# ANTIMICROBIAL ACTIVITY AND INTERACTION OF *TODDALIA ASIATICA* COUMARINS WITH TWO KNOWN DRUGS

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## DECLARATION

This thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for degree or any other award.

**Sign**……. **Date**……

**Name**: Onjero Isaiah **Reg. no**. SCH/G/01/10

## CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by Masinde Muliro University of Science and Technology a thesis entitled **"Antimicrobial activity and interaction of *Toddalia asiatica* Coumarins with two known drugs."**

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# DEDICATION

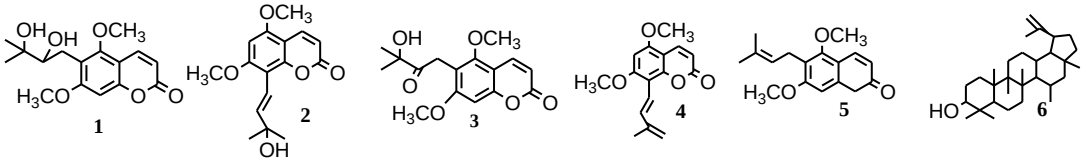
This thesis is dedicated to my wife Cecilia, parents Elisha and Deborah, children Bright, Immaculate and Adrian and all who patiently waited for me to log off.

# ACKNOWLEDGEMENT

All thanks to the Almighty God who is the source of my strength and the reason for my living. I am greatly indebted to my supervisors, Dr. Bernard Juma and Dr. Jackson Cheruiyot for the supervision and guidance during the entire time of my research. Special thanks to Dr. Baraza who contributed in running NMR and MS. Thanks to Kakamega forest staff and Mr. Osogo for their great assistance in plant identification/collection and in carrying out the bioassays, respectively. Sincere thanks to; Opanga, Rahab, Wekesa, Ondeko, Sande and the entire Chemistry department, for their guidance and support in ensuring that this work became a success. Finally, my most profound appreciation goes to my loving family; Elisha (Dad), Debora (Mum), David (Uncle), Cecilia (Wife), Bright and Adrian (sons), Immaculate (daughter), Elphas (Father), Immaculate (Mother), Ann, Jael, Hezron, Zadock, Floice, Roselida, James, Joseph, Dickson, Tonny and all of you who have in one way or the other acted as a spring board to my academics.

## ABSTRACT

Plant-derived compounds could exhibit a direct and/or an indirect antimicrobial activity as antimicrobial resistance modifying compounds. Their effectiveness could further be increased when combined with other bioactive compounds or antibiotics (synergy). A systematic search for plant-derived bioactive compounds, including those which can synergistically act with other bioactive compounds or antibiotics, as resistance modifying agents represents a potential approach to overcoming microbial resistance. Synergistic interaction of isolated coumarins from *T. asiatica* had not been reported prior to the current study, thus the basis for this work. It was also of interest to investigate possible synergistic effects of the isolated coumarins with known antimicrobials. This study evaluated the antimicrobial activity of the crude, isolated and combined coumarins from *Toddalia asiatica.* Further to this, combination of the pure compounds and known antimicrobials were investigated for possible synergy. The stem and root bark from the plant were collected from Kakamega forest in Western Kenya and after drying separately, subjected to sequential solvent extraction with organic solvents (n-Hexane, dichloromethane and methanol). Chromatographic separation of the crude extracts followed by characterization using spectroscopic techniques lead to the identification of six compounds namely: toddalolactone (**1**), coumurrenol (**2**), 5, 7-dimethoxy- 6 - (3'-methyl-,3'-hydroxyl–but–2-none) coumarine (**3**), toddaculin (**5**), and lupeol (**6**), [from the stem bark] and 5, 7- dimethoxy – 8 - (3'-methyl-but-1',3'-diene) coumarine (**4**) [from the root bark]. This is the first time compounds **3** and **6** are being reported from *T. asiatica* stem bark. Compound **3** (coumarin) had never been reported prior to this work. The crudes and isolated pure compounds **1, 2, 3** and **4** were evaluated for their antimicrobial activity against *Staphylococcus aureus, Escherichia coli*, *Penicillium digitatum* and *Rhizopus stolonifer* using both dilution and disc diffusion susceptibility methods. Antimicrobial activity of combined compounds **2** and **3** was also investigated together with their combination with standard drugs gentamycin and fluconazole against the same microbes. Analysis of variance (ANOVA) was used to analyze the disc diffusion results. The individual isolated compounds and all the crude extracts (apart from that of stem bark hexane) showed considerable activity against all the organisms tested. Combinations of compound **3** and fluconazole displayed additive effect in inhibiting the growth of *Penicillium digitatum* with lower MIC of 125$\mu$g/mL compared to that of fluconazole alone at 250 $\mu$g /mL. Combination of compounds **2** and **3** also showed additive effect in inhibiting *Rhizopus stolonifer* lowering the MIC from 500$\mu$g/mL (for both molecules) to 250 µg /mL. Interaction in antibacterial activity between compounds **2** and **3** lowered the MIC in action against *Staphylococcus aureus* to 250$\mu$g/mL compared to individual compounds with MIC of 500$\mu$g/mL while showing additive effect. Statistical data analysis of the bioassays using the Statistics Package for Social Scientists (SPSS) illustrated significant variation of bioactivity between pure and combined compounds **2** and **3** ($p<0.05$)**.** The Spectrum of activity observed in the present study is indicative that drug combination could be a possible method to obtain effective medicines against microbial resistance.

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# ACRONYMS AND ABBREVIATIONS

| | | |
|---|---|---|
| ANOVA | - | Analysis of Variances |
| CC | - | Column Chromatography |
| CFU | - | Colony forming unit |
| COSY | - | Correlation spectroscopy |
| CQ | - | Choloroquine |
| DEPT | - | Distortionless Enhancement by Polarisation Transfer |
| DHA | - | Dihyroatemisinin |
| DOMC | - | Division of Malaria Control |
| EtOAc | - | Ethyl acetate |
| H.I.V | - | Human Immune Virus |
| HMBC | – | Heteronuclear multiple bond correlation spectroscopy |
| HMQC | - | Heteronuclear multiple Quantum correlation spectroscopy |
| IR | - | Infra red |
| KEMRI | - | Kenya Medical Research Institution |
| MeOH | - | Methanol |
| M.I.C | - | Minimum Inhibitory Concentration |
| MMUST | - | Masinde Muliro University of Science and Technology |
| Rf | - | Retention factor |
| TLC | - | Thin Layer Chromatography |
| UV | - | Ultra Violet spectroscopy |
| VIS | - | Visible |
| W.H.O | - | World Health Organization |

# CHAPTER ONE

# INTRODUCTION

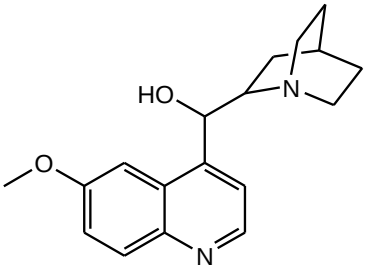
## 1.0 BACKGROUND INFORMATION

### 1.1 Plant sources as antimicrobials

Since the beginning of civilization, traditional medicine has been an important part of the human society (You *et al*. 2014). The medicine has been used globally as treatments of various diseases, including allergies, gastrointestinal symptoms, skin conditions, respiratory and urinary problems (Lin *et al*, 2014). Plant biodiversity together with the ability of medicinal plants to display a wide range of biological activities has enabled them to act as a source of new drug candidates for most diseases. Through bioactivity-guided fractionation of plant extracts scientists have discovered some bioactive compounds from plants, majority of which have suffered resistance (Abreu *et al*, 2017).

The use of herbal medicines has grown globally, due to the fact that the side effects and prices of these medicinal herbs are often less than those of synthetic drugs and many ordinary medicines (Lin *et al*, 2014). The discovery of quinine (**7)** isolated in 1820 from the bark of *Cinchona* species (Rubiaceae) and artemisinin **(8**) isolated from the herb plant *Artemisia annua* (Kachur *et al* 2010), made a lot of interest to shift to plants as sources of medicine. Some of the clinical agents that have been extensively used to combat microbial diseases include the penicillin (**9**), glycopeptide antibiotics for example chloroamphenic (**10**), fluconazole (**11**) and nystatin (**12**) mainly from plants.

Nevertheless the downside of their usage is the existence of resistant strains and the high cost of these medicines (Pang *et al*, 2016).

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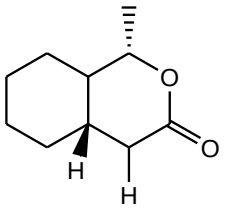


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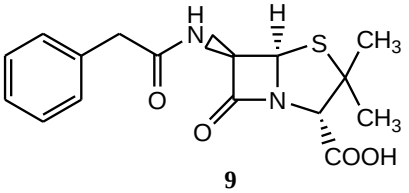


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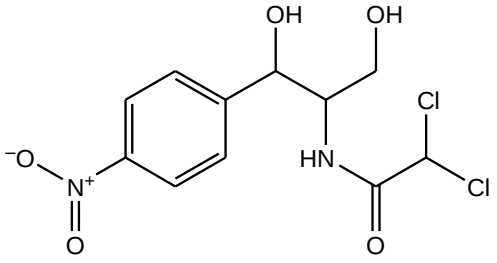


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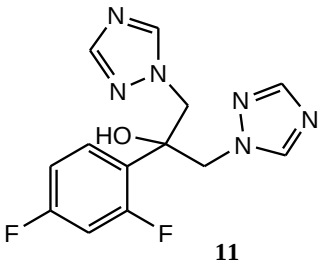


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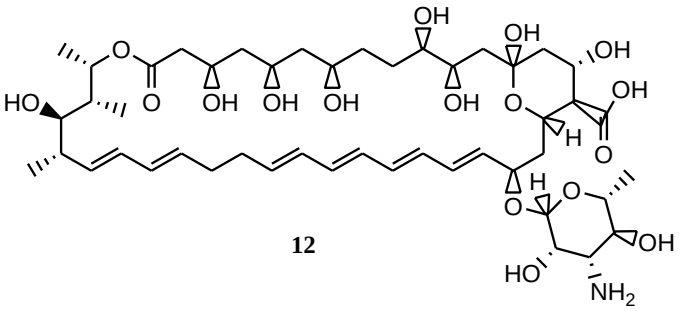


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This study focuses on isolating antimicrobial agents from *Toddalia asiatica*, a plant species from the Rutaceae family. Then based on the bioassay guided fractionation investigate the synergistic effect of the isolated coumarins and that of isolated coumarins combined with known drugs so as to find a way of improving the activity. This plant was selected based on its ethno botanical information. It has been widely discussed as one of the medicinal herbs traditionally used by different ethnicities in Eastern Africa for medication of myriad diseases, including cough, bronchitis, cold, fever, influenza, malaria, bacterial infections, stomach ache and

snake bite (Orwa *et al*. 2015).

Previous phytochemical studies on the plant have led to the isolation and identification of coumarins, alkaloids and terpenoids (Shi *et al*. 2012). Although some scientific information about its antibacterial activity is available, there are no reports about the investigation of the synergistic interactions of combined coumarins from this plant with the aim of improving its bioactivity and fighting drug resistance.

## 1.2. Microbial opportunistic infections

Most microbial infections are either bacterial or fungal (Ahmed *et al*., 2014). In humans these infections can range from superficial to deeply invasive or disseminated, and have increased dramatically in recent years making them the leading causes of mortality and morbidity (Orwa *et al*, 2015). Opportunistic infections are those caused by bacterial, viral, fungal or protozoan pathogens that take advantage of a host with weakened immune system. People living with diseases such as HIV/AIDS can face serious health threats from these opportunistic infections (Kamatenesi *et al*, 2014).

Patients subjected to high level immunosuppression are greatly affected by almost any type of bacterial or fungal organism making the host to suffer devastating consequences. The opportunistic infections such as skin infection that is characterized by pus-filled abscesses or respiratory infections such as sinusitis are caused by *Staphylococcus aureus* while gallbladder and biliary duct infections are caused by *E. coli*. If these infections are not identified and treated early enough they can lead to death (Pang *et al*, 2016).

Most pathogens such as *Staphylococcus* species, *Penicillium digitatum* and *Rhizopus stolonifer* that were traditionally regarded as low grade pathogens are increasingly important causes for most infections (Aliero and Ibrahim, 2012). There is need for much emphasis to be placed on measures such as drug combinations to help fight these infections.

## 1.3 Antimicrobial resistance

The number of antimicrobial resistant pathogens has increased worldwide (Smith 2011), making antimicrobial resistance a global public health threat (WHO, 2016). The propensity of microorganisms to acclimate to adverse climatic conditions enables them to survive at clinically relevant concentrations of current antimicrobials, resulting in the growth of resistant strains. The adverse conditions are mainly caused by the misuse and overuse of antimicrobials (Pang *et al*, 2016).

Microbes develop immunity through rapid mutation and lateral transfer of resistance genes. Resistance genes (via plasmids, transposons) may be transmitted between people of the same or linked bacterial species, among members of the commensal or pathogenic micro biota and between various environmental habitats, thereby spreading resistance (Amuka *et al*, 2014). Some of the drug resistant microbes include: Gram negative *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus* species (VRE) (Pang *et al*, 2016).

Plants are useful sources of new and biologically active molecules with

antimicrobial properties (Pang *et al*, 2016). These antimicrobial activity can be attributed either to direct action against microbes or to synergistic activity with antimicrobials. Various academic studies have demonstrated the *in vitro* synergistic activity of plant active compounds against multidrug-resistant bacteria (Wagner and Ulrich-Merzenich, 2009).

This development in synergy research increases the possibility of developing new antibacterial agents of plant origin as therapy for treating infections. Synergistic interaction is the evaluation of interactions between multiple bioactive constituents. Due to increasing cases of drug resistance these interactions have gained popularity in many scientific disciplines (Hemaiswarya *et al*, 2008). The interactions between multiple agents can be classified as antagonistic, additive/non- interactive, or synergistic (Satish *et al*, 2005).

i. Additive/non-interactive combinations indicate that the combined effect of two substances is a pure summation effect
ii. Antagonistic interaction results in a less than additive effect.
iii. Synergy is the positive interactions, known as potentiation occurs when the combined effect of constituents is greater than the expected additive effect (Teethaisong *et al*. 2014).

Artemisinin-based combination therapies a good case study are now the front-line treatment recommendation by the World Health Organization (WHO 2016).

## 1.4 Statement of the problem

Resistance by microorganisms to a number of known drugs is alarming, making

it a high priority concern (WHO, 2016). Plants have been shown as an important source of active agents with a number of them acting as leads in development of useful drugs (Saleem *et al*. 2010). A study of the synergistic interactions of isolated active compounds from plants used as traditional medicine could lead to the discovery of compound combinations with improved activity and less prone to suffer resistance to microbes. Moreover these could help scientists develop drugs that will be affordable and more environmental friendly. (Kachur *et al*, 2010). Synergistic interaction of isolated coumarins from *T. asiatica* had not been reported prior to the current study, therefore formed the basis for this work. Further, it was of interest to investigate possible synergy of the isolated coumarins with known antimicrobials.

## 1.5 Objectives

### 1.5.1 General Objective

To study the antimicrobial activity of coumarins of *T. asiatica,* establish the interaction amongst themselves and with two known drugs

### 1.5.2 Specific Objectives

i. To isolate and characterize coumarins from *T. asiatica*.
ii. To test the antimicrobial activities of crude extracts and isolated pure compounds from *T. asiatica*.
iii. To investigate antimicrobial synergistic effect of coumarins isolated from *T. asiatica* and in combination with two known drugs.

## 1.6 Research Questions

i. Do the Crude extracts and isolated pure compounds from different parts of *T. asiatica* exhibit antimicrobial activities?

ii. Can some of the pure isolated bioactive compounds from *T. asiatica* display synergistic effects amongst themseleves?

iii. Can some of the pure compounds from *T. asiatica* display synergistic effects with known antimicrobials?

## 1.7 Significance of the study

Bacterial and fungal infections have become a concern for health professionals due to the emergence of resistant strains that have been reported for all known classes of antimicrobial drugs. Example of bacteria and fungi that have developed resistance to several antimicrobials include: *Staphylococcus aureus*, *Escherichia coli*, *Penicillium digitatum* and *Rhizopus stolonifer*. The problem of microbial resistance could be solved by establishing combinations of molecules that may work synergistically. It may not be easy for these kinds of combinations to suffer resistance because microbes don't respond to the multiple agents easily (Hemaiswarya *et al*, 2008). Combinations involving different isolated compounds, known drugs and extracts, besides reducing the effective dose of a drug, also potentially reduce side effects of medicines (Thakur *et al*., 2016). Studies have revealed *in vitro* synergistic effects between plant extracts and antibiotics with a significant reduction of minimum inhibitory concentration (MIC) in antibiotics (Stefanović and Comic 2012; Yang *et al*., 2005; Wagner and Ulrich-Merzenich, 2009). For example a fourfold reduction in the MIC of gentamicin when combined

with the phytochemicals, protocatechuic acid, quercetin, caffeic acid on one hand and by the same factor for sulfadiazine in combination with the same compounds on another, in their action against *Pseudomonas aeruginosa* have been revealed (Sakharkar, *et al*. 2009). Further studies on combinations of antibiotics and phytochemicals may provide new therapeutic options for antimicrobial infections. Therefore this study tried to evaluate interactive effects between the antimicrobials gentamicin and fluconazole in combination with the coumarins isolated from *T. asiatica* against the bacteria *Staphylococcus aureus* and *Escherichia coli* and the fungi *Penicillium digitatum* and *Rhizopus stolonifer*. This was the first study of interactive effect of the prenylated coumarins against the tested microbes.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.0 INTRODUCTION

## 2.1 Traditional medicine

Traditional plant use, is of tremendous importance in most rural African communities (Kamatenesi *et al*, 2014). According to World Health Organization (WHO) approximately 80% of the world's inhabitants use traditional medicine for their health care (WHO, 2016). In Kenya, most people especially in rural areas rely on traditional medicine to treat many diseases (Nyambati, 2013). The use of medicinal plants has proved to be a good alternative to modern drugs for the majority of populations particularly in developing countries that cannot afford to pay for conventional drugs. Plant derived drugs are more environmental friendly and have served as very important agents for fighting the resistant strains (Munyendo *et al*, 2011).

