnap software (Syngene). Illumination with ultraviolet light causes the intercalated dye to fluoresce with a pale pink colour.

2.3.5 Isolation and purification of the plasmid DNA

E. coli J53 harbouring plasmid PKM101 was grown with shaking (New Brunswick incubator; 165 rpm) for 16h at 37°C in Luria-Bertani broth containing amoxicillin (50 μg/mL). Plasmid PKM 101 was extracted using an alkaline lysis procedure and the resulting DNA purified by adsorption chromatography (QIAprep Spin Miniprep Kit,

Qiagen). The stepwise protocol of plasmid purification using Qiagen mini kit is as follows:

A.) Bacteria culture, harvest and lysis

Four vials of each 25 mL of PKM 101 overnight cultures were pelleted using Biotech centrifuge 6000 x *g* for 15 mins at 4^{o}C, after which they were combined into two vials. The harvested bacterial pellet was resuspended in buffers, PI, P2 and P3 accordingly as stated in the kit manual.

B). The bacterial lysate mixture was centrifuged at $\geq$20,000 x *g* for 30 mins at 4^{o}C, and the supernatant re-centrifuged at $\geq$20,000 x *g* for additional 15 mins at 4^{o}C.

C). The supernatant was carefully pipetted on onto an already equilibrated the QIAGEN tip- column by applying 4 mL of QIAGEN buffer QBT (equilibration buffer) and allowed to empty by gravity flow. After the supernatant is applied unto the column, it was allowed to enter the silica resin by gravity flow, and the column washed twice with 2 x 10 mL of buffer QIAGEN QC (wash buffer). The buffer is allowed equally to elute by gravity flow. This was followed by elution of the DNA by applying 5 mL of buffer QIAGEN QF (elution buffer) into a clean 15 mL vial. The DNA was precipitated by adding 3.5 mL of isopropanol to the eluted DNA and mixed carefully. The resultant mixture was centrifuged at $\geq$15,000 x *g* for 30 mins at 4^{o}C and carefully decanted. The DNA pellet was washed with 2 mL of 70% ethanol and centrifuged at $\geq$15,000 x *g* for 10 mins and decanted carefully. The final DNA pellet was allowed to stand and air-dried for 5 - 10 mins, then dissolved in 1 mL of elution buffer and stored at 4^{o}C, for future use.

2.3.6 Restriction Endonuclease Fragmentation Assay

Restriction endonucleases bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition site. For example, the restriction enzyme *ApaI* recognizes the sequence 5´...GTGCAC...3´. The recognition site of the restriction enzyme *HpaI*: C C G G and *SphI*: 5`…G C A T G C…3` can therefore cut the plasmid DNA into smaller DNA fragments of known sizes. A DNA ladder or size meter run on the same gel and can be used to determine by comparison of the fragments of known sizes to estimate the sizes of the unknown DNA fragments. The restriction enzymatic activities are strongly dependent on the local DNA conformation at the different restriction sites.

10 µg of PKM101 was digested with *Hpa1*-DNA restriction endonuclease (Fischer Scientific; 10U for 2h at 37°C) before purification using QIAquick PCR Purification Kit, Qiagen. The expected fragment sizes of *HpaI*-digested PKM101 are: 7.8, 6.7, 5.3, 4.6, 3.7, 2.7, 2.6 and 2.0 kb (Langer et al, 1981). The restriction digestion of plasmid pAKlux3 was also conducted with *SphI*. The expected fragment sizes of *SphI*-digested pAKlux3 are: 8.03, 2.86 and 1.07 Kb.

2.3.7 Binding of the digested DNA with the compounds

Binding of each of the test compounds (final concentration of 10x MIC) to 0.5 µg of *HpaI*-digested PKM 101 DNA was performed at 37°C for 1h in a total volume of 20 µL. Samples were electrophoresed in 1 x TAE buffer in an 0.8% agarose gel at a constant voltage of 65V for 1.5h. The binding of the test compounds to the DNAs in the absence of the restriction enzymes were also prepared. All the solutions were incubated at 37 °C for 1 h; and the samples were analysed by agarose gel electrophoresis.

The gel was subsequently stained with ethidium bromide (VWR International) for 30 minutes to visualise the location of the DNA. Images of the DNA profiles were captured with a G: BOX gel documentation system (Syngene). Images were inverted (black to white) with GeneSnap software (Syngene).

CHAPTER THREE

RESULTS

3.0 Bioassay-guided Isolation and Purification

3.0. 1 *Mallotus philippinensis*

Preliminary screening of the initial three plant extracts: hexane (A), chloroform (B) and methanol (C), indicated that only the chloroform extract had appreciable biological activity. The three fractions were subjected to both antibacterial and anti-plasmid assays after fractionation as a guide to aid isolation of active compounds.

3g of the chloroform extract was fractionated using VLC with an increasingly polar gradient of 100% hexane to 100% ethyl acetate and 100% methanol, which yielded 21 fractions. These fractions were monitored by TLC using the solvent system containing hexane-ethyl acetate-formic acid (4:6:1) solution. The spots were visualised by long (365 nm) and short (254 nm) wavelengths (Figure 9) as well as spayed with 1% vanillin-sulphuric acid and heated until a colouration was observed.

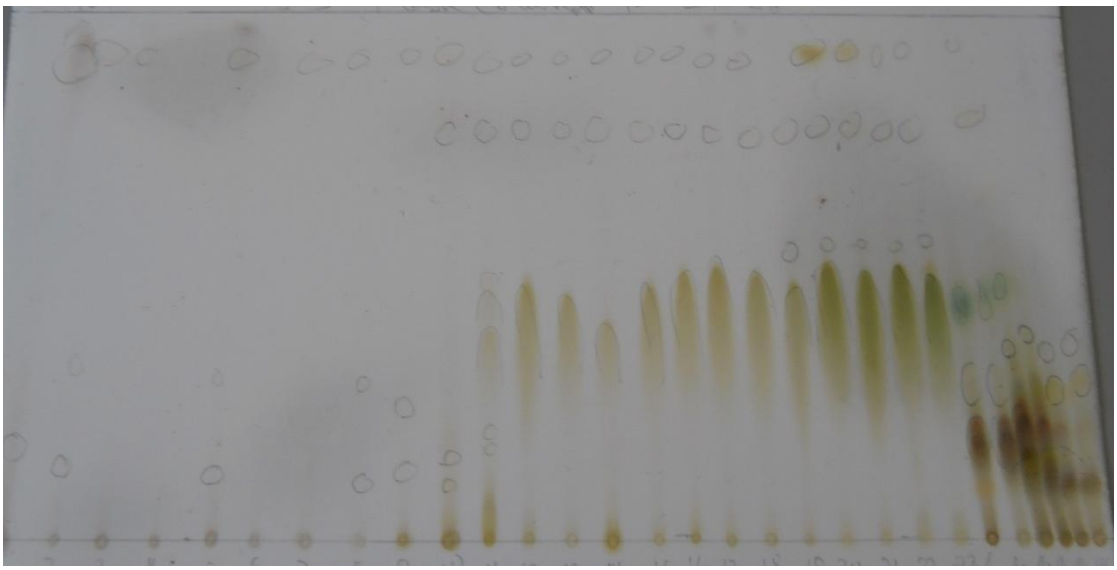


Figure 9: TLC of VLC fractions using the solvent system containing hexane-ethyl acetate-formic acid (4:6:1) solution

The TLC profile of the extracts was dominated by purple and yellow bands which appeared at the different R_f values. Less prominent violet/purple spots also appeared at the top of the plate indicating the presence of nonpolar components which were fluorescent under long UV light. This implied that the extracts constituted flavonoids and phenolic compounds. Similar fractions were pooled together into 10 fractions F, G, H, I, I_2, J, K, M, O, P and were tested for antibacterial activity against SA1199B and XU 212 and anti-plasmid activity against PKM101. Fractions F and H were inactive against the bacteria while fraction G was active only at 512 mg/L. The other fractions I-P were all active, Fr-K was most potent fraction at 8 mg/L against SA1199B and 2 mg/L against XU212 (Table 7). Fractions J and K were the only fractions with anti-plasmid activity, so both were pooled to gain enough material (10.9mg) to subject to column chromatography using 100% toluene and 100% acetone.

All fractions obtained were monitored by TLC (Figure 10), and those that showed single spots were submitted to NMR analysis which yielded a pure compound, BM-1.

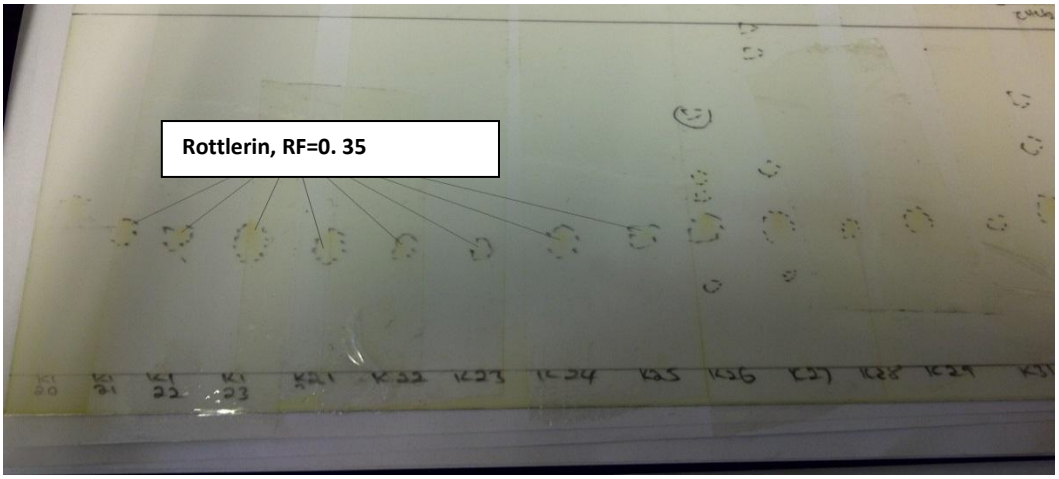


Figure 10: TLC profile showing the R_f value of rottlerin at 0.35 (hexane-ethyl acetate-formic acid (4:6:1) solution

The other samples did not show anti-plasmid activity, because of that, they were not further fractionated. The hexane extract did not show any significant antiplasmid activity but antibacterial activity was observed. Compound BM-2 was isolated purified for phytochemical and antibacterial studies. Notwithstanding, all isolated compounds were re-tested for antibacterial and antiplasmid activities.

Table 7: MICs (mg/L) of norfloxacin and VLC fractions of *M. phillipinensis* against MRSA

	SA1199B	XU212
Norfloxacin	32	16
Mat Fr-F	>512	>512
Mat Fr-G	512	>512
Mat Fr-H	>512	>512
Mat Fr-I	512	512
Mat Fr-I-3	64	32
Mat Fr-J	64	16
Mat Fr-K	32	8
Mat Fr-M	32	32
Mat Fr-O	128	64
Mat Fr-P	128	64

3.0.2 *Cannabis sativa* L.

Only the chloroform extract of this plant showed anti-plasmid activity. 5g of the chloroform extract was fractionated by VLC using a gradient solvent system from 100% chloroform to 100% ethyl acetate in 5% increments, then up to 50% methanol in ethyl acetate in 10% increments, (50% methanol in ethyl acetate) and a final wash of 100% methanol. This yielded 17 fractions that were pooled to give 9 fractions for TLC analysis.

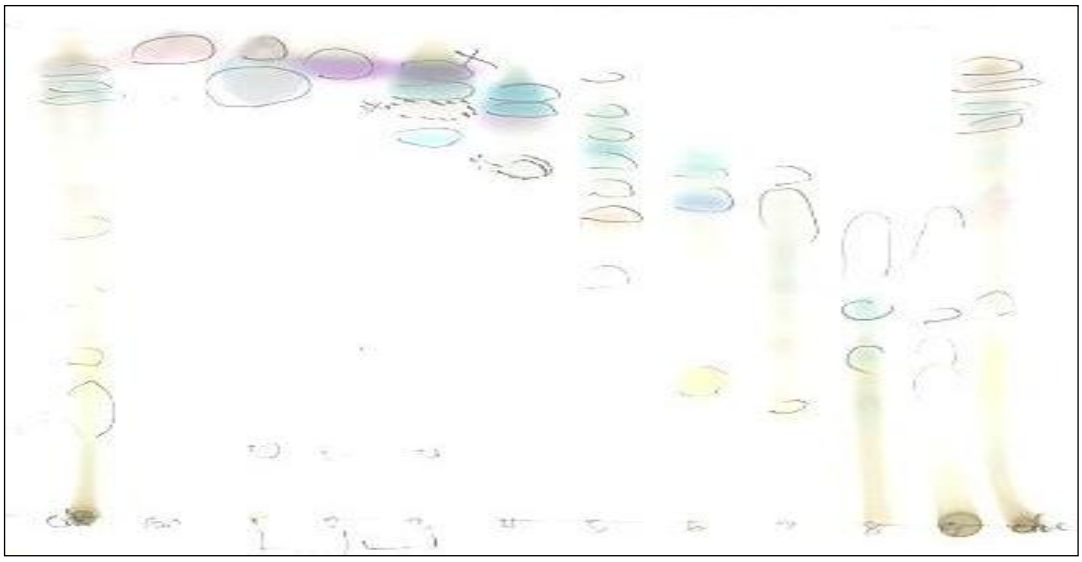


Figure 11: TLC of *C. sativa* fractions developed in a solvent mixture of hexane and ethyl acetate (6:4).

The TLC profile (Figure 11) was developed in a solvent mixture of hexane and ethyl acetate (6:4), which showed a variety of purple, blue and light yellow spots on the TLC plate after spray with 1% vanillin-sulphuric acid and heating until a colouration was observed indicative of phenolic compounds. Only fraction 9 showed anti-plasmid activity against TP114. Fraction 8 and 9 were pooled because they had similar TLC profiles and an SPE separation was carried out eluting with toluene, chloroform and

finally 100% methanol. This yielded 10 fractions monitored on silica TLC plate with a solvent system of chloroform/toluene (6:4) (Figure 12).

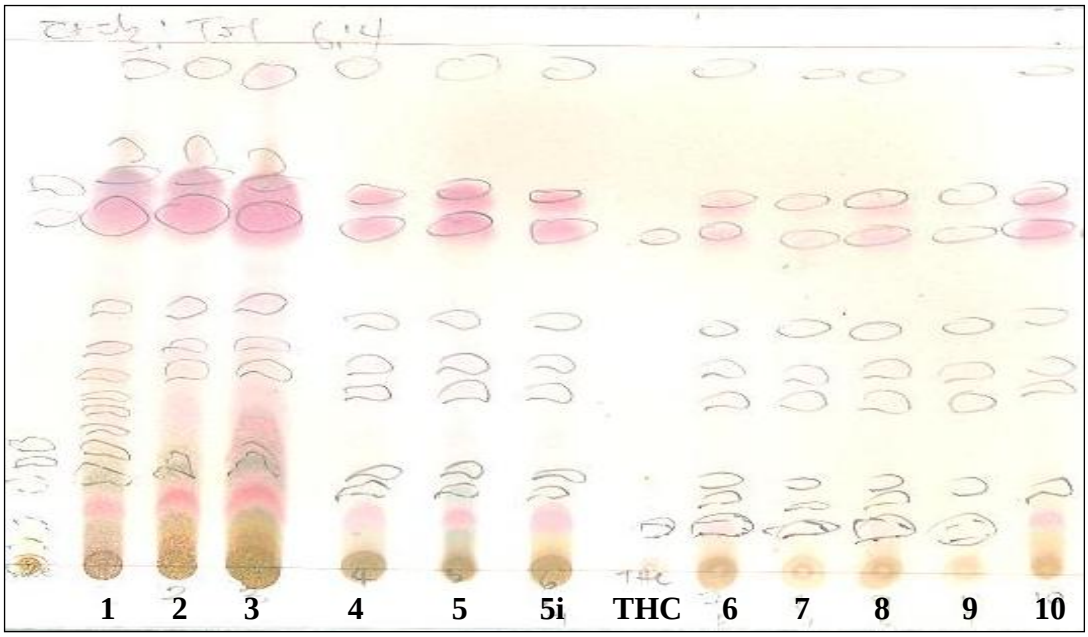


Figure 12: TLC using solvent mix of chloroform and toluene (6:4) of SPE fractions of pooled 8&9 fractions.

The combined fractions 1-3 were further isolated in appreciable quantity, with the aid of column chromatography eluting with 100% chloroform to 100% methanol. The resulting fractions were monitored by TLC using solvent mix of chloroform and methanol (95:5) (Figure 13) as to note which of the fractions showed minimal number of spots and as such fractions 4-8 were submitted for NMR analysis, to yield BM-3 (fr 4), BM-4 (fr 5) (Figure 13). Fraction 6 was a complex mixture and the NMR profile is presented in Appendix 1. Fractions 7 and 8 were produced in very small amounts that were not detected by the NMR.

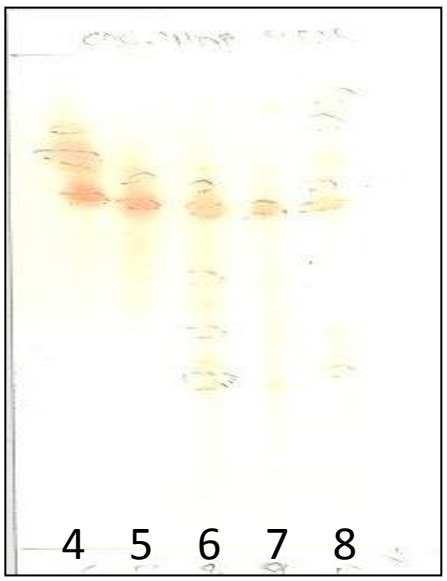


Figure 13: TLC of fractions 4, 5 and 6 developed in solvent mixture of chloroform and methanol (95:5)

3.0.3 *Evodia rutaecarpa*

10g of the methanolic extract of *E. rutaecarpa* was dissolved in methanol (10mL) and poured into a separating funnel. 200mL of 0.05N sulphuric acid was added, followed by 200mL of 30% ammonia and stir vigorously. After shaking and allowing for phase separation, the lower phase containing the compound of interest is collected into a 500mL beaker. Using a different separating funnel, the collected solution was washed successively four times with chloroform (50mL), and with each wash, the organic bottom was collected and combined together. The bulk solution was poured into a round bottom flask and evaporated using the rotary evaporator. The extract obtained weighed 1.2g and was assayed for antiplasmid activity (Appendix 11). The basified (EvB) extract exhibited antiplasmid activity against plasmid TP114 as seen in appendix 11, and was taken for further separation and isolation of the active compound using VLC, which yielded 11 fractions and monitored by TLC in the

solvent system hexane: ethyl acetate (6:4) in figure 14. The spots were visualised under long and short wave UV light as well as spraying with Dragendorff's reagent and heating until a colouration was observed. Dominant yellow colours were observed (Figure 14). VLC fractions 5, 6 and 7 were active against TP114 plasmid however; they were in very small amounts and were not processed further except for fraction 6. Fraction Ev-6 (56 mg) was fractionated by silica column chromatography (toluene: ethyl acetate) to obtain 2 compounds BM-5 (toluene: ethyl acetate 9:1) and BM-6 (toluene: ethyl acetate 8:2). Fractions 7 and 8 were pooled to afford Ev-8 and subjected to HPLC analysis (reverse phase adsorbent, gradient elution of water-methanol (90:10/0-10 min) and (95:5/10-20 min, flow rate of 1 mL/min) to give compound BM-7.

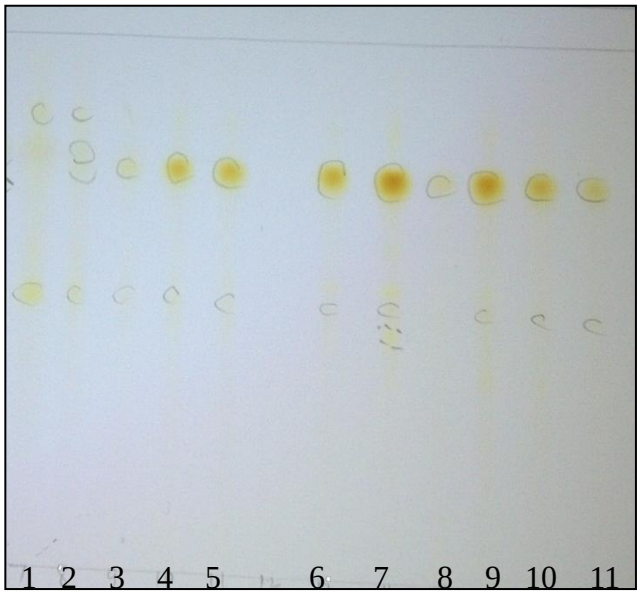


Figure 14: TLC of VLC fraction of alkaloids in basified extract of *Evodia* in hexane: ethyl acetate (6:4)

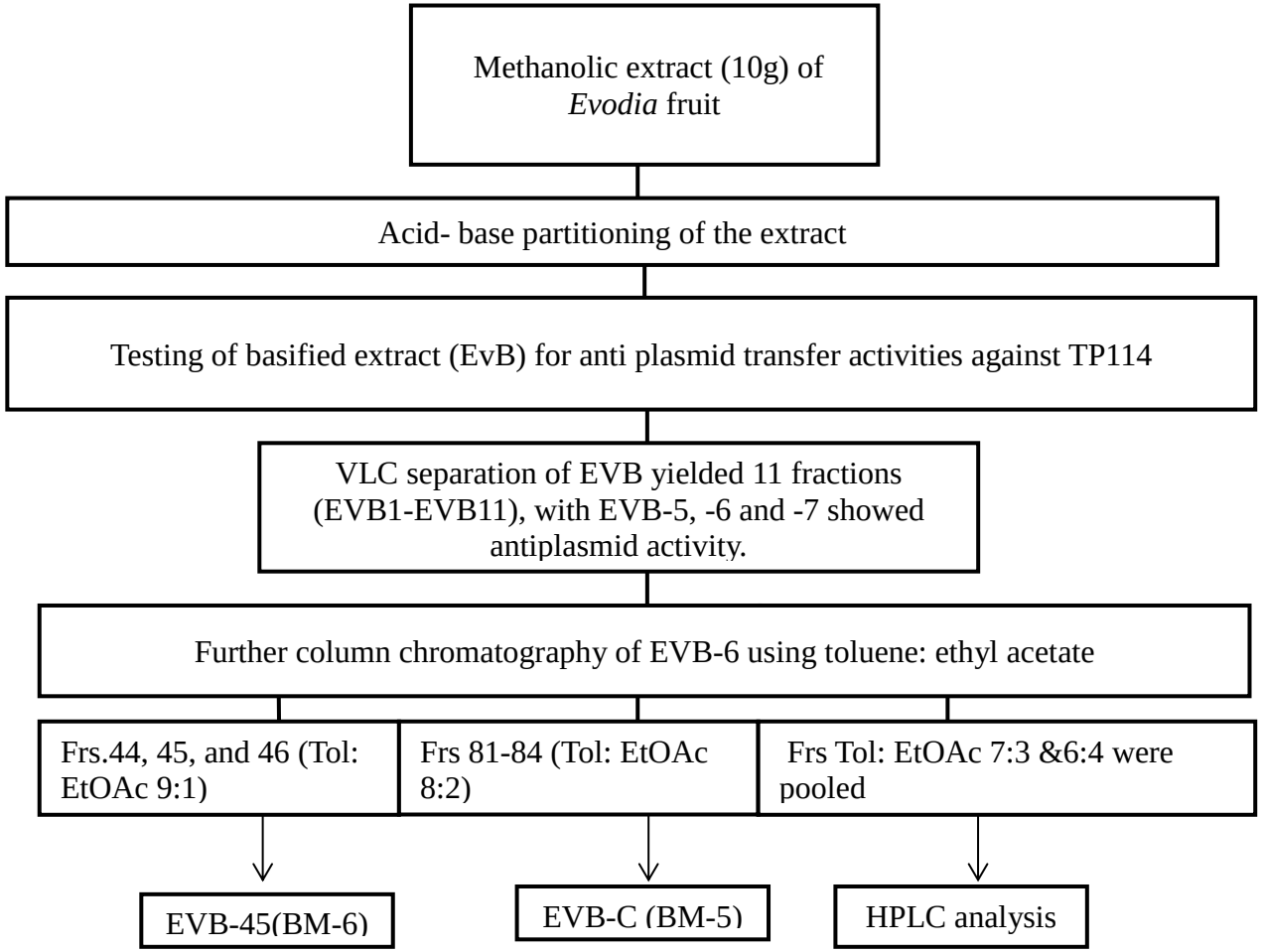


Figure 15: Isolation and purification scheme for alkaloid extract of *Evodia*

3.0.4 *Capsicum annuum* L.

700mg of *C. annuum* methanol extract were fractionated using solid phase extraction as described above. A step gradient from 100% distilled water to 100% methanol was applied as eluent system, followed by a step gradient up to 100% ethyl acetate in order to wash off all the material from the column. The fractions Ca-SPE 1 to Ca-SPE 6 were analysed using reverse phase TLC and the solvent mixture of methanol and water in the ratio 8:2 with a few drops of acetic acid to facilitate the separation. After the separation was completed, the plate was viewed under UV 254 nm and 365 nm.

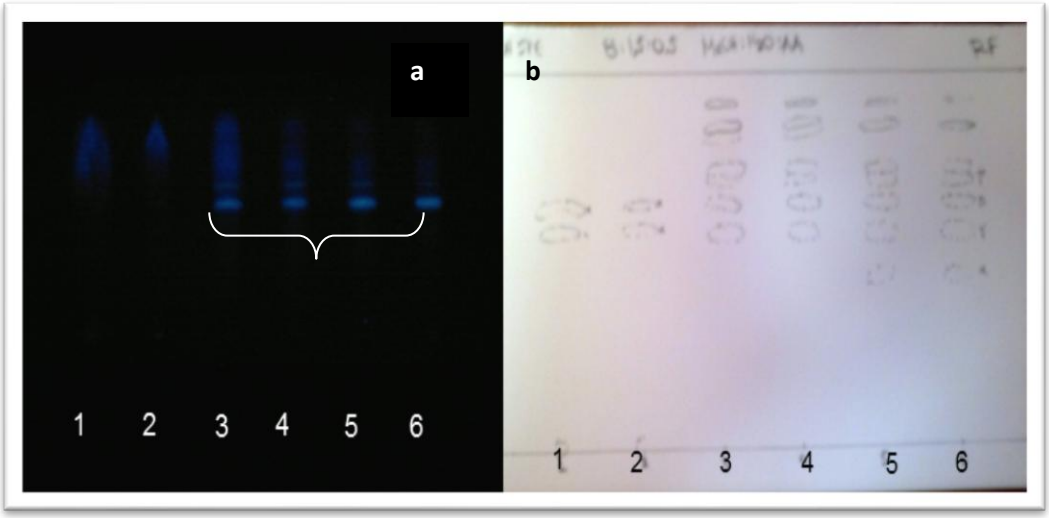
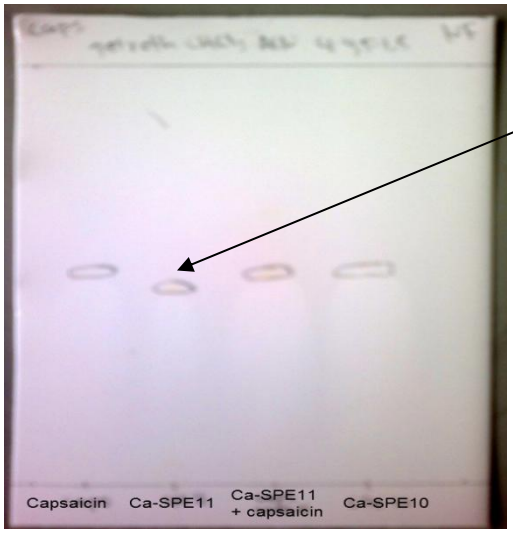


Figure 16: Reverse phase TLC profile of Ca-SPE 1 to Ca-SPE 6 fractions from *C. annum* methanol extract in methanol and water (8:2) viewed under (a) long wave 365 nm and (b) short wave 254 nm.

The TLC (Figure 16) revealed that fractions Ca-SPE 1 and Ca-SPE 2 had similar chemical profile and thus they were combined to generate fraction Ca-SPE A. Likewise, Ca-SPE 3, Ca-SPE 4, Ca-SPE 5 and Ca-SPE 6 appeared to contain similar compounds and they were combined into one fraction named Ca-SPE B. A second TLC was applied to fractions Ca-SPE 1 to Ca-SPE 10 using reverse phase TLC in methanol and water (9:1) as the solvent system with a few drops of acetic acid, which

showed that fractions Ca-SPE 8 and Ca-SPE 9 had similar chemical constitution and therefore they were combined to form Ca-SPE C.

All the SPE fractions of the methanol extract of *C. annuum* were evaluated for their ability to inhibit the conjugal transfer of TP114, using the broth mating assay (section 2.3.2). Subsequently, the active fractions were additionally tested for their effect on the transfer frequency of PKM 101 and PUB 307. A reference sample of pure capsaicin, obtained from Sigma-Aldrich UK was also tested along with the fractions, since this compound is considered the major active constituent of the plant. Among the fractions, Ca-SPE 11 exhibited the highest activity against the transfer of TP114 (Appendix 12a, 12b and 12c), followed by fractions Ca-SPE 12, Ca-SPE 10, Ca-SPE B and Ca-SPE A. All these fractions were submitted for proton NMR, which indicated that Ca-SPE 10 as a pure compound, BM-8. The ^{1}H NMR spectrum of Ca-SPE 11 (Appendix 9) revealed the presence of major signals of capsaicin in a mixture of other compounds. In order to determine if capsaicin was present in Ca-SPE 11, TLC was employed using the solvent system petroleum ether/chloroform/acetonitrile (4:4, 5:1, 0.5) and sprayed with vanillin-sulphuric acid spray.



Ca-SPE 11 had R_f value of 0.39, which is lower than capsaicin combined with Ca-SPE 11 and Ca-SPE 10 with R_f

Figure 17: TLC analysis of Ca-SPE 10, -11, capsaicin + Ca-SPE 11 combined in petroleum ether/chloroform/acetonitrile (4:4, 5:1, 0.5)

From the TLC profile (Figure 17), it was possible to visualise the various spots and all samples recorded identical R_f values of 0.45, confirming the presence of capsaicin in combined fraction Ca-SPE 11 + Capsaicin, except for pure capsaicin with R_f value of 0.39. This indicated that the mixed fraction contained capsaicin and possibly the presence of another capsaicinoids. Thus, CA-SPE 11 was further subjected to analytical HPLC for separation of the capsaicinoids.

The high performance liquid chromatography of Ca-SPE 11 was conducted with mobile phase solvent mixture of methanol-water from A-B 30% - 70% to A-B 70% - 30% for 20 minutes. The flow rate was 1 mL/min. With gradient elution, the standard solution contained capsaicin and dihydrocapsaicin while samples 2 and 3 were two different fractions of Ca-SPE 10. Sample 4 was Ca-SPE 11 for evaluation.

3.1 Phytochemical analysis of selected medicinal plants

3.1.1 Mallotus philippinensis

Compound BM-**1**, (1.3mg) was isolated from the chloroform extract of *M. phillipinensis*. The ^{1}H, ^{13}C, $^{13}CDEPT135$, ^{1}H-^{1}H COSY, NOESY, HMQC and HMBC experiments were used as the basis for structure elucidation. The isolated compound was a known compound named rottlerin and its structure was confirmed by careful analysis of its NMR and mass spectra. The molecular formula was determined as $C_{30}H_{28}O_8$ and calculated as an ion of *m/z* 516.18 which corresponded to the ESI-MS *m/z* 515.17 $[M-H]^+$ (Appendix 2).

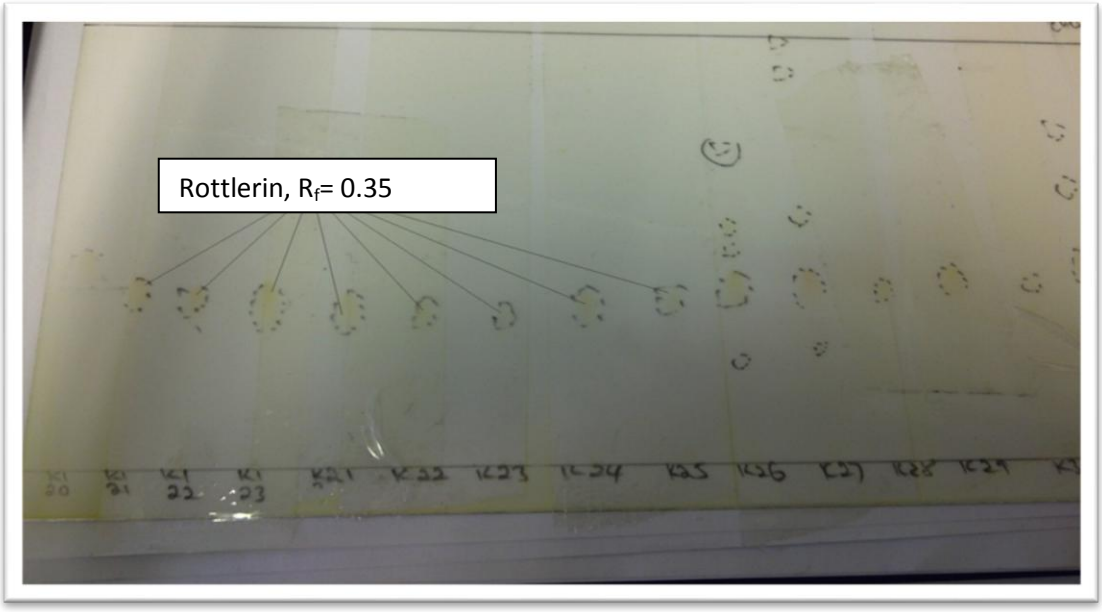


Figure 18: Silica TLC profile showing the R_f value of rottlerin at 0.35 (hexane-ethyl acetate-formic acid 4:6:1)

The TLC of rottlerin in solvent mixture of hexane and ethyl acetate-formic acid in the ratio of 4:6:1, showed an R_f value of 0.35 (Figure 18).

The structure of rottlerin is represented in Figure 19.

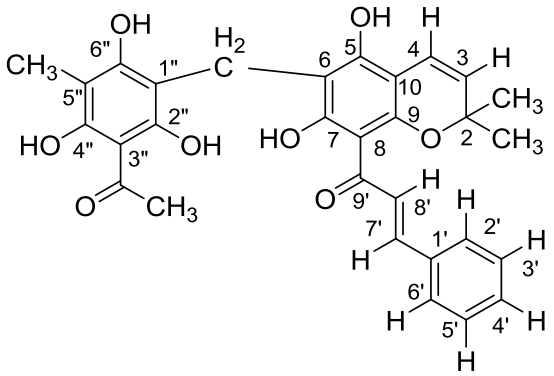


Figure 19: Structure of rottlerin.

The ^{1}H spectrum of rottlerin as depicted in Figure 20 recorded 28protons with the signals δ1.54, 2.08 and deshielded δ2.71 at the lower ppm correspond to the three methyl groups present in the compound. The ^{1}H-NMR spectrum showed the deshielded signals of five aromatic protons at δ 7.41-7.61 ppm indicative of a phenyl ring. The protons H-2', H-4' and H-6' appeared as one peak in the environment, as well as H-5' and H-3' and thus appear as a multiplet peak in the spectrum. The characteristic methylene ($-CH_2$) bridge connecting the acetophenone unit to an aromatic ring was a singlet proton resonance at δ 3.80, and 15.8 ppm in the ^{13}C spectrum. The ^{13}C spectrum (Figure 21) showed 21 carbon signals in total from which 9 were quaternary carbons. Details of the 1D and 2D NMR experiments can be found in Appendix 3a-d.

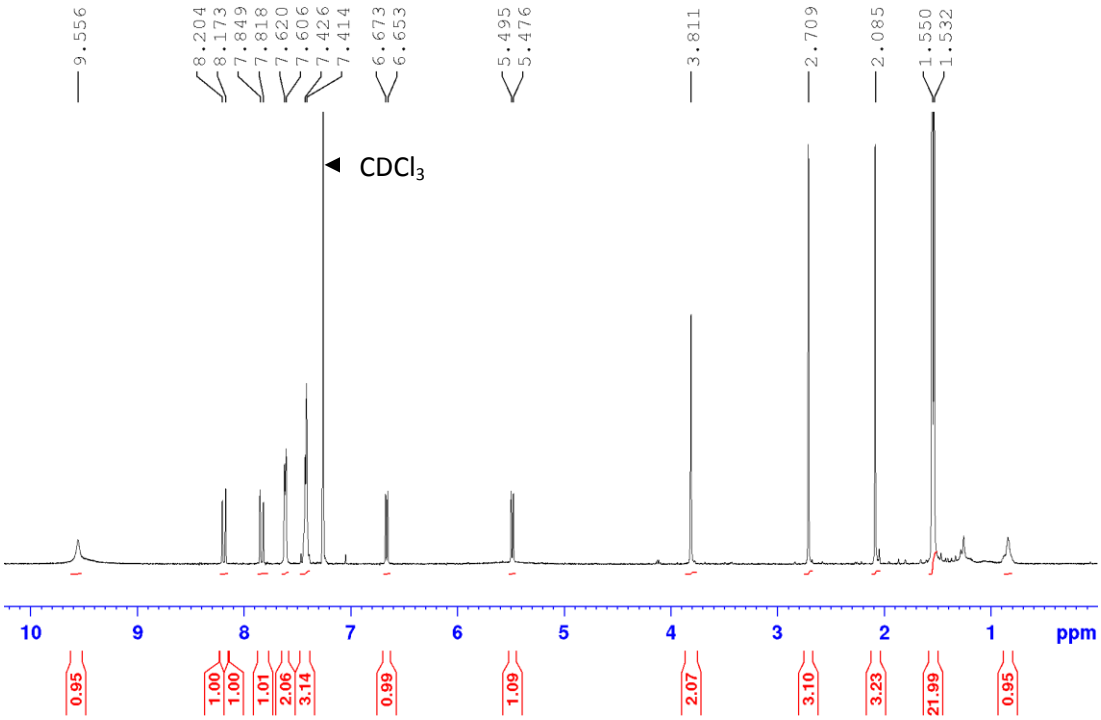


Figure 20: ^{1}H NMR spectrum of rottlerin in $CDCl_3$ (500MHz)

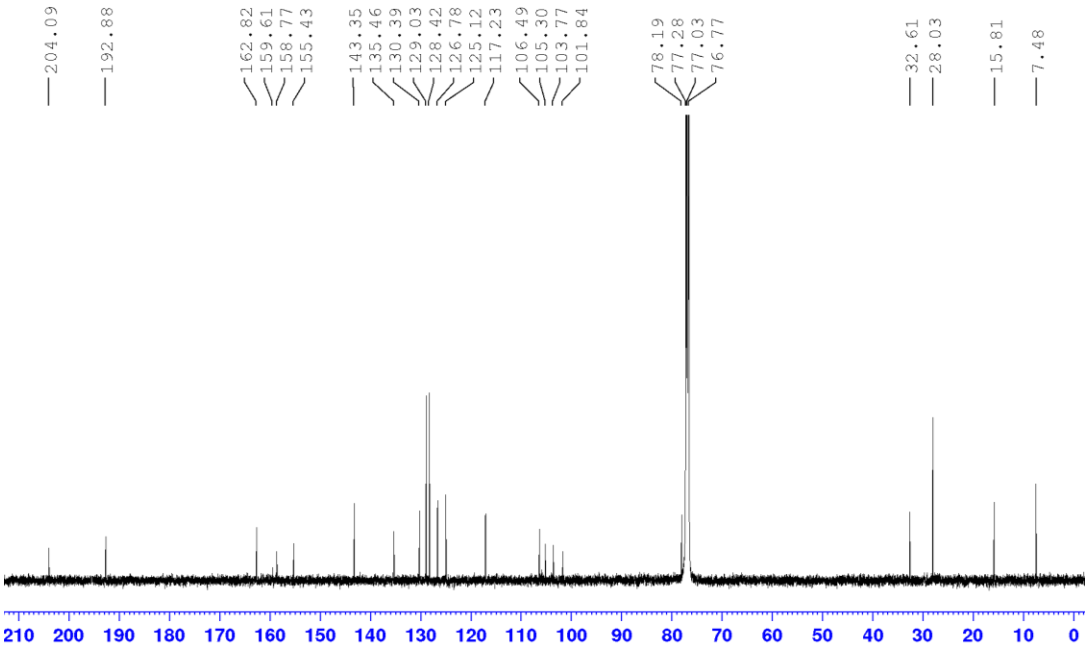


Figure 21: ^{13}Carbon spectrum of rottlerin in $CDCl_3$ (500MHz)

The values of the ^{1}H and ^{13}C signals of rottlerin as determined from 1D experiments corresponded to the chemical shifts recorded for the structure of rottlerin by (Hong *et al.,* 2010), as seen in Table 8.

Table 8: ^{1}H NMR (500 MHz) and ^{13}C (125 MHz) rottlerin

Position	^{13}C NMR	^{1}H NMR	^{13}C NMR and ^{1}H NMR (Hong *et al.,* 2010)	
C-2	78.2		78.1	
C-3,	125.1	5.49(d) 1H *J=9.5*	125.1	5.49(d)1H *J=9.9*
C-4	117.2	6.66(d) 1H *J=10*	117.2	6.66(d)1H *J=9.9*
C-5	158.8		158.6	
C-6, C-8	105.3		105.3,C-8=104.9	
C-7	159.6		159.0	
C-9	155.4		155.4	
C-10	103.8		103.7	
C-7’	126.8	8.19(d) *J=15.5*	126.8	8.17(d)1H *J=15.7*
C-8’	143.4	7.83(d) *J=15.5*	143.3	7.82(d)1H *J=15.7*
C-9’	192.8		192.9	
C-1’	135.5		135.4	
C-2’ C-4’ C-6’	128.4	7.61(d) 3H	128.4,C-4=130.4	7.58-7.61(m)2H
C-3’C-5’	129.0	7.41(d) 2H	129.0	7.40-7.42(m) 3H
C-1’’	106.5		106.7	
C-2’’	162.8		162.8	
C-3’’	105.3		105.6	
C-4’’	158.8		158.2	
C-5’’	103.8		103.0	
C-6’’	161.0		160.4	
Bridge CH_2	15.8	3.80(s) 2H	16.0	3.79(s) 2H
$\underline{CO}CH_3$	204.1		204.3	
$CO\underline{CH_3}$	32.6	2.71(s) 3H	32.7	2.71(s) 3H
C-2CH_3	28.0	1.54(s) 6H	27.8	1.53(s) 6H
C-5’’CH_3	7.5	2.08(s) 3H	7.9	2.08(s)3H
2’’, 4’’-OH		9.60(s) 1H		9.4-9.6 2H

Compound BM-**2** was known as the red compound (Figure 22) from the paper of (Hong *et al.,* 2010) gives the Kamala plant its characteristic red colour. It was isolated from the hexane extract of *M. philippinensis* in our laboratory. It had a characteristic red colour and a molecular formula $C_{21}H_{20}O_4$. The ^{1}H, ^{13}C, COSY, HMQC, HMBC experiments were the basis for its structure elucidation.

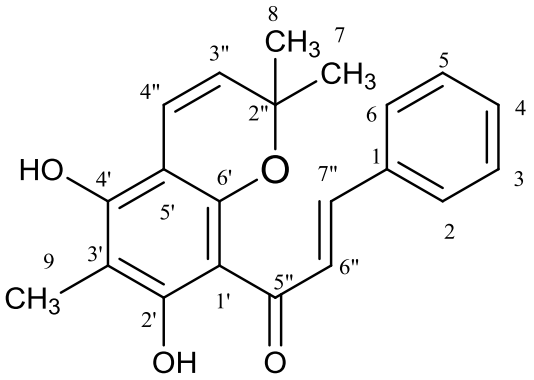


Figure 22 : Structure of the red compound, BM-2

The examination of the proton NMR spectrum (Figure 23) showed that there were 20 protons present in this compound. The first two peaks which appear at lower ppm in the spectrum corresponded to the three methyl groups that were present in the compound. One hydroxyl group was deshielded and corresponded to the H-2′ position whereas the other hydroxyl group present corresponded to the H-4′ position.

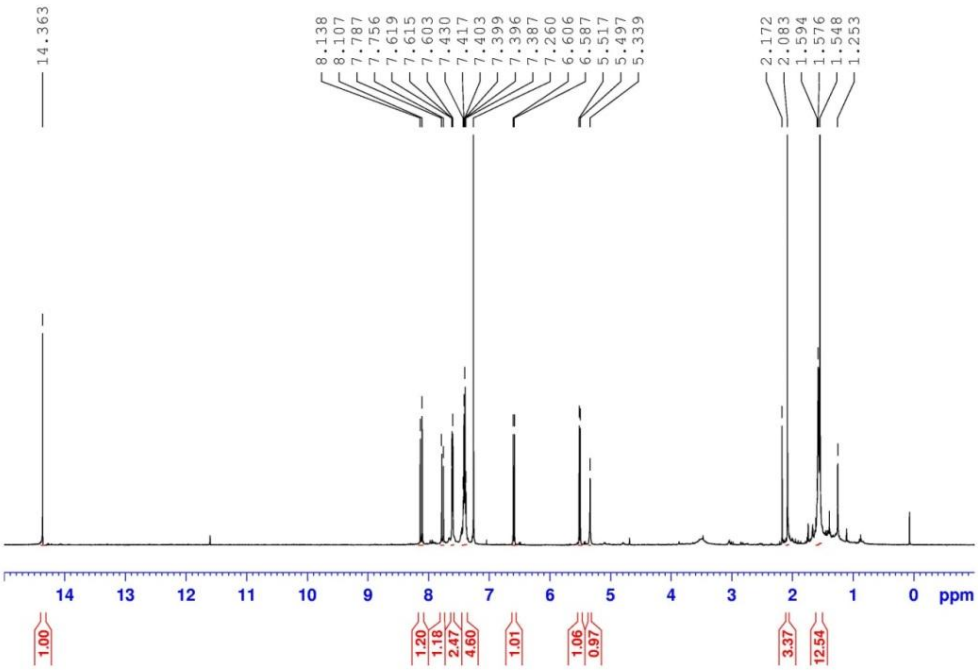


Figure 23: ^{1}H NMR spectrum of the red compound in $CDCl_3$ (500MHz)

The ^{1}H NMR spectrum showed the deshielded signals of five aromatic protons at 7.38-7.61 ppm indicating the presence of 1 phenyl ring. The protons H-2, H-4 and H-6 appeared as one peak in the ^{1}H spectrum as they were in the same environment. Protons H-5 and H-3 were also in the same environment and thus, they appeared as one peak in the spectrum. The H-6´´ and H-7´´ hydrogens displayed *trans* isomerism with coupling constant value, *J*=15.5 respectively.

