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Phytochemical and Pharmacological Properties of Crude Extracts and Pure Compounds from Leaves of *Vernonia galamensis* and *Croton macrostachyus*

By

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Phytochemical and Pharmacological Properties of Crude Extracts and Pure Compounds from Leaves of *Vernonia galamensis* and *Croton macrostachyus*

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This is to certify that the thesis presented by Geremew Tafesse, entitled: *Phytochemical Screening and Comparative Study on Antibacterial and Ileum Muscle Contractile Activities of Vernonia galamensis and Croton macrostachyus Leaves Extracts* and submitted in fulfillment of the requirements for the Degree of Philosophy (Biomedical Sciences) complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Abbreviations

$[Ca^{2+}]_i$ = Intracellular Calcium Ion Concentration

AAU = Addis Ababa University

ACh = Acetylcholine

AMP = Ampicillin

ARA = Adrenoceptor Agonist

ATCC = American Type Culture Collection

ATP = Adenosine Tri-Phosphate

CAF = Chloramphenicol

CEE = Croton Ethanol Extract

CEaE = Croton Ethylacetate Extract

CHE = Croton Hexane Extract

CIP = Ciproflaxin

CME = Croton Methanol Extract

CMRA = Cholinergic Muscarinic Receptor Agonist

DMCMB = Department of Microbial, Cellular and Molecular Biology

EHNRI = Ethiopian Health and Nutrition Research Institute

ERY = Erythromycin

ET-1 = Endothelin-1

FDA = Food and Drug Administration

GI = Gastrointestinal

HIV/AIDS = Human Immunodeficiency Virus/Acquired Immuno Deficiency Syndrome

HPLC = High Pressure Liquid Chromatography

HTS = High Throughput Screening

IBS = Irritable Bowel Syndrome

IL-9 = Interleukin-9

MDR = Multidrug Resistance

MIC = Minimal Inhibitory Concentration

MoE = Ministry of Education

MS = Mass Spectroscopy

NAG = N-Acetylglucoseamine

NAMA = N-Acetlymuramic Acid

NABSA = Network for Analytical and Bioassay Services in Africa

NMR = Nuclear Magnetic Resonance

PDGF = Platelet Derived Growth Factor

Prep-TLC = Preparative Thin Layer Chromatography

RA = Receptor Agonist

SARS = Severe Acute Respiratory Syndrome

Th-2 = T- Helper 2

TLC = Thin Layer Chromatography

TWAS = The Academy of Sciences for the Developing World

UB = University of Botswana

VAE = Vernonia Acetone Extract

VEE = Vernonia Ethanol Extract

VHE = Vernonia Hexane Extract

VME = Vernonia Methanol Extract

Abstract

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Geremew Tafesse, Thesis for Degree of Doctoral Philosophy, Addis Ababa University, July 2014

*Human beings rely on medicinal plants to combat infections since long time ago. Vernonia galamensis and Croton macrostachyus are used in different parts of Ethiopia to treat wound and/or intestinal worms traditionally. The main objective of this research was to proof the traditional uses of these plants through testing the effect of their leaf extracts on selected pathogenic bacteria and on the contractility of ileum muscle. Crude extraction was done by subsequent method using n-hexane, acetone/ethylacetate, ethanol and methanol. Bioactive compounds were isolated using column chromatography, NMR and MS. Crude extracts were tested against Escherichia coli, Salmonella typhi, Shigella boydii and Staphylococcus aureus at load of 3mg/disk, 6mg/disk and 9mg/disk followed by testing the isolated compounds using disk diffusion method. The effect of six crude extracts on ACh induced guinea pig ileum contractility was tested at concentrations of 80µg/ml, 160µg/ml and 320µg/ml in a dose dependent manner. Acute toxicity tests were done for each crude extract on albino mice. Each crude extract showed antibacterial activities at loads of 6mg/disk (200mg/ml) and 9mg/disk (300mg/ml) with MIC of 125mg/ml. At a load of 9mg/disk both extracts showed strong activity against S. aureus and S. boydii. Vernolide (**C-I**), vernoguinoside (**C-II**), vernodalin (**C-III**), compound IV (**C-IV**) from VAE, methyl laurate (**C-VI**) and creptoxide (**C-VII**) from CEaE were isolated. **C-I** and **C-II** were not reported from V. galamensis so far. **C-II** and **C-IV** showed strong antibacterial activities against S. typhi and S. boydii (P=0.69; 0.89) while **C-VI** and **C-VII** showed moderate to strong activity without significant differences with the respective standards against all tested bacteria. CEaE and VAE significantly increased the ACh induced contraction of guinea pig ileum in a dose dependent manner while CME and VME reduced it (P<0.00). All extracts showed no toxicity at all tested doses. Results showed that these plants possess antibacterial and intestinal muscle contractile properties, which might validate their traditional uses. However, further studies must be carried out on their mechanism of actions.*

Key Words: Phytochemicals, Antibacterial, Contractility, *Vernonia galamensis, Croton macrostachyus*

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Chapter I

General Introduction

1.1 Background of the Study

Although the term pharmacognosy has been in use for more than a century, the history of phytotherapy (using plants for treatment of human and animals' ailments), is almost as long as the history of civilization. The Assyrian, Egyptian, Chinese and Greek records of great olden days make references to the use of herbal drugs. Through the monasteries and their schools of medicine, the Knowledge of medicinal plants spread over Europe and then in the whole Western World (Waksmundzka-Hajnos *et al.*, 2008).

Pharmacognosy is part of the pharmaceutical sciences focusing on the components of natural products that show biological activity thereby used in therapy (Ahmad *et al.*, 2006). Phytotherapy aims at complete investigations of materials from medicinal plants by the use of physical, chemical and biological methods in order to use them as optional medicines. Therefore, it focuses on the study of components of the plant, including its structure and pharmacological properties responsible for therapy. Pharmacognosy is generally based on the go-ahead treatment of the natural sources of drugs taking into account their biochemical transformations that allows synthesis of new biologically active substances (Ahmad *et al.*, 2006; Waksmundzka-Hajnos *et al.*, 2008).

The isolation of the first biologically active compounds (basically alkaloids) from plants (e.g., morphine, strychnine, narcotine, caffeine, etc.) in the 19^{th} century made phytotherapy important in modern science. The first synthesized sulfonamides and antibiotics from plants being used in therapy by 1935 had marked the golden age of

phytotherapy until the age of chemotherapy began. However, numerous synthetic drugs produced by means of chemotherapy exert harmful and often irreversible side effects; while in the plant world, strongly active substances coexist with other compounds and mitigate these negative side effects. This phenomenon forced a return of phytotherapy in recent years (Waksmundzka-Hajnos *et al.*, 2008).

In recent years, World Health Organization has urged to further focus on phytotherapy through screening plant materials for the presence of biologically active compounds that have therapeutic potential. Though only a few percent of 250,000 plant species have been investigated with regard to their usefulness in medicine so far, it is firmly believed that a great, yet not fully revealed, therapeutic potential exists in plants (WHO, 1996, 2002; Waksmundzka-Hajnos *et al.*, 2008).

Several investigations have shown that chemicals obtained from higher plants usually affect the physiology and survival of almost all animals in one or another way. Such physiological effects could have positive or negative consequences on the survival of the animal. It could be based on such general assumption that human beings rely on plant materials as sources of medicines for varieties of ailments since ancient times. It took long time, however, until investigators gave scientific testimonies for the effects and mode of action of most plant extracts. The mechanism of flavonoids (usually found in red wine) on reducing coronary heart disease was not known until reported by Hertog and other researchers in 1993 (Rauha, 2001).

Although large number of higher plants have been claimed to possess medicinal value, only around 25% of them have been exploited in modern medicines. Controversially, about 80% of the world populations (especially in developing countries) use such medicinal plants either by themselves or with the help of traditional healers, for most probable reason that herbal medicines offer a holistic approach which is lacking in modern ones (Anokbonggo, 1992 cited in Mushiga *et al.*, 2004). As a result, the development and usage of herbal medicines is not a concern to the developing nations only but became a global scene too (WHO, 2002). Regardless of these facts, among the whole plant species only 15% have been investigated for phytochemicals and only 6% have been studied for their biological activities (Cragg *et al.*, 1997) suggesting that the attention given to herbs is still less in spite of the problem in finding effective medicines.

Phytochemicals that are claimed for their medicinal values usually inhibit the growth of pathogenic organisms thus, serving as antimicrobial agents or may expel out parasitic worms as antiparasitic agents. They can also be involved in correcting blood circulation, preventing fertility, maintain fetus development, or other therapeutic activities. To give some examples, *Achyranthes aspera* has been investigated for possession of antimicrobial and anti-inflammatory activities (Goyal *et al.*, 2007); while plants like *Chassalia coriaceae* reported to possess agents for treatment of diseases such as diarrhea and dysentery (Gurib-Fakim *et al.*, 2005).

In another investigation, it is reported that the extract obtained from *Aspila africana* leaves re-enforced norepinephrine induced vascular smooth muscle contraction (Dimo *et al.*, 2002 in Oluyemi *et al.*, 2007). Phytochemicals obtained from *Monida whitei* were found to

augment potassium chloride (KCl) and adrenaline induced contractions of rats' vas deferens (Watcho *et al*, 2006). Extracts from *Ruta graveolens* and *Cannabis sativa* (Sailani and Moeini, 2007) and *Achyranthes aspera* (Goyal *et al.*, 2007) are reported to reduce the number of sperm in adult male rats. These are some examples out of many other phytochemicals that were scientifically investigated for their biological activities.

The mechanism by which phytochemicals alter the normal physiology of an animal could vary. For instance, the anti-fertility and/or antiimplantation effects of these chemicals can be through delaying ovulation, delaying gamete maturation, or through blockage of implantation sites on the uterine wall. The abortifacient effect of such agents, on the other hand, is mostly through uterotonic activities. The effect of phytochemicals to result in any of the aforementioned phenomena is suggested to be usually hormonal: estrogenic (Oluyemi *et al.*, 2007) and oxytocic (Mitra *et al.*, 1999) respectively.

Various studies have been made on the antimicrobial activity of plant extracts especially on their action to kill or suppress the growth of agents of such infections. A commonly known tropical and warm temperate plant, *Ocimum gratissmum*, had been reported to have an inhibitory effect on the growth of some bacterial species when its leaf extract obtained through steam distillation was applied (Adebolu and Oladimeji, 2005). The crude extracts made from *Citrus aurantifolia* and *Titonia diversifolia* are also reported to show inhibition on growth of different bacterial species though the later was less potent (Taiwo, *et al.*, 2007). Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of

the side effects often associated with synthetic antimicrobials (Iwu *et al.*, 1999 in Idu *et al.*, 2007).

Many plants extracts have also been reported to show anthelminthic properties by which their ability to act against helminthes parasites can be expressed in terms of either killing and/or pushing out them from the intestine. The latter is usually accompanied with increasing the contractility of the intestinal muscle (Amos *et al*., 2003; Keiser and Utzinger, 2008 in Behnke *et al*., 2008).

Like most other smooth muscles, intestinal muscle has at least one important feature that makes it best fit to its functions since it is arranged in tubular form with both circular and longitudinal portions. This feature can then determine the propulsive action of the muscle (Barreto, 2002), thereby letting the passage and/or expelling out of its content easily. One of the probable reasons for this property could be the ability of such a muscle to show rhythmic contraction and relaxation to allow peristaltic movements (Guyton and Hall, 2006). So any factor that affects such rhythmic contraction in one or another way will alter the passage and/or expelling out of materials from the lumen of the intestine (Kahle, *et al.*, 1986; Despopoulos & Silbernagl, 2003; Duthie and Gardiner, 2004).

Vernonia spp and *Croton spp* are some of the higher plant groups with the ability to cure different ailments traditionally. Among the claimed ailments to be treated by these plants the most common are skin diseases (rushes), fungal infection, diarrhea and intestinal worm (Geyid *et al.*, 2005). *V. amygdalina* for instant is reported to have antihelminthic activity specially to expel out ascaris. Likewise many other related species such as *Vernonia*

colorata (Rabe *et al.*, 2002), *Croton zambesicus* (Reuben *et al.*, 2008) and *C. regelianus* (Torres *et al.*, 2008) have been reported to possess antimicrobial properties.

People in different parts of Ethiopia are believed to use different plants such as *Vernonia amygdalina, V. galamensis* and *Croton macrostachyus* as a traditional medicine to treat different bacterial and/or intestinal infections (Geyid *et al.*, 2005). They use *V. amygdalina* to wash their skin whenever they get skin rush, which give a clue that this plant may possess antimicrobial property. Similarly, *Croton macrostachyus* is claimed, in areas where it grows, to serve against infections of skin (*Mich*) that may suggest its antibacterial activity. This plant is also claimed to be used against intestinal nematode parasites (especially ascaris) in different areas of the country (personal information).

Though these and other traditional medicinal purposes have been reported for these plants, few or no experimental proofs have been made available for *Vernonia galamensis,* a highly related species to *V. amygdalina,* and for *Croton macrostachyus*. Therefore, the main purposes of this research work are to give experimental proofs for possession of antibacterial and intestinal muscle contractile activities in *Vernonia galamensis* and *Croton macrostachyus*. The research tries to screen out the phytochemical agents for such activities from each plant and hence the thesis is organized in six chapters: Chapter I: General Introduction dealing with the back ground of the study and statement of the problem; Chapter II: Reviews of Literatures on chemical screening from natural products for medicinal purposes and other related works; Chapter III: Phytochemical Screening of *V. galamensis* and *C. macrostachyus* Leaves; Chapter IV: Antibacterial Activities of *V. galamensis* and *C. macrostachyus* Leaves Extracts; Chapter V: Guinea pig Intestinal Muscle

(Ileum) Contractile Properties of *V. galamensis* and *C. macrostachyus* Leaves Extracts; and Chapter VI: Acute Toxicity Test, General Conclusion and Recommendations.

1.2 Statement of the Problem

The world was and still facing enormous problems due to number of ailments since time immemorial. Among these ailments infectious diseases took tremendous part affecting both human and livestock throughout the world. The agents for infectious diseases are various including viruses, bacteria, fungi, protozoans and other parasites. These agents can harbor different body parts of their hosts, which could be internal or external and can lead to a more complicated situations that might end up with mortality (Beaglehole *et al*., 2002; WHO, 2002).

Internally infectious agents can affect cells, tissues, organs or even body system. Skin, eye, mouth and nostrils are the most vulnerable external part of the animal body to be affected by such agents especially by bacteria, fungi and viruses (Gilbert and Allison, 2004). An animal body may respond to effects of infections to get rid of them by its immune system. However, most of the time the effect of infection outweigh and become beyond the immune system of the animal leading to complications. As a result different effects can be observed including irritations, rushes, inflammations, gastrointestinal disorders and the likes. If situations reach these levels the body has to get external supports that can help to boost and complement its immune systems so that it can get relief of infection (Roitt and Delves, 2001; Folds, 2008).

Disorders such as that of GI tract can also be caused by parasites like helminthes or by conditions like IBS, constipation or others. These may lead to a change in its motility either by increasing or decreasing the state of contraction of its muscles. The animal body also reacts to correct this situation either by developing the immune system or through changing the status of its muscle contraction. For instance, it increases its muscle contractility to push parasites out. If the body becomes unable to do this by itself it needs external agents to correct the situation (Roitt and Delves, 2001; Tams, 2003).

One of the best ways for the host's body to get relief from infections or to correct disorders is the use of natural products. Human beings had been used natural products especially higher plants to treat themselves and livestock from different disorders, such as infectious diseases and GI disorders starting a very long time ago. Through time human beings made some progresses to produce and use synthetic drugs to combat infections and disorders (Dewick, 2002).

However, due to hindering factors such as affordability, effectiveness and safety, people in most parts of the world rely on natural products especially higher plants to treat themselves and livestock from different diseases and/or disorders. Moreover, resistances developed by organisms like microbes and helminthes to many synthetic drugs, and side effects posed by such drugs on the host have been forcing many researchers to find alternative drugs from natural sources especially from higher plants. Large numbers of *in vivo* and *in vitro* tests have been conducted on the extracts obtained from higher plants to validate their traditional uses in treating infectious diseases and GI disorders. In spite of such tremendous works, however, infection and GI disorder are still great problems

especially to the developing nations (Geerts and Gryseels, 2000; Dickson and Gagnon, 2004; Cseke *et al*., 2006).

Higher plants like *Vernonia galamensis* and *Croton macrostachyus* have been claimed to be used traditionally to treat infections (wound) and to expel out intestinal worms (Geyid *et al.*, 2005). Therefore, the present study is designed to give experimental proofs, for the effects of extracts from the leaf of these two plants on selected pathogenic bacteria and on intestinal muscle (ileum) contraction, so as to validate their traditional uses. Thus, the study was conducted on three components: phytochemical screening of the leaves of these plants, evaluating the antibacterial properties of the crude extract as well as the isolated compounds from them, and evaluating the effect of crude extracts of each plant on guinea pig ileum contraction.

1.3 Research Questions

The following are the major questions.

1. Do crude extracts from *V. galamensis* and *C. macrostachyus* leaves have antibacterial properties?
2. What is/are the exact phytochemical(s) showing such properties?
3. Which of the extract and/or compound from leaves of each plant is more potent in its antibacterial activity?
4. Do crude extracts of *V. galamensis* and *C. macrostachyus* leaves have ileum contractile properties?
5. Which of the crude extract from leaves of each plant is more potent in its ileum contractile activity?

6. Is the effect of each extract concentration dependent?
7. What is the toxicity profile of the extract from each plant?

1.4 General Objectives

- To evaluate the antibacterial activity of the extracts from leaves of each claimed plants (*V. galamensis* and *C. macrostachyus*)
- To screen the phytochemical(s) that has (have) the antibacterial activity from each plant leaves
- To identify the most potent extract and compound (s) for antibacterial activity from each plant
- To evaluate guinea pig ileum contractile activity of crude extracts from leaves of each plant (*V. galamensis* and *C. macrostachyus*)
- To identify the most potent crude extract for ileum muscle contractile activity
- To assess the toxicity profile of the leaf extracts of each plant

1.4 Significance of the Study

- Findings of the present study will provide information about the safety of these plants leaves to individuals or communities who use them as traditional medicine.

- Results of the study also provide scientific information to those interested to extract each plant for medicinal purposes.

- Findings of the study also serve as a base line for the development of antimicrobial drugs from these plants with further detailed study of the safety and efficacy in preclinical trials.

- Results of the study also provide information to governmental and other bodies that are involved in conservation of natural resources.

- The study will also serve as a landmark for other researchers that might be interested in doing related researches on these plants.

- The study can also provide information for policy makers and implementers of herbal medicine practices.

Chapter II

Reviews of Related Literatures

2.1 Chemical Screening from Natural Products for Medicinal Uses

2.1.1 Historical Overview of Medicine from Natural Products

A natural product refers to a chemical compound or substance produced by a living organism found in nature that usually has a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design (Clardy and Walash, 2004; Newman and Cragg, 2007). Natural products are believed to possess huge classes of chemicals that serve human and other animals in a number of ways. Among these, medicines (drugs) obtained from them dated back to times immemorial (Waksmundzka-Hajnos *et al.*, 2008).

Humans used chemicals from natural products especially from plants and animals for the purpose of curing themselves or domestic animals since the down of medicine. Large numbers of compounds derived from microorganisms, plant and animal have been used to treat a variety of human diseases including cancer, diabetes, cardiovascular diseases, infections etc. In areas of cancer and infectious diseases for instance, 40% and 75% new drugs respectively were originated from natural products until 2002 (Gilani, 1998; Dickson and Gagnon, 2004; Newman *et al.*, 2007).

Even if the use of natural products as sources of drugs to treat different human ailments had taken a very long time, their involvement in drug discovery took time nearly half a century. Modern drug discovery from natural products became a wide activity in 1950s though started before 1940 and lagged then until 1970. The period between 1970 and

1989 is considered as a time where the discovery of drugs from natural products had reached the highest level (Newman *et al*., 2003; Clardy and Walash, 2004).

After 1990, however, its history had been changed in such a way that the discovery of drugs from these sources declined since many pharmaceutical companies discouraged due to factors mainly related to time and markets (Lam, 2007; Ganesan 2008). Though the time between 1990 and 2000 was a dreadful period in the short history of drug discovery from natural products, good news had appeared after 2000. The years after 2000 are accepted as a time of renaissance for the drug development from natural products since its discovery became re-revolutionized (Gertsch, 2009 in Harvey, 2010).

2.1.2 The Need for Medicinal Plants as a Source of Drugs

Natural products continue to play an important role in the discovery and development of new drugs, as clinically useful drugs, as starting materials to produce synthetic drugs, or as lead compounds from which a totally synthetic drug is designed. Natural and synthetic compounds are, in many respects, harmonizing as a road for new drug discovery since natural products often possess complex structural features not easily accessible by total synthesis (Lam, 2007; Newman *et al.*, 2007).

It has been estimated that 80% of the world population (especially in the developing nations) used to rely on medicinal plants during the late 20^{th} century, which is almost the same these days. The main reasons for such high proportion of medicinal plants usage usually goes with the availability, safety and affordability. Since people using medicinal

plants for different ailments live for long period with a particular plant they knew well that it has little no side effects that they can handle (WHO, 2002).

Most synthetic drugs are unaffordable and less accessible to people in developing countries. In such nations there are few or no mechanisms to lower the cost of western medicines. Seventy percent of drug costs were covered for 80-100% of the population in Europe as opposed to 35% in Latin America and less than 8% in Africa by different bodies (WHO, 1996**;** Pecoul, 1999), which may suggest that the cost for synthetic drugs is high even for people of the developed world. Therefore, it will not be surprising if people tend to depend on on medicinal plants for their relief. However, these are not the only reasons for medicinal plants to be choices of priority by large number of people. Other possible reasons can be overlooked in the following sections.

2.1.2.1 Advantages of Medicinal Plants over Synthetic Drugs

Beside their role to serve as platforms for developing front-line drugs, natural products have been considered as a major tools for making out the logic of biosynthesis (Altmann, 2001 and Newman *et al.*, 2002 in Clardy and Walash, 2004). This could be seen from their involvement in the development of new chemical entities especially between 1981 and 2002. Within this period 5% of the 1,031 approved drugs by the US Food and Drug Administration (FDA) were from natural products, and another 23% were natural-product-derived molecules (Newman *et al.*, 2003).

Comparative studies revealed that natural products have some similarities as well as differences with synthetic drugs both of which make them to be essential in the

development of new and novel drugs. Having similar mode of action and possessing similar chemical entities/functional groups and the ability by which natural products can give principal molecules for synthetic drugs or the later can be produced by modifying the former are their main similarities (Clardy and Walash, 2004; Newman and Cragg, 2007).

Besides, both synthetic drugs and natural products show a minor (≈10%) violation of Lipinski's 'rule-of-five', which states that any drug that has to be administered should have a molecular mass of <500, number of hydrogen-bond donors <5, number of hydrogen-bond acceptors <10 and calculated octanol-water partition coefficient to be <5. These properties are so important for a given drug or target substance because having such values can make it to act effectively on a biological material. The other similarity lies on the fact that natural product libraries have the same hit detection process as synthetic libraries (Lipinski, 2004 in Newman and Cragg, 2007).

As they have similarities to synthetic drugs, natural/medicinal plants products also show differences including the fact that they (1) have high chemical diversity, biological specificity and other molecular properties; (2) have a great number of chiral centers and increased steric complexity; (3) bear high number of oxygen atoms while synthetic drugs tend to contain higher number of nitrogen-, sulfur-, and halogen-containing groups; (4) have less (lower) ratio of aromatic ring atoms to total heavy atoms than synthetic drugs; (5) have higher number of solvated hydrogen bond donors and acceptors with a greater molecular rigidity as compared to synthetic drugs; (6) have libraries with broader distribution of molecular properties such as molecular mass, octanol-water coefficient and

diversity of ring systems; and (7) act on multiple targets while synthetic drugs only on one (Feher & Schmidt, 2003; Clardy and Walash, 2004).

Though medicinal plants are so important in the development of novel drugs both in terms of safety and efficacy, there are some limitations still hindering their application. However, there are a lot of advantages that could outweigh these limitations. The existence of unmatched chemical diversity with diverse biological potency, having a nearly 100-fold hit-rate over synthetic drugs, occupying complimentary regions of chemical spaces, and their ability of using combined techniques make them more advantageous than synthetic drugs. Moreover, it is possible to optimize the regulation of biosynthesis through genome mining in medicinal plants than in synthetic drugs making the production of new derivatives with superior qualities and quantities possible. In addition, the ability being going straight from 'hit' to drug without modification, medicinal plants enable us to understand pathways (mechanism of actions) involved in disease curing processes, which are so difficult in synthetic drugs (Dickson and Gagnon, 2004; Newman and Cragg, 2007).

2.1.2.2 Problems Related to Drug Resistance

One major role that makes medicinal plants a choice of today's source for drug development is their effectiveness against many drug resistance pathogens and other disease causing parasites like helminthes. Drug resistance usually refers to the ability of infectious organisms to withstand the effect of conventional drugs so that continue their effect or damage to the tissue of their host (Sheldon, 2005; Flohr *et al*., 2007).

Although not all disease causing organisms are resistant to a given drug, many of them became resistant especially to antibiotics and antihelminthic drugs. Those pathogens resistant to vancomycin (enterococci bacteria and *Streptococcus pneumoniae*), methicillin (*Staphylococcus aureus*), cefotaxim (*Klebsiella pneumoniae*), ceftazidime (*Pseudomonas aeruginosa*), metronidazole, ampicillin and clarithromycin (*Helicobacter pylori*) and clotrimazole, fluconazole and ketoconazole (*Candida albicans*) are few examples to this end (Nostro, 2006).

Many parasitic helminthes, especially those affecting livestock, developed resistances to anthelminthic drugs such as mebendazole, thiabendazole, pyrantel, levamisole, morantel and ivermectin (Kaplan, 2004). Helminthes that parasitize humans such as *Necator americanus* and *Ancylostoma duodenale* became less sensitive to the benzimidazoles and pyrantel, respectively (De Clercq *et al.*, 1997; Flohr *et al*., 2007).

Though several factors can bring about drug resistance, genetic reversion, mutations, acquisition of genes from other microorganisms, presence of side resistance and lack of universality are widely accepted (Geerts and Gryseels, 2000; Nostro, 2006**)**. These all lead to different mechanisms. For instance, intrinsic impermeability or alterations in the bacterial outer membrane, extrusion of drugs from cells by multidrug resistance (MDR) pumps, the production of drug-inactivating enzymes, and modification of target are the major mechanisms by which bacteria overcome drug action (Sharma *et al.*, 2005 in Nostro, 2006; Sheldon, 2005).

Anthelminthic drug resistance usually occurs by preventing ion channels, interfering with acetylcholine receptors, decreasing the permeability of body wall, preventing uncoupling of oxidative phosphorylation, or inhibition of glucose and arachidonic acid metabolisms. Therefore, since phytochemicals have different pharmacokinetics, medicinal plants possess chemical agents that effectively overcome such problems (Martin, 1997; Geerts and Gryseels, 2000).

2.1.3 Factors that Hinder the Use of Medicinal Plants

Those medicinal plants with aforementioned advantages in using them to treat different ailments as well as in drug development also have some limitations that importantly hinder their application. As in other natural products, drug development from medicinal plants; a) require high skill to build up and maintain a high quality libraries; b) are labor expensive and time consuming; c) requires large scale fermentation; and d) lack methodology for dereplication. These pose a great difficulty to prepare many analogs while synthetics do with in a short period (Mendonça-Filho, 2006).