Studies have shown that more than 1200 plant species from 160 families have been used as traditional medicine in about 30 countries (Kamatenesi *et al*. 2014). In Kenya plants that have been used alone or in combination for treating various diseases include: *Mormodica friesiorum* (Cucurbitaceae), *Myrsine africana* (Myrsinaceae), *Azadirichta indica* (Meliaceae), *Warbugia ugandensis* (Canellaceae), *Ajuga remota* (Acanthaceae), *Toddalia asiatica* (Rutaceae), *Teclea nobilis* (Rutaceae), among others (Abreu *et al*, 2017).

Medicinal plants are rich in a wide variety of secondary metabolites whose main function is to primarily defend the plants against predators and pathogens and provide reproductive advantage (Imbenzi *et al*, 2014). Plant secondary metabolites include coumarins, tannins, terpenoids, alkaloids, quinoids, flavonoids and phenolic compounds which have been reported to have different biological activities (Aziz *et al*, 2010).

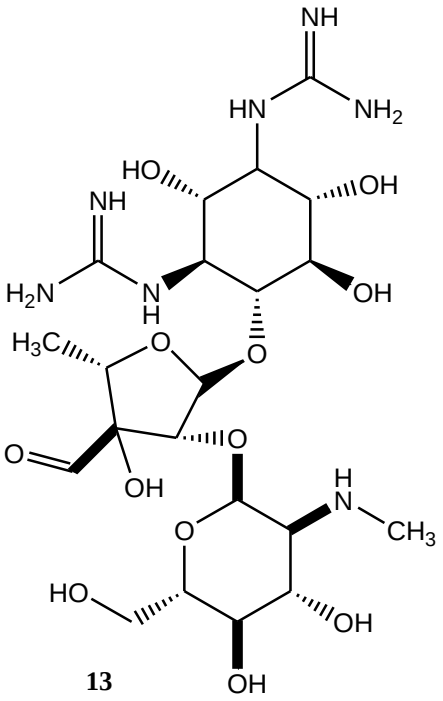
Numerous studies have been done to most plant species and the bioactivities reported (Abreu *et al*, 2017), nevertheless till today most of the plants have not been developed or commercialized as suitable drugs by the pharmaceutical industries. It is therefore probable that some of them may contain yet undiscovered powerful bioactive compounds (Orwa *et al*, 2015). This study centered on evaluating the antimicrobial activities of crude, isolated compounds, combined pure compounds and combined known drugs with compounds from *Toddalia asiatica.*

## 2.2 Phytochemicals currently used to fight bacterial and fungal infections.

An antimicrobial refers to a substance which inhibits the growth of micro-oganisms (Aliero and Ibrahim, 2012). It was not until 1940 that first best-known antimicrobial penicillin (**9**) was discovered from a blue green mould of the soil called *Penicillium notatum* (Kozlovskii *et al* 2013). This discovery marked the beginning of the development of antibacterial compounds produced by living organisms. Another antibiotic, streptomycin (**13**) was isolated in 1944 from a species of soil bacteria called *Streptomyces griseus*, particularly *Tubercle bacilli*, and has proved to be very valuable against tuberculosis (Kozlovskii *et al* 2013). Chloromycetin (**14**) was isolated from *S. venezuelae*. It has a powerful action on a

wide range of infectious bacteria both Gram positive and negative (Kamali and Amir, 2010). About 2,000 antibiotics have been characterized out of which approximately 50 are used therapeutically (Abreu *et al.* 2017).

Of the few antimicrobials being used, most of them suffer the challenge of resistance, toxicity and affordability (Ahmed *et al*, 2014). There is need to search for new antimicrobial agents to fight the development and spread of drug resistant pathogens.

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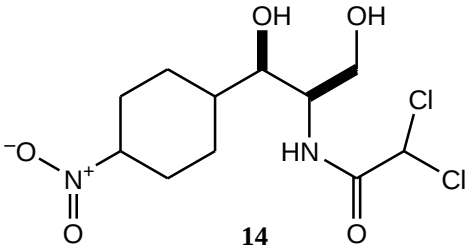


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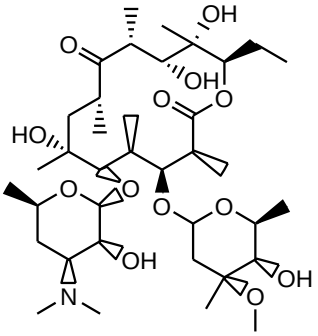
## 2.3 Antimicrobial Resistance

Antimicrobial resistance is the ability of a microbial organism to endure an antibiotic or an antifungal attack. It is a scenario where the antimicrobial drug kills the defenseless microbe, leaving behind or "selecting," those that can resist it. The rogue microbes then multiply, expanding their number to become the predominant microorganism (Aliero and Ibrahim 2012). The antimicrobial does not technically cause the resistance, but lets it happen by fostering an environment where an

existing variant can thrive (Kamali and Amir, 2010). Antimicrobial medications develop immunity when disease-causing microbes meddle with the mechanism of action of antimicrobials. For example, penicillin (**9**) kills bacteria by affixing to their cell walls, demolishing a key part of the wall, breaking down the wall, and drying the bacterium (Stefanovic' and Comic 2012). Resistant microbes, however, either modify their cell membranes so that penicillin cannot bind or generate enzymes that disintegrate the antibiotic (Kozlovskii *et al.* 2013).

Antibiotics such as erythromycin (**15**) are used to destroy ribosomes (cell structures that allow proteins to be produced). Resistant bacteria have slightly modified ribosomes that the drug cannot bind to (Stefanović and Comic, 2012). The ribosomal route is also away bacteria become antibiotic resistant to the likes of tetracycline (**16**), streptomycin (**13**) and gentamicin (**17**) (Pang *et al*. 2016).

Bacterial resistance may also happen through a process of microbial gender called transformation; one bacterium takes up DNA from some other bacterium. Penicillin resistant gonorrhea is the result of transformation. Most frightening, however, is the resistance that comes from a small DNA circle called a plasmid that can wander from one type of bacterium to a nother. A single plasmid then produces arrange of different resistances (Pang *et al,* 2016).



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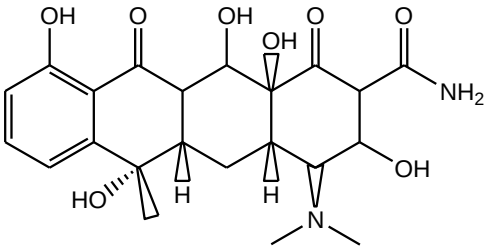

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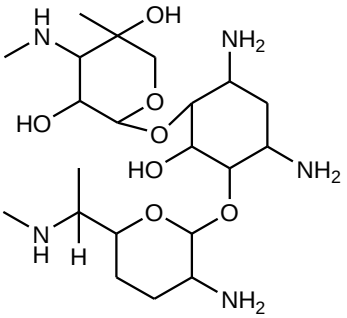

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Drug resistant diseases increase risk of mortality and are often linked with extended hospital stays and sometimes complications (Smith, 2011). Illogical use of antimicrobials, low quality generic drugs, and sub-optimal dose are the major causes of antimicrobial resistance (Pang *et al*, 2016).

### 2.3.1 Drug resistant pathogens

The main known resistant microbes are the Methicillin resistant *Staphylococcus aureus* (MRSA), Extended Spectrum beta-lactamase (ESBL), Multidrug resistant *A. baumannii* (MRAB) and Vancomycin resistant *Enterococci* (VRE) and these possess potential threats to human health (Ahmed, *et al*., 2014). Some microbes such as *Staphylococcus* species have emerged as an increasingly important cause of most infections (Aliero & Ibrahim, 2012).

The only compound that has been used effectively for some time against this *Staphylococcus* is an older antibiotic vancomycin. However, some clinical strains of *S. aureus* have developed resistance to it apart from the reported undesirable effects on humans (Ahmed *et al*., 2014). Therefore, this research attempted to find active compounds from *T. asiatica* that can act as leads to drug agents that may help fight opportunistic infections caused by *S. aureus*, *E. coli*, *P. digitatum* and *R. stolonifer*.

### 2.3.2 Fighting drug resistance

Many measures to combat drug resistance have been carried out for years (WHO, 2016). One of the strategies is typically aimed at reducing total opioid stress through more careful use of medications and improving the way medicines are used. This is done by enhancing scheduling, follow-up and clinical adherence (Rafiq *et al*, 2017). The second approach requires the use of medications and combinations of drugs that are potentially less likely to promote tolerance or have properties that do not allow the production and dissemination of resistant parasites (Teethaisong *et al*, 2014). Combination of antimicrobial drugs and structure modification of active compounds is a strategy that has received much attention recently. The aim of this intervention is to develop bioactive compounds that can withstand resistant pathogens (Pang *et al*, 2016). This research focused on the combination of pure isolated bioactive coumarins from *T. asiatica* with the view of determining their synergistic effect.

### 2.3.3 Synergistic interaction

The assessment of encounters between multiple bioactive constituents has grown in popularity in a number of scientific disciplines (Wagner and Ulrich-Merzenich, 2009).

However, it is widely accepted that associations between multiple agents can be categorised as antagonistic, additive/non-interactive, or synergistic interaction. Additive/non- interactive combinations suggest that the combined effect of two substances is a mere summation effect, while an antagonistic relationship results in less than an additive effect. Positive interactions, known as potentiation or synergy, happen when the cumulative effect of the components is greater than the anticipated additive effect (Thakur *et al*. 2016).

Studies have revealed *in vitro* synergistic effects between plant extracts and antibiotics with a significant reduction of minimum inhibitory concentration (MIC) in antibiotics (Stefanović and Comic 2012; Yang *et al*., 2005; Wagner and Ulrich-Merzenich, 2009). A fourfold reduction in the MIC of gentamicin when combined with the phytochemicals, protocatechuic acid, quercetin, caffeic acid on one hand and by the same factor for sulfadiazine in combination with the same compounds on another, in their action against *Pseudomonas aeruginosa* have been revealed (Sakharkar, *et al*. 2009).

A 34-fold reduction of MIC of *Klebsiella pneumoniae* resistant in combination tests involving ethanol extract of *Punica granatum* rind with ciprofloxacin was demonstrated by Rafiq and coworkers (2017). Allicin, a phytochemical present in garlic, has been shown to work synergistically in combination with *β*-lactam antibiotics against *Staphylococcus* spp. and *P. aeruginosa* (Cai *et al.*, 2007). Betoni *et al*. (2006) reported synergistic interactions between eight Brazilian herbal extracts with antibiotics against *S. aureus* isolated from human infections. Cooperation and/or antagonism inside botanical arrangements have been addressed and widely analysed in a

number of bioactivities (Martensoon *et al*. 2005) including; artemisinin-based combination therapy, which is now the World Health Organization first line treatment protocol for malaria (WHO, 2006). Artemisinin-based combination therapy has reduced malaria-associated morbidity and mortality globally. Some scientists also proposed that artemisinin serves to kill *Plasmodium falciparum* parasites by stimulating the *P. falciparum* food vacuole in trioxane-bridge in a heme- dependent manner.

This disturbance triggers the development of free radicals that inhibit heme detoxification, eventually creates more reactive oxygen species and killing the parasite (Bhattarai, *et al*, 2007). In addition to artemisinin, there are about 30 additional flavonoids and sesquiterpenes within *A. annua*, some of which have mild anti-plasmodial intervention (Barnes *et al*. 2005). Since botanical arrangements are multi-factorial instead of mono specific in nature, both *in vitro* and *in vivo* studies assessing the activity of *A. annua* extracts also found that the amount of artemisinin in the extracts does not fully explain the effectiveness of the extract's efficacy against *P. falciparum* (Bhattarai, *et al*, 2007).

Certain chemicals, including rosmarinic acid and arteannuin B, display distinct combination effects when measured against susceptible and resistant *P. falciparum* strains. Rosmarinic acid was synergistic against the resistant strain, but displayed an antagonistic behaviour in the tolerance strain. Likewise, arteannuin B had an additive/indifferent relationship in the chloroquine sensitive strain, but a synergistic association with the resistant strain, contributing to a three-fold increase in the production of artemisinin's agility (Bhattarai, *et al*, 2007). Because arteannuin B arbitrarily enhances the activity of artemisinin in the chloroquine-resistant strain, it is

likely to target the chloroquine resistance mechanism of the parasite, illustrating the promise of combination therapy not only to develop drug-resistant pathogens, but also to provide insight into the mechanisms by which parasites gain resistance as a whole. Cancer cells and pathogenic organisms may easily become immune to single-compound medicines, many cancers and resistant bacterial infections are now being managed with complicated drug combinations that affect multiple targets to combat resistance (Brooks & Brooks, 2014).

There are several forms of synergy that may include; pharmacodynamic synergy which comes from attacking multiple pathways, including proteins, substrates, metabolites, ion channels, ribosomes, and signal cascades (Brooks & Brooks, 2014). The second type happens when there are compounds that enhance the solubility of bioactive substances, both within and between species. This is an important type of coordination that is often overlooked. There are several examples of how mixture constituents improve the solubility of active constituents. Hypericin from Saint John's Wort (*Hypericum perforatum* L. (Hypericaceae), is poorly soluble in water. If hypericin is paired with *H. perforatum* mixture constituents such as procyanidin B2 and hyperocide, solubility and oral bioavailability of hypericin are improved to great extent. Researchers have examined the role of extremely abundant carbohydrates, amino acids, choline, and organic acids commonly found in bacterial, mammalian, and plant cells (Choi, *et al*, 2011). There is also a form of coordination which exists when inactive solution constituents are used to neutralize the unwanted side effects of a toxic and bioactive element. Such method of synergy is not intended to improve the potency of the active compound (s) *per se*, but rather to reduce the negative effects that the active agent triggers. In a given study, an extract of staghorn sumac (*Rhus hirta* (L.) Sudw

(Anacardiaceae) was combined with a 5-fluorouracil (5-FU) commonly used to treat breast and colon cancer. In conjunction with 5-FU, the *R. hirta* extract was found to shield normal cells from *in vitro* 5-FU toxicity (Brooks & Brooks, 2014). This chemo protective effect was partly attributed to the presence of antioxidants in the *R. hirta* extract, which reduced oxidative stress and cell damage caused by 5-FU treatment (Brooks & Brooks, 2014). This research thus explored the synergistic effect of two isolated, prenylated coumarins from *T. asiatica* stem bark methanol extract.

## 2.4. The Rutaceae Family

Rutaceae, commonly known as citrus or Rue, is asmall family of flowering medicinal plants placed in the order Sapindales (Stephen *et al*., 2012, Karunai *et al*, 2012). It is a large family consisting of 158 genera and 1,900 species that have diverse morphological features (Rajkumar *et al*., 2010). This family is largely known for its economic importance since many species are sources of foods, spices, essential oils, herbal medicines, horticultural items and pharmaceuticals (Cardoso-Lopes *et al*., 2010). The largest numbers are found in Africa and Australia often in semi-arid woodlands. In Kenya, there are seven genera of the Rutaceae family; *Clausena, Diphasiospsis, Fagaropsis, Teclea, Toddalia, Vepris* and *Zanthoxylum* (Balasubramaniam *et al*, 2011).

Most species in the Rutaceae family are shrubs and trees, a few are herbs which are sometimes armed with spines and prickles. A distinct characteristic of this family is the presence of glands containing aromatic oils on the stems, leaves, flowers and fruits generally. The leaves are opposite and compound, while flowers mainly divide into four or five parts. Plant species in the rutaceae family are traditionally

used for the treatment of rheumatic pain, arthritis, malaria and stomach ailments (Aziz *et al*, 2010).

### 2.4.1 The Genus *Toddalia*

*Toddalia* is a monotypic genus of family Rutaceae (Duraipandiyan and Ignacimuthu, 2009). It consists of flowering plants in the citrus family containing *Toddalia asiatica* as the only single species. This species is commonly known as the orange climber (Orwa *et al.,* 2015).

### 2.4.2 The species *Toddalia asiatica*

*T. asiatica* is a single species belonging to the Rutaceae family, Toddalioideae subfamily. This species is commonly known as the orange climber (Orwa *et al*., 2015). Other names used in Kenya include; Nyalwet-kwach (Luo), Katemwe (Tugen), Mkuro / Chikombe za Chui (Digo), Mdakakomba (Swahili), Mwikunya (Kikuyu) and Olebarmonyo (Maasai) (Stephen *et al*, 2012). The botanical synonyms of *Toddalia asiatica (L.) Lam.* includes *Toddalia ambigua Paullinia asiatica*, *Toddalia effuse* and *Toddalia aculeate* (Karunai *et al.,* 2012).

### 2.4.3 Botanical information

*T. asiatica* is native of tropical Asia from India and Sri-Lanka to Malaysia. It is also found in China and tropical Africa (Nyahanga, *et al*, 2013). *T. asiatica* (Figure **2.1**) is a liana with woody, corky, thorny stems (Figure **2.2**) that climb on trees, reaching up to 10 meters in length. It has a shiny green citrus-scented leaves, yellow-green flowers, and orange fruits about half a centimetre wide that taste like orange peel. The leaves (Figure **2.3)** are palmately compound, trifoliate with oblong acute

leaflets. Flowers are white or greenish yellow in terminal or auxiliary panicles, while the fruits are sub-globose, fleshy, orange coloured with small seeds covered by mucilage.

These seeds are dispersed by birds and monkeys that eat them (Praveena and Suriyavathana, 2013). *Toddalia asiatica* always occurs in forests near rivers or streams. It grows fairly well in clay soils in Maasai land. The species prefers forest edges at low and medium altitudes ascending to 1,700m (Nyahanga, *et al*, 2013).