In the ^{13}C NMR spectrum (Figure 24), there exhibited 18 signals, which by ^{1}H NMR, and ^{13}C DEPT-135 corresponded to 9 quaternary carbons, 3 methyls and 9 methine carbons. The two carbons of the methyl groups at the 7 and 8 positions of the compound were shown as one peak at 27.9 ppm. There were 3 carbons that appeared as one peak at 128.8 ppm.

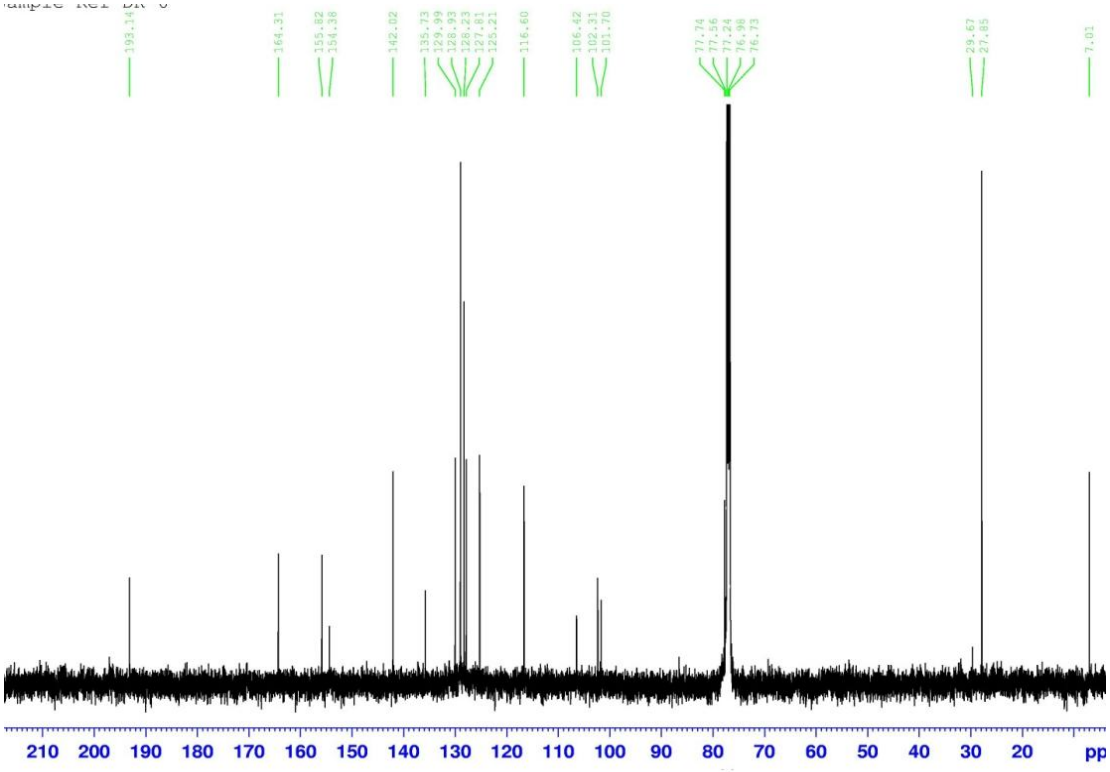


Figure 24: ^{13}Carbon spectrum of the red compound in $CDCl_3$ (125MHz)

The ^{1}H and ^{13}C resonances (Table 8) of the red compound were assigned by correlations of the HMQC data (Table 9): δ 7.40 (H-2,4,6) with δ 128.2 (C-2,4,6), δ 7.61 (H-3) with δ 130.0 (C-3), δ 7.61 (H-5) with δ128.9 (C-5), δ 5.51 (H-3′′) with δ 125.2 (C-3′′), δ 6.60 (H-4′′) with δ 116.6 (C- 4′′), δ 8.12 (H-6′′) with δ 127.8 (C-6′′) and δ 7.77 (H-7′′) with δ 142.0 (C-7′′), while the COSY data (Table 9) showed the correlations between protons H-3′′ and H- 4′′, H-2, and 4, 6 with the H-3, 5 and between H- 6′′ and H- 7′′.

On the basis of the above data which were in agreement with published literature (Hong *et al.*, 2010) represented in Table 8, BM-**2** was assigned as the red compound.

Table 8: ^{1}H NMR (500 MHz) and ^{13}C (125 MHz) of the red compound BM-**2** in $CDCl_3$

Position	$^{\delta}H$	$^{\delta}C$	^{13}C and ^{1}H (Hong *et al.*, 2010)	
1	_	135.7	NL	
2,4,6	7.41 (m)	128.2	128.2	7.56-7.61, m
3	7.61 (m)	130.0	128.9	7.36-7.41, m
5	7.61 (m)	128.9	128.9	7.36-7.41, m
Me 2′′ (x2)	2.08 (s)	27.8	27.8	2.07, s
Me 3′	1.58 (s)	7.0	7.1	1.53, s
1′	-	77.7	77.6	
C-2′-OH	14.36 (OH)	164.3	164.2	
3′	-	101.7	101.9	
4′	5.34 (OH)	106.4	106.3	
5′	-	155.8	156.2	
6′	-	102.3	102.6	
2′′	-	154.4	154.3	
3′′	5.51 (d)	125.2	125.1	5.48, d, *J*=9.9
4′′	6.60 (d)	116.6	116.6	6.60, d, *J*=9.9
5′′	_	193.1	193.1	
6′′	8.12 (d)	127.8	127.7	8.12, d, *J*=15.7
7′′	7.78 (d)	142.0	142.0	7.75, d, *J*=15.7

NL= Not listed

Table 9: NMR data (COSY, HMQC, and HMBC) of the red compound

Protons	Cosy	HMQC	HMBC	
			2*J*	3*J*
H-2,4,6		128.2 (CH)	142.0 (CH)	128.9(CH)
H-3		130.0 (CH)	-	130.0 (CH)
			-	
			-	
H-5	H-3, 5	128.9 (CH)	-	128.2 (CH)
H-1′	H-2,4,6		154.4 (C)	-
H-2′	H-2,4,6		-	-
H-3′			135.7 (C)	-
H-4′		-	128.2 (CH)	-
H-5′		-	125.2 (CH)	-
H-6′		-	164.3(C),	-
H-2′′		125.2 (C)	106.4 (C)	-
H-3′′		116.6 (C)		77.7(C),
H-4′′		127.8 (C)		102.3(CO)
H-5′′	H-4′′	142.0 (C)		77.7 (C)
H-6′′	H-3′′	27.9 (C)		-
H-7′′		7.0 (C)		-
$(Me)_2$ 2′′				155.8 (C)
Me 3′				77.7 (C)

3.1.2 *Cannabis sativa* L.

Compounds BM-**3** and BM-**4** were obtained from the chloroform extract of *Cannabis sativa*, following bioguided isolation, to determine active fractions. The ^{1}H, ^{13}C, ^{13}C DEPT135, COSY, NOESY and HMBC experiments were the basis for their structure elucidation. BM-**3** (Figure 25) was identified as tetrahydrocannabinolic acid (THCA) on the basis of the NMR data from literature and ESI-MS value of *m/z* 357.4 $[M-H]^{+}$ (Appendix 4) which corresponded to the molecular formula of $C_{22}H_{30}O_4$ and molecular mass of *m/z* 358.54 (Hazekamp *et al.*, 2005).

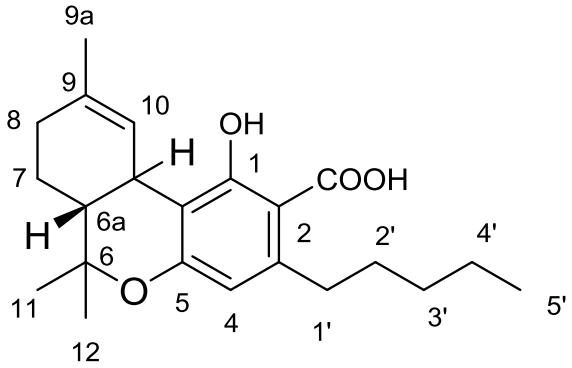


Figure 25: Structure of tetrahydrocannabinolic acid

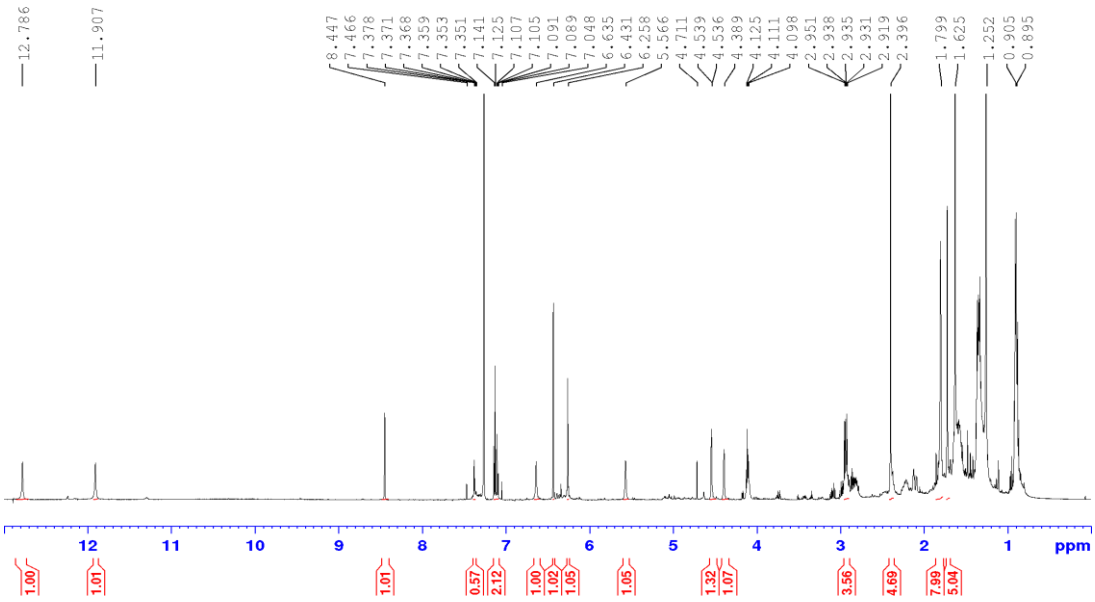


Figure 26: ^{1}H NMR of tetrahydrocannabinolic acid in $CDCl_3$ (500MHz)

The ^{1}H NMR of THCA has been previously published by several authors, and they are in agreement with our proton spectral data (Table 10). The spectral data (Figure 26) showed major signals of a typical cannabinoid at H-4, δ6.26; H-10, δ6.43, two angular methyl groups (3H each, s at δ 1.63) located at position 11 indicate on structure a methyl (α) and position 12-methyl (β), one tertiary methyl group (3H, t, *J*= 7.5Hz, at δ 0.9, H-5'), and the aromatic olefinic protons at δ 6.26, δ 6.43, and δ 7.04 - 7.11 ppm.

In the ^{13}C NMR spectrum (Figure 27), a downfield shift of 148.4 ppm was found for C-2, and the other aromatic carbons, C-3, C-4, C-5, C-6, and C-7, showed shifts at103.8, 159.4, 113.0, 163.6 and 78.6 ppm, respectively. The chemical shifts of the aliphatic side chain were found upfield; C-1, C-2', C-3', C-4' and C-5 at 14.1- 36.9 ppm. The ^{1}H and ^{13}C NMR assignments are represented in Table 11.

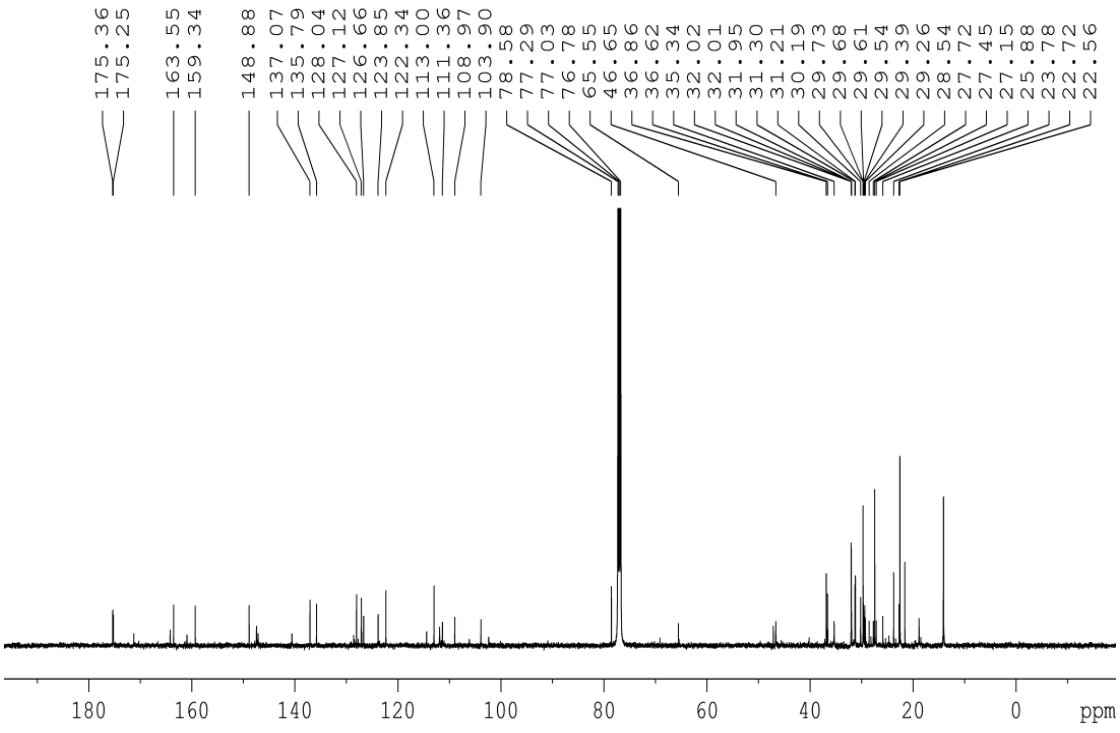


Figure 27: ^{13}C NMR of tetrahydrocannabinolic acid in $CDCl_3$ (125MHz)

Table 10: ^{1}H (500MHz) and ^{13}C (125 MHz) of tetrahydrocannabinolic acid in $CDCl_3$

Position	^{1}H	^{13}C	^{1}H (400MHz) in $CDCl_3$ (Choi *et al.*, 2004)	^{13}C (100MHz)
1		163.6		164.7
2		103.9		102.3
3		147.3		146.9
4	6.26 (s) 1H	113.0	6.24 H (s)	112.3
5		159.4		159.8
6		78.6		78.8
6a	1.67	46.7	1.67 (m)	45.6
7	1.79 (m)	25.9	1.92, 1.35(t), 2H,	25.0
8	1.80	31.3	2.17 2H (m)	31.2
9		135.8		133.8
9a	2.39	21.6	2.38	21.5
10	6.43 H	122.8	6.39 1H	123.6
10a	2.95 (s) H	31.9	3.21 1H	31.2
11	1.63 (m) 3H	27.5	1.44 3H (s)	27.4
12	1.63 (m) 3H	27.5	1.41 3H (s)	27.4
1’	2.93 (m)	36.9	2.94, 2.78, 3H (t)	36.5
2’	1.36 (m)	22.6	1.35 (m)	22.5
3’&4’	1.36 (m)	31.2	1.35 (m)	32.0
5’	0.91 (t) 3H	14.1	0.90 3H (t ,6.9 Hz)	14.1
C1-OH	12.79 (s) 1H		12.19 (1H, s)	
COOH	11.91 (s) 1H	175.4		176.2

Compound BM-**4** (Figure 28) was obtained as dark brown solid. The proton NMR (Figure 28) corresponded with those earlier reported by (Choi *et al.,* 2004; Bastola *et al.,* 2007) and identified BM-**4** as cannabinolic acid (CBNA), a major cannabinoid present in *C. sativa*.

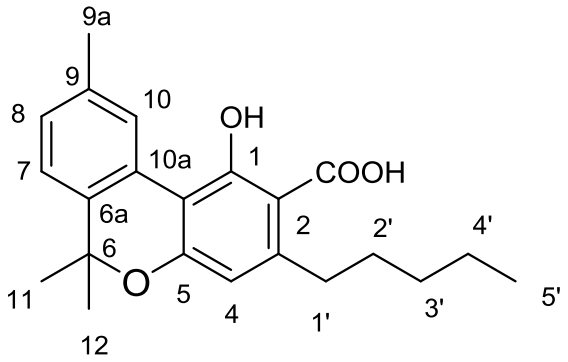


Figure 28: Structure of cannabinolic acid (CBNA)

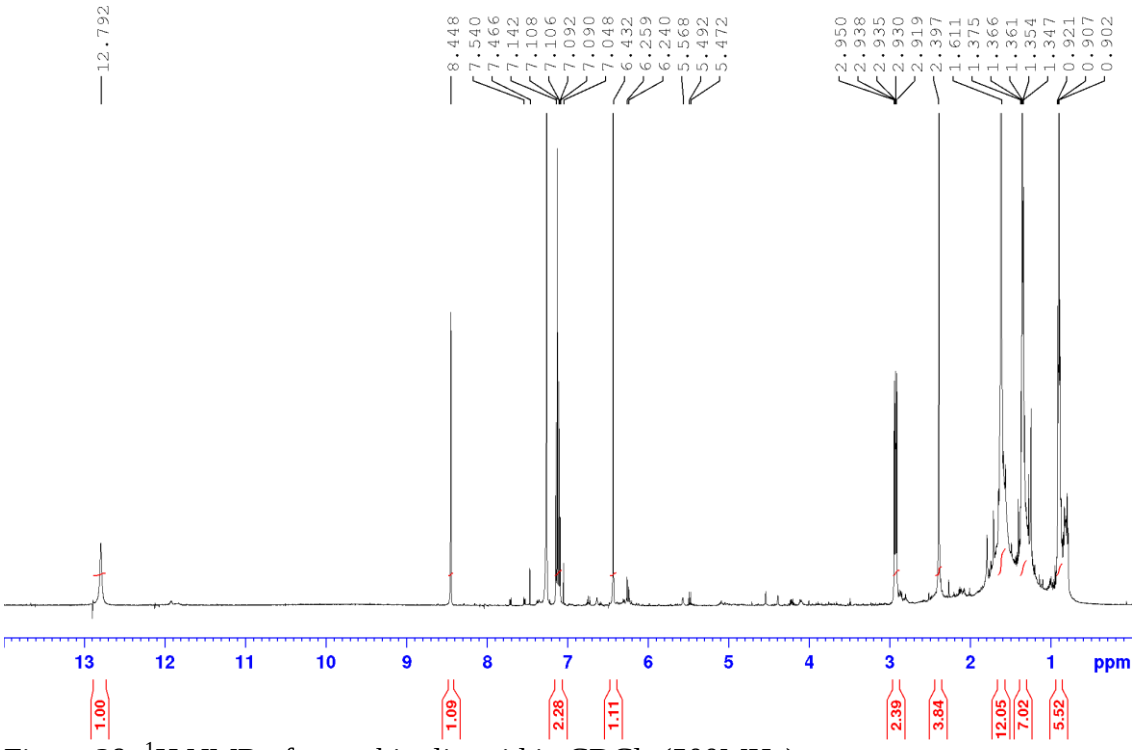


Figure 29: ^{1}H NMR of cannabinolic acid in $CDCl_3$ (500MHz)

The molecular formula was determined as $C_{22}H_{26}O_4$ and ESI-MS analysis showed an ion peak at *m/z* 353.0 $[M-H]^+$ (Appendix 4b) which corresponded to the expected molecular mass of *m/z* 354.45.

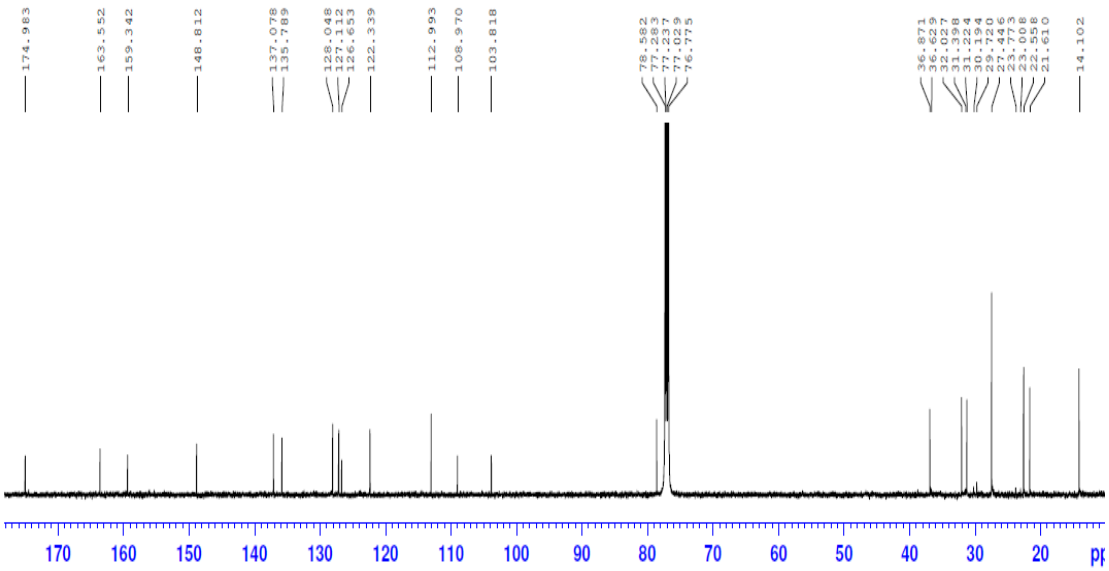


Figure 30: ^{13}C spectrum of cannabinolic acid in $CDCl_3$ (125 MHz)

Based on the ^1H (Table 11) and ESI-MS spectral data (Appendix 4b) of the isolated BM-4 which corresponded to available literature, the compound was assigned as cannabinolic acid. Given that there is no available ^{13}C data on cannabinolic acid, the ^{13}C NMR Peak assignment was done by comparison to the NMR spectrum of CBN [Choi, 2004] (Table 11).

Table 11 : ^{1}H (500MHz) and ^{13}C (125MHz) NMR of Cannabinolic acid in $CDCl_3$

	^{1}H	^{13}C	^{1}H (300MHz $CDCl_3$) (Bastola *et al.*, 2007)
1		108.9	
2		103.8	
3		148.8	
4	6.43 (s) 1H	113.0	6.40 H (s)
5		159.4	
6		78.6	
6a		135.8	
7	7.16(m) 8Hz,7.5Hz	122.4	7.11 2H, (d)
8	7.11(m)	128.12	7.11 2H, (d)
9		126.7	
9a	2.40 (s) 3H	21.5	2.38 3H (t)
10	8.45 (s) H	127.1	8.40 H (s)
11	1.61 (s) 3H	27.3	1.60 3H (s)
12	1.61 (s) 3H	27.5	1.60 3H (s)
1’	2.93 (m)	36.9	2.96 2H (t)
2’	1.35 (m)	22.6	1.32 4H (m)
3’ and 4’	1.35 (m)	31.2	1.32 4H (m)
5’	0.90 (t) 3H	14.1	0.83, 3H, t
C1-OH	5.49 (s) 1H		5.13 1H (s)
COOH	12.79 (s) 1H	174.9	NL

NL= Not listed

The summary of ^{13}C DEPT135, COSY and HMBC NMR analysis of tetrahydrocannabinolic acid and cannabinolic acid are presented in Appendix 5 and 6.

3.1.3 *Evodia rutaecarpa* (Juss) Benth

The crude methanol extract of *E. rutaecarpa* fruit was subjected to acid–base partitioning and the resultant acidic, basic, methanolic and aqueous partitions were tested for plasmid transfer inhibition. By using the plasmid transfer inhibition assay to guide separations, the bioactive basic fraction was separated by vacuum liquid chromatography, in combination with column chromatography on silica gel F_{254}. From the VLC analysis, fractions 6, 7 and 8 were active against the plasmid TP114. Fr-6 was re-chromatographed on silica gel to obtain two major constituents from *Evodia rutaecarpa* namely BM-**5**; evodiamine, BM-**6**; rutaecarpine, while fractions 7 and 8 were pooled using TLC and subjected to HPLC analysis to obtain BM-**7** named evosugar. The physical properties of the isolated compounds (BM **5-7**) are listed in Table 12.

Table 12: Compound names, molecular formula and physical properties of agents isolated from *Evodia rutaecarpa*

Compound	Name	Molecular formula /mass	Yield (mg)	Colour	Solubility
BM-5	Evodia-mine	$C_{19}H_{17}N_3O$ / *m/z* 303	1.4 mg	Light yellow powder	$CHCl_3$
BM-6	Rutae-carpine	$C_{18}H_{13}N_3O$ / *m/z* 287	3.18 mg	Light yellow powder	$CHCl_3$
BM-7	sucrose	$C_{12}H_{22}O_{11}$	9.6 mg	Brown solid	H_2O

A number of NMR experiments named ^1H, ^{13}C, ^{13}CDEPT 135, ^1H-^1H COSY, NOESY, HMQC and HMBC analysis were employed to elucidate the chemical structures of the compounds.

BM-**5** (Figure 31) was obtained as light yellow crystalline powder and in the very small quantity (1.4 mg). The NMR of BM-**5** were in line with the proton spectrum (Figure 32) and ^{13}C NMR profile as reported by (Liu *et al.*, 2005) for evodiamine.

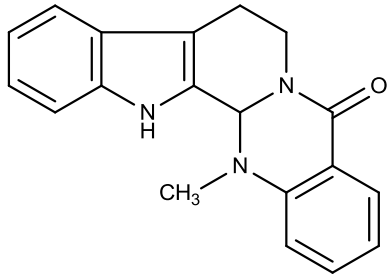


Figure 31: Structure of evodiamine

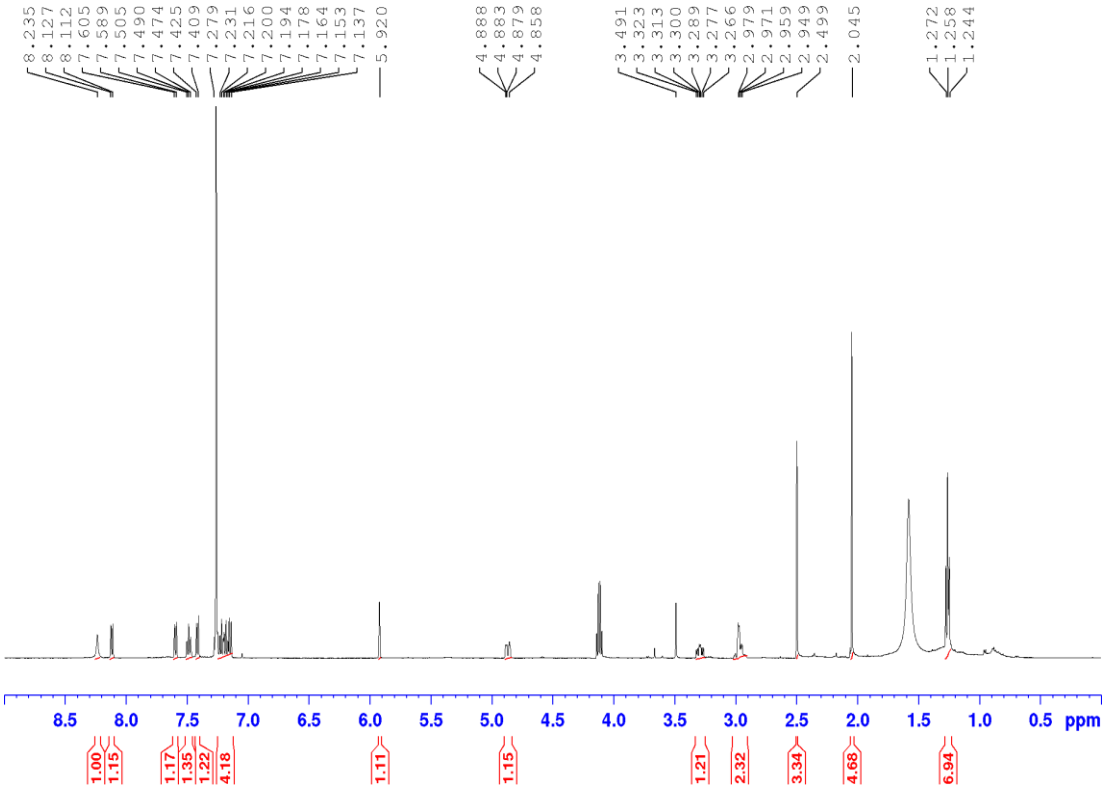


Figure 32: ^{1}H spectrum of evodiamine in $CDCl_3$ (500MHz)

The ESI-MS spectrum of evodiamine (Figure 33) showed an ion peak at *m/z* 302.2 $[M-H]^+$ which corresponded with the molecular mass of *m/z* 303.36 and molecular formula of $C_{19}H_{17}N_30$.

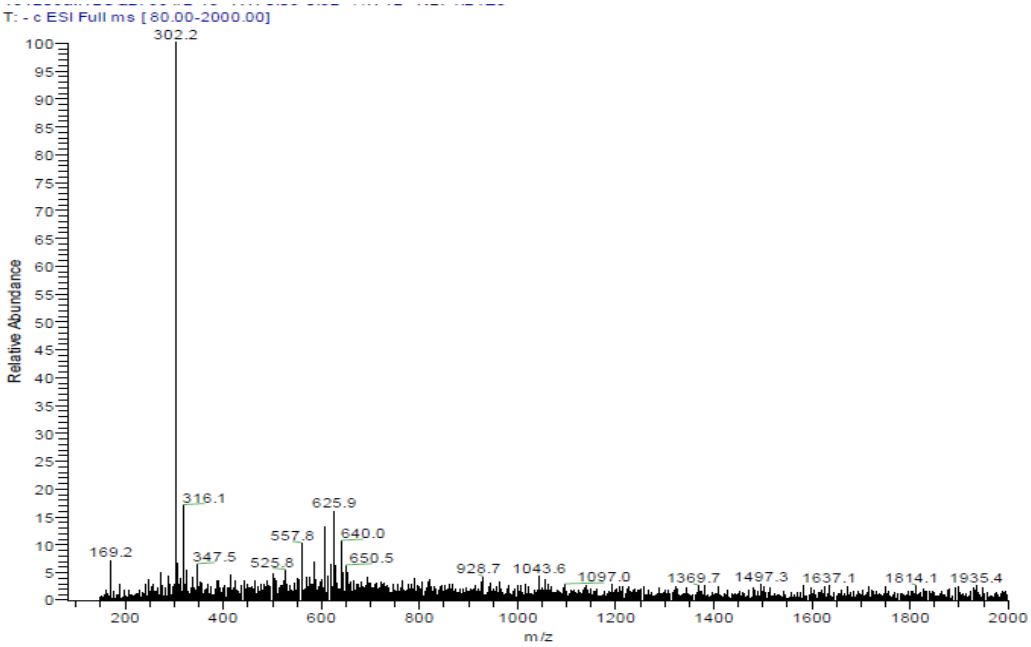


Figure 33: ESI-MS spectrum of evodiamine showing peak ion at *m/z* 302

The ^{1}H and ^{13}C NMR data (Table 13) confirmed the characteristic protons assignments of a quinolone alkaloid: H-16, H-18, δ 7.13-7.23, m,H-17, δ 7.47-7.51, H-19, δ 8.12, (s, 1H), and N-Me δ2.50, (s, 3H), H-3, δ 5.92 (s, 1H); H-5, δ3.27-3.32 (m, 2H); H-6, δ 2.95-3.03 (m, 2H) and N-H, 8.24 (s, 1H). The full details of ^{13}C DEPT 135, COSY, NOESY and HMBC spectra of evodiamine are given in the Appendix 7a-d

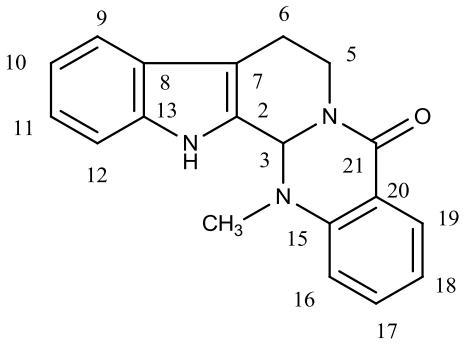


Figure 34: Structure of evodiamine

Table 13: ^{1}H (500MHz) and ^{13}C (125MHz) NMR of evodiamine in $CDCl_3$

Position	^{13}C	^{1}H	^{13}C (100MH_Z)	^{1}H (400MH_Z) in $CDCl_3$ by Liu *et al*., 2005
2	126.3		125.3	
3	68.8	5.92, s, 1H	68.4	5.95 (1H,s)
5	39.5	3.27-3.32, m, 1H	39.1	3.01(2H, m)
6	19.7	2.95-3.03, m, 2H	19.3	
7	118.9		117.8	
8	123.8		121.9	
9	118.9	7.42, d, *J*= 8, 1H	117.8	7.16-8.17 (8H, Ar-H, m)
10	123.8		121.7	
11	113.7		113.7	
12	111.3	7.13-7.23, m, 4H	110.8	7.16-8.17 (Ar-H, m)
13	136.7		136.4	
15	150.7		150.1	
16	122.5	7.15, d, *J*= 8, 1H	122.3	7.16-8.17 (Ar-H, m)
17	133.0	7.47-7.51, t, 1H, *J*= 7.5	132.4	
18	129.0	7.13-7.23, m	128.4	
19	128.2	8.12, d, *J*= 7.5, 1H	128.2	7.16-8.17 (Ar-H, m)
20	120.1		120.3	
21	164.8		164.5	
N-CH_3	37.3	2.50, s, 3H	36.4	2.54 (3H, s)
N-H		8.24, s, 1H		8.24(1H, br)

Compound BM-**6**, was isolated as light yellow powder from two bioactive fractions in small amounts of 1.46 and 1.72 mg respectively and a total of 3.18 mg. The molecular formula of BM-**6** was determined to be $C_{18}H_{13}N_3O$ and experimental ESI-MS ion of *m/z* 288.2 $[M+H]^+$ which corresponded with molecular weight of *m/z* 287.32 indicative that BM-6 could be rutaecarpine (Figure 35).

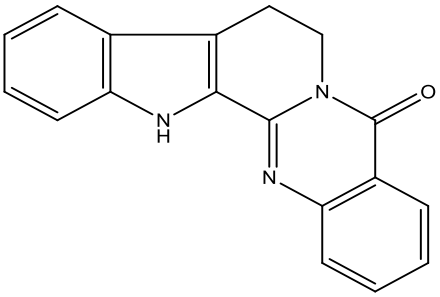


Figure 35: The structure of rutaecarpine.

The ^{13}C DEPT 135, COSY, NOESY, HMQC and HMBC spectra of rutaecarpine, are shown in Appendix 8a-d. The ESI-MS spectrum peak at *m/z* 288.2 $[M+H]^+$ is represented in Figure 36.

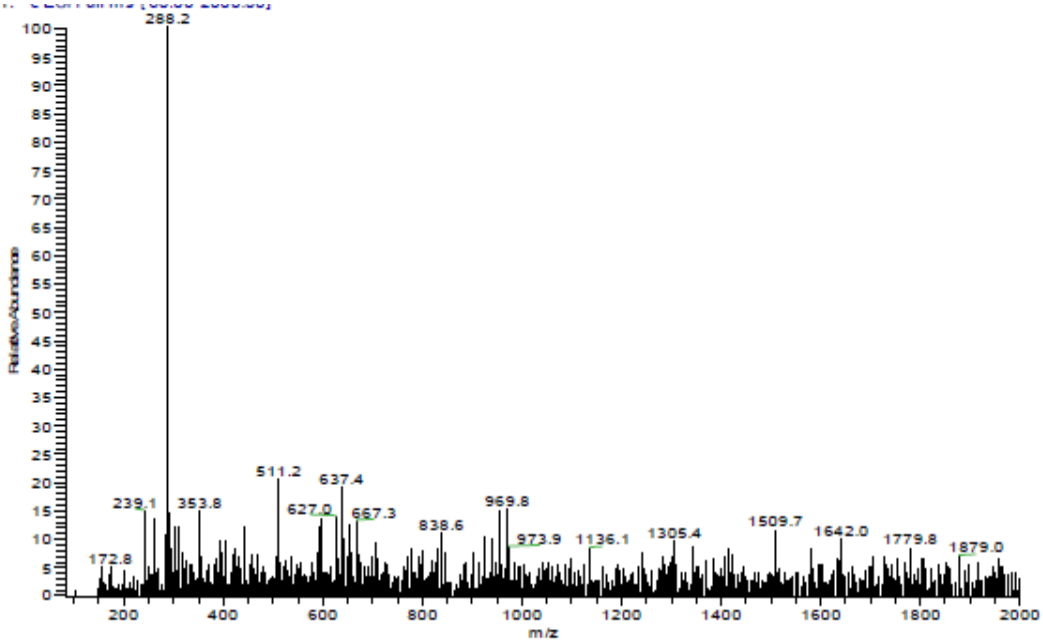


Figure 36: ESI-MS spectrum peak of rutaecarpine at *m/z* 288.2

The ^{1}H NMR spectrum (Figure 37) showed signals of due to a quinolone skeleton and conjugated protons; H-9, δ 7.18-7.21, t, *J*=7, 1H; H-11, δ 7.33-7.36, t, 1H; H-16, δ 7.42-7.46, m, 2H; H-17, δ 7.64-7.74, 3H; H-18, δ 8.31, dd, 1H; H-19, δ 8.33, dd, 1H; which resembled those of evodiamine.

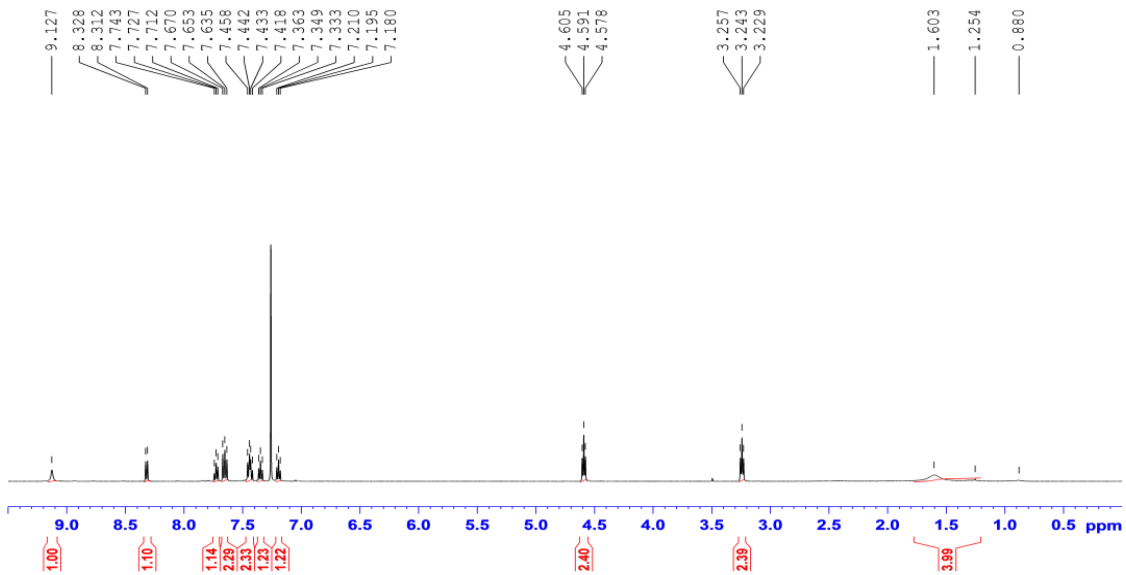


Figure 37: ^{1}H NMR spectrum of rutaecarpine in $CDCl_3$ (500 MHz)

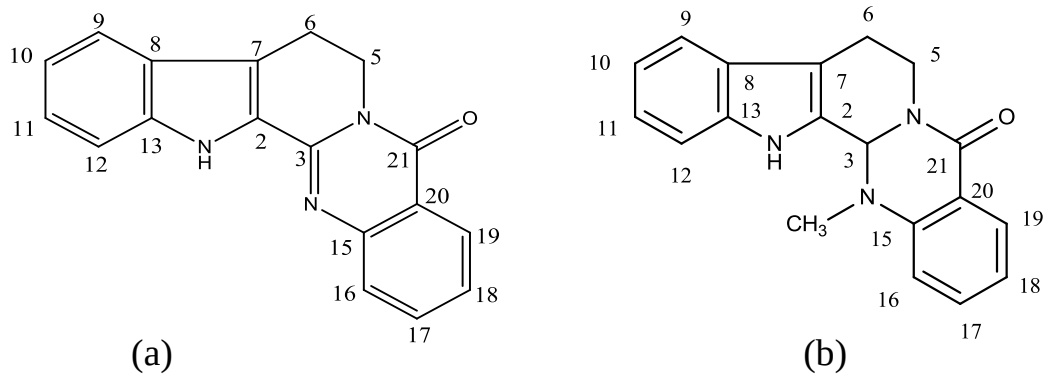


Figure 38: Structures of rutaecarpine (a) and evodiamine (b)

Other prominent signals included an olefinic proton at H-3 (δ 5.92, s, 1H), and protons H-5 (δ 4.58-4.61, t, *J*=7, 2H), H-6 (δ 3.22-3.26, t, *J*=7, 2H) and N-H at δ9.13 (s, 1H). Both rutaecarpine and evodiamine (Figure 38) are highly similar in their

chemical structure, except for the absence of N-Me in rutaecarpine. All of the spectral data of rutaecarpine including the ^{13}C NMR data given in Figure 39 and Table 14 are in agreement with those of Liu *et al.*, 2005.

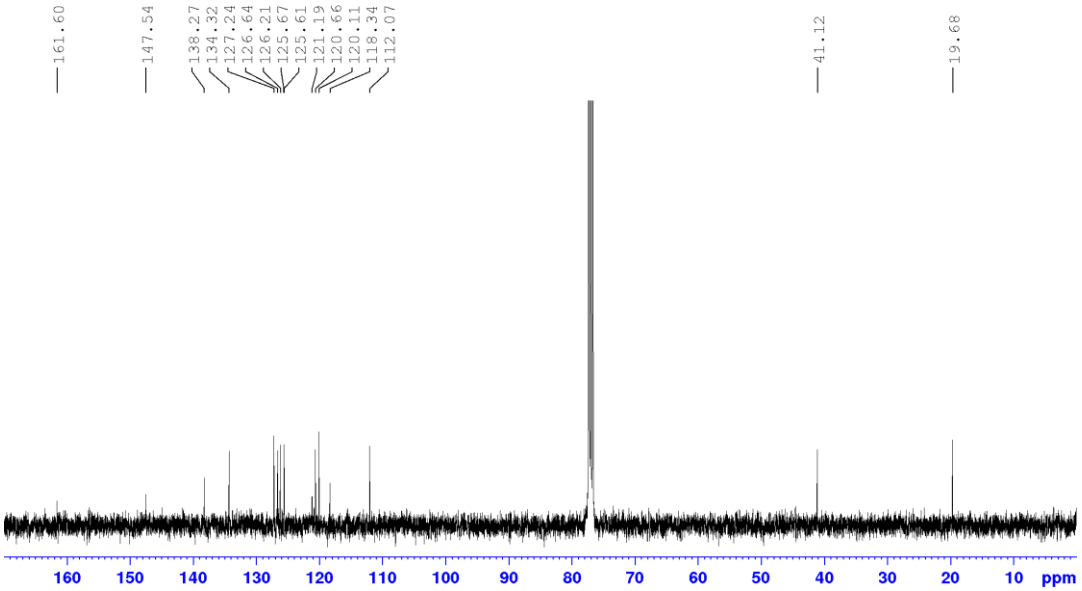


Figure 39: ^{13}C NMR of rutaecarpine in $CDCl_3$ (125MHz)

Table 14: ^{1}H (500MHz) and ^{13}C (125MHz) NMR of rutaecarpine in $CDCl_3$

Position	^{13}C	^{1}H	^{13}C (100MHz) in $CDCl_3$ by Liu *et al.*, 2005	^{1}H (400MHz)
2	127.3		127.3	
3	144.9		145.5	
5	41.1	4.58-4.61, t, *J*=7, 2H	41.7	4.63 (2H, t)
6	19.6	3.22-3.26, t, *J*=7, 2H	20.1	3.35 (2H, t)
7	112.0		112.6.	
8	125.5		125.7	
9	118.3		112.6	
10	120.0		121.0	
11	120.5	7.33-7.36, t, *J*=7 1H	120.4	
12	118.3	7.18-7.21, t, *J*=7	118.7	7.12-7.18 (7H, m Ar-H)
13	138.2		138.8	
15	147.6		147.7	
16	126.	7.42-7.46, m	126.0	
17	134.3	7.64-7.74,H	134.9.	
18	127.3	8.31, dd, *J*=8 1H	127.1	8.33–8.36
19	126.3	8.33, dd, 1H	126.8	(1H, m Ar-H)
20	121.0		121.0	
21	161.6		162.0	
N-H		9.13, s, 1H		9.64 (1H, s, br)

Compound BM-**7**, was obtained from the HPLC analysis of bioactive fractions 7 and 8. The mobile phase that gave good separation results was a gradient elution of water-methanol/90:10 for 0-10min and a 95:5 mix for 10-20 min. The chromatogram is shown in Figure 40.