Other factors that can effectively hinder the use of medicinal plants usually go with both intrinsic and extrinsic situations. Intrinsic factors refer to the genetic makeup of a particular plant claimed to have medicinal property which influences the chemical composition as well as the growth and development of the plant. Since genetic makeup of plants determines not only the presence or absence of chemicals with medicinal properties but also the relative amount of such agents, only those having genes with superior quality for production of high amount of the active constituents can only be selected (Mendonça-Filho, 2006).

Some plants may be chemodemes by which they are similar in phenotypes but different in their genotypes. These plants seem identical in external appearance but differ in their chemical constituents posing a problem in selecting the actual one (Evans, 2009). Species differences, organ specificity, and diurnal and seasonal variation are also among the intrinsic factors determining the quality of medicinal plants for selection (Mendonça-Filho, 2006).

Chemical composition of medicinal plants and hence their medicinal properties can also be affected by external factors such as interference by humans or other organisms, climatic conditions, soil type and even ways of handling them during collection. Adulteration, contamination, drying condition, time of collection, transportation and storage are among external factors to affect obtaining of such agents from medicinal plants (Farnsworth, 1993, Mendonça-Filho, 2006).

2.1.4 The Role of Phytochemical Screening and Bioassay Guided Fractionation in Drug Development from Medicinal Plants

Nowadays, medicines of natural origin are getting attention not only for their high effectiveness and low toxicity but also for the fact that they become the widely used commercial products. In United States alone, the market value of herbal preparations is estimated at several dozen million dollars per year. In order to obtain the expected therapeutic effect, however, there should be a high duty for efficient purity control of plant material, and further for the evaluation of their identity and chemical composition (Waksmundzka-Hajnos *et al.*, 2008).

Considering the threat to mankind by incurable diseases like HIV/AIDS and other new emerging diseases such as SARS, bird flu (H5N1) etc, there is an urgent need to revolutionize and strengthens researches in herbal medicine so as to facilitate drug discovery. Many synthetic drugs owe their discovery and potency as a result of a mimic of structures from isolated products from plants. For example, the drug taxol (paclitaxel), one of the most known powerful anticancer drugs for breast and ovarian cancer, was first isolated from the bark of the yew tree *Taxus brevifolia* and now became an approved drug. Paclitaxel is a mitotic inhibitor used in cancer chemotherapy (Barre *et al.*, 1997; Tavares *et al.*, 2006 in Jagessar *et al.*, 2008).

Drug discovery from higher plants often starts with selection of the plant part followed by chemical screening that in turn followed by one or more biological assays (Mendonça-Filho 2006**;** O'Neill and Lewis, 2009). The selection may be based on various situations including plants used in traditional medical systems such as herbalism, folklore, and shamanism; and the use of databases. Biological assay helps to investigate the bioactive phytocompounds as either a full or partial identification to the level of a family of known compounds (Miles *et al.*, 1998 in Mendonça-Filho 2006).

In pharmacological research and drug development higher plants are important for both providing bioactive phytocompounds that are used directly as therapeutic agents and as starting materials for the synthesis of drugs or as models for pharmacologically active compounds. Between the years 1980 and 2006, for example, among all the drugs investigated 50.4% of them were either purely natural origin, naturally derived or semi-

synthetic (Fig. 1). Therefore, to ensure their availability for the future, regulation on the exploitation and exportation of plants is essential (Newman *et al.*, 2008).

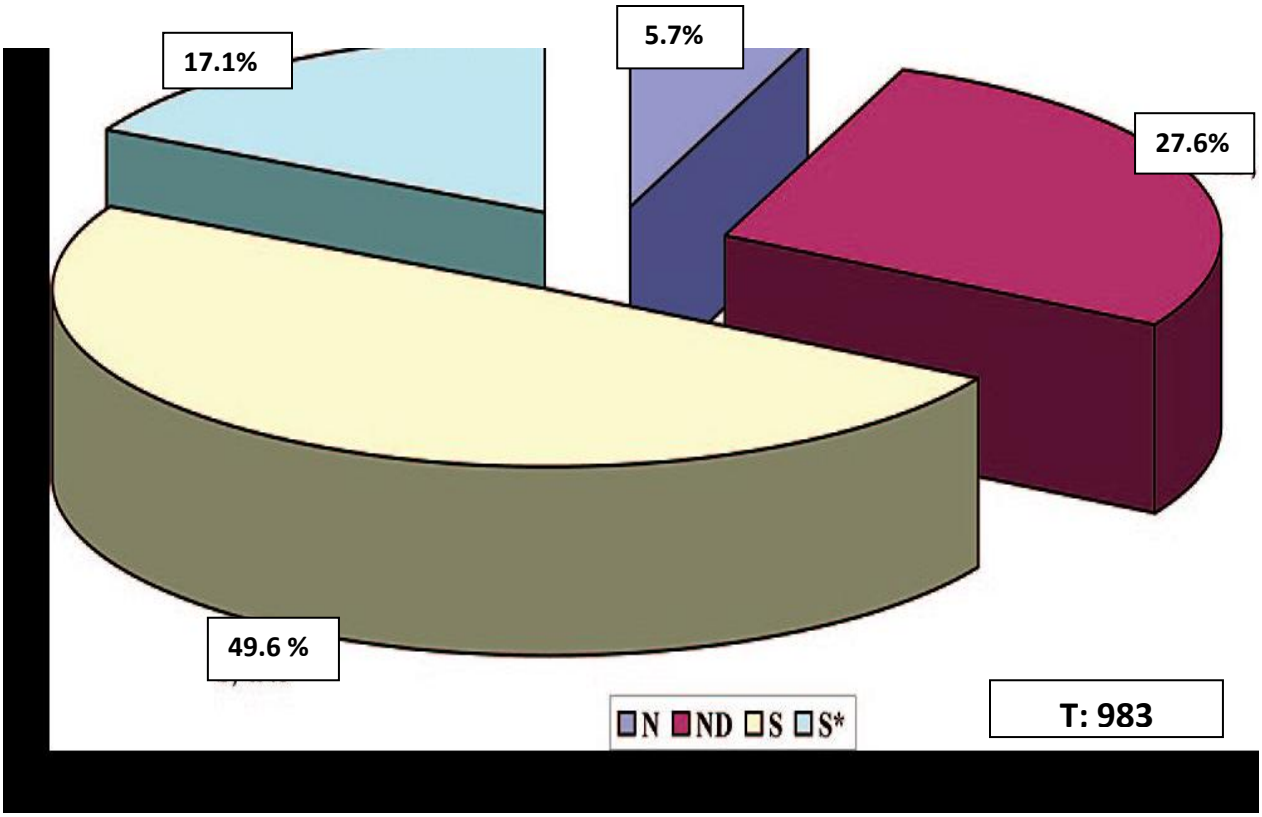


Fig. 1 Percentage of drugs investigated from 1981–2006: T= total drugs investigated; N= natural product; ND= derived from a natural product; S*= semi-synthetic; S= totally synthetic drug (Newman, 2008).

A tremendous step made by biotechnology in the 1970s and 1980s has enclosed the way for emerging of new era for the pharmaceutical industry as a result of which many enzymes and receptor proteins of therapeutic interest were made available in large quantities by recombinant expression. Such mechanism was based on *in vitro* assays and became agreeable to large scales of operation, which brought the concept of high-throughput (HTS) screening standard for lead discovery (Ganesan, 2008).

Techniques of phytochemical screening involve different methods and instruments including chromatography (TLC, HPLC, and Liquid chromatography), MS, NMR and others which usually accompanied with bio-guided fractionation. Bioassay guided technique helps to fractionate and re-fractionate extracts until a pure active agent is isolated from them. Phytochemical screening in general and bioassay guided fractionation in particular helped a lot in isolation of large number of drugs and drug leads from natural products especially from higher plants (Ode *et al.*, 2011).

Bioassay guided fractionation became an important way for drug discovery especially after the foundation of HTS in 1990s. The success of the HTS approach is based on a large and diverse compound collection, which in the early days, comprised in-house archives and natural product extracts. Neither the chemical diversity nor the total number of compounds, were appropriate to feed HTS, and being considered as one of a deficiencies for it. However, the establishment of the science of combinatorial chemistry in the late 1980s and early 1990s with an unanticipated consequence of HTS chemical synthesis tried to combat such deficiencies (Mendonça-Filho, 2006).

Industrially, combinatorial chemistry emerged as the preferred option in past two decades since it had competed with natural product extracts and purified bioactive phytocompounds for HTS resources. Though it has not produced a wealth of high-quality drug candidates, its integration with other mechanisms for lead generation is now rightly considered as the correct strategy to discover novel analogs (Hall *et al.*, 2001 in Mendonça-Filho, 2006). Because the obtained compounds sometimes become more potent than the natural product in showing drug-like properties or may display new biological activities not

seen with the original molecule (Breinbauer *et al.*, 2004 in Mendonça-Filho, 2006; Halpin and Harpury, 2004).

Understanding of the role of specific biological targets in diseases progression, the development of bioassays capable of discovering modulators of the target, the design, miniaturization and automation of bioassays, are some of the key points to employ HTS. It also needs an understanding of the macro-and micro-structure of the biological target so that the sample selection strategy is optimized. Due to capability of engineering custom-built robots, retrieval and bioassay of millions of samples per annum and the development of software systems HTS enable scientists to make sense of the mass of data (O'Neill and Lewis, 2009).

HTS also makes the ability of identifying undesirable or desirable compounds in natural product extract libraries, dereplication, to be rapid and easier. Dereplication is important to prevent the unnecessary use of resources for the isolation of compounds of little or no value for development from extracts used in the screening process giving better chance to focus on samples containing the most promising leads (Hook *et al*., 1997 in Mendonça-Filho, 2006).

2.1.4.1 Phytochemical Screening of Plants for their Antimicrobial Activities

A need to develop new drugs to delay, prevent or treat infectious diseases became apparent following the increasing resistance of bacteria (Eloff *et al.* 2005). Plants are the best candidates of antimicrobial agents since they have been used for centuries to combat infectious diseases without significant resistance development (Cowan, 1999). It is thus

likely to develop extracts from higher plants despite a little success in developing new compounds from them.

After collective preliminary activities (collection, botanical identification and proper drying) during phytochemical screening, the next duty will be extraction of the sample with appropriate solvents. Though different solvents can be used ethanol, methanol and water are the most useful ones during primary extraction of medicinal plants for antimicrobial activities (Mendonça-Filho, 2006).

The primary extraction methods are very variable but the idea is to investigate activities cited in popular use, and to choose the same extraction method. This is especially useful to corroborate the *in vivo* activity found in popular use. For initial drop test screening, the obtained crude extract from primary extraction must be re-suspended in water at a higher concentration (i.e. 1 g mL^{-1}) in order to avoid false negative results. Since crude extracts have complex compositions primary separation is used to facilitate the identification process (Mendonça-Filho, 2006).

For effective result of antimicrobial test micromolecules can be separated from macromolecules (proteins and carbohydrates) by very simple techniques such as ethanol precipitation (30% v/v) with centrifugation (10 000g for 10 min) and filtration systems such as Centricon and Amicon (Millipore). Supernatant separated by this means contains micromolecules that possess antimicrobial activity. To initiate the bioactive phytocompound identification process, bioguided chromatography techniques such as bioautography through solvent separation is essential (Fig. 2). Bio-guided fractionation and

purification confirms previous results leading to isolation of a bioactive phytocompound (Mendonça-Filho, 2006).

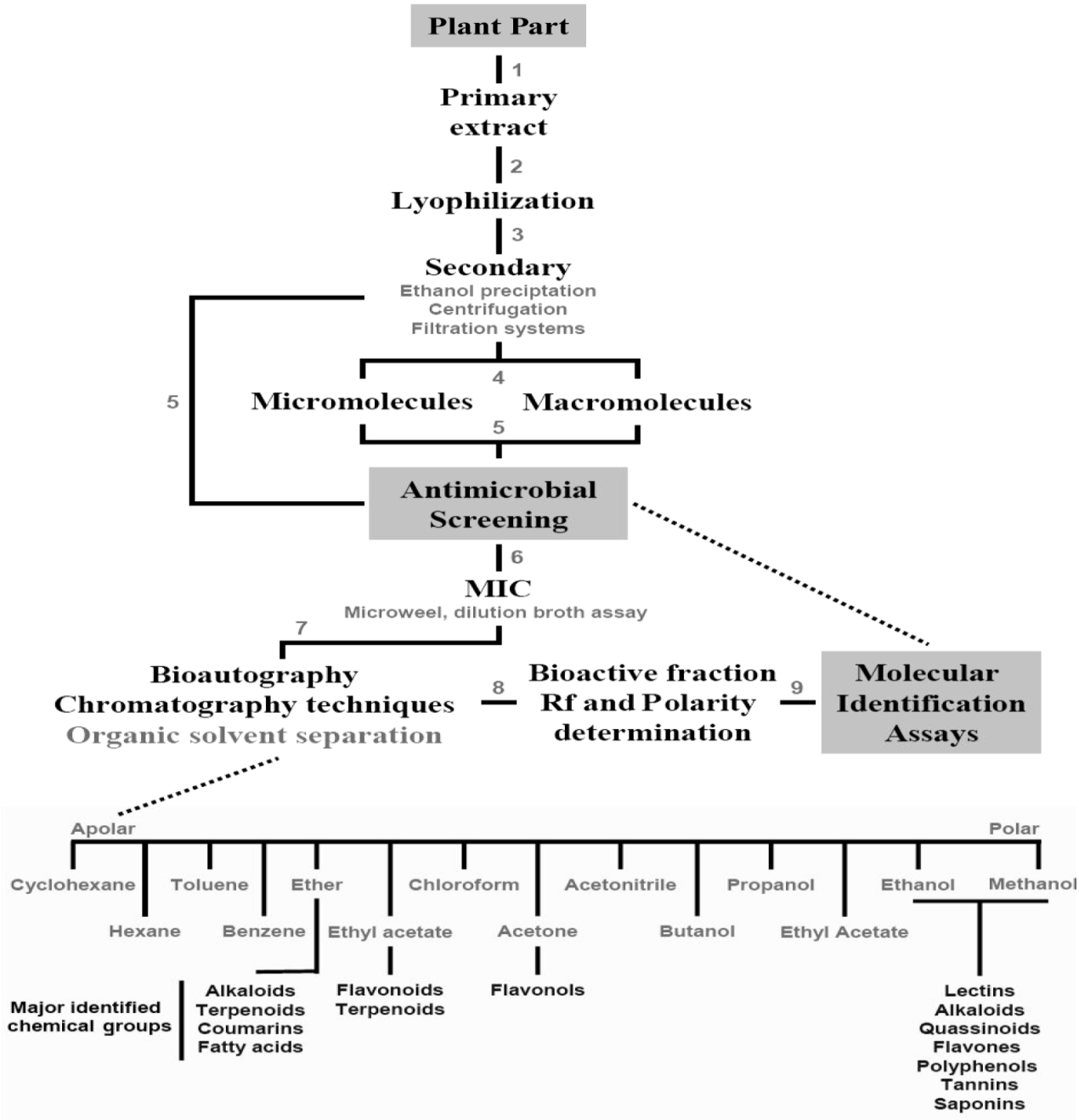


Fig. 2 Flow chart showing standardization of bioactive phytocompounds from extraction to identification (Mendonça-Filho, 2006)

Quite a number of researches have been made confirming the screening of antimicrobial compounds from higher plants. The antimicrobial activities of two flavonoids (apigenin 7-*O*-β- glucoside and apigenin 7-*O*- β- glucuronide) and the *n*-butane extract isolated from the aerial parts of *Launea resedifolia* were tested against eleven bacteria and one fungus. The result of this study showed that these compounds found to be most powerful against *Morganella morgani*; *Streptococcus* Sp; *Enterobacter* Sp. and *Proteus mirabilis* (Moussaoui *et al.*, 2010).

Antimicrobial test made using extracts from *Phyllanthus acidus* against *Escherichia Coli*, *Staphylococcus aureus* and *Candida albicans* showed effective activity with highest activity of ethanol extract followed by ethyl acetate extract then chloroform extract and lastly hexane extract (Jagessar *et al*., 2008).

Different solvents can be employed for primary extraction and bioassay-guided fractionation during antimicrobial activity tests. Crude methanol extract obtained from *Ficus polita* and some of its fractions have showed effective activities against *Salmonella typhi, Escherichia coli and Candida albicans* (Kuete *et al.*, 2011). Although ethanol, methanol and water are the best solvents for extraction of phytochemicals for antimicrobial activity other solvents can also show better results. A compound fractionated from leaf of *Combretum woodii,* combretastatin B5 (Fig. 3) using chloroform as fractionating solvent and acetone as a solvent for primary extraction, showed significant activity against *Staphylococcus aureus* with lower activities against *Escherichia coli* and *E. faecalis* (Eloff *et al.*, 2005).

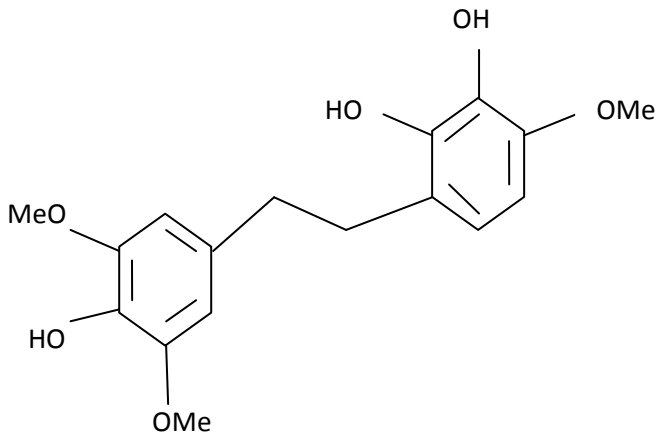


Fig. 3 Structure of combretastatin B5 (2', 3', 4-trihydroxyl-3, 5, 4'-trimethoxyl bibenzyl) [Eloff *et al.,* 2005].

Four compounds: (5S,6R,8aR) – 5 – (carboxymethyl)-3, 4 ,4a, 5, 6, 7, 8, 8a -octahydro-5,6,8a tri-methylnaphthalene carboxylic acid (**1**), methyl 3, 4, 5-trihydroxybenzoate (**2**), enzene-1, 2, 3-triol (**3**) and 2, 3- dihydroxypropyltriacontanoate (**4**) were fractionated from *Entada abyssinica* stem bark using ethyl acetate (Fig. 4). They were tested against Gram (+) bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*); Gram (-) bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi*, *Proteus mirabilis* and *Shigella flexneri*); and yeasts (*Candida albicans*, *C. albicans*, C*. albicans, C. parapsilosis, C. lusitaniae, C. tropicalis, C. krusei, C. guillermondi, C. glabbrata and Cryptococcus neoformans*). The result of this study showed that these compounds have a significant effect on the tested organisms better than the crude methanol extract though they were more effective on the bacteria than the yeasts (Teke *et al.*, 2011).

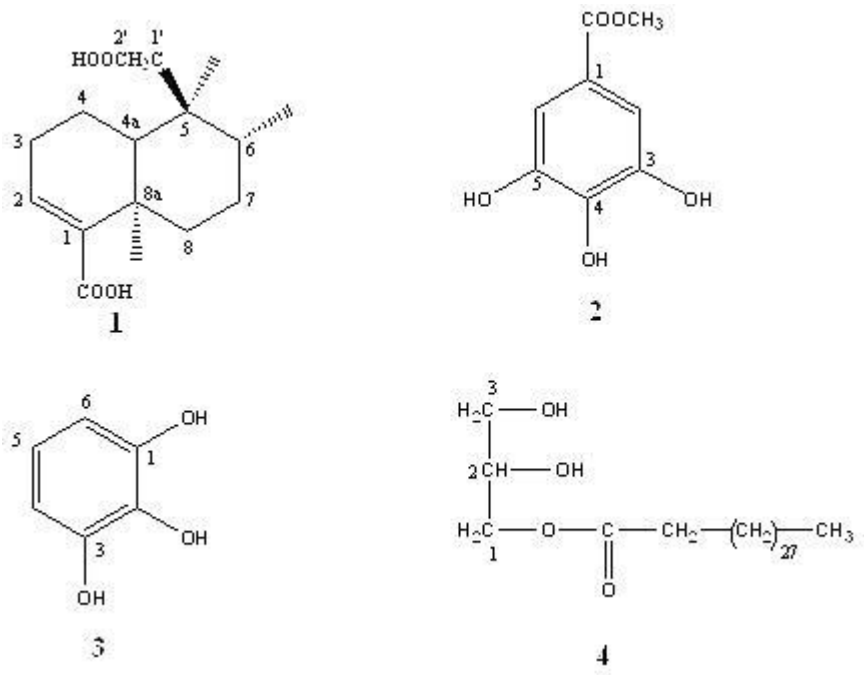


Fig. 4 Examples of compounds isolated from *Entada abyssinica* ethyl acetate fraction (Teke *et al.*, 2011).

Antimicrobial activity of medicinal plants can vary even using similar solvent but with different conditions. Among extracts from the leaves of *Ocimum gratissimum* using cold water, hot water and steam distillation, only the later showed inhibitory effects on the selected bacteria *S. aureus, E. coli, Salmonella typhimurium* and *S. typhi* (Adebolu and Oladimeji, 2005).

The antimicrobial activities of medicinal plants can also depend on the microbial strains. The effect of extracts from *Disthemonanthus benthamianus* (Caesalpiniaceae) and *Zanthoxylum anthoxyloides* (Rutaceae), used as chewing sticks in Nigeria, on some microbes were investigated. *D. benthamianus* extract inhibited the growth of *Pseudomonas aeruginosa*, *Escherichia faecalis* and *Bacillus cereus* with MIC ≤ 2.64 mg mL−1 and *S. aureus* and *S. epidermidis* with MIC ≤ 0.44 mg mL−1. *Z. anthoxyloides* extract was

notably effective against *Candida albicans* and *S. aureus* with the MIC ≤ 2.52 mg/ml and 0.28 mg/ml respectively. However none of them were active against *E. coli* (Adebiyi *et al.*, 2009).

2.1.4.2 Phytochemical Screening of Plants for their Intestinal Muscle Contractile Activities

Many plants extracts have been reported to show anthelminthic properties either by killing the parasites or facilitating their expulsion from the intestine through increasing GI motility. Extracts obtained from some plants were reported to expel out adult worms or their eggs especially that of *Trichuris trichiura* and *Ascaris lumbricoides,* from the intestine of human patients through increasing intestinal motility (Schapiro, 1925 and Hall, 1925 in Behnke *et al*., 2008; Amos *et al*., 2003).

The most persistent human intestinal worm *Trichuris trichiura* was expelled by extracts from *Carica papaya*, *Ficus* spp. and *Ananas comosus* more effectively than by the synthetic drugs. Such expulsion was due to the ability of the extracts to increase intestinal muscle contractility thereby pushing down the parasite (Caldwell *et al*., 1929 and Keiser and Utzinger, 2008 in Behnke *et al*., 2008). Treatment of mice infected with *Trichuris muris* with papaya latex resulted in expulsion of eggs, which were assisted with enhanced intestinal motility (Stepek *et al*., 2006). Extracts from E*laeophorbia drupifera* and *Brassica oleraceae* leaves have shown a dose dependent contraction on intestinal muscle (duodenum, jejunum and ileum) preparation from rabbit (Eno and Azah, 2004) and rat (Jung *et al*., 2000) respectively.

The effect of plant extract on intestinal muscle contraction may vary with the site of the intestine or with its dose. The methanol extract and aqueous infusion of *Cassia sieberiena* leaves increased the contraction of the colon segment of rat intestine at a dose of 2-8 mg/ml but relaxed it when the dose increased. However, none of them contracted (rather relaxed) the ileum (Akomolafe *et al*., 2003). A contractile activity was also observed on ileum and colon of guinea pig by extract from *Elaeagnus angustifolia* and *Raphanus sativus* leaves at low concentration which was abolished at higher concentrations (Gilani and Ghayur, 2004; Mohammed *et al*., 2006).

2.2 Mechanism of Actions of Substances on Target Cells

Substances that have medicinal value whether synthetic or natural believed to choose specific site(s) while acting. These sites of action may differ depending on factors like the type of target cell/tissue, size of the compound, ability of the compound to pass through the membrane of the target cell or to regulate the cascade of processes within the target cell and so on (Hooper, 2001).

2.2.1 Mechanism of Actions of Antimicrobial Agents

The mechanisms of actions of antimicrobial agents on their targets are generally grouped in to five categories. These mechanisms include inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, affecting the cell membrane sterols and inhibition of unique metabolic steps (Hahn, 1979; Cocito *et al*., 1997; Hooper, 2001).

In inhibition of cell wall synthesis antimicrobial agents (especially those on bacteria) inhibit the synthesis of peptidoglycan, a rigid and shape maintaining molecule of the cell, through blocking different steps. Fosfomycin involves in blocking of the changing of N-acetylglucosamine (NAG) in to N- acetylmuramic acid (NAMA), a precursor of peptidoglycan; and β- lactam antibiotics prevent peptidoglycan from forming cross linking with D- alanine. This will then help the antibacterial agents to penetrate in to the cell of a bacterium (Arthur *et al*., 1993; Hooper, 2001).

Many antibiotics are known to show a mechanism of actions by inhibition of protein synthesis through different interferences such as preventing peptide bond formation (chloramphenicol and streptogramins), preventing charged tRNA delivery (tetracycline), preventing polypeptide translocation or movement of mRNA (erythromycin and streptomycin) etc (Chopra *et al*., 1992; Cocito *et al*., 1997). Antibacterial agents such as quinolones, novobiocin and rifampicin are known to exert mechanism of action through inhibition of DNA synthesis (Ng *et al*., 1996).

2.2.2 Mode of Action of Intestinal Muscle Contracting/Relaxing Agents

The mode of actions of substances (agents) that affect the normal state of intestinal muscle contractility thereby its motility generally depend on their effect to mobilize calcium ion and to increase or decrease its intracellular concentration within the muscle cells. To do this they usually interfere with different channels. Those agents that serve as agonist (stimulating contraction) increase intracellular calcium ion concentration, $[Ca^{2+}]_i$ through opening of L-type Ca^{2+} channels either as membrane depolarizing or as receptor agonists (Amrani *et al.*, 1995; Kuriyama *et al.*, 1998). While those known as antagonists (stimulating

relaxation) decrease $[Ca^{2+}]_i$ through either blocking calcium channels or opening potassium channels (Karaki *et al.*, 1997).

Norepinephrine, maitotoxin, ionophores and acetylcholine are among those agonists known to stimulate contraction through membrane depolarization ((Taylor and Stull, 1988; Ohizumi *et al.*, 1983 in Karaki *et al.*, 1997). The agents that comprise receptor agonists (RA) are generally classified as adrenoceptor agonists (ARA), cholinergic muscarinic receptor agonists (CMRA), prostanoids, endothelin-1 (ET-1), histamine, ATP, angiotensin-II, PDGF and neuropeptide-Y (Takayanagi and Onozuka, 1990; Chen and Rembold, 1995). Carbacol and acetylcholine are the most commonly known agonists that act through cholinergic muscarinic receptor to manifest their contractile effect in intestinal muscle (Vogalis *et al*., 1991; Ozaki *et al.*, 1993; Karaki *et al*., 1997).