**Figure 2.1** *Toddalia asiatica* plant

(Source: Photo by Isaiah Onjero May 2017)

**Figure 2.2** *T. asiatica* Stem

(Source: Photo by Isaiah Onjero May 2017)

**Figure 2.3** *T. asiatica* Leaves

(Source: Photo by Isaiah Onjero
May 2017)

### 2.4.4 Ethno botanical uses of *T. asiatica* species

All parts of the plant have been used as traditional medicine (Nyahanga, *et al*, 2013). The crop is well recognized for its antipyretic effects. It is used for sprains, intercostal neuralgia, cough, malaria, dysentery, gastralgia, poisonous snake bites and furuncle infections (Orwa *et al,* 2015). Clean root bark is used in the diagnosis of hill fever. Root is used in dental analgesic, odontalgia, vomiting,persistent fatigue, dyspepsia, colic, flatulence, bronchitis, diarrhoea, cuts, filthy ulcers, seizures, gonorrhoea, convalescence after febrile or debilitating

infections, irregular blood motions and arthritis. Root bark is bitter, astringent, acrid, digestive, carminative, constipating, diaphoretic, expectorant, antibacterial, vulgar, aromatic, tonic, stimulant, antiperiodic, antidiarrhoeal, antipyretic and diuretic. Fresh leaves are eaten raw for intestinal pain (Karunai *et al*, 2012).

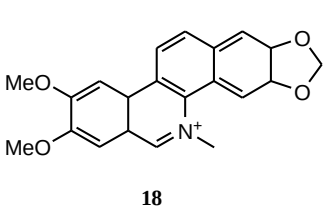
The leaves are burned and the ash is used as tooth powder and in decayed teeth. The flowers are valuable as agents used to relieve wasp-stings (Linn *et al*, 2014). The unripe fruit is treated with oil to make arthritis relaxing liniment. Fruits are used for vomiting or discomfort in lungs (Orwa *et al*, 2015). Roots and leaves are cooked and used orally or by inhalation for common cold and coughing. Such fruits are also used for culinary reasons in the form of pickles (RajKumar *et al*, 2010 and Balasubramaniam *et al*, 2011).

### 2.4.5 Phytochemistry of *Toddalia asiatica*

The genus is known for its extraordinarily array of secondary metabolites such as alkaloids, flavonoids, coumarins, limonoids, lignans and volatile oils (Shi *et al*, 2012). The metabolites possess a wide spectrum of biological activities with some of them having proven medically useful (Praveena and Suriyavathana, 2013). Such metabolites do not only support organisms, but also have tremendous biochemical functions in humans and animals. These include cancer prevention, antibacterial, antifungal, antioxidative, hormonal activity, enzyme activation and much more (Venkatesh *et al.,* 2011). The accompanying parts describe some of the biochemical properties (including the physiological and biological activity) of these phytochemicals.

### *2.4.5.1 Alkaloids*

Alkaloids are nitrogen compounds that are physiologically active (Ahmad *et al.*, 2006). A plant containing alkaloids almost never produces one type of alkaloid but rather a whole array of closely related components (Duraipandiyan and Ignacimuthu, 2009). Plant alkaloids have served as models for modern synthetic drugs (Shi *et al*, 2012). They are widely distributed in this genus except for acridones and furoquinolines alkaloids that have not been widely reported. The several bioactive alkaloids isolated from the genus have showed mild to high antimalarial activity (Rajkumar *et al*, 2010. Some of the alkaloids that have been reported from the genus *Toddalia* include; nitidine **(18),** aporphinoid alkaloid magnoflorine **(19)**, N- methyl-4-hydroxy-7-methoxy- 3- (2, 3- epoxy- 3- methylbutyl)- 1H -quinolin- 2- one (**20**), 3- (2, 3- dihydroxy- 3- methylbutyl)- 4, 7- dimethoxy- 1- methyl- 1H- quinolin- 2-one (**21**), toddaquinoline (**22**), flindersine (2, 6- dihydro- 2, 2- dimethyl- 5H- pyrano [3,2-c] quinoline-5-one-9cl) (**23**) skimmianine (**24**) and berberine (**25**) (Boyd M.R 1988, Duraipandiyan & Ignacimuthu 2009).

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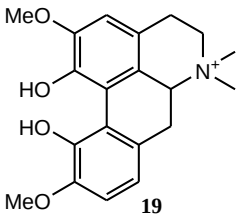


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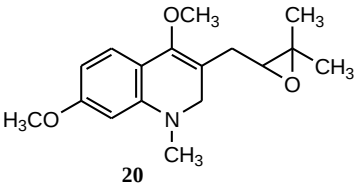


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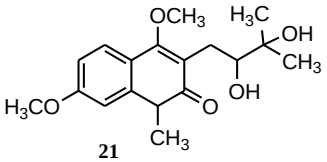


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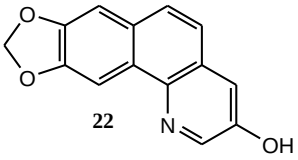


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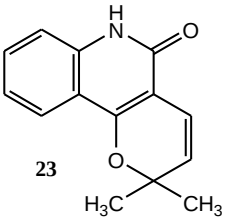


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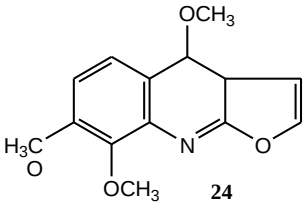


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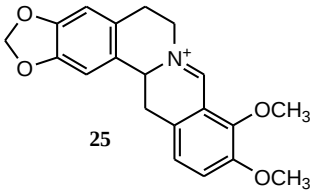


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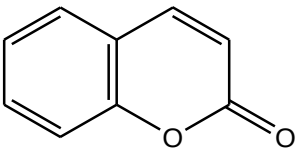
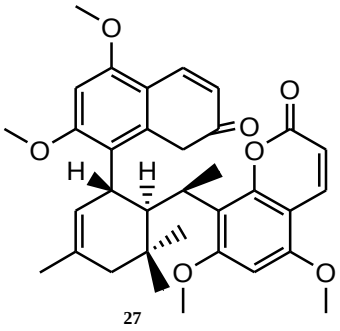
### 2.4.5.2 Coumarins

Coumarins **26** are widely distributed in several plants, including: *Carissa spinarum*, *Urtica dioica, Warburgia ugandensis*, *Senna didymobotrya*, *Physalis peruviana*, *Bidens Pilosa*, *Leonotis nepetifolia* and *Toddalia asiatica* (Phatchana & Yenjai, 2014). They are unsaturated aromatic lactones, which occur freely or combined with the sugar glucose (Coumarin glycoside). Coumarins are distributed in different parts of plants and biosynthetically originate from the Shikimic acid pathway Fig **2.4** (Linn *et al*, 2014).




**26**

Some of the coumarins from the genus *Toddalia* include: toddalosin (**27**), 5-methoxysuberenon (**28**), toddalenone (**29**) and 5, 7-dimethoxy-8-(3'-hydroxy-3'-methyl-1'-butene) (**30**) isolated from the roots. Other including norbraylin (**31**), 5, 7, 8- trimethoxycoumarin (**32**), bergapten (**33**) and luvangetin (**34**), from the stem have been reported (Munyendo *et al*, 2011).



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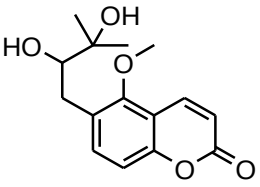

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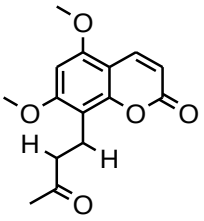

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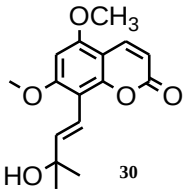

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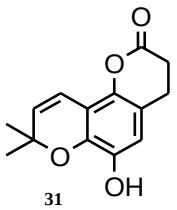

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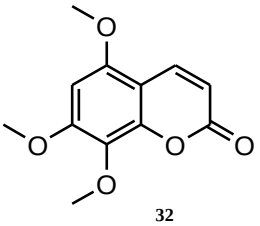

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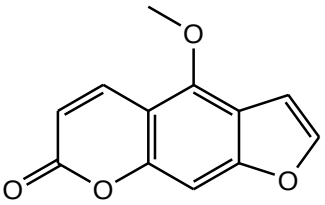

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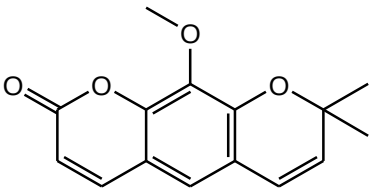

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#### 2.4.5.2.1. Biosynthesis of coumarins

Biosynthesis also referred to as anabolism is an enzyme catalyzed reaction in which substrates are transformed to complex compounds (macromolecules) in living organisms. The conversion involves various metabolic pathways some of which are located within a cell organelle while others involve enzymes located within various cell organelles. Examples of common biosynthetic pathways are the production of lipid membrane components.

This study focuses on coumarins. Coumarins (benzo-α-pyrones) are a group of secondary metabolites found mostly in higher plants such as Rutaceae, Moraceae, Apiaceae and Fabaceae plant families (Bourgaud *et al*. 2006). The compound umbelliferone (a 7-hydroxycoumarin) is a coumarin of higher plants that acts as the parent compound for a large number of natural products.

Umbelliferone can undergo subsequent biochemical modifications such as prenylation at C6 or C8 followed by the closure of a furan ring. These modifications generate prenylated and furanocoumarins, compounds involved in plant defense against pathogens and in plant environmental adaptation (Bourgaud *et al*. 2006). *Toddalia asiatica* a member of the Rutaceae, can synthesize various classes of coumarins, it is rich in prenylated coumarins such as Toddaculin (Vazquez *et al*. 2012).

Umbelliferone a phenylpropanoid can also be synthesized from L-phenylalanine produced via the shikimate pathway. The Phenylalanine is converted into cinnamic acid then hydroxylated by cinnamate-4-hydroxylase to produce 4-coumaric acid.

The 4-coumaric acid produced is then hydroxylated again by cinnamate or coumarate 2- hydroxylase to give 2, 4-dihydroxy-cinnamic acid (umbellic acid). Umbellic acid then undergoes a bond rotation of the unsaturated bond adjacent to the carboxylic acid group followed by an intramolecular attack from the hydroxyl group of C-2' to the carboxylic acid group thereby closing the ring. This forms the lactone umbelliferone (Bourgaud *et al*. 2006).

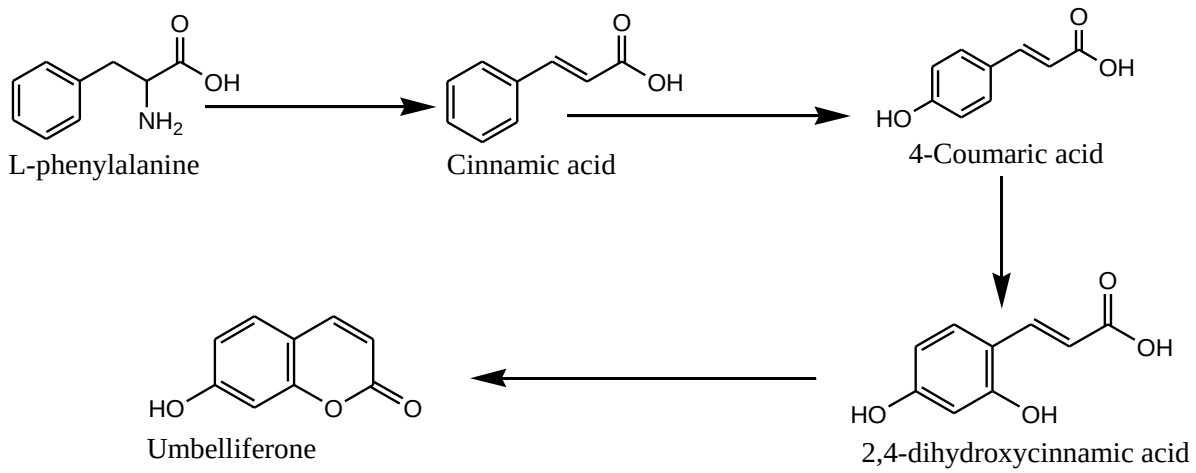
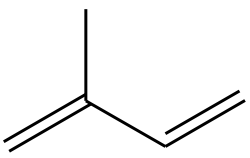


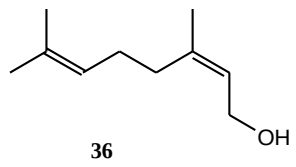

**Figure 2.4:** Biosythetic pathway for umbelliferone

**2.4.5.3 Terpenoids**

They are biosynthetically formed from isoprene units, which have a molecular formula of $C_5H_8$ (Ahmad *et al.,* 2006). The basic molecular formulae of terpenes, then, are multiples of it, $(C_5H_8)_n$, where n is the number of attached isoprene units



The isoprene units may either be linked together "head-to-tail" to create linear chains or grouped to form loops. Terpenes are classified as monoterpenes, sesquiterpenes, diterpenes, sesterpenes, triterpenes, tetraterpenes, and rubber, based on the overall number of carbon atoms or isoprene units in the molecule. Monoterpenes consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. They may be linear (acyclic) or may contain rings (Ahmad *et al.*, 2006). Different terpenoids have been isolated from *T. asiatica*, they include: include β-amyrin (**43**), F-pinene (**44**), C-pinene, p- cymene (**45**), d-limonene (**46**), linolyl acetate (**47**) (Rajkumar *et al*. 2008).

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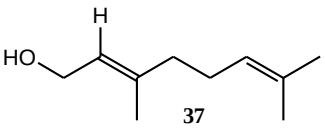


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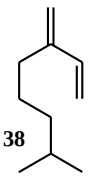

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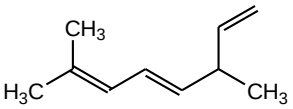


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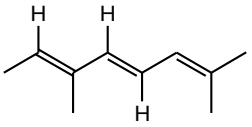


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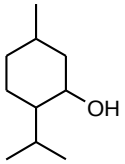


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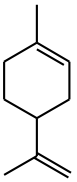

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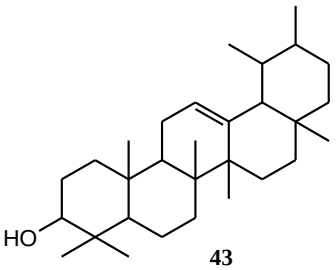


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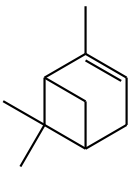

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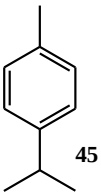

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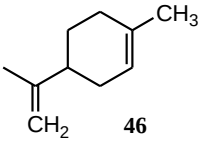


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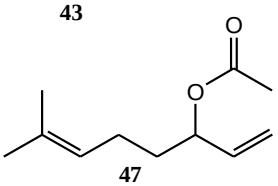
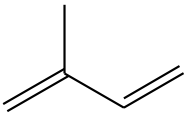



**47**



**35**

### 2.4.6 Antimicrobial activity of *Toddalia asiatica*

#### 2.4.6.1 Crude extracts

Munyendo *et al.,* (2011) reported the stem extract of *T. asiatica* to have the highest antibacterial activity, with the gram positive organisms being more susceptible than the gram negative. Essential oils from the fruit have showed significant antimicrobial effects against *Trichophyton* and *microsporum* species (Rajkumar *et al*. 2008). The leaves extracts of hexane, chloroform, ethyl acetate, methanol and water have been studied for their antimicrobial activity against selected bacteria and fungi using disc- diffusion method, where the ethyl acetate extract possessed the highest antimicrobial activity (Karunai *et al*. 2012). The leaf extracts have been found to be effective against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella Newport, Staphylococcus aureus, Aspergillus fumigatus, Aspergillus niger, Microspermum gypsum* and *Rhizophus stolonifer* up to 1:20 dilution (Orwa *et al*. 2015).

#### 2.4.6.2 Compounds from *Toddalia asiatica*

##### 2.4.6.2.1 Bioactivity of alkaloids

Nitidine (**8)** and magnoflorine **(19)** were tested for HIV-inhibitory properties where Nitidine inhibited the cytopathic effects of HIV infection effectively at a concentration range of 1-10 while magnoflorine revealed low anti-HIV activity at 25-50 pg/ml (Boyd M.R 1988)**.** N- methyl-4-hydroxy-7-methoxy-3-(2,3-epoxy-3-methylbutyl)-1H-quinolin-2-one (**20**) and 3-(2,3-dihydroxy-3-methylbutyl)-4,7-dimethoxy-1-methyl-1H-quinolin-2-one (**21**) were found to possess strong antibacterial activity against three bacteria viz., *L. lactis* (NCC946, *B. cereus*

(M.1.16) and *E. coli* (0157:H7) (Sharma *et al*.1982). Flindersine (**23**) an active compound isolated from the ethyl acetate extract of the plant has been reported to be active against: *Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis, Enterococcus faecalis, Trichophyton simii, Epidermophyton floccosum, Magnaporthe grisea* and *Candida albicans* (Duraipandiyan and Ignacimuthu, 2009).

The following MIC values of the compound Flindersine against bacteria were reported *Staphylococcus aureus* (62.5_g/ml), *Bacillus subtilis* (31.25_g/ml), *Pseudomonas aeruginosa* (250_g/ml), *Staphylococcus epidermidis* (62.5_g/ml), *Enterococcus faecalis* (31.25_g/ml), *Acinetobacter baumannii* (125_g/ml) and fungi *Trichophyton rubrum* 57 (62.5_g/ml), *Trichophyton mentagrophytes* (62.5_g/ml) and *Candida albicans* (250_g/ml) indicating that this is one of the most active compounds in the plant (Duraipandiyan and Ignacimuthu 2009).

**2.4.6.2.2 Bioactivity of coumarins**

Compound **30 (**5, 7-dimethoxy-8-(3'-hydroxy-3'- methyl-1'-butene) isolated from the roots of *T. asiatica* has been reported to have moderate activity against the chloroquine-sensitive K39 and chloroquine- resistant V1/S strains of *P. falciparum* strains with $IC_{50}$ values of 16.2mg/mL and 8.8mg/mL, respectively (Nyahanga *et al*, 2013). Despite the numerous reports on the bioactivity of compounds isolated from *T. asitica*. There is no report of the antimicrobial synergistic assay of coumarins isolated from the plant. It was therefore important to test the synergistic interactions of coumarins from isolated from the plant and those of known standards.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.0 Material, reagents and equipment

## 3.1 Reagents

Solvents used were hexane, dichloromethane, ethyl acetate and methanol and were purchased from Kobian Ltd, Nairobi, Kenya. The solvents were distilled on a rotary evaporator to remove impurities, before using them for extraction, fractionation and crystallization. The distilled solvents were stored in 2.5 litre clean glass bottles.