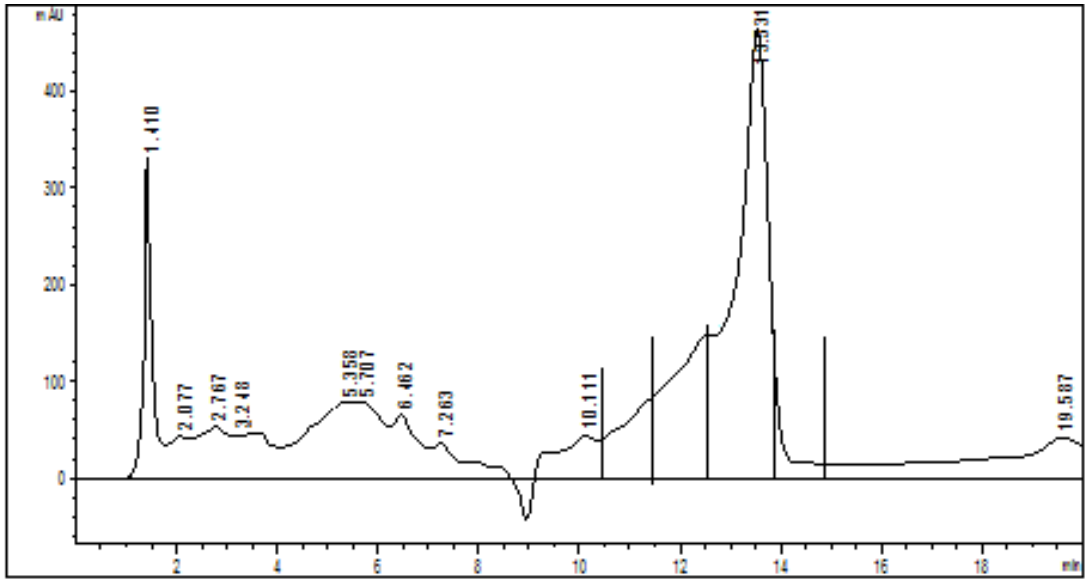


Figure 40: Chromatogram showing a major peak at 13.33min at 280 nm

Identification of the major peak signal at 13.33 min was performed by proton NMR (Figure 41), ^{13}C NMR (Figure 43) and low resolution ESI-MS (Figure 44). The molecular mass of sucrose was *m/z* 342.21 which corresponded with the measured ion peak of *m/z* 364.0 $[M\text{-}Na]^+$, confirming the compound as sucrose.

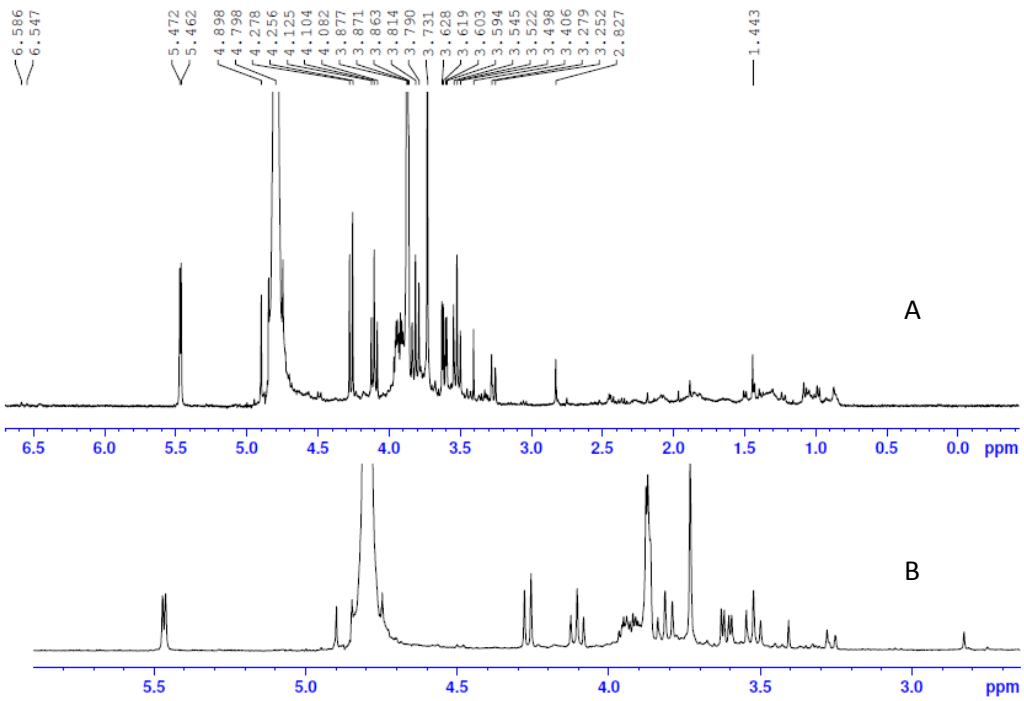


Figure 41: ^{1}H NMR of isolated sucrose (a) and expanded section 3.0-5.5ppm (b) from *E. rutaecarpa* in D_20 (500MHz)

Taken together the results of the analysis suggested that the compound could be sucrose, naturally isolated from *Evodia* fruit. To confirm the identified substance as sucrose, some table sugar was submitted also for NMR analysis (Figure 42), and both spectra established that the compound was indeed sucrose.

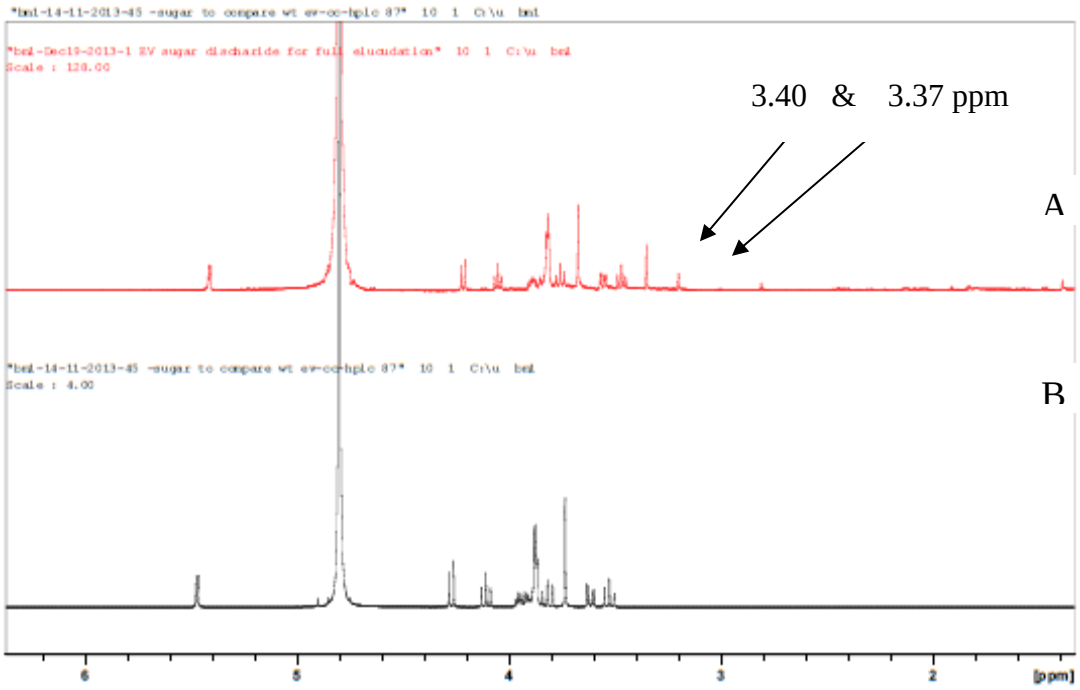


Figure 42: Comparison of ^{1}H NMR (D_20 500MHz) of sucrose (a) and table sugar (b) showing similar signals at ppm range 6 - 3.4 ppm but distinct between 3.37 and 3.40ppm in (a) with the presence of very low level of impurities downfield in the aliphatic region.

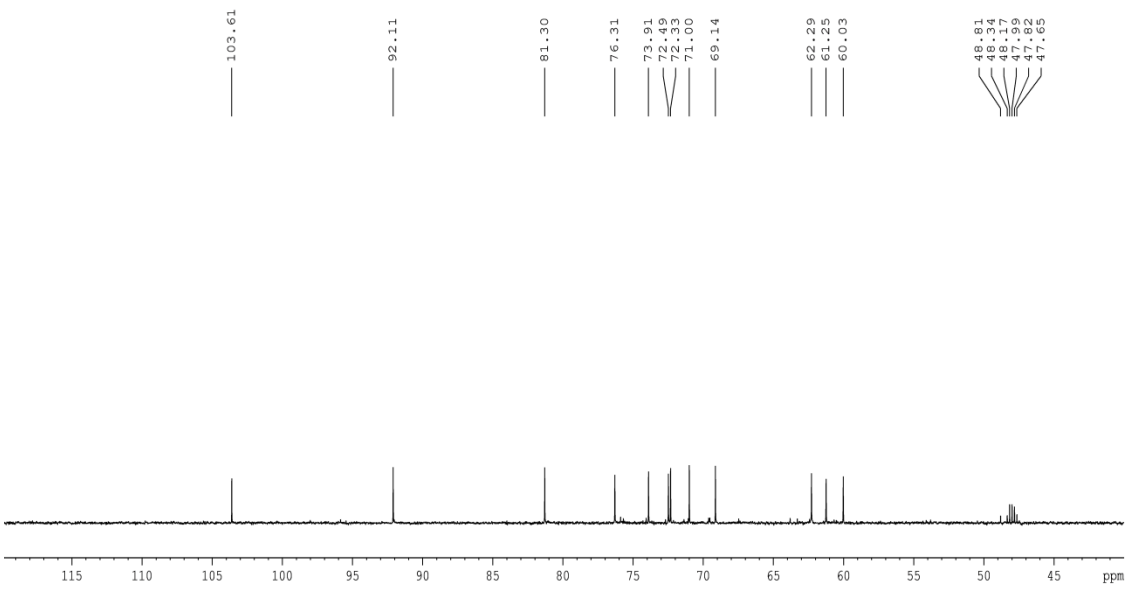


Figure 43: ^{13}C (125 MHz) spectrum of sucrose isolated from *Evodia* fruit

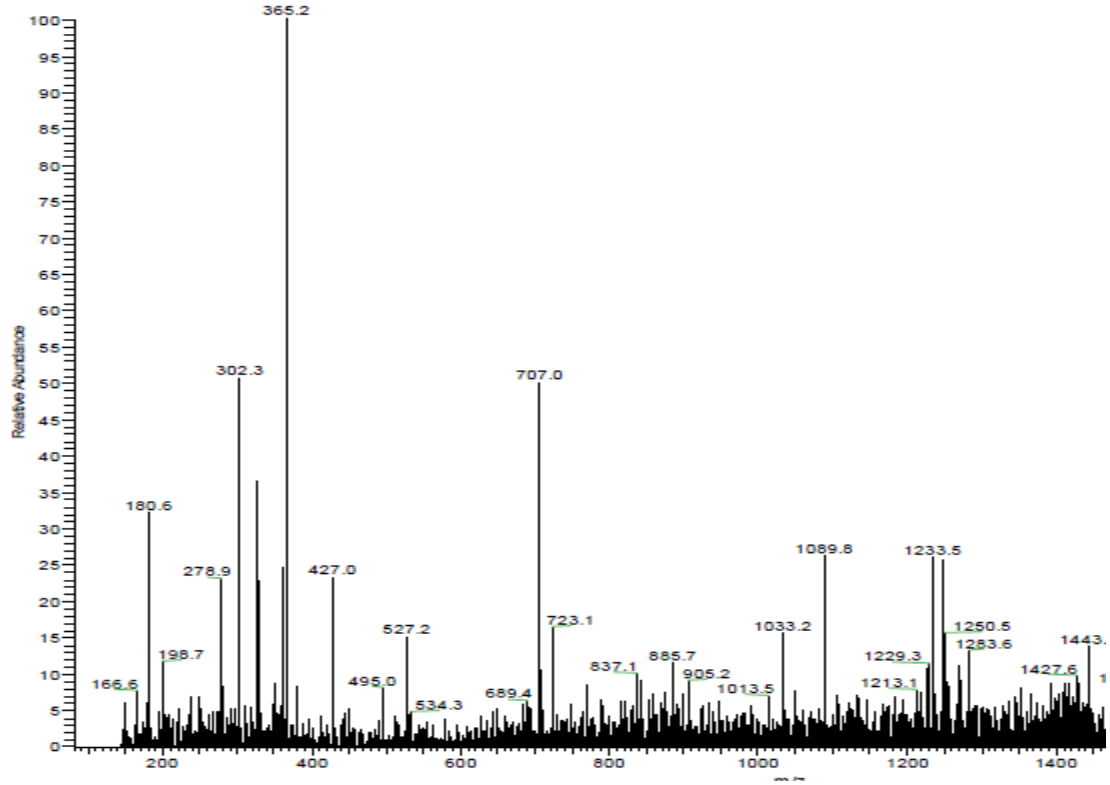


Figure 44: ESI-MS of the sucrose isolated from *Evodia* fruit

3.1.4 *Capsicum annuum* L.

A total of 18 sub-fractions were obtained from reverse phase solid-phase extraction starting from 100% distilled water in 5% increments until 100% ethyl acetate elution of the *C. annuum* methanol extract. By using TLC analysis, combined fractions gave Ca-SPE A, B and C, were tested, alongside fractions Ca-SPE 7 and 10-18 for their antiplasmid activity against TP114 in order to determine the active fractions, which yielded Ca-SPE 10, 11 and 12. The 3 resultant bioactive fractions were submitted for ^{1}H NMR profiling. From the analysis, Ca-SPE-10 indicated a pure compound which was identified using literature data as capsaicin, BM-**8** (Figure 45).

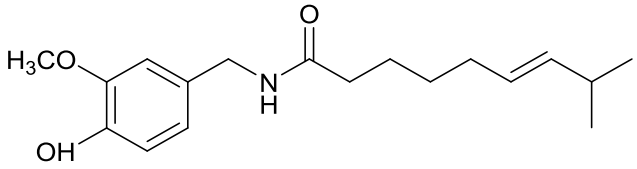


Figure 45: Structure of capsaicin.

The ^{1}H NMR spectrum of BM-8 (Figure 46) was compared to ^{1}H NMR of pure capsaicin (Figure 47) to confirm that the compound was capsaicin. The ^{1}H NMR spectrum of capsaicin showed the presence of three aromatic protons (δ 6.78, s, 1H, H-2'; δ 6.65, 2H, d, *J*=8.5, H-5') indicative of a phenyl ring. The ^{1}H NMR spectrum showed a triplet at δ2.14, t, 2H, *J*=7.5 as expected of capsaicin arising from the H-2 methylene. The multiplet at δ5.26-5.28 corresponded with the signal for olefinic protons, H-6 and H-7, and OMe group (δ3.76), OH (δ5.16) and the NH amide moiety (δ5.20) corresponded with data earlier reported in literature. In the downfield region, the methyls of the isopropyl group at δ0.70- 0.88 (6H dd, H-9 and H-10 *J*=6.5 with fine splitting), δ1.69 (1H, s, H-3), δ1.31 (3H, d, *J*=8, H-4) and δ1.94 (2H, dd, *J*= 8, H-5) were equally observable and strong indication of capsaicin (Table 15).

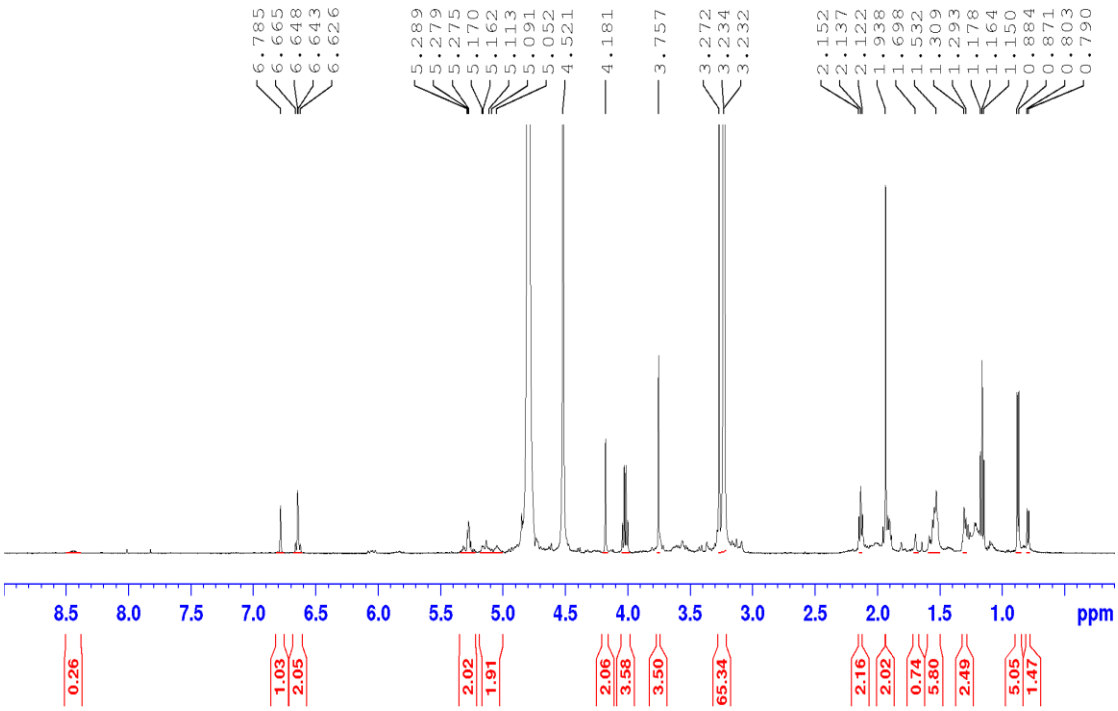


Figure 46: ^{1}H NMR of BM-8 in CD_3OD (500MHz)

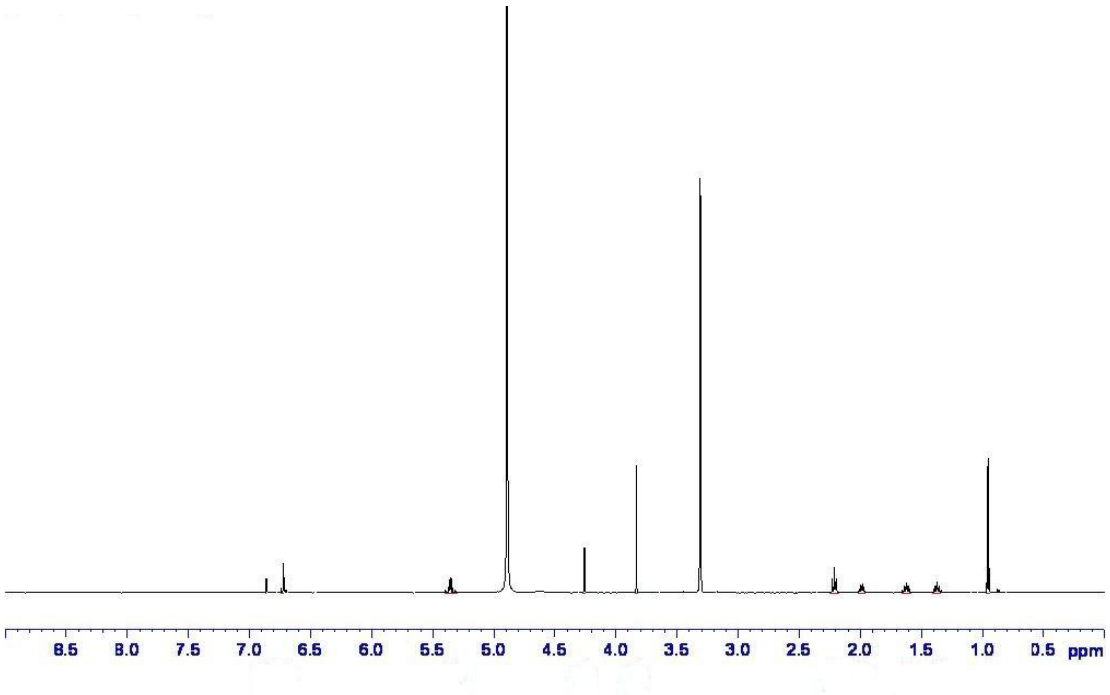


Figure 47: ^{1}H NMR of standard capsaicin in CD_3OD (500 MHz)

The molecular formula of capsaicin (BM-8) was determined as $C_{18}H_{27}NO_3$ and the measured mass of the molecular ion at *m/z* 303 $[M+2H]^+$ which corresponded to an expected mass of *m/z* 305.

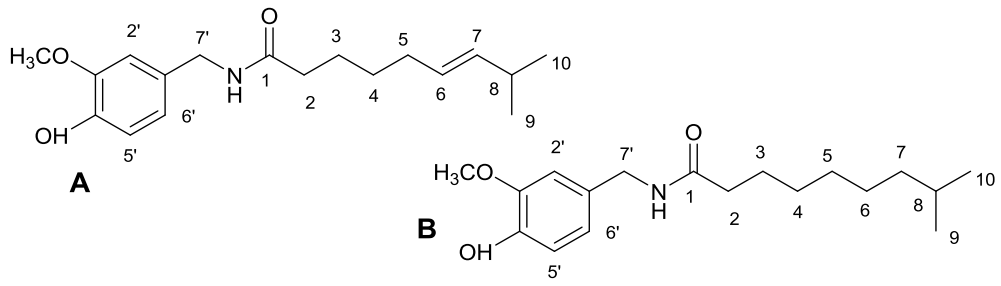


Table 15: NMR of capsaicin BM-8 and dihydrocapsaicin BM-9 in CD_3OD (^1H 500MHz, ^{13}C 125MHz)

	Capsaicin				Dihydrocapsaicin		
	^{13}C	^1H	^{13}C (100MH_Z) in $CDCl_3$ Kobata *et al*., 1998	^1H (400MH_Z)	^1H	^1H (100MH_Z) in $CDCl_3$ Kobata *et al*., 2009	^{13}C (400MH_Z)
1	173.1		172.9				173.1
2	37.0	2.14, t, *J*=7.5, 2H	36.7	2.19	2.33	2.19	36.9
3	27.1	1.69, s, 1H	25.3	1.65	1.54	1.64	25.9
4	28.4	1.31, *J*=8, 2H	29.3	1.38	1.29	1.31	29.6
5	33.3	1.94, 2H,	32.5	1.98	1.29	1.29	27.2
6	127.9	5.26-5.28, m, 2H	126.5	5.30	1.29	38.9	126.0
7	139.1	5.26-5.28, m	138.1	5.37	1.25		
8	30.1	2.15, t, *J*=7.5 2H	31.0	2.20	1.62	1.50	28.0
9, 10	23.1	0.70-0.88,dd, *J*=7.5 6H	22.7	0.95	0.91	0.86	22.7
1’	131.6		130.3				130.3
2’	112.5	6.78, s, 1H	110.7	6.79	6.85	6.84	114.5
3’	149.0		146.8				145.2
4’	146.8		145.2				146.8
5’	116.1	6.65, d, *J*=8.5, 2H	114.4	6.74	6.71	6.79	110.8
6’	121.4	6.65, d, *J*=8.5, 2H	120.7	6.80			120.8
7’	43.9	4.20	43.5	4.33	4.14	4.33	43.6
OCH_3	56.4	3.76, s, 3H	55.9	3.85	3.84	55.9	3.85
OH		5.16		5.87	5.35		5.89
NH		5.20		5.84	4.59		

The ^{1}H NMR spectrum of Ca-SPE 11 (Appendix 9) revealed the presence of major signals of capsaicin in a mixture of other compounds. Using TLC with solvent system of petroleum ether/chloroform/acetonitrile 4:4, 5:1, 0.5 v/v, after spraying with 1% vanillin–sulphuric acid spray, it was possible to visualise the various spots of reference capsaicin, Ca-SPE 11, combined capsaicin + Ca-SPE 11 and Ca-SPE 10 (Figure 48).

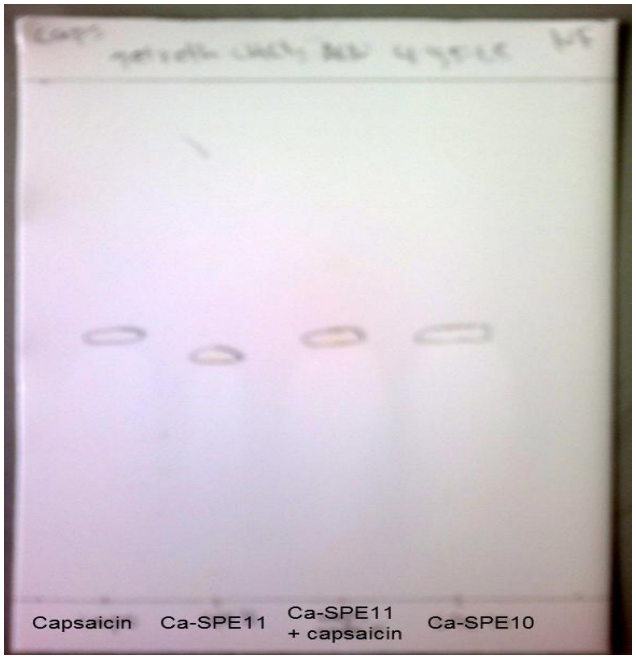


Figure 48: TLC analysis of capsaicin, Ca-SPE 11, capsaicin+ Ca-SPE 11 combined and Ca-SPE 10 under UV 254nm.

All of the samples recorded identical R_f value of 0.45 which confirmed the presence of capsaicin except Ca-SPE-11, which recorded an R_f value of 0.39. This suggested that the fraction Ca-SPE 11 contained a mixture of capsaicin and the presence of another capsaicinoid.

High performance liquid chromatography of Ca-SPE 11 was conducted with mobile phase solvent mixture of methanol-water from A-B 30%- 70% v/v to A-B 70%-30% for 20 min. Flow rate was 1 mL/min. The chromatogram is shown in Figure 49.

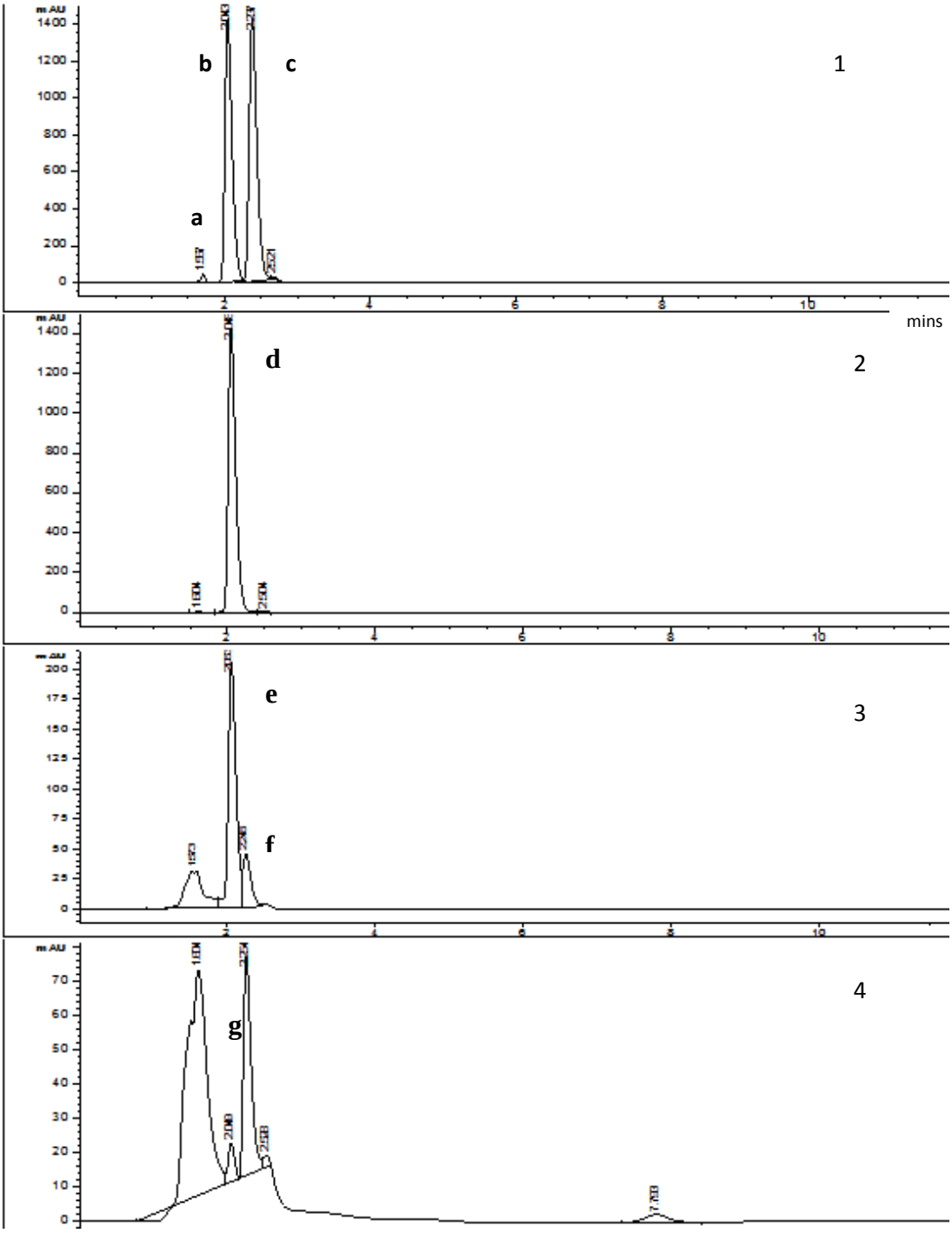


Figure 49: HPLC chromatogram of capsaicin and dihydrocapsaicin

Using gradient elution, the chromatogram showed baseline separation and retention times of minor solvent peak (1a at 1.57 min), pure capsaicin (1b at 2.05 min) and dihydrocapsaicin (1c at 2.25 min).

Samples 2 and 3, different samples were derived from Ca-SPE 10 and showed peaks (2d at 2.05 min) and (3e at 2.05 min) which corresponded to the retention times of the pure capsaicin. Sample 4, which is Ca-SPE 11, showed a major peak (4f at 2.25 min) which corresponded with dihydrocapsaicin (1c at 2.25 min) and a minor peak (4g at 2.04 min) showing capsaicin. Thus, the HPLC analysis of Ca-SPE-11 indicated presence of compound BM-9 (Figure 50), suggested to be dihydrocapsaicin (DHC)

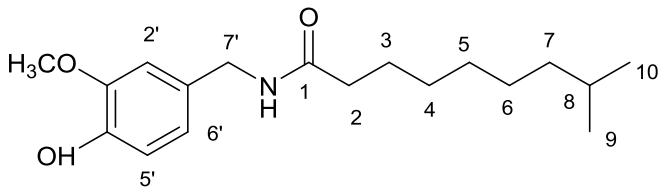


Figure 50: Structure of dihydrocapsaicin

The ^{1}H NMR of dihydrocapsaicin (Table 15) corresponded with the available data when compared with the literature (Li *et al.*, 2009). The spectral data were similar to those of capsaicin, confirming that dihydrocapsaicin is a dihydro derivative of capsaicin, and possessing a structural resemblance with capsaicin, except for the absence of olefinic protons (-CH=), δ 5.28, at positions H-6, H-7. The molecular formula of DHC was determined as $C_{18}H_{29}NO_3$, and the measured mass showed ion peak at *m/z* 308 $[M-H]^+$ which was in line with the calculated molecular mass of dihydrocapsaicin, *m/z* 307.

3.1.5 Physical properties of the compounds

Rottlerin: dark orange red solid, $R_{f\,=}$ 0.35 in hexane-ethyl acetate-formic acid (4:6:1). Molecular mass: *m/z* 516. Molecular formula: $C_{30}H_{28}O_8$. ^{1}H NMR: H-3 (5.49, d), H-4(6.66, d), H-7^1 (8.19, d), H-8^1 (7.83, d), H-2',4',6' (7.61, m), H-3',5' (7.41, m), H-CH_2 (3.80, s), H-COCH3(2.71, s), H-2CH_3 (1.54, s), C-5''CH_3 (2.08, s), H-2'',4''-OH (9.60, s). ^{13}C: C-2 (78.2), C-3 (125.1, C-4 (117.2), C-5 (158.8), C-6, 8 (105.3), C-7 (159.6), C-9 (155.4), C-10 (103.8), C-7^1 (126.9), C-8^1 (143.4), C-1' (135.5), C-2',4',6' (128.4), C-3,5' (129.0), C-9^1(193.1), C-1''(106.5), C-2''(162.8), C-3'' (103.8),C-4'' (158.8), C-5'' (103.8), C-6'' (161.0), C-CH2 (15.8), C-5''CH_3 (7.5), C-2CH_3 (28.0), CO$\underline{CH_3}$ (32.6), $\underline{CO}CH_3$ (204.1).

Red compound: red solid. Molecular formula: $C_{21}H_{20}O_4$. Molecular mass: *m/z* 336. ^{1}H NMR: H-3, 5 (7.61, m), H-2,4,6 (7.41, m), H-2' (14.36), H-4' (5.34), H-3'' (5.51, d), H-4'' (6.60, d), H-6'' (8.12, d), H-7'' (7.77, d), (H-Me2'' x2 (2.08, s), H-Me3' (1.58, s). ^{13}C: C-1 (135.72), C-3 (129.99), C-5 (128.93), C-2, 4, 6 (128.23), C-1' (77.74), C-2' (164.31), C-3' (101.70), C-4' (106.42), C-5' (155.82), C-6' (102.31), C-2'' (154.38), C-3'' (125.21), C-4'' (116.60), C-5'' (193.14), C-6''(127.81), C-7''(142.02), C-Me2'' x2 (27.25), C-Me3' (7.01).

Tetrahydrocannabinolic acid: Dark brown solid. Molecular formula: $C_{22}H_{30}O_4$. Molecular mass: *m/z* 358.5. ^{1}H NMR: H-4 (6.27, s), H-6 (1.17), H-7 (1.79), H-8 (1.80), H-10 (6.43, m), H-10a (2.99, s), H-11 (1.63, s), H-12 (1.63, s), H-1' (2.93, dd), H-2' (1.36, dd), H-3' & 4' (1.36, dd), H-5' (0.90 , dd), C1-OH (12.70, s), COOH (11.90, s). ^{13}C: C-1 (163.6), C-2 (103.9), C-3 (147.3), C-4 (113.0), C-5 (159.4), C-6 (78.6), C6a (46.7), C-7 (25.9), C-8 (31.3), C-9 (135.8), C-10 (122.8),

C-10a (31.9), C-11 (27.3), C-12 (27.5), C-1’ (36.9), C-2’ (22.6), C-3’& 4’ (31.2), C-5’ (14.1), COOH (175.4).

Cannabinolic acid: Dark brown solid. Molecular formula: $C_{22}H_{26}O_4$. Molecular mass: *m/z* 354. ^{1}H NMR: H-4 (6.43, s), H-8 (7.11,m), H-9 (7.16, m), H-9-Me (2.40, s), H-10 (8.43, s), H-11 (1.63, s), H-12 (1.63, s), H-1’ (0.9, dd), H-2’ (1.35, dd), H-3’ & 4’ (1.35, dd), H-5’ (2.93, dd), C1-OH (5.50, s), COOH (12.79, s). ^{13}C: C-1 (159.4), C-2 (103.8), C-3 (148.8), C-4 (113.0), C-5 (108.9), C-6 (78.6), C-7 (163.6), C-8 (122.6), C-9 (126.7), C9-Me (21.5), C-10 (127.1), C-11 (27.3), C-12 (27.5), C-1’ (36.9), C-2’ (22.6), C-3’& 4’ (31.2), C-5’ (14.1), COOH (174.9).

Evodiamine: Light yellow powder. Molecular formula: $C_{19}H_{17}N_3O$. Molecular mass: *m/z* 303. Soluble in chloroform. ^{1}H NMR: H-3 (5.92, s), H-5 (3.27 -3.23, m), H-6 (2.95 – 3.03, m), H-9 (7.42, d, *J*= 8), H-16 & 18 (7.13-7.23, m), H-17 (7.47-7.51, t, *J*= 7.5), H-19 (8.12, d, *J*= 7.5), N-CH_3 (2.50, s), N-H (8.24, s). ^{13}C: C-2 (133.1), C-3 (68.8), C-5(39.5), C-6 (19.7), C-7(113.7), C-8 (129.0), C-9 (118.9), C-10 (120.1), C-11 (122.5), C-12 (111.3), C-13 (136.7), C-15 (150.7), C-16 & 18 (122.5), C-17 (126.2), C-19 (128.2), C-20 (120.1), C-21 (164.8), N-CH_3 (37.3).

Rutaecarpine: Light yellow powder. Molecular formula: $C_{18}H_{13}N_3O$. Molecular mass: *m/z* 287.3. ^{1}H NMR: H-3 (5.92, s), H-5 (4.58- 4.61, t), H-6 (3.22 – 3.26, t), H-9 (7.18 -7.21, m), H-11 (7.33 -7.36, t), H-16 (7.42 -7.46, m), H-17 (7.64 -7.74, m), H-18 (8.31, dd), H-17 (8.13, s), H-18 (8.33, dd), N-H (9.13, s). ^{13}C: C-2 (127.3), C-3 (144.9), C-5 (41.1), C-6 (19.6), C-7(112.0), C-8 (125.5), C-8 (125.5), C-9 (118.3),

C-10 (120.0), C-11 (120.5), C-12 (111.8), C-13 (138.2), C-15 (147.6), C-16 (126.3), C-18 (127.3), C-17 (134.3), C-19 (126.8), C-20 (121.0), C-21 (161.6).

Capsaicin: white solid. Molecular formula: $C_{18}H_{27}NO_3$. Molecular mass: *m/z* 305. ^{1}H NMR: H-2 (2.24), H-3 (1.69), H-4 (1.31), H-5 (1.94), H-6 (5.36-5.28)), H-8 (2.15), H-9 & 10 (0.70-0.88), H-2' (6.78), H-5' (6.65), H-7' (4.20), OCH_3 (3.76), OH (5.16), NH (5.20). ^{13}C: C-1 (173.1), C-2 (37.0), C-3 (27.1), C-4 (28.4), C-5 (33.3), C-6 (127.9), C-7 (139.1), C-8(30.1), C-9 & 10 (23.1), C-1' (131.6), C-2' (112.5), C-3' (149.0), C-4 (146.8), C-5 (116.1), C-6' (121.4) C-7' (43.9), OCH_3 (56.4).

Dihydrocapsaicin: white solid. Molecular formula: $C_{18}H_{29}NO_3$. Molecular mass: m/z 307. ^{1}H NMR: H-2 (2.33), H-3 (1.54), H-4 (1.29), H-5 (1.29), H-6 (1.29), H-7 (1.25), H-8 (1.62), H-9 & 10 (0.91), H-2' (6.85), H-5' (6.71), H-7'(4.14), OCH_3 (3.84), OH (5.35), NH (4.59). ^{13}C: C-1 (173.1), C-2 (36.9), C-3 (25.9), C-4 (29.6), C-5 (27.2), C-6 (126.0), C-8 (28.1), C-9 & 10 (22.7), C-1' (130.3), C-2' (114.5), C-3' (145.2), C-4 (146.8), C-5 (110.8), C-6' (120.8) C-7' (43.6), OCH_3 (55.9).

3.2 Antibacterial activities of the compounds

3.2.1 Minimum inhibitory concentration (MIC) of standard antibiotics against multidrug-resistant *S. aureus* and Gram-negative bacteria

The results found in Table 16 and 17, show the MICs of all the antibiotics used for antibacterial assay, for a wide range of multidrug-resistant *S. aureus*, Gram-positive and Gram-negative bacterial strains.

Table 16: Antibacterial activities of standard antibiotics against some Gram-positive bacteria and multidrug-resistant *S. aureus*

	Minimum inhibitory concentration (mg/L)				
Gram-positive bacteria	Nor	Tet	Ery	Oxa	Cip
SA 1199B	32				
ATCC 29523	0.25				
Bacillus subtilis BsSOP01	0.25				
XU212		16			
EMRSA 15		0.25			
EMRSA 16		0.25			
RN 4220			32		
MRSA 346724				<0.25	
MRSA 774812				<0.25	
MRSA 274829				128	
MRSA 12981				8	
Enterococcus faecalis 13379					<0.06
Enterococcus faecalis 12697					<0.06

Nor= Norfloxacin, Tet= Tetracycline, Ery= Erythromycin, Oxa= Oxacillin, Cip=Ciprofloxacin. Results are outcome of three independent experiments, n=3

Table 17: MIC of ciprofloxacin against selected Gram-negative bacteria

Gram-negative bacteria	Ciprofloxacin (mg/L)
E. coli NCTC 10418	≤0.06
*Pseudomonas aeruginosa*10662	≤0.03
Klebsiella pneumoniae 342	≤0.03
Proteus sp P10830	32

Whilst the above panel of bacteria tested showed their various MIC to the antibiotics, the antibiotics have broad-spectrum bacterial activity and are available for use in the treatment of infections. However, some of bacterial strains have become resistant to the antibiotics, for example SA1199B to norfloxacin.

3.2.2 Minimum inhibitory concentration (MIC) of rottlerin (BM-1) and the red compound (BM-2) against multidrug-resistant *S. aureus* and Gram-negative bacteria

The two polyphenolic compounds, rottlerin and the red compound extracted from *M. philippinensis* showed antibacterial activities against multidrug-resistant *S. aureus* and Gram-negative bacteria with MIC ranged from 1 to 512 mg/L (Table 18). The most potent activity of rottlerin with an MIC value of 1 mg/L was observed against *E. faecalis* 12697, followed by *E. feacalis* 13379, SA1199B, MRSA 274829 and MRSA 12981 with MICs of 2 mg/L respectively. *E. faecalis* strains are known for their high level of resistance to the antibiotic vancomycin. The activity of rottlerin towards SA1199B (2 mg/L) was remarkably 16-fold lower than the MIC of antibiotic norfloxacin.

Table 18: Antibacterial activities of rottlerin and the red compound against multidrug-resistant *S. aureus* and Gram-negative bacteria

Strains	Minimum inhibitory concentration (mg/L)	
	Rottlerin	Red compound
SA1199B	2	32
ATCC 29523	4	32
Bacillus subtilis BsSOP01	4	NT
XU212	16	32
EMRSA 15	16	32
EMRSA 16	32	32
RN 4220	8	32
MRSA 346724	8	NT
MRSA 774812	8	NT
MRSA 274829	2	NT
MRSA 12981	2	NT
Enterococcus faecalis 13379	2	NT
Enterococcus faecalis 12697	1	NT
E. coli NCTC 10418	512	256
Pseudomonas aeruginosa 10662	512	256
Klebsiella pneumoniae 342	512	256
Proteus sp P10830	512	NT

NT= Not tested

Furthermore, rottlerin showed antibacterial activity against erythromycin–resistant *S. aureus* RN4220 and this compares favourably with erythromycin (8 mg/L). MRSA 346724 and MRSA 774812 strains had equally MIC value of 8 mg/L, while XU212 and EMRSA-15 recorded MIC value of 16 mg/L. Only EMRSA-16 showed an MIC of 32 mg/L towards rottlerin. The MICs of the red compound for all of the multidrug resistant strains that were tested (SA1199B, ATCC 29523, XU212, EMRSA-15, EMRSA-16 and RN4220) were 32 mg/L. The antibacterial activity appeared broad

with similar MIC values but with lesser range of activity against the same strains of bacteria compared to rottlerin. Rottlerin and the red compound had slight activity against all the Gram-negative strains including *E. coli* NCTC 10418, *P. aeruginosa* 10662, *K. pneumoniae* 342, and *Proteus sp* P10830 in the range of 256 - 512 mg/L and no inhibition was recorded or seen at lesser concentrations. Ciprofloxacin was used as control and showed an MIC range of 32 - 0.06 mg/L against Gram-negative bacteria.

3.2.3 MIC determination of evodiamine BM-5, rutaecarpine BM-6 and sucrose BM-7 against multidrug-resistant *S. aureus* and Gram-negative bacteria

The antibacterial activities of the quinolone alkaloids, evodiamine (BM-**5**) and rutaecarpine (BM-**6**), and the sucrose (BM-**7**) isolated from *Evodia* fruit against multidrug-resistant *S. aureus* and Gram-negative bacteria are listed in Table 19.

Table 19: Antibacterial activities of quinolone alkaloids and isolated sugar against multidrug-resistant *S. aureus* and Gram-negative bacteria

Strains	Minimum inhibitory concentration (mg/L)		
	Rut	Evo	Sucrose
SA 1199B	8	128	128
ATCC 29523	128	128	128
XU212	64	128	128
EMRSA 15	128	128	128
EMRSA 16	128	128	128
RN 4220	NT	NT	NT
E. coli NCTC 10418	≥128	≥128	≥128
P. aeruginosa 10662	64	128	128
K. pneumoniae 342	128	128	128

Rut= rutaecarpine, Evo= evodiamine

The MIC values of the alkaloids against *S. aureus* ATCC 29523, EMRSA-15, EMRSA-16, Gram-negative *E. coli*, and *K. pneumoniae* were 128 mg/L, except for rutaecarpine which showed varied activity with MIC of 8 mg/L towards SA1199B, four-fold better than the MIC of norfloxacin; and MIC of 64 mg/L towards *S. aureus* XU212 and *P. aeruginosa* strains respectively.

3.2.4 MIC determination of ferulenol (BM-10) against multidrug-resistant *S. aureus* and Gram-negative strains

Ferulenol BM-**10** (Figure 51) was obtained as a gift from Professor Giovanni Appendino, Italy. It is a major constituent, a coumarino-sesquiterpene compound from *Ferula communis*.

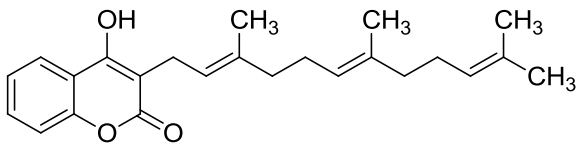


Figure 51: Structure of ferulenol

The antibacterial importance of the member compounds from *Ferula spp*, especially the anti-mycobacterial properties have been documented by other researchers (Galal *et al.*, 2001; Appendino *et al.*, 2004). However, little investigation has been done to access the antibacterial activity of ferulenol against multidrug-resistant strains of *S. aureus.* As a result, ferulenol was tested (128 mg/L) for its antibacterial potential against a wide range of multi-resistant *S. aureus* strains; SA1199B, SA 13373, ATCC 29523, XU212, RN4220, EMRSA-15, and -16, MRSA 346724, MRSA 774812, MRSA 274829, and MRSA 12981. The study extended to *E. faecalis* 12697, *E. faecalis* 13379, *B. subtilis* BsSOP01, *E. coli*, *P aeruginosa* and *K. pneumoniae* (Table 20). Ferulenol displayed good antibacterial against the above wide range of multi-drug resistant Gram-positive bacteria with MIC values raged from of 0.25 - 32 mg/L,

having comparably higher activity than norfloxacin (32 mg/L), tetracycline(16 mg/L), and oxacillin (8 mg/L), which are commonly used to treat infections caused by Gram-positive bacteria. However, the MIC of ferulenol against the Gram-negative bacteria was 256 mg/L which indicate that the Gram-negative bacteria were insensitive to ferulenol unlike the Gram-positive strains.