Chapter III

Phytochemical Screening of *Vernonia galamensis* and *Croton macrostachyus* Leaves

Abstract

*Vernonia galamensis and Croton macrostachyus are groups of higher plants belonging to the genus Vernonia (Asteraceae family) and genus Croton (Euphorbiaceae family), respectively. Previous works have shown that these plants were screened for compounds with medicinal properties against many infectious diseases. The objective of this study was to screen out crude extracts having antibacterial and ileum contractile properties from these plants using successive solvent systems targeting on isolation of active compounds with antibacterial activities. Finely powdered leaves of each plant were subsequently extracted with four solvents of increasing polarity. After conducting preliminary antibacterial tests for each crude extract the one that gave a better result was selected for fractionation and isolation of active compounds. Bioactivity guided fractionation was performed through column chromatography and Prep-TLC. The isolated pure compounds were identified and elucidated using ^{1}H and ^{13}C NMR and MS. Four crude extracts were obtained where only one (VAE from V. galamensis and CEaE from C. macrostachyus) from each plant was selected for fractionation. Fractionation and Prep-TLC of VAE resulted in four active compounds: compound **I** (vernolide), **II** (vernoguinoside), **III** (vernodalin) and **IV** (to be identified). Compounds **I** and **II** are isolated for the first time here from V. galamensis. CEaE gave two active compounds: compound **VI** (methyl laurate) and **VII** (creptoxide). Compound **V** (from VAE) and compounds **VIII** and **IX** (from CEaE) were also isolated none of which showed antibacterial activities. Findings of the present study showed that both V. galamensis and C. macrostachyus contain bioactive compounds like other members of Vernonia and Croton genera respectively. However, identification and structural elucidation of C-**IV** needs further work. Furthermore fractionation using different solvent system may be useful in order to isolate more active compounds.*

Key Words: Phytochemical Screening, Column Chromatography, Prep-TLC, ^{1}H and ^{13}C NMR, Bioactivity, Polarity

3.1 Introduction

Phytochemical screening is a way of extracting active chemical substances from plants using different procedures. A large number of medicinal plants have been successfully screened for their active agents that showed great properties against many infectious diseases. Among these are *V. galamensis* and *C. macrostachyus* which belong to the genus Vernonia and the genus Croton respectively.

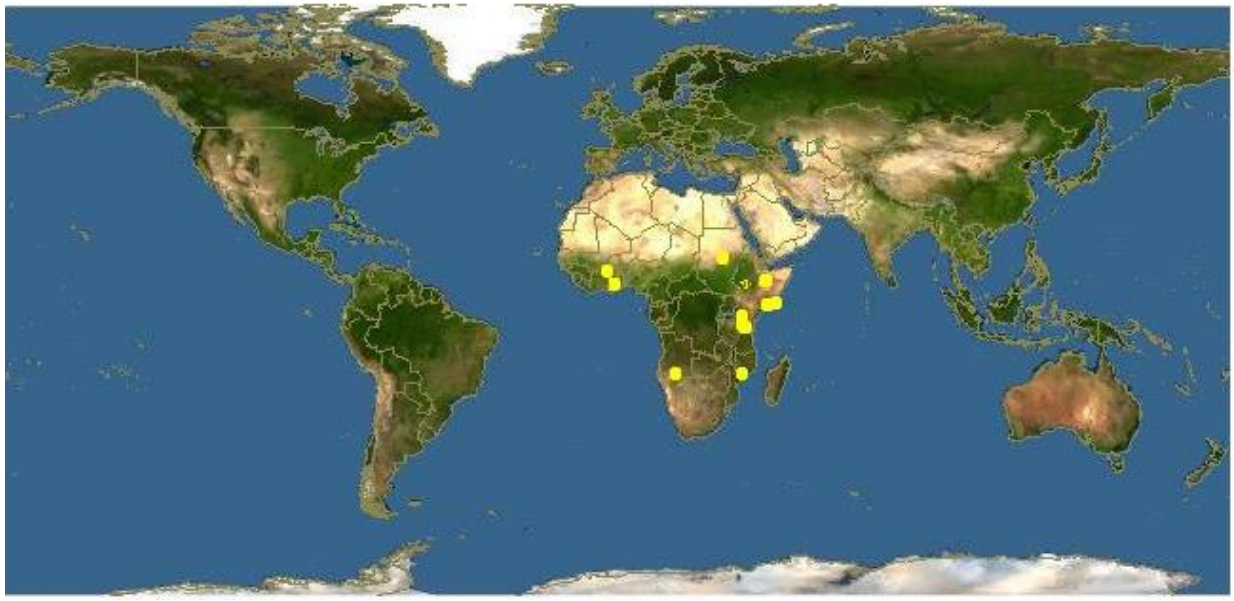
3.1.1 Botanical Description of Genus Vernonia and *Vernonia galamensis*

The genus name for Vernonia is derived after the English botanist William Vernon who had collected and identified it in the late 1600s (Kelley and Jones, 1979 and Quattrocchi, 1999 in Toyang and Verpoorte, 2013). Some species are known as *Ironweed*. Some member species of the genus are edible and are of economic values. They are known for having intense purple flowers though some are grayish. There are numerous distinct subgenera and subsections in this genus. The genus Vernonia comprises about 1000 species of herbs and shrubs in the family Asteraceae (Compositae) (Gilbert 1986; Perdue 1988).

Even though it is highly abundant in tropical regions, Vernonia distribution is cosmopolitan being found both in Old and New worlds. Members of the genus Vernonia grow in a wide range of habitats and climatic conditions. They grow well in areas including tropical forest; tropical savannahs; dry and marsh habitats; wet and even frosty regions. Morphologically member species of the genus are composed of liana, herbaceous, shrubs and trees (Kelley and Jones, 1979 in Toyang and Verpoorte, 2013; Yeap et al. 2010).

Vernonia galamensis Less, Astraceae/Compositae is one member species of the genus Vernonia and has 4 widely distributed sub-species/varieties namely *galamensis*, *petitiana*, *australis* and *ethiopica.* It is a tropical, indeterminate annual plant. It requires a well-drained soil and can grow under low rainfall and marginal conditions that makes it most suitable for dry land farming (Gilbert 1986; Perdue 1988; Baye and Gudeta, 2002).

In Africa the distribution of *V. galamensis* includes Kenya, Ethiopia, Eritrea, Sudan, Somalia, Uganda, Tanzania, Malawi and Angola (Fig. 5). The species is known to naturally grow as a weed in fields or in woodlands under a wide range of agro-ecological conditions of Africa. It prefers to grow at the altitude ranging between 1250 and 2050 m a.s.l, a rain fall less than 600mm and soil pH from 5.1 to 8.5. The most suitable soil type for it is sandy loam with organic matter content ranging from 0.2% to 12.9%. In Ethiopia *V. galamensis* is found in various areas including Arsi, Bale, Borena, Harargie, Shewa, Sidama, Wello and Tigray (Baye, 2000; Baye and Becker, 2005).



(a)

(b)

Fig. 5 (**a**) Geographical Distribution of *V. galamensis* (Yellow marks) in Africa (Source: *Global Biodiversity Information Facility*), and (**b**) the whole plant and its shoot with purple flower (Picture taken from the field; November 2010)

3.1.2 Traditional and Medicinal Values of Vernonia

Vernonia includes several species with different values by which some can be used as food (e.g. *V. amygdalina* and *V. colorata*) while others have ethnomedicinal values (e.g. *V. amygdalina, V. condensate, V. cineria, V. quineensis* and *V. conferta*). Some species such as *V. galamensis* are used industrially for their oil content (Baye and Becker, 2005; Toyang and Verpoorte, 2013). The leaf of several species of *Vernonia* including *V. calvoana*, *V. amygdalina* and *V. colorata* are used as food and/or as ingredient in different areas specially in most West African and Central African countries such as Cameroon, Nigeria and others. In these areas they are known by their common names like bitter leaf, *ewuro, ndole* and *onugbu*. Members of the genus are generally characterized by their sweet and bitter tasted leaves and by having purple or whitish/gray flowers (Perdue 1988; Baye and Gudeta, 2002; Arhoghro, *et al*., 2009).

The medicinal value of the genus can be categorized as ethnomedicinal related for uses to humans, ethnoveterinary for their use in treating livestock and zoopharmacognosy when their use is connected to self-medication of wild animals. According to a review by Toyang and Verpoorte (2013), among 109 species of Vernonia identified by different researchers, 103 are used in ethnomedicine, 12 in ethnoveterinary while two species are used in zoopharmacognosy. Similar species of the genus are reported to treat diseases both in human and animals. *V. amygdalina* is one of the best studied species which is used to control gastrointestinal parasites in both animals and man (Toyang *et al.*, 2007; Yeap *et al.*, 2010; Toyang and Verpoorte, 2013).

V. amygdalina is well known as a medicinal plant with several uses attributed to it including diabetes, fever reduction and recently a non-pharmaceutical solution to persistent fever, headache, stomachache, hypertension and joint pain associated with AIDS by using its infusion being taken as needed. Due to this its leaves are exported from several African countries and purchased in grocery stores aiming to serve African clients for about $1.50/225gm. The roots of *V. amygdalina* have been used for gingivitis and toothache due to its proven antimicrobial activity (Ajibesin *et al*., 2008; Mensah *et al*., 2008; Gbolade, 2009).

V. galamensis is used as an oilseed in East Africa. It is grown in many parts of Ethiopia, especially around the city of Harar, with an average seed yield of 2 to 2.5 tons per hectare. It is reported that the Ethiopian varieties of genus Vernonia have the highest oil content up to 41.9% with 80% vernolic acid, and is used in paint formulations, coatings plasticizers, and as a reagent for many industrial chemicals (Baye and Becker, 2005).

3.1.3 Bioactive Compounds from Vernonia

Vernonia is believed to be the source of many terpenes including monoterpenes and sesquiterpenes with a medicinal potential to treat infections (Zhang *et al.*, 2005; Chaturvedi, 2011). In North America, the most identified species of Vernonia (e.g. *V. altissima*, *V. condensata*, *V. fasciculata* and *V. flaccidifolia*) reported to be effectively serving as a blood purifier and uterus toner and contain sesquiterpene lactone, which enables to prevent atherosclerosis (Elujoba *et al*., 2005; da-Silva *et al*., 2013).

As in the other higher plants terpenoids/terpenes are the largest class of secondary metabolites in the genus Vernonia. They have been produced in large amount due to their great role in interaction of plants with their environment. They generally believed to possess a wide range of biological activities including antibiotic, antimalarial, insecticidal and herbicidal (Zhang *et al.*, 2005; Roberts, 2007; Kaur *et al.*, 2009). Monoterpenes and sesquiterpenes are the main constituents of essential oils of the Asteraceae family including genus Vernonia that is known to play a major role in its medicinal importance (Ogunbinu *et al.*, 2009).

Flavonoids (especially the flavones and phenolic compounds) are one of the major classes of secondary metabolites encountered in Vernonia following terpenoids. They have been isolated from different species of the genus and reported to exhibit various bioactivities. For instance, flavones and phenolic compounds from *V. amygdalina* and *V. cinerascens* have been reported to possess a potent antioxidant and urease inhibitory properties (Igile *et al.*, 1994; Ahmad *et al.*, 2006).

Even though Vernonia has not been generally considered to be a source of alkaloids some of its member species have been reported to contain these compounds. Among these species, *V. ambigua, V. blumeoides, V. oocephala* (Aliyu *et al.*, 2011), *V. amygdalina* (Ayoola *et al.*, 2008; Sharma *et al.*, 2010), *V. cineria* (Maheshwari *et al.*, 2007), *V. colorata* (Neuwinger, 1996), *V. condensate* (Risso *et al.*, 2010), *V. kotschyana* (Deeni and Hussain, 1994) and *V. patula* (Saha and Paul, 2012) are reported for possession of alkaloids. However, the role of these secondary metabolites and their biological activities is not reported yet (Toyang and Verpoorte, 2013).

Bioactive compounds isolated from Vernonia other than alkaloids, flavonoids and terpenoids include coumarins, which have antiprotozoal effects (Oketch-Rabah *et al.*, 1997) and sucrose ester with insecticidal activity (Simonovska *et al.*, 2006 in Toyang and Verpoorte, 2013).

3.1.4 Botanical Description of Genus Croton and *Croton macrostachyus*

Genus Croton is readily recognized by a suite of characters that includes conspicuous stellate or scale like trichomes, narrow or condensed inflorescences of unisexual flowers, watery to colored sap, frequent petiolar glands, and senescent leaves that turn orange before dehiscing (Berry *et al.*, 2005). Furthermore, like other several early branching lineages in subfamily Crotonoideae genus Croton has inaperturate pollen and a strong synapomorphy, which is unusual feature among the angiosperms (Nowicke, 1994).

Croton macrostachyus Delil belong *to* one of the largest genera of family Euphorbiaceae, called Croton under the subfamily Crotonoideae, which comprises more than 1200 species of herbs, shrubs, trees, and occasionally lianas. Croton is ecologically prominent and important elements of secondary vegetation in the tropics and subtropics (Webster, 1993; Govaerts *et al.*, 2000).

C. macrostachyus Delil is shrub or deciduous tree that grows up to 30 m high at an altitude of 200-2000 m a.s.l. The plant is native to Eritrea, Ethiopia, Kenya, Nigeria, Tanzania, Uganda and Madagascar (Fig. 6). It is crown rounded and opens with large spreading branches and has large green leaves that turn orange before falling, with more or less furry texture and slightly toothed margin. *C. macrostachyus* is a dioecious (or at least on

separate shoots) with creamy to yellow-white flowers. Its fruits are green when young and grey at maturity. The name *macrostachyus* is given to it from the Greek words *macro-* (large) and *–stachyus* (spike), referring to "a large spike" (Hedberg *et al.,* 1995; Schmelzer and Gurib-Fakim, 2008).

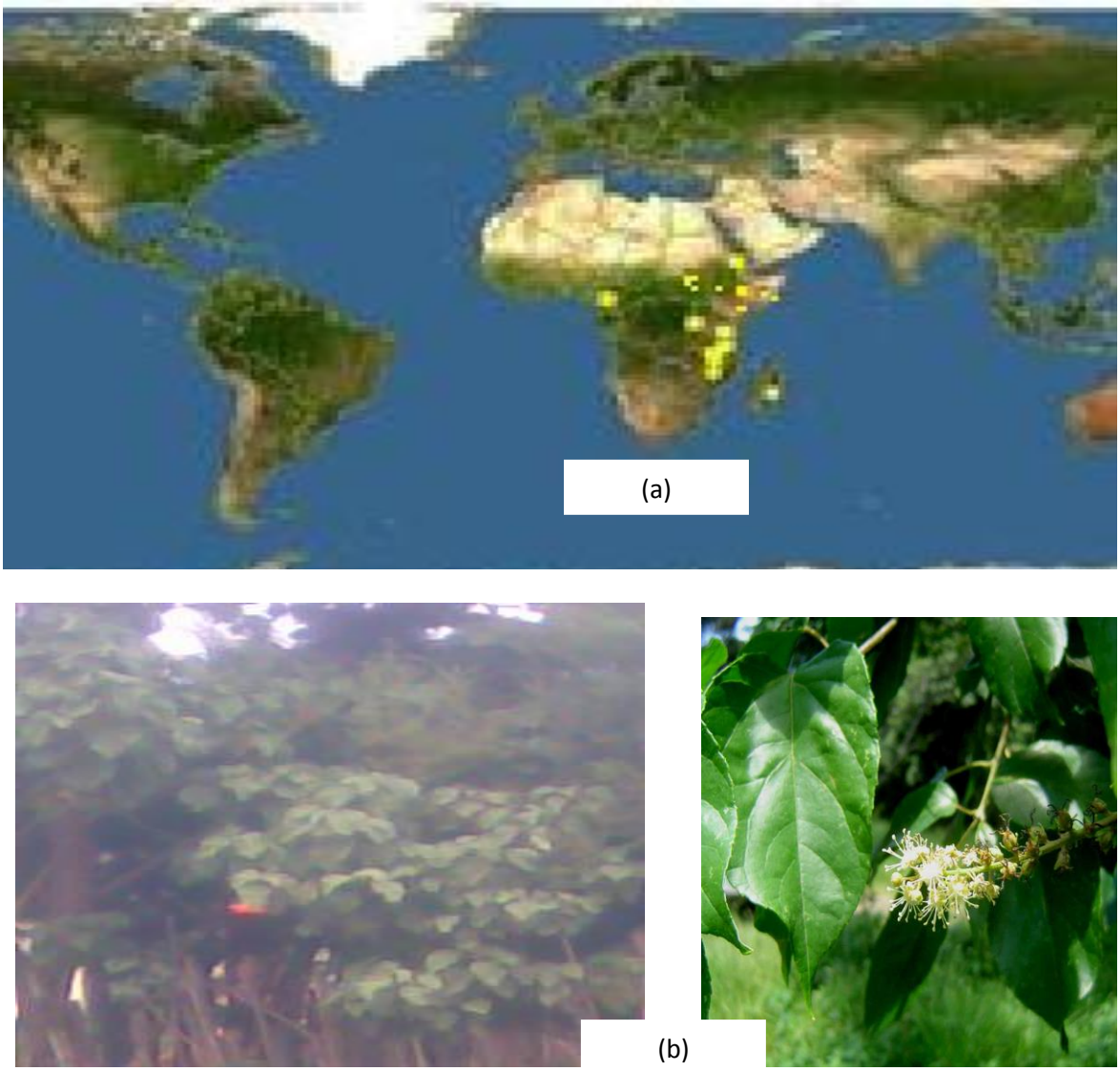


Fig 6 (**a**) Geographical Distribution of *C. macrostachyus* (Yellow marks) in Africa (Source: *Global Biodiversity Information Facility*) and (**b**) the whole plant and its branches showing the broad leaves and spiny flowers (picture taken from the field; November 2010)

3.1.5 Traditional and Medicinal Values of Croton

The genus Croton is used for treatment of several human health problems including diabetes, malaria, dysentery, stomachache, ascariasis and taeniasis in different areas (Kloos *et al.*, 1978; Kasa, 1991; Giday *et al.*, 2007; Mesfin *et al.*, 2009). Abdominal pain, gonorrhea, wounds, ringworm infestation, hemorrhoids, venereal diseases, cough, rheumatism and as a purgative in cases of ascariasis are also among diseases traditionally treated by different species of Croton (Abebe, 1986; Mazzanti *et al.*, 1987; Yirga *et al.*, 2011). There are also reports for the analgesic, anti-inflammatory, mitogenic, molluscicidal and larvicidal activities of extracts from different species of Croton (Tachibana *et al.*, 1993; Albert *et al.*, 2009; Karunamoorthi and Ilango, 2010).

3.1.6 Bioactive Compounds from Croton

The biological activities reported for genus Croton secondary metabolites include anti-hypertensive, anti-cancer, antiplasmodial/antimalarial, anti-inflammatory, antimicrobial, antispasmodic, antiviral, antiulcer, myorelaxant and cytotoxic. Several species of the genus Croton are aromatic, indicating the presence of volatile oil constituents (Salatino *et al.*, 2007; Lima, *et al*., 2010). Essential oils extracted from some member species of genus Croton (e.g. *C. cajucara* and *C. nepetaefolius*) have various bioactivities including antinociceptive, gastroprotective, antimicrobial, antiparasitic, cardiovascular, intestinal, myorelaxant and antispasmodic (Magalhães *et al.*, 1998; Hiruma-Lima *et al.*, 2000; Lahlou *et al.*, 2000; Anthony *et al.*, 2005).

Bioactive compounds isolated from genus Croton include terpenes/terpenoids (monoterpenes, sesquiterpenes, diterpenes and triterpens), alkaloids and flavonoids. For instance, lupeol, a triterpene, is one of a bioactive compound isolated from the genus Croton. Other bioactive compounds such as crotin (a chalcone), crotepoxide (a cyclohexane diepoxide), fatty acids and saponins are also reported from Croton (Salatino *et al.,* 2007; Schmelzer and Gurib-Fakim, 2008).

3.1.7 Specific Objectives

- Extracting crude extracts from the leaves of *Vernonia galamensis* and *Croton macrostachyus*
- Fractionating and isolating the bioactive compounds (antibacterial) from the crude extract of each plant
- Identifying and elucidating the structure of the isolated bioactive compounds using ^{1}H and $^{13}CNMR$ spectra (1D and 2D)

3.2 Materials and Methods

3.2.1 Chemicals and Instruments

All solvents used were analytical grade reagents and included methanol and ethyl acetate from SkyLabs, South Africa; n-hexane and ethanol from SMM instrument, South Africa; while chloroform from Sigma-Aldrich, Germany and acetone from Gaborone Chemicals, Botswana. Sulfuric acid and vanillin were from Sigma-Aldrich, Germany. Thin layer chromatography (TLC) made of aluminum plate coated with silica gel 60 F_{254}, silica gel 100 (0.2 – 0.5 mm) for adsorption of samples, silica gel 60 F_{254} (0.04 – 0.063 mm) for column chromatography, silica gel $HF_{254+366}$ for preparative TLC (Prep-TLC) and sephadex- LH-20 were all from Merck, UK.

Whatmann N^{O} 1 filter paper (27 cm) and Rota-Vapor R-210 with Vacuum Pump V-700, Buchi, were used for extraction and concentration of extracts respectively. UV 254/365, UVGL- 58, Miner-Alight, was used for TLC analysis and cutting bands from Prep-TLC. 1D (^{1}H and ^{13}C) and 2D (DEPT, HBMC, HMQC and COSY) NMR, Brucker, Avance DPX 300 were used to identify compounds.

3.2.2 Collection of Plant Materials

Fresh leaves of *Vernonia galamensis* were collected from Bule-Hora (Hageremariam) Woreda, Borena Zone, about 450 km while that of *Croton macrostachyus* from Dilla Woreda, Gedeo Zone, 360 km from Addis Ababa, Ethiopia, in November 2010 at altitude of about 1870 m and 1550 m a.s.l. respectively. Each plant was identified by the help of botanists and specimens were kept in the Herbarium of Addis Ababa University under voucher number GT 005/2010 and GT 006/2010 respectively. Leaves of each plant were

cleaned of any external contaminants and dried under shade for about 15 days with a careful and continuous follow up to avoid any contamination. Leaves were then ground using a general purpose blender to an appropriate size for extraction with the help of mesh (0.5 mm).

3.2.3 Crude Extraction

Subsequent extraction method was employed to get crude extracts from each plant sample using four different solvents with increasing polarity according to the methods in Rimando *et al*. (2001) and Sigh, (2008). 700 g of *V. galamensis* and 1000 g of *C. macrostachyus* leaves powder were macerated for 24 hrs in n-hexane with the ratio of 1:7 and 1:5 (w/v) respectively. After 24 hrs filtration was made using a double layer filter paper (Whatmann No 1) giving filtrates and residues. Residues were then macerated in acetone (for *V. galamensis*) and ethylacetae (for *C. macrostachyus*) for another 24 hrs with similar ratio as of n-hexane. These processes were repeated using ethanol and methanol for two subsequent days (24 hrs each). All filtrates were concentrated using Rotavapor to obtain crude extracts (Fig 7).

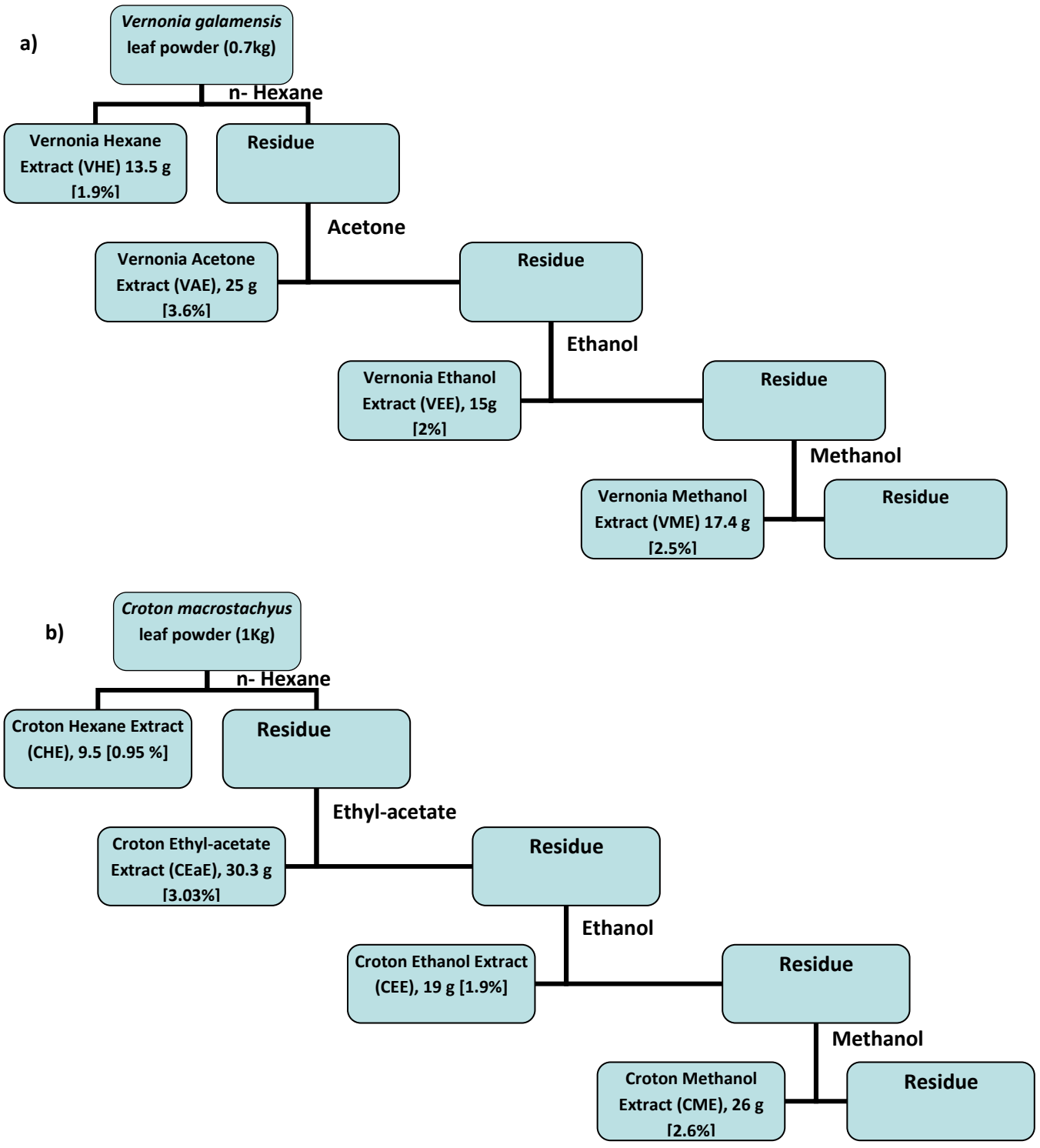


Fig. 7 Flow chart of crude extraction of the leaves of a) *Vernonia galamensis* and b) *Croton macrostachyus*

3.2.4 Bioactivity Fractionation and Isolation of active compounds

A preliminary bioactivity tests (antibacterial and intestinal muscle contractility tests) were made for each crude extract to select the most active ones. Based on the preliminary test VAE and CEaE were selected since they showed the best results and then subjected to fractionation according to methods in Shahverdi *et al*. (2005) and Erasto *et al*. (2006) with slight modifications. Fractionation was done for each using flash column chromatography that was packed with silica gel 60 $F_{254.}$ Samples were adsorbed on silica gel 100 (1:2) and then applied to the column followed by addition of solvents with increasing polarity (100% n-hexane to 100% chloroform then to 100 % ethyl acetate ending with methanol:ethylacetate [1:3]).

Fractions of small volume (50 ml) were collected and then TLC analysis was made for each using silica gel 60 F_{254} TLC plate. Each fraction was spotted and let to develop on TLC paper in appropriate solvent system followed by spraying vanillin sulfuric acid to identify possible bioactive compounds families. Based on their TLC profile fractions of similar bands were combined and concentrated. After concentration each combined fraction was subjected to column chromatography packed with sephadex using chloroform/methanol (1:1) for separation of compounds based on their molecular sizes. Once again fractions were collected and then subjected to TLC analysis.