## 3.2 Chromatographic methods

Chromatographic techniques that were employed in the separation procedure included column chromatography (CC) on normal silica gel 60G (Merck, 70-230 mesh) and Sephadex LH-20. In order to monitor the separation of compounds, analytical thin layer chromatography (TLC) pre-coated plates were used (silica gel 60 F254 (Merck). To qualitatively determine presence or absence of compounds the TLC plates were exposed to iodine vapour or sprayed with vanillin.

## 3.3 Spectroscopic methods

Mass spectra were obtained on electron impact mass spectra (EI-MS) using a Finnigan GC-MS in Potsdam Germany. NMR spectra were obtained using Bruker Avance ($^{1}$H 600MHz, $^{13}$C NMR 150 MHz) in Potsdam Germany. Solvents used were deuterated $CDCl_3$, $CD_3OD$ and $(CD_3)_2CO$.

Chemical shifts were given in (ppm) values with trimethylsilane (TMS) used as the internal standard. Homonuclear Correlation Spectroscopy (COSY), Heteronuclear

Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectra were obtained using the standard Bruker software.

## 3.4 Plant Material Collection and identification

The root and stem barks of *T. asiatica* were collected from Kakamega forest. The plant was identified at the Department of Biological Sciences Herbarium, Masinde Muliro University. Herbarium species was deposited in the University herbarium for reference (voucher specimen no. MMUST/TA/003/2018)

## 3.5 Extraction and Isolation of Compounds

The plant materials were dried separately under shade, then ground to fine powder ready for extraction. The powdered plant materials (1kg) were sequentially extracted with solvents of increasing polarity starting with hexane, dichloromethane and methanol for 24 hours after which the extract was filtered and concentrated using a rotary evaporator at $45^{o}$C and low pressure. The filtered extracts were combined to form one extract for each of the solvents. For every plant extract, TLC analysis was done using various solvent systems.

The developed plates were sprayed with vanillin reagent or exposed to iodine vapour. The samples were stored in refrigerator for use when required. The crude extracts were assayed for antibacterial and antifungal activity. The organic extracts were then subjected to column chromatography packed with silica gel. Further fractionation of the fractions using CC, re-crystallization and cleaning led to isolation of pure compounds.

### 3.5.1 *T. asiatica* stem hexane extract

34g of the crude hexane extract was subjected to fractionation by column

chromatography gradient elution starting with hexane then followed by a mixture of EtOAc/Hex. This afforded 7 combined fractions (1-7). Fraction 2 (eluted with 10% EtOAc/Hex) was subjected to further fractional crystallisation to obtain compound **5** (0.26g) as cream crystals. Similarly Fractions 7 (eluted with 15% EtOAc/Hex) was subjected to further fractional crystallisation to give compound **6** (0.06g) as white crystals.

### 3.5.2 *T. asiatica* stem dichloro-methane extract

26g of the crude dichloromethane extract was subjected to fractionation by column chromatography gradient elution with n-hexane containing increasing amounts of EtOAc. This afforded 8 combined fractions (1-8). Fraction 7 (eluted with 75% EtOAc/Hex) was subjected to fractional crystallization to obtain compound **1** (0.22g) as colorless crystals.

### 3.5.3 *T. asiatica* stem methanol extract

26g of the crude methanol extract was subjected to fractionation by column chromatography gradient elution starting with hexane then followed by a mixture of EtOAc/MeOH. This afforded 7 combined fractions (1-7). Fractions 5 (eluted with 10 % EtOAc/MeOH) was subjected to further fractional crystallization to obtain compound **2** (0.8g) as colourless crystals. Fractions 6 (eluted with 10 % EtoAc/ MeOH) was also subjected to further fractional crystallization give compound **3** (0.3g) as grey crystals.

### 3.5.4 *T. asiatica* root hexane extract

24g of the crude hexane extract was subjected to fractionation by column chromatography Gradient elution with n-hexane containing increasing amounts of

EtOAc. This afforded 6 combined fractions (1-6). Fractions 3 (eluted with 20 % EtOAc/Hex) was subjected to further fractional crystallization to obtain compound **4** (0.6g) as colorless crystals.

## 3.6 Physical and Spectroscopic data for the isolated compounds

The physical and spectroscopic data for the isolated compounds included; colour, melting point, $^{1}$H-NMR, $^{13}$C NMR and MS spectral data, respectively.

### 3.6.1 Toddalolactone (1)

White crystals, m.p 139-140 $^{0}$C, MS; *m/z*: 309 $[M+ H]^{+}$, 250, 220, 219, 207, 161, 95, 77, 59, 43, $^{1}$H-NMR (600 MHz, $CDCl_3$): δ 6.24 (1H, d, J=9.6Hz, H-3),8.04 (1H, dd, J=9.6,9.6Hz, H-4), 6.77 (1H, s, H-8), 2.86 (2H, J=1.2, H-1'), 3.66 (1H, m, H-2'), 1.26 (3H, s, H-4$^{'}$), 1.27 (3H, s, H-5'), 3.99 ($OCH_3$-5), 3.89 ($OCH_3$-7); $^{13}$C NMR (150 MHz, $CDCl_3$): δ 163.8 (C-5), 163.4 (C-2), 157.8 (C-7), 156.2 (C-8a),141.2 (C-4) 120.3, (C-6), 112.6 (C-3), 108.4 (C-4a), 96.3 (C-8), 78.3 (C-2$^{'}$), 74.0 (C-3$^{'}$), 63.8 ($OCH_3$-5), 56.7 ($OCH_3$-7), 27.1 (C-1$^{'}$), 25.5 (C-4$^{'}$), 25.5 (C-5$^{'}$).

### 3.6.2 Coumurrenol (2)

$^{1}$H-NMR (600 MHz, $CDCl_3$): δ 6.15 (1H, d, J=9.6Hz, H-3), 8.10 (1H, d, J=10.2Hz, H-4), 6.58 (1H, s, H-6), 6.83 (2H, s, H-1'), 1.39 (3H, s, H-4'), 1.39 (3H, s, H-5'), 3.98 (3H, s, $OCH_3$-5), 3.99 (3H, s, $OCH_3$-7); $^{13}$C NMR (150 MHz, $CDCl_3$): δ163.4 (C-5), 163.1 (C-2), 157.4 (C-7), 154.5 (C-8a), 142.9 (C-1$^{'}$), 140.9 (C-4), 115.3(C-2'), 107.6 (C-8), 104.8 (C-4a), 92.3 (C-6), 72.1 (C-3'), 56.8 ($OCH_3$-5), 56.8 ($OCH_3$-7), 30.1 (C-4'), 30.1 (C-5').

### 3.6.3. 5, 7-dimethoxy-6-(3'- hydroxy - 3'- methyl-2'-oxo-butyl) coumarine (3)

$^{1}$H-NMR (600 MHz, $CDCl_3$): δ 6.21 (1H, d, J=9.6Hz, H-3), 7.85 (1H, d, J=10.2, H-4), 6.66 (1H, s, H-8), 3.92 (2H, s, H-1'), 1.50 (3H, s, H-4'), 1.50 (3H, s, H-5'), 3.76 (3H, s, $OCH_3$-5), 3.81 (3H, s, $OCH_3$-7); $^{13}$C NMR (150 MHz, $CDCl_3$): δ161.4 (C-2), 112.9 (C-3), 138.8 (C-4), 107.4 (C-4a), 156.0 (C-5), 126.0 (C-6), 160.9 (C-7),95.7 (C-8), 114.2 (C-8a), 31.6 (C-1'), 212.3 (C-2'), 77.0 (C-3'), 27.0 (C-4'),27.0 (C-5'), 63.7 (OCH3-5), 56.4 ($OCH_3$-7).

### 3.6.4 Gleinadiene (4)

M.p 120-121 $^{0}$C, $^{1}$H-NMR (600 MHz, $CDCl_3$): δ 6.20 (1H, d, J=9.6Hz, H-3), 7.91 (1H, d, J=9.6Hz, H-4), 6.32 (1H, s, H-6), 7.27 (1H, d, H-1'), 6.67 (1H, d, H-2'), 5.11 (2H, d, H-4'), 2.02 (3H, s, H-5'), 3.93 (3H, s, OCH3-5), 3.79 (3H, s, $OCH_3$-7); $^{13}$C NMR (150 MHz, $CDCl_3$): δ 161.2 (C-2), 110.9 (C-3), 138.7 (C-4), 107.2 (C-4a), 153.5 (C-5), 90.3 (C-6), 161.1 (C-7), 103.8 (C-8), 155.6 (C-8a), 135.7 (C-1'),117.1 (C-2'), 143.3 (C-3'), 117.0 (C-4'), 18.3 (C-5'), 56.0 ($OCH_3$-5), 55.9 ($OCH_3$-7).

### 3.6.5 Toddaculin (5)

White crystals, m.p 94-96 $^{0}$C, MS; *m/z*: 275.4 $[M+ H]^{+}$, 297.1, 571.4, 573.6, 845.4, $^{1}$H-NMR (600 MHz, $CDCl_3$): δ 6.22 (d, J=9.6, H-3), 7.86 (d, J=9.6, H-4), 6.62 (s, H- 8), 3.36 (d, J=6.9, H-1'), 5.16 (t, H-2'), 1.78 (s, H-4'), 1.68 (s, H-5'), 3.88 (s, $OCH_3$- 5), 3.87 (s, $OCH_3$-7); $^{13}$CNMR (150 MHz, $CDCl_3$): δ 161.3 (C-2), 161.7 (C-7), 155.2 (C-5), 154.7 (C-8a), 139.0 (C-4), 132.1 (C-31), 122.2 (C-21), 120.3 (C-6), 122.2 (C- 21), 112.3 (C-3), 107.1 (C-4a), 95.4 (C-8), 63.1 ($OCH_3$-7), 56.1 ($OCH_3$-5), 25.7 (C- 4'), 17.8 (C-5').

**3.6.6 Lupeol (6)**

White crystals, M.p 215-216 $^{0}$C, MS; *m/z*: 425.6 [M+], 112.8,143.2, 157.3, 177.0, 241.2, 255.3, 269.3, 281.2, 1H-NMR (600 MHz, $CDCl_3$): δ 1.68 (s, H-1), 1.50 (H-2), 3.21 (1H, m, H-3), 0.69 (1H, s, H-5), 1.39 (H-6), 1.39 (H-7), 1.27 (H-9), 1.26 (H-11), 1.43 (H-12), 1.62 (H-13), 1.68 (H-15), 1.52 (H-16), 1.35 (H- 18), 2.39 (1H, m, H-19), 1.93 (H-21), 1.42 (H-22), 0.97 (3H, s, H-23), 0.90 (3H, s, H-24), 0.83 (3H, s, H-25), 1.03 (3H, s, H-26), 0.95 (3H, s, H-27), 0.79 (3H, s, H-28), 4.69 (1H, s, H-29), 1.68 (3H, s, H-30); 13C NMR (150 MHz, CDCl3): δ 151.0 (C- 20), 109.3 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 48.3 (C-18), 48.0 (C-19), 43.0 (C-17), 42.8 (C-14), 40.8 (C-8), 40.0 (C-22), 38.9 (C-4), 38.7 (C-1), 38.0 (C-13), 37.2 (C-10), 35.6 (C-16), 34.3 (C-7), 29.8 (C-21), 28.0 (C-2), 28.0 (C-23), 27.4 (C-15), 25.1 (C-12), 20.9 (C-11), 19.3 (C-30), 18.3 (C-6), 18.3 (C-28), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.5 (C-27).

## 3.7 Bioassays

Antimicrobial bioassay was done in collaboration with the Department of Biological sciences Microbiology laboratory at Masinde Muliro University of Science and Technology. A combination of agar well diffusion and serial dilution technique with some modification (Sen and Batra, 2012) was applied to all crude extracts, some isolated compounds and some combined compounds for both antibacterial and antifungal activity. The microbes *Escherichia coli*, *Staphylococcus aureus*, *Rhizopus stolonifer* and *Penicillium digitatum* were obtained by MMUST microbiology laboratory as standard reference strains. Gentamycin and fluconazole were used as standards while the solvents hexane, ethyl acetate, dichloromethane and methanol were used selectively as controls.

### 3.7.1 Tested microbial Strains

Bacterial test strains were chosen in terms of their gram-staining properties as gram positive / negative and in consideration to the ethno botanical exploitation of the plant as follows;

- **Gram positive**; *Staphylococcus aureus* (ATCC 5923)
- **Gram negative**; *Escherichia coli* (ATCC 5922)

Similarly the fungal test organisms were chosen categorically as saprophytic microorganisms;

- *Penicillium digitatum* (ATCC 48811)
- *Rhizopus stolonifer* (ATCC 14037)

### 3.7.2 Antibacterial tests for crude and isolated compounds

Concentrations of 1000 $\mu$g/mL for all the crude extracts and four isolated compounds were prepared by dissolving one gram of the sample in one milliliter of the suitable solvent. The mixture of nutrient agar and distilled water in a ratio of 1:26 agar (grams) to water (mL) was sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes then cooled for 5 minutes. 20 mL of the molten agar was distributed to each sterile plates of 100 mm diameter and the agar allowed to solidify. A 20 $\mu$L of each sample was placed in wells bored through the agar, at the middle of the plate. The bacterial strains *Escherichia coli* and *Staphylococcus aureus* were then streaked by spread plate method using a sterile loop uniformly on the agar followed by incubation of the plates for 24 hours at 37 $^{\circ}$C. Concentrations of the samples were varied by serial dilutions to make 500, 250 and 125 $\mu$g/mL for crude extracts and isolated compounds (Sen and Batra, 2012).

Zones of growth inhibition were observed and measured in millimeters as represented in table **4.5 - 4.8** and figures **4.8 - 4.10**. The experiment was done in triplicate and the mean value rounded up to the next millimeter. The lowest concentration of tested compounds that inhibited visible bacterial growth on the culture plates was defined as MIC.

### 3.7.4 Combination /Synergistic assay

Prior to performing the synergy test, the minimum inhibitory concentrations (MICs) of isolated compounds and antimicrobials were determined using micro dilution plate method with resazurin in Mueller-Hinton broth (Satish *et al*, 2005). Microplate wells were filled with 100 µL of Müeller-Hinton broth that had isolated compounds concentrations ranging from 0.1mg.mL$^{-1}$to 10 mg.mL$^{-1}$and $10^6$CFU.mL$^{-1}$of microbial suspensions. A control of microbial growth in this solvent was done with 100 µL of microbial suspension and 100 µL of Müeller-Hinton broth with DMSO at the highest concentration used in the preparation of the extract. After 24 h at 37 ºC, 4 µL of *p*-iodonitrotetrazolium was added to each well, and the plate was incubated for an additional 2 h at 37 ºC. A change in the color of the medium from yellow to pink-violet was used as an indication of microbial growth. The minimal inhibitory concentration of the antimicrobials was determined by the same procedure, for concentrations ranging from 0.1 mg.mL$^{-1}$ to 500 mg.mL$^{-1}$.

The checkerboard method is a traditional board testing technique that involves the analysis of square matrix of increasing concentrations of two compounds which is commonly used for measurement of interactive inhibition. These method was used

to determine the interactions between the isolated compounds and the antimicrobial drugs (Palaniappan and Holley, 2010). Synergistic interactions involving the isolated compound **2** and **3** plus the antimicrobial gentamycin and fluconazole were tested. The concentrations of the agents used started from twice their MIC value and were serially diluted in five-fold steps. The effects of combinations were evaluated by calculating the FIC (Fractional Inhibitory Concentration) index for each combination using the formula displayed below (Satish *et al*, 2005):

$$FICI = \frac{MICa \text{ in combination}}{MICa} + \frac{MICb \text{ in combination}}{MICb} \quad (1)$$

Where: MICa is MIC of **2** and MICb is MIC of 3

Interpretation of the FICI was as follows: FICI ≤ 0.5 synergy; FICI > 0.5–1, additivity; FICI > 1–4 indifference and FICI > 4 antagonism. The action of antimicrobial agents was considered to be:

***Synergistic*** - if their joint effect was stronger than the sum of effects of the individual agents

***Additive*** - if their joint effect was equal to the sum of effects of the individual agents

***Indifferent*** - if their joint effect was equal to the effect of either individual agent

***Antagonistic*** - if their joint effect was weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent (Satish *et al*, 2005). The mean FICI of all combination was used to

categorize results as synergy, additivity, indifference and antagonism.

### 3.8 Method of data analysis

Bioactivity of crude extracts, isolated and combined compounds and combination of isolated compounds and known drugs towards *Escherichia coli, Staphylococcus aureus, Rhizopus stolonifer* and *Penicillium digitatum* were presented as zones of inhibition in millimeters with values expressed as mean ± Standard error of the mean(SEM). Statistical significance was determined using one way Analysis of Variance (ANOVA). Significant differences in activity between the test-samples and positive control was done by comparison of means by Student-Newman-Keuls (SNK) test ($\alpha$= 0.05). Samples with values of $p < 0.05$ were considered statistically significant. The related effectiveness was also tested by applying linear correlation between control and tests.

# CHAPTER FOUR

# RESULTS AND DISCUSSIONS

## 4.1 Plants extracts

The plant parts of *T. asiatica* were subjected to sequential solvent extraction using hexane, dichloromethane (DCM) and methanol. The percentage yields of the extracts obtained from powdered 1kg stem and 0.8 kg root bark are shown in Table **4.1**.