Table 20: Antibacterial activities of ferulenol against multidrug-resistant *S. aureus* and Gram-negative bacteria

Strains	MIC of Ferulenol (mg/L)
SA1199B	0.5
ATCC 29523	4
B. subtilis BsSOP01	1
XU212	32
EMRSA-15	16
EMRSA-16	16
RN 4220	8
MRSA 346724	1
MRSA 774812	1
MRSA 274829	1
MRSA 12981	0.5
SA13373	1
Enterococcus faecalis 13379	0.25
Enterococcus faecalis 12697	0.25
E.coli NCTC 10418	256
P. aeruginosa 10662	256
K. pneumoniae 342	256
Proteus sp P10830	256

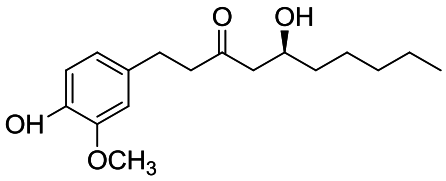
3.2.5 MIC determination of capsaicin and capsaicin-like compounds against multidrug-resistant *S. aureus* and Gram-negative bacteria

The antibacterial activities of capsaicin and capsaicin-like compounds were assessed against the panel of multi-drug resistant Gram-positive and Gram-negative bacteria (Table 21).

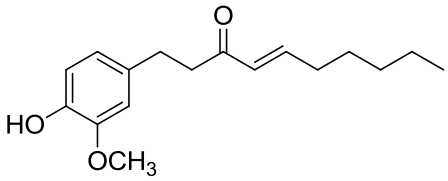
Table 21: Antibacterial activities of capsaicin and capsaicin-like compounds against multidrug resistant *S. aureus* and Gram-negative bacteria.

Strains	Minimum inhibitory concentration (mg/L)				
	Cap	DHC	Noni	6-gin	6-sho
SA1199B	256	512	256	16	16
ATCC 29523	256	128	256	128	128
B. subtilis BsSOP01	128	256	128	64	NT
XU212	256	256	64	16	16
EMRSA-15	256	512	128	64	32
EMRSA-16	256	512	512	512	512
RN4220	256	512	128	128	8
MRSA 346724	256	512	256	128	16
MRSA 774812	256	512	512	512	512
MRSA 274829	256	256	512	64	64
MRSA 12981	256	512	128	512	128
E. faecalis 13327	256	128	128	8	NT
E.coli NCTC 10418	512	512	512	512	128
P. aeruginosa 10662	512	512	512	512	128
K. pneumoniae 342	512	512	512	512	128
Proteus sp P10830	512	512	512	512	512

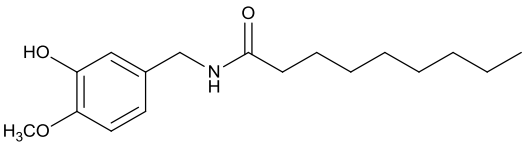
Cap= capsaicin, DHC= dihydrocapsaicin, Noni= nonivamide, 6-gin= gingerol, 6- sho= shogaol, NT= Not Tested



6-gingerol



6- shogaol



Nonivamide (pseudocapsaicin)

Capsaicin had constant MIC value of 256 mg/L, which was poorly active to all of the bacteria tested, including *B. subtilis* with an MIC of 128 mg/L (Table 21). DHC and nonivamide, both derivatives of capsaicin, had similar poor activity with MIC values of 512 - 64 mg/L, against the MRSA and the Gram-negative bacteria. Only 6-gingerol and 6-shogaol were active against SA1199B and XU212 at 16 mg/L and MRSA 274829 at 64 mg/L. The most potent activity was shown by 6-shogoal against RN4220 with MIC of 8 mg/L, four-fold lesser than the MIC of erythromycin (32 mg/L). All the compounds tested were not active against the Gram-negative bacteria (128 - 512 mg/L) compared to the antibiotic ciprofloxacin.

3.3 The antiplasmid activities of selected compounds on the bacterial plasmid conjugal system in *E. coli.*

3.3.1. MIC determination of the compounds against *E. coli* NCTC 10418

In order to carry out the plasmid transfer inhibition assay, the MIC of all of the test samples were determined against *E. coli* NCTC 10418 and sub-inhibitory concentration (SIC) of the respective agents were determined. The MIC results showed that the test samples had no inhibition against *E. coli* at 512 mg/L (Appendix 11).

The use of sub-inhibitory concentrations (SIC), which is ¼ of MIC, allowed the growth of the bacterium sub-optimally as one of the required conditions for the demonstration of plasmid transfer inhibition because transfer can obviously only occur within a living bacterial host. Given that most of the compounds had MIC of 512 mg/L and therefore SIC values of 128mg/L, the test concentrations were adjusted to optimal concentration of 100 mg/L and 10 mg/L. This enabled for ease of testing of the compounds at the same SIC value or lower dilutions, when applicable.

3.3.1.1 Determination of conjugation efficiency of the plasmids

To determine the maximum conjugation efficiency of the mating system between the plasmid-donor harboring and recipient *E. coli*, the transfer frequency was measured in colony forming unit per mL (cfu/mL) of the bacterial population. The transfer frequency, also known as conjugation frequency or efficiency, transfer or conjugation rate is calculated:

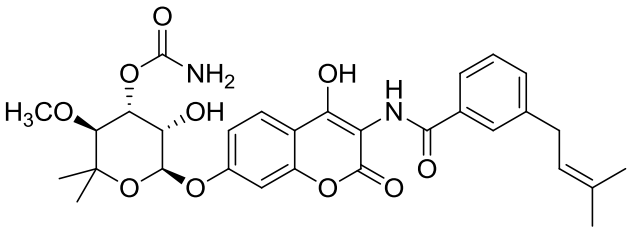
$$\text{Transfer frequency cfu/mL} = \frac{\text{Number of transconjugants}}{\text{Total number of donor carrying cells}}$$

The transfer frequency equals conjugation frequency as a fraction of 1. So a value of 0.1 indicates a transfer frequency of 1 in 10, a value of 0.01 indicates 1 in 100, and a value of 0.001 indicates 1 in 1000. For clarity of expression in the course of this project, the transfer frequency was further expressed as a percentage value relative to the control 100% (Transfer frequency (%) = sample/control x 100). Transposition of the degree of transfer therefore implies that the transfer frequency of the plasmid is inversely proportional to the rate of inhibition of the drug, which means that the lower the transfer frequency of the plasmid, the higher the extent of inhibition by the drug, and vice versa.

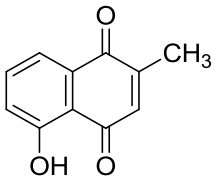
The transfer frequency values (%) were categorized into three levels of inhibition in order to determine the degree of inhibition demonstrated by the test compounds relative to the plasmid free control. The three levels of inhibition were: active, if transfer frequency value falls between the range 0-10%, moderate, when the values are within 15 - 50%; and poor or no activity when the values are 50% and above. In some cases, no inhibition may be observed and the transfer frequency value may become higher than the control, thus enhancement of the plasmid transfer.

3.3.2 The inhibitory activities of promethazine, novobiocin and plumbagin against the conjugal transfer of five plasmid strains.

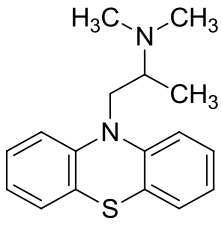
The anti-plasmid activities of promethazine, plumbagin and novobiocin were conducted based on their previous reports on anti-plasmid and direct antibacterial activities of the compounds against some bacteria (Molnar and Nakamura 1988; Amaral *et al.,* 1992; Monlar *et al.,* 1992; Monlar *et al.,* 2003). The ability of these compounds to inhibit plasmid transfer was attributed to probable ability to inhibit plasmid replication and partition systems.



Novobiocin (MIC=64 mg/L)



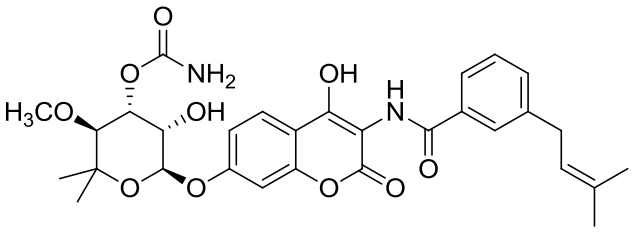
Plumbagin (MIC=32 mg/L)



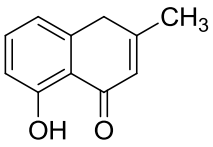
Promethazine (MIC=128 mg/L)

The MICs of these compounds were determined against model *E. coli* NCTC 10418 and the results were promethazine (128 mg/L), novobiocin (64 mg/L) and plumbagin (32 mg/L). Plumbagin had the lowest MIC value of 32 mg/L, followed by novobiocin (64 mg/L) and promethazine (128 mg/L) against *E. coli* 10418, but all were less potent compared to the MIC of ciprofloxacin antibiotic at 0.03mg/L.

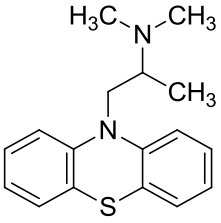
Promethazine was tested at SIC value of 32 mg/L, while for ease of handling, novobiocin and plumbagin were tested at an optimal value of 10 mg/L respectively, for their abilities to inhibit the transfer of resistant plasmids between the plasmid-containing donors PKM 101, TP114, PUB 307, R6K and R7K; and recipients *E. coli* ER 1973 and JM101 (Figure 52).



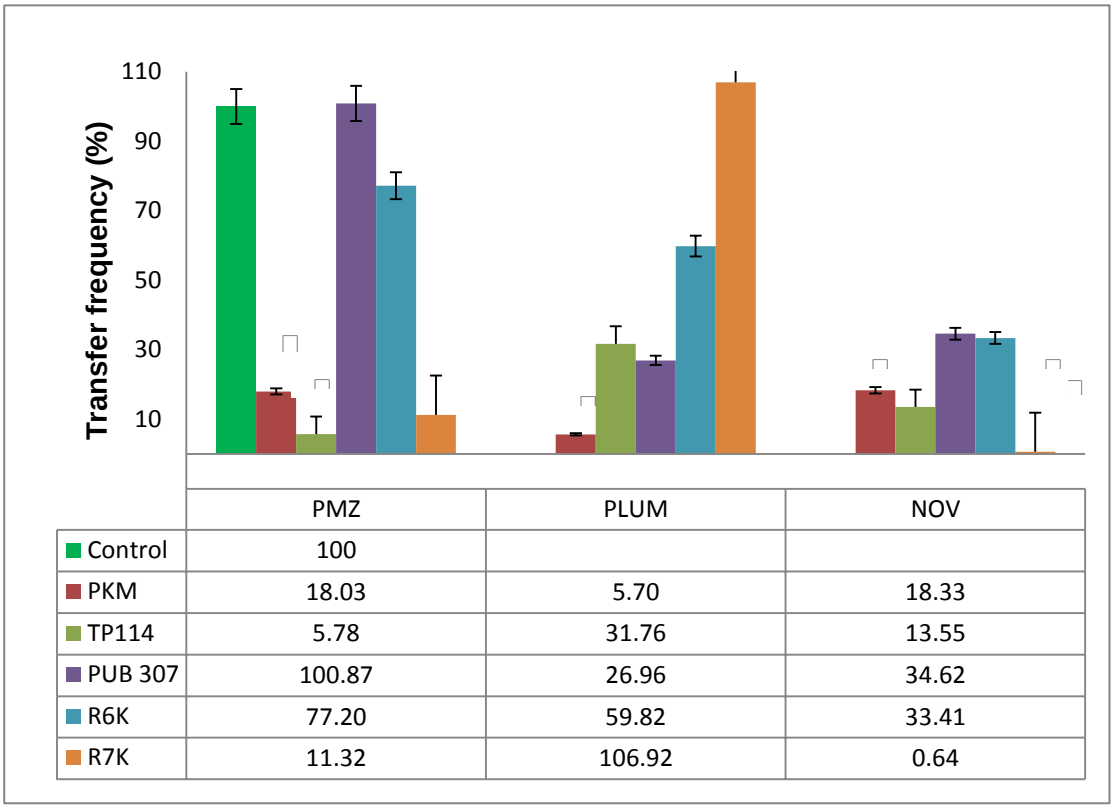
Novobiocin 10 mg/L



Plumbagin 10 mg/L



Promethazine 32 mg/L



	PMZ	PLUM	NOV
Control	100		
PKM	18.03	5.70	18.33
TP114	5.78	31.76	13.55
PUB 307	100.87	26.96	34.62
R6K	77.20	59.82	33.41
R7K	11.32	106.92	0.64

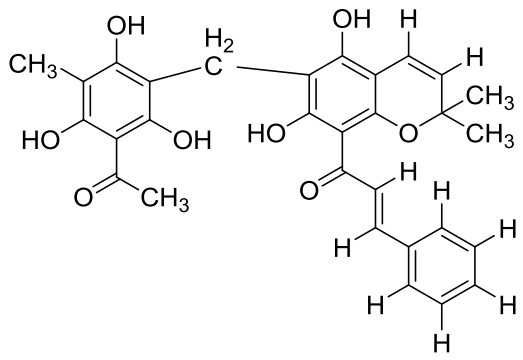
Figure 52: The antiplasmid activities of promethazine (PMZ), novobiocin (Nov) and plumbagin (Plum). Each replicate was used as a data point, N=6. □ indicates ($p<0.05$).

The results (Figure 52) showed a moderate degree of inhibition by promethazine (18.03%), and novobiocin (18.33%), while plumbagin was active with a lower

transfer frequency (5.70%) towards amoxicillin-resistant PKM 101. The levels of inhibition by the three compounds against PKM 101 were statistically significant compared to the control. Promethazine and novobiocin recorded significant reduction with low transfer frequencies of 5.7% and 13.6% respectively, than plumbagin with 31.76% against the kanamycin-resistant TP114. Plasmids PUB307 and R6K bearing amoxicillin resistance, appeared to be unsusceptible to promethazine but susceptible to the treatment of plumbagin and novobiocin with moderate inhibition. Promethazine and novobiocin with transfer rates of 0.64% and 11.31%, demonstrated anti-plasmid activity against amoxicillin-conferring R7K strain, whereas no inhibition was in R7K when it was treated with plumbagin.

3.3.3 The inhibitory activities of rottlerin (BM-1) against the conjugal transfer of resistant (R) - plasmids.

The ability of rottlerin to inhibit the transfer of bacterial plasmids; PKM 101, TP114, PUB307, R6K, and R7K was evaluated at SIC values of 100 and 50 mg/L (Figure 53). The results as shown in Figure 53A showed that rottlerin had a remarkable transfer frequency of less than 1% at 100 mg/L towards PKM 101, but a higher transfer frequency of 37.40% at 50 mg/L, indicating a concentration–related activity.



Rottlerin (SIC 100 mg/L)

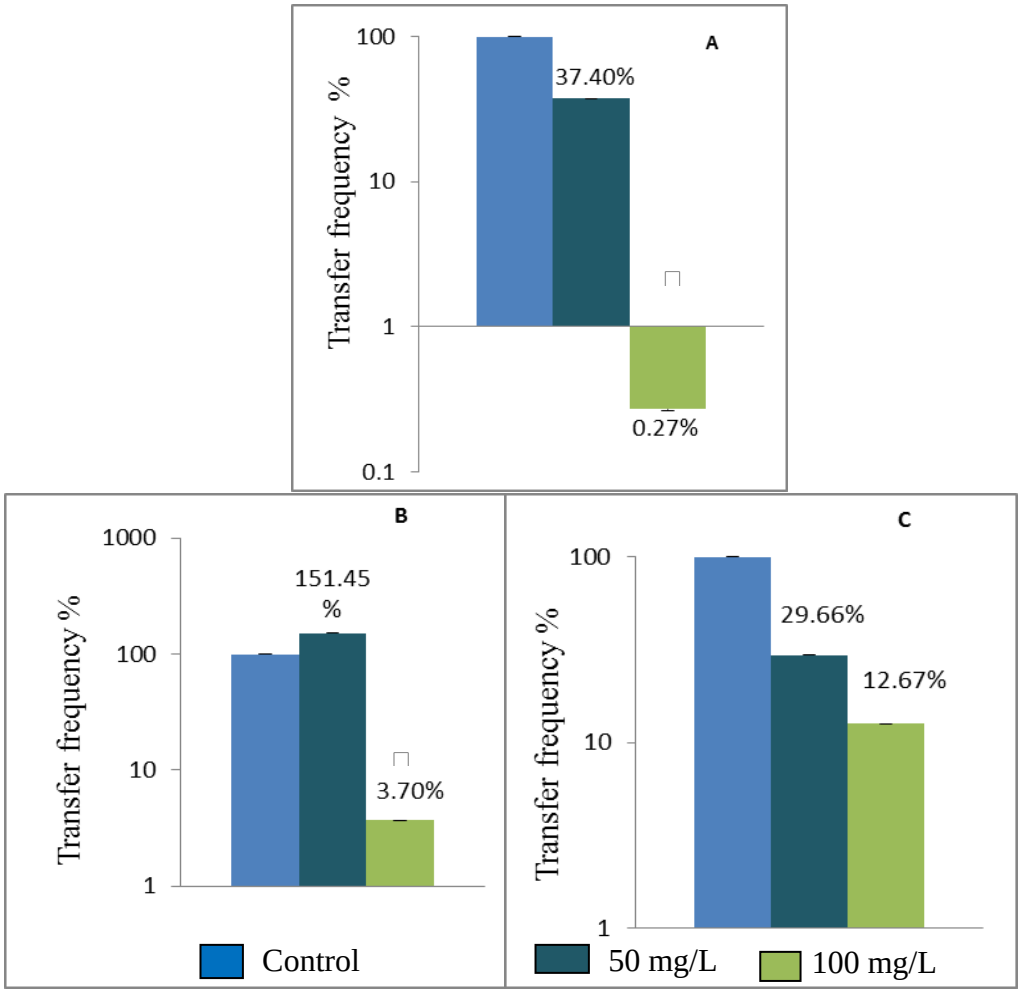


Figure 53A-C: The anti-plasmid activities of rottlerin. A=PKM 101, B=TP114, C=PUB 307. N=6

Rottlerin was equally active at 100 mg/L against TP114 as seen in a low transfer frequency of 3.7% (Figure 53B) but a slightly higher transfer frequency value of 12.67% towards PUB 307 (Figure 53C).

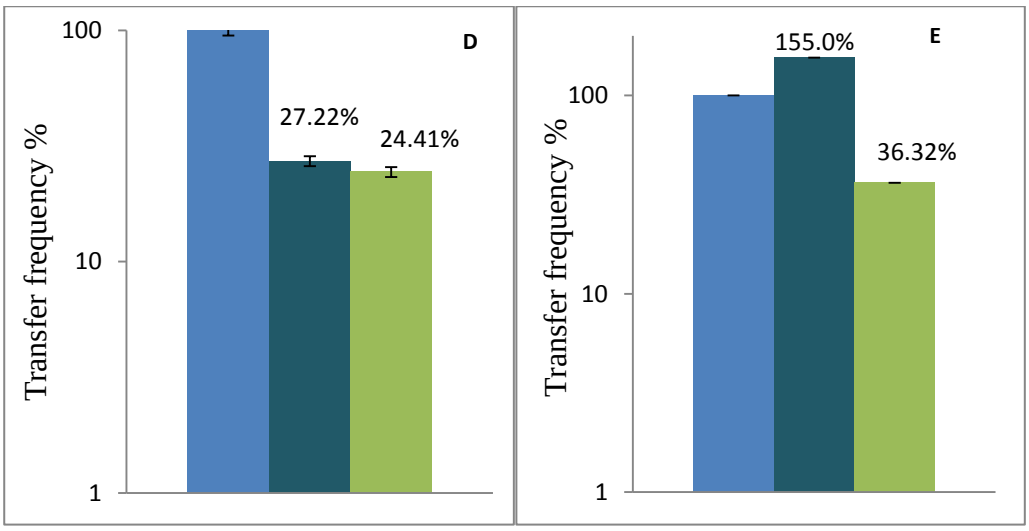
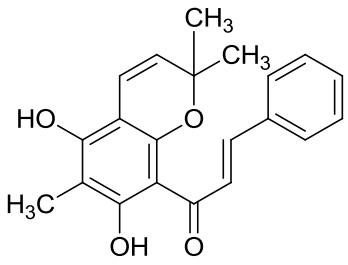


Figure 53D-E: The antiplasmid activities of rottlerin against plasmids R7K (D) and R6K (E). Each replicate was used as a data point, N =6.

Rottlerin recorded transfer frequency values of 24.41 and 36.32% towards R7K and R6K (Figure 53D-E). On the contrary, at a lesser concentration of 50 mg/L, the transfer frequency of plasmids TP114 (Figure 53C) and R6K (Figure 53E) were enhanced by rottlerin, while, the transfer frequencies of all the other plasmids PKM 101, PUB307, R7K ranged between 27.22% - 39.40%.

3.3.4 The inhibitory activities of the red compound (BM-2) against the conjugal transfer of R-plasmid.

The inhibition of conjugal transfer of plasmids PKM 101, TP114 and PUB 307 by the red compound (BM-2) was investigated in *E. coli* at sub-inhibitory concentration of 100 mg/L (Figure 54).



Red compound (SIC 100 mg/L)

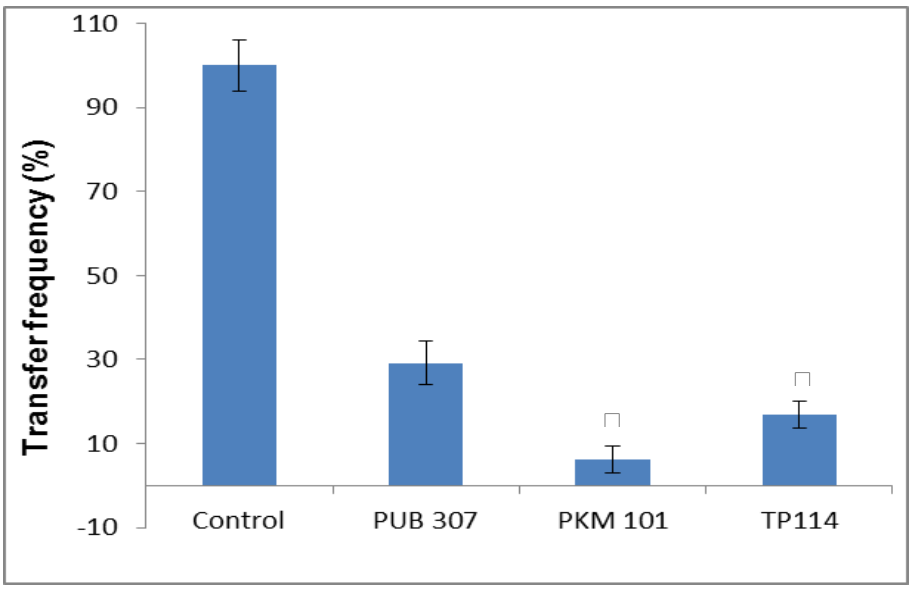
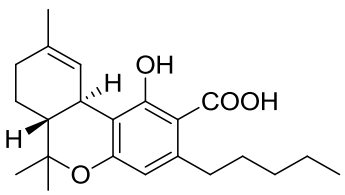


Figure 54: The antiplasmid activities of BM-2, red compound against plasmids PUB 307, PKM 101 and TP114. N =6. indicates ($p<0.05$).

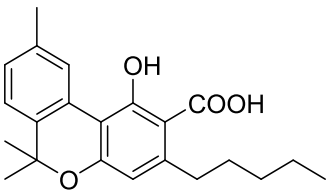
The red powder had significant inhibition ($p<0.05$) on the transfer of PKM 101, with a transfer frequency value of 6.23%, and a moderate inhibitory activity against TP114, with a transfer frequency of 17.03%. The transfer frequency of 29.16% was observed towards PUB 307.

3.3.5 The anti-plasmid activities of cannabinoids (BM-3 & 4) on the inhibition of plasmid conjugal transfer

The VLC fractions (VSc1-9) of the chloroform extract of *C. sativa* were tested for plasmid conjugation inhibition using the plasmid TP114 at a SIC value of 100 mg/L (Figure 55). Promethazine and cannabidiol were used as positive controls to the test samples. Fraction 9 (VSc-9) showed an active reduction of TP114 with a transfer frequency value less than 10%. As a result, the bioactive semi pure fraction VSc-9 was further subjected to phytochemical analysis, which yielded two major cannabinoids namely; tetrahydrocannabinolic acid, BM-3 (THCA) and cannabinolic acid, BM-4 (CBNA).



Tetrahydrocannabinolic acid (THCA)



Cannabinolic acid (CBNA)

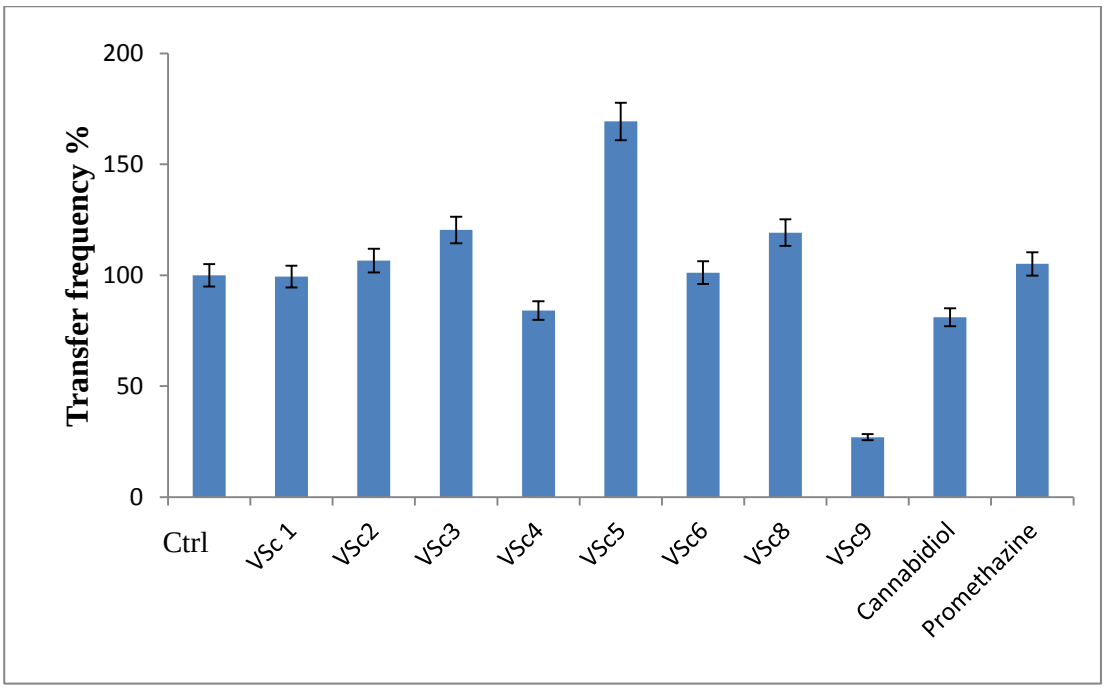
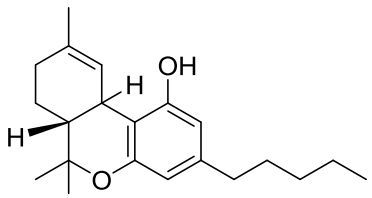
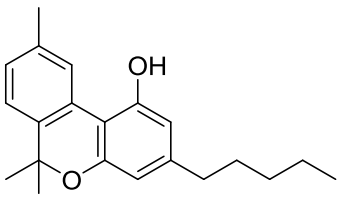


Figure 55: The antiplasmid activities of VLC fractions (VSc1-9) from *C. sativa* against TP114 at 100 mg/L. N=6

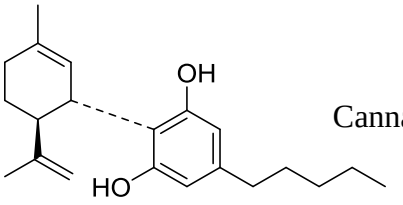
To establish the antiplasmid activities of the individual cannabinoids, pure samples of 9-tetrahydrocannbinol (THC), cannabinol (CBN), and cannabidiol (CBD) were purchased and tested against plasmids PKM 101, TP114 and PUB 307 at 100 mg/L were determined (Figure 56A-C).



Δ9-tetrahydrocannabinol



Cannabinol



Cannabidiol

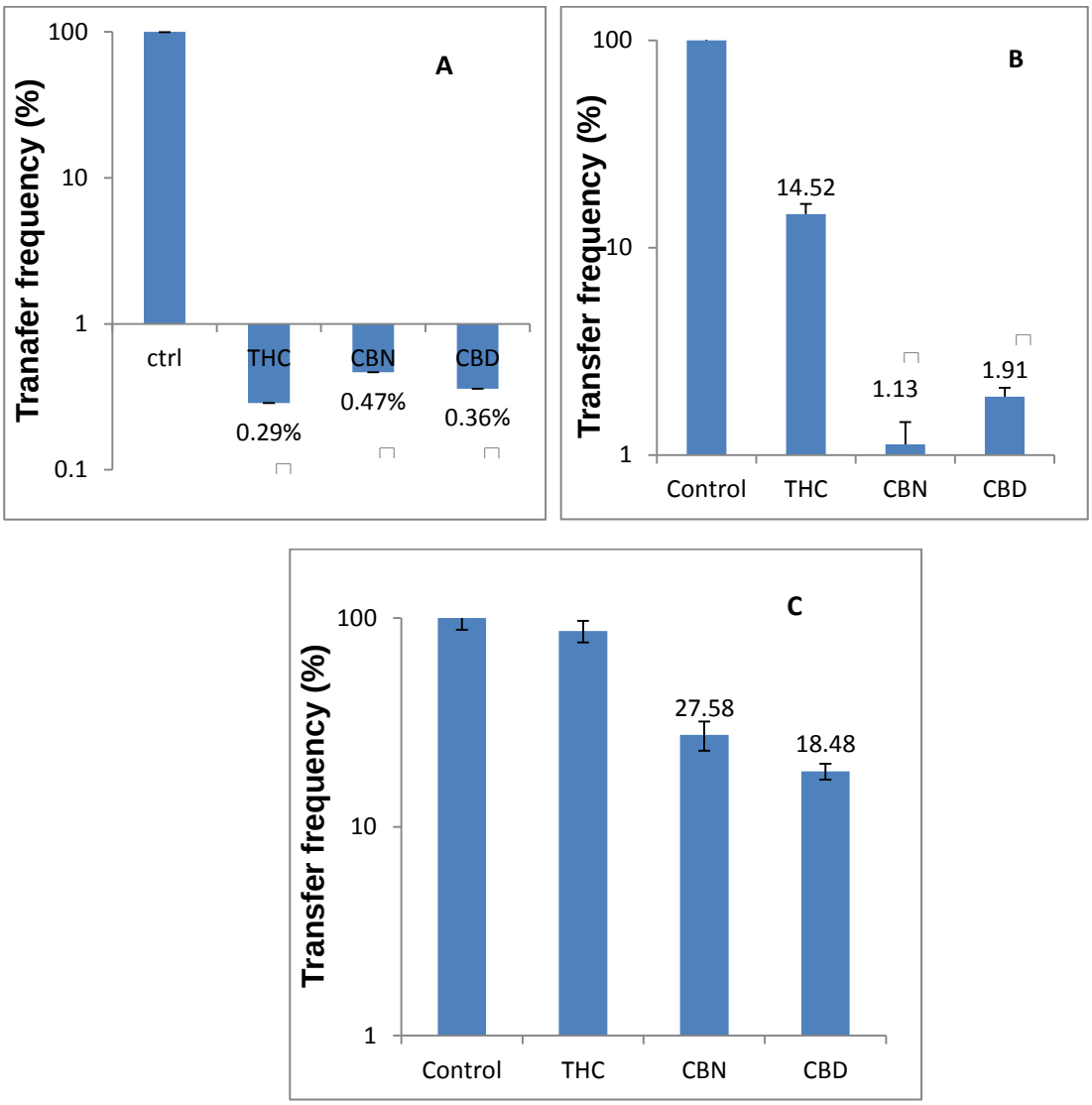


Figure 56: The anti-plasmid activities of Δ9-THC, CBN and CBD against three different R-plasmids at 100 mg/L. THC = Tetrahydrocannabinol, CBN = Cannabinol, CBD = Cannabidiol. A = PKM101, B = TP114 and C = PUB307. N =6. □ indicates ($p<0.05$).

As shown in Figure 56, the plasmid transfer inhibition ability of THC, CBN, and CBD were evaluated independently on PKM 101, TP114 and PUB 307 at 100 mg/L. The effect of THC, CBN and CBD on PKM 101 resulted into very low transfer frequencies, 0.29% - 0.47% than 1%, an indication that these compounds are active inhibitors of PKM 101 transfer activities in *E. coli*. Significant inhibition was noted

against TP114 with 1.13% and 1.91% transfer frequencies in the presence of CBN and CBD; followed by THC with transfer frequency of 14.53%. These cannabinoids displayed promising potential against PKM 101 and TP114 transfer frequencies, which may perhaps suggest plasmid-specific activity.

Some cannabinoid derivatives synthesized by Dr Proma Khondhar, University College London were also tested for plasmid conjugal transfer inhibition activity against TP114 at SIC value of 100 mg/L. The compounds designated Sc1- 6 showed various transfer frequencies compared to the control (Figure 57).

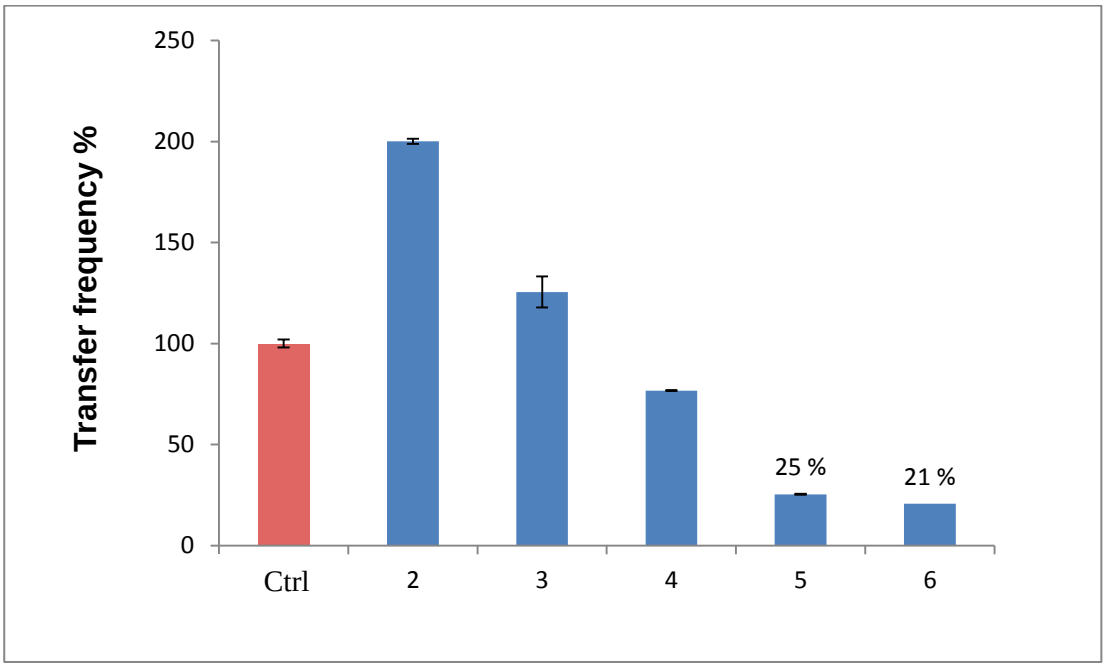


Figure 57: The anti-plasmid activities of synthetic cannabinoids against the conjugal transfer of TP114. N=6

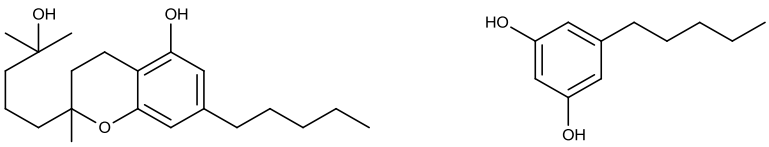


Figure 58: Structures of cannabigerol-type cannabinoid (CBG-1) and olivetol

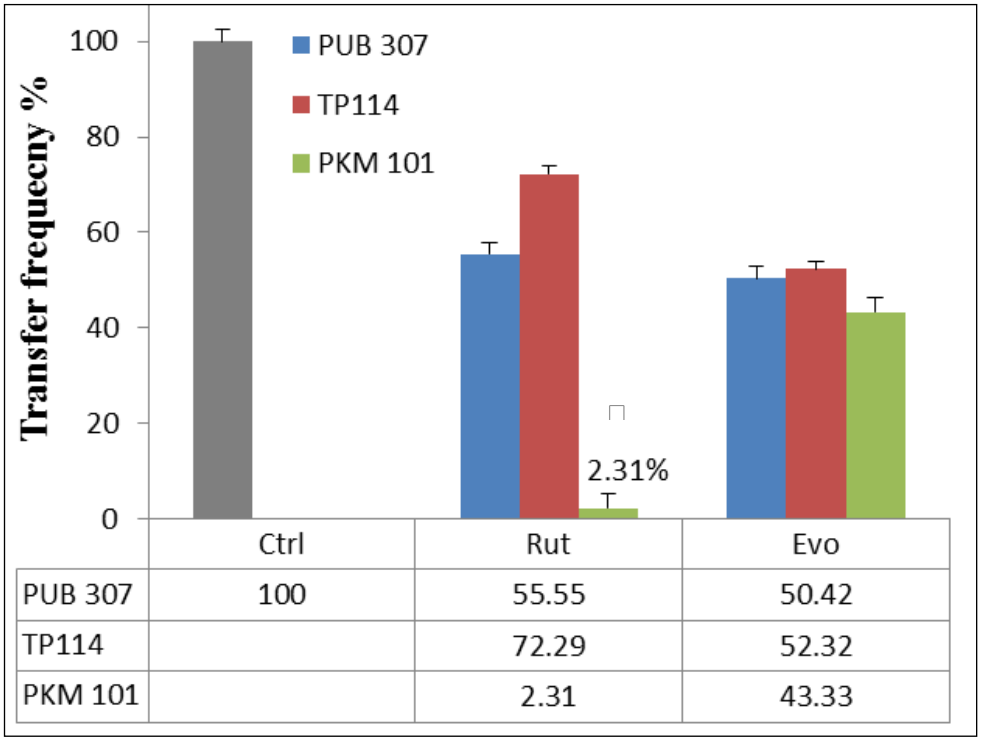
Both compounds, designated as CBG-1 (Fr 5) and olivetol (Fr 6) in Figure 58 recorded transfer frequencies of 25.44% and 20.75% respectively, while the transfer

frequencies of the compounds Sc1-Sc4 demonstrated little or no inhibition against the plasmid TP114.

3.3.6 The inhibitory activities of quinolone alkaloids (BM-5, 6 & 7) from *Evodia* on the inhibition of plasmid conjugal transfer

The acidic, basic and methanol extracts of *E. rutaecarpa* were assessed for their possible antiplasmid activities using TP114. The results of the antiplasmid activities of the VLC sub-fractions from bioactive EVB extract is summarised in Appendix 11. The fraction Ev6 recorded 21.06 % inhibition against the plasmid transfer compared to the other fractions tested and the control. This suggested the presence of active principles in the fractions.

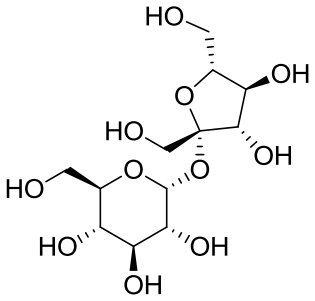
Further bioassay-guided separations resulted in the isolation of quinolone compounds namely; evodiamine, BM-5 and rutaecarpine, BM-6. The anti-plasmid effect of pure evodiamine and rutaecarpine were re-investigated on only three plasmid strains namely PKM 101, PUB 307 and TP114 due to limited amount of compounds (Figure 59).



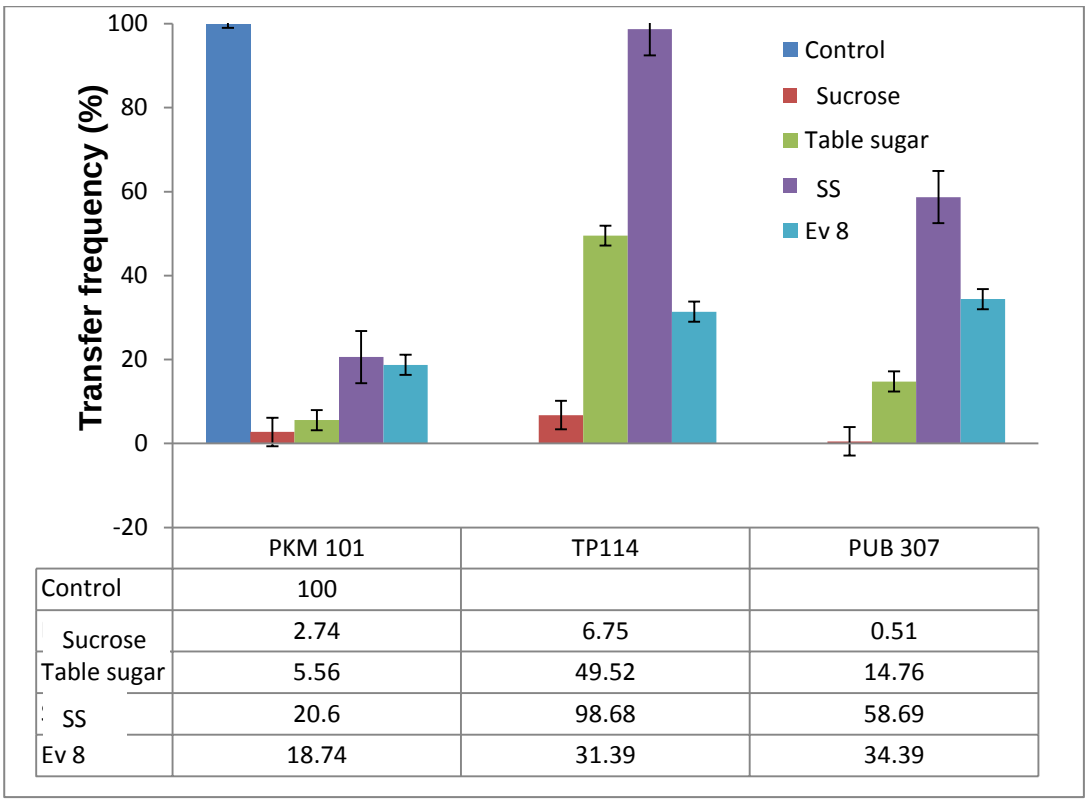
	Ctrl	Rut	Evo
PUB 307	100	55.55	50.42
TP114		72.29	52.32
PKM 101		2.31	43.33

Figure 59: Anti-plasmid activities of quinolone alkaloids against TP114 and PUB 307. N =6. □ indicates ($p<0.05$). Rut= Rutaecarpine. Evo= evodiamine

The pooled fraction, Ev 7/8 (EV8) reduced the transfer of TP114 to 27.61% (Appendix 11) and was further analysed using HPLC separation, which yielded the major compound BM-7, sucrose. A further assay was conducted to validate the anti-plasmid activity of the semi-purified fraction Ev-8, the isolated sucrose (BM-7), standard sucrose purchased from Sigma, UK, and table sugar, all were assayed against TP114, PKM 101 and PUB 307 at 100 mg/L. The results are represented in Figure 60.



Sucrose (SIC 100 mg/L)



	PKM 101	TP114	PUB 307
Control	100		
Sucrose	2.74	6.75	0.51
Table sugar	5.56	49.52	14.76
SS	20.6	98.68	58.69
Ev 8	18.74	31.39	34.39

Figure 60: Anti-plasmid activities of the isolated sucrose, table sugar, standard sucrose (SS) and Ev-8. N =6. □

3.3.7 The anti-plasmid activities of various disaccharides against PKM 101, TP114 and PUB 307

The results obtained from Figure 60 showed that plasmid transfer frequencies were lowered by the sucrose from *Evodia* fruit, Ev-8, table sugar and the standard sucrose, especially the plasmid PKM 101. Therefore further evaluation of a wide range of disaccharides (Table 22) were purchased from Sigma, UK, and assayed for potential antiplasmid activity against PUB 307, TP114 and PKM 101 at 100 mg/L.

Table 22: Anti-plasmid activities of various disaccharides against PKM 101, TP114 and PUB 307 at 100 mg/L

Disaccharides	Transfer frequencies (cfu/mL)		
	PKM 101	TP114	PUB 307
Control	$5.2 \times 10^{-4} \pm 7.38$	$4.8 \times 10^{-4} \pm 22.72$	$2.9 \times 10^{-4} \pm 14.67$
Sucrose	$1.6 \times 10^{-6} \pm 19.50$	$1.1 \times 10^{-4} \pm 29.89$	$1.5 \times 10^{-5} \pm 4.34$
Lactose	$2.1 \times 10^{-6} \pm 19.52$	$3.9 \times 10^{-4} \pm 26.49$	$9.4 \times 10^{-5} \pm 13.22$
Maltose	$5.3 \times 10^{-6} \pm 36.85$	$9.6 \times 10^{-5} \pm 38.60$	$2.9 \times 10^{-5} \pm 19.26$
Cellobiose	$7.4 \times 10^{-6} \pm 8.49$	$2.6 \times 10^{-6} \pm 28.49$	$3.6 \times 10^{-4} \pm 5.07$
Meliobiose	$4.8 \times 10^{-6} \pm 39.96$	$1.7 \times 10^{-4} \pm 22.34$	$3.0 \times 10^{-5} \pm 7.80$
Maltulose	$1.2 \times 10^{-5} \pm 15.50$	$4.9 \times 10^{-4} \pm 29.00$	$2.9 \times 10^{-4} \pm 32.18$
Rutinose	$6.9 \times 10^{-6} \pm 21.72$	$8.4 \times 10^{-4} \pm 30.39$	$2.6 \times 10^{-4} \pm 26.04$
Turanose	$1.4 \times 10^{-4} \pm 12.64$	$4.2 \times 10^{-4} \pm 45.79$	$2.4 \times 10^{-5} \pm 27.50$
Mennobiose	$6.6 \times 10^{-6} \pm 11.18$	$5.0 \times 10^{-4} \pm 23.39$	$1.9 \times 10^{-4} \pm 10.10$
Xylobiose	$2.1 \times 10^{-5} \pm 6.55$	$1.4 \times 10^{-4} \pm 32.76$	$1.2 \times 10^{-4} \pm 11.76$

Premating experiment showing total number of cells (cfu/mL)

PKM 101	TP114	PUB 307	ER 1473
1.80×10^{-6}	5.50×10^{-8}	3.00×10^{-7}	5.20×10^{-8}

Results shown as average of two independent experiments ± SD

Overall, the results (Table 22) showed that conjugation took place in the presence of these disaccharides without significant reduction in the transfer rates of the trans-conjugants of PKM 101, TP114 and PUB 307. Cellulobiose was the only disaccharide that demonstrated a mild reduction of 2.6×10^{-6} cfu/mL on TP114 compared to the control (4.8×10^{-4}). Premating counts of the plasmids indicated the initial start number of the donor and recipient plasmids without drug treatment, used in the experiment

3.3.7.1 The anti-plasmid activity of evocarpine (BM-11) on the inhibition of plasmid conjugal transfer

Evocarpine (BM-11) (Figure 61) is one of the major quinolone alkaloids from *Evodia* fruit, and obtained as a gift from Professor Franz Bucar of Austria. Evocarpine, like related quinolones, have been shown as an effective antibacterial and anti-mycobacterial agent. As a result, the antiplasmid potential of evocarpine was tested at 100 mg/L on our panel on R-plasmids: PKM 101, TP114, PUB307, R7K and R6K (Figure 60). R1-drd-19 was selected for both amoxicillin and kanamycin resistance, in order to assess any specificity inhibition of the compound.