Fractions of similar profile on the TLC were combined together and prep-TLC was made for each using appropriate solvent system to get bands. Prep-TLC was examined under UV light of 254/365 nm wave length and then each band was carefully cut out and dissolved separately in chloroform and then filtered using a 9 mm Whatmann $N^{\underline{O}}$ 1 filter paper to

separate from the silica gel. The obtained filtrate was left to dry until the solvent completely evaporate, which was then weighed and subjected to NMR analysis (Rimando *et al*., 2001).

3.2.5 Data Presentation

1D spectrum (^{1}H NMR and ^{13}C NMR) was used to get the number of proton and carbon respectively while 2D spectrum (DEPT, HMBC, HMQC and COSY) to confirm and plot results for each compound. The structure of each compound was identified based on available literatures and constructed using Chem-Draw® software.

3.3 Results and Discussion

3.3.1 Extracts and Compounds Isolated from *V. galamensis* Leaves

Subsequent extraction of the leaf powder of *V. galamensis* (0.7 kg) resulted in four crude extracts (Fig. 7a) namely VHE (Vernonia Hexane Extract, 13.5 g), VAE (Vernonia Acetone Extract, 25 g), VEE (Vernonia Ethanol Extract, 15 g) and VME (Vernonia Methanol Extract, 17.4 g). Among these, VAE showed the best antibacterial activity during preliminary test. Bioactivity guided fractionation of VAE resulted in identification of five compounds (compounds **I** – **V**).

Fractionation of VAE using Hexane: Ethylacetae (7:3) resulted in 11 fractions (Fractions **A – K**) among which fraction **E** and fraction **J** were active. Fractionation of **E** using sephadex with chloroform/methanol (1:1) solvent system gave three fractions (E1 – E3). Fraction E_3 (40 mg) was a pure white crystal and resulted in compound **I**. Compound **II** (34 mg), **III** (7 mg) and **V** (5 mg) were obtained from fraction E_2 after Prep-TLC as band 2, 1 and 3 respectively. Compound **IV** (35 mg) is obtained from fraction **J** after separation using sephadex with a 1:1 chloroform/methanol solvent system followed by Prep-TLC developed as band 3. The ^{1}H and ^{13}C readings of those compound that have antibacterial activities are given in Appendix A and their proposed structures are shown in Fig. 8.

Compound **I** (Fig. 8a) is a 19-C with chemical formula $C_{19}H_{22}O_7$ and Mol. Wt. 362. It is sesquiterpenes lactone, vernolide that was previously isolated from *V. amygdalina* by Erasto *et al.* (2006), *V. colorata* by Rabe *et al*. (2002) and other member species of the genus Vernonia as reported by different researchers cited in Chaturvedi (2011) and Toyang and Verpoorte (2013). Compound **II** (Fig. 8b) is a 31-C with chemical formula $C_{31}H_{46}O_8$ and

Mol. Wt. 546.7. It is a steroid, vernoguinoside previously isolated from *Vernonia guineensis* by Tchinda *et al*. (2002) and Donfack *et al.* (2012) except it has hydrogen (H) instead of glucose unit attached to the oxygen at carbon 3. Compound **III** (Fig. 8c) is a 19-C with chemical formula $C_{19}H_{24}O_7$ and Mol. Wt. 364.4. It is a sesquiterpenes lactone, vernodalin formerly isolated from *V. amygdalina* by Jisaka *et al*. (1993) and *V. colorata* by Rabe *et al.* (2002).

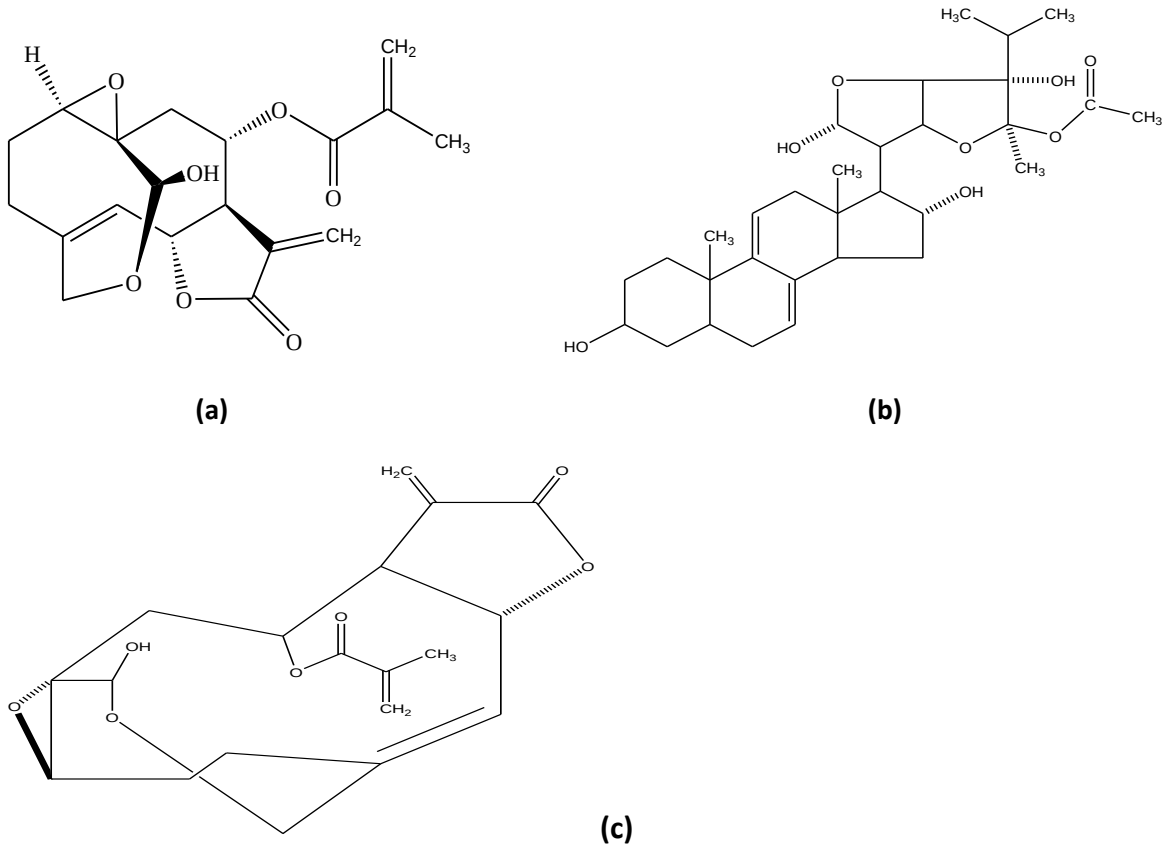
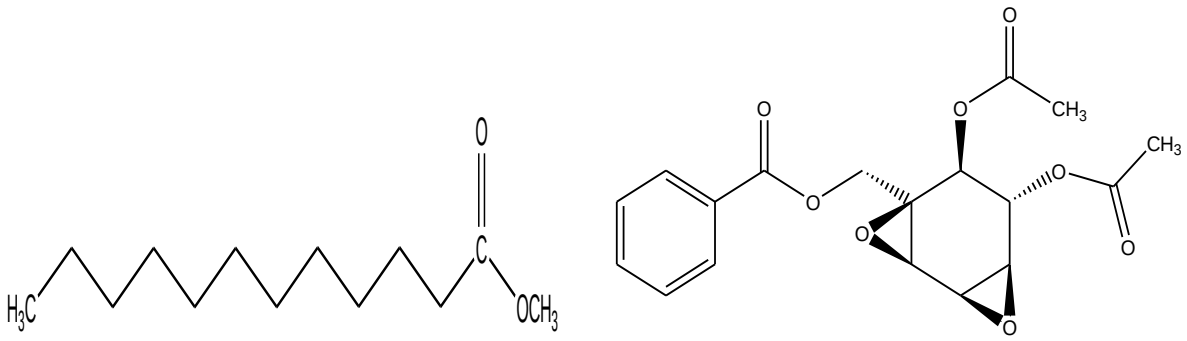


Fig. 8 Proposed structure of compounds isolated from *V. galamensis* leaf extract (VAE): (a) compound **I** (vernolide), (b) compound **II** (vernoguinoside), and (c) compound **III** (vernodalin)

3.3.2 Extracts and Compounds Isolated from *C. macrostachyus L*eaves

Four crude extracts were collected after subsequent extraction of the leaves powder of *C. macrostachyus* (1 kg). These are CHE (Croton Hexane Extract, 9.5 g), CEaE (Croton Ethylacetae Extract, 30.3 g), CEE (Croton Ethanol Extract, 19 g) and CME (Croton Methanol Extract, 26 g) (Fig. 7b). Since CEaE gave the best antibacterial activity during preliminary test, it was subjected to bioactivity fractionation in order to get pure compounds responsible for this activity. Column chromatography of this crude extract resulted in 12 main fractions (fractions **A–L**).



(a) **(b)**

Fig. 9 The proposed structure of compounds isolated from *C. macrostachyus* leaf extract CEaE): (a) compound **VI** (methyl laurate) and (b) compound **VII** (creptoxide)

Fractionation of fraction **F** using sephadex with chloroform/methanol (1:1) solvent system produced two sub fractions F1 and F2. Fraction F1 was a pure compound with a whitish crystal (57 mg) and gave compound **VI**. Prep-TLC made for fraction F2 resulted in three bands in which the third band gave compound **VII** (32 mg), while the second band gave compound **VIII** (17.5 mg). Fraction D also gave two fractions D1 and D2 after fractionation using sephadex. During Prep-TLC separation, faction D2 resulted in four bands among which the third band was pure and gave compound **IX** (4.6 mg).

The proposed structure of these compounds with antibacterial activities is given in Fig. 9 and their ^{1}H and ^{13}C reading is shown in Appendix B. Compound **VI** (Fig. 9a) is a 13-C compound, with a formula of $C_{13}H_{26}O_2$ and Mol. Wt. 214.3. It is a lauric acid derivative known as methyl laurate (methyl dodecanoate). Compound **VII** (Fig. 9b) is an epoxide with 18 carbons. Its chemical formula is $C_{18}H_{18}O_8$ and Mol. Wt. 362.3. The structure of compound **VII** is the same with that of crotepoxide isolated from *Kaempferia pulchra* (Stevenson *et al*., 2007 in Prasad *et al*., 2010) and *Croton macrostachyus* (Coggan *et al*., 1969; Habtamu *et al.*, 2012).

Chapter IV

Antibacterial Activities of *Vernonia galamensis* (VAE) and *Croton macrostachyus* (CEaE) Leaves Extracts

Abstract

*V. galamensis is best known for its traditional use to treat chest-pain, diabetes and gastrointestinal tract diseases, external infection and wound repair while C. macrostachyus is involved in stopping bleeding in child birth, inducing abortion, treating skin infections, management of helminthes and venereal diseases. The objective of this study was to evaluate the antibacterial properties of two crude extracts (VAE and CEaE) and isolated compounds from each on selected pathogenic bacteria (E. coli, S. typhi, S. boydii and S. aureus). Each crude extract was tested at concentrations of 100mg/ml, 200mg/ml and 300mg/ml using disk diffusion method. Each pure compound was also tested using the same method. Two and half mg per ml of standard drugs and 1 ml of 3% Tween 80 were used as positive and negative control, respectively. MIC was also determined for them. Results of the study indicated that both crude extracts showed antibacterial activities at higher concentrations (200mg/ml and 300mg/ml) with MIC of 125mg/ml. Both crude extracts showed significant antibacterial activities, as compared to the negative control (P = 0.00) with highest results against S. aureus and S. boydii at 300 mg/ml that were not significantly different from the drugs erythromycin and ciproflaxin (P = 0.27 and 0.16; 0.39 and 0.61 respectively) with MIC of 125mg/ml. Compounds **I** and **IV** showed growth inhibitory activity against all tested bacteria except E. coli whereas compound **II** was active only against S. typhi and S. boydii. The antibacterial activities of these compounds and that of C-**VI** and C-**VII** were significantly different from the negative control (P= 0.00). The antibacterial activities of C-**II** against S. typhi and S. boydii, and C-**IV** against S. boydii were not significantly different from the drugs chloramphenicol and ciproflaxin (P= 0.69, 0.89 and 1) respectively. C-**VI** showed activity with no significant differences with the respective standard drugs against S. aureus (P= 0.4), E. coli (P= 0.85), S. typhi (P= 0.1) and S. boydii (P = 0.43). The antibacterial activity of C-**VII** was significantly different from the standard drugs against S. typhi and S. boydii (P= 0.01 and 0.02 respectively) but not against S. aureus and E. coli (P= 0.67 and 0.26 respectively). The findings of this study indicated that both V. galamensis and C. macrostachyus contain phytochemicals with antibacterial properties in their leaves, which might validate their traditional uses. However, further study on the mechanism of actions and other related properties is required for the safe use of these plants for related health problems.*

Key Words: Pathogenic bacteria, Antibacterial, Disk diffusion, MIC, Standard drugs, Tween 80, *V. galamensis, C. macrostachyus*

4.1 Introduction

4.1.1 Ethnomedicinal Uses of *Vernonia galamensis* and *Croton macrostachyus*

Those plants especially from the wild have successful defense mechanisms against many diseases and/or insect pests as evidenced by the scarcity of infective diseases in them, which provide an indication for production of effective drugs from them (Konno *et al*., 2004; Stepek *et al*., 2007; Hemaiswarya *et al.*, 2008). Medicinal plants are also preferred for their synergistic effect with some drugs. The synergism of *Epigallocatechin gallate* (from green tea) with both ampicillin and sulbactam that shows effective activity against S*taphylococcus aureus* can be taken as good example (Zhao *et al.*, 2002).

Ethnomedicinal uses of *V. galamensis* include treating of chest-pain, diabetes and gastrointestinal tract diseases in Tanzania, external infection and wound repair in Ethiopia (Chabra *et al.*, 1989; Teklehaymanot and Giday, 2010). In vitro studies carried out on *V. galamensis* indicated that its extract had antiulcer, analgesic, sedative (Johri *et al.*, 1995) and anti-diabetic activities (Autamashih *et al.*, 2011).

C. macrostachyus is reported to have ethnomedical uses in relation to reproductive biology such as stopping bleeding in child birth, inducing abortion and serving as a purgative**.** In Ethiopia, *C. macrostachyus* has folk medicinal uses as purgative and vermifuge, treatment of various skin infections, management of helminthes and venereal diseases and induce abortion (Abate, 1989 in Giday *et al*., 2007; Schmelzer and Gurib-Fakim, 2008).

4.1.2 Antimicrobial Activities of Vernonia

Large numbers of species within the genus Vernonia have been studied *in vitro* for their antimicrobial activities. Out of 109 studied species of Vernonia 23 of them have been reported to show *in vitro* antimicrobial activities (antibacterial, antifungal and antiviral) among which the report for *V. amygdalina* is outstanding (Table 1). Although *V. galamensis* has been reported to treat external infections and wound (Teklehaymanot and Giday, 2010), no *in vitro* test was made for its antimicrobial activity. Antimicrobial compounds isolated from the genus Vernonia include flavonoids, steroids and triterpens (Toyang and Verpoorte, 2013).

Table 1: Summary of the number of in vitro tests performed for antimicrobial activity of different species of Vernonia

Species	No of Ref.	Species	No of Ref.	Species	No of Ref.
adoenisi	3	*glaberrima*	2	*oocephala*	1
ambigua	1	*guineesis*	1	*pogosperma*	3
amygdalina	15	*incana*	1	*polyanthes*	1
auriculifera	2*	*karaguensis*	1	*scorpiodes*	3
blumeoides	1	*kotschyana*	1	*thomsooniana*	1
cineria	5	*lasiopus*	2	*tweediana*	1
colorata	1	*leopoldii*	1	*venosa*	1
cruda	1	*miobicola*	2		

Compiled from Toyang and Verpoorte (2013); * Kiplimo *et al.* (2011)

4.1.3 Antimicrobial Activities of Croton

Different reports have been made available for some compounds isolated from Croton for their *in vitro* antimicrobial activities. Antimicrobial compounds isolated from Croton include flavonoids, alkaloids and terpenes (Junior *et al*., 2011). Sesquiterpene oxide obtained from the bark *C. stellulifer* has been reported to possess antimicrobial property against some bacterial strains such as *Escherichia coli, Staphylococcus aureus, S. epidermidis, Proteus vulgaris*, and fungal species *Candida albicans* and *Aspergillus fumigatus* (Martins *et al.*, 2000). Some other examples of in vitro test for antimicrobial activities of the genus Croton is given in Table 2.

Table 2: Summary of *in vitro* tests for antimicrobial activities of different species of Croton

Croton spp.	**Plant part**	**Compound/Extract**	**MIC Value**	**Reference**
urucurana	Stem Bark	Essential oil	1.25 – 10 mg ml^{-1}	Simionatto *et al.*, 2007
campestris	Stem Leaves	Flavones, Flavonols, Alkaloids, Terpenes	≥ 1024 µg ml^{-1}	Junior *et al.*, 2011
campestris	Leaves	Hexane CE*		Matias *et al.*, 2010
gibsonianus	Leaves	MeOH CE	2.5 mg ml^{-1}	Vinayaka, *et al.*, 2010
zoxburghii	Bark and Leaves	Aqueous and Alcoholic CE	625 µg ml^{-1}	Panda *et al.*, 2010a
pullei	Stem	Hexane and MeOH CE	0.08 – 5mgml^{-1}	Peixoto *et al.*, 2013
zoxburghii	Bark and Leaves	Acetone, Ethanol, MeOH and Aqueous CE	0.3 – 2.5 mg ml^{-1}	Panda *et al.*, 2010b

* CE = Crude Extract

4.1.4 The Bacterial Strains

In this study four bacterial species; *Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Shigella boydii* were selected based on their ability to cause frequent and series infection in human.

Escherichia coli is a Gram-negative facultative bacterium classified under the Family Enterobacteriaceae as gamma-probacteria. It is commonly found in the lower intestine of warm-blooded animals in health and disease (Prescott, 2002; Tortora *et al.*, 2010). Most *E. coli* strains are harmless, but some, such as serotype 0157:H7 can cause serious food poisoning in humans (Vogt and Dippold*,* 2005). *E. coli* K1 strain RS218 is reported to cause neonatal meningitis (Rode *et al.*, 1999; Xie *et al.*, 2006).Within the gut of their host the harmless strains can produce vitamin K2 as a benefit. *E. coli* is not always confined to the intestine but can also survive outside the body of its host making it an ideal indicator to test environmental samples for fecal contamination (Bentley *et al*., 1982 and Fang *et al*, 2000 in Zahera *et al.*, 2011).

Shigella boydii is one of the four species of the genus Shigella that are known to cause bacillary dysentery. It belongs to gamma proteobacteria in the family *Enterobacteriaceae.* It is a Gram-negative, non-motile, non-spore forming, rod-shaped bacterium, closely related to *Escherichia coli. S. boydii* is also known as serogroup C bacteria and believed to be the main cause of diarrhea in developing countries next to *S. dysenteraie* (Kotloff *et al.*, 1999; Tortora *et al.*, 2010). Like other shigellosis causing bacteria this species is adapted to the human intestine (Janda & Abbott, 1998 in Woodward *et al.*, 2005) where the severity

of the illness and the case fatality rate are functions of the age and pre-existing nutritional state of the host in addition to serotype (Chin, 2000 in Woodward *et al.*, 2005).

Salmonella typhi is a gram-negative, rod-shaped bacillus belonging to the Family Enterobacteriaceae (Prescott, 2002). It is anaerobic, unable to form spores, and spontaneously motile. The ability to grow and multiply outside living host organisms, gives *S. typhi* greater survival chances (Gray and Paula, 2002). It is one reason of GI tract inflammation in human causing a series disease called typhoid fever, which is manifested more at early and elder age. It has evolved remarkable mechanisms for persistence in the host that help to ensure its survival and transmission and hence to become human specific pathogen (Mirza *et al.*, 1996; Parry *et al.*, 2005).

Like many Salmonella strains, *S. typhi* is showing a growing resistance to the antibiotics that have been used to combat it. It has also developed multi-drug resistance (MDR) as a part of its fundamental genetics that may help it to become permanently resistant to the drugs (WHO, 2005). Antibiotics like penicillin, cephalosporin, chloramphenicol and co-trimoxazole are readily available for typhoid treatment in developing countries (Mermin *et al.,* 1999). Unfortunately, some strains of *S. typhi* become resistant to all of the above mentioned agents (Newman *et al.,* 2006).

Staphylococcus aureus is a Gram-positive bacterium existing as a normal inhabitant/commensal of the respiratory tract and skin of humans living in anterior nares (Tortora *et al.*, 2010). It is an opportunistic pathogen common in individuals with diabetes, malnourished persons and children, and has been a leading cause of hospital and

community-acquired infections over the past decades (Burnett *et al.*, 1996; Shittu *et al.,* 2006).

Boils, scalded skin syndrome and toxic shock syndromes are some of the diseases caused by *S. aureus* which up on spreading in the body can lead to staphylococcal pneumonia or multi-organ failure due to its high capability to produce toxins (Klodkowaska-Farner *et al.*, 1995; Lappin and Ferguson, 2009). *S. aureus* has become resistant to many antibiotics used since the 1960s including macrolide, cephalosporin, penicillin, glycopeptides, fluoroquinolones and methicillin (Morris *et al.,* 2006; Newman *et al.,* 2006).

4.1.5 Specific Objectives

- To evaluate the antibacterial activities of crude extracts (VAE and CEaE) of leaves of *V. galamensis* and *C. macrostachyus* respectively.
- To test for the antibacterial activities of isolated bioactive compounds from the crude extract of each plant.
- To assess the MIC value of each effective crude extract and the isolated bioactive compound(s).

4.2 Materials and Methods

4.2. 1 Bacteria Used

Four bacterial species selected on the bases of their pathogenicity to cause frequent and series infections in human were used. Standard bacterial strains *Staphylococcus aureus* (ATCC25223), *Salmonella typhi* (ATCC13311), *Escherichia coli* (ATCC23923) and *Shigella boydii* (ATCC9207) were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia.

4.2.2 Antibacterial Activities of Crude Extracts and Isolated Compounds

Disk diffusion method was employed for antibacterial sensitivity test according to methods of Onyeagba *et al.* (2004) and Taiwo *et al.* (2007) with some modification. Absorbent filter paper was used to prepare disks with a diameter of 6 mm each. The paper disks were dispensed in batches in screwed capped bottle and sterilized at 160^0C for 1 h. The four bacterial strains were made to grow and activated on their selective media: S.S agar for *Salmonella typhi* and *Shigella* boydii, Malliton-Salt agar for *Staphylococcus aureus* and Mackonkey agar for *Escherichia coli*. These plates were incubated at 37^OC for 24 hrs.

Few colonies of each strain were transferred with a sterile inoculating loop to a liquid medium (nutrient broth) until turbidity was adjusted to that of McFarland 0.5 turbidity standard. Two groups (four in each) plates containing Muller-Hinton agar were prepared where the four bacterial strains were streaked using sterile cotton swabs (Tadeg *et al.* 2005; Taiwo *et al.*, 2007). One group of plates was used for testing *V. galamensis* leaves extract (VAE) and the other for *C. macrostachyus* leaves extract (CEaE). The external surface of each plate was divided in to six quadrants.

The crude extract of each plant (VAE and CEaE) was dissolved in 3% Tween 80 at concentrations of 100 mg/ml, 200 mg/ml and 300 mg/ml. A total of 24 disks (12 for each plant) were impregnated with 30 µL of the crude extract from each measured concentration. Three of these disks were kept on different quadrant of each plate. On the other two quadrants a susceptibility disk (CAF for *S. typhi*; ERY for *S. aureus;* AMP for *E. coli* and CIP for *S. boydii*) at 2.5mg/ml each and on the rest, a disk immersed in 1ml of 3% Tween 80 were kept as positive and negative controls respectively. All plates were then incubated at 37^{O}C for 24 hrs after which zone of inhibition was measured.

Similar protocol was employed for pure compounds except that only one concentration was tested for each due to availability of insufficient amount for the tests. Compound **I** was tested at a concentration of 20 mg/ml. Compound **II** and **IV** were tested at concentration of 16 mg/ml while compound **III** and **V** were tested at a concentration of 7 mg/ml and 5 mg/ml respectively. Each procedure was repeated three times and average value of zone of growth inhibition was taken to present results. Compounds **VI** to **VIII** was tested at a concentration of 16 mg/ml while compound **IX** at 4 mg/ml using the disk diffusion method.

4.2.3 Determination of MIC of Crude Extracts (VAE and CEaE) and Isolated Compounds

Minimum Inhibitory Concentration (MIC) of crude extracts of each plant (VAE and CEaE) and pure compounds that showed effective antibacterial activities was determined according to methods in Taiwo *et al.* (1999), Adebolu and Oladimeji (2005) and Doughari *et al*. (2008). The disk diffusion method was employed as in the susceptibility tests except the disks were immersed in each prepared concentration of the samples. Each crude extract

was tested at concentrations of 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml and 15.6 mg/ml.

Compound **I** was tested at concentrations of 20 mg/ml, 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml and 0.65mg/ml whereas compounds **II, IV, VI** and **VII** were tested at 16 mg/ml, 8 mg/ml, 4mg/ml, 2mg/ml, 1mg/ml, 0.5mg/ml and 0.25mg/ml to determine the MIC to inhibit the growth of each bacterium. Compounds which did not have effect on a particular bacterial strain were excluded from the MIC test. All procedures were repeated three times in order to confirm results.

4.2.4 Data Analysis

Results were recorded by measuring (in mm) zones of growth inhibition by the controls, each crude extract and each pure compound on each bacterium, and taking the average (Mean ± SEM) value of three tests. One way ANOVA (Tukey) was used to compare results with 95% confidence intervals where P-value less than 0.05 showing significant difference.

4.3 Results and Discussion

4.3.1. Antibacterial Activity of *V. galamensis* Leaves Extract (VAE)

The crude acetone extract of *Vernonia galamensis* leaves (VAE) showed antibacterial activity at concentrations of 200mg/ml and 300mg/ml but not at 100mg/ml (Table 3; Appendix C). In most cases, the crude extract at a concentration of 300mg/ml showed a better antibacterial activity against *S. aureus, E. coli, S. typhi* and *S. boydii* with mean inhibition of 24.3± 0.8, 16.3± 0.6, 22± 0.8 and 27.7± 0.9 mm respectively (Fig. 10; Table 4).

Table 3: Comparative growth inhibitory level of crude extracts of leaves of *V. galamensis* (VAE) and *C. macrostachyus* (CEaE) on tested bacteria as compared to that of the standard drugs

Test Material*	**Effect Level**					**Remarks**
	Conc.	**Test organisms**				
		S. typhi	*E. coli*	*S. aureus*	*S. boydii*	Drugs used: Chloramphenicol (for *S. typhi*), Ampicillin (*E. coli*), Erythromycin (for *S. aureus*), and Ciproflaxin (for *S. boydii*)
Tween 80	1 ml	-	-	-	-	
Drug	2.5 mg/ml	++++	+++	+++	++++	
VAE	100 mg/ml	-	-	-	-	
	200 mg/ml	++	+	++	++	
	300 mg/ml	+++	++	++++	++++	
CEaE	100 mg/ml	-	-	-	-	
	200 mg/ml	++	++	++	++	
	300 mg/ml	+++	+++	++++	++++	

Among the five pure compounds isolated from VAE three of them (Compounds **I**, **II** and **IV**) showed the best antibacterial activities against the tested bacterial strains (Fig. 10). Compound **I** inhibited the growth of *Staphylococcus aureus*, *Salmonella typhi* and *Shigella boydii* with mean inhibition values of 17.7±0.8, 18.7±0.8 and 18±0.9 mm respectively. Compound **II** showed a growth inhibitory effect on *Salmonella typhi* and *Shigella boydii* with mean value of 29±0.8 mm each, but not on the others. The mean zones of growth inhibitions on *S. aureus, S. typhi* and *S. boydii* recorded by compound **IV** were 15.3±0.8, 24.3±0.8 and 30±0.84 mm respectively. Compound **III**, which had a negligible effect on *S. aureus* (5±0.8 mm), and compound **V** did not show any effect on all tested bacteria. Moreover all compounds did not show any effects on *Escherichia coli* (Table 4).