**Table 4.1**: Percentage yields of crude extracts of *T. asiatica*

| Crude Extract | Stem bark % =(x/1000*100%) | Root bark % =(x/800*100%) |
|---|---|---|
| | *T. asiatica* | *T. asiatica* |
| Hexane | 3.52 | 2.4 |
| DCM | 2.75 | 1.6 |
| MeOH | 3.94 | 2.8 |

Methanol stem bark extract produced the highest yield with approximately 3.37 % followed by hexane extract with 2.96 % and DCM root bark extract at 2.18 %. The percentage yield of the methanol extracts was generally high compared to the other extracts, implying the plant materials studied could be endowed with components that are mainly polar (Table **4.1**).

## 4.2 Isolation of the crude extracts

Using the percentage yields data (Table **4. 1**) and bioassay data of crude extracts (Table **4.5-4.8**) only four crude extracts were subjected to isolation: stem; [hexane, DCM and methanol extracts] and root bark: [hexane extract], the other crude extracts (DCM and methanol root bark extracts) could not be followed given that the quantities obtained were negligible.

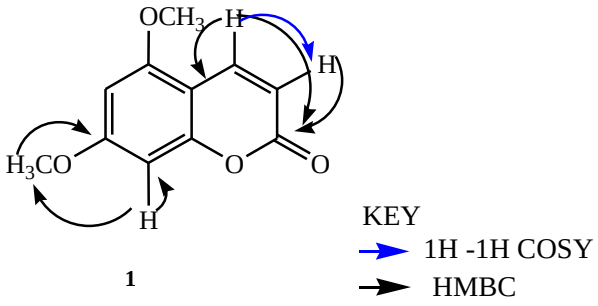
## 4.3 Structure Elucidation

The structure elucidation of compounds **1-6** were done by interpretation of their $^{1}H$, $^{13}C$, COSY, HMBC, HSQC NMR and mass spectrometric data. Spectral and physical data obtained for the isolated compounds yielded sufficient data that allowed structure determination and was in agreement with literature.

### 4.3.1 Toddalolactone (1)

Compound **1** was isolated as colourless crystals with melting point of 139-140 $^{o}C$. Its molecular ion peak was seen at *m/z* 308 [M+] in the EIMS spectrum. Its structure was determined by comparison of its spectroscopic data (Table **4.2** and **4.3**) with those from literature and isolated compounds **2-5** which indicated that it was a coumarin. Its $^{1}H$ NMR spectrum (Table **4.2** & appendix **1)** displayed a pair of doublets at δ 8.04 (J = 9.6 Hz) and 6.24 (J = 9.6 Hz) characteristic of H-4 and H-3 in a coumarin basic structure and a singlet occurring at $\delta_H$ 6.77 which could be attributed to *α*-benzopyrone protons at H-3, H-5 and H-6/8, respectively. The singlets at δ 3.99 and 3.89 were due to the presence of two methoxy groups at C-5 and C-7 in the aromatic ring, respectively. This was confirmed from the $^{13}C$ NMR by the signals at $\delta_C$ 63.8 and $\delta_C$ 56.7, respectively. Furthermore the $^{13}C$ NMR spectrum (Table **4.3** and appendix **1**) showed two olefinic signals at $\delta_C$ 112.6 (C-

3) and 141.2 (C-4). The resonance structure of the coumarin nucleus decreases electron density at C-4 making its signal to shift downfield at $\delta_C$ 141.2. Conversely the electron density at C-3 increases raising its shielding effect hence its signal shifts up field at $\delta_C$ 112.63. The carbonyl at C-2 occurred at $\delta_C$ 163.39. The quaternary carbons at C-4a, C-8a, C-6 and C-7 were assigned chemical shifts at $\delta_C$ 108.4, 156.2, 120.3 and 157.8, respectively. The assignments of $^{1}$H- and $^{13}$C-NMR data were confirmed by HMQC and HSQC spectrum. The HMBC spectrum revealed H-4 at δ 8.04 correlated with carbon signal for C-2 (163.4ppm), C-4a (108.4 ppm) and C-8a (156.2 ppm) (Fig **4.1**).




**1**

**Figure 4.1:** $^{1}$H-$^{1}$H COSY and HMBC correlation for compound **1**

From this it was possible to deduce a 5, 7-dimethoxy coumarin basic structure.

This was further supported by the fragmentation peak at *m/z* = 219 in the EIMS spectrum which was typical of a 5, 7 – dimethoxy - coumarine ion formed due to the loss of a 2 - methyl, butan - 2, 3 –diol group (Fig **4.2**).

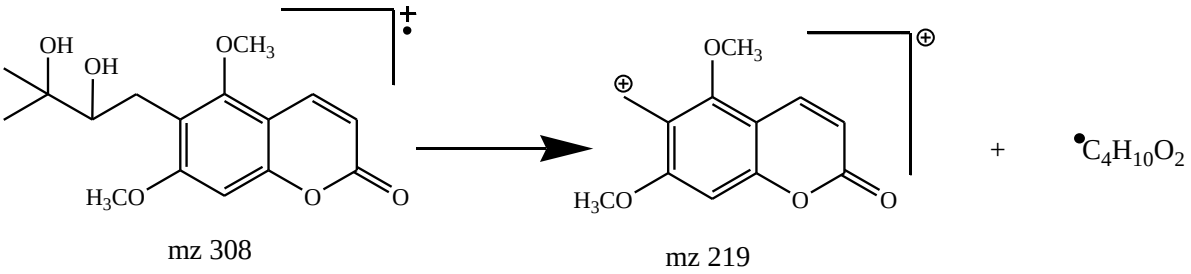
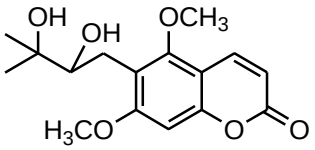



**Figure 4.2:** Fragmentation of compound **1**

This showed that Compound **1** had a 5, 7-dimethoxy coumarin basic structure as in the other four isolated compounds (Table **1**). The remaining signals in the $^{1}$H NMR spectra of **1** (Table **4.2**) belonged to a substituted prenyl. The signals were consistent with two methyl groups at 1.26 (3H, s, H-4') $\delta_C$ 25.54 and 1.27 (3H, s, H-5') $\delta_C$ 25.54 (the protons at C-5' appear to be more deshielded by the electron withdrawing hydroxyl groups, making it to resonate downfield at $\delta_H$ 1.27 than that at C-4'), a methylene at 2.86 (2H, s, H-1') $\delta_C$ 27.14, a methine group at $\delta$ 3.66 (1H, m, H-2') $\delta_C$ 78.25 a quaternary carbon at 74.04 (C-3') characteristic of a 3-methly, butan 2, 3-diol substituent/prenyl group.

A study of the HMQC and HMBC spectra revealed correlation between $\delta$ 2.86 (H-1') showed cross-peak with carbon signals at 120.3 ppm (C-6), 78.3 ppm (C- 2') and 74.0 ppm (C-3'). The aromatic proton at H-8 $\delta$ 6.67 ( H-2' ), fairly up field due to the shielding effect of the methoxy group was found to correlate with C-6 (120.3 ppm), C-4' (25.5 ppm) and C-3' (74.0 ppm) placing the prenyl group at position 6 in the ring system. This could further be confirmed by the fact that the methoxy at C-5 ($\delta_c$ 63.8) was downfield shifted compared to that at C-7 ($\delta_c$ 56.7) implying that the former was *ortho* disubstituted (Juma *et al*., 2001). Through this data compound **1** was identified as Toddalolactone, previously isolated from *T. asiatica* (Nyahanga, *et al*., 2013).



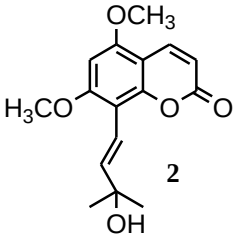

**1**

**4.3.2 Coumurrenol (2)**

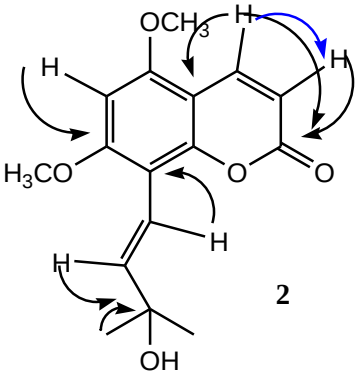
Compound **2** was isolated as colourless crystals. As in the case of compound **1,** it was possible to conclude a coumarin basic structure from spectroscopic data available (Table **4.2** and **4.3**). The $^{1}H$ NMR spectrum of **2** displayed signals that were in agreement with those of a 5,7-dimethoxy-8-substituted coumarin unit [$\delta_H$ 8.10 (1H, d, J 9.6 Hz, H-4), 6.15 (1H, d, J 9.6 Hz, H-3), 6.58 (1H, s, H-6),3.98 (3H, s, 5- $OCH_3$), and 3.99 (3H, s, 7-$OCH_3$); $\delta_C$ 163.42 (C, C-5), 163.13(C, C-2), 157.43 (C, C-7), 154.49 (C, C-8a), 140.87 (CH, C-4), 110.94 (CH, C-3), 107.59 (C, C-8), 104.82 6(C, C-4a), 92.26 (C, C-6), 56.75 (CH3, 7-$OCH_3$), and 56.75 ($CH_3$, 5-$OCH_3$)] (Table **4.2 & 4.3**). This was further confirmed by the COSY, HMBC and HSQC (Fig **4.3** and appendix **2).**

The remaining resonances in the 1D NMR data of **2** (Table **4.2** and **4.3**) were consistent with two methyl singlets at $\delta_H$ 1.39 (Me-4') and $\delta_H$ 1.39 (Me-5'), two methine signals at $\delta_H$ 6.83 (H, d, H-1') and $\delta_H$ 6.83 (H, d, H-2') and one quaternary carbon signal at $\delta_C$ 72.10 (C-3') and the hydroxyl singlet at δ 3.33 (OH-3') indicating the presence of a methylbuten-1-ol derivative moiety. The COSY, HMQC and HMBC spectrum (Fig **4.3**) revealed the proton signal at δ 6.83 (H-1') showed cross-peaks with carbon signals at 107.59 ppm (C-8), 154.49 ppm (C-8a), 115.29 ppm (C-$2^1$) and 72.10 ppm (C-3'). The proton signal at δ 6.58 (H-6) showed cross- peaks with carbon signals at 157.43 ppm (C-7), 163.42 ppm (C-5) and

104.82ppm (C-4a). In addition the two methoxy groups resonated at $\delta_C$ 56.75. From this it was possible to confirm that the prenyl group was attached to C-6 of the coumarin. From the data available and correlations with those of similar compounds in literature compound **2** was identified as Coumurrenol. This compound was previously isolated from *T. asiatica* (Nyahanga, *et al*., 2013).




**2**




KEY

1H -1H COSY

HMBC

**2**

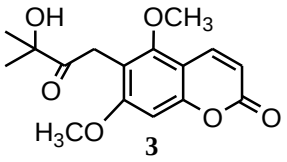
**Figure 4.3:** $^1$H-$^1$H COSY and HMBC Correlations for compound **2**

#### 4.3.3 5, 7-dimethoxy-6 – (3' - methyl, 3'- hydroxyl - but-2'-none) coumarine (3)

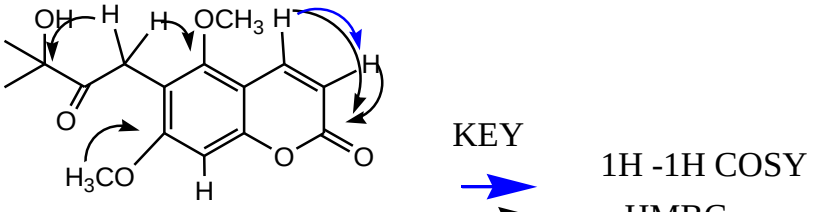
Compound **3** was isolated as colourless crystals. The $^1$H NMR spectrum (Table **4.2**) data suggested that compound **3** had a 5, 7-dimethoxy coumarin basic structure as in co- isolated compounds **1** and **2**. The remaining signals could be associated with a 5- carbon moiety displaying signals consistent with an OH group [$\delta_H$ 3.47 (1H, s, OH- 3')], two methyl groups at 1.50 (3H, s, H-4')] and 1.52 (3H, s, H-5')], a methylene at 3.92 (2H, s, H-1')] (appendix **3**) carbonyl carbon at $\delta_C$ 212.3 (C-2'), a quaternary carbon at 77.0 (C-3'), a methylene carbon at 31.6 (C-1') and two methyl

group carbons both at 27.0 (C-4' & C-5') characteristic of a 3-methly, 3-hydroxy-butan-2- none substituent.

The COSY, HMQC and HMBC spectrum (Fig **4.4)** revealed the proton signal at $\delta_H$ 3.92 (H-1') showed cross-peaks with carbon signals at 156.0 ppm (C-5) and 160.6 ppm (C-7). The methoxy at C-5 ($\delta_C$ 63.7) occurred downfield compared to that at C- 7 ($\delta_C$ 56.4) showing the prenyl was *ortho* disubstituted. From this it was possible to confirm that the prenyl group was attached to C-6 of the coumarin.The structure of this compound was elucidated as 5, 7-dimethoxy-6-(3'-hydroxy-3'-methylbutan-2'- oxo) coumarine. This is the first time the compound is being reported to occur in *T. asiatica* stem bark.




**3**




**3**

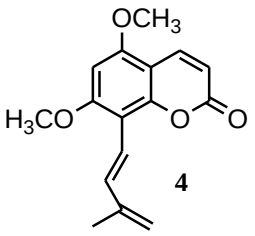
**Figure 4.4:** $^1$H-$^1$H COSY and HMBC correlations for compound **3**

**4.3.4 Gleinadiene (4)**

Compound **4** was isolated as colourless crystals with melting point of 120-121 $^oC$. The $^1$H NMR spectrum (Table **4.2**) data suggested that compound **4** had a 5, 7-dimethoxy coumarine basic structure similar to compounds **1** and **2** and **3**. The remaining signals in the 1D NMR spectra of **4** (Appendix **4**) were consistent

with a pair of doublets at δ 6.67 (J = 16.2 Hz) and 7.27 (J = 16.2 Hz) were assigned to H-2' and H-1', respectively. The coupling constant 16.2Hz assigned to H-2' and H-1' indicates that the structure has *trans*-configuration. A methyl group signal at δ2.02 and a two doublets due to two methylene protons at δ 5.11 and 5.03 (H-4') characteristic of a 3'-methylbut-1', 3'-dienyl side chain. The HMBC spectrum revealed H-4 at δ 7.91 correlated with carbon signal for C-2 (161.2 ppm) and C-5 (153.5 ppm). The proton signal at δ 7.27 (H-1') showed cross-peaks with carbon signals at 103.8 ppm (C-8), 117.1 ppm (C-2') and 143.3 ppm (C-3') H-2' at δ 6.67 found to correlate with C-1' (135.7 ppm) and C-3' (143.3 ppm). The proton signal at δ 6.32 (H-6) showed cross-peaks with carbon signals at 161.1 ppm (C-7), 103.8 ppm (C-8) and 107.2 ppm (C-10). In addition, proton signal at δ 6.20 (H-3) correlated with carbon signal at 155.6 ppm (C-9) and 107.2 ppm (C-10).

Basing on this data, the prenyl group is attached to C-8 of the coumarine skeleton. The structure of this compound was established as gleinadiene based on the similarity of its spectral and physical data to those of the same compound in literature. Gleinadiene was previously isolated from *M. gleinei* root (Kumar. *et al*. 1987).This is the first time the *trans*-isomer of this compound is being isolated from the root bark of *Toddalia asiatica*




**4**

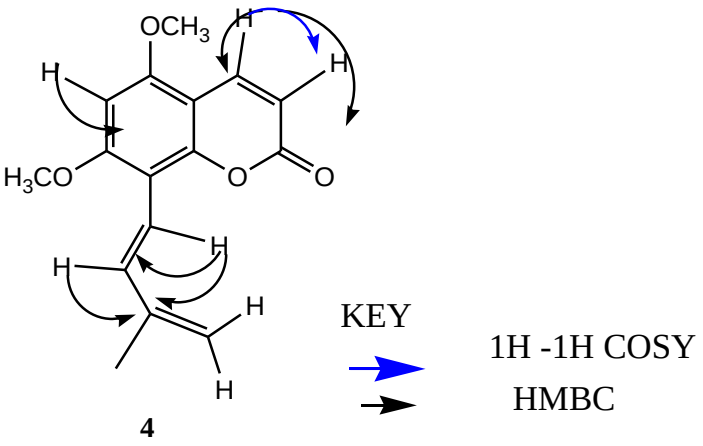
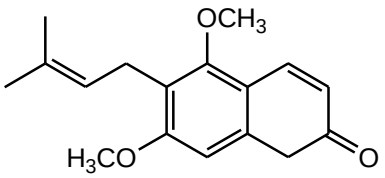



**Figure 4.5:** $^{1}$H-$^{1}$H COSY and HMBC correlations for compound **4**

#### 4.3.5 Toddaculin ( 5)

Compound **5** was isolated as colourless crystals with melting point of 94-96$^{o}$C. Its molecular ion peak was seen at *m/z* 275.4 [M+] in the EIMS spectrum. The $^{1}$H NMR spectrum (Table **4.2**) data suggested that compound **3** had a 5, 7-dimethoxy coumarin basic structure as in isolated compounds **1, 2, 3** and **4**. The remaining resonances in the 1D NMR data of **5** (Table **4.2**) were consistent with two methyl singlets at $\delta_H$ 1.781 (Me-4') and δ1.683 (Me-5'), one methine at δ 5.16 (1H, t, H-2'), and one methylene δ 3.36 (2H, d, H-1'), indicating the presence of a methylbut-3-ene moiety. One of the two methoxy groups C-7 ($\delta_C$ 63.13) occured downfield indicating that the prenyl group was *ortho*- disubstituted with the methoxy's as in compounds **1** and **3** this enabled the assignment of the prenyl group to C-6. The structure of this compound was therefore established as toddaculin based on the similarity of the data obtained to that in literature. This compound has been reported from *T. asiatica* stem bark (Nyahanga, *et al*., 2013).