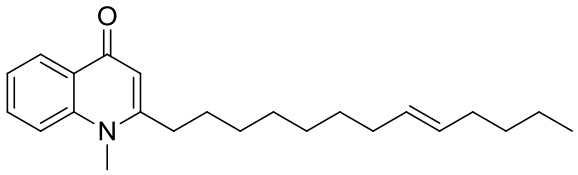


Figure 61: Evocarpine (SIC 100 mg/L)

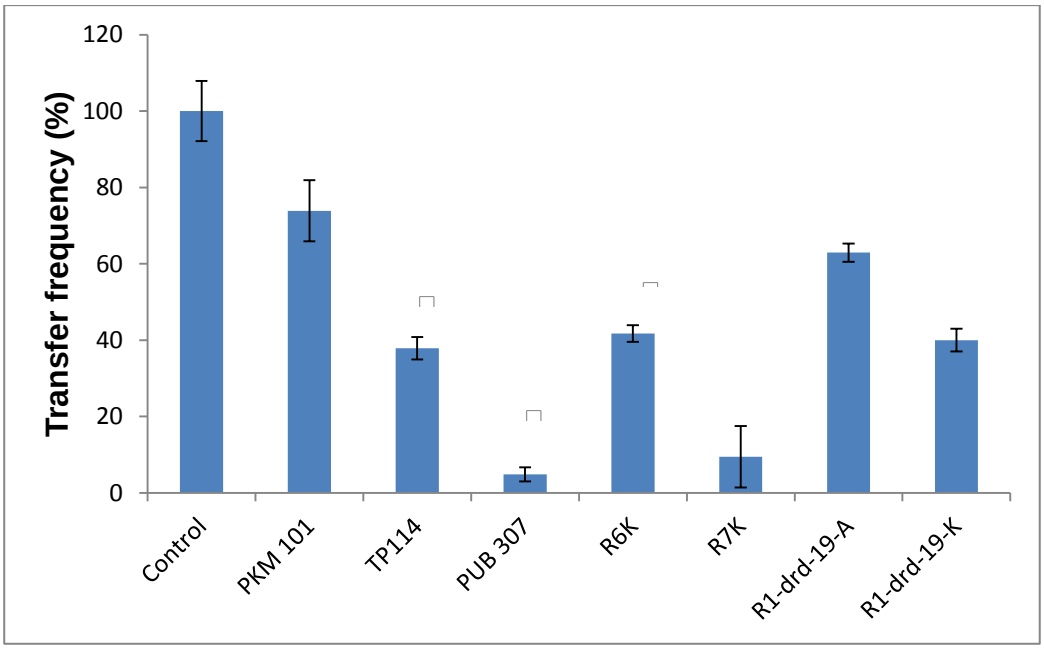
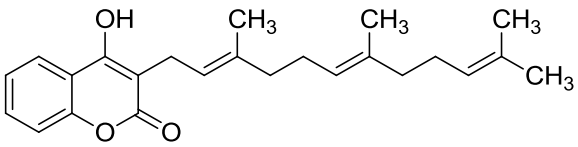


Figure 62: Selective anti-plasmid activity of evocarpine (R1-drd-19-A selecting for amoxicillin and R1-drd-19–K for Kanamycin). N =6. □ indicates ($p<0.05$).

In Figure 62, evocarpine showed robust activity against the transfers of PUB307 and R7K with transfer low frequencies of 4.85% and 9.45% respectively. The level of inhibition against R6K and TP114 was moderate ($P < 0.05 = 0.04$) while evocarpine showed poor anti-plasmid activity against PKM 101, R1-drd-19-A and R1-drd-19-K relative to the control.

3.3.8 The anti-plasmid activity of ferulenol (BM-10) on the inhibition of R-plasmid conjugal transfer.

The inhibitory activity of ferulenol (BM-10) was assayed against plasmids PKM 101, TP114, PUB 307, R7K and R6K at a sub-inhibitory concentration of 100 mg/L (Figure 63).



Ferulenol (SIC 100 mg/L)

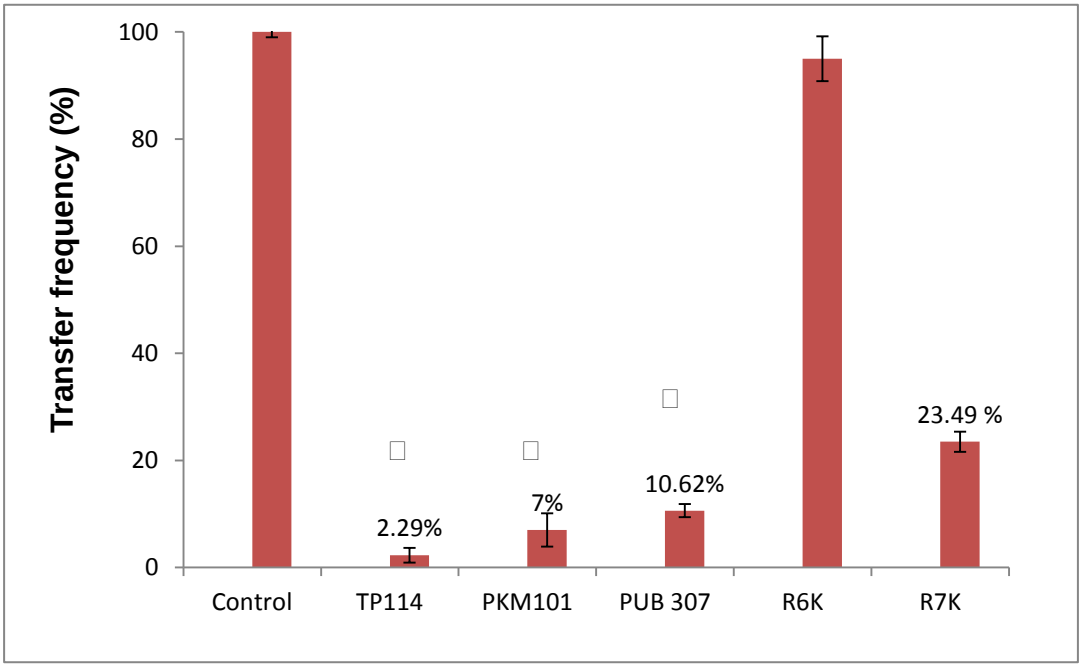


Figure 63: Selective anti-plasmid activity of ferulenol against various plasmids at a sub-inhibitory concentration of 100mg/L. N =6. □ indicates ($p<0.05$).

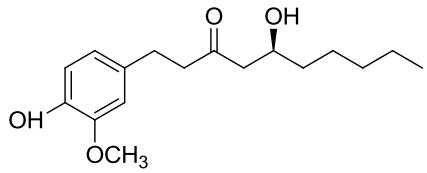
Ferulenol showed selective inhibition against the plasmids, with the most significant activities against TP114, PKM 101 and PUB307 at transfer frequencies of 2.29%, 7.0% and 10.62% respectively. The transfer of R6K was antagonised in the presence of evocarpine; while the transfer frequency of R7K (23.49%) did not represent any significant inhibition of the plasmid transfer by evocarpine.

3.3.9 The antiplasmid activity of capsaicin, dihydrocapsaicin (BM-8 & -9) and capsaicin-like compounds on the inhibition of R-plasmid conjugal transfer

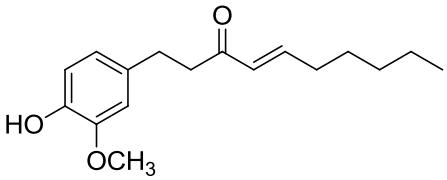
The anti-plasmid activities of the crude methanol extract of, and bioactive SPE sub-fractions of *C. annum*, Ca-SPE 10, 11 and 12 against PKM 101, PUB307 and TP114 are shown in (Appendix 12a-12c). A reference sample of pure capsaicin (a major active constituent of the plant) purchased from Sigma-Aldrich was tested along with the fractions. Ca-SPE 11 exhibited moderate activity against the transfer of TP114, followed by fractions Ca-SPE 12, and then Ca-SPE 10. A similar trend of anti-plasmid activity was exhibited by the reference capsaicin and methanol crude extract from *C. annum* and Ca-SPE-10. Again, Ca-SPE 11 was the most fraction candidate against transfer of PKM 101 and PUB 307 corresponding to that of methanol crude extract from *C. annuum*, Ca-SPE 10 and the pure capsaicin. Other tested fractions showed poor inhibitory activity towards transfer frequency of the plasmids.

Bioassay-guided isolation and identification of the capsaicinoids that could be responsible for the anti-plasmid activity obtained in semi-pure fraction CAP-SPE-11 yielded capsaicin, BM-8 and dihydrocapsacin, BM-9.

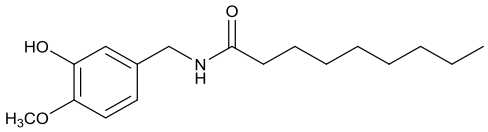
Other synthetic capsaicin-like compounds: nonivamide, 6-gingerol and 6-shogaol, together with capsaicin and dihydrocapsacin were assayed to assess their anti-plasmid activities at SIC value of 100 mg/L.



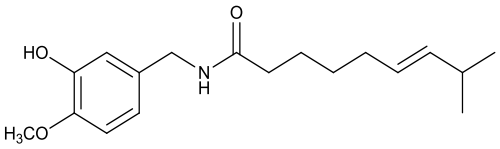
6-gingerol



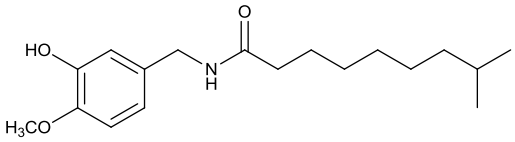
6- shogaol



Nonivamide



Capsaicin



Dihydrocapsaicin

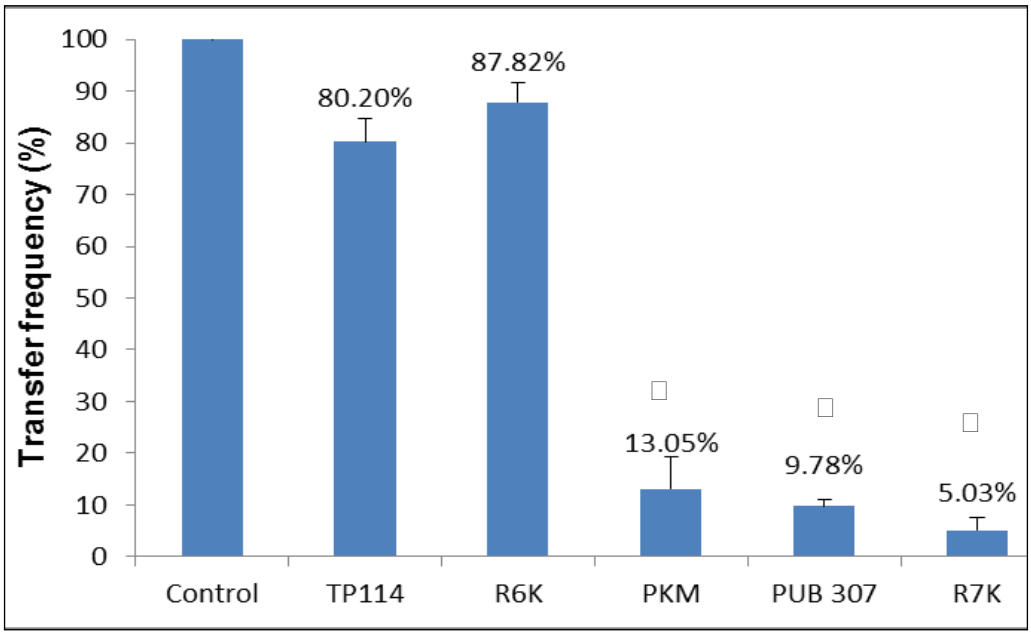
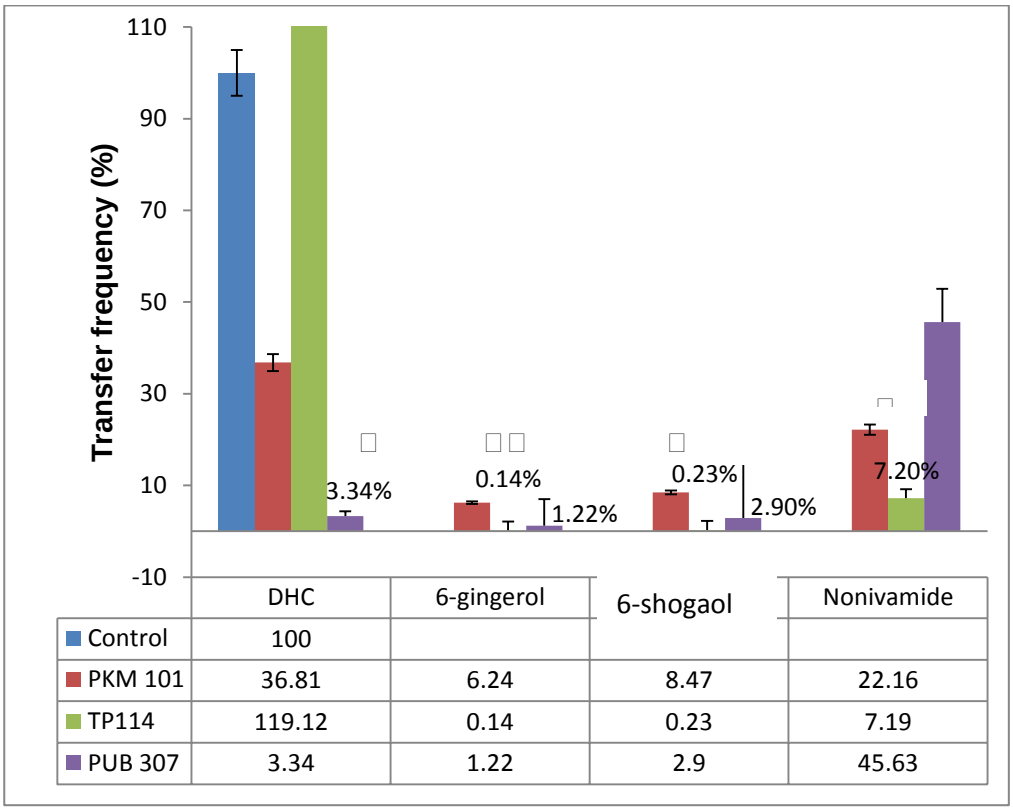


Figure 64: Anti-plasmid activity of capsaicin against R-plasmids. N =6. □ indicates ($p<0.05$).

From the results in Figure 64, capsaicin showed selective but active inhibition of resistant plasmids R7K, PUB307 and PKM 101 at low transfer frequencies of 5.03%, 9.78% and 13.05% respectively. On the contrary, the transfer of TP114 and R6K

were enhanced suggesting that the plasmid may contain substrate for capsaicin, hence the antagonism of plasmid inhibition.

Furthermore, the anti-plasmid activity of dihydrocapsaicin (DHC), nonivamide, 6-gingerol and 6-shogaol were tested equally against PKM 101, TP114 and PUB307.



	DHC	6-gingerol	6-shogaol	Nonivamide
Control	100			
PKM 101	36.81	6.24	8.47	22.16
TP114	119.12	0.14	0.23	7.19
PUB 307	3.34	1.22	2.9	45.63

Figure 65: Antiplasmid activities of DHC, nonivamide, 6-gingerol and 6-shogaol as evaluated by unpaired student's t-test. N =6. □ indicates ($p<0.05$).

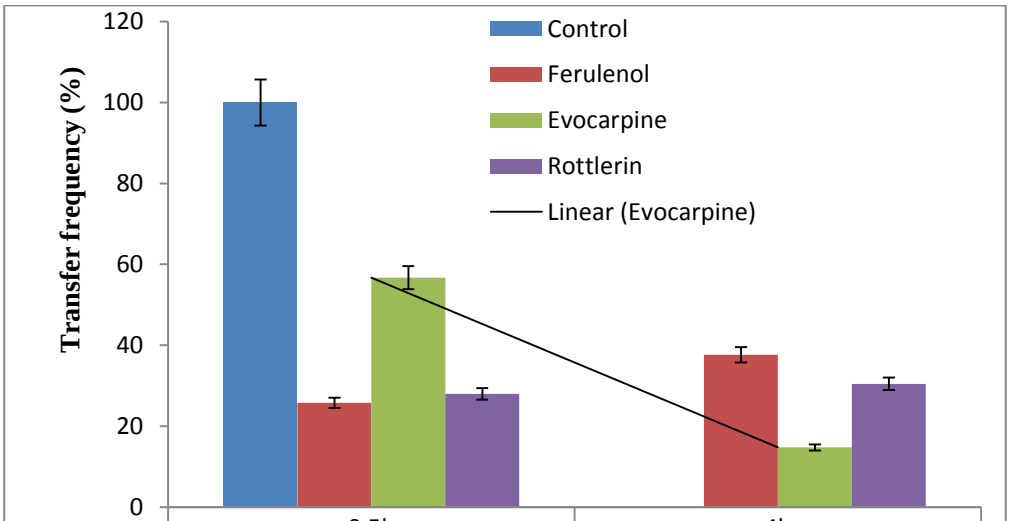
DHC actively reduced the level of transfer of PUB 307 to 3.33% but had a moderate inhibitory effect against PKM 101 at 36.81%. There was no inhibition against TP114, rather an increased rate of transfer frequency above the control was noted (Figure 63).

Nonivamide, an analogue of capsaicn, recorded the highest inhibitory activity agaisnt TP114 at 7.19%, followed by a moderate activity against PKM 101 and PUB 307 at 22.16% and 45.63% transfer frequencies. The inhibitory effects of 6-gingerol and 6-shogaol on the tested plasmids were noteworthy. For example, 100% inhibition of

transfer frequency of TP114 was recorded for the compounds. PUB307 was the most sensitive plasmid to the treatment of DHC (3.34%), 6-gingerol (1.22%), and 6-shogaol (2.90%). A similar pattern of inhibition was exhibited by the four compounds against TP114, within the range of 0.14%-7.20%. In the case of PKM 101, the effect of 6-gingerol and 6-shogoal reduced the rate of transfer of the plasmid, but inhibition was moderate by DHC and nonivamide. All the tested plasmids (PKM 101, TP114 and PUB307) showed significant reductions of their transfer frequencies in the presence of 6-shogaol and 6-gingerol.

3.3.10 The influence of time on the inhibitory effect of selected compounds against the conjugal transfer of PKM 101.

The effect of time on the inhibitory potentials of rottlerin, ferulenol, and evocarpine was investigated on the PKM 101 mating system (Figure 66).



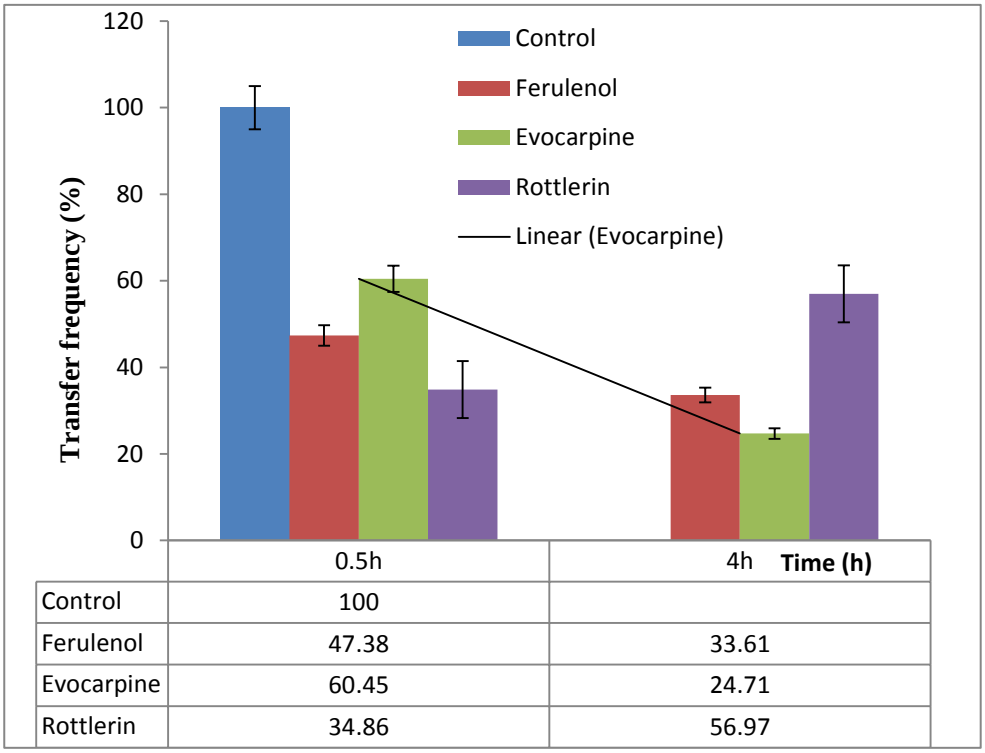
	0.5h	4h
Control	100	
Ferulenol	25.77	37.64
Evocarpine	56.72	14.77
Rottlerin	28.01	30.48

Figure 66: The influence of time on the inhibitory effect of compounds on plasmid transfer of PKM101. T1=0.5h, T2=4H.

The transfer frequencies was observed after 30min (T1 = 0.5 h) and 4 hours (T2 = 4 h) intervals relative to the control experiment. The result after 30 min of incubation of the donor-recipient mixture with the compounds recorded various transfer frequencies for the three compounds, ferulenol, evocarpine and rottlerin against plasmids PKM 101 at 25.77%, 28.01%, and 56.72% respectively. After 4h of incubation, there was no significant reduction in the transfer frequencies of PKM in the presence of ferulenol (37.64%) or rottlerin (30.48%), rather a slight increase in the transfer frequencies was observed. However, evocarpine showed a marked reduction of PKM 101 from 28.01% to 14.77% after 4 h. This suggested that PKM 101 could be sensitive to evocarpine when exposed for a longer period. The control experiment showed that maximum conjugation was experienced over the time period, while changes seen in the levels of transfer were due to the effect of the compounds.

3.3.11 The influence of time on the inhibitory effect of selected compounds against the conjugal transfer of TP114

The effect of ferulenol, evocarpine, and rottlerin on the inhibition of TP114 over time is shown in Figure 67.



	0.5h	4h
Control	100	
Ferulenol	47.38	33.61
Evocarpine	60.45	24.71
Rottlerin	34.86	56.97

Figure 67: The influence of time on the inhibitory effect of compounds on plasmid transfer of TP114. T1=0.5h, T2=4H.

Figure 67 showed that plasmid TP114 was affected in the presence of rottlerin and ferulenol at different transfer frequencies 34.36% and 47.38%, after 30 mins. The transfer rate of ferulenol was slightly decreased to 33.61%, whereas the inhibition effect of rottlerin was diminished after 4h. A level of inhibition against TP114 was seen in the presence of evocarpine after the first 30 mins, with a transfer frequency of 60.45% but decreased to 24.71% after 4h exposure. The effect of rottlerin on both PKM 101 (Figure 66) and TP114 (Figure 67) could suggest that the inhibition activity of the compound was initiated probably at a later period after the first 30 mins of incubation.

3.4. Gel Mobility Shift Studies

3.4.1 Purification of PKM 101, PBCSK and pAKlux3 DNA

The result of gel electrophoresis of the 100-bp DNA ladder, isolated PKM 101 and PBCSK DNA, and their binding effect with actinomycin-D are shown in Figure 68.

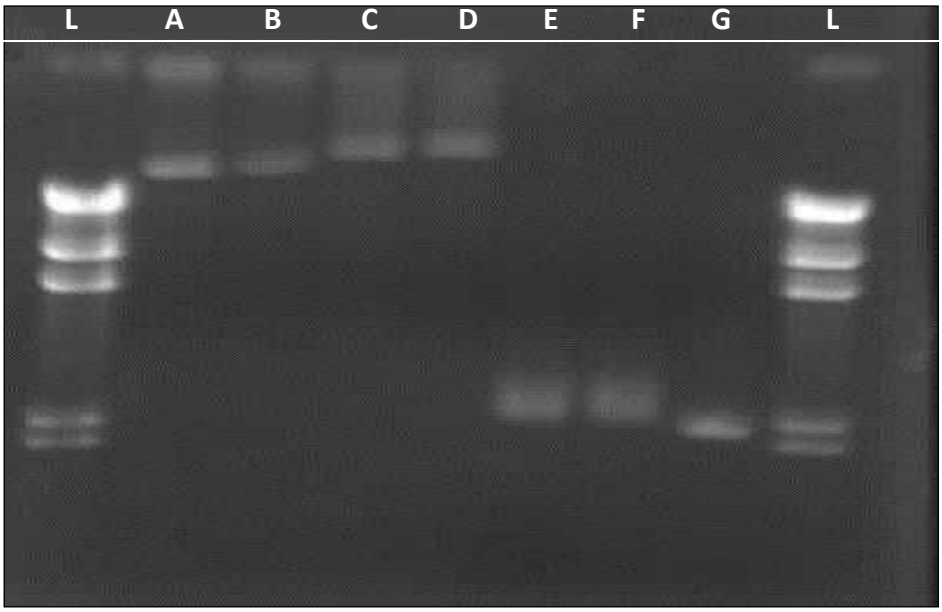


Figure 68: DNA binding assay of DNA from PKM 101 and PBCSK with actinomycin-D electrophoresed on 0.8% agar-rose gel, final concentration of 100 mg/L

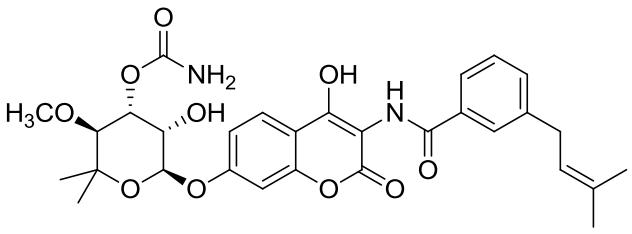
Lanes: (L) ladder (A) DMSO + PKM 101, (B) PKM 101, (C) PKM 101 treated with actinomycin-D at 10mg/L, (D) PKM 101 treated with actinomycin-D at 100mg/L, (E) Pbcsk treated with actinomycin-D at 10mg/L, (F) Pbcsk treated with actinomycin-D at 100mg/L, (G) DMSO+ PBCSK.

PKM 101 was chosen for the DNA binding assay considering the molecular weight for ease of purification, and its susceptibility to most of the compounds that were tested for anti-plasmid activity. Actinomycin-D, an anticancer drug notably for its intercalative mode of action, was used as a positive control at 100 and 10 mg/L concentrations. From the result (Figure 68), single bands of PKM 101 were observed

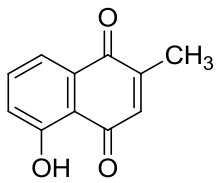
in lanes A-B of purified PKM 101 while lanes C-D showed a decrease in mobility of PKM 101-actinomycin bound complexes at both 100 and 10 mg/L. Similar decrease in mobility was observed with PBCSK when treated with actinomycin–D (Lanes E-F), compared to Lane G showing the parent PBCSk in DMSO.

3.4.2 Gel mobility shift assay of digested and undigested pAklux

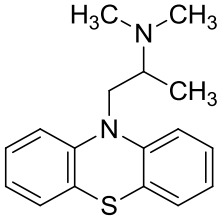
This experiment was done to investigate the nature of binding of the tested compounds either to the whole undigested DNA or to any specific fragment of the DNA upon its digestion. Also, digestion of pAKlux3 was to enhance the DNA-compound interactions with the smaller DNA fragments and for easy visualisation of any shift any particular fragment. Therefore, the purification and digestion of pAklux3 with *SphI*-restriction endonuclease was completed, and both the digested and whole DNA were incubated with novobiocin (64 mg/L), plumbagin (32 mg/L) and promethazine (128 mg/L). Actinomycin-D (100 mg/L) served as a control experiment (Figure 69).



Novobiocin (64 mg/L)



Plumbagin (32 mg/L)



Promethazine (128 mg/L)

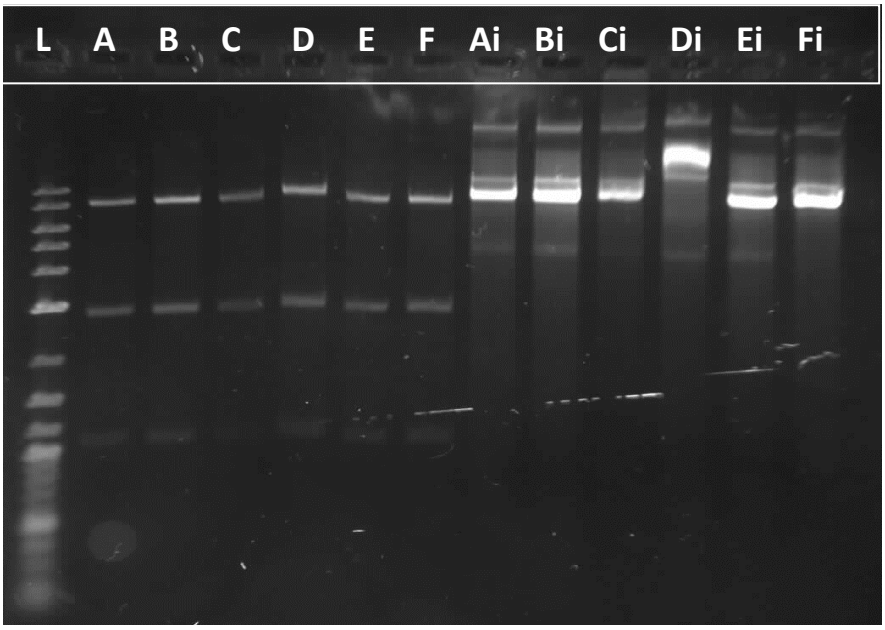


Figure 69: DNA binding assay of, *SphI*-digested and undigested pAKlux3

Lanes: (L) ladder, SphI-digested (A-F) and undigested (Ai-Fi) products of pAKlux3 A = novobiocin, B = plumbagin, C = promethazine, D = actinomycin-D, E = plasmid + elution buffer (EB), F = plasmid + DMSO

Three bands corresponding to DNA fragments 8.03, 2.86 and 1.07 kb of the ladder (L) were obtained and resolved by the gel electrophoretic analysis (Figure 69, Lanes A-C, E-F). In Lane D, there was a slight decrease in the DNA mobility of fragment 1.07 kb when interacted with actinomycin-D, showing the binding of actinomycin-D to that particular region of pAKlux3. In Lane C, the binding of promethazine to the undigested products of pAKlux3 in the presence of actinomycin-D (Lane Di) are shown in Figure 69, Lanes Ai-Fi. The results indicated coiled, super coiled and nicked portions of the whole DNA resolved across the gel. The binding of actinomycin-D to pAKlux (Lane Di) showed a marked decrease in the migration of the band through the gel. The intensity of the fluorescence in bands C and Ci, were less compared to other bands that showed more intense brightness in the gel. This

could be as a result of the binding of promethazine to the DNA and therefore, less adherence of ethidium bromide to the DNA strands and fluorescence under UV light.

3.4.3 Gel mobility shift assay of digested pAklux3 with selected synthetic and natural products

These selected compounds had a broad-range of inhibition against R-plasmids as described earlier, and therefore were assessed for binding to pAKlux3 DNA: rottlerin, red compound, ferulenol, evocarpine, rutaecarpine, capsaicin, dihydrocapsaicin, 6-gingerol, 6-shogaol, and nonivamide (Appendix 13).

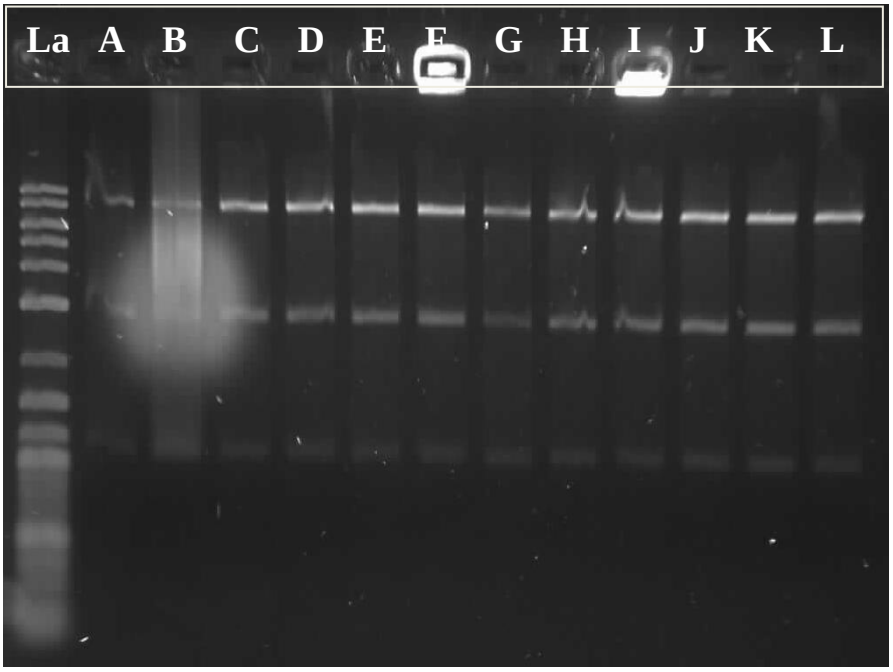


Figure 70: DNA binding assay of digested pAKlux3 with samples A-J. Lanes: (La) ladder, A= rottlerin, B = red compound, C = ferulenol, D = evocarpine, E = rutaecarpine F= capsaicin, G= dihydrocapsaicin, H= 6-gingerol, I= 6-shogaol, J= nonivamide, K= DNA + Elution buffer (EB), L= DNA + DMSO

In Figure 70 Lanes A-J, the gel showed three bands of DNA fragments similar to Figure 69. However, no mobility shift was observed among the bands in the presence of the compounds, when compared to the parent pAKlux3 (Figure 70, Lane K). But in the first and second bands of dihydrocapsacin-DNA bound complex, there was

decreased fluorescence of the bands, and this may suggest that binding occurred. Hence, the ethidium bromide dye could not be absorbed intercalated more into the DNA. The activity of the compounds that did not bind to pAKlux3 DNA as expected, might be due to a different mechanism of action.

3.4.4 Gel mobility shift assay of digested PKM 101 with selected synthetic and natural products

An *HpaI*-digested PKM 101 was assayed in the presence of the 10 compounds: rottlerin, red compound, ferulenol, evocarpine, rutaecarpine, capsaicin, dihydrocapsaicin, 6-gingerol, 6-shogaol, and nonivamide (Appendix 13).

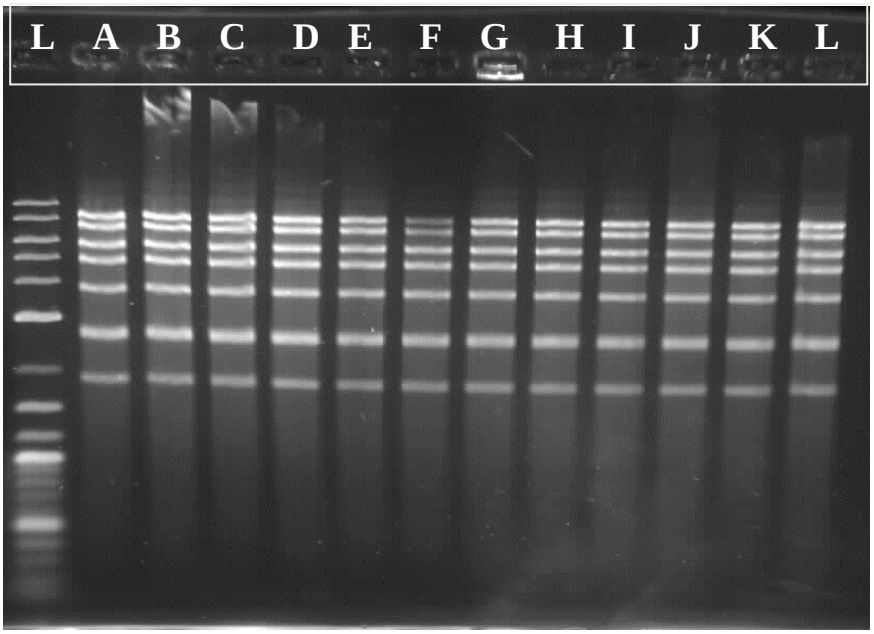


Figure 71: DNA binding assay of *HpaI*-digested PKM 101 with samples A-J. Lanes: (L) ladder, A=rottlerin, B=red compound, C=ferulenol, D=evocarpine, E= rutaecarpine F=capsaicin, G=dihydrocapsaicin, H=6-gingerol, I=6-shogaol, J= nonivamide, K= DNA + Elution buffer (EB), l= DNA + DMSO

The expected sizes of *Hpal*-PKM 101 (Figure 71, Lane K); corresponded to 7.8, 6.7, 5.3, 4.6, 3.7, 2.7, and 2.6 Kb fragments of the DNA ladder (L). The seven fragments

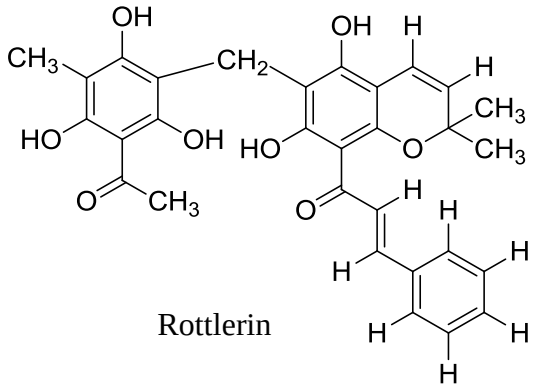
showed similar pattern of migration as shown in Lanes A-J, which were binding interactions between PKM 101 and the compounds compared to the control (Lane K, PKM 101). This may suggest that the compounds did not bind to any of the DNA fragments with the exception of capsaicin-DNA complex in Lane F observed in the first two bands.

CHAPTER FOUR

4.0 DISCUSSION

4.1 Antibacterial and antiplasmid properties of rottlerin and the red compound isolated from *Mallotus philippinensis*

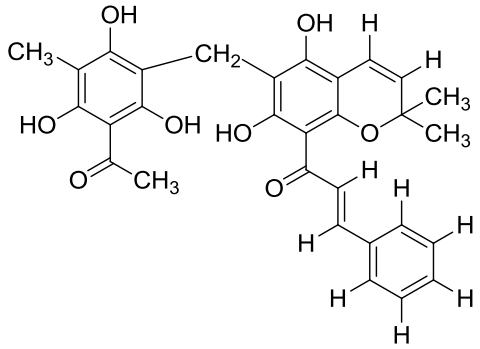
Rottlerin, also called mallotoxin or kamalin, is chemically known as 5, 7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene.



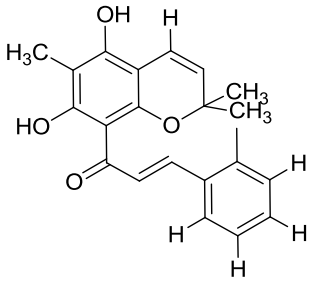
Rottlerin

It is a natural occurring polyphenolic compound found in the genus *Mallotus*. The *Mallotus* genus is a valuable source of phytochemicals such as coumarins and isocoumarins, terpenes, steroids, flavonoids, ligans, chalcone and dimeric chalcones derivatives. This array of phytochemicals has demonstrated diverse therapeutic potentials, including antibacterial (Zaidi *et al.,* 2009), antimycobacterial (Hong *et al.,* 2010), antifungal (Kulkarni *et al.,* 2014b), anti-leukaemic (Khan *et al.,* 2013), antitumor, anticancer and anti-allergic properties (Daikonya *et al.,* 2002; Tanaka *et al.,* 2008; Sharma 2011; Chan *et al.,* 2013; Gangwar *et al.,* 2014). These data, therefore justify the ethnomedicinal uses of *Mallotus* for the treatment of various ailments.

Rottlerin is structurally similar to that of the red compound, except for the presence of the acetophenone moiety. The acetophenone unit is composed of a phloroglucinol backbone, and connected to an aromatic ring via a deshielded CH_2 group, a cinnamoyl group Ar-CH=CH-CO, and geminal methyl pair, together with olefinic resonances forming a dimethyl-pyran unit. These moieties are very similar to the structural features seen in the red compound, although this has an aromatic–bearing methyl group, instead of a benzylic methylene in the case of rottlerin.



Rottlerin



The red compound

The red compound is chemically known as 8-cinnamoyl-5, 7-dihydroxy-2, 2, 6-trimethylchromene, and gives the Kamala plant its characteristic red colour. The red compound, isorottlerin and rottlerin are naturally occurring compounds, while the red compound primarily serves as a precursor to various chalcone and phloroglucinol derivates from *Mallotus* species (Daikonya *et al.,* 2002, 2004, Furusawa *et al.,* 2005).

Rottlerin and the red compound have been previously isolated from *M. philippinensis* and tested for their anti-mycobacterial activities (Hong *et al.,* 2010) and antibacterial properties against *Helicobacter pylori* (Zaidi *et al.,* 2009; Hong *et al.,* 2010). The present study therefore, reports the antibacterial activities of rottlerin and the red compound, against a panel of methicillin-resistant *S. aureus* strains, and some

multidrug-resistant Gram-negative bacteria, for the first time. Rottlerin showed a good potency of 2 mg/L against RN 42290 (MsrA), MRSA 274829, MRSA 12981 and NorA over-expressing SA1199B, which compared favourably with norfloxacin, erythromycin and oxacillin. The observed antibacterial activity of rottlerin against these clinically-relevant strains is noteworthy and highlights the potential of rottlerin as a good candidate for antibacterial drug development, against the MRSA. Methicillin-resistant *S. aureus* has become a great health concern because of causing many hospital-acquired infections and *S. aureus* 'exceptional' wide-range resistance to tetracycline's, macrolides, aminoglycosides, fluoroquinolones, especially glycopeptides such as vancomycin, which is used in its treatment (Stapleton and Taylor 2002; Gibbons 2008). In addition, high level resistance in strains such as SA1199B, XU212 and RN4220 strains are promoted by the expression of efflux pumps, NorA, TetK and MsrA; and these mechanisms have become increasingly important in the current threat of multidrug resistance. The XU212 overexpresses TetK (tetracycline) efflux pump, which reduces the transport and recognition of tetracycline (Gibbons 2008). Multidrug-resistant SA1199B, overexpresses the NorA efflux transporter, in addition to a gyrase mutation, thereby confers a high level of resistance to certain fluoroquinolones (Poole 2000); RN4220 possesses the MsrA macrolide efflux pump, which binds to macrolides, expelling them from their drug-binding sites (Ross *et al.,* 1995), while epidemic strains of EMRSA are notable for cases of bacteraemia in the UK hospitals (Gould *et al.,* 2009).

Rottlerin was active against ATCC 29523, *B. subtilis* BsSOP01, XU212, EMRSA-15, and EMRSA-16, MRSA 346724 and *E. faecalis 13379* and 12697 at MIC values ranging between 1-8 mg/L, which is not as potent compared to the antibiotics. The activity of rottlerin towards these strains may have been reduced by the presence of

resistance genes harboured by the organisms, for example EMRSA -15 and -16 strains which harbour *mecA* genes responsible for their resistance phenotypes (Cox *et al.,* 1995).

The antibacterial activity of the red compound had constant MIC value of 32 mg/L against XU212, EMRSA-15, and -16, SA119B, ATCC 29523 and erythromycin-resistant RN4220, though the reason for this is not understood but it is likely that its chemical uniqueness gave rise to similar range of inhibition against the various organisms. In comparison, rottlerin showed improved antibacterial activity compared to the red compound (rottlerin > red compound) especially against the MRSA, and this could be attributed the presence of the acetophenone moiety. The sensitivity of SA1199B, RN4220 and XU212 to both compounds is interesting, indicative of similar mode of antibacterial action of rottlerin and the red compound against these bacteria. However, this is out of scope of this study. The high MIC of rottlerin and the red compound towards the Gram-negatives: *E. coli, K. pneumoniae, P. aeruginosa* and *Proteus sp* P10830 is almost certainly be due to poor penetration of the compounds through the bacterial outer membrane (Cox and Wright 2013b), as these compounds are large polyphenolic structures, with hydroxyl groups and phloroglucinol moieties, which contribute to their lipophilic and hydrophilic functions.

Rottlerin was reported recently with IC_{50} values of 9 and 8 µM when screened against HL-60 and MIAPCa-2 cells, respectively (Jain *et al.,* 2013). Based on the IC_{50} values, it can be estimated that ≥ 5 mg/L of rottlerin may perhaps show a strong effect to the cells. Further in vivo studies conducted by (Shaikh *et al.,* 2012) reported a weak toxic effect at the dose of 1000 mg/kg in mouse animal model. Given the potential antibacterial activity displayed by the red compound and rottlerin, further research is

needed from preclinical and clinical studies on human to justify the toxic nature of these compounds as drug leads. Their structures can equally be modified to improve both their antibacterial and cytotoxic activities.