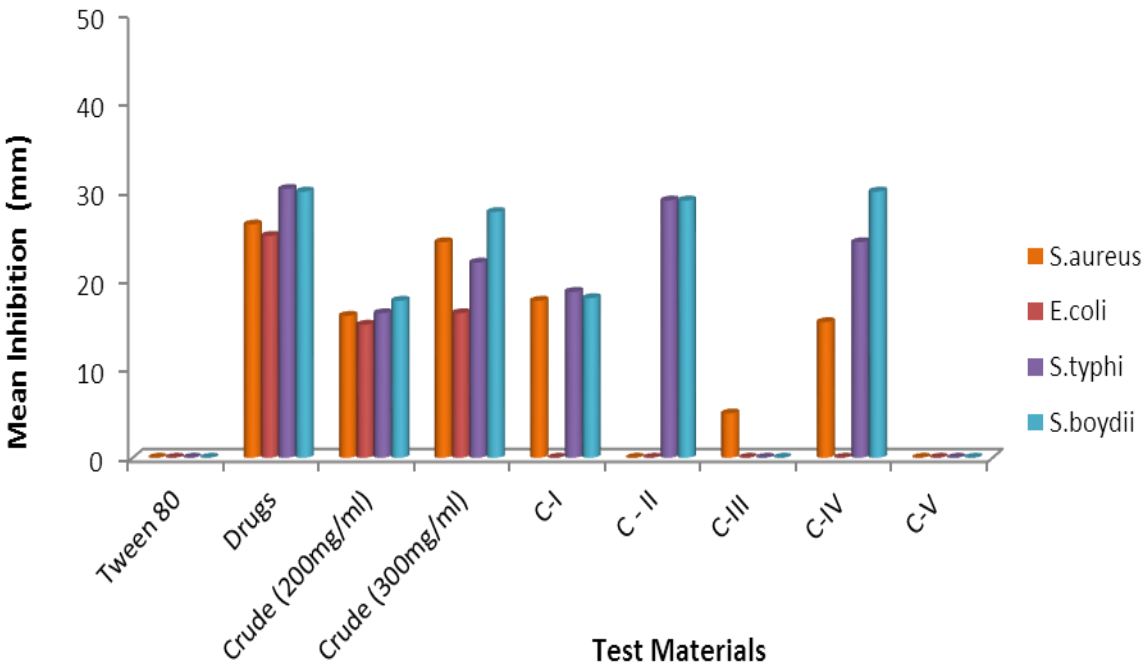


Fig. 10. Mean bacterial growth inhibition of the crude acetone extract of *Vernonia galamensis* leaf (VAE) and pure compounds isolated from it as compared to the standard drugs and the negative control (3% Tween 80).

As shown in Fig. 10, the crude extract of *V. galamensis* leaf (VAE) at a concentration of 300mg/ml and some pure compounds isolated from it showed better inhibitory activities as compared to the negative (3% Tween 80) and the positive (respective drugs) controls. The growth inhibitory activities of the crude extract at a concentration of 300mg/ml are significantly different from that of 3% Tween 80 ($P < 0.05$) but not to the standard drugs ($P > 0.05$) for *S. aureus* ($P = 0.27$) and *S. boydii* ($P = 0.16$) (Table 4).

The significant difference between the crude extract of VAE at 300mg/ml and the negative control (3% Tween 80) and the insignificant difference between the extract and the positive controls (erythromycin and ciproflaxin respectively), indicated that the extract has inhibitory effect on *S. aureus* and *S. boydii* comparable to that of the standard antibacterial drugs. Although the crude extract of VAE at concentration of 200mg/ml and 300mg/ml is significantly different to that of the standard drugs, the significant difference that exists between them and the negative control ($P = 0.00$) may suggest that they have some inhibitory effect to all bacterial strains tested. The MIC values of the crude acetone extract of *V. galamensis* leaves (VAE) were 250 mg/ml for *E. coli* and 125 mg/ml for the rest tested bacterial strains (Table 6).

The growth inhibitory effect of all pure compounds except C-III was significantly different from the negative control ($P = 0.00$). However, only compounds **II** and **IV** were not significantly different from the standard drugs (CAF and CIP), in their effect on *S. typhi* and *S. boydii*.

Compound **II** did show no significant difference to that of chloramphenicol (P = 0.69) in its inhibitory effect against *S. typhi* and there were no significant differences between compounds **II** and **IV** (P = 0.89 and 1.00, respectively) to that of ciproflaxin on their effect on *S. boydii* (Table 4). Compound **I** (vernolide) was active against *S. aureus, S. typhi* and *S. boydii* with MIC value of 2.5mg/ml (Table 7) while compound **II** and **IV** were active against *S. typhi* and *S. boydii* with MIC value of 1mg/ml (Table 8).

In this study both the crude extract (VAE) and some pure compounds from the acetone extract of the leaves of *V. galamensis* showed antibacterial activities comparable with other similar works for other species of the genus Vernonia. The results of this study are in agreement with those of Jisaka *et al.* (1993), Akinpelu (1999), Taiwo *et al.* (1999), Oboh and Masodje (2009), Adesanoye *et al.* (2012), who reported antibacterial activities of *V. amygdalina* and to the work of Toyang *et al.* (2012) who reported antimicrobial property of *V. guineensis*.

In addition, extract obtained from *V. galamensis* seeds have also shown antibacterial activity against a gram-positive bacterium, *Bacillus subtilis* but not on a gram-negative bacterium, *Escherichia coli* (Mbugua *et al*., 2007). This might support the result of VAE that showed little effect, but none of the isolated compounds have an effect on *E. coli* in the present study.

Some of the compounds (**I, II** and **IV**) isolated from the leaf of *V. galamensis* in this study showed antibacterial activities on some of the tested bacterial strains. As reported by Erasto *et al*. (2006) and Rabe *et al*. (2002), vernolide had antibacterial activity with MIC of

0.5 mg/ml on most tested bacteria but not on *E. coli*. The antibacterial activity of compound **I** (vernolide) on tested bacteria except *E. coli* in the present study is in agreement with this report.

Vernoguinoside, previously isolated from *V. guineensis* (Donfack *et al*., 2012) and *V. anthelmintica* (Hua *et al*., 2012) was reported to have antimicrobial activities. The antibacterial activity of compound **II** (vernoguinoside) on *S. typhi and S. boydii* in the present study is in agreement with the results reported by these authors. Compound **III** (vernodalin) had no antibacterial activities on tested bacteria except showing a non-significant effect on *S. aureus*. In a previous work it had been reported that this compound have antibacterial activity with MIC of 8 mg/ml (Jisaka, *et al*., 1993). Though it is not conclusive, the lack of activity by this compound in the present study might be either the tested amount or it may act on bacteria other than those tested.

Table 4: Growth inhibition (Mean ± SEM) of the four bacterial strains by crude acetone extract (VAE) of *V. galamensis* leaves and the isolated compounds.

Test Material	Conc.	*S. aureus*		*E. coli*		*S. typhi*		*S. boydii*	
		Mean± SEM	**P- value**	**Mean± SEM**	**P- value**	**Mean± SEM**	**P- value**	**Mean± SEM**	**P- value**
Tween 80	1ml	0±0.8	0.00	0±0.62	0.00	0±0.84	0.00	0±0.86	0.00
Drug*	2.5mg/ml	26.3 ±0.8		25±0.62		30.3±0.81		30±0.84	
Crude	200mg/ml	16±0.8	0.00	15±0.62	0.00	16.3±0.83	0.00	17.7±0.85	0.00
Crude	300mg/ml	24.3±0.8	0.27	16.3±0.62	0.00	22±0.83	0.00	27.7±0.85	0.16
C-I	20mg/ml	17.7±0.8	0.00	0±0.64	0.00	18.7±0.82	0.00	18±0.85	0.00
C - II	16mg/ml	0±0.8	0.00	0±0.64	0.00	29±0.83	0.69	29±0.84	0.89
C-III	7mg/ml	5±0.8	0.00	0±0.62	0.00	0±0.84	0.00	0±0.84	0.00
C-IV	16mg/ml	15.3±0.8	0.00	0±0.64	0.00	24.3±0.81	0.00	30±0.84	1.00
C-V	5mg/ml	0±0.8	0.00	0±0.62	0.00	0±0.84	0.00	0±0.86	0.00

*Drugs Used: Erythromycin (for *S. aureus*), Ampicilin (for *E. coli*), Chloramphenicol (for *S. typhi*) and Ciproflaxin (for *S. boydii*)

P-value indicate statistical differnces between the effect of each extract/compound with the respective drug used; P-value < 0.05 is taken as significance difference

4.3.2 The Antibacterial Activity of *C. macrostachyus* Leaves Extract (CEaE)

The ethylacetae crude extract of *C. macrostachyus* leaves (CEaE) showed antibacterial activities against all tested bacterial strains at the concentrations of 200 mg/ml and 300 mg/ml but not at 100 mg/ml (Appendix D; Table 3). The MIC of this extract was 125 mg/ml to all bacterial strains tested (Table 6). The mean zones of grwoth inhibitions by CEaE at 200 mg/ml againts *S. aureus, E. coli, S. typhi* and *S. boydii* are 20.3±0.6, 15.7±1.5, 18±1.0 and 16±1.0 mm whereas 25.7±0.6, 18.3±1.2, 20±1.0 and 26.3±1.23 mm at 300 mg/ml respectively.

Out of the four compounds isolated from CEaE only compounds **VI** (25.7±0.6, 21±1.0, 25.3±0.6 and 26±2.0) and **VII** (26±1.0, 20±1.0, 23±2.0 and 22.3±1.5) showed antibacterial activities against all tested bacteria while compounds **VIII** and **IX** lack any effect (Fig. 11). The growth inhibitory effect of the crude extract of *C. macrosyhthachus* leaves (200 mg/ml and 300 mg/ml), compounds **VI** and **VII** was significantly different from the negative control, Tween 80 (P = 0.00). Compound **VI** showed better inhibitory activities against all bacteria than compound **VII** with respective values of 25.7, 21, 25.3 and 26 mm (Table 5).

The MIC value of compound VI is 0.5 mg/ml for all bacterial strains tested except *E. coli* (1 mg/ml). The similar MIC value (0.5 mg/ml) was recorded for coompound VII on *S. typhi* and *S. boydii* which was 1 mg/ml for *E. coli* and *S. aureus* (Table 8).

Although there is significant difference between the crude extract (CEaE) at a concentration of 200 mg/ml and the respective drugs in their effect on all tested bacteria (P = 0.00), it showed no significant differences with the antibiotic drugs erytromycin (P = 0.39) and ciproflaxin (P = 0.61) in its effect on *S. aureus* and *S. boydii* respectivelly at 300 mg/ml (Table 5).

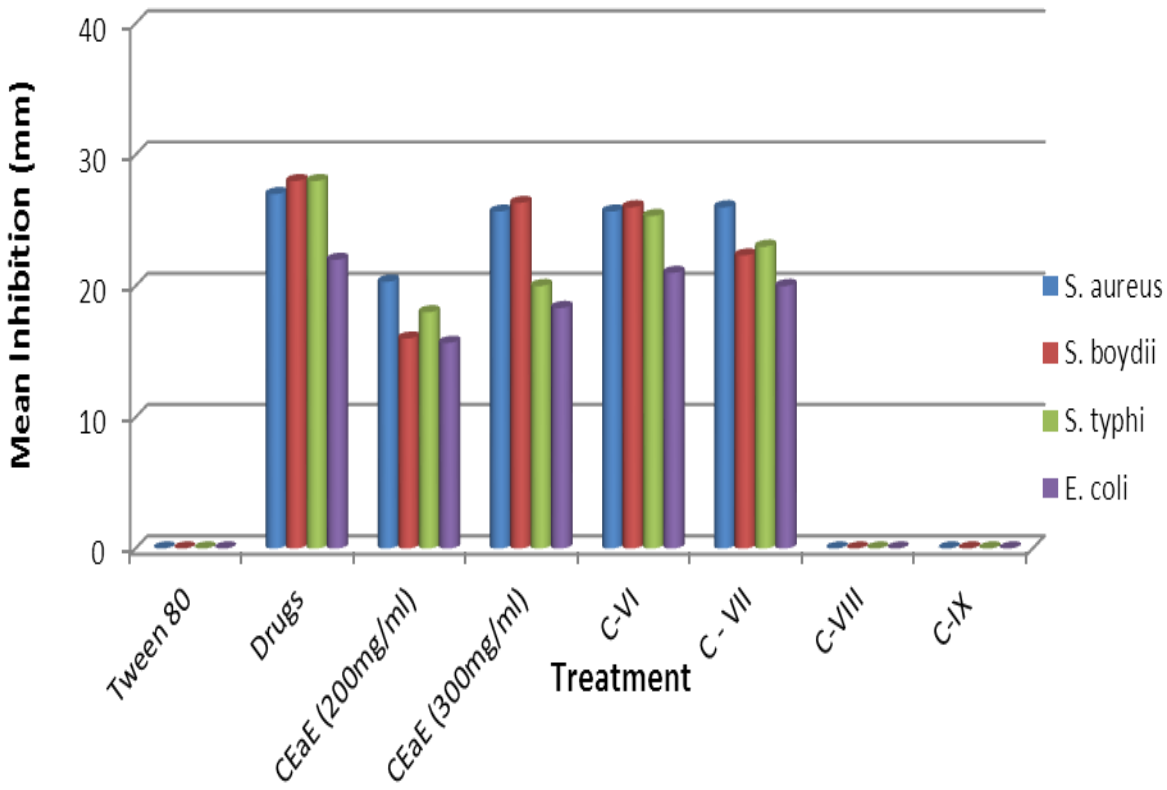


Fig. 11 Mean bacterial growth inhibition of the crude ethyl acetate extract of *Croton macrostachyus* leaves (CEaE) and pure compounds isolated from it as compared to the standard drugs and the negative control (3% Tween 80).

Table 5: Growth inhibition (Mean ± SEM) of the four bacterial strains by crude ethyl acetate extract (CEaE) of *C. macrostachyus* leaves and the isolated compounds**.**

Test Material	Conc.	*S. aureus*		*E. coli*		*S. typhi*		*S. boydii*	
		Mean± SEM	**P- value**	**Mean± SEM**	**P- value**	**Mean± SEM**	**P- value**	**Mean± SEM**	**P- value**
Tween 80	1ml	0±0.0	0.00	0±0.0	0.00	0±0.0	0.00	0±0.0	0.00
Drug*	2.5mg/ml	27±1.0		22±1.0		28±1.0		28±1.0	
Crude	200mg/ml	20.33±0.6	0.00	15.67±1.53	0.00	18±1.0	0.00	16±1.0	0.00
Crude	300mg/ml	25.67±0.6	0.39	18.33±1.16	0.01	20±1.0	0.00	26.33±1.15	0.61
C-VI	16mg/ml	25.67±1.2	0.40	21±1.0	0.85	25.33±0.58	0.10	26±2.0	0.43
C -VII	16mg/ml	26±1.0	0.67	20±1.0	0.26	23±2.0	0.01	22.33±1.53	0.02
C-VIII	16mg/ml	0±0.0	0.00	0±0.0	0.00	0±0.0	0.00	0±0.0	0.00
C-IX	4mg/ml	0±0.0	0.00	0±0.0	0.00	0±0.0	0.00	0±0.0	0.00

*Drugs Used: Erytromycin (for *S. aureus*), Ampicilin (for *E. coli*), Chloramphenicol (for *S. typhi*) and Ciproflaxin (for *S. boydii*)

P-value indicate statistical differnces between the effect of each extract/compound with the respective drug used; P-value < 0.05 is taken as significance difference

Unlike VAE, CEaE and some compounds isolated from *C. macrostachyus* leaves showed antibacterial activities on all bacterial strains tested. Similar antibacterial activities have been reported for other members of the genus Croton in previous times. Crude extracts obtained from the leaves and stem of *C. macrostachyus* had been reported to show effective inhibitory activities against both Gram negative and Gram positive bacteria (Taniguchi and Kubo, 1993). The crude hydro-alcoholic extract of *C. campestris* leaf has been reported to show antibacterial activities on *S. aureus* and *E. coli* (Junior *et al*., 2011). Martins *et al*. (2000) had reported that essential oil from *C. stellulifer* have growth inhibitory activities against *E. coli, S, aureus, S. epidermidis* and *Streptococcus faecalis.* The crude methanol extract from *C. pullei* has also shown inhibitory activity of *S. aureus* (Peixoto *et al*., 2013).

In this study, the crude extract of *C. macrostachyus* leaves has the least activity against *Escherichia coli* which can be supported by Panda *et al*. (2010a) that had reported similar results for the aqueous and alcoholic extracts of *C. roxburghii* having a higher antibacterial against *Staphylococcus aureus* than *E. coli*. The antibacterial test performed on *Staphylococcus aureus, S. epidermidis, Escherichia coli, Bacillus subtilis* and *Salmonella setubal* using essential oil of *C. urucurana* stem bark convince that this plant has antibacterial phytochemicals (Simionatto *et al*., 2007).

The works mentioned so far may serve as supportive evidences for the presence of phytochemicals with antibacterial property in *C. macrostachyus* leaves reported in the present study. The better antibacterial activities of the crude extract of *C. macrostachyus*

leaves against *S. aureus* and *S. boydii* than *S. typhi and E. coli* at concentration 300 mg/ml, in the present study, might suggest that the extract has a selective property and active at high concentration in its antibacterial activity.

In the present study, among four isolated compounds from *C. macrostachyus* leaves, two of them (compounds **VI** and **VII**) showed antibacterial activities against all tested bacterial strains. Compound **VI** (methyl laurate) showed the best antibacterial activities in this study as other lauric acid derivatives such as lauric acid carbohydrate esters do in the previous study (Nobmann *et al*., 2009).

In addition, many other lauric acid derivatives such as D-laurate A, T-laurate and lauroysucrose have been reported for their effective antimicrobial activities (Riháková *et al*., 2001). Moreover, lauric acid derivatives such as methyl caprate and methyl laurate have been reported to involve in manufacturing of detergents and surfactants (Thompson *et al*., 1990; Cermak and Isbel, 2004) due to their ability to fight microbes. These could support the positive result of antibacterial activity observed for methyl laurate in the present work. Crotepoxide isolated from *Kaempferia rotunda* was reported to act against pneumonia, bronchitis and dysentery (Partha *et al*., 2007 and Lotulung *et al*., 2008 in Prasad *et a*l., 2010). The antibacterial activity of compound **VI** (crotepoxide) observed in the present study might go with its ability to treat such diseases.

The mechanism of action of antibacterial agents is believed to depend mostly on their lipophilicity and water solubility, which may give them the ability to penetrate the bacterial cell. Terpenes and steroids have the ability to alter the permeability of bacterial cell

membrane due to their lipophilic features, which play crucial role in their antimicrobial effects (Trombetta *et al*., 2005). Since active compounds isolated from the leaves of *V. galamensis* in the present work are terpenes and steroid, their antibacterial activities might be due to these features. Increased membrane permeability had also been reported for some lauric acid derivatives antimicrobial agents such as lauric acid carbohydrate esters, D-laurate A and T-laurate (Riháková *et al*., 2001; Nobmann *et al*., 2009). The antibacterial activities of methyl laurate isolated from the leaves of *C. macrostachyus* might also be due to its ability to penetrate the bacterial cell membrane. The crude extract of *V. galamensis* leaves was active but none of the isolated compounds have shown activities against *E. coli*, which might suggest that the crude extract has a synergic effect on this bacterium.

Table 6: Minimum Inhibitory Concentration (MIC) of crude extracts of *V. galamensis* (VAE) and *C. macrostachyus* (CEaE) leaves

Test Bacteria	**Activity**					
	500mg/ml	250mg/ml	125mg/ml	62.5mg/ml	31.25mg/ml	15.625mg/ml
Salmonella typhi	++	+	+	-	-	-
Shigella boydii	++	+	+	-	-	-
Staphylococcus aureus	++	+	+	-	-	-
Escherichia coli	+	+	+*	-	-	-

*only for CEaE

Table 7: Minimum Inhibitory Concentration (MIC) of Compound **I** (vernolide)

Test Bacteria	**Activity**						
	20mg/ml	10mg/ml	5mg/ml	2.5mg/ml	1.25mg/ml	0.65mg/ml	0.325mg/ml
Salmonella typhi	++	+	+	+	-	-	-
Shigella boydii	++	+	+	+	-	-	-
Staphylococcus aureus	++	+	+	+	-	-	-
Escherichia coli	NT	NT	NT	NT	NT	NT	NT

*NT= Not Tested

Table 8: Minimum Inhibitory Concentration (MIC) of Compounds **II, IV, VI** and **VII**

Test Bacteria	**Activity**						
	16mg/ml	8mg/ml	4mg/ml	2mg/ml	1mg/ml	0.5mg/ml	0.25mg/ml
Salmonella typhi	+++	++	++	+	+	+[(a)]	-
Shigella boydii	+++	++	++	+	+	+[(a)]	-
*Staphylococcus aureus****	+++	+++	++	+	+	+[(b)]	-
*Escherichia coli***	+++	+++	++	+	+	-	-

***Not tested for compound **II** ****Not tested for compounds **II** and **IV** [(a)]only compounds **VI** and **VII** [(b)]only for compound **VI**

Chapter V

Guinea Pig Ileum Contractile Activities of *Vernonia galamensis* and *Croton macrostachyus* Leaves Crude Extracts

Abstract

Many species of both Croton and Vernonia have been reported to contain phytochemicals with the ability to increase or decrease GI motility through contracting or relaxing its muscle. C. macrostachyus and V. galamensis are claimed to be medicinal in treating GI problems like expelling out worms (parasites), reliving constipation and the like. The objective of this work was to test the effect of crude extracts from each plant on contraction of guinea pig ileum. Six crude extracts (CEaE, CEE and CME from C. macrostachyus; VAE, VEE and VME from V. galamensis) were tested for their effect on ACh (80ng/ml) induced guinea pig ilial muscle contraction at organ bath concentrations of 80µg/ml, 160µg/ml and 320µg/ml. CEaE, CEE, VAE and VEE showed contractile activities while CME and VME relaxation of guinea pig ileum in a dose dependent manner. At 160µg/ml and 320µg/ml organ bath concentrations tensions (g) recorded by CEaE (3.5±0.03, 4.7±0.04 and 4.9±0.05), CEE (4.7±0.05 and 4.9±0.05) and VAE (3.0±0.03 and 3.1±0.04) respectively, were higher with significant differences from the control ACh (P=0.00). At 80µg/ml, however, CEaE, CEE, CME and VAE showed a non-significant contractile effects as compared to ACh (P=0.1, 0.08, 0.1 and 0.07 respectively). CEaE, CEE and VAE increased the ACh induced contraction of guinea pig ileum dose dependently (8.6%, 31.9% and 34.7% for CEaE; 13.2%, 28.9% and 32.7% for CEE; 13%, 33.3% and 35.5% for VAE). CME completely reduced the ACh induced guinea pig ileum contraction by 20% at the maximum concentration (320µg/ml). However, VEE showed increased contraction at low while decreasing it at high concentration. VME reduced such contraction dose dependently by 36%, 50% and 200%, suggesting that it has a relaxant effect. Findings of the study revealed that each plant possess chemical agents having both contractile as well as relaxing effects varying with the type of the solvent used and concentration, which might validate their traditional uses in treating GI disorders.

Key Words: Guinea pig, Ileum, GI motility, Contraction/Relaxation, ACh, Tension, Crude Extracts, *V. galamensis, C. macrostachyus*

5.1 Introduction

There is a relationship between intestinal muscle contractility and its motility which can be intensified as the contractility increased. Intestinal motility generally depends on the extent of its muscle contraction and relaxation. In other word, high intestinal motility is governed by increased muscular contraction while low intestinal motility is often associated with reduced contraction (relaxation) of the associated muscles. One major advantage of increased intestinal motility is to aid the movement of intestinal contents through the lumen (Guyton and Hall, 2006; Ohama *et al*., 2007).

The small intestine and its motility have long remained interesting. The discovery of the peristaltic reflex at the beginning of the 20th century and the first description of the interdigestive migrating motor complex in 1969 prompted a number of researchers to conduct studies into the patterns of small intestinal motility and the mechanisms involved in its regulation, but the findings long failed to interest the clinician. In more recent years, however, the bridge between basic science and clinical management of patients with disordered small intestinal motility has become stronger. Many of the studies on small intestinal motility published during the past years reflect this fact (Collins *et al*., 2001; Smout, 2004; Tanović *et al*., 2006).

Abnormal intestinal motility may lead to obstruction (blockage) associated with symptoms of bloating, pain, nausea and vomiting. Abnormal intestinal motility usually occurs due to either weak or strong but disorganized (unsynchronized) contraction of the muscles, which hinder the downstream movement of its contents. The main causes of abnormal intestinal

motility are IBS (irritable bowel syndrome), bacterial overgrowth, and parasitic infection (Khan and Collins, 2005; Ohama *et al*., 2007).

Intestine is one of the most ideal places where infection and inflammation are frequently observed since it can highly be contaminated with pathogenic organisms and/or inflammatory mediators coming with food or others. Various studies have shown that there is a significant correlation between intestinal motility and infection and/or inflammation (Vallance *et al.*, 1997). Moreover, there has been a great link between mucosal inflammation and GI motor dysfunction, which is caused by enteric infection both in human and other mammals (Venkova *et al.*, 1999). In vivo test conducted on rats have shown that intestinal muscle contraction was increased after infection by *Hymenolepis dimenuta* (Dwinell *et al.*, 1998).

Infection of GI tract of murine by *Schistosoma mansoni* resulted in altered intestinal motility with increased muscle contraction during its chronic stage (Moreels *et al.*, 2001). Increased intestinal muscle contractility was also observed during infection of mice by *Trichinella spiralis* that is associated with high production of T-helper (Th-2) cells (Vermillion and Collins, 1988; Vallance *et al.*, 1997). These and other studies suggested that the normal contraction of intestinal muscle can be affected by factors such as infection especially by worms. Such intestinal contraction is believed to be a natural response of a GI tract that might help to expel out infectious agents. One of such a response is due to cytokines like IL-9. Vaccination of *Trichuris muris*-infected mice with anti-IL-9 can prevent

the expulsion of the worm (Richards *et al*., 2000) suggesting that activation of such cytokines can enhance worm expulsion.

The effect of medicinal plants extracts on intestinal worms can be in two general ways; by killing or paralyzing and expelling out of the intestinal lumen alive or dead. Expulsion of worms from the intestine is dependent on the extent to which an extract is able to increase the contractility of the muscle because hypercontractility leads to high level of peristaltic and other waves resulting in efficient pushing down of worms (Richards *et al*., 2000; Despopoulos & Silbernagl, 2003; Duthie and Gardiner, 2004).

Therefore, the use of medicinal plants that can normalize intestinal muscle contraction will maintain its motility thereby helping the intestine to push its contents and even to expel out infectious agents. Ethnobotanical studies conducted in different parts of Africa including Ethiopia have suggested that large numbers of plant species are used to get relief from intestinal helminthes (worms) due to their effect on increasing the intestinal muscle contraction and its motility to the level sufficient to expel them out. As reported by Mohammed *et al.* (2006) crude extract from *Elaeagnus angustifolia* had shown guinea pig intestinal muscle contraction. The aqueous extract of the leaf of *Peninanthus longifolius* had shown a dose dependent contractile activity on guinea pig ileum (Akah, *et al*., 2001). Elenoside, an arylnaphthalene lignan isolated from *Justicia hyssopifolia* has been reported to show high intestinal muscle (duodenum, jejunum, and Ileum) contractility in rats (Navarro *et al*., 2006). The aqueous and methanol extracts from *Anthocleista vogelii* have

shown a dose dependent contraction when tested on ileum of albino rats (Ateufack *et al*., 2010).