**5**

**Table 4.2**: $^{1}$H NMR of compounds **1-5** from *T. asiatica* root bark

| **Carbon** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| **2** | - | - | - | - | - |
| **3** | 6.24d,(9.6) | 6.15d(9.6) | 6.21d(9.6) | 6.20d(9.6) | 6.22d(9.6) |
| **4** | 8.04dd(9.6) | 8.10d(10.2) | 7.85d(9.6) | 7.91d(9.6) | 7.86d(9.6) |
| **5** | - | - | - | - | - |
| **6** | - | 6.58s | - | 6.32s | - |
| **7** | - | - | - | - | - |
| **8** | 6.77s | - | 6.66s | - | 6.62s |
| **1'** | 2.86 | 6.83s | 3.92s | | 3.36d(6.9) |
| **2'** | 3.66m | 6.83s | - | 7.27d(16.2) | 5.16t(6.9) |
| **3'** | - | - | - | 6.67d(16.2) | - |
| **4'** | 1.26s | 1.39s | 1.50s | - | 1.78s |
| **5'** | 1.27s | 1.39s | 1.52s | 5.11d | 1.68s |
| **5-OCH$_3$** | 3.99s | 3.98s | 3.76s | 2.02s | 3.88(s) |
| **7-OCH$_3$** | 3.89s | 3.99s | 3.81s | 3.93s | 3.87(s) |
| **OH** | 4.852s | - | | 3.79s | |
| **OH** | 4.854s | 3.3 | 3.47s | | |

"s", "d", and "m" represents singlet, doublet and multiplet

**Table 4.3**: $^{13}C$ NMR data of compounds **1-5** from *T. asiatica* stem and root bark

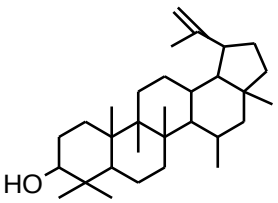
| Carbon | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **2** | 163.39 | 163.13 | 161.4 | 161.2 | 161.29 |
| **3** | 112.63 | 110.94 | 112.90 | 110.9 | 112.26 |
| **4** | 141.24 | 140.87 | 138.82 | 138.7 | 138.98 |
| **4a** | 108.44 | 104.82 | 107.37 | 107.2 | 107.11 |
| **5** | 163.80 | 163.42 | 155.98 | 153.5 | 155.22 |
| **6** | 120.31 | 92.26 | 125.90 | 90.3 | 120.30 |
| **7** | 157.81 | 157.43 | 160.89 | 161.1 | 161.70 |
| **8** | 96.33 | 107.59 | 95.69 | 103.8 | 95.38 |
| **8a** | 156.21 | 154.49 | 114.20 | 155.6 | 154.66 |
| **1'** | 27.14 | 115.29 | 31.60 | 135.7 | 22.70 |
| **2'** | 78.25 | 142.87 | 212.30 | 117.1 | 122.15 |
| **3'** | 74.04 | 72.10 | 76.96 | 143.3 | 132.05 |
| **4'** | 25.54 | 30.10 | 26.96 | 117.0 | 25.71 |
| **5'** | 25.54 | 30.10 | 26.93 | 18.3 | 17.83 |
| **5-$OCH_3$** | 63.80 | 56.75 | 63.66 | 56.0 | 56.05 |
| **7-$OCH_3$** | 56.73 | 56.75 | 56.41 | 55.9 | 63.13 |

### 4.3.6 Lupeol (6)

Compound **6** was isolated as white needle shaped crystals with melting point of 215- 216$^{O}$C. Its molecular ion peak was seen at *m/z* 425.6 [M+] in the EIMS spectrum. The $^{1}H$ NMR spectrum showed seven tertiary methyl singlets at δ 0.79 (Me-28), 0.83 (Me-25), 0.90 (Me-24), 0.95 (Me-27), 0.97 (Me-23), 1.03 (Me-26), 1.68 (Me-30) and one secondary hydroxyl group at δ 3.21 (OH-3).

It also showed olefinic protons for the methylene group ($CH_2$) at δ 4.69 and 4.57 as broad singlets, (Table **4.4).** $^{13}C$ NMR of the compound showed 30 signals for the terpenoid of lupine skeleton which was represented by seven methyl groups resonating at $\delta_C$ 14.5 (C-27), 15.34 (C-24), 16.0 (C-26), 16.1 (C-25), 18.3 (C-28) and 19.3 (C-30), 28.0 (C-23). The carbon bonded to the hydroxyl group C-3

appeared at δ 79.0, while the alkenic carbons appeared at $\delta_C$ 150.9 (C-20) and 109.3 (C-29). The resonance at $\delta_C$ 150.98 represented a quaternary carbon for C-20. Other quaternary carbons were assigned to C-5, C-8, C-10, C-14 and C-17, respectively. That appearing at $\delta_C$ 109.31 was assigned to C-29. The $^{13}$C-NMR spectrum also showed peaks at $\delta_C$ 38.70, 27.98, 18.3, 34.27, 20.92, 25.13, 27.44, 35.58, 29.84 and 40.00 assigned to methylene carbons at C-1, C-2, C-6, C-7, C-11, C-12, C-15, C-16, C-21 and C-22 respectively. The structure of this compound was determined as Lupeol based on the similarity of the data obtained to that in literature. The presence of lupeol in the *T. asiatica* is being reported for the first time, however the compound has been reported in many plants such as *T. nobilis*, *Heritiera utilis*, *Strobilanthus callosus* and *Strobilanthus ixiocephala* roots (Chepkirui, 2012).




**6**

**Table 4.4:** $^{1}H$ and $^{13}C$ NMR chemical shift, together with DEPT data for compound **6**.

| C | $^{1}H$ NMR($CDC_{l3}$) | | $^{13}C$ NMR | ($CDC_{l3}$) | DEPT |
|---|---|---|---|---|---|
| | Obs | Lit | obs | lit | |
| 1 | 1.68 (s) | 1.67 (s) | 38.70 | 38.9 | $CH_2$ |
| 2 | 1.50 | 1.52 | 27.98 | 27.6 | $CH_2$ |
| 3 | 3.21 (1H,m) | 3.20 (1H,m) | 79.00 | 79.2 | CH |
| 4 | | | 38.86 | 39.1 | Q |
| 5 | 0.69 (1H,s) | 0.66 (1H,s) | 55.29 | 55.5 | CH |
| 6 | 1.39 | 1.38 | 18.31 | 18.5 | $CH_2$ |
| 7 | 1.39 | 1.38 | 34.27 | 34.5 | $CH_2$ |
| 8 | | | 40.83 | 41.0 | Q |
| 9 | 1.28 | 1.28 | 50.43 | 50.6 | CH |
| 10 | | | 37.16 | 37.4 | Q |
| 11 | 1.26 | 1.25 | 20.92 | 21.2 | $CH_2$ |
| 12 | 1.43 | 1.43 | 25.13 | 25.3 | $CH_2$ |
| 13 | 1.62 | 1.62 | 38.04 | 38.3 | CH |
| 14 | | | 42.83 | 43.0 | Q |
| 15 | 1.68 | 1.68 | 27.44 | 27.7 | $CH_2$ |
| 16 | 1.52 | 1.52 | 35.58 | 35.8 | $CH_2$ |
| 17 | | | 42.99 | 43.2 | Q |
| 18 | 1.35 | 1.35 | 48.30 | 48.5 | CH |
| 19 | 2.39 (1H,m) | 2.45 (1H,s) | 47.98 | 48.2 | CH |
| 20 | | | 150.98 | 151.2 | Q |
| 21 | 1.93 | 1.97 | 29.84 | 30.1 | $CH_2$ |
| 22 | 1.42 | 1.41 | 40.00 | 40.2 | $CH_2$ |
| 23 | 0.97 (3H,s) | 0.96 (3H,s) | 27.98 | 28.2 | $CH_3$ |
| 24 | 0.90 (3H,s) | 0.88 (3H,s) | 15.36 | 15.6 | $CH_3$ |
| 25 | 0.83 (3H,s) | 0.82 (3H,s) | 16.11 | 16.4 | $CH_3$ |
| 26 | 1.03 (3H,s) | 1.03 (3H,s) | 15.97 | 16.2 | $CH_3$ |
| 27 | 0.95 (3H,s) | 0.94 (3H,s) | 14.54 | 14.8 | $CH_3$ |
| 28 | 0.79 (3H,s) | 0.79 (3H,s) | 18.31 | 18.2 | $CH_3$ |
| 29 | 4.69 (1H,s) | 4.73 (1H,s) | 109.31 | 109.6 | $CH_3$ |
| 30 | 1.68 (3H,s) | 1.68 (3H,s) | 19.30 | 19.5 | $CH_3$ |

### 4.3.7 Possible derivatisation of the prenyl substituent group

The isolated compounds **1-5** showed interesting substitutions on the prenyl group that seems to suggest a systematic biosynthetic pathway to 3-methylbutan-1, 3-dienyl isoprenyl derivative moety on compound **4**. The proposed derivatisation starts with the isoprene unit. The isoprene ending **A** seems to undergo a number of

oxidation processes leading to derivative **B**. This is then reduced probably by biological reducing agent NADPH to **C**. It is clear that **C** loses two water molecules through D to form E (Figure **4.6**)

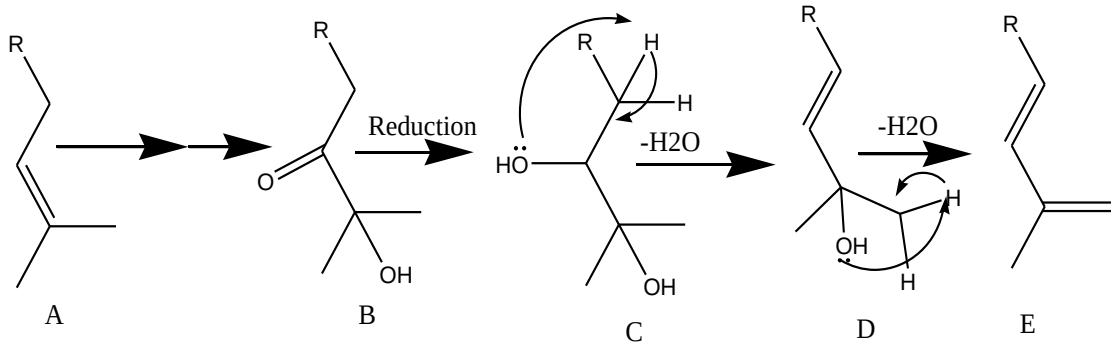



**Figure 4.6:** Proposed pathway for compound **2** and **4**

## 4.4 Biological activity of *T. asiatica*

### 4.4.1 Antimicrobial activity of crude extracts from *T. asiatica*

When the antimicrobial potential of stem and root bark crude extracts was evaluated according to their zone of inhibition against various pathogens/saprophytes, both the methanol and DCM *T. asiatica* stem bark extracts showed activity against *S. aureus* with inhibitions zones of 16.7 and 11.0 mm at 1,000 $\mu$g/mL (Table **4.5**). The methanol extract was more potent in this test displaying a minimum inhibition concentration (MIC) of 250 $\mu$g/mL.

**Table 4.5**: Zones of inhibition of crude extracts against *S. aureus*

| Plant | Plant Part | Crude Extract | Zone of inhibition (mm) at various Concentrations | | | |
|---|---|---|---|---|---|---|
| | | | 1000 $\mu$g/mL | 500 $\mu$g/mL | 250 $\mu$g/mL | 125 $\mu$g/mL |
| *T. asiatica* | Stem bark | Hex | - | - | - | - |
| | | DCM | 11.00±0.58 | 8.33±0.33 | - | - |
| | | MeOH | 16.67±0.67 | 14.33±0.33 | 12.67±0.33 | - |
| | Root bark | Hex | - | - | - | - |
| Standard | Gentamycin | | 25.33±0.67 | 22±0.00 | 18±0.58 | 13.67±0.88 |

Against the gram negative bacteria *E. coli*, DCM extract showed higher inhibition of 12.3 mm compared to the more polar methanol extract with an inhibition zone of 9 mm at 1,000 $\mu$g/mL (Table **4.6**). Both extracts recorded an MIC of 500 $\mu$g/mL compared to that of the standard at 125 $\mu$g/mL.

**Table 4.6:** Zones of inhibition of crude extracts against *E. coli* bacteria

| Plant | Plant Part | Crude Extract | Zone of inhibition (mm) at various Concentrations | | | |
|---|---|---|---|---|---|---|
| | | | 1000 $\mu$g/mL | 500 $\mu$g/mL | 250 $\mu$g/mL | 125 $\mu$g/mL |
| *T. asiatica* | Stem bark | Hex | | - | - | - |
| | | DCM | 12.33±0.33 | 9.33±0.67 | - | - |
| | | MeOH | 9.00±0.58 | 7.67±0.33 | - | - |
| | Root bark | Hex | - | - | - | - |
| Standard | Gentamycin | | 24±0.00 | 18.67±0.88 | 17.67±0.58 | 12.33±0.67 |

Tests against the two fungi, *P. digitatum* and *R. stolonifer* showed the methanol extract to be more potent with inhibition zones of 21.0 mm and 18.3, respectively, at 1000 μg/mL (Table **4.7** and **4.8**) compared to the standard with an inhibition of 16.3 mm at 1000 μg/mL

**Table 4.7:** Zones of inhibition of crude extracts against *Penicillium digitatum*

| Plant | Plant Part | Crude extract | Zone of inhibition (mm) at various concentrations | | | |
|---|---|---|---|---|---|---|
| | | | 1000 $\mu$g/mL | 500 $\mu$g/mL | 250 $\mu$g/mL | 125 $\mu$g/mL |
| *T. asiatica* | Stem Bark | Hex | - | - | - | - |
| | | DCM | 13.00±0.00 | 11.00±0.58 | - | - |
| | | MeOH | 21.00±1.00 | 19.67±0.58 | 15.5±0.5 | - |
| | Root Bark | Hex | 10.67±0.67 | 8.33±0.33 | - | - |
| Standard | Fluconazole | | 16.33±0.88 | 14±0.00 | 12.33±0.88 | - |

For antifungal activity against *R. stolonifer*, methanol stem and hexane root extracts displayed an MIC of 250 $\mu$g/mL compared to the standard 500 $\mu$g/mL (Table **4.8).** The methanol extract showed a lower MIC value (250 $\mu$g/mL) than that of the standard (500 $\mu$g/mL) (Table **4.8**) while in the case of *P. digitatum* the same MIC value of 250 $\mu$g/mL was recorded as for the standard. The hexane stem bark extract showed no activity against all the organisms tested but the root bark hexane extract inhibited the growth of the two fungi. For this extract, the highest zone of inhibition of 12.0 mm was seen against *R. stolonifer* at 1000 $\mu$g/mL at an MIC value of 250 μg/mL (Table **4.8**).

**Table 4.8**: Zones of inhibition of crude extracts against *R. stolonifer* fungi growth

| Plant | Plant Part | Crude extract | Zone of inhibition (mm) at various concentrations | | | |
|---|---|---|---|---|---|---|
| | | | 1000 $\mu$g/mL | 500 $\mu$g/mL | 250 $\mu$g/mL | 125 $\mu$g/mL |
| *T. asiatica* | Stem bark | Hex | - | - | - | - |
| | | DCM | 7.45±0.58 | 6.24±0.25 | - | - |
| | | MeOH | 18.33±0.88 | 15.67±0.33 | 13.67±0.33 | - |
| | Root bark | Hex | 12.00±0.58 | 10.33±0.33 | 8.33±0.33 | - |
| Standard | Fluconazole | | 23.67±1.45 | 20±0.58 | - | - |

The plant's methanol extract was more potent against *P. digitatum* with an inhibition of 15.5 mm than *R. stolonifer* where it had an inhibition of 13.7 mm at an MIC of 250 $\mu$g/mL. For the antibacterial activity only the methanol extract was active, recording an MIC of 250 $\mu$g/mL against *S. aureus* (Fig **4.7**). DCM extract recorded an MIC of 250 $\mu$g/mL against the four microbes, whereas the hexane root bark extract recorded no antibacterial activity. (Fig **4.7** – **4.8**).

### 4.4.2 Antimicrobial activity of isolated compounds

Compounds **1, 2, 3** and **4** were subjected to antibacterial and antifungal tests against *S. aureus*, *E. coli*, *P. digitatum* and *R. stolonifer* bacteria and fungi, respectively. The bioactivities were taken as zones of inhibitions at concentrations of between 1000 - 125 $\mu$g/mL).

#### 4.4.2.1 Antibacterial activity of isolated compounds

Compounds **1**, **2**, **3** and **4** all showed appreciable activity against both gram positive and negative bacteria. Toddalolactone (**1**), the most polar compound, showed the

highest potency with inhibition zones of 18 and 16 mm against *S. aureus* and *E. coli*, respectively, at a concentration of 1,000 $\mu$g/mL (Table **4.9**). At the same concentration the standards recorded zones of inhibition of 26 and 24 mm, respectively. This compound had lower MIC values of 250 $\mu$g/mL against *S. aureus* and 500 $\mu$g/mL against *E. coli* compared to gentamycin at 125 $\mu$g/mL. Gleinadiene (**4**) recorded inhibition zones of 13 and 10 mm against *S. aureus* and *E. coli*, respectively, at MIC value of 500 $\mu$g/mL for both organisms (Table **4.9).** Coumurrenol (**2**) showed low bioactivity against *S. aureus* with a zone of inhibition of 9mm at 500 $\mu$g/mL, significantly different ($p < 0.05$) from that of Gentamycin 14mm at 125 $\mu$g/mL (Table **4.9**). Compound **3** recorded the lowest inhibition of 8mm at 500 $\mu$g/mL against *S. aureus* compared to the other compounds. Compound **3** was the only compound with a carbonyl group on the prenyl substituent. The low activity exhibited by the compound may greatly be attributed to the carbonyl group.