The antiplasmid studies of rottlerin and the red compound revealed that the compounds affected the conjugal transfer of R-plasmids, namely PKM 101, TP114, PUB307, R6K. These plasmids belong to incompatibility (Inc) groups that are broad host and they serve as vehicles of conferring resistance genes to many antimicrobial classes especially genes encoding extended-spectrum β-lactamases (ESBLs) and 16S rRNA methylation resistance to aminoglycosides, in Enterobacteriaceae (Carattoli 2009). Apart from TP114 that expresses kanamycin (aminoglycoside) resistance (Kmr), all the other plasmids harbour amoxicillin (β-lactam) resistance (Amr), which illustrates the very promiscuous nature of plasmids due to their 'talented' ability of self-transmissibility to transfer between bacteria from different groups with genes conferring resistance to one or more classes of antibiotics.

Interestingly, the antiplasmid activity of rottlerin was pronounced and widespread against PKM101, PUB307 and TP114, of different incompatibility groups. The red compound equally inhibited the conjugal transfers of TP114 and PUB307, in a moderate manner. The transfer frequencies of the transconjugants of the plasmids showed that maximum conjugation was achieved during the mating. This is important because researchers have noted that the rate of transfer is essential for the stable maintenance of conjugative plasmids in a bacterial population (Haft *et al.,* 2009). Plasmids may be lost during bacterial reproduction by failing to segregate into one of the daughter cells during binary fission, but especially for the purpose of this study,

the conjugative plasmids carry antibiotic resistance genes or virulence genes that are able to spread via a successful transfer between the bacteria population.

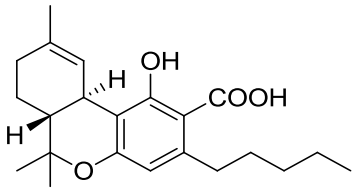
The polyphenolic compounds were applied in sub-inhibitory concentrations that did not kill the bacteria but targeted the plasmids. This could be by a way of interfering with the conjugative machinery or proteins, or destabilising the plasmid replication process. Earlier reports showed that plasmid curing agents such as ethidium bromide and SDS can intercalate the DNA (Hahn and Ciak 1976), indirectly damage the DNA, such as quinolones (Michel-Briand *et al.,* 1986), or affect the architecture of the cell membranes, as seen with phenothiazine, thereby disrupting plasmid partition (Monlar *et al.,* 2003). The full mode of antiplasmid action of the compounds is yet to be elucidated. To the best of our knowledge, this is the first report on the inhibitory effect of natural compounds from *M. phillipinensis* on the conjugal transfer of R-plasmids.

Many researches therefore support that natural agents interfering with the process of bacterial conjugation remains a potential way of combating the spread of plasmid-mediated antibiotic resistances (Fernandez-Lopez *et al.,* 2005; Williams and Hergenrother 2008), given that currently available antiplasmid agents are toxic (Amábile-Cuevas and Heinemann 2004). Some non-toxic antiplasmid natural products have been isolated, such as the nor-diterpene compound, 8-epidiosbulbin-*E*-acetate (EEA) isolated from the bulbs of *Dioscorea bulbifera* (Shriram *et al.,* 2008) and the phenolic compound bharangin isolated from *Pygmacopremna herbacea* (Roxb) (Marie-Magdeleine *et al.,* 2010).

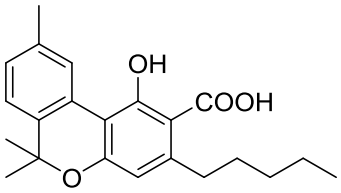
The planar nature of rottlerin and the red compound, rich in olefinic and hydroxyl groups may promote ease of penetration into the plasmid DNA, and largely affect the process of conjugation. This is in line with a number of antiplasmid compounds, that are either natural or synthetic, in that their antiplasmid action depended upon the chemical structures of the compounds, their aromaticity, and their ability to interact with the DNA gyrase (Spengler *et al.*, 2006) Spengler, Miczak *et al.*, 2003; Molnar, Molnar *et al.*, 2004; Takacs, Cerca *et al.*, 2011).

4.2 Antibacterial and antiplasmid properties of natural and synthetic cannabinoids

The bioassay-guided analysis of the chloroform extract of *Cannabis* resin yielded two acidic cannabinoids, tetrahydrocannabinolic acid, (THCA) and cannabinolic acid (CBNA).

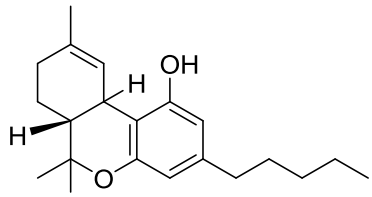


Tetrahydrocannabinolic acid (THCA)

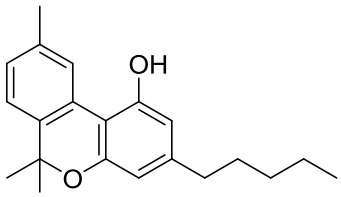


Cannabinolic acid (CBNA)

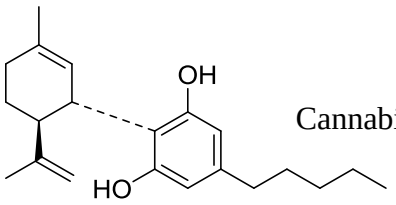
Detailed characterisation of the five most common cannabinoids from *C. sativa*; Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), and cannabinol (CBN) and their carboxylated counterparts including THCA and CBNA have been documented by many researchers (Choi *et al.*, 2004; Hazekamp *et al.*, 2005; Lone *et al.*, 2012).



Δ9-tetrahydrocannabinol (THC)



Cannabinol (CBN)



Cannabidiol (CBD)

Some of the biological activities exhibited by *C. sativa*, such as anti-inflammatory, antitumor, and neuroprotective effects, have been attributed mainly to the presence of psychoactive Δ9-THC, and less related to other non-psychoactive cannabinoids such as CBD and CBN. A recent study revealed the potential of cannabidiol and cannabidiolic acid, as anti-cancer agents (Takeda *et al.*, 2012; Takeda 2013).

This study did not investigate the antibacterial activities of the cannabinoids against MRSA, because it has been investigated in our research team in collaboration with Professor Appendino's group. The antibacterial activities of the five major cannabinoids, (CBD, CBC, CBG, Δ9-THC and CBN) were evaluated against the multidrug methicillin *S. aureus* (MRSA) and standard strains; SA1199B, ATCC 25923, XU212, RN4220, EMRSA-15 and -16 (Appendino *et al.*, 2008). According to Appendino *et al.*, 2008, the antibacterial activities of the five main cannabinoids had MIC values in the 0.5-2 mg/L range, with exceptional activity against SA-1199B, EMRSA-15 and EMRSA-16. The mechanism of the antibacterial activity of the cannabinoids is not known. However, the activity is proposed to be dependent on the presence of the prenyl moiety and its relative position, which is not the case with the

carboxylated resorcinyl moieties of the acidic counterparts (Appendino *et al.,* 2008) or the *n*-pentyl moiety of the abnormal cannabinoids with lesser antibacterial activity (Razdan *et al.*, 1974).

The antiplasmid activities of these cannabinoids; Δ9-THC, CBN, CBD, including a semi-purified extract of *Cannabis*, showed various transfer frequencies (0.29-27.58%) of three broad host plasmids, PKM 101, TP114 and PUB 307, with varying transfer frequency values. The three cannabinoids significantly reduced PKM 101 transfer to a near-zero transfer rate. Similarly, TP114, which determines kanamycin resistance, was affected by CBN and CBD, but was marginally inhibited by Δ9-THC. Numerous evidence abounds on cannabinoid-mediated effects in various biological systems, especially with the discovery of CB_1 and CB_2, the endocannabinoid receptors (Izzo *et al.,* 2009). It is not strange that plant cannabinoids were able to target plasmid DNA, influence the inhibition of bacterial conjugation or related activities, since they are known to have the ability to modulate several physiological and pathophysiological activities (Izzo *et al.,* 2009) .

Neither CBD nor CBN showed a marked effect on the transfer of PUB307, while that of THC was completely opposite to the expected inhibition. This indicated that the activity of these compounds is plasmid-specific, which is clearly seen in the selective inhibition effect of the individual cannabinoids on PKM 101 and TP114, over PUB 307. It is possible that PUB 307 may undergo modification in the presence of the cannabinoids or contain a substrate which likely is antagonised by the effects of these compounds. However, the scope of available data is insufficient to provide an

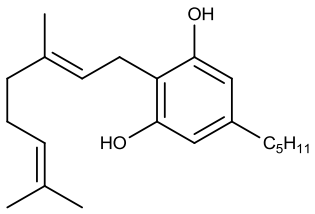
explanation as to what is modified in the presence of the compounds such that plasmid transfer efficiency is either enhanced or reduced.

A key feature of these cannabinoids, the tricyclic aromatized structure, has been related to the tricyclic skeleton of phenothiazine derivatives, which exert antiplasmid action (Spengler *et al.,* 2006b). The electronic configuration of the phenothiazine skeleton, in addition to the presence of the heteroatoms, is considered primarily responsible for the plasmid curing activities of the phenothiazine compounds. Despite the similarity in their structural backbone of cannabinoids to phenothiazine-type compounds, the cannabinoids under investigation are rich in functional groups: phenolic oxygen of the monoterpene unit, the alkyl side chain, and the prenyl unit; which may contribute to their antiplasmid effect.

The Monlar group have demonstrated the plasmid curing of F'lac from model *E. coli* K12LE140 by some ring substituted phenothiazines and cannabinoid derivatives (CBNA, CBN, CBD, CBDA, 8-THC, 9-THC, THCA) (Molnar *et al.,* 1992). In general, all of the cannabinoids profiled produced a weak effect except for THCA, which showed 30% elimination of the F'*lac* plasmid. Despite there being no further explanation by the authors for the decreased activity of the cannabinoids; it is possible that the very low sub-MIC concentrations applied could induce plasmid loss as expected. Conversely, the inhibition of conjugal transfer of resistance bearing plasmids occurred at low concentrations of these cannabinoids, and even in strains that confer broad range antibiotic resistance. The weak curing effect shown by the rest of the cannabinoids against *E. coli* K12LE140 may be due to the inefficient

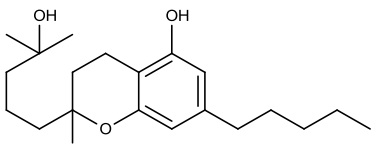
penetration of the compounds, since the bacterial cell wall/cell membrane has been implicated as a barrier resulting in weak plasmid elimination (Molnar *et al.,* 1992). In essence, the successful permeation of these cannabinoids through the *E. coli* membrane was crucial to their antiplasmid effect, and could be attributed to the structural and electronic features which are typical of cannabinoids. Regardless of the difference in the method and plasmid strains used in Monlar's work, the potent activity shown by THCA corresponded with our results, which showed the presence of THCA as the major responsible antiplasmid agent, isolated from the bioactive semi-pure *Cannabis* extract against TP114.

The cannabigerol (CBG) and CBG-derivatives constitute an important class of the major cannabinoids, either obtained synthetically or from *C. sativa* (Appendino*et al.*, 2008; Radwan *et al.*, 2009; Appendino *et al.*, 2011).

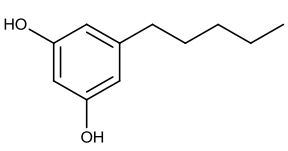


Cannabigerol

Cannabigerol has demonstrated anti-proliferative and antibacterial activity, and is a potent cannabinoid agonist (Izzo *et al.,* 2009). Two synthetic compounds, cannabigerol CBG-1 and olivetol also inhibited the conjugal transfer of plasmid TP114 between *E. coli* strains.



CBG-1

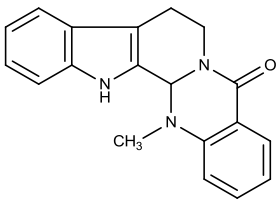


olivetol

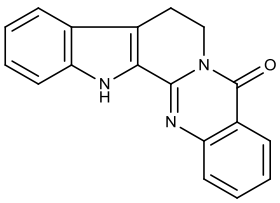
CBG-1 was strongly inhibitory, compared to olivetol. CBG-1 consisted of the basic olivetol moiety that is often found in the cannabinoids, and which may have contributed, alongside with other functional groups to the antiplasmid activity of the cannabinoids. The olivetol core in itself has been proposed as a poorly active pharmacophore, with respect to its antibacterial activity (Appendino. *et al.,* 2008). These findings highlight the untapped potential of phytocannabinoids and their analogues as antiplasmid drug leads, and templates for combinatory chemistry, besides their richly investigated pharmacological and psychotropic activities.

4.3 The antibacterial and antiplasmid properties of quinolone alkaloids from *Evodia rutaecarpa*

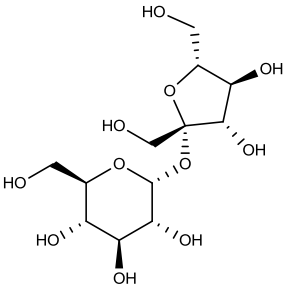
Evodiamine and rutaecarpine, two indolequinazoline alkaloids were isolated from the dried fruits of *E. rutaecarpa*, including naturally occurring sugar. This is the first time sucrose is reported from the fruit of *Evodia rutaecarpa*.



Evodiamine



Rutaecarpine



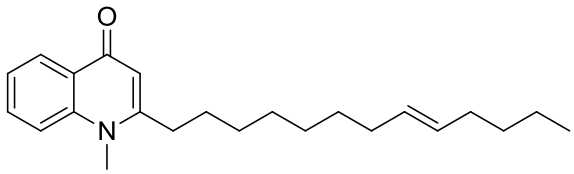
Sucrose

Numerous papers have reported the isolation of evodiamine and rutaecarpine from the unripe, fresh and dried fruit of the *E. rutaecarpa*, *E. fructus* and sub-species *officinalis* (Dode) Huang (Yuan-Qing Tang *et al.,* 1996; Adams *et al.,* 2005; Liu *et al.,* 2005; Yang *et al.,* 2006b; Shin *et al.,* 2007; Pei-ting Zhang *et al.,* 2013; Wang *et*

al., 2013; Zhang *et al.,* 2013; Zhang *et al.,* 2014). These reports suggest that the *Evodia* fruit may be a richer source of alkaloids including evocarpine, compared to other parts of the plant. Rutaecarpine has also been isolated from two new plant families apart from the Rutaceae and they include *Taxus chinensis* (Taxaceae) and *Winchia calophylla* from family Apocynaceae (Guan *et al.,* 2000; Zhu *et al.,* 2005)

From available literature, quinolone alkaloids possessed potent antibacterial activity against *Helicobacter pylori* (Hamasaki *et al.,* 2000; Tominaga *et al.,* 2002; Tominaga *et al.,* 2005) and some extremely drug-resistant *Mycobacterium tuberculosis* strains (Adams *et al.,* 2005). However, none of the studies on the antimicrobial activities of quinolone alkaloids has explored the activity of evodiamine and rutaecarpine against multidrug-resistant *S. aureus* strains.

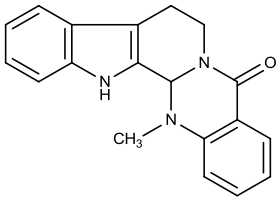
From the present study, the antibacterial activity of evodiamine, rutaecarpine and the isolated sugar showed a wide range of MIC values ranged 8-128 mg/L. Rutaecarpine was the most active alkaloid with an MIC value of 8 mg/L against NorA-expressing SA1199B and the XU212 strain expressing the TetK efflux mechanism. Evodiamine and the isolated sugar showed slight inhibitory activity against the panel of bacteria, both Gram-positive and Gram-negative. In a study by Pan's group, some quinolone alkaloids showed similar range of antibacterial activity (8-128 mg/L) (Pan *et al.,* 2014) against a methicillin-resistant *S. aureus* ATCC 33591 and other standard strains and their MIC values compared well with our results on the antibacterial activities of evodiamine and rutaecarpine. Evocarpine was potent with an MIC of 8 mg/L, which was 16-fold more active compare to the activity of oxacillin against the MRSA strain.



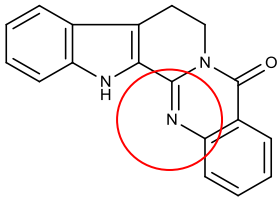
Evocarpine

It is suggested that the chemical nature of evocarpine: 1-Methyl-2-(8-tridecenyl)-4(1H)-quinolinone, with a characteristic 13-carbon monounsaturated aliphatic side chain at the position-2, conferred an antibacterial advantage on the compound, compared to others with shorter or longer side chains. A major structural difference that is common with quinolone compounds lies with the alkenyl chain substitution at C-2, which somewhat influences their level of activity. In another study, an array of isolated *N*-methyl-4(1H)-quinolone alkaloids, demonstrated good activity (4-28 mg/L) against a standard *S. aureus* ATCC 25923, *S. epidermidis* and *B. subtilis* , and within these quinolones, the compound with the thirteen carbon aliphatic chain had a remarkable antibacterial activity (Wang *et al.,* 2013). Previous studies on the antibacterial and antimycobacterial effects of 1-methyl-2-alkenyl-4(1H)-quinolones, and their potential structural–activity-relationships were evaluated by Bucar, Gibbons and Bhakta's collaborative groups (Guzman *et al.*, 2011; Wube *et al.*, 2011; Wube *et al.,* 2012).

The antibacterial activity of rutaecarpine is not strange as it has been documented for its various biological activities such as its cardioprotective (Jia and Hu 2010), antithrombotic (Sheu *et al.,* 2000), anti-inflammatory (Chiou, W. F. *et al.,* 2011; Liao, J. F. *et al.,* 2011), including antibacterial activities (Wang *et al.,* 2013). The major structural difference between rutaecarpine and evodiamine is the presence of an additional double bond at position C3-N (rutaecarpine) which may have enhanced the anti-staphylococcal activity of rutaecarpine.



Evodiamine



Rutaecarpine

Results for the inhibition of R-plasmid transfer of the quinolones from *E. rutaecarpa*, showed various levels of reduction towards conjugative transfer frequencies. The semi-purified fractions EV6, EV-8 and EVB respectively, recorded 21.06%, 27.61% and 26.61% inhibition towards the transfer of TP114 between the donor and recipient *E. coli*, compared to isolated evodiamine (55.55%). Similar result was reported of the crude extract of *Alpinia galanga*, which demonstrated higher plasmid curing activity than the isolated single entity, 1'-acetoxychavicol acetate (Latha *et al.*, 2009). The enhanced antiplasmid activities of the bioactive extracts may be due to synergistic effects of the chemical constituents present in the extracts. Synergy in whole plant medicines or crude extract treatments has been very common by means of adjuvant substances in the plant, which usually enhance the activity of the components responsible for the effect (Gilbert and Alves 2003). In this case, the combinatorial effect of the crude extracts may possibly facilitate transport across the bacterial cell wall which aided maximum absorption of the drug, interference with the conjugation process, or provide other signals to the hosts or recipient cells that result in higher efficacy of the crude drug compared with their isolated components. However, the exact mechanism of action of the crude extract is not known.

Evodiamine and rutaecarpine are both widely known for various pharmacological activities, but did not vigorously affect the transfer frequencies of plasmids TP114

and PUB307. Only the plasmid transfer of PKM 101 was actively reduced by rutaecarpine with a transfer frequency of 2.31%. With the exception of rutaecarpine that demonstrated a specific inhibition of PKM 101, the overall results suggest that the independent effects of the evodiamine and rutaecarpine could not diminish the plasmid-coded conjugative machinery of PUB307 and TP114. Due to their large size, it is possible that the compounds were weakly absorbed into the bacterial cell or degraded, which reduced their efficiency on the targeted plasmid. The ability of bacteria to degrade, transform or even mineralise diverse organic compounds and their derivatives, including indole and quinolone derivatives has been extensively reviewed (Fetzner 1998).

The antiplasmid effect shown by sucrose from *Evodia* fruit on plasmid PKM 101, TP114 and PUB307 could be largely attributed, in addition to sucrose, to the presence of possibly saturated and unsaturated fats and other residues. This is because fatty acids dehydrocrepenynic acid (DHCA) from *Sistotrema semanderi*-FX21 and linoleic acid from *Mollisia ventosa*-FX14 have been shown to have antiplasmid effect on plasmid R388 and other plasmid transfers between *E. coli isolates* (Fernandez-Lopez et al., 2005). Though, the saturated fatty acids as seen in the NMR spectrum (Figure 42) were minor but showed activity against the transfer of these R-plasmids resulting in low transfer frequencies of PKM 101 (2.74%), TP114 (6.75%) and PUB307 (0.5%). Equally, the parent bioactive extract EV7/8 demonstrated moderate inhibition against these plasmids, contrary to both table sugar and standard sucrose, which showed varying transfer frequencies against PKM 101 and TP114, but did not achieve the anticipated antiplasmid effect.

Marshall and Bullerman (1994) showed that low concentration of sucrose usually are not inhibitory to microorganisms, but provide readily available carbohydrate energy and stimulate growth. Thus, the antiplasmid activity of the isolated sugar and the semi-pure extract could be connected with the inhibitory effect of the saturated fatty acid residues, since sucrose esters of fatty acids are shown to possess some antimicrobial effect (Marshall and Bullerman 1994). Studies conducted on conjugal inhibitory activities of a collection of fatty acids also revealed that some bioactive fractions with antiplasmid activity during screening of NatChem library contained unsaturated fatty acid, mainly linoleic acid (Fernandez-Lopez *et al.,* 2005).

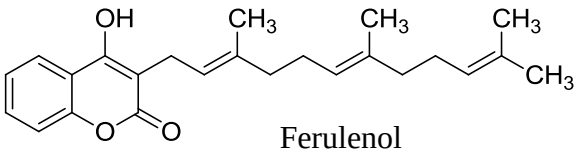
Evocarpine showed a robust antiplasmid activity which was evident by the low transfer frequencies of broad host R-plasmids, PUB 307 and R7K at 4.85% and 9.45% respectively. The level of inhibition against R6K, TP114 and R1-drd-19-K was moderate but insignificant except towards R6K. Evocarpine exhibited poor anti-plasmid activity against PKM 101 and R1-drd-19-A in comparison to the control. These results suggested that evocarpine is a selective plasmid inhibitor, even though the mechanism by which evocarpine leads to selective decreased efficiency of plasmid transfer is not yet clear. Generally, bacterial conjugation involves the cell to cell fusion via the pili between the donor and the recipient, which is by the type IV secretion system (T4SS), also known as the mating pair formation (Mpf) apparatus, encoded on Gram-negative conjugative elements. (Lawley *et al.,* 2003). It is likely that evocarpine itself might affect some general function or specific component of the cell surface, such as electrochemical potential, by influencing the intracellular or periplasmic space pH, which could lead to unstable mating pair formation.

Overall, the antibacterial and antiplasmid activities of these metabolites (evodiamine, rutaecarpine and evocarpine) from the quinolone family are noteworthy and should

be further investigated, particularly given their characteristic 4-quinolone structural component, which shows a good relation with the fluoroquinolones class of antibiotics. The quinolones, possess remarkable ability to inhibit both DNA gyrase and topoisomerase IV (Drlica and Zhao 1997), a feature accounting for their wide use in drug development and clinical practice, as antibacterials from 4(1H)-quinolone-3-carboxylic acid agents (Guzman *et al.*, 2011; Ahmed and Daneshtalab 2012), anticancer from 2-phenyl-4-quinolones (Sissi and Palumbo 2003), antitumor (Xia *et al.*, 1999) and antiviral (Kumar *et al.*, 2012) activities derived from quinolone-related compounds. However, the safety of evodiamine and rutaecarpine has not been fully studied on either animals or humans. Few studies conducted showed high toxicity of rutaecarpine and evodiamine against CCRF-CEM and multidrug-resistant CEM/ADR5000 cells with IC_{50} values ranging from 0.76 to 1.37 mg/L (Adams *et al.*, 2007). This thus implies that MIC generated in this study could be toxic to bacteria. Evaluation of acute toxicity of evodiamine in mice and *Drosophila melanogaster* [LD_{50} values of 77.79 mg/kg and 3.58mg/L, respectively (Yang *et al.*, 2006a) indicated that the MIC obtained equally may be toxic. Notwithstanding, these findings are necessary in addition to the antibacterial potential of these quinolones to provide rationale for their drug development. These quinolones are potential templates for modifications of their structures essential for effective antibacterial and antiplasmid drug design.

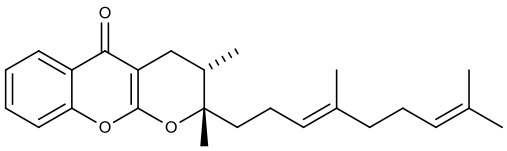
4.4 The antibacterial and anti-plasmid properties of Ferulenol

Ferulenol, a prenylated 4-hydroxyl-coumarin, is naturally isolated from *Ferula communis* L.; in the Apiaceae family, commonly known as giant Fennel.

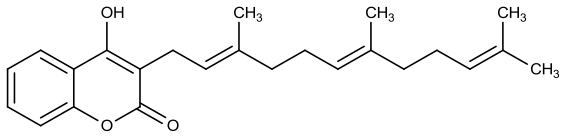


Ferulenol

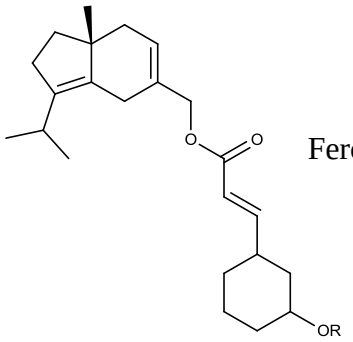
There are two distinguishable chemotypes of *F.* communis: i) prenylated coumarins, which contain daucane esters; and ii) a poisoinous chemotype, containing prenylated coumarins, thus an excellent source of ferulenol (Appendino *et al.,* 2004; Mamoci *et al.,* 2011). Earlier reports have been obtained on various biological activities of ferulenol including antimicrobial activity. Three antibacterial sesquiterpenes namely 14-(*o*-hydroxycinnamoyloxy)-dauc-4,8-diene, ferulenol and ferchromone from rhizomes of *F. communis* were evaluated by (Al-Yahya *et al.,* 1998) while (Appendino *et al.,* 2004) showed the antimycobacterial activities of ferulenol towards fast-growing stains of *Mycobacterium*.



14-(*o*-hydroxycinnamoyloxy)-dauc-4,8-diene



Ferulenol



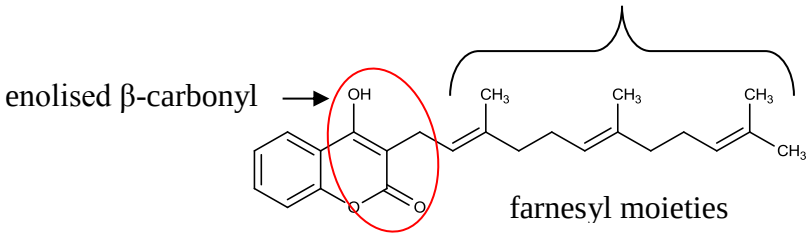
Ferchromone

In this study, ferulenol exhibited excellent antibacterial activity with an MIC of 0.5 mg/L, 16-fold better than the standard norfloxacin towards SA1199, and this activity was similar to the earlier reported MIC of 0.63 mg/L exhibited against standard *S. aureus, B. subtilis*, *Streptococcus durans* and *Enterococcus faecalis* by Al-Yahya's group. Ferulenol, also demonstrated good to moderate activity (0.5 – 32mg/L) against

other problematic MRSA strains such as XU212, *S. aureus* 13373, SA12981 and erythromycin-resistant RN4220 strain, which are currently associated with healthcare and MRSA infections (Gould *et al.*, 2012). However, it was inactive against *E. coli* and *P. aeruginosa*, which was presumably due to poor penetration through the cell walls of the Gram-negative bacteria.

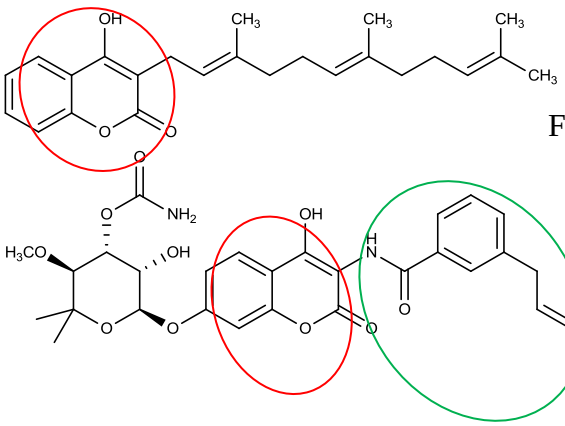
Previously from *F. hermonis* (Galal *et al.*, 2001) and *F. kuhistanica*, (Tamemoto *et al.*, 2001), some daucane sesquiterpenes with various aromatic ester moieties were characterised which demonstrated good MIC values of 6.25 mg/L and 8–16 mg/L against the MRSA strains. Taken together these results, they therefore highlight the potential of coumarins from the genus *Ferula*, as an interesting source of antibacterial leads, again with the outstanding antistaphylococcal activity of ferulenol against clinically relevant MRSAs.

Ferulenol has been associated with hepatocyte cytotoxicity in livestock due to its binding to tubulin (Bocca *et al.*,2002) and anticoagulant properties linked to inhibition of vitamin K epoxide reductase enzyme (Silverman 1981). It exerts taxol-like and dose-dependent cytotoxicity against various human tumor cell lines but none of these mechanisms support its antibacterial activities. Appendino's group correlated its mode of action to the presence of two structural elements, the enolized β-carbonyl and farnesyl moieties (Appendino *et al.*, 2008), separately found in two classes of antibacterial agents.



It is also possible that ferulenol could act on DNA gyrase, which is a typical mode of action for aminocoumarins used clinically, for example novobiocin (Peterson 2001).

The antiplasmid activity of ferulenol was studied to evaluate its ability to inhibit conjugal transfer of five R-plasmids bearing resistance markers, mainly amoxicillin and kanamycin. Ferulenol, when compared to novobiocin, exhibited an excellent broad host activity (2.29 – 10.62%) against the plasmids, especially TP114, PKM 101 and PUB307. The lipophilic phenolic moieties of the molecule probably enhanced the membrane permeability of ferulenol, leading to dislocation of conjugation steps or the correct assembly of the plasmid DNA thereby inhibiting plasmid transfer. The antiplasmid action of ferulenol can be quickly related to the inhibition of plasmid DNA, given that coumarins are known to have extensive biological activity due to their effect by inhibition of the bacterial DNA gyrase (Chatterji *et al.*, 2001). Inhibition of DNA gyrase and complex interaction with the supercoiled form of the plasmid DNA has been shown to lead to the cessation of plasmid replication in the bacterial cells (Wolfson *et al.*, 1982; Hooper *et al.*, 1984; Monlar *et al.*, 1992). Unfortunately, plasmids R6K and R7K were not refractory to ferulenol, contrary to the effect of novobiocin on them.



Ferulenol

Novobiocin

It is possible that novobiocin, possessing a hydroxyl at C-4 and a subsistent at C-3, effectively affected R6K and R7K, rather than ferulenol, indicating the importance of

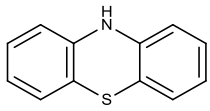
both molecules to specific plasmid activity. Probably, it can be assumed that ferulenol and novobiocin exerted similar mode of antiplasmid action, by targeting the inhibition of plasmid DNA in vitro, but the failure to achieve plasmid conjugal inhibition in some of the conjugation systems, for example R6K; may perhaps reflect to a bio-target different from the plasmid DNA gyrase.

4.5 The anti-plasmid properties of promethazine

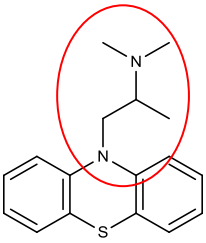
The antiplasmid activity of heterocyclic drugs has been extensively studied, and two drugs with outstanding effects on plasmid replication have been discovered. Promethazine and chlorpromazine eliminated the tetracycline, chloramphenicol, streptomycin and sulfonamide resistance of an *E. coli* strain (Mandi *et al.,* 1975b; Molnar *et al.,* 1977). This finding resulted in systematically synthetized phenothiazine and acridine derivatives that have extensively been studied for their biological activity and positive antiplasmid effects (Mandi *et al.,* 1975a; Molnar *et al.,* 1976; Molnar *et al.,* 1977; Barabas and Molnar 1980; Molnar and Nakamura 1988; Ford *et al.,* 1989; Molnar *et al.,* 1992; Kristiansen *et al.,* 2003; Spengler *et al.,* 2003; Takacs *et al.,* 2011).

Our results showed promethazine as an effective broad host anti-conjugative agent against the resistance plasmids, PKM 101 (18.03%), TP114 (5.7%) and R7K (0.64%). R6K and PUB307 were poorly affected by this molecule, however, the activity exerted by promethazine on these plasmids below the MIC concentration, showcases promethazine, not only as plasmid curing agent (Spengler *et al.,* 2006) but also as a conjugative inhibitor. The antiplasmid action of promethazine has been

linked to the π-electrons of the phenothiazine skeleton, which could target molecules essential for plasmid replication.



Phenothiazine



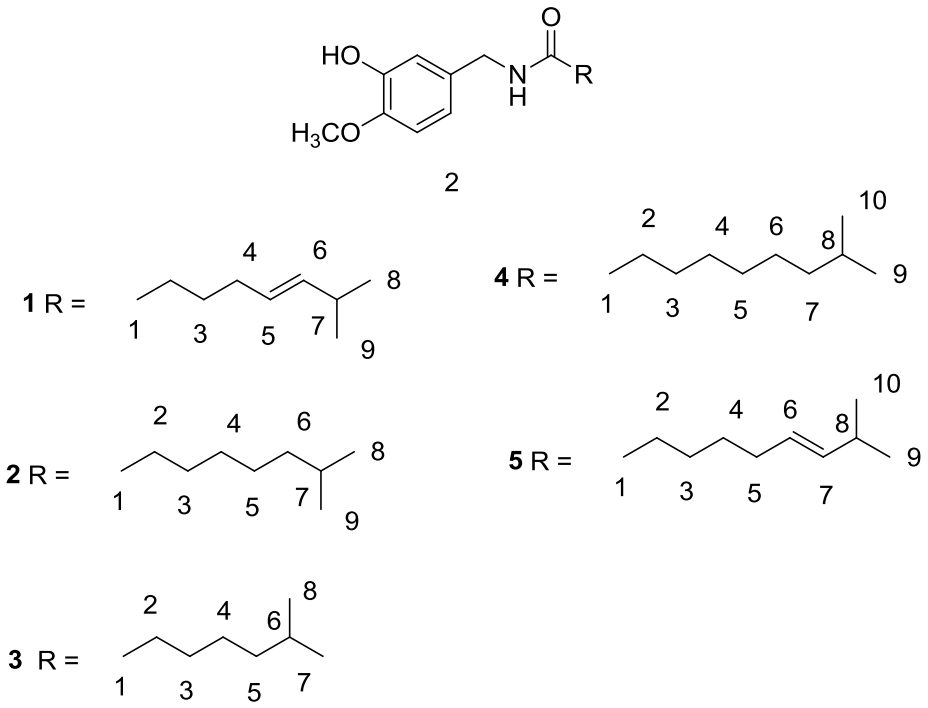
Promethazine

A cationic amide group may promote the permeability of promethazine into the plasmid cell (Spengler *et al.,* 2006). Therefore, the marginal effect of promethazine on R6K and PUB307 might be as a result of possible modification of the phenothiazine such that there was inefficient penetration and thus, inhibition could not be achieved.

Promethazine has eliminated antibiotic resistance plasmids of different *E. coli* at varying frequencies, cured lactose fermentation of *E. coli*, has tumour inducing ability of *Agrobacterium tumefaciens* and nodule formation of *Rhizobium meliloti* (Monlar *et al.,* 2003). In addition, promethazine has demonstrated various in vivo effects, either as a stand-alone drug or in combination with gentamycin (Molnár *et al.,* 1990), and other drugs namely imipramine and ciprofloxacin (Hooper *et al.,* 1984; Spengler *et al.,* 2006). These findings do appear to indicate a lack of toxicity on the part of promethazine, which can serve as an adjuvant for drug design in the future, to develop related substances which are potent in eliminating drug resistance-carrying plasmids of bacteria, but with no cytotoxic effects.

4.6 The antibacterial and anti-plasmid properties of capsaicin and capsaicin–like compounds.

Capsaicin and dihydrocapsaicin are found primarily in the fruit of the *Capsicum* genus and account for up to 90% of the total pungency of the pepper (Garces-Claver *et al.*, 2007). They are the main capsaicinoids generally isolated from the fruit of *Capsicum annuum* or related species, followed by 12-20 minor capsaicinoids and their analogues; a few examples include nordihydrocapsaicin, homodihydrocapsaicin and homocapsaicin (De Marino *et al.*, 2006; Reyes-Escogido, Maria *et al.*, 2011).



1R=capsaicin, 2R=dihydrocapsaicin, 3R=nordihydrocapsaicin, 4R=homodihydrocapsaicin, 5R= homocapsaicin

Growing interest in capsaicin has led to its characterization with various spectrophotometric and chromatographic methods such as silica gel column chromatography, normal-phase thin-layer chromatography (TLC), HPLC, LC-MS, UV-Vis and GC. High-performance liquid chromatography (HPLC) has been widely

used for the characterization of capsaicin and its analogues (Kobata *et al.*, 1998; Materska and Perucka 2005; Garces-Claver, A. *et al.*, 2007; Peng *et al.*, 2009). In this study, capsaicin was isolated via solid phase extraction chromatography, while Reverse-phase HPLC was applied to identify dihydrocapsaicin, in addition to mass spectrometric determination, proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR), which were applied for the comparative analysis of the capsaicinoids purity and structure. Due to their similarity in structures, the detection of dihydrocapsaicin from the capsaicinoid mixture was only successful by the use of HPLC, a situation that has been often encountered by researchers (Peng *et al.*, 2009; Butnariu *et al.*, 2012).

Capsaicin is a phenolic alkaloid and contains a vanillyl moiety. It is chemically designated as trans-8-methyl-*N*-vanillyl-6-nonenamide. It is a decylenic acid amide of vanillyl-amine and known with various synonyms, such as *N*-[(4-hydroxy-3-methoxybenzyl]-8-methyl-trans-6-nonenamide, *N*-[(4-hydroxy-3-methoxyphenyl) methyl]-8-methyl-trans-6-nonenamide, *N*-(3-methoxy-4-hydroxybenzyl)-8-methyl non-trans-6-enamide, *trans*-8-methyl-*N*-vanillyl-6-nonenamide, isodecenoic acid vanillylamide and 8-methylnon-6-enoyl-4-hydroxy-3-methoxybenzylamide (Arora *et al.*, 2011).

The molecular structure of capsaicin was resolved by Nelson and Dawson, in 1919, and exhibits typical *cis/trans* isomeric forms, though mostly found in its *trans* isomeric form (Reyes-Escogido, Maria *et al.*, 2011). The capsaicin molecule is basically divided in to three regions: (A) an aromatic ring, (B) an amide bond, and (C) the aliphatic (hydrophobic) side chain (Figure 72).

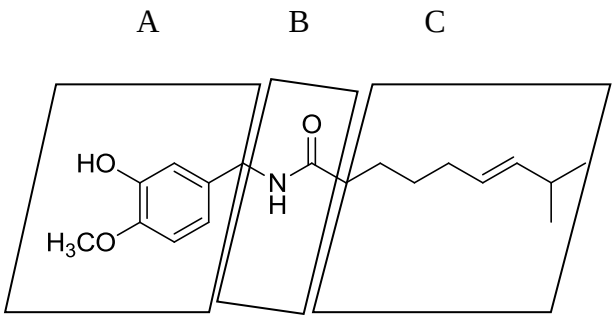
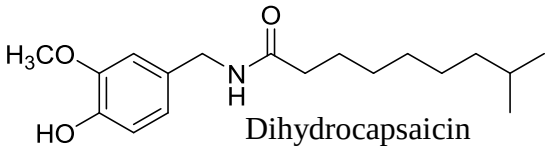


Figure 72: Regions of the molecule of capsaicin: A-aromatic ring, B-amide bond, C-hydrophobic side chain

Each of these features such as: substituents in the aromatic ring, the phenolic 4-OH group, and the H-bond donor/acceptor properties are important to the bioactivity of capsaicin, as a potent agonist (Arora *et al.,* 2011). It is been shown that lateral chain lengths higher or between 8 and 9 carbons atoms are equally important for the bioactivity of capsaicinoids (Barbero *et al.,* 2010). The forms of different natural capsaicinoids depend on the number of lateral chain carbons (R), which ranges from 9-11 in length, with the absence or presence of double bonds located at different positions along the chain (Reyes-Escogido *et al.,* 2011). In general, capsaicinoids are all amides formed from the condensation of vanillylamine and fatty acids of different chain lengths.

Dihydrocapsaicin (8-methyl-N-vanillynonenamide/-N-(4-hydroxy–3-methoxybenzyl) –8-methylnonanamide), is a reduced 6, 7 dihydro-derivative of capsaicin, with a molecular formula of $C_{18}H_{29}NO_3$ and molecular weight of 307.43.



Dihydrocapsaicin

During hydrogenation, capsaicin turns into dihydrocapsaicin, without the carbon-carbon double bond, resulting in a difference in the degree of unsaturation of the 9-

carbon fatty acid side chain (Garces-Claver *et al.,* 2007). This is the major structural difference between dihydrocapsaicin and capsaicin.

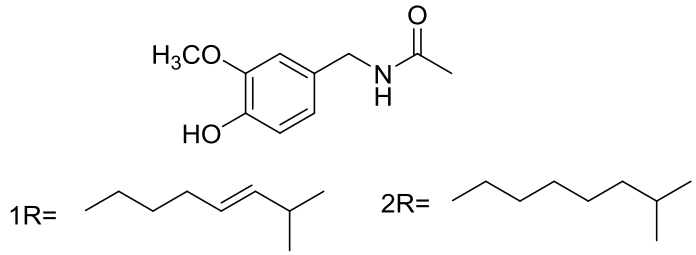
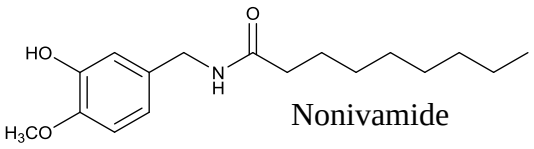


Figure 73: Structure of capsaicin (1R), and dihydrocapsaicin (2R)

Nonivamide is a naturally occurring analogue of capsaicin and has a molecular mass of nonivamide *m/z* 294 (Reilly *et al.,* 2001). Before, it was identified as naturally present in *Capsicum* species after many years, it was first synthesized and used as an adulterant of capsaicin, thereby regarded as synthetic capsaicin, a term that remains in current parlance (Constant *et al.,* 1996).



Nonivamide

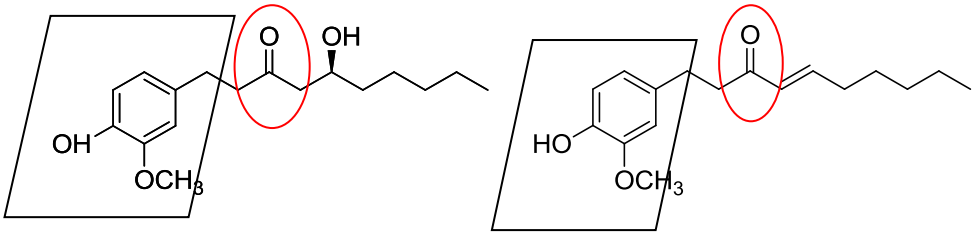
Nonivamide was isolated from *Capsicum oleoresin*, which was exact when compared with an authentic sample of synthetic nonivamide (Constant *et al.*, 1996).

From this study, capsaicin, dihydrocapsacin and synthetic nonivamide demonstrated moderate anti-staphylococcal activities, with the MIC range of 64 - 512 mg/L. These multidrug-resistant Gram-positive bacteria, especially the MRSA's, possess various efflux pumps that contribute greatly to their high level of resistance to a given drug or class of drugs, like tetracycline, fluoroquinolones and macrolides. Capsaicin appears

only to be active slightly against the norfloxacin–resistant SA1199B but remained inactive towards other Gram-positive and Gram-negative species, indicating that these resistant strains may confer various resistance mechanisms present in the bacteria which contributed to the poor uptake of the capsaicinoids. However, capsaicin (25 mg/L), when combined with ciprofloxacin, potentiated the activity of ciprofloxacin from 8 mg/L to 2 mg/L after 8 hours exposure, in the presence of SA1199B. This suggests that capsaicin possess ability as an inhibitor of NorA efflux pump (Kalia *et al.*, 2012).

Antimicrobial data on *Capsicum* species and pure capsaicin have been previously obtained by several researches against showing the scope of antimicrobial effect of capsaicinoids against *Helicobacter pylori* (Zeyrek Yildiz and Oguz 2005; Koffi-Nevry *et al.*, 2011), *Salmonella typhimurium*, *Pseudomonas aeruginosa* (Careaga *et al.*, 2003), fungi (Soumya and Bindu 2012; Fieira *et al.*, 2013), *E. coli*, *Psuedomonas solanacearum*, and *Bacillus subtilis* (Wei *et al.*, 2006; Voukeng *et al.*, 2012; Noumedem *et al.*, 2013).

The primary pungent agents of ginger are gingerol, with other gingerol analogues, shogaol, zingerone and paradol (Singh *et al.*, 2010). Both 6-gingerol and 6-shogaol consist of phenylakylketones or vanillyl ketone moieties, which show some resemblance with the vanillylamide (aromatic) moieties of capsaicinoids (Figure 74).



6-gingerol 6-shogaol

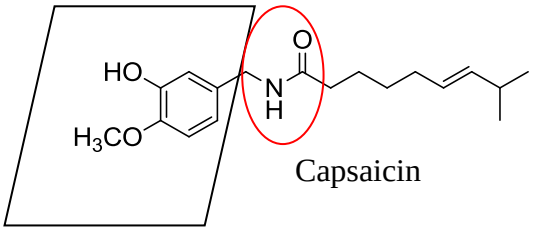
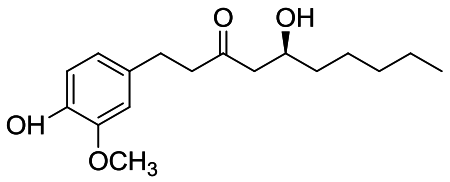


Figure 74: Structures showing resemblances of gingerol and shogaol with capsaicin

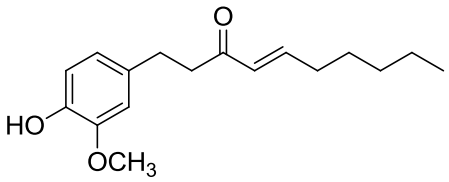
Interestingly, 6-gingerol and 6-shogaol showed better anti-staphylococcal activity against the multidrug MRSA 274829 and XU212 with an MIC range of 16 - 64 mg/L, compared with oxacillin and erythromycin respectively. They were marginally active against SA1199B with an MIC of 16 mg/L. Only 6-shogaol demonstrated good activity with an MIC of 8 mg/L, against RN4220 MsrA macrolide efflux while 6-gingerol did not show any activity against this organism. It is possible that the presence of MrsA pump undermined the effect of 6-gingerol by expelling them from their drug-binding sites (Ross *et al.*, 1995).