The contractile activity of a given plant extract may depends on different factors one of which is its dose (concentration). The crude extract of *Lavandula stoechas* tested on guinea pig ileum has shown a spasmogenic (contractile) effect at a lower dose (≤ 10 mg/ml) where its effect changed to spasmolytic (relaxation) as the concentration increased (Jabeen *et al*., 2007). The extract of *Andrographis paniculata* leaves has shown a decreased GI motility and intestinal muscle (ileum) contraction in rats at low concentration (16 – 32 mg/kg and 1 -4 mg/ml respectively. However, the same extract increased the GI motility and increased the contraction of ileum in the same animal at a higher concentration (64 – 500 mg/kg and 8 – 32 mg/ml respectively) (Nwinyi, *et al*., 2012).

The effect of extracts from the same plant may also depend on the solvent used for extraction and fractionation. For instance, the crude ethanol extract of *Gratiola officinalis* has shown a high spasmogenic activity while fractions obtained from it using different solvent systems have different effects on rabbit intestinal muscle. But its aqueous, ethyl acetate and n-butanol fractions have shown a high spasmogenic response while the n-hexane and chloroform fractions had spasmolytic effects (Muhammad *et al*., 2012). In the same manner methanol crude extract from *Onosma griffithii* have shown contractile effect on rabbit jejunum while all fractions (n-hexane, ethyl acetate and chloroform fractions) from it had relaxing effects (Ali *et al*., 2011).

The effect of plant extract on intestinal muscle contraction can also show regional differences. A relaxant effect of extracts obtained from *Cassia sieberiena* on the ileum of rat was reported while contracting its colon dose dependently (Akomolafe, *et al*., 2003). Despite its regional differences the crude extract from E*laeophorbia drupifera* leaves also showed a dose dependent contraction on the whole of the small intestine of rabbit (Eno and Azah, 2004). Radish (*Brassica oleraceae*) extract also showed a dose dependent contraction on duodenum, jejunum and ileum of rat with the largest contractile response observed on the ileum (Jung *et al*., 2000).

Some plants are also reported for their effect on intestinal motility through relaxation rather than contraction. Hydroalcholic extract of *Tecoma stans* (yellow bells) leaves has shown a spasmolytic activity on ilia of rats (Gharib-Naseri *et al*., 2007). In another instant, Kumar et al. (2007) had reported that the chloroform fraction of *Sarcostemma brevistigma* have shown spasmolytic activity on ileum of guinea pigs through inhibiting contraction induced by acetylcholine and histamine. Extract prepared from *Carica papaya* seeds has shown a concentration dependent inhibition of contraction of jejunum in rabbit (Adebiyi and Adaikan, 2005). These examples mentioned so far are only few to show the effect of medicinal plants on intestinal muscle contraction and on its motility.

5.2 Intestinal Muscle Contractile Activity of Croton

Traditionally members of Croton are used to treat ailments related to GI tract including inflammation, constipation, indigestion and infection beside others. Most of them are taken by human or by their livestock as anthelminthic and/or to facilitate digestion as well

as bolus movement. The mechanism of these treatments is mainly related to the ability of the phytochemicals from these plants to increase intestinal motility through increasing intestinal muscle contractility. Many research works have been conducted to validate this. *Croton macrostachyus* seed, for instance, had been reported for possessing a laxative property (Mazzanti *et al*., 1987) that could be related to its effect on intestinal muscle contraction. Magalhães *et al*. (2004) have also reported the antispasmodic effect of the extract from *C. nepetaefolius* seeds on guinea pig ileum. An extract from *C. tiglium* had shown a concentration dependent contraction of a rabbit jejunum (Hu *et al*., 2010; Hu *et al*., 2012; Liu *et al*., 2012). Rodrigues *et al*. (2009) also reported that *C. zehntneri* has the ability to give a relief to gastrointestinal disturbance, which could be related to its effect on the motility of the later.

5.3 Intestinal Muscle Contractile Activity of Vernonia

Many research works have shown the presence of phytochemicals in Vernonia having a remarkable effect on contraction of smooth muscles including the intestine. Among these the effects of aqueous extract of the leaves of *V. amygdalina* on contraction of rat uterus, rabbit jejunum (Kamatenesi-Mugisha *et al*., 2005 in Ijeh and Ejike, 2011) and guinea pig ileum (Owu *et al*., 2008) are reported. Alawa *et al*. (2003) also reported that the traditional usage of *V. amygdalina* as anthelminthic is associated with its ability to expel out worms through increasing intestinal muscle contraction.

5.4 Specific Objectives

- To evaluate the effect of different crude extracts of *V. galamensis* leaves on intestinal muscle contraction in a dose dependent manner.
- To evaluate the effect of different crude extracts of *C. macrostachyus* leaves on intestinal muscle contraction in a dose dependent manner.
- To assess the extent to which each plant extract affects the intestinal muscle contraction induced by agonist acetylcholine.

5.5 Materials and Methods

5.5.1 Preparation of Plant Extracts

Six crude extracts (three from each plant: VAE, VEE, and VME from leaves of *V. galamensis*; CEaE, CEE and CME from leaves of *C. macrostachyus*) were prepared through subsequent extraction technique. All of them were tested for their effect on ACh induced guinea pig intestinal (ileum) muscle contraction. Due to their limited quantities pure compounds or fractions were not tested. Each crude extract (10 mg) was dissolved in 1ml of distilled water which gave a stock solution of 10 mg/ml concentration. Three different amounts (0.2 ml, 0.4 ml and 0.8 ml) were taken to be added separately to a 25 ml organ bath, which gave an organ bath concentration of 80µg/ml, 160µg/ml and 320µg/ml respectively (Tafes**se** *et al*., 2006; Mulatu and Mekonnen, 2007).

5.5.2 Guinea Pig Ileum Preparation

Guinea pigs of either sex weighing 460 g – 480 g were used in this study. Each guinea pig was starved for 18 h prior to the experiment allowing only water and handled according to guidelines for the care and use for experimental animals (National Academy of Science, 2011). After 18 h of starvation each animal was sacrificed by gentle blow on the head and the abdomen was cut open to get the small intestine. About 15 cm of ileum was taken and kept in Tyrode solution from which short segment (1.5 – 2 cm) was cut out during each test (Jabeen *et al*., 2007). Tyrode solution was prepared immediately prior to the experiment with a composition of 8 g NaCl, 0.2 g KCl, 0.1 g $MgCl_2$, 1 g $NaHCO_3$, 0.05 g NaH_2PO_4, 0.2 g $CaCl_2$ and 1 g D-glucose at pH of 7.4. Six guinea pigs were used to test each crude extract (Gharib-Naseri *et al*., 2007; Mulatu and Mekonnen, 2007).

5.5.3 Guinea Pig Ileum Contractility Test

Effect of each plant crude extract on ileum contractility was determined according to methods in (Mekonnen, 1999), Gharib-Naseri *et al*. (2007) and Jabeen *et al*. (2007). A 25 mL organ bath containing Tyrode solution was maintained at a temperature of 37^0C and aerated with 5% CO_2 and 95% O_2. The ileum segment (1.5 – 2 cm) was hanged in 25 mL organ bath by tying it to both sides (to the bottom of the organ bath and to a transducer (Grass FT03) on opposite side) using a tread. The transducer was then connected to a polygraph (Grass 79D) to record contraction. The tension on the transducer was maintained at 1 g before tying of the tissue. The tissue was then allowed to equilibrate for about 30 – 40 min before addition of the contraction agonist, acetylcholine (ACh) and the extract subsequently (Gharib-Naseri *et al*., 2007; Jabeen *et al*., 2007).

Acetylcholine (ACh) solution was prepared by measuring 10 mg of acetylcholine chloride and dissolving it in 10 ml of solvent (1 ml of distilled water and 9 ml of Tyrode solution) that gave 1 mg/ml stock solution. 1 ml of this solution was diluted in 9 ml of Tyrode Solution to give 0.1mg/ml (100µg/ml) of ACh solution. Then 1 ml was taken and 9 ml of Tyrode solution was added to it which gave 0.01 mg/ml (10µg/ml). This was used as test concentration that was added successively to the organ bath with interval of 5 min and flashing of the tissue after 30 sec of contact to select the one that produces a submaximal contraction of the tissue to avoid desensitization by a maximum concentration. The additions were made in increasing amounts (0.1, 0.2, 0.4 and 0.8 ml) that gave an organ bath concentration of 40ng/ml, 80ng/ml, 160ng/ml and 320ng/ml of ACh respectively. ACh

that gave submaximal contraction of the ileum (0.2 ml or 80ng/ml of organ bath) was chosen for testing the extracts (Mekonnen, 1999; Tafesse *et al*., 2006).

After resuming normal contraction a specific extract was added to the organ bath in a dose dependent manner (80µg/ml, 160µg/ml and 320µg/ml of organ bath concentration) followed by addition of ACh (80ng/ml) after 5 min and washing (flashing) the tissue after 30 sec. After flashing out the tissue the recovery period was 5 min before addition of the next dose of the extract. At the end of each experiment 0.2 (80ng/ml) of ACh was added to the organ bath to make sure the tissue (ileum) resume contraction (Mekonnen, 1999; Tafesse *et a*l., 2006).

Tensions of the contraction were recorded by measuring the distance (in cm) from the base line on the polygraph paper produced by the agonist (ACh) alone and the extract with ACh; and converting the results into tension of contraction by taking that 1 cm is equivalent to 1 g of tension. Similar procedures were applied for every extract and each was tested on six segments of ileum from six guinea pigs. The effect of each extract on the ACh induced contraction of ileum was recorded by calculating the average change in twitch tension using the following formula (Eno and Azah, 2004):

$$\text{Twitch Tension (\%)} = \frac{\text{TREA - TRA}}{\text{TREA}} \times 100$$

Where, TREA = Average tension recorded by extract in presence of ACh
TRA = Average tension recorded by agonist (ACh) alone

5.5.4 Data Analysis

Data were recorded by taking the average (mean ± SEM) value of tension (g) and average value of twitch tension (%) from six independent tests. Comparisons were made between the effect of ACh with and without the extracts (at different doses) using One Way ANOVA (Tukey) test. 95% confident interval was taken and P-value ≤ 0.05 was considered as significant difference.

5.6 Results and Discussion

5.6.1 The Effect of *C. macrostachyus* Leaves Crude Extracts on Contraction of Guinea pig Ileum

Different crude extracts obtained from the leaves of *C. macrostachyus* resulted in an increase and decrease of ACh induced contraction of guinea pig ileum. Extracts CEaE and CEE showed a contractile effect at all tested concentrations, while CME increased ACh induced contraction of guinea pig ileum at low concentrations (80µg/ml and 160µg/ml) but decreased it at a higher concentration (320µg/ml). The polygraph record of the effect of each crude extract on ACh induced guinea pig ileum contraction is shown in Appendix E. As shown in Fig. 12 the tensions of contraction recorded by extracts CEaE and CEE were higher than those recorded by ACh alone while CME showed the least at organ bath concentrations of 160µg/ml and 320µg/ml.

Table 9: Tension and twitch tension recorded during guinea pig ileum contraction by the ACh alone and leaves of C. *macrostachyus* crude extracts in presence of ACh**.**

Extract	**Mean ± SEM of Tension (g) and [P-value]; n = 6**				**Twitch Tension (%)**		
	Doses				**Doses**		
	ACh alone 80ng/ml	80µg/ml + ACh	160µg/ml + ACh	320µg/ml + ACh	80µg/ml + ACh	160µg/ml + ACh	320µg/ml + ACh
CEaE	3.2±0.10	3.5±0.03 [0.10]	4.7±0.04 [0.00]	4.9±0.05 [0.00]	8.6	31.9	34.7
CEE	3.3±0.04	3.8±0.04[0.08]	4.7±0.05 [0.00]	4.9±0.05 [0.02]	13.2	28.9	32.7
CME	2.4±0.03	2.9±0.06 [0.10]	3.1±0.04 [0.00]	2.0±0.07 [0.00]	17.2	22.6	-20

All crude extracts (CEaE, CEE and CME) at low organ bath concentration (80µg/ml) have insignificant effect on ACh induced guinea pig ileum contraction as compared with that induced by the agonist (ACh) alone (P= 0.1, 0.08 and 0.1 respectively). At a higher organ bath concentrations (160µg/ml and 320µg/ml), however, CEaE and CEE showed contractile effect on ACh induced contraction of the guinea pig ileum that are significantly different from that induced by ACh alone (P= 0.00). In presence of ACh, tensions (g) recorded by CEaE (3.5±0.03, 4.7±0.04 and 4.9±0.05), CEE (4.7±0.05 and 4.9±0.05) and respectively at 160µg/ml and 320µg/ml organ bath concentrations, were higher with significance differences with the control ACh (P= 0.00). At 80µg/ml + ACh, however, CEaE, CEE, CME and VAE showed contractile effects which are not significantly different from ACh alone (P=0.1, 0.08, 0.1 and 0.07 respectively) (Table 9).

The statistical analysis have revealed that although extracts CEaE and CEE (+ACh) showed significant difference with ACh alone in their contractile activities, there is no significant difference between themselves (P= 0.12). The effect of extract CEE (+ACh) is significantly different from that of ACh alone as well as between the concentrations 160µg/ml and 320µg/ml (P= 0.02).

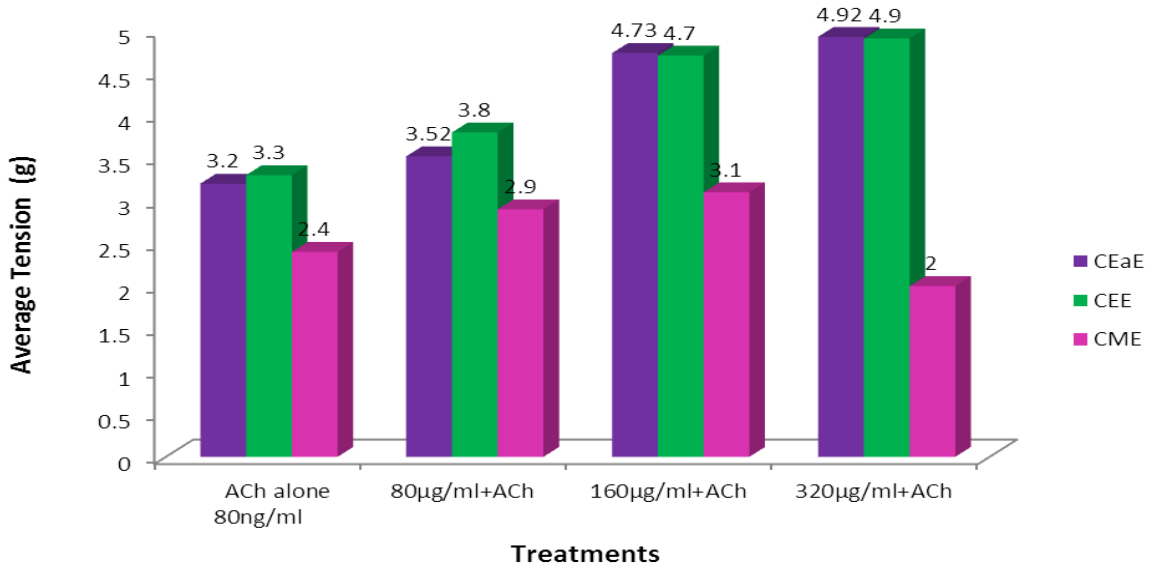


Fig. 12 Tension of contraction of guinea pig ileum by ACh alone and by different organ bath concentration of *C. macrostachyus* leaves crude extracts in the presence ACh (each point represent the average tension value of six experiments).

The change in tension of contraction also revealed that CEaE (+ACh) has increased the contraction of the ileum induced by ACh at high concentrations. It increased the ACh induced contraction by 31.9% and 34.7% at concentrations 160µg/ml and 320µg/ml respectively. CEE (+ACh) also increased ACh induced contraction of guinea pig ileum by 28.9% and 32.7% at these respective concentrations. Although CME increased ACh induced guinea pig ileum contraction at the organ bath concentrations of 80µg/ml and 160µg/ml by 17.2 and 22.6 respectively, it decreased it by 20% at 320µg/ml (Table 9, Fig. 13).

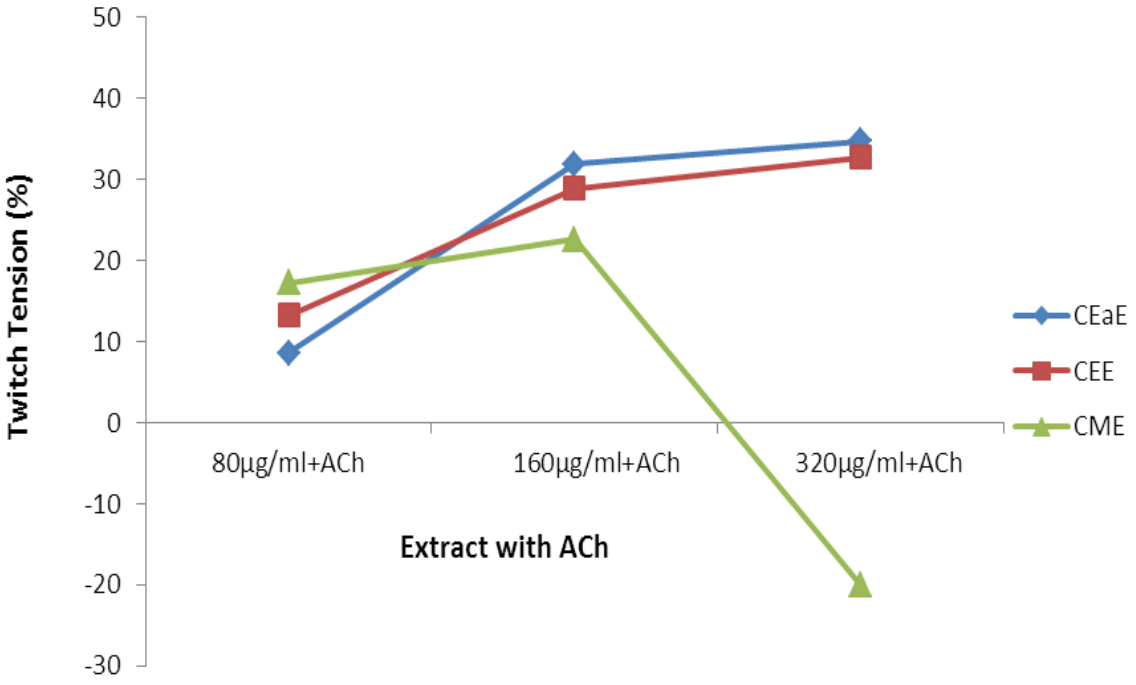


Fig. 13 The effect of different crude extracts of *C. macrostachyus* leaf on ACh induced contraction of guinea pig ileum (each point indicates the Mean ± SEM value of change in contraction tension of six experiments).

5.6.2 The Effect of Crude Extracts of *V. galamensis* Leaves on Contraction of Guinea pig Ileum

Crude extracts obtained from *V. galamensis* leaves showed different results in which some increased the agonist (acetylcholine) induced contraction of guinea pig ileum while others decreased it. The polygraph record of the effect of each crude extract on ACh induced guinea pig ileum contraction is shown in Appendix F. VAE + ACh (3.0±0.03 and 3.1±0.04) and VEE + ACh (3.2±0.06 and 2.3±0.04) recorded a higher tension of contractions at 160µg/ml and 320 µg/ml with ACh as compared to the agonist (ACh) alone while VME+ACh

showed the least (even less than the ACh) at all tested organ bath concentrations (Table 10, Fig. 14).

Table 10: Tension and twitch tension recorded during guinea pig ileum contraction by the ACh alone and crude extracts of leaves of *V. galamensis* in presence of ACh.

Extract	(Mean ± SEM)Tension (g) and [P-value]; n = 6				Twitch Tension (%)		
	Doses				Doses		
	ACh alone 80ng/ml	80µg/ml + ACh	160µg/ml + ACh	320µg/ml + ACh	80µg/ml + ACh	160µg/ml + ACh	320µg/ml + ACh
VAE	2.0±0.04	2.3±0.05 [0.07]	3.0±0.03 [0.00]	3.1±0.04 [0.00]	13	33.3	35.5
VEE	1.4±0.06	3.2±0.04 [0.00]	3.2±0.06 [0.00]	2.3±0.04 [0.00]	56.3	56.3	39.4
VME	1.5±0.03	1.1±0.06 [0.00]	1.0±0.04 [0.00]	0.5±0.03 [0.00]	-36	-50	-200

At a bath concentration of 80µg/ml (+ACh), VAE has little contractile effect on guinea pig ileum that was not significantly different from the contractile effect of the ACh alone (P= 0.07). In presence of ACh this extract showed high contractile activities on this intestinal segment at 160µg/ml and 320µg/ml organ bath concentration which are significantly different from that of ACh alone (P= 0.00). Extract VME (+ACh) showed a significantly different (P= 0.00) contractile activities at all tested concentrations to ACh alone better than VAE (+ACh). In contrast the effect of extract VEE (+ACh) was significantly different form the effect of ACh alone (P= 0.00) and has generally shown relaxant activities on ileum of guinea pig at all tested concentrations (Table 10).

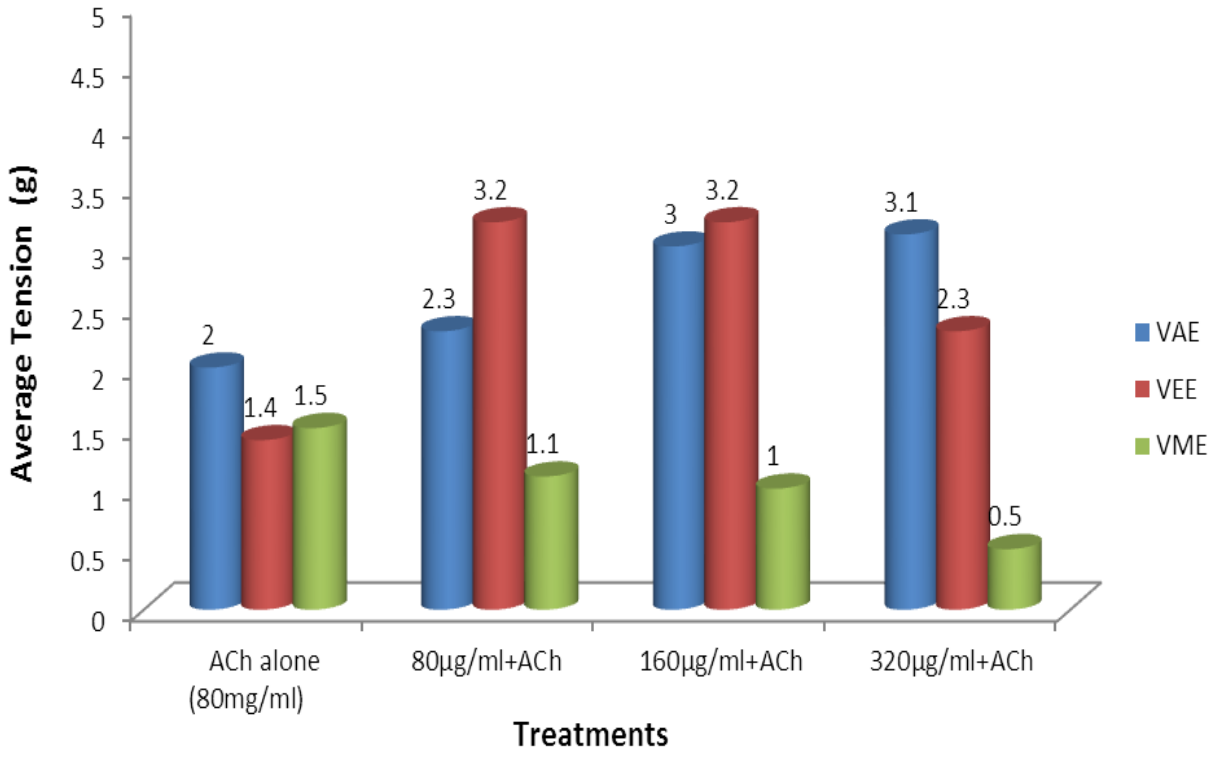


Fig. 14 Tension of contraction of guinea pig ileum by ACh alone and by different organ bath concentration of *V. galamensis* leaves crude extracts in presence ACh (each point represent the average tension value of six experiments).

At all tested organ bath concentrations (80µg/ml, 160µg/ml and 320µg/ml) extract VEE (+ACh) has increased the contraction of guinea pig ileum with twitch tension of 56.3%, 56.3% and 39.5% respectively better than VAE (+ACh). However, the contractile effects were not dose dependent and even decreased as concentration increased. In contrary, extract VAE (+ACh) has a dose dependent contractile effects on this muscle strip that were higher at an organ bath concentration of 160 µg/ml and 320 µg/ml with twitch tension of 33.3% and 35.5% respectively. Unlike the two extracts, extract VME (+ACh) has abolished

the ACh induced contraction of the ileum dose dependently by 36%, 50% and 200% at the three tested concentrations respectively (Table 10; Fig 15).

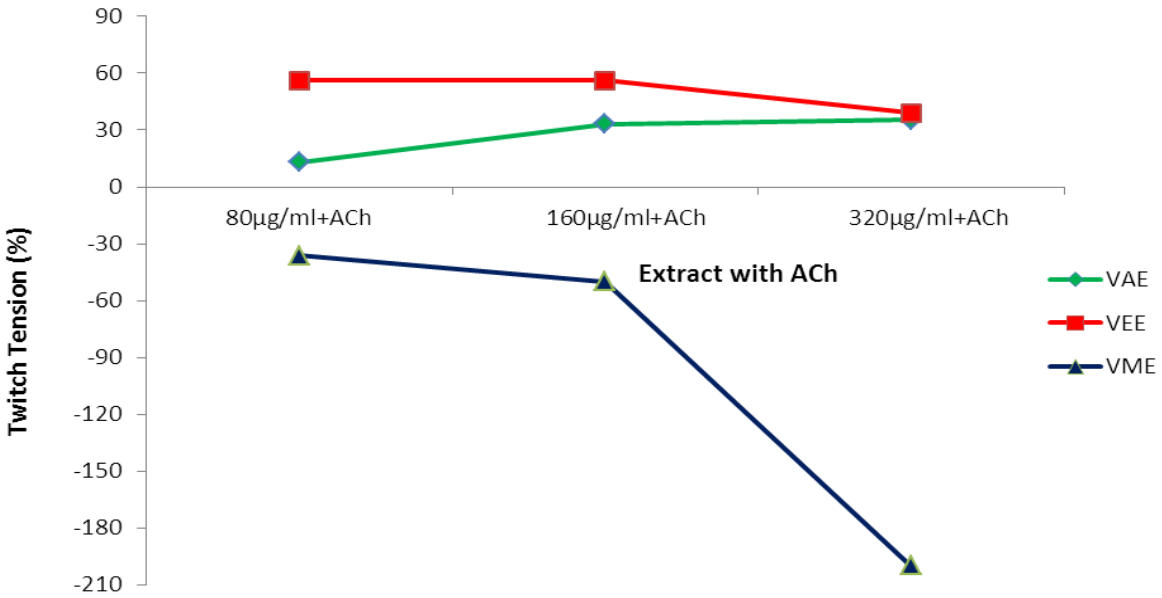


Fig. 15 The effect of different crude extracts of *V. galamensis* leaves on ACh induced contraction of guinea pig ileum (each point indicates the average value of tension of contraction of six experiments).