**Table 4.9**: Zone of inhibition of pure compounds against *S. aureus* and *E. coli*

| Compounds | **BIOACTIVITY (Zone size in mm)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1000$\mu$g /mL | | 500$\mu$g /mL | | 250$\mu$g /mL | | 125 $\mu$g /mL | |
| | *S.*a | *E.c* | *S.a* | *E.c* | *S. a* | *E.c* | *S.a* | *E.C* |
| **1** | 18 | 16 | 16 | 13 | 8 | - | - | - |
| **2** | 10 | 9 | 9 | 8 | - | - | - | - |
| **3** | 9 | 10 | 8 | - | - | - | - | - |
| **4** | 13 | 10 | 12 | - | - | - | - | - |
| Gentamycin | 26 | 24 | 22 | 20 | 18 | 18 | 14 | 12 |

**Key**: ***S.a*** - *S. aureus*, ***E. c***- *E. coli*

The high activity of the methanol crude extract could be attributed to polar compounds like compound **1** or other non-isolated compounds. Compound **1** was more active (MIC 250 $\mu$g /mL) than compound **3** at (MIC 500$\mu$g) (Table **4. 9**).

The two have the same basic structure but differ in substitution pattern of the prenyl group at C-6. Compound **1** has 1', 2'- dihydroxy group on the prenyl unlike **3** with a carbonyl at the C – 2 position. This showed a 1', 2'- dihydroxy substitution is necessary for enhanced activity. On the other hand, compounds **2** and **4** have the substituent prenyl group attached to C - 8 of the coumarin structure. Compound **2** with hydroxyl group was less active than compound **4** with the conjugated structure. The conjugation seems to favor the activity more than the single hydroxyl group.

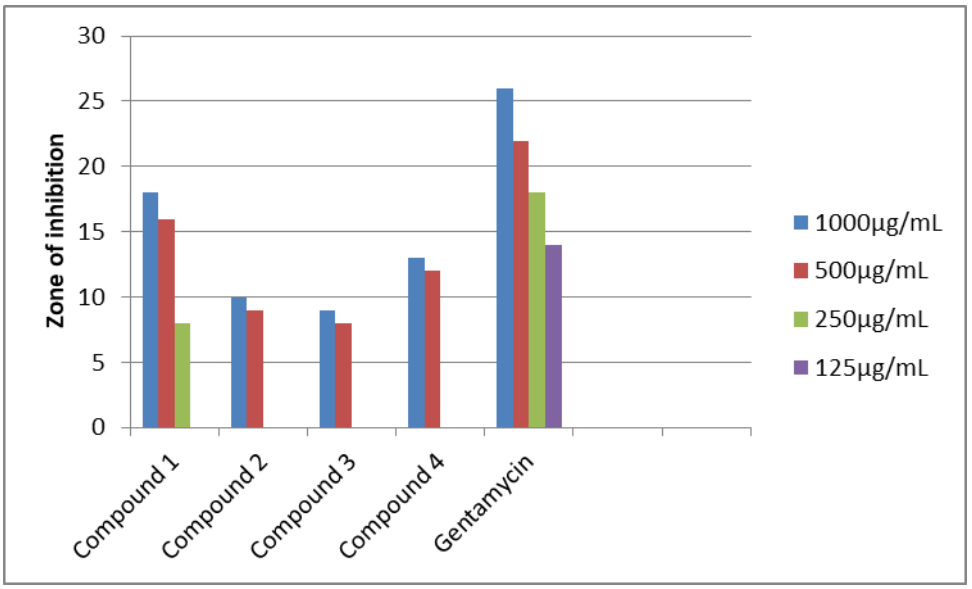



**Figure 4.7**: A graph on zone of inhibition by pure compounds against *S. aureus*

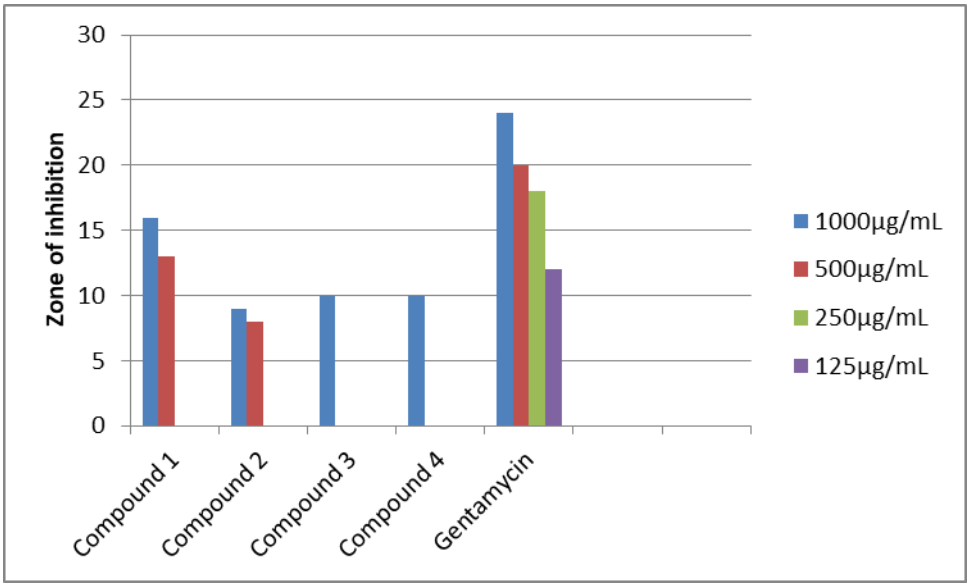



**Figure 4.8**: Agraph on Zone of inhibition by pure compounds against *E.coli*

All compounds except **1** and **4** displayed moderate antibacterial activity (15 – 19 mm) (Table **4.9**) which can be approximated to 3+ according to the antimicrobial activity index (AI) (Rajakaruna *et al*, 2002). This can be considered moderate activity given the activity index varies as: $4^+$ = (20-24 mm) for the highest, $3^+$ = (15-19 mm), $2^+$ = (10-14 mm), whereas $1^+$ = (8 – 9 mm) is the lowest according to the scale.

#### 4.4.2.2 Antifungal activity of isolated compounds

Toddalolactone (**1)** showed the highest zone of inhibition of 18 mm and 16 mm at a concentration of 1000 $\mu$g/mL towards *P. digitatum* and *R. stolonifer*, respectively (Table **4.10**). Compound (**2**) showed the lowest inhibition of 8 mm against *P. digitatum* with MICs of 500 $\mu$g/mL, significantly different ($p < 0.05$) from that of Fluconazole (250 $\mu$g/mL) (Table **4.10**). Compound **3** and **4** displayed an MIC of 500 $\mu$g/mL for both fungi.

**Table 4.10:** Zone of inhibition of pure compounds against *P. digitatum* and *R. stolonifer*

| Compounds | **BIOACTIVITY (Zone size in mm)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1000 $\mu$g /mL | | 500 $\mu$g /mL | | 250 $\mu$g /mL | | 125 $\mu$g /mL | |
| | *P.d* | *R.s* | *P.d* | *R.s* | *P.d* | *R.s* | *P.d* | *R.s* |
| 1 | 18 | 16 | 16 | 14 | - | - | - | - |
| 2 | 14 | 13 | 10 | 8 | - | - | - | - |
| 3 | 10 | 12 | 8 | 9 | - | - | - | - |
| 4 | 13 | 14 | 12 | 13 | - | - | - | - |
| Fluconazo -le | 16 | 24 | 14 | 20 | 12 | - | - | - |

Key: *P.d- Penicillium digitatum, R.s – Rhizopus stolonifer*

Compound **1** registered the highest bioactivity index in the range of 3+ for the strains tested. Compound **3** showed the lowest activity in the order of 2+ against *Penicillium digitatum*

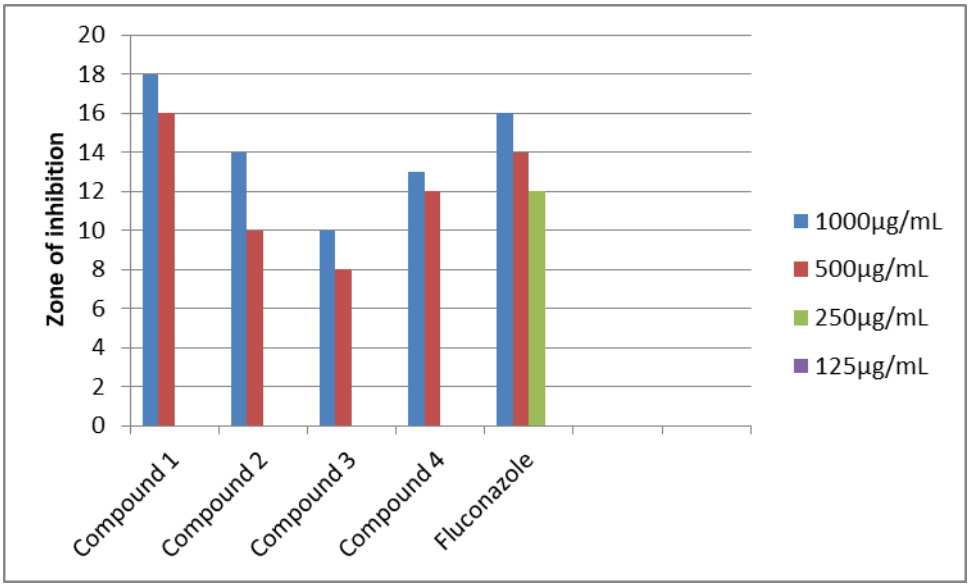



**Figure 4.9**: A graph on zones of inhibition by pure compounds against *P. digitatatum*

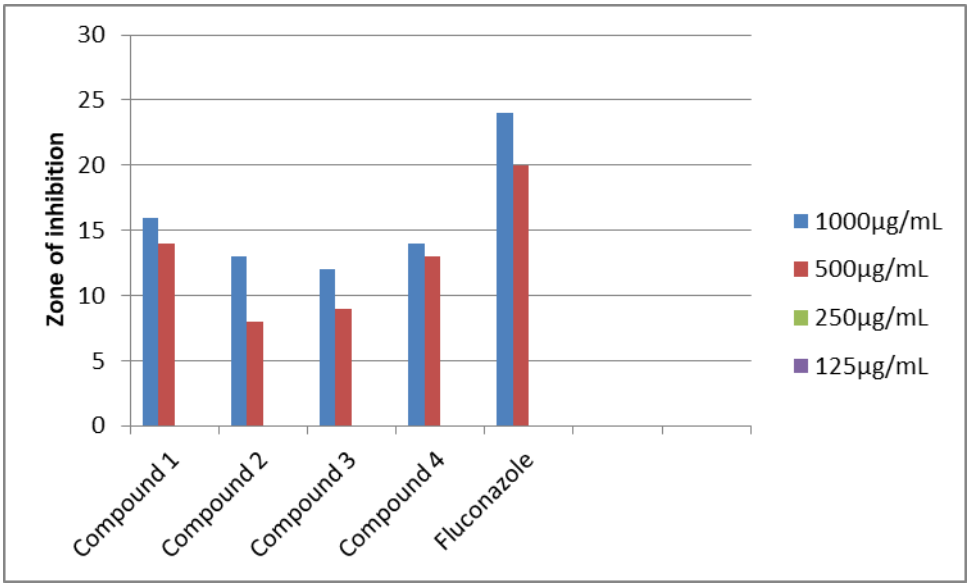



**Figure 4.10**: A graph on zones of inhibition by pure compounds against *R. stolonifer*

The carbonyl group attached to C-2' of the prenyl group of compound **3** may be enhancing the antifungal activity better than the antibacterial activity. Similar to antibacterial activity, it seems the hydroxyl groups at C-2' and C-3'could be enhancing the activity in compound **1** greater than the conjugation in compound **4**. The position of the prenyl group seems also to have no effect on the activity of the compounds.

Compounds **1**, **2**, **3** and **4** all showed some activitiy against the fungi tested. Compound (**1**) was the most potent recording inhibition of 18 mm and 16 mm against the two fungi, respectively, at a concentration of 1,000$\mu$g/mL. These results were comparable with those of Fluconazole though with a lower MIC (250$\mu$g/mL) (Table **4.10**).

### 4.4.3 Antimicrobial Synergistic effect

The following combinations were considered in the study: a mixture of isolated pure compounds **2** and **3**, compound **2** with gentamycin, **3** with gentamycin, **2** with

fluconazole and **3** with fluconazole.

#### 4.4.3.1 Antimicrobial Synergistic effect

Interaction in antimicrobial activity between two isolated compounds **2** and **3** and in combination with antimicrobial agents, gentamycin and fluconazole was evident. Against *S. aureus*, the MIC of combined compounds **2** and **3** improved to 250$\mu$g/mL compared to individual compounds both with MIC at 500$\mu$g/mL (Table **4.11**).

**Table 4.11**: Minimum Inhibitory Concentration of isolated and combined compounds against *S. aureus* and *E. coli*

| Compound | Minimum Inhibitory Concentration in $\mu$g /mL | |
|---|---|---|
| | *S. aureus* | *E. coli* |
| 2 | 500 | 500 |
| 3 | 500 | 1000 |
| 2 +3 | 250 | 500 |
| 2+Gentamycin | 125 | 500 |
| 3+Gentamycin | 250 | 500 |
| Gentamycin | 125 | 250 |

Interaction of compounds against *E. coli* showed that combination of compounds **2** and **3** and the two compounds with gentamicin produced indifferent effect (FIC 1.5, 3.0 and 2.5, respectively). Table **4.12**. Combination of gentamycin with compound **3** however showed indifference effect (FIC 1.25) with the lowest MIC value of 125µg/mL. Its combination with compound **3** also showed indifferent effect in the test against *S. aureus*.

**Table 4.12**: Synergism between combined compounds **2**, **3** and Gentamycin

| Test Organism | **BIOACTIVITY** | | |
|---|---|---|---|
| | Compound **2** + **3** | Compound **2** + G | Compound **3** + G |
| *S. aureus* | 1.0a | 2.5[i] | 1.25[i] |
| *E.coli* | 1.5[i] | 2.5[i] | 3.0i |

*The most active combination, G-gentamycin, s- synergy, i-indifferent, a-additivity

Against *R.stolonifer*, the MIC of combined compounds **2**and **3** improved to 250$\mu$g/mL compared to individual compounds both with MIC at 500$\mu$g/mL (Table **4.13**).

**Table 4.13:** Minimum Inhibitory Concentration of isolated and combined compounds against *P.digitatum* and *R. stolonifer*

| Compound | Minimum Inhibitory Concentration in $\mu$g /mL | |
|---|---|---|
| | *P. digitatum* | *R. stolonifer* |
| 2 | 250 | 500 |
| 3 | 500 | 500 |
| 2 +3 | 250 | 250 |
| 2+ Fluconazole | 125 | 250 |
| 3+ Fluconazole | 250 | 500 |
| Fluconazole | 250 | 250 |

Combination of compound **3** with fluconazole **(**Table **4.14)** gave additive effect (FIC 1.0) while mixtures of compounds **2** with **3** and compound **3** with fluconazole both gave indifference effect with FIC indices of 1.5 each in the test against *P. digitatum* (Tables **4.14**). The best result against this organism was in the combination of compound **3** with fluconazole with an MIC of 125 µg/mL. In the test against *R.*

*stolonifer* combinations of compounds **2** with **3** showed improved activity when compared with individual compounds with FIC of 1.0 (additive). Combinations of both compound **2** and **3** with Fluconazole showed indifference effects with FIC indices of 3.0 and 1.5, respectively (Table **4 .14**).

**Table 4.14:** Synergism between combined compounds 2, 3 and Fluconazole.

| **Test Organism** | **BIOACTIVITY** | | |
|---|---|---|---|
| | Compound **2** + **3** | Compound **2** + F | Compound **3** + F |
| *P. digitatum* | $1.5^{i}$ | $1.5^{i}$ | $1.0^{a}$ |
| *R. stolonifer* | $1.0^{a}$ | $3.0^{i}$ | $1.5^{i}$ |

*The most active combination, F - Fluconazole, a- additivity, s-synergy

Generally *S. aureus* was susceptible to most of the plant extracts used in this study. For the other organisms there was variation in susceptibility unique to each extract. The various substituents in the coumarin nucleus, strongly influence the biological activities against the organisms tested. For instance the high bioactivity reported in compound **1** against all microbs could be attributed to the presence of the dihydroxyl groups on the prenyl substituent moiety. When coumarins **2** and **3** were combined an additive effect was recorded against *S. aureus* and *R. stolonifer*. Synergistic interactions of compounds if exploited could lead discovery of combination of molecules that would provide leads to drugs with better activities (Stefanović and Comic 2012).

# CHAPTER 5

# CONCLUSION AND RECOMMENDATIONS

## 5.1 Conclusion

The study guided by both ethnobotanical, biological activity studies together with chromatographic isolation of active principles:

i. Led to isolation and characterisation of six compounds, toddalolactone (**1**), coumurrenol (**2**), 5, 7- dimethoxy- 6- (3' -methyl- 3'- hydroxy- but- 2-none) coumarin (**3**), Gleinadiene (**4**) toddaculin (**5**) and lupeol (**6**), from *T. asiatica*. Compounds **3** and **6** are being reported for the first time from *T. asiatica* stem. Compound **3** had never been reported prior to this work.

ii. The individual isolated compounds and all the crude extracts (apart from that of stem bark hexane) showed considerable activity against *S. aureus*, *E. coli*, *P. digitatum* and *R. stolonifer*.

iii. Combinations of compound **2** with **3** and compound **3** with fluconazole displayed additive effect in inhibiting the growth of *Rhizopus stolonifer* and *Penicillium digitatum*, respectively. Interaction in antibacterial activity between compounds **2** with **3** lowered the MIC in action against *Staphylococcus aureus* to 250$\mu$g/mL compared to individual compounds with MIC of 500$\mu$g/mL while showing additive

## 5.2 Recommendations

These results validate the ethnobotanical uses of *T. asiatica*, its worthwhile recommending that:

- The bioassay should be extended to other micro-organisms because a wide range of microbes have suffered resistance.
- Synergistic studies of most of the other known compounds could be done as this would lead to development of drug agents that may help fight microbial resistance.
- Toxicity studies on the crude and pure active compounds should be carried out to establish their safe levels for use by humans. This will go a long way in availing information to assist in calculation of the safety dosage required for complete microbial clearance
- Structure modifications could be done on some of the isolated compounds from the plant with the aim of studying structure activity relationships. This may lead to discovery of more portent molecules.