Gingerol and shogaol are considered to be the active constituents of ginger and ginger-based preparations used in folklore medicines (Singh *et al.*, 2010; Mishra *et al.*, 2012), but they unexpectedly had slight activity against the Gram-positive and Gram-negative bacteria relative to the antibiotics. This suggests that gingerol and shogaol could not overcome the resistance mechanisms present in these bacteria, which either chemically modified the compounds and render them inactive, or made the target sites unrecognisable by the compounds.

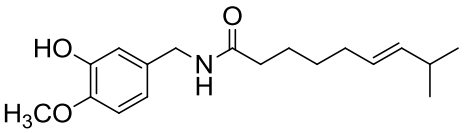
To the best of our knowledge, the inhibition effect of capsaicinoids, gingerol and shogaol on the conjugal transfer of R-plasmids has not been studied before, but various reports have shown a wide range of biological activities of these compounds, as analgesic, antiemetic, antipyretic, anti-arthritic, anti-inflammatory, anti-microbial, anticancer, and antioxidant activities (Park *et al.,* 2008; Singh *et al.,* 2009; Singh *et al.,* 2010; Arora *et al.,* 2011; Mishra *et al.,* 2012).



6-gingerol



6-shogaol



Capsaicin

From the anti-plasmid results, capsaicin was able to actively inhibit the conjugal transfer of amoxicillin-resistant PKM 101, PUB307 and R7K, while kanamycin-resistant TP114 and amoxilicin-resistant R6K remained unaffected. Dihydrocapsaicin was the most active against the conjugal transfer of PUB307, with a transfer frequency of 3.33%. PKM 101 was moderately inhibited by DHC, while TP114 was totally unaffected. The effect of capsaicin on these plasmids is remarkable such that it exhibited a broad range of activity over unrelated plasmid incompatibility groups; Inc N, W and P, while DHC seems plasmid specific. Nonivamide only had a minor inhibition effect on plasmid PUB307 transfer. The prevalent inhibitory activity of capsaicin over DHC might be presumably related to the difference in saturation of the

alkyl side chain. The strong inhibition effect of nonivamide on TP114 was rather surprising being that capsaicin and DHC only showed a minimal effect. Interestingly, both capsaicin and dihydrocapsaicin had an inhibitory effect on PKM 101 was consistent, which shows that PKM 101 was particularly sensitive to the capsaicinoids and highlights the potential of the capsaicinoids as new class of drug leads.

The effect of gingerol and shogaol was very active against the transfer of the plasmids, PKM 101, TP114 and PUB307. These results suggest the ability of the compounds and the responsible constituents to block the plasmid conjugation processes. Not only are these broad host plasmid conduits of various antibiotic resistance determinants in *E. coli,* but each of these plasmids codes similar pattern for conjugal replication and transfer, which employs the type IV secretion system (Grindley *et al.,* 1972; Wiletts 1977; Schroder and Lanka 2005) Consequently, it is possible that gingerol, and its dehydrated derivative, shogaol, had interfered with the plasmid DNA transfer and replication (Dtr) establishment in the cells, or secretion proteins coupled to DNA, which are actively transported substrates involved in bacterial conjugation (Schroder and Lanka 2005).

To further illustrate the effect of gingerol on conjugation type IV secretion systems (T4SS), gingerols inhibited of the growth of *Helicobacter pylori* CagA+ strains in vitro leading to its chemopreventative effects (Mahady *et al.,* 2003). *H. pylori*, is a human pathogen associated with the cancer-associated genes (cag) that localize on the pathogenicity island (PAI) genes, six of which show homologies to genes encoding T4SS components. Therefore, *H. pylori* uses the T4SS encoded CagA and associated CagA secretion proteins to target the gastric epithelial cells, leading to

various gastric diseases and cancer (Mahady *et al.*, 2003; Haniadka *et al.*, 2013). This explanation therefore sheds light on the potential effect of gingerols on the plasmid property, which is the T4SS-coded conjugal plasmid transfer; and more importantly the ability of gingerols to reverse horizontal antibiotic resistance spread in bacteria.

The ability of the unsaturated fatty acids, linoleic acid and dehydrocrepenynic acid (DHCA) is also a C_{18}, *cis*-unsaturated (9,12,14) fatty acid, with the peculiarity of having a triple bond between C-12 and C-13), to inhibit conjugal plasmid transfer by targeting Dtr plasmid systems was studied in Inc W plasmid group (Fernandez-Lopez *et al.*, 2005). The study suggested that the presence of the carboxylic group, chain length and presence of the double bond may be essential for the inhibitory activity, with polyunsaturated fatty acids being more potent than monounsaturated acids. Thus, the conclusion that unsaturated fatty acids of capsaicinoids, gingerol and analogues may be responsible for the observed antiplasmid activity is in agreement with these findings. The activities of capsaicinoids, gingerol analogues and relationship to plasmid conjugation inhibition require further investigation, which was beyond the scope of this study.

4.7.1 Kinetic studies of selected antiplasmid compounds

The presence of the compounds rottlerin, evocarpine and ferulenol, in the R-plasmid mating systems, showed inhibitory effects on their transfer rates over time. The sensitivity of the plasmids PKM 101 and TP114 towards the compounds after 30 mins incubation or an extended period of exposure was reflected in the various transfer frequencies. The effect of the compounds was observed not long after introduction into the mating mixture but achieved different degrees of inhibition, which either increased or fluctuated over time. Earlier data on the kinetics of plasmid conjugation in *E. coli* have reported that an exponential increase in the number of transconjugants is normally achieved after the initial 20 – 30 mins of the lag phase, while a lag time of 50 mins occurs in *P. aeruginosa* (Nakamura *et al.*, 1976; Cullum *et al.*, 1978) The most newly formed transconjugants are only able to mate after approximately 90 mins (Cullum *et al.*, 1978). These observations might be responsible for a moderate level of inhibitory effect of the compounds seen within the initial 30 mins. Furthermore, an active level of inhibition showed, especially by evocarpine after 4h, suggesting that longer than 30 mins exposure of the mating system in the presence of the compounds is essential and that equally such an effect can be sustained for up to 4 hours. The effect of ferulenol and rottlerin was relatively moderate towards the conjugation activities of PKM 101 and TP114 with no considerable changes over the time duration investigated, except an unexpected higher transfer frequency of TP114 in the presence of rottlerin at 4 hours. In our earlier experiments, rottlerin was shown to actively inhibit TP114 transfer, after 24 hour incubation. It is possible that a period of 0 - 4 hours may not be sufficient time to implicate an effective inhibition of the compound that would reduce the conjugation efficiency of the plasmid. In addition, a number of environmental,

cellular and host plasmid-specified conditions may have contributed to the diminished effect of rottlerin. Given that conjugation is a cellular process, F-pili are believed to retract when the cultures are cooled below 25^{o}C (Novotny and Fives-Taylor 1974; Novotny and Fives-Taylor 1978; Firth *et al.,* 1996). Notwithstanding that the frequency and mechanisms of gene transfer between micro-organisms in nature are still under investigation, the kinetics of plasmid transfer is a useful tool in studying and answering questions related to the use of recombinant bacteria.

Overall, various methods for the estimation of plasmid transfer rates, both in surface and liquid medium have been reported. They are : basic use of end-point differential models (Simonsen *et al.,* 1990; Dunn *et al.,* 1995; Zhong *et al.,* 2012), mechanistic models (Andrup and Andersen 1999), PCR quantification (Wan *et al.,*) and the most widely used traditional method of determining conjugation efficiency, being described by its transfer frequency (Andrup and Andersen 1999). However, each of these methods does not guarantee accurate measurement and may be sensitive to physical, chemical and biological conditions. There is still no agreement on a single quantitative measure of the rate of plasmid transfer (Zhong *et al.,* 2012).

4.7.2 DNA binding studies of the anti-plasmid compounds

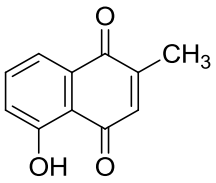
The binding of these anti-plasmid conjugative compounds was conducted for the purpose of understanding the possible mechanisms of action of the compounds based on the proposed mechanism that these compounds bind to the bio-target, plasmid DNA. From literature data, multiple mechanisms of action for anti-plasmid compounds are closely associated with increased plasmid membrane permeability, inhibition of DNA gyrase and complex formation plasmid DNA that causes cessation of plasmid replication in the bacterial cells (Monlar *et al.,* 1992), dislocation or

blocking of the coupling proteins, the pilus formation or the entire T4SS conjugative machinery (Lawley *et al.*, 2003).

In this study, the gel mobility shift assay was used to resolve the DNA-compound complexes formed as a result of the interaction of the compounds with the DNA of *HpaI*-digested PKM 101, and *SphI*-digested pAKlux3. Results showed that none of the compounds showed any migration shift with the DNA-compounds complexes, indicative of no binding effect of the compounds, except promethazine and dihydrocapsacin binding to some of the fragments of pAKlux3 DNA and capsaicin binding to *HpaI*-digested PKM 101. Actinomycin D-DNA complex showed a pronounced shift in its DNA migration along the gel. Actinomycin-D is well known for its high intercalation affinity to DNA, the stabilization of cleavable complexes of topoisomerases I and II with DNA (Koba and Konopa 2005), and that was also well demonstrated from our findings. Unfortunately but surprisingly, most of the anti-plasmid compounds: rottlerin, the red compound, ferulenol, evocarpine, 6-gingerol, 6-shogaol, rutaecarpine and nonivamide, did not show direct binding to the plasmid DNAs from PKM 101 and pAKlux3. Given the planar and cyclic nature of these compounds, presumably, they were expected to intercalate between the DNA base pairs or fuse into the minor groove of the DNA. These results suggested that the compounds might be acting via an alternative mechanism for their inhibitory activities, most likely the inhibition of type IV secretion system (T4SS) bio target. The importance of molecules that can bind to DNA cannot be overemphasised, and typical examples include anthracyclines which show both antineoplastic and antibacterial properties, and possess intercalative unit as well as a groove-binding side chains (Palchaudhuri and Hergenrother, 2007).

The low intensity fluorescence of the bands under the UV absorption, may suggest that promethazine, dihydrocapsaicin and capsaicin interacted with the DNA. Further experiments can be performed to verify the nature of binding, hence decreased the fluorescence of the intercalator ethidium bromide. The interaction of promethazine with DNA was not in agreement with a previous study that, with exception of methylene blue and chlorpromazine, phenothiazine compounds (promethazine inclusive) did not intercalate into the *E. coli* DNA (Barabas and Molnar 1980). The authors concluded that the plasmid curing ability of phenothiazines is not necessarily related to their intercalation ability. However, the binding of promethazine to PAKlux3 require further investigation to determine if the process took place by intercalation or any other mode of binding.

It has been earlier shown that plumbagin, notably for its anticancer and tubulin binding effect (Acharya *et al.,* 2008), also induced plasmid loss presumably by inhibiting DNA gyrase (Bharathi and Polasa 1991).



Plumbagin

But from this study, plumbagin did not show any DNA binding effect to the plasmidic DNA. Similar results were observed with novobiocin showing no binding, but this does not agree with the existing findings of several researchers that novobiocin binds to type II topoisomerases, including DNA gyrase, inhibits the enzyme-catalysed hydrolysis, of ATP (Shen *et al.,* 1989), and is able to induce changes in plasmid molecules of bacteria (Sioud *et al.,* 1988). These findings on novobiocin do support the DNA binding effect but it is likely that we were unable to

detect this due to the limitation of the method applied, the electrophoretic mobility shift assay (EMSA). Most likely, the compounds may have bound to some molecules of the plasmidic DNA in such an unspecific manner and were minor not to cause any visible shift in DNA migration which was easily detectable by the method. The gel mobility shift assay is a popular, fast analysis that allows rapid screening of DNA-binding agents, including highly purified proteins or crude extracts, but it is not without limitation. Whilst the EMSA is mostly applied to protein-DNA analysis than to plant compound-DNA interaction, perhaps, the most important limitation is that samples are not at chemical equilibrium during the electrophoresis step. Rapid dissociation during electrophoresis can prevent detection of complexes, while even slow dissociation can result in an underestimation of binding density (Sidorova *et al.*, 2005). Of course, further DNA binding analysis of these bioactive compounds exhibiting plasmid conjugation inhibitory activities can be demonstrated taking advantage from other DNA classical procedures, such as DNA melting point (Hadjivassileva *et al.*, 2007) and DNA footprinting assays (Carey *et al.*, 2013).

5.0 SUMMARY

Undoubtedly natural products remain an invaluable source of classes of antimicrobial compounds waiting to be developed for clinical use. In this study, new plant-derived anti-staphylococcal agents (rottlerin, red compound, ferulenol, and rutaecarpine) had outstanding activity towards a panel of MRSA possessing multidrug efflux mechanisms and hence could be potential leads for the development of antibacterial agents. The antibacterial activity of some of the compounds may not be significant *in vitro* against some of the Gram-positive and Gram-negative strains, but considering that they are of natural source and possess interesting chemical templates, that could be harnessed to improve on their antibacterial activity. Also their resistance modifying action might be of great interest, which may enhance and complement their antiplasmid activities. Thus, investigation of resistance modifying activities of these natural compounds and their synthetic analogues on existing antibiotics is strongly recommended.

It has also been demonstrated that plant-derived compounds are active inhibitors of plasmid conjugal transfer, a highly efficient antibiotic resistance mechanism common in Gram-negative bacteria. These plasmids have an uncommonly broad host range, from Inc groups PKM 101 (IncN), TP114 (IncI2), PUB307 (IncP), R7K (IncW), R6K (IncX) and R1-drd-19 (Inc IncF11), which confer cross-resistance to all classes of antibiotics currently in use. A common characteristic of these plasmids is that all code for the essentials of bacterial conjugative Type-IV secretion system (T4SS) machinery (Bhatty *et al.*, 2013), which obviously underlies bacterial conjugation. It therefore follows that, the use of these compounds that affect T4SS-typed conjugative plasmids carrying these resistance and virulence genes directly or indirectly, present

one of the intriguing strategies and anti-plasmid leads that are much needed to fight against plasmid-mediated antibiotic resistance.

Common features observed among these anti-plasmid agents and many other curing agents are their large size, planar structure, saturated or unsaturated aliphatic side chains and a high degree of lipophilicity. These qualities are likely to be of importance for their permeability into the bacterial membrane and interference with the plasmid DNA replication and conjugative transporters. Also, the presence of the functional groups is often associated with the cytotoxicity of the compounds and such will be crucial to their use in drug development of the next generation of anti-plasmid agents.

The antiplasmid and antibacterial action of the compounds outlined through this study is of great contribution to the role of natural product in the on-going search for antimicrobial drug leads. Thus, further studies are required to unravel the mode of action, improve their toxicity and druggability profiles which are necessary for the development of novel drug.

FUTURE STUDIES

Experiments described in this thesis provide a good preliminary foundation on the role of natural product in the inhibition of plasmid–mediated antibiotic transfer in Gram-negative bacteria, *E. coli*. However, due to time constraints and use of agar-based method of conjugation, it was not possible to screen as many plant extracts for identification of more bioactive plasmid inhibitors. Equally, natural product anti-plasmid agents require more research to uncover the potentials towards containing antibiotic resistance spread. Therefore, future work should aim to:

- Screen as many medicinal plants and natural and chemical libraries in search of anti-plasmid inhibitor leads.
- Study the resistance modifying activities of these natural compounds and their synthetic analogues
- Develop a high-through-put plasmid conjugation screening assay.
- Study more into understanding the mechanism of action of the anti-plasmid agents and their cytotoxicity.
- Determine if the compounds with plasmid inhibition activities bind to DNA using other DNA binding methods.
- Examine if the anti-plasmid compounds interfere with plasmid gene expression by monitoring *lac* light production.
- Whether the compounds interfere or induce DNA damage using RecA reporter.

Scientific meetings attended with presentations

- British Society for Antimicrobial Chemotherapy Spring meeting, UK. 20th March, 2014. Poster: *In vitro* screening of natural inhibitors of plasmid mediated antibiotic resistance in *E. coli*.

- UCL School of Pharmacy Research Day, Friday 20 September 2013. Oral presentation: The effect of natural and synthetic inhibitors on conjugal transfer of plasmid-coded antibiotic resistance genes in *E. coli*.

- International Plasmid biology Conference, Sept 12- 16, 2012, Santander, Spain. Oral presentation: Natural and Synthetic Cannabinoids as potential inhibitors of plasmid mediated antibiotic resistance.
- International Congress on Natural Products Research, Jul 28 – Aug 1, 2012, New York City, USA. Poster: Rottlerin: An antibacterial agent and inhibitor of plasmid-mediated antibiotic resistance transfer. *Planta Medica* 2012; 78 - PI165.DOI: 10.1055/s-0032-1320853

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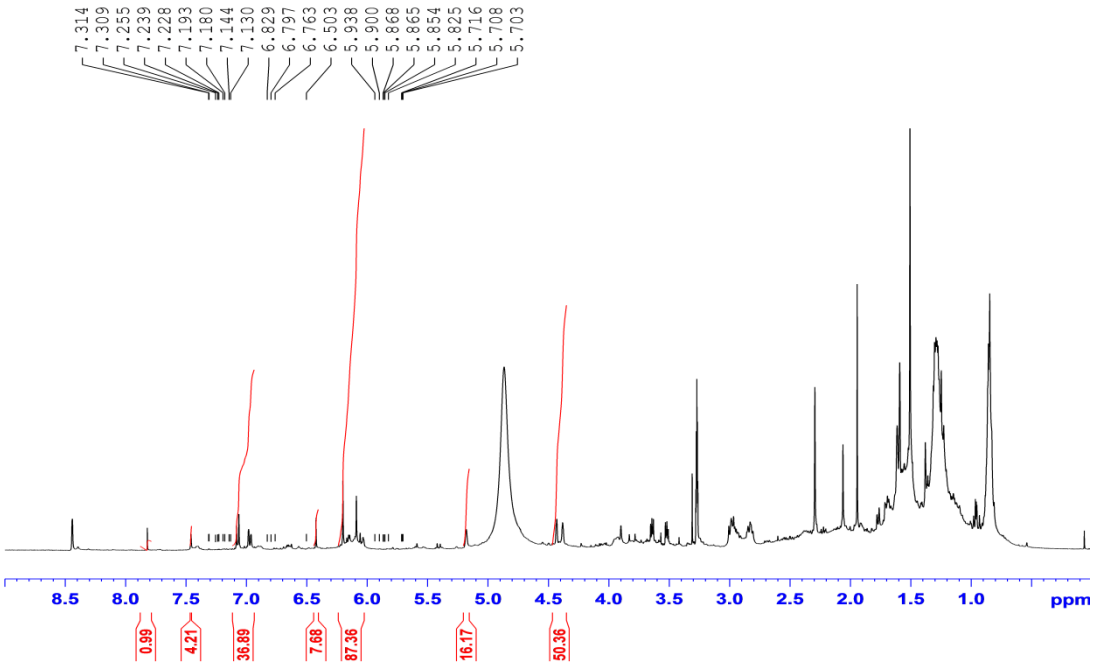
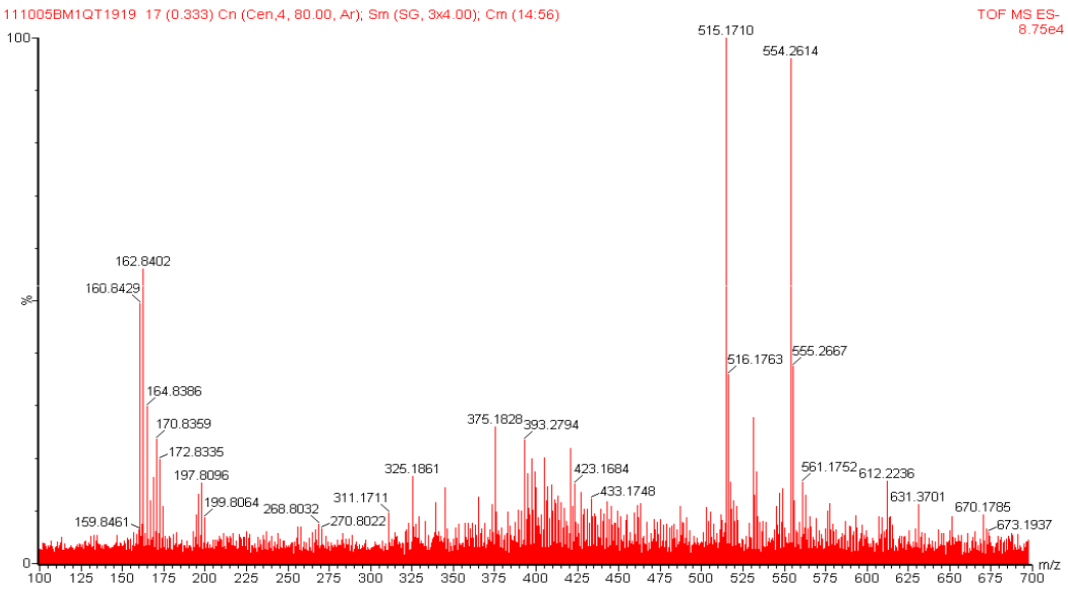
Evodia rutaecarpa. http://kampo.ca/herbs-formulas/herbs/goshuyu

7.0 APPENDIX

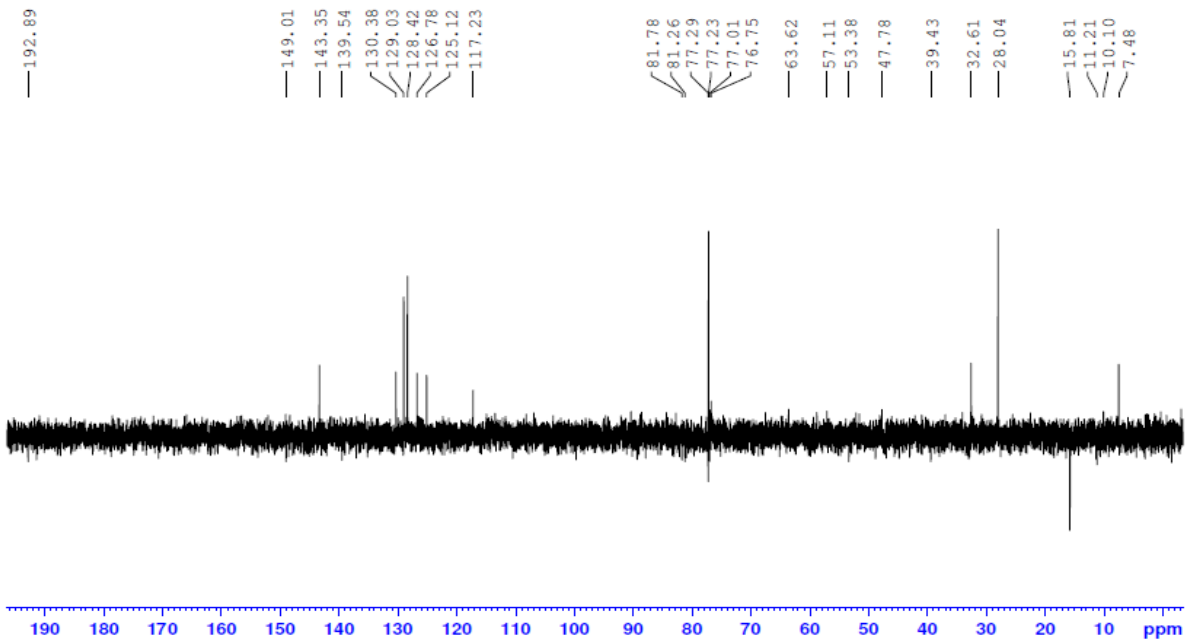
Appendix 1: ^{1}H NMR spectrum of Can-6 in $CDCl_3$ (500MHz) showing mixture of constituents



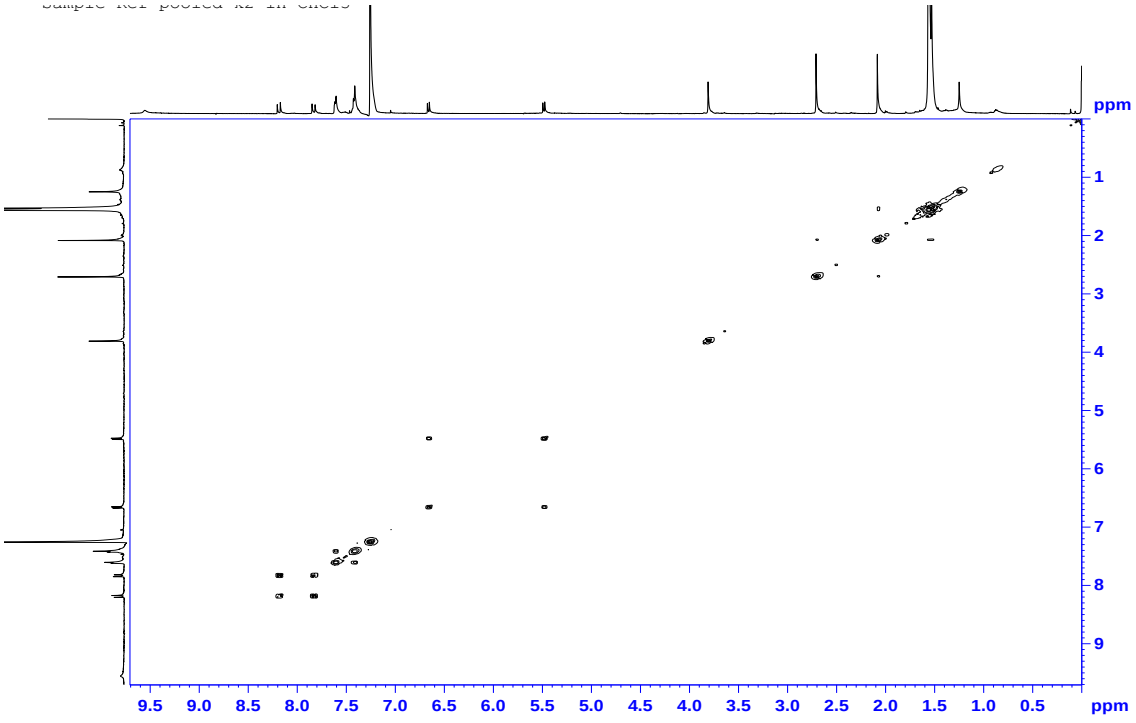
Appendix 2: The ESI-MS of rottlerin showing molecular ion at *m/z* 515 and 516 m/z



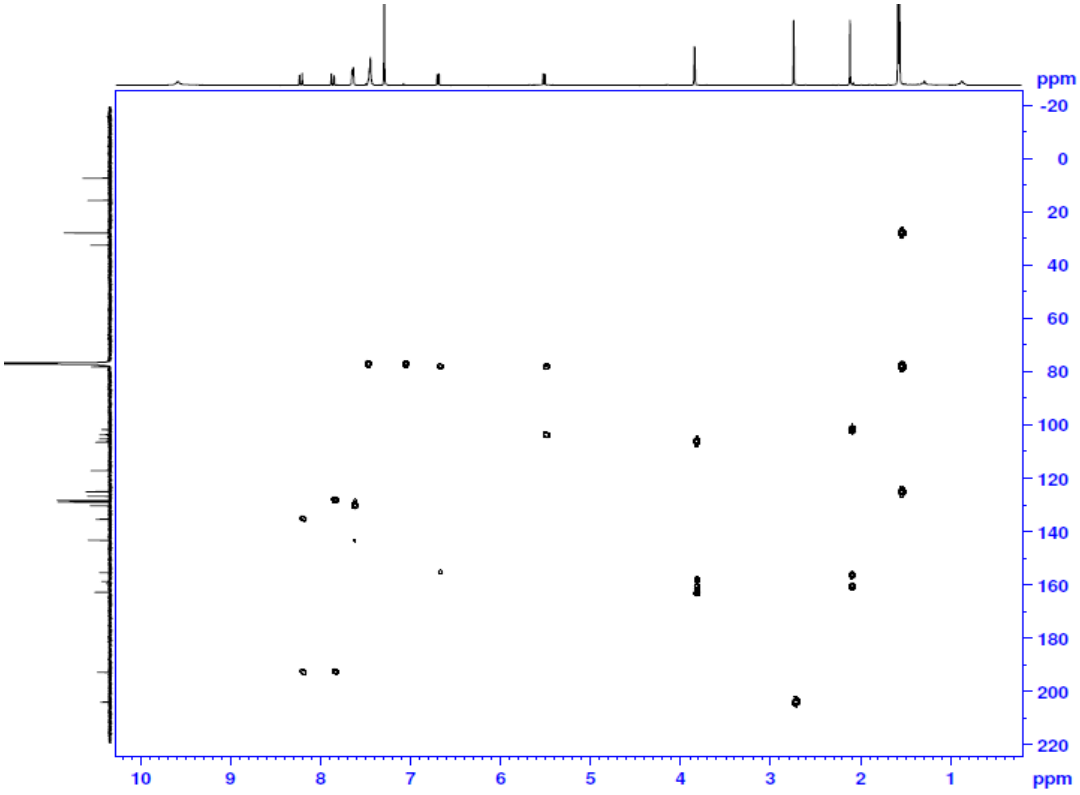
Appendix 3a: ^{13}C-DEPT 135 spectrum of rottlerin in $CDCl_3$ (500MHz)



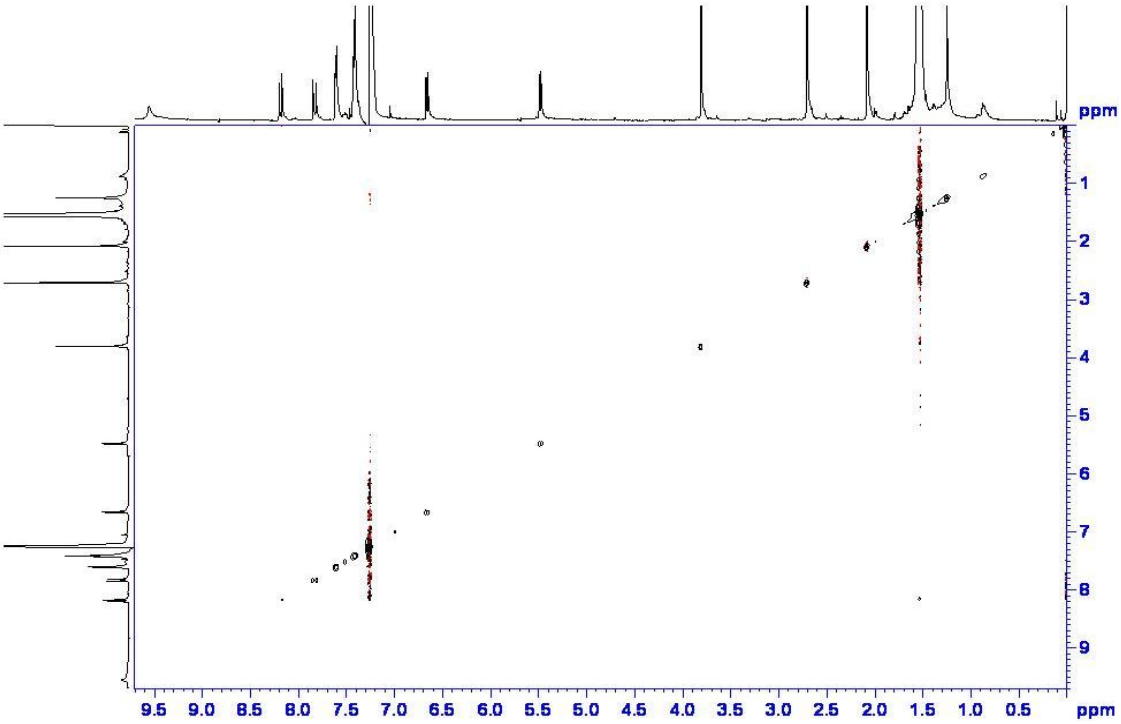
Appendix 3b: ^{1}H-^{1}H COSY (500MHz) spectrum of rottlerin in $CDCl_3$



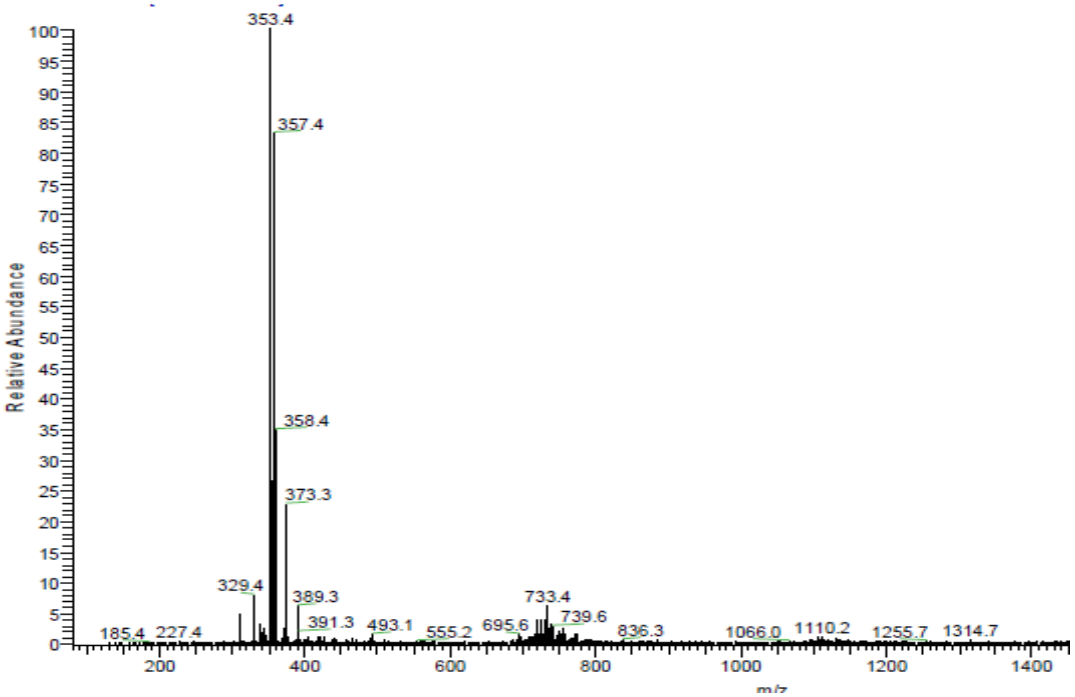
Appendix 3c: The HMBC spectrum of rottlerin at 500MHz in $CDCl_3$



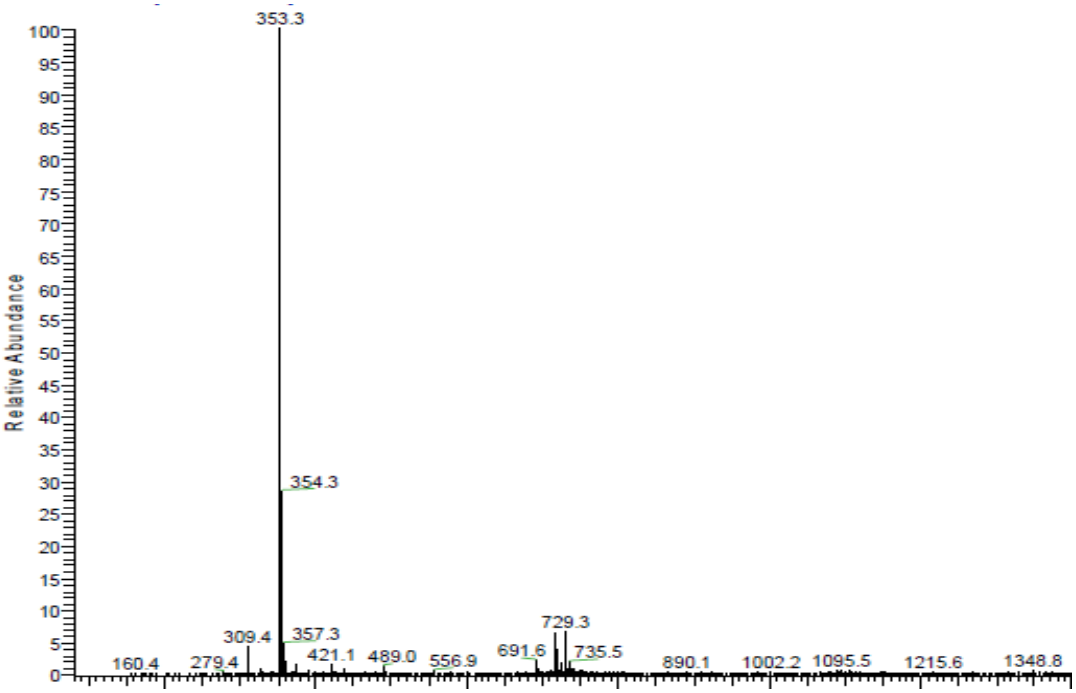
Appendix 3d: NOESY spectrum of rottlerin at 500MHz in $CDCl_3$



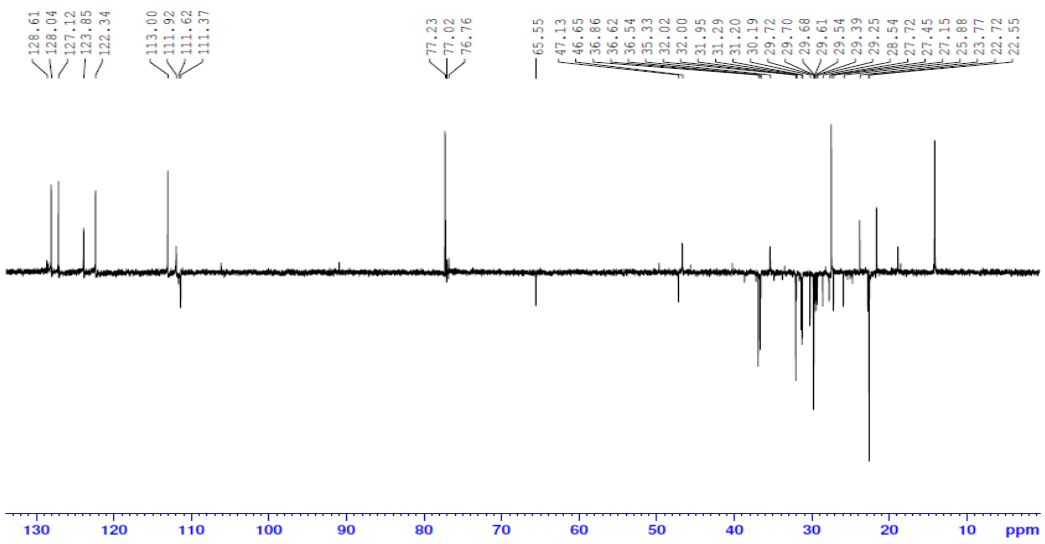
Appendix 4a: ESI-MS spectrum of tetrahydrocannabinolic acid [negative mode)



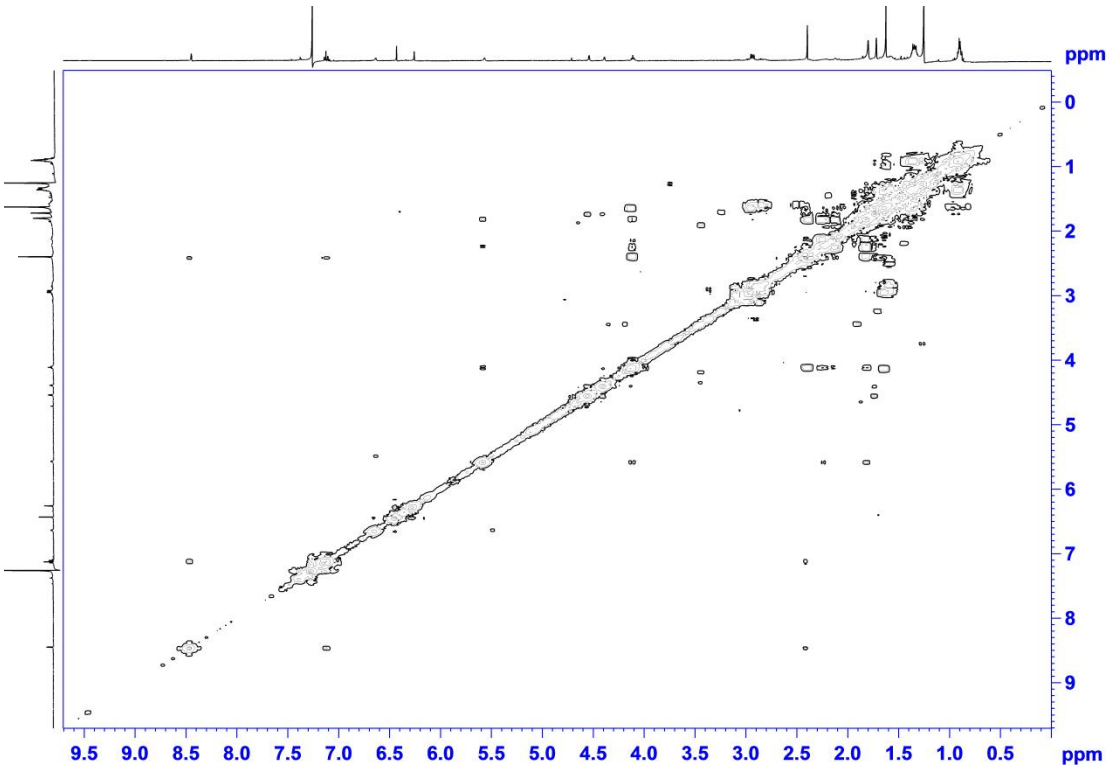
Appendix 4b: ESI-MS spectrum of cannabinolic acid [negative mode]



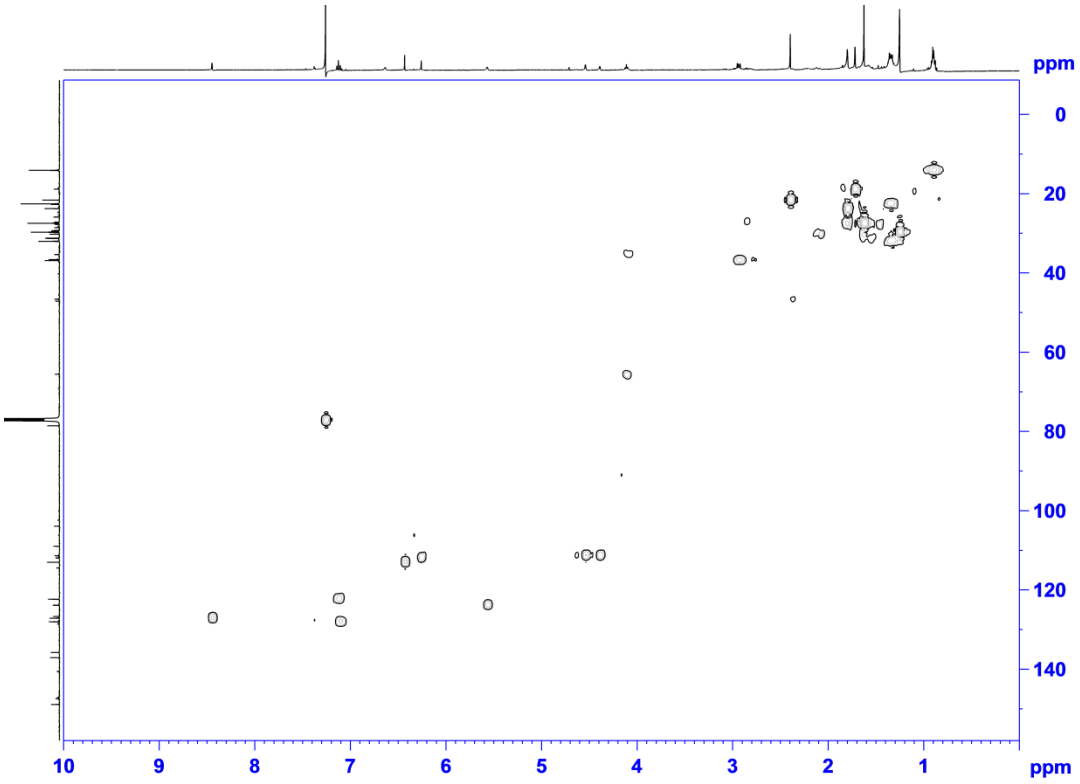
Appendix 5a: ^{13}CDEPT 135 of tetrahydrocannabinolic acid in $CDCl_3$ (500MHz)



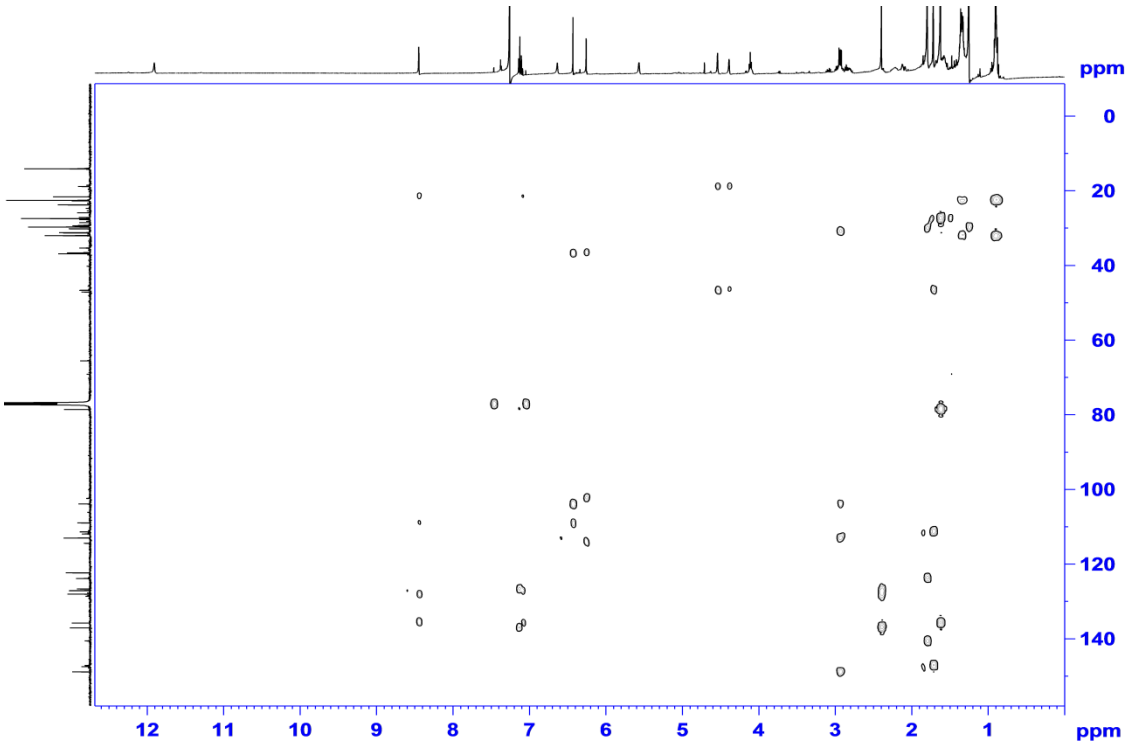
Appendix 5b: ^{1}H-^{1}H COSY (500MHz) spectrum of THCA in $CDCl_3$