The findings of this study clearly indicate that both *C. macrostachyus* and *V. galamensis* contain phytochemicals in their leaves that have different effects on guinea pig ileum contraction. The results obtained in the present study is in agreement with results reported by other works for many plants (Gharib-Naseri *et al*., 2007; Jabeen *et al*., 2007; Ateufack *et al*., 2010; Ali *et al*., 2011). The results also concur with those reports for the genus Croton (Mazzanti *et al*., 1987; Liu *et al*., 2012) and the genus Vernonia (Owu *et al*., 2008; Ijeh and Ejike, 2011).

As have been seen from the twitch tension records extracts CEaE, CEE, VAE and VEE increased the agonist (ACh) induced contraction of guinea pig ileum as their concentration increased, which suggest that these extracts have a dose dependent effect. In contrast, extracts CME and VME reduced the contraction of ileum as their concentration increases indicating that they have a relaxant effect.

Such differences might be due to the polarity of solvents used. Phytochemicals extracted by acetone, ethylacetate or ethanol are less polar than those extracted by methanol. Extracts/fractions made using less polar solvent like acetone and ethylacetate have been reported to show high contarctile activities on intsetinal muscle while those extracted by a highly polar solvent showed relaxation (Thaina *et al*., 2005; Hu *et al*., 2012; Liu *et al*., 2012). This might suggest that extract CME contains high polar compounds that could induce relaxation as concentration increases. Extracts CME and VME increased the ACh induced contration of the giunea pig ileal muscle at low concentrations while decreased it at high concentartion suggesting that they have a biphasic action (Hu *et al*., 2010; Hu *et al*., 2012).

The intestinal muscle contractile effect of a plant extract may also show regional differences as reported for methanol crude extract of *Onosma griffithii.* This extract contracted the rabbit jejunum while relaxing its ileum (Ali *et al*., 2011). The relaxing activities of extracts CME and VME observed in the present study might be due to these regional differences.

The effect of substances on contraction of intestinal muscle is believed to be due to an increase or a decrease in the intracellular calcium ion concentration, $[Ca^{2+}]_i$ of contracting

cells. They increase contraction through competing contractile agonists resulting in opening of Ca^{2+} channels. Their relaxation effect comes while they occupy muscarinic receptors as antagonists, which brings about closing of Ca^{2+} channels while opening K^{+} channels (Vogalis *et al*., 1991; Ozaki *et al.*, 1993; Karaki *et al*., 1997). The increased contractile activities of some extracts on guinea pig ileum in the present study might be due to their ability to occupy muscarinic receptors (CMAR) as agonists leading to high entry of calcium ions to the sarcoplasm resulting in high contraction. Some extracts also showed a decreased contraction of guinea pig ileum in the present study, which might be due to their action of occupying muscarinic receptors giving no place for acetylcholine. This leads to blockage of Ca^{2+} channels making no further entry of calcium while opening K^{+} channels thereby pumping out calcium. The overall result of these is decreasing contraction and leading to relaxation.

Chapter VI

Toxicity Test, General Conclusion and Recommendations

6.1 Acute Toxicity Test for Crude Extracts of *Leaves of V. galamensis* and *C. macrostachyus*

6.1.1 Introduction

The term toxicity refers to a harmful effect of substances to living organisms. Such substances could be chemicals or physical exposures like radiation. Toxicity also include a cascade of events starting with exposure to toxin, its distribution and metabolism, its interaction with cellular macromolecules (usually DNA or protein) and the expression of its end point (Hodgson, 2004). Experimental animals such as rodents, guinea pigs and ferrets are the principal choices for toxicity tests. Their genetic constitution, the facility and duration of controlled exposure, and the possibility of detailed examination of all tissues following necropsy, make these animals a choice of priority (Cunny and Hodgson, 2004).

Although medicinal plants are traditionally chosen by large number of people throughout the world due to their affordability, easy access, manageable side effects and effectiveness, it does not mean that they don't have toxic effects. Like anything else plants can also contain chemicals that could be toxic to other organisms especially to mammals. Hence, caution should be made to evaluate the toxicity profile of a given plant before validating its medicinal values.

6.1.2 Methods

Acute toxicity test of crude extracts of *V. galamensis* (VAE, VEE and VME) and *C. macrostachyus* (CEaE, CEE and CME) leaves were performed on eight weeks old experimental mice weighing 34 – 40 g according to the methods in Mirghazanfari *et al*. (2012) and Singh and Singh (2012). Each extract was prepared at doses of 500 mg/kg, 1000 mg/kg, 1500 mg/kg and 2000 mg/kg body weight and administered orally using a gavage.

Twenty five groups of mice (six individuals in each) were kept in independent cages and served as experimental groups for each extract while another one group of the same number of individual served as a control taking 0.2 ml vehicle. Groups 1 - 6 received 500 mg/kg, 7 – 12, 1000 mg/kg, 13 – 18, 1500 mg/kg while groups 19 – 24 received 2000 mg/kg body weight of extracts VAE, VEE, VME, CEaE, CEE and CME respectively. The last (control) group received 0.2 ml of vehicle. Each test was performed three times to confirm the result. After treatment all mice were monitored for 14 days with continuous physical observations for mortality, hair erection, weight gain/loss, and for behavioral signs such as sleepiness/dizziness, over activeness and changes in water and food intake (Mirghazanfari *et al*., 2012).

6.1.3 Results

All mice in both the experimental and control group did not show any form of physical signs of acute toxicity. No mortality, hair erection, dizziness or over activeness were recorded among all experimental as well as control groups. All mice showed normal activity and no significant changes in their intake of food and water were observed until the end of

the experiment. Moreover, the weight gained by all animals was insignificant (Appendix G). The results, in general, indicated that these two plants have no chemicals that induce death or other symptoms of acute toxicity up to a dose of 2, 000 mg/kg body weight and hence validate their safety at least in animal model and probably also justify their traditional medicinal uses.

6.2 General Conclusion and Recommendations

6.2.1 General Conclusion

Infections, especially those caused by bacteria, and gastrointestinal problems have been major health threats to humans and livestock since long period of times. Human beings are trying to overcome these problems both traditionally as well as using scientifically proven methods. The use of synthetic drugs to get rid of bacterial infections has been reported posing problems mainly due to the resistance developed by bacteria. Besides, due to affordability and accessibility of these drugs, people opt to rely on traditionally available substances around them. In this regard higher plants take the major part of the sources.

People, especially those living in developing nations like Ethiopia have used and are still using medicinal plants designing their own methods of applications. Scientific proof is, then, crucial to validate the traditional application of such plants along with suggesting the possibility of developing drugs from them.

Vernonia galamensis and *Croton macrostachyus* are members of higher plants claimed for various traditional uses including treating microbial infections and giving reliefs to gastrointestinal problems. In Ethiopia, these plants are used to heal wounds and get rid of intestinal worms in different rural and suburban areas.

The findings of the present study might give an insight that these plants contain secondary metabolites that could combat infectious agents like bacteria and give reliefs to GI tract complaints. Problems associated with GI tract include worm burden, constipation and

intestinal cramp. The use of these plants could alleviate such problems by increasing or decreasing intestinal muscle contraction.

The preliminary tests on the extracts of less polar solvents (ethyl acetate for *C. macrostachyus* and acetone for *V. galamensis*) gave the best antibacterial activities over non polar (n-hexane) and more polar solvents (ethanol and methanol). These results could suggest that less polar compounds have better antibacterial activities due to their ability to pass the cell membrane of the bacteria easily.

The effectiveness of some crude extracts and isolated pure compounds against some tested bacterial strains in the present work might suggest their specificity of actions. The isolated compounds like compounds **IV**, **VI** and **VII** showed the best antibacterial activities than the others. While some like compound **III** (vernodalin) was less effective, where its concentrations might not be enough to act, but others (compounds **V**, **VIII** and **IX**) completely lack activities. Though people traditionally apply these plants externally, the effects of some compounds on *S. typhi* and *S. boydii* recorded in the present study might suggest that *C. macrostachyus* and *V. galamensis* have phytochemicals which could combat internal pathogenic bacteria. The mechanisms of actions of antibacterial agents are various as mentioned earlier. Although the possible antibacterial mechanism of action of compounds in the present study is discussed comparing with previous works, detailed study is needed to fully confirm this.

In the present study, crude extracts of both *C. macrostachyus* and *V. galamensis* leaves showed contrasting effects on contraction of guinea pig ileum in which those extracted

with less polar solvents (ethyl acetate and acetone respectively) having a contractile effects while those extracted with high polar solvent (methanol) relaxing it. This finding could validate the traditional uses of these two plants in different parts of the country to get relief from GI problems (intestinal worms and/or constipation). However, further study is needed to compare their effect with those of intestinal muscle contracting and/or relaxing agents in order to know their mechanism of actions. Further studies might also be needed to isolate pure compounds from each extract responsible for these activities.

These plants did not show any sign of acute toxicity up to a dose of 2000 mg/kg body weight, suggesting that they can be applied safely both externally and internally, though further study is needed for chronic toxicity test. In general, findings of the present study suggest that these two plants (*C. macrostachyus* and *V. galamensis*) contain secondary metabolites that could help to combat life threatening pathogens such as *E. coli*, *S. aureus, S. typhi* and *S. boydii* all of which are great problems of many people of the world especially in developing nations. Phytochemicals from these plants could also help to solve problems related to gastrointestinal motility if detailed studies are conducted.

In general, findings of this study seem to be novel for the following:

1) Vernolide and vernoguinoside are reported from *V. galamensis* for the first time.
2) It is the first work to report methyl laurate from *C. macrostachyus.*
3) The antibacterial properties of methyl laurate and creptoxide are also reported for the first time.

6.2.2 Recommendations

The present research work attempted to fill the gap of knowledge about the medicinal importance of *V. galamensis* and *C. macrostachyus*. The following recommendations are forwarded:

1. Since the present study focused only on the antibacterial activities of leaf extracts and/or active compounds similar studies need to be performed on other parts of each plant.
2. In order to widen the knowledge of antimicrobial effect these plants, similar researches have to be carried out on pathogenic microbes other than those used in the present study.
3. Only crude extracts from each plant were tested for their effect on acetylcholine induced guinea pig ileum. Hence, further study has to be conducted in order to isolate and test active principles for such activity.
4. Further study is needed focusing on testing the intestinal muscle contractile property of extracts from each plant on other experimental animals and other intestinal part for existence of species as well as regional differences.
5. The intestinal muscle contractile effect of each plant has to be compared with synthetic agents currently used to contract and/or relax GI tract muscle.
6. The possible mechanism of actions of both the antibacterial and ileum muscle contractile activities of each extract and/or active compounds must be studied.
7. Great effort and attention should be given to conserve these plants through confirming and educating people about their medicinal values.
8. It is also recommendable to check the toxicity profile of each plant using other animal models.

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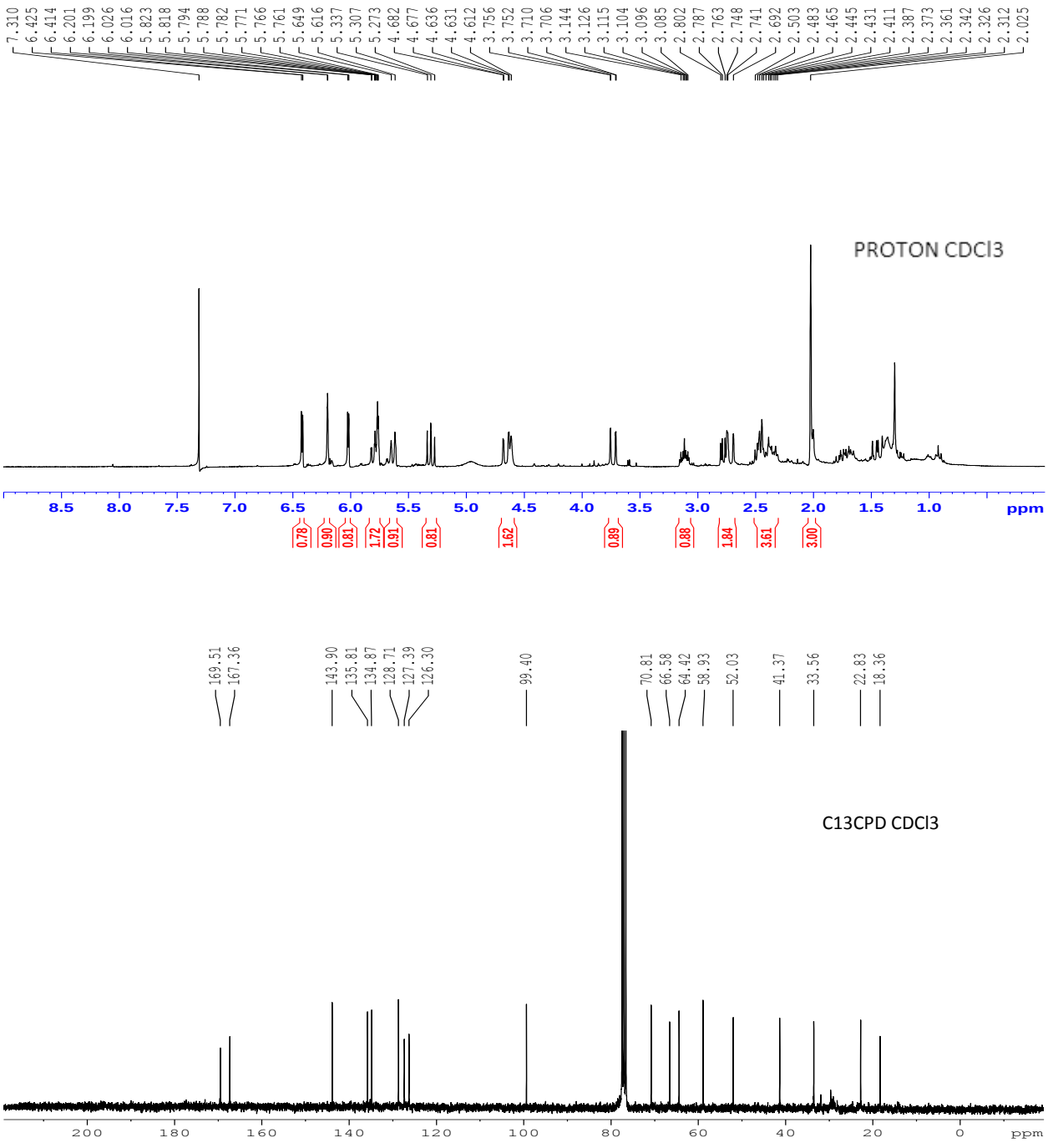
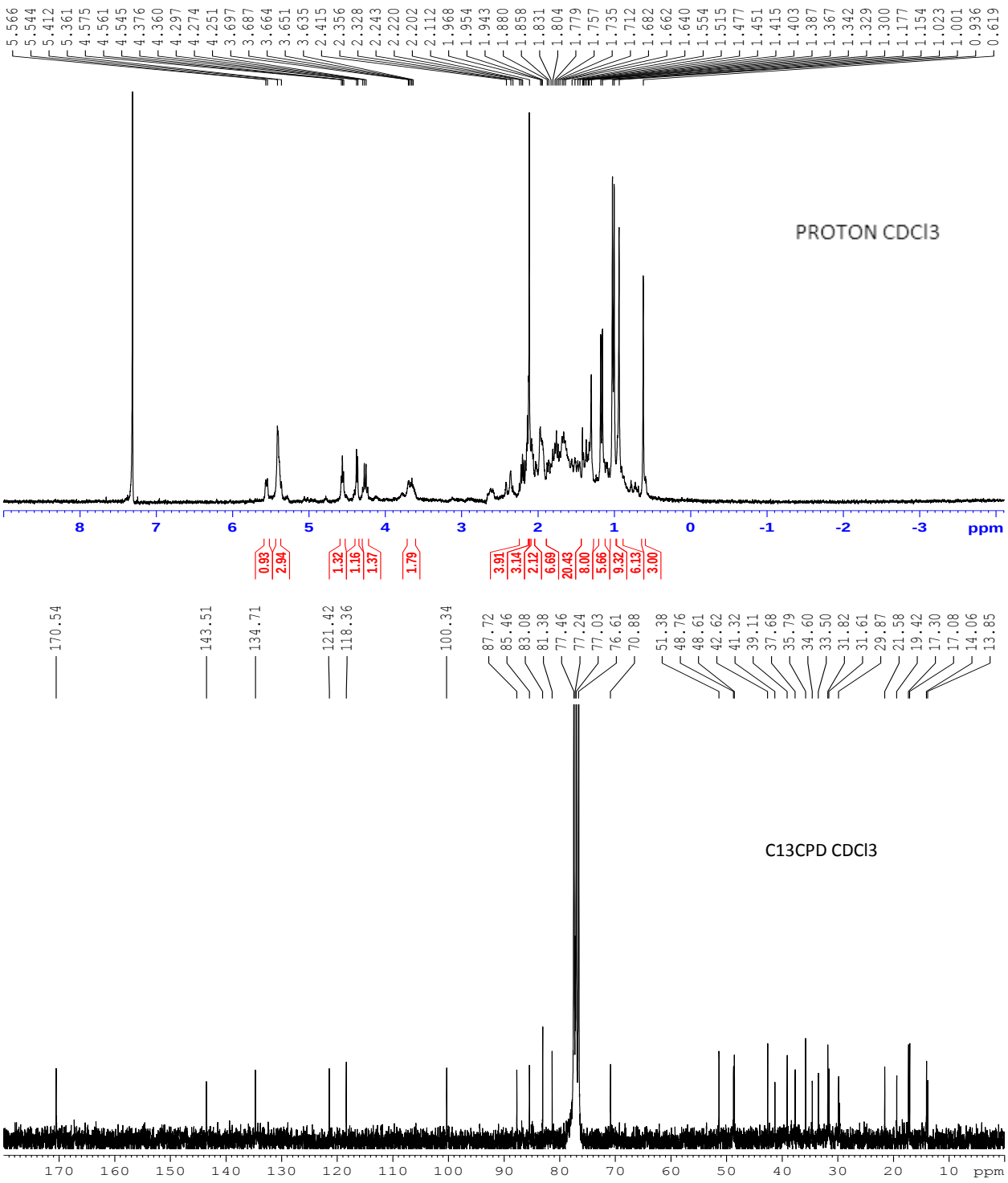
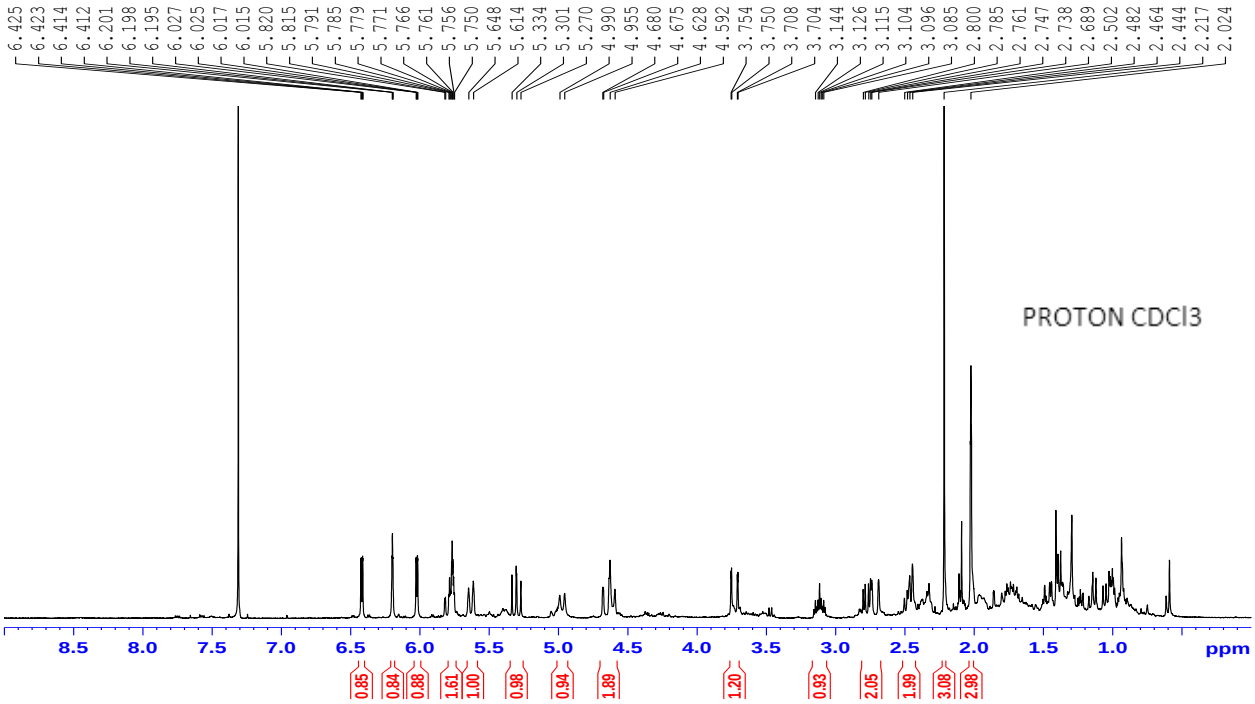
Appendix A: 1H-NMR and 13C-NMR of active pure compounds isolated from VAE (Vernonia Acetone Extract)