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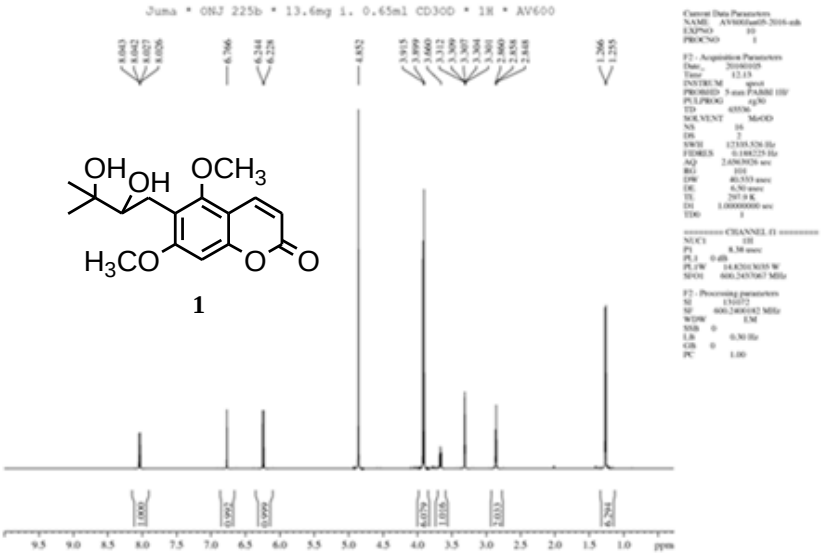
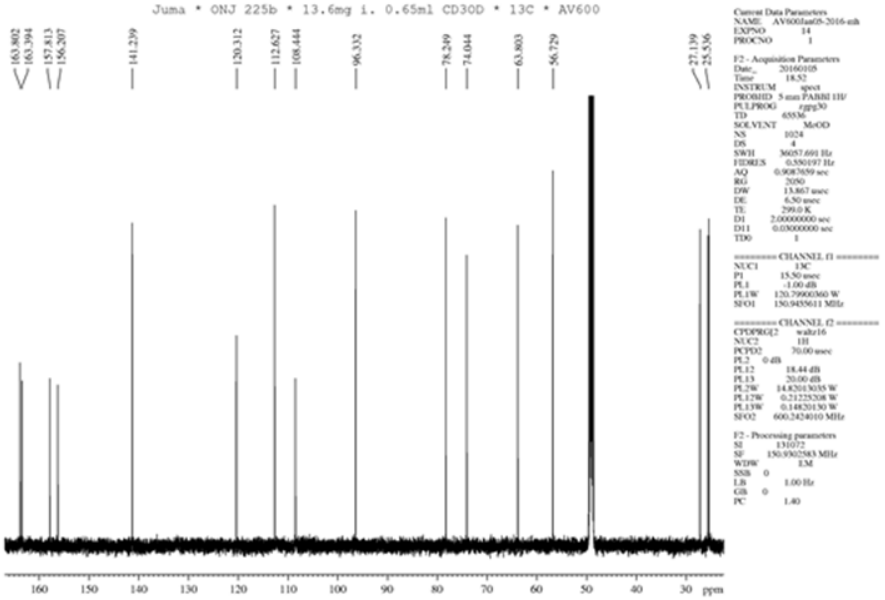
# APPENDICES

## APPENDIX 1: NMR SPECTRA FOR COMPOUND 1

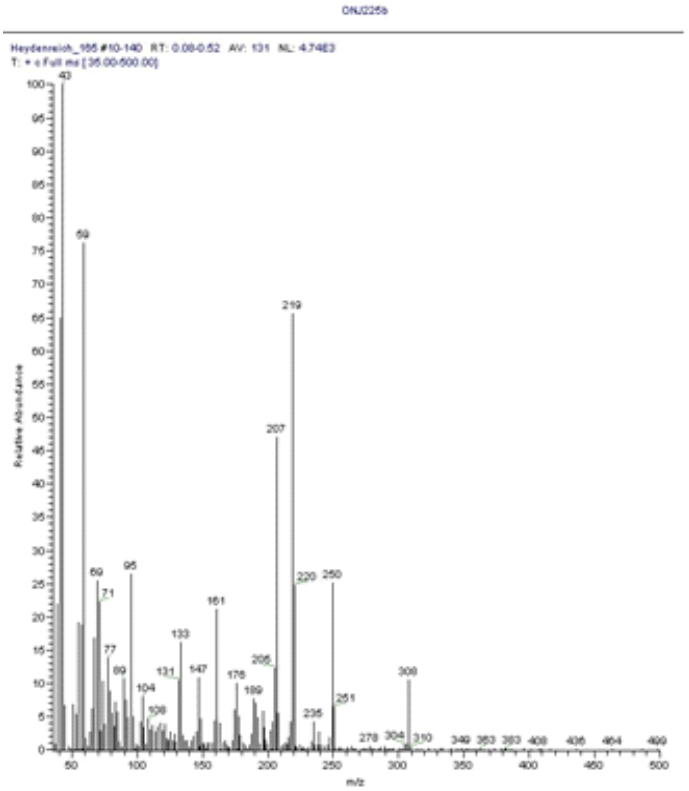
### $^{1}$H NMR spectrum for compound 1




### $^{13}$C NMR spectrum for compound 1

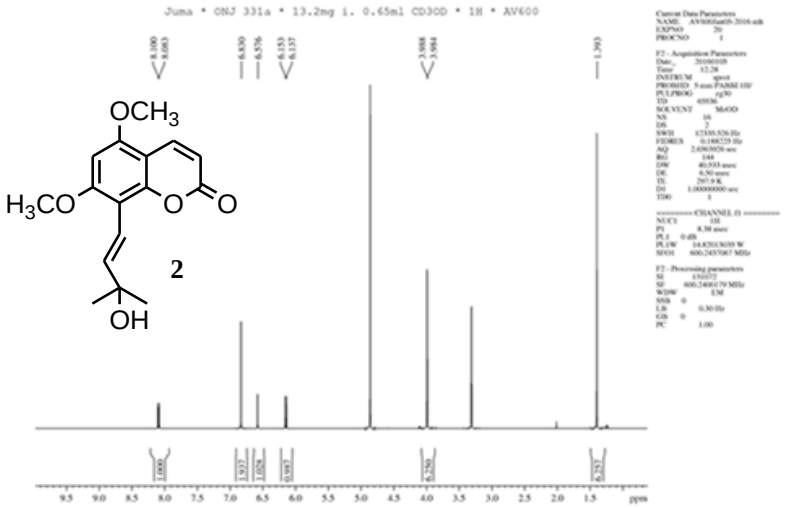


**MS spectrum for compound 1**

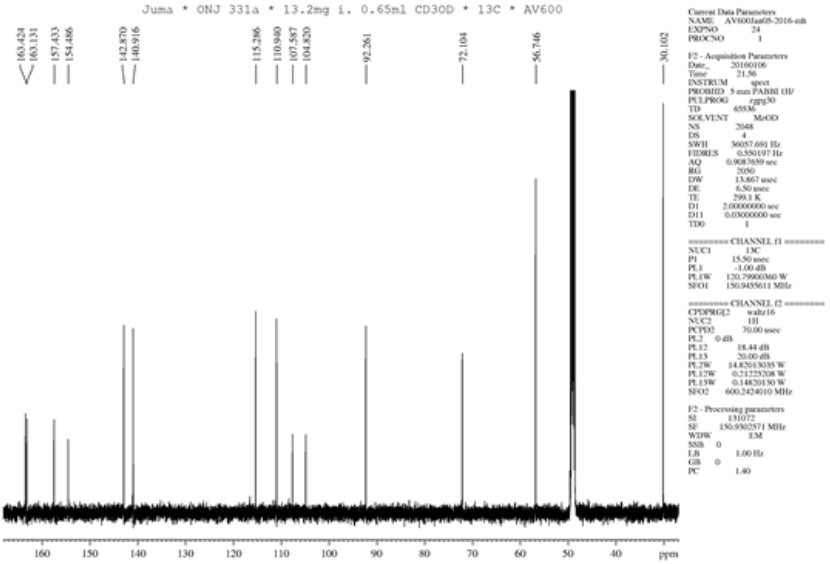



## APPENDIX 2: NMR SPECTRA FOR COMPOUND 2

**$^{1}$H NMR spectrum for compound 2**

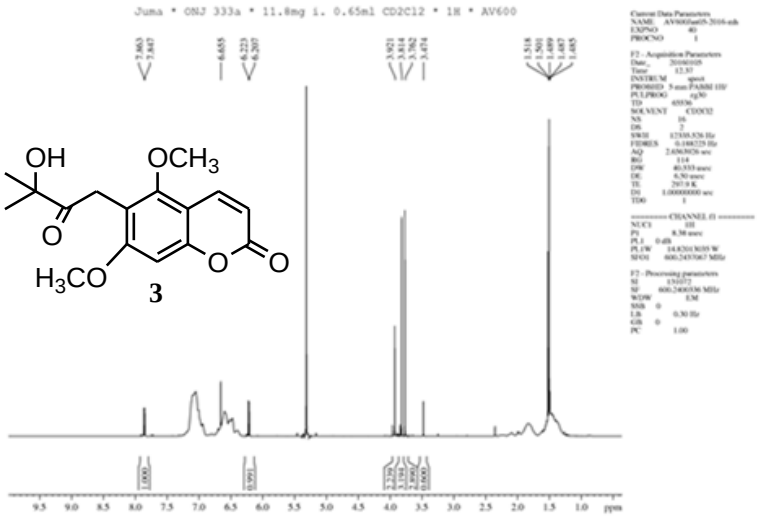


**$^{13}$C NMR spectrum for compound 2**

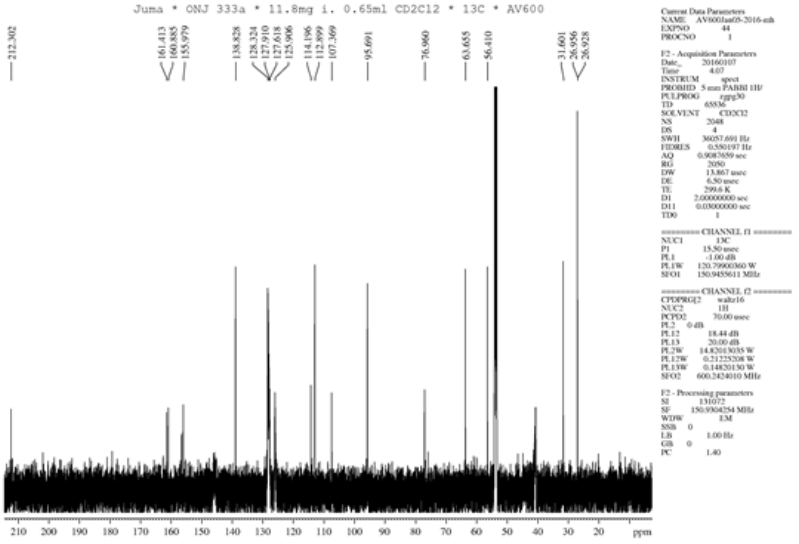



## APPENDIX 3: NMR SPECTRA FOR COMPOUND 3

**1H NMR spectrum for compound 3**

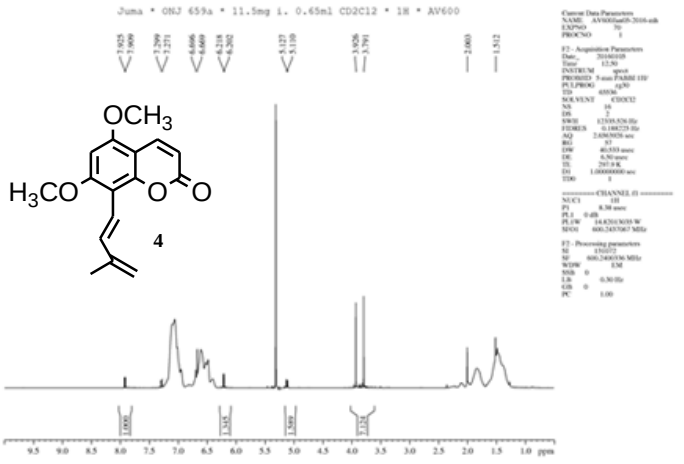


## $^{13}$C spectrum for compound 3



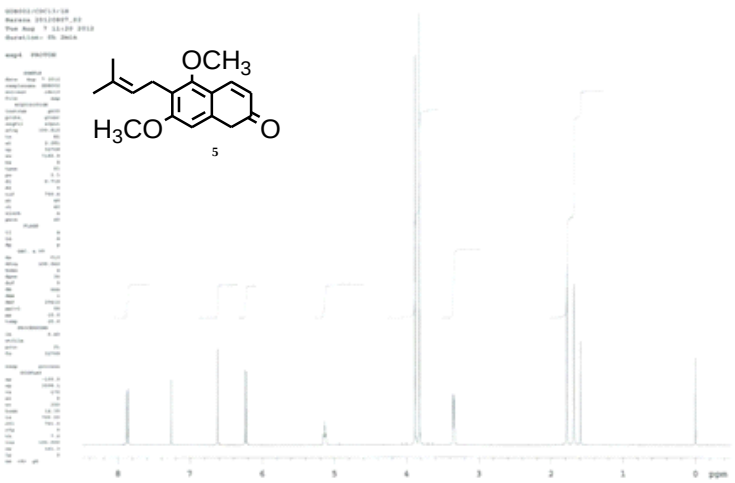

## APPENDIX 4: NMR SPECTRA FOR COMPOUND 4

## $^{1}$H NMR spectrum for compound 4

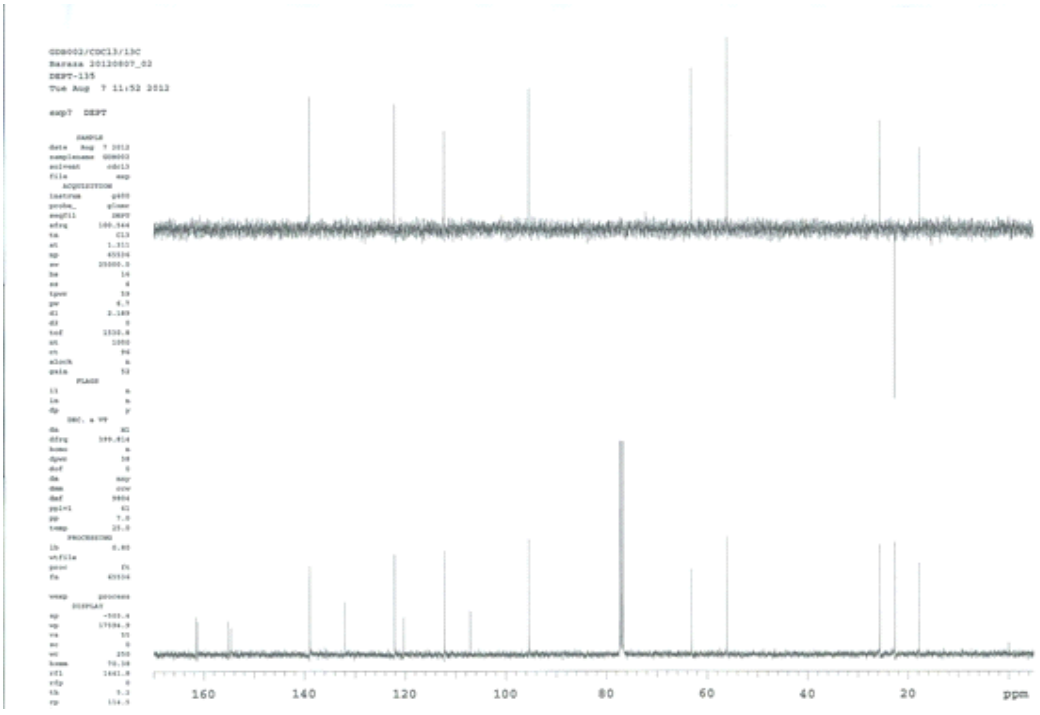


## APPENDIX 5: NMR SPECTRA FOR COMPOUND 5

### $^{1}$H NMR spectrum for compound 5

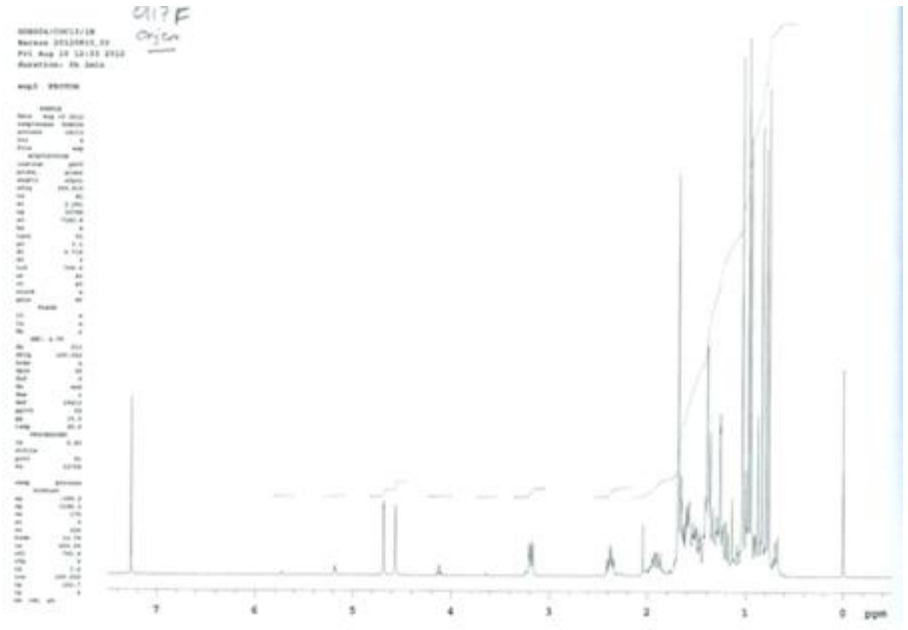



### $^{13}$C and DEPT spectrum for compound 5



## APPENDIX 6: NMR SPECTRA FOR COMPOUND 6

### $^{1}$H NMR spectrum for compound 6



### $^{13}$C NMR and DEPT spectrum for compound 6