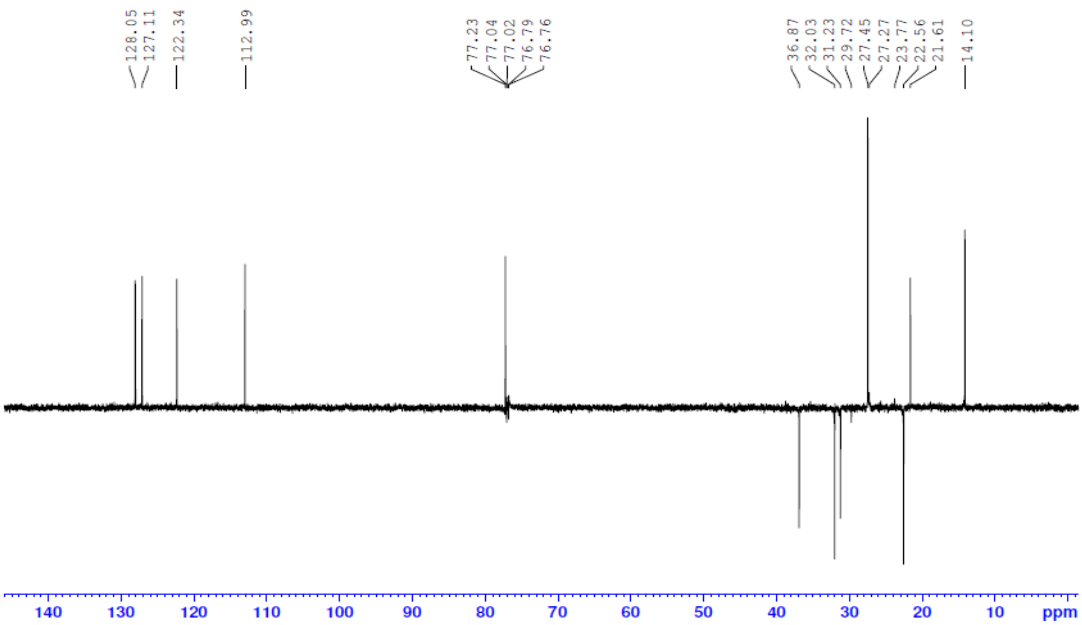
Appendix 5C: HMQC of THCA at 500MHz in $CDCl_3$



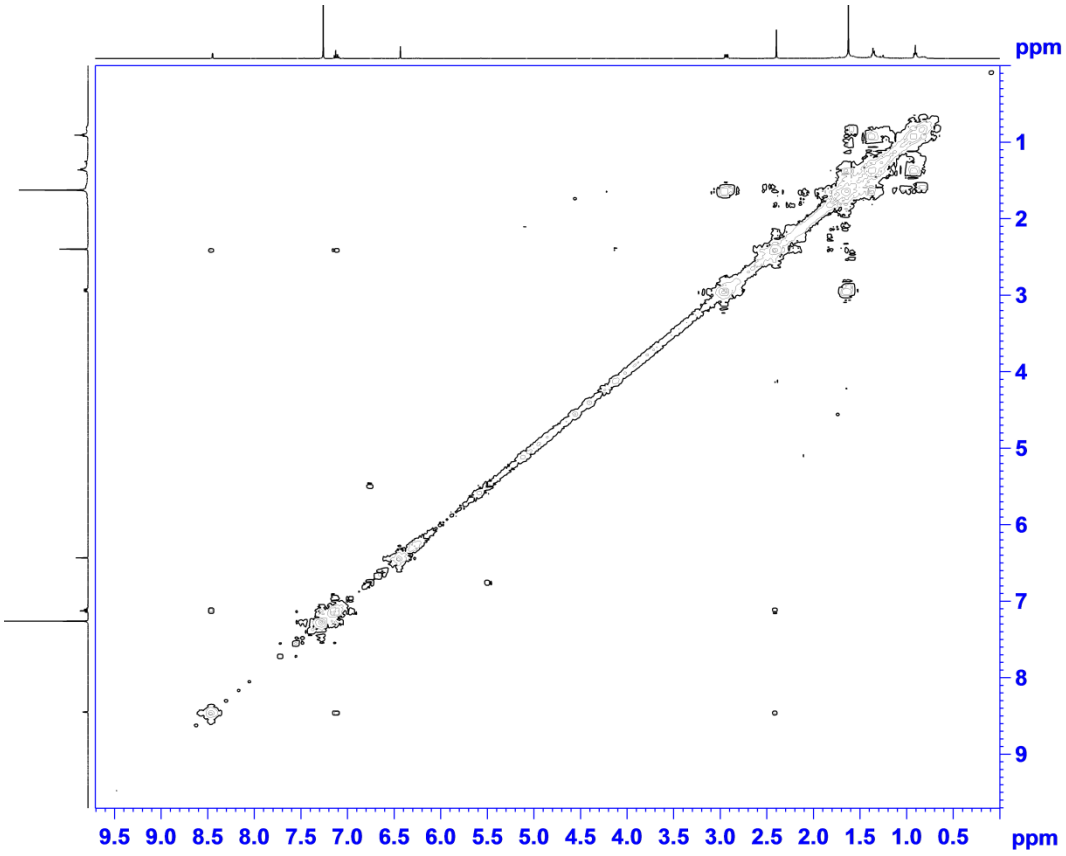
Appendix 5d: HMBC of THCA at 500MHz in $CDCl_3$



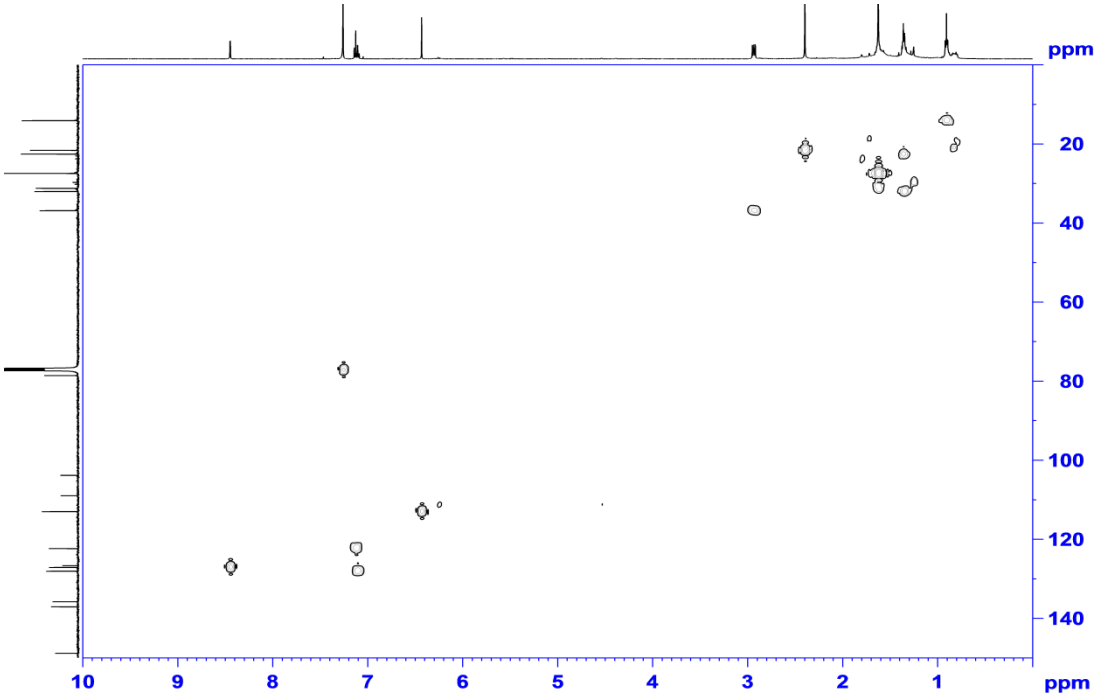
Appendix 6a: ^{13}CDEPT 135 of cannabinolic acid (CBNA) at 500MHz in $CDCl_3$



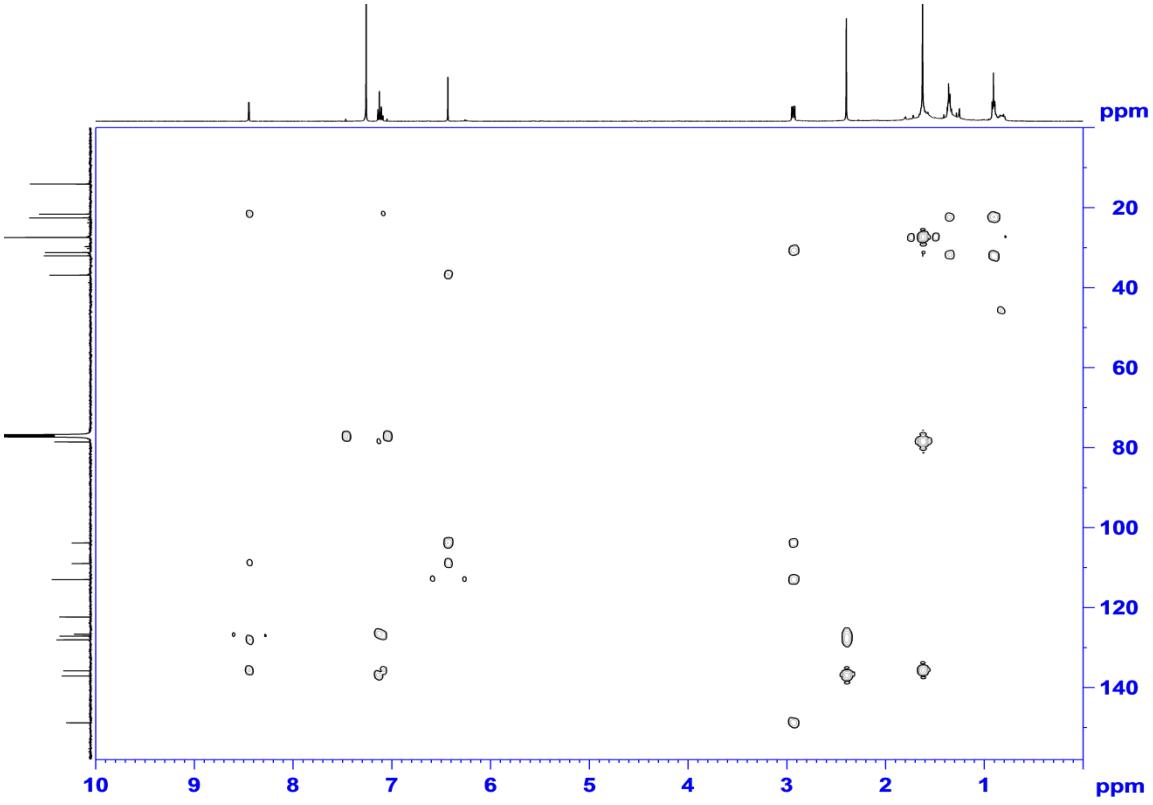
Appendix 6b: ^{1}H-^{1}H COSY (500MHz) spectrum of CBNA in $CDCl_3$



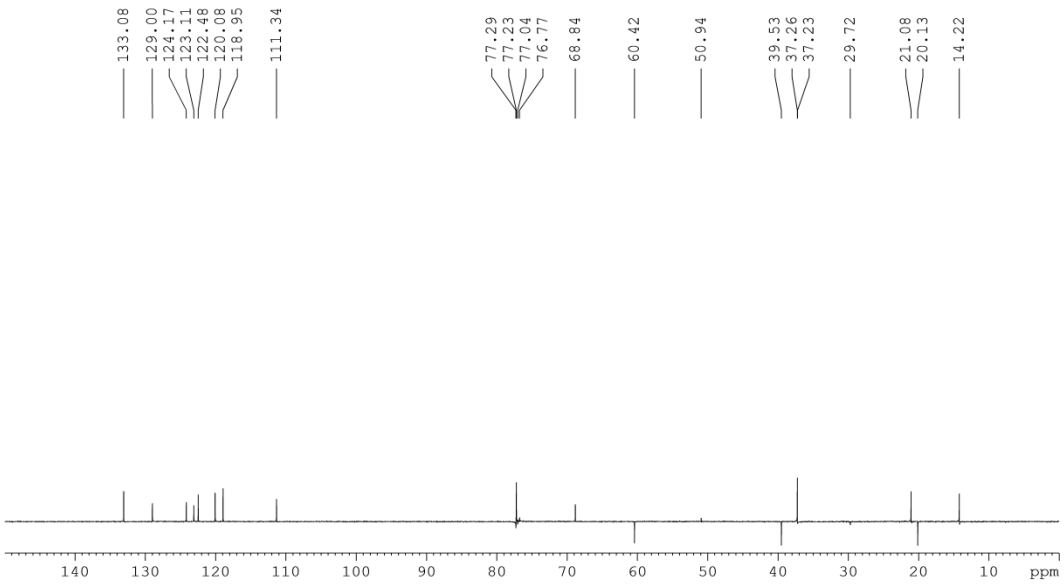
Appendix 6c: HMQC of CBNA at 500MHz in $CDCl_3$



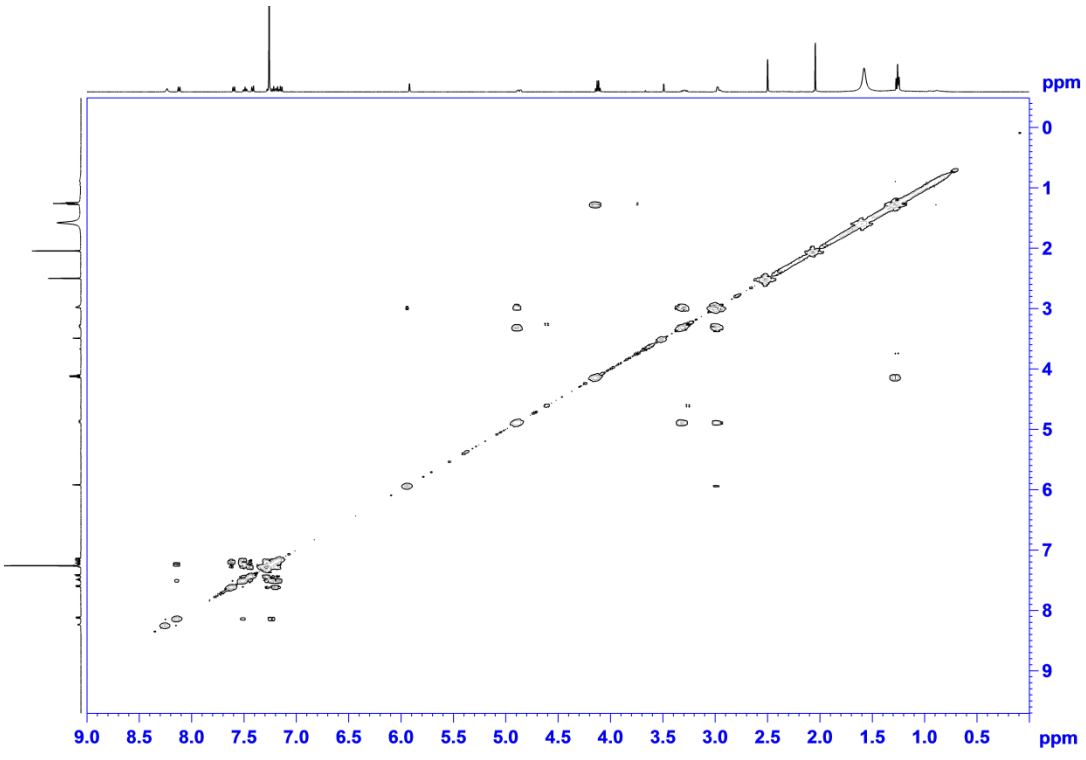
Appendix 6d: HMBC of CBNA at 500MHz in $CDCl_3$



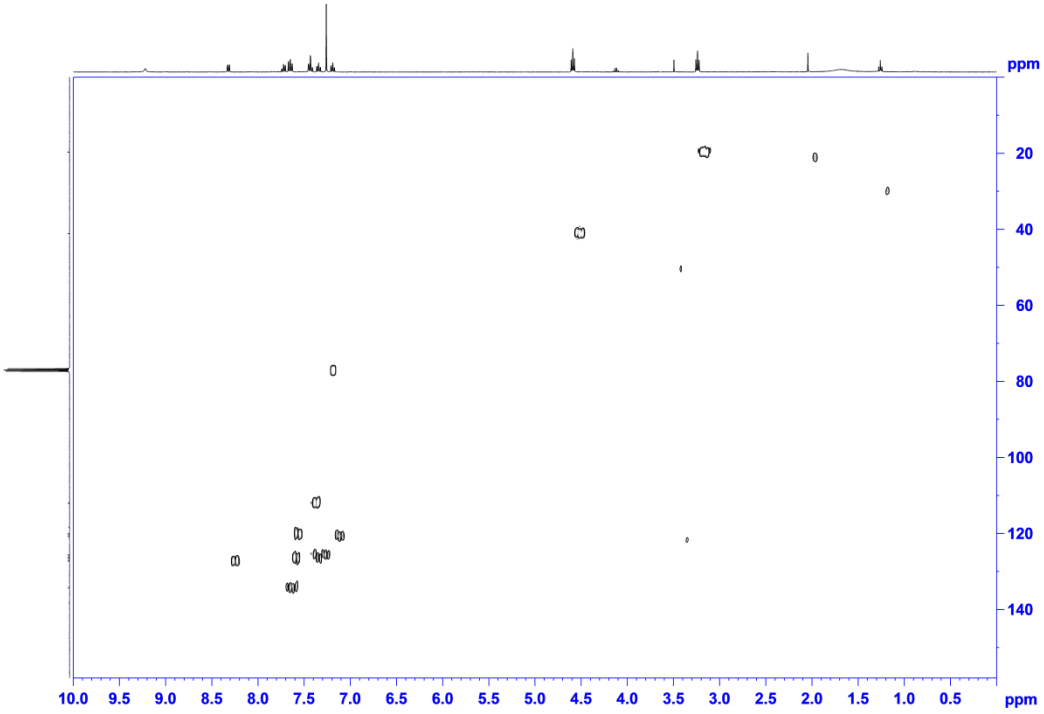
Appendix 7a: ^{13}CDEPT 135 of evodiamine at 500MHz in $CDCl_3$



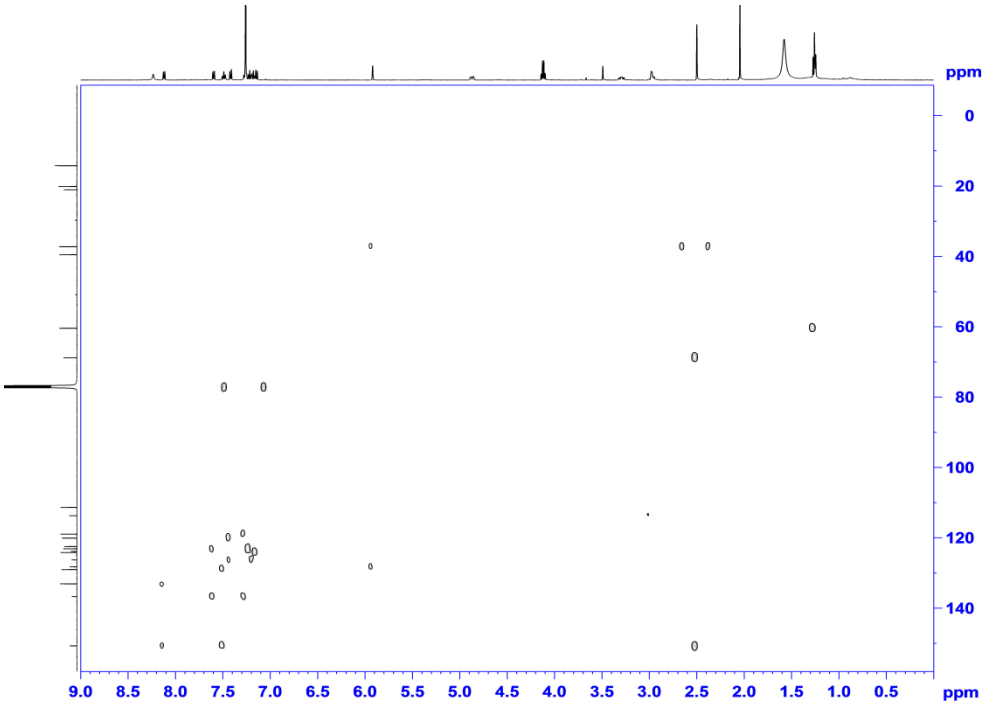
7b: ^{1}H-^{1}H COSY (500MHz) spectrum of evodiamine in $CDCl_3$



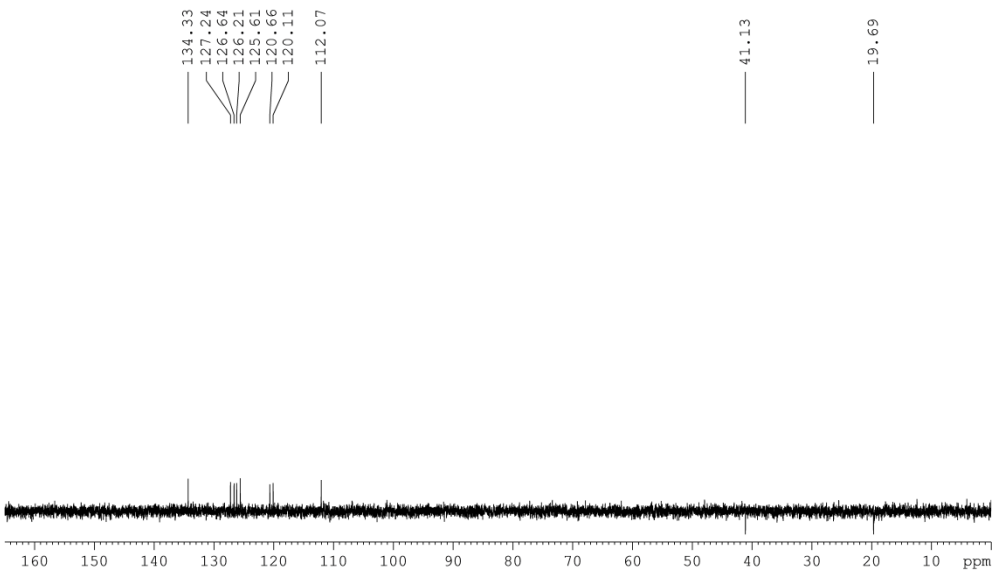
7c: HMQC of evodiamine at 500MHz in $CDCl_3$



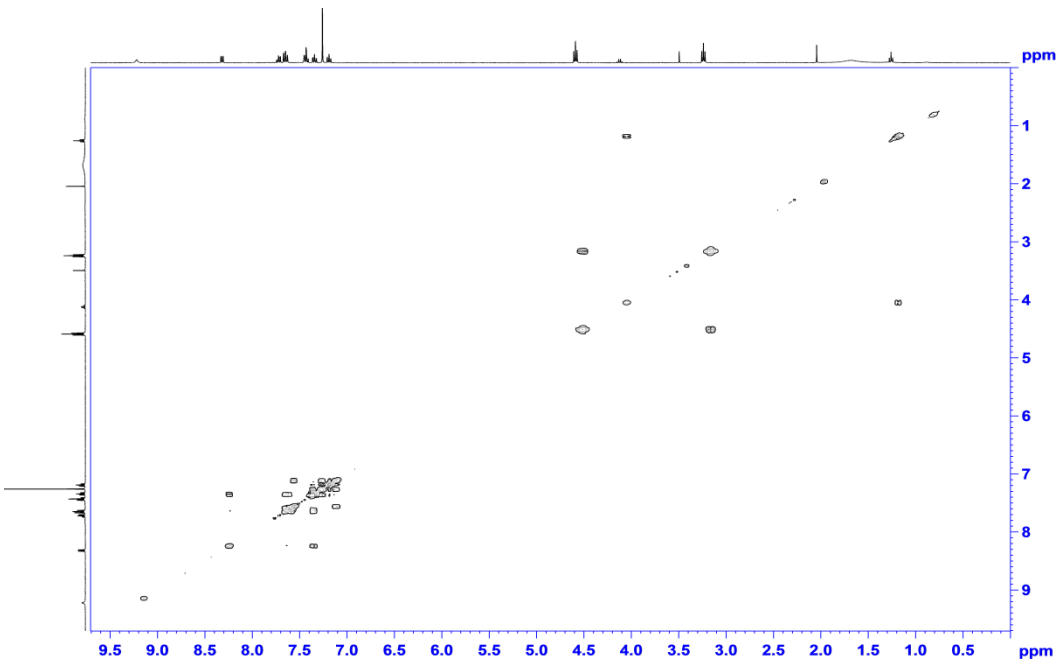
Appendix 7d: HMBC of evodiamine at 500MHz in $CDCl_3$



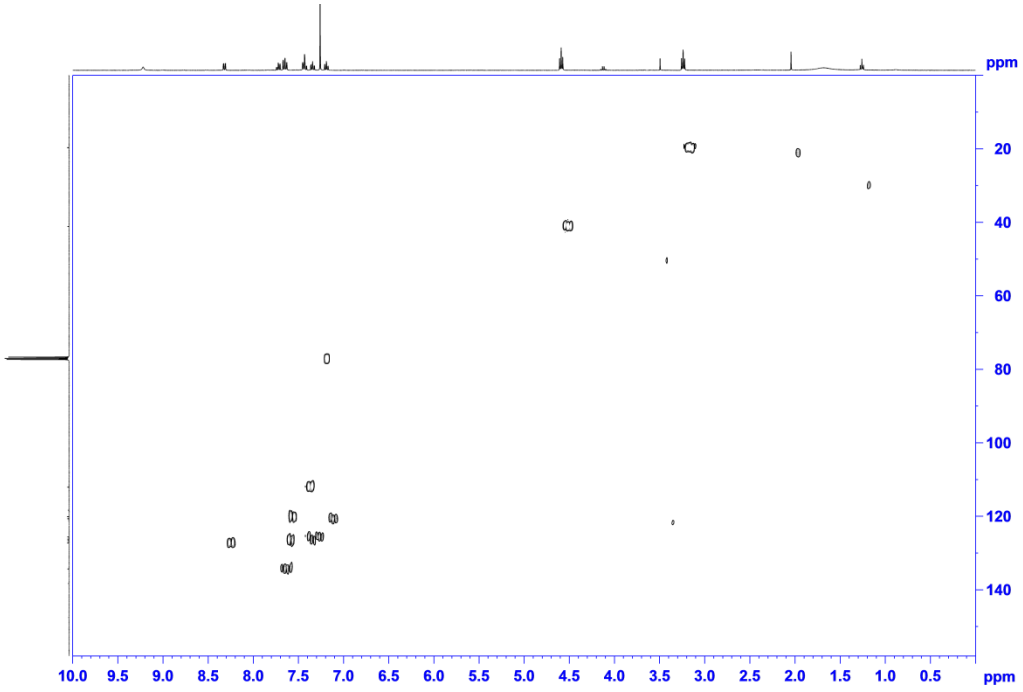
Appendix 8a: ^{13} CDEPT 135 (500MHz) spectrum rutaecarpine in $CDCl_3$



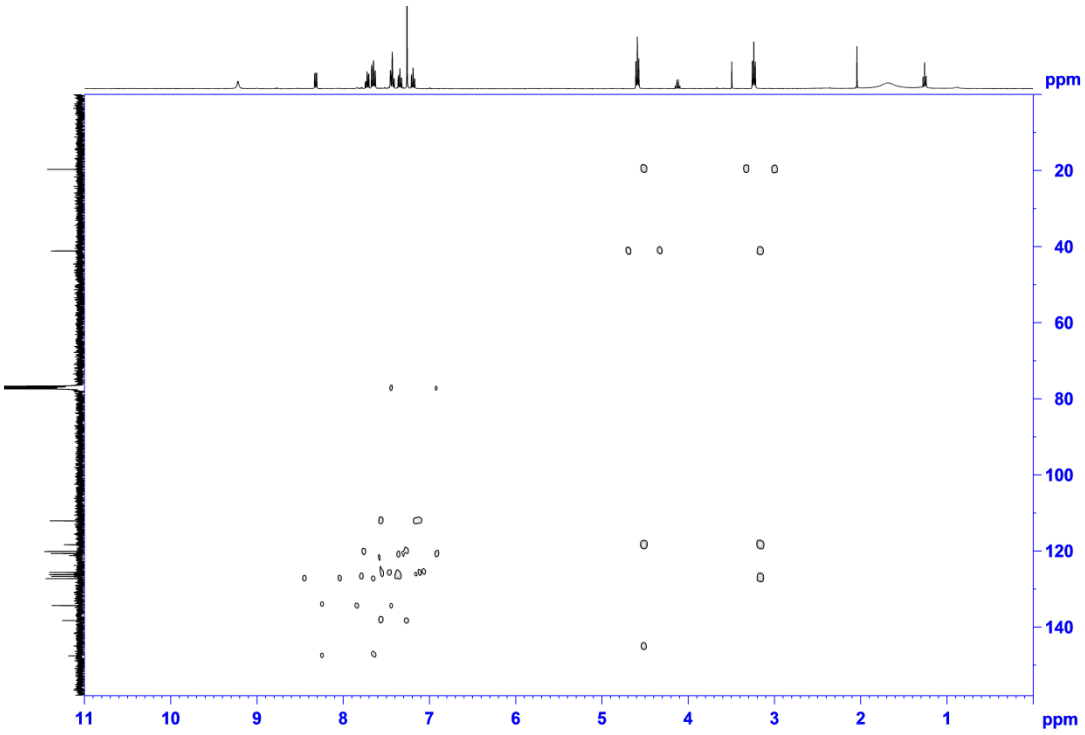
Appendix 8b: ^{1}H-^{1}H COSY (500MHz) spectrum of rutaecarpine in $CDCl_3$



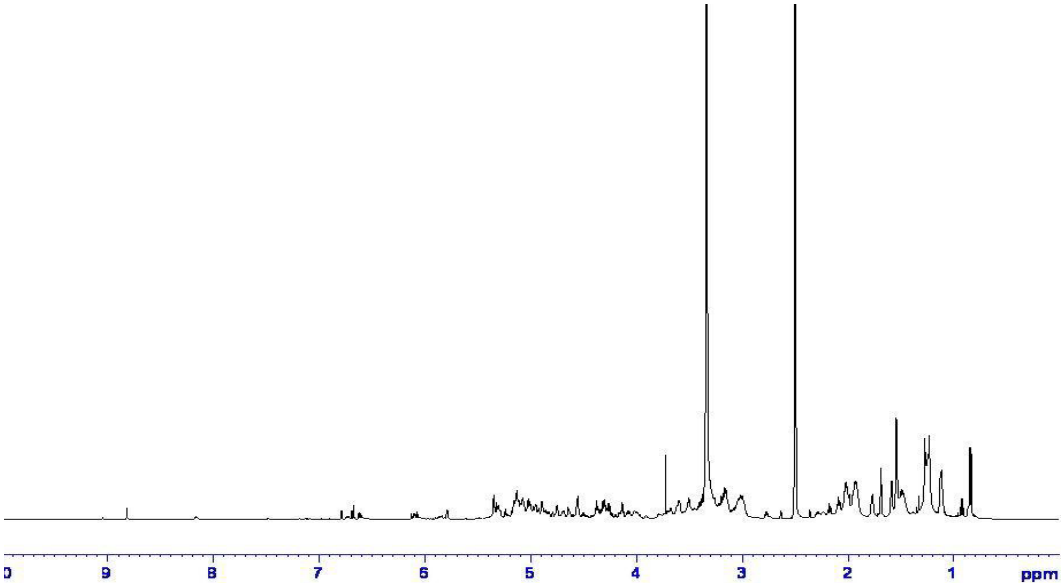
Appendix 8c: HMQC NMR of rutaecarpine at 500MHz in $CDCl_3$



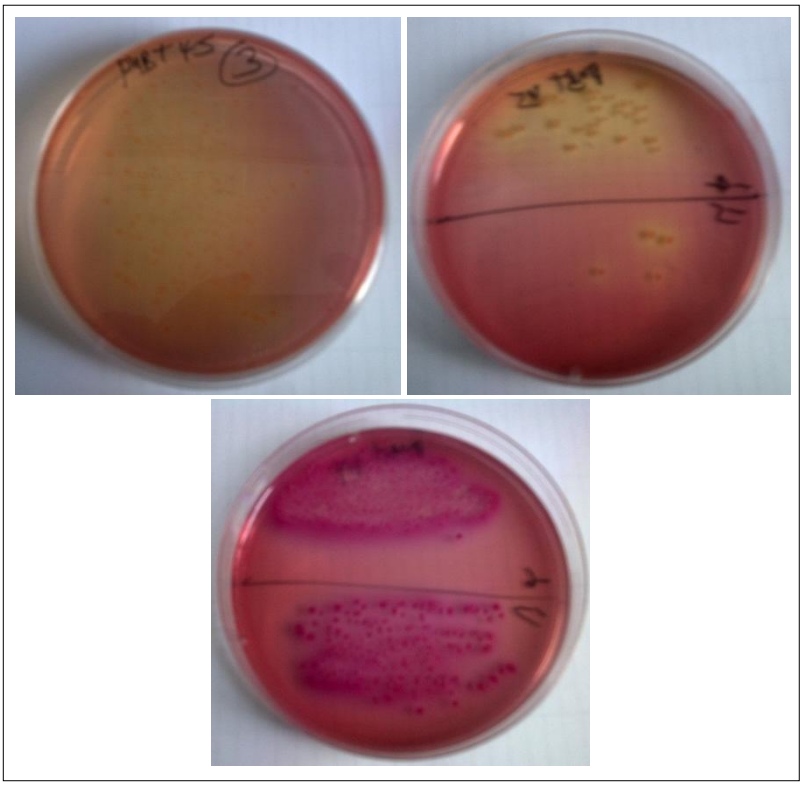
Appendix 8d: HMBC of rutaecarpine at 500MHZ in $CDCl_3$



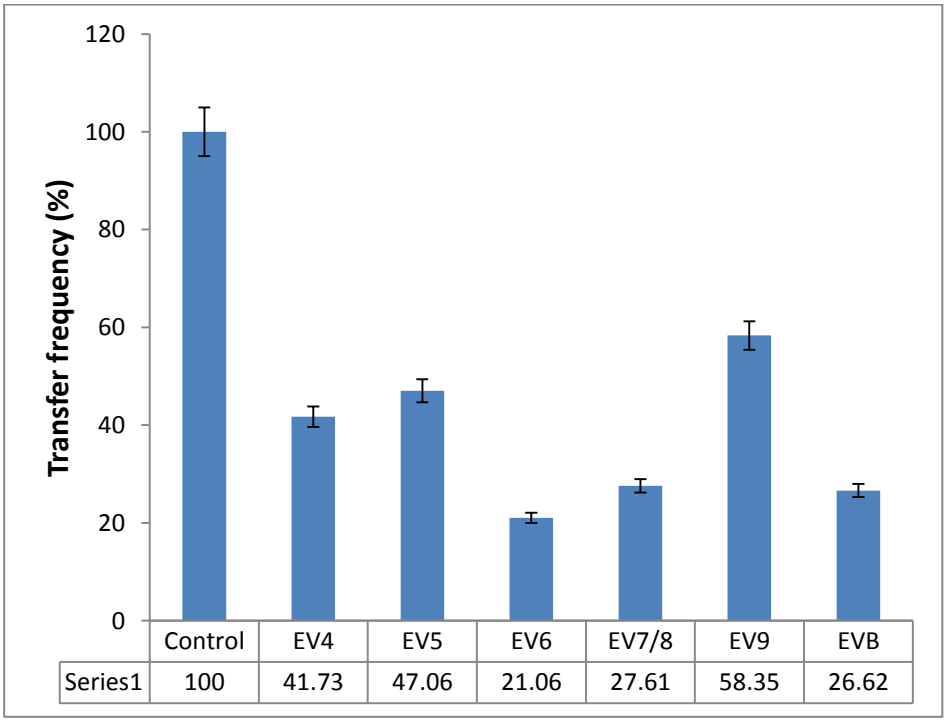
Appendix 9: ^{1}H NMR spectrum of impure fraction Ca-SPE 11 from *C. annuum* at 500MHz in D_20



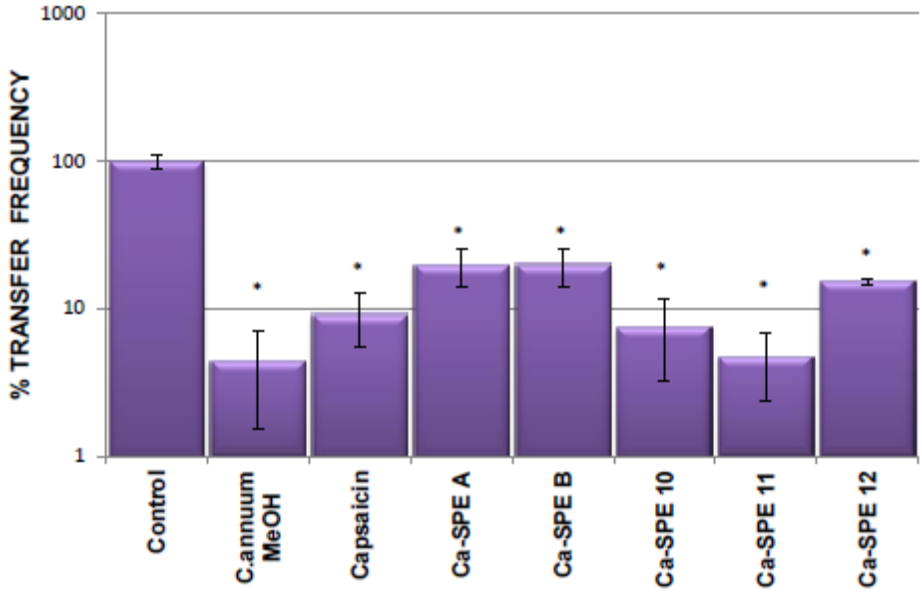
Appendix 10: Typical bacterial plasmid colony forming plates (A, B, C), showing the pattern of reduction of the transconjugants and total donor (cfu/mL) in a dilution dependant manner. Example seen is the effect of rutaecarpine on PUB307.



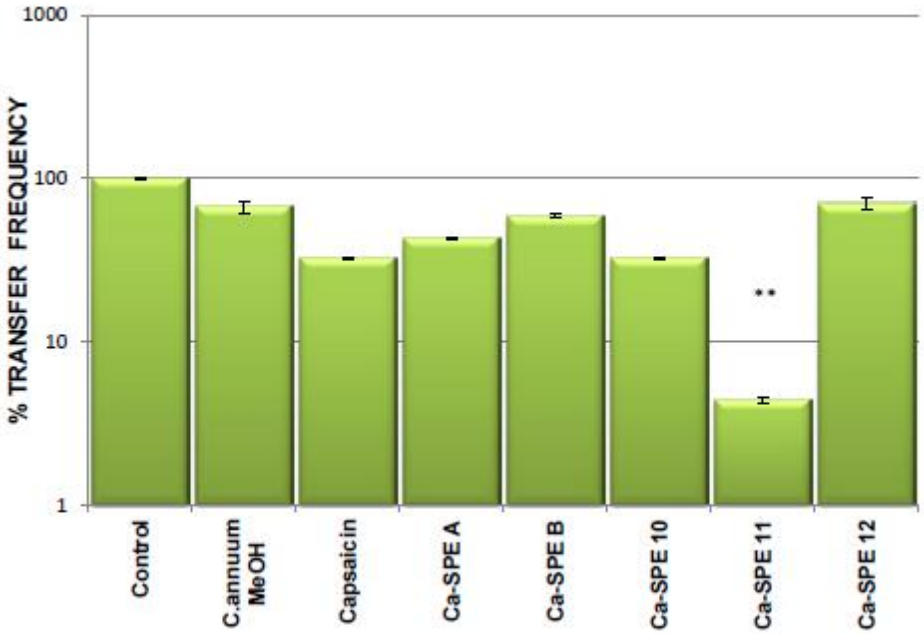
Appendix 11: Anti-plasmid activities of EVB-VLC sub fractions 4-9 against TP114



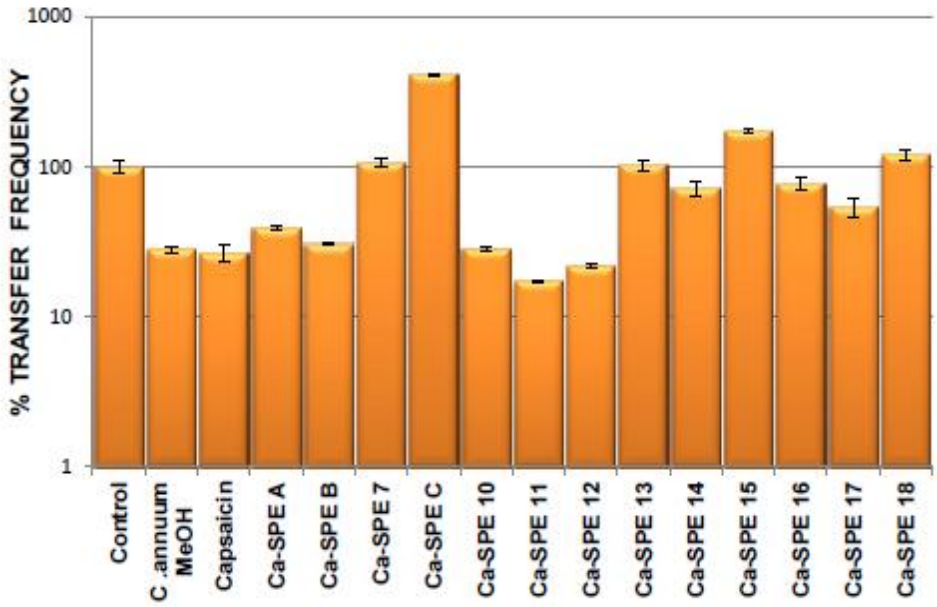
Appendix 12a: Anti-plasmid activity of SPE fractions of *Capsicum annum*.



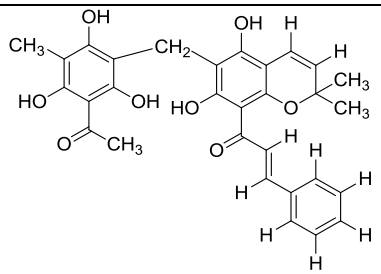
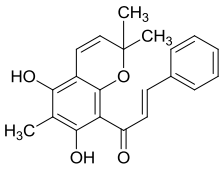
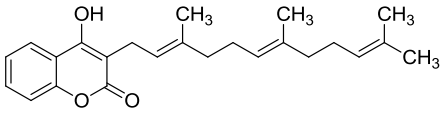
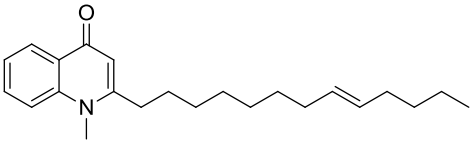
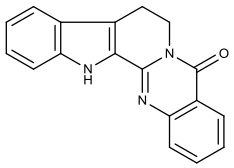
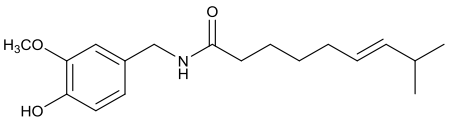
Appendix 12b: Anti-plasmid activity of SPE fractions of *Capsicum annum*

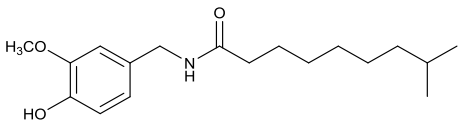
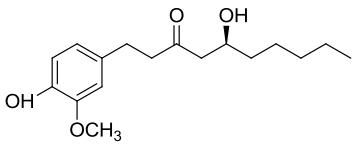
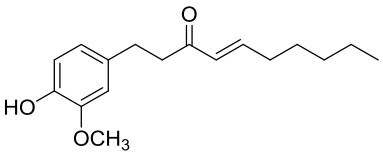
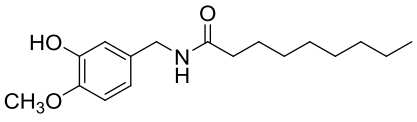


Appendix 12c: Anti-plasmid activity of SPE fractions of *Capsicum annum*



Appendix 13: Compounds assayed for DNA binding and their MIC and SIC values

	MIC (mg/L)	SIC (mg/L)	
Rottlerin	>512	100	
Red compound	>512	100	
Ferulenol	>512	100	
Evocarpine	>512	100	
Rutaecarpine	>512	100	
Capsaicin	>512	100	

Dihydrocapsaicin	>512	100	
6-gingerol	>512	100	
6-shogaol	>512	100	
Nonivamide	>512	100	

Appendix 14: Weight (g) and yield (%) of solvent extracts of the plants

Plants	**Weight after extraction/yield (%)**		
	Hexane Extract (g)	**Chloroform extract (g)**	**Methanol extract (g)**
M. philippinensis (680g)	14.1g (2.07%)	62.3 (9.62%)	165.0 (24%)
C. sativa (500g)	Hexane not used	98.1 (19.62%)	101.0 (20.20%)
E. rutaecarpa (500g)	Hexane not used	126.2 (25.24%)	96.3 (19.26%)
C. annuum (500g)	10.8 (2.16%)	71.1 (14.22%)	201.4 (40.28%)