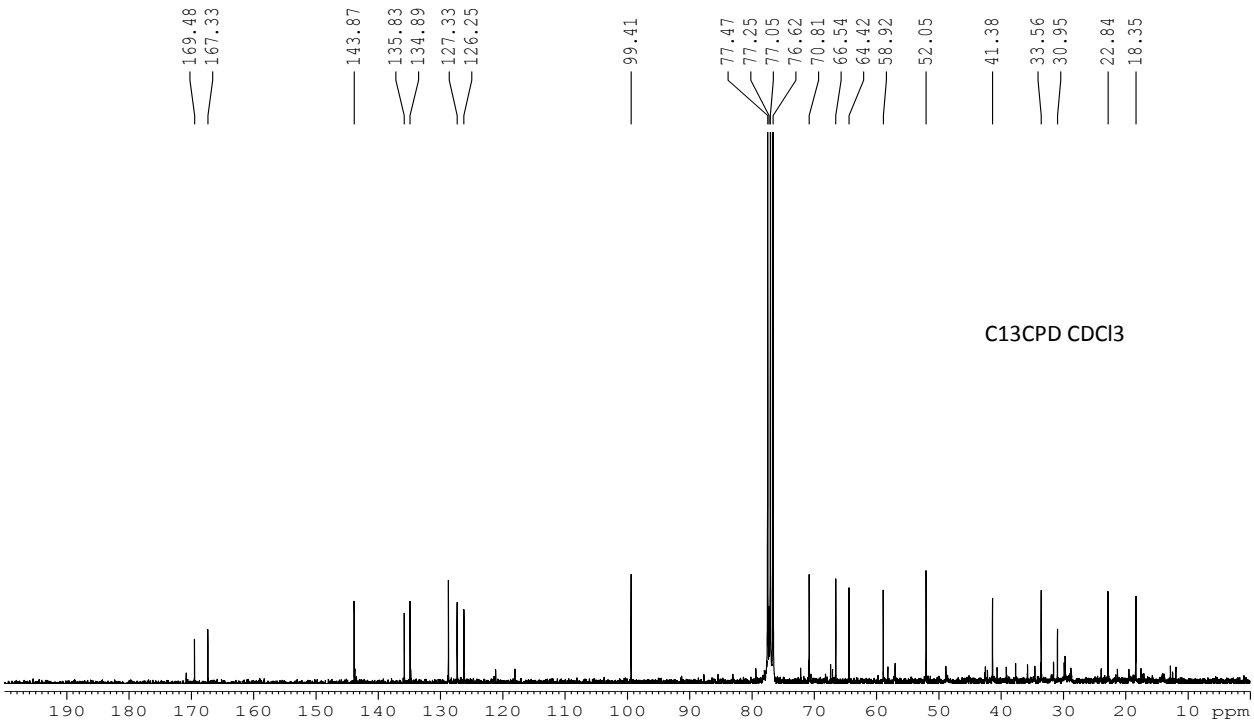


a) Compound **I**

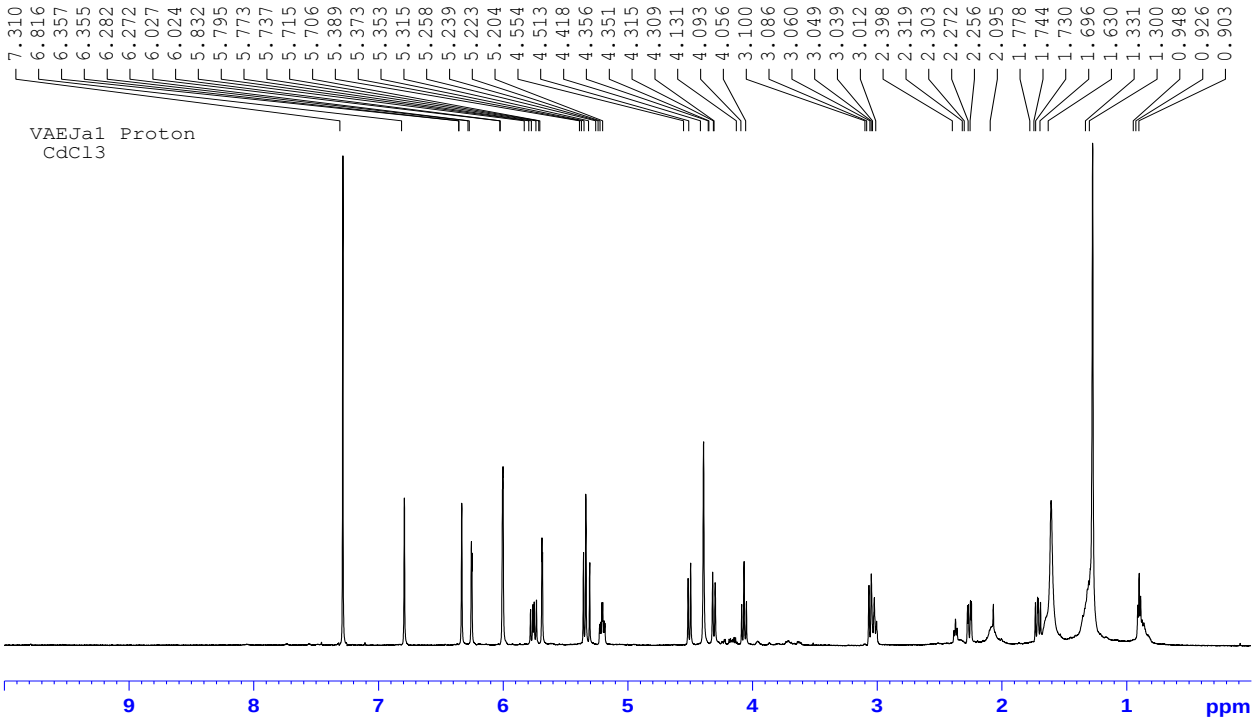


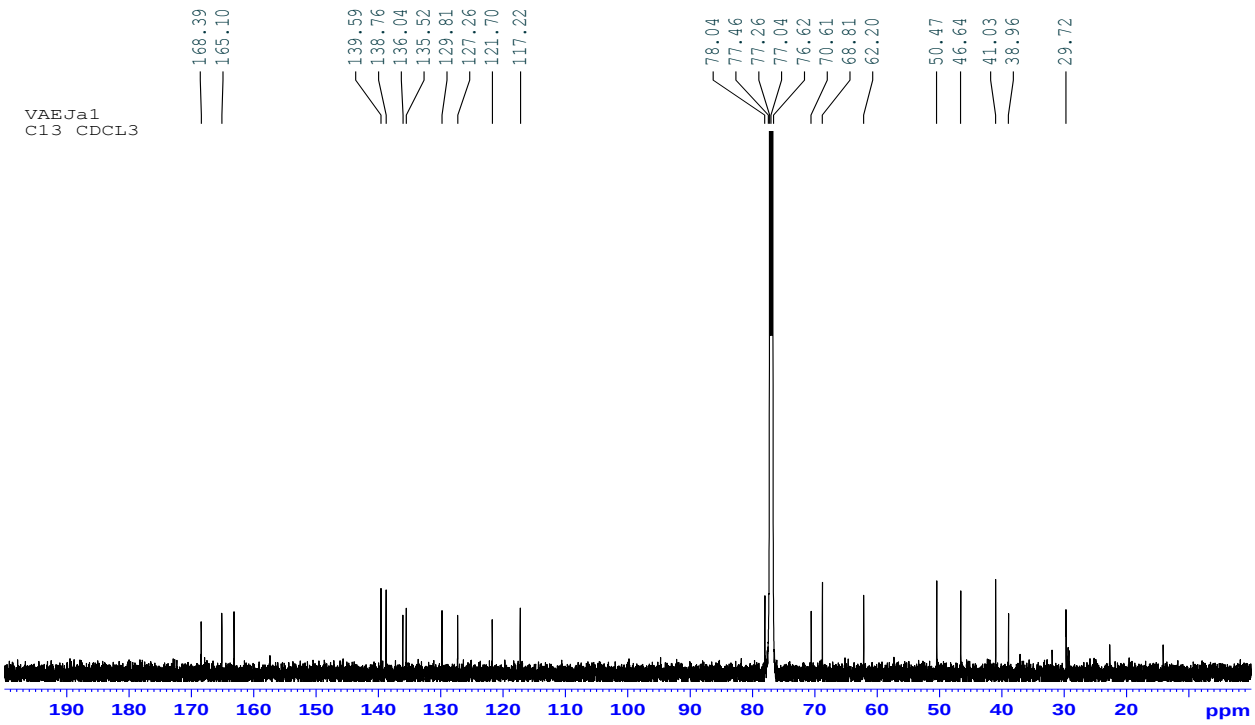
b) Compound **II**





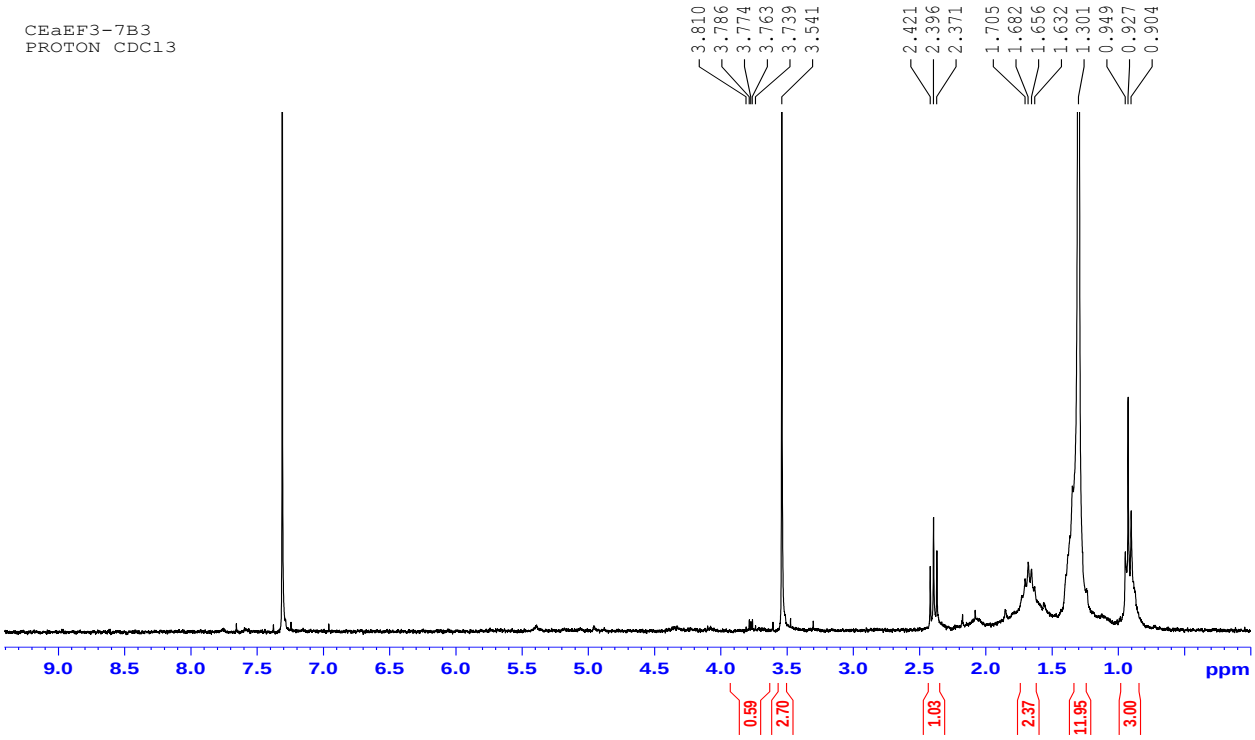
c) Compound **III**

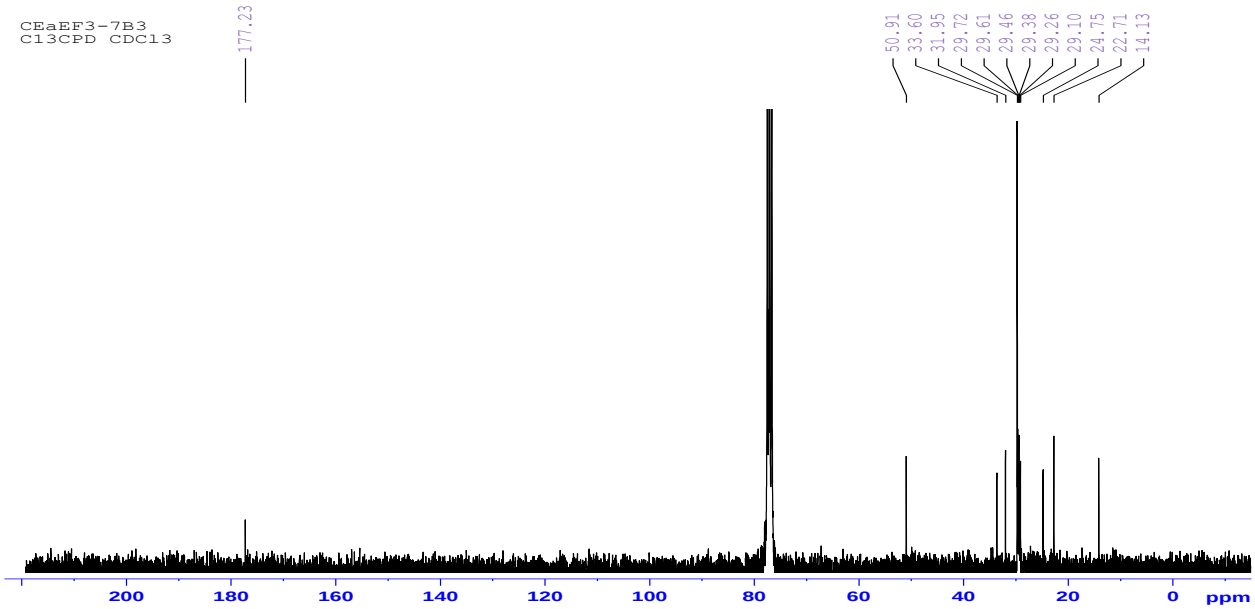




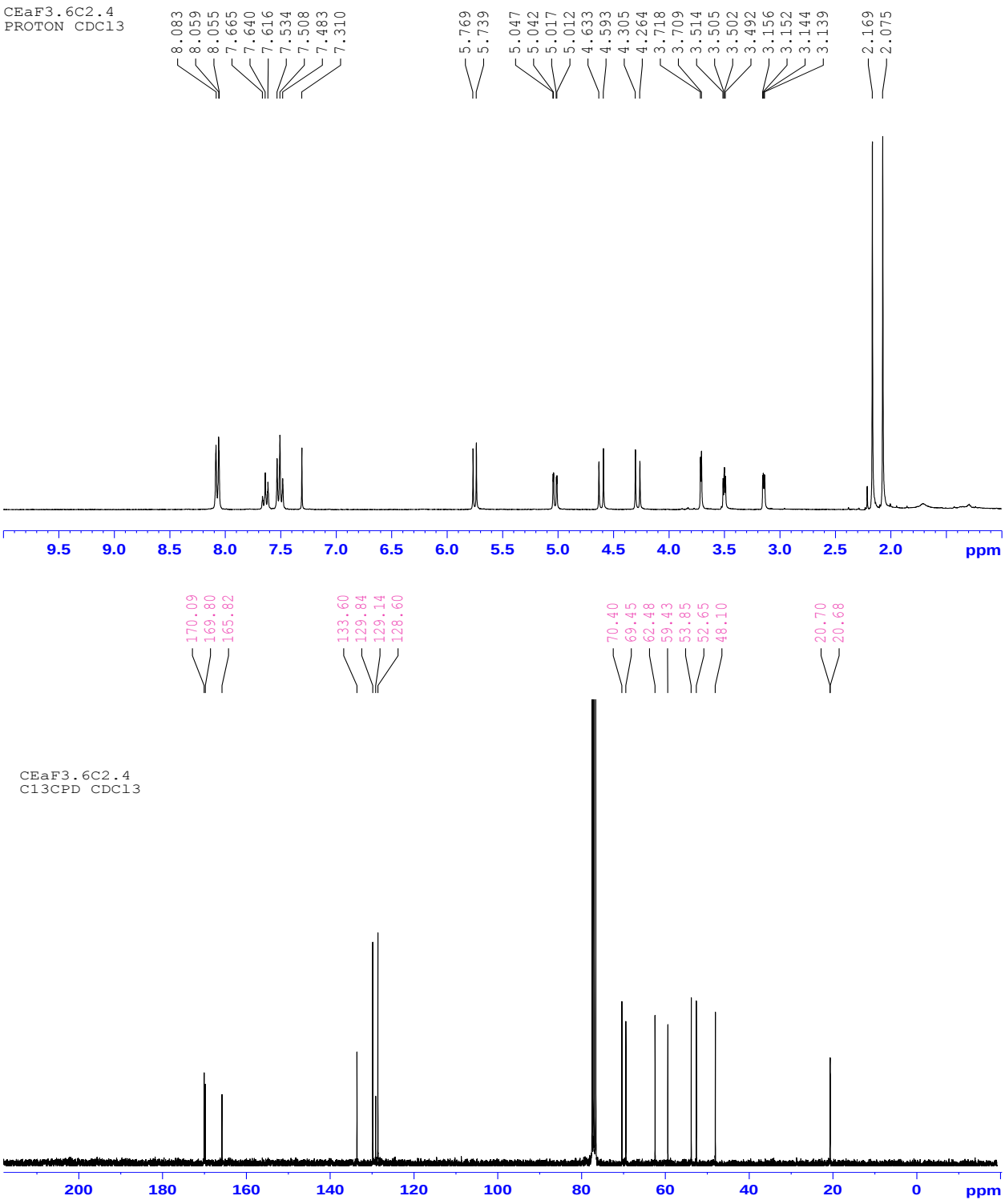
d) Compound **IV**

Appendix B: 1H-NMR and 13C-NMR of active pure compounds isolated from CEaE (Croton Ethylacetate Extract)



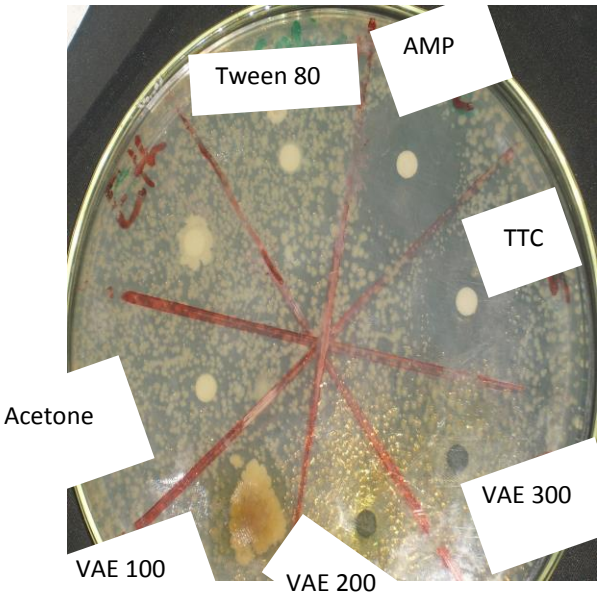


a) Compound **VI**

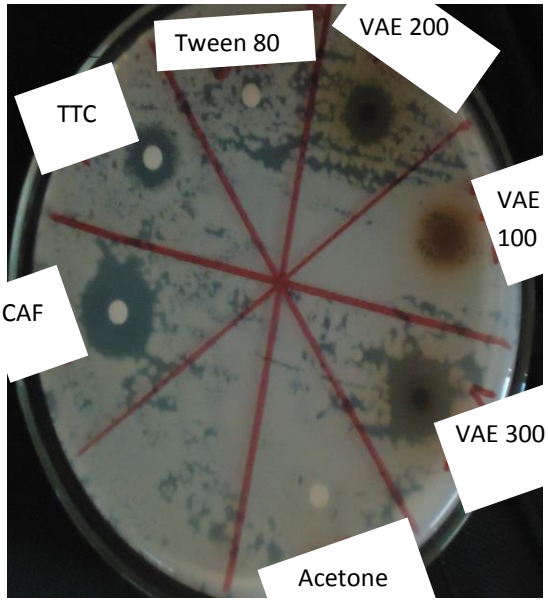


b) Compound **VII**

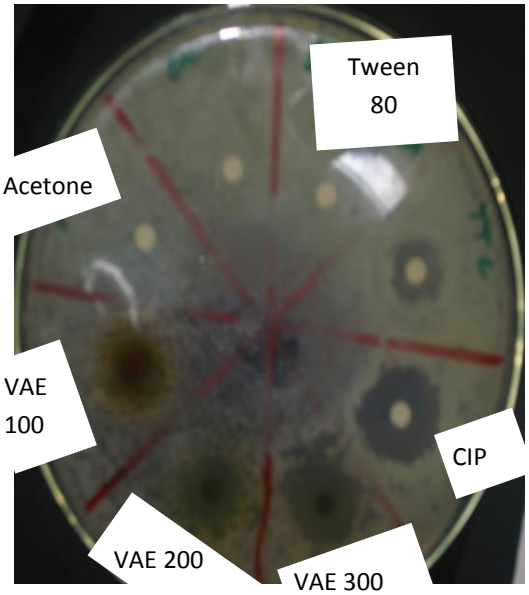
Appendix C: Pictures showing bacterial growth inhibition by VAE (Vernonia Acetone Extract) and pure compounds isolated from it (a – d crude extract; e – h pure compounds) on tested bacteria



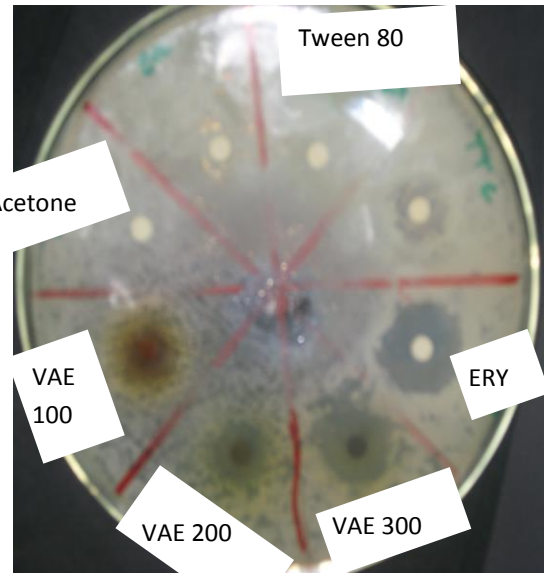
a) *Escherichia coli*



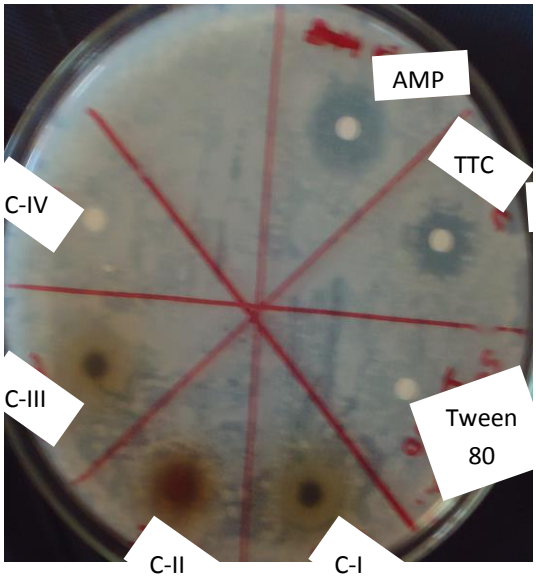
b) *Salmonella typhi*



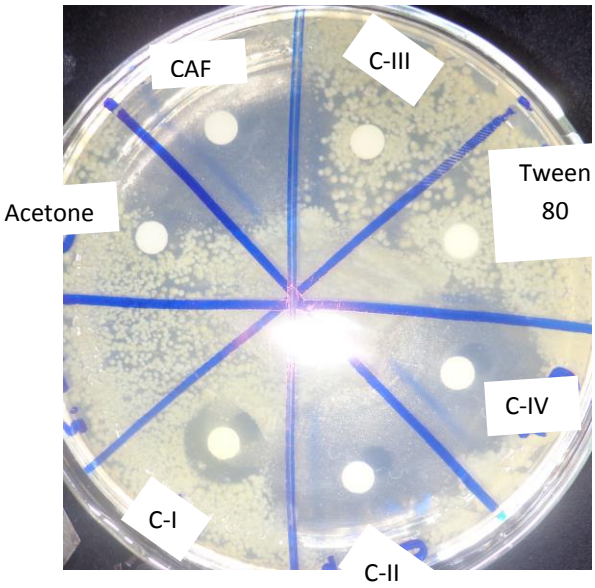
c) *Shigella boydii*



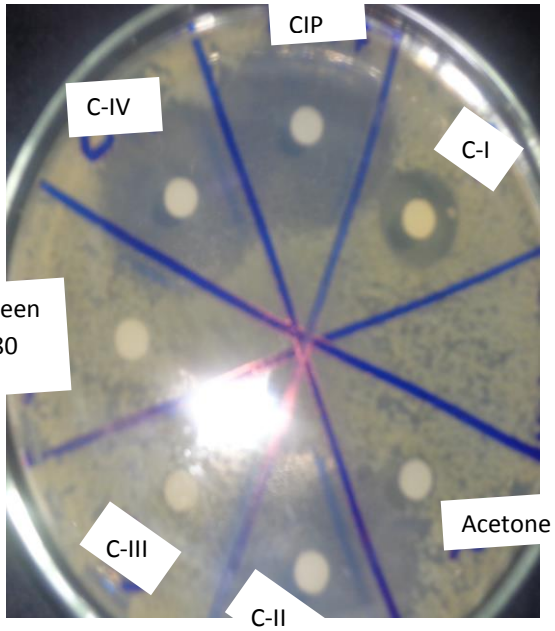
d) *Staphylococcus aureus*



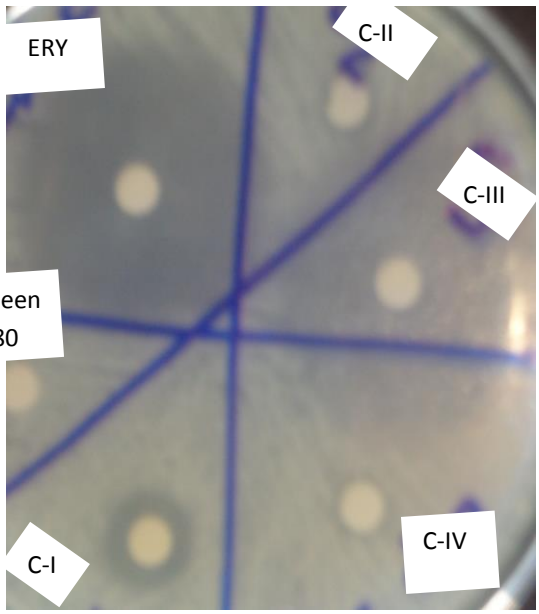
e) *Escherichia coli*



f) *Salmonella typhi*

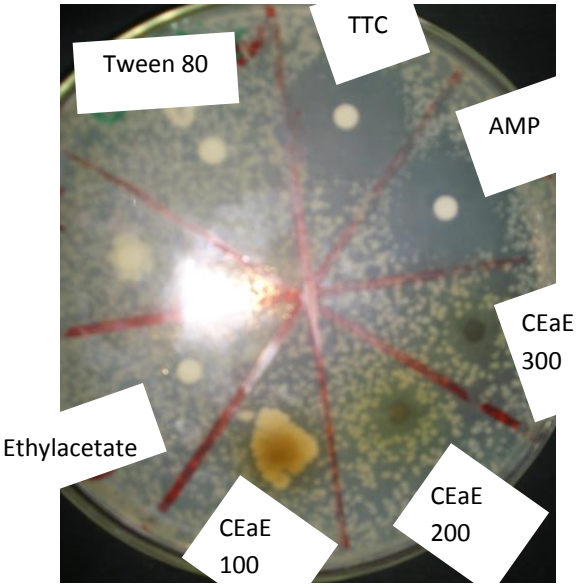


g) *Shigella boydii*

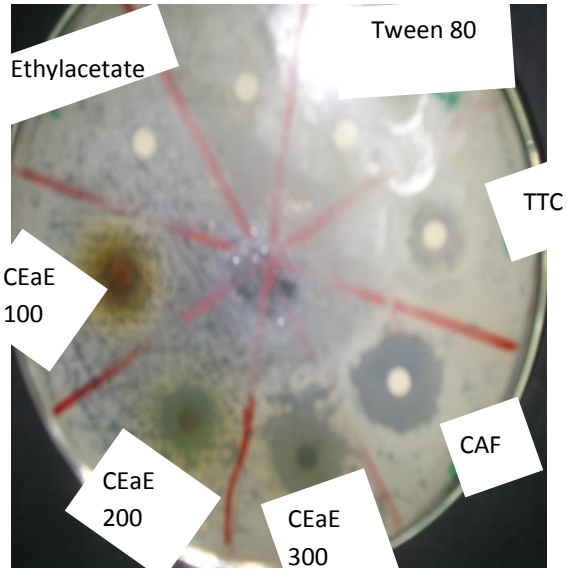


h) *staphylococcus aureus*

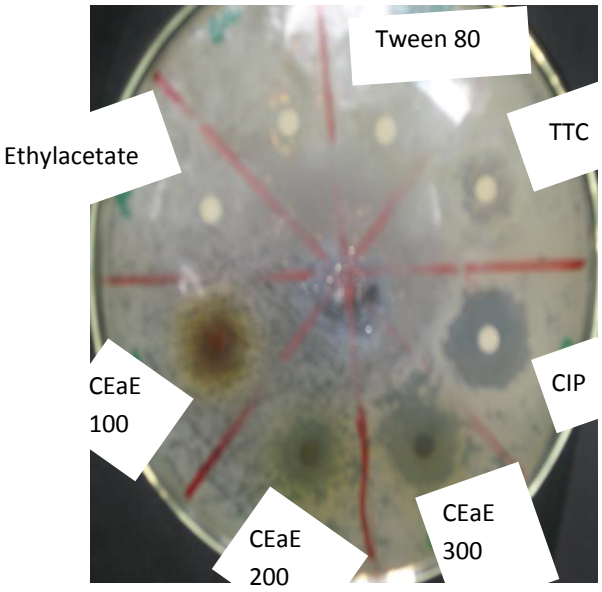
Appendix D: Pictures showing bacterial growth inhibition by CEaE (Croton Ethylacetate Extract) and pure compounds isolated from it (a – d crude extract; e – h pure compounds) on tested bacteria



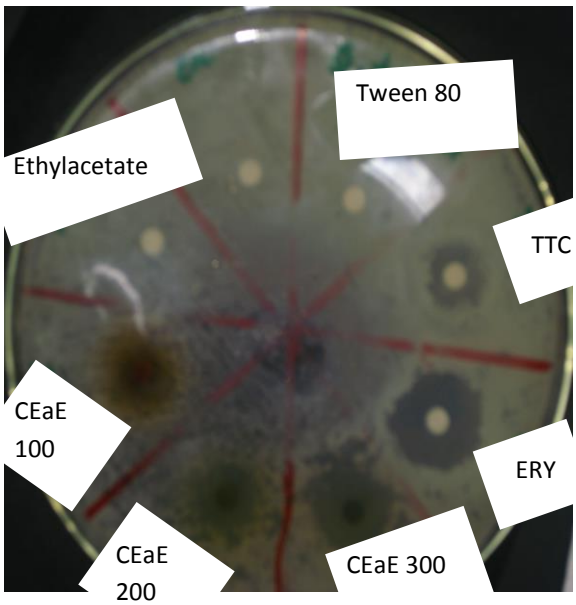
a) *Escherichia coli*



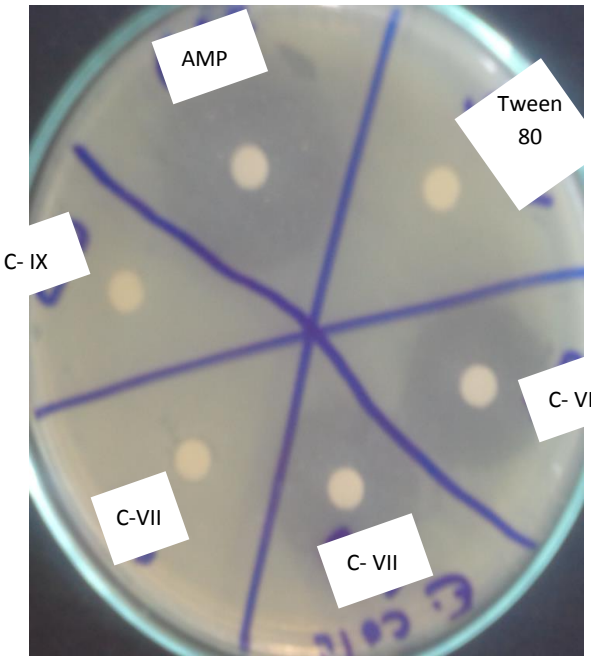
b) *Salmonella typhi*



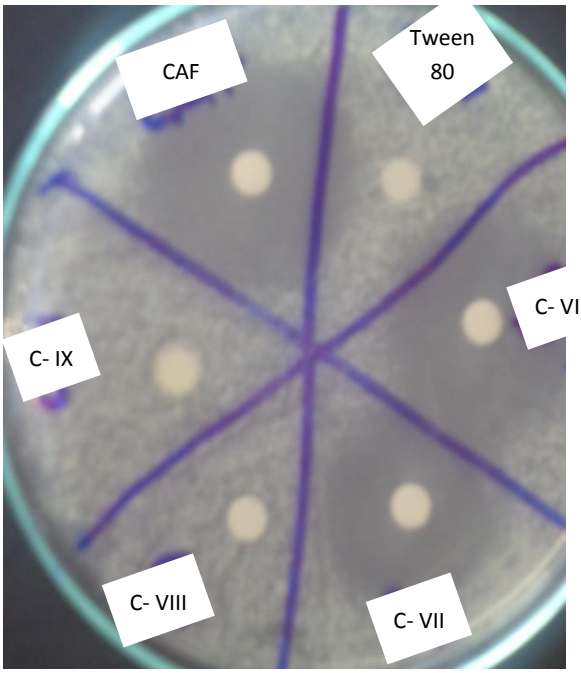
c) *Shigella boydii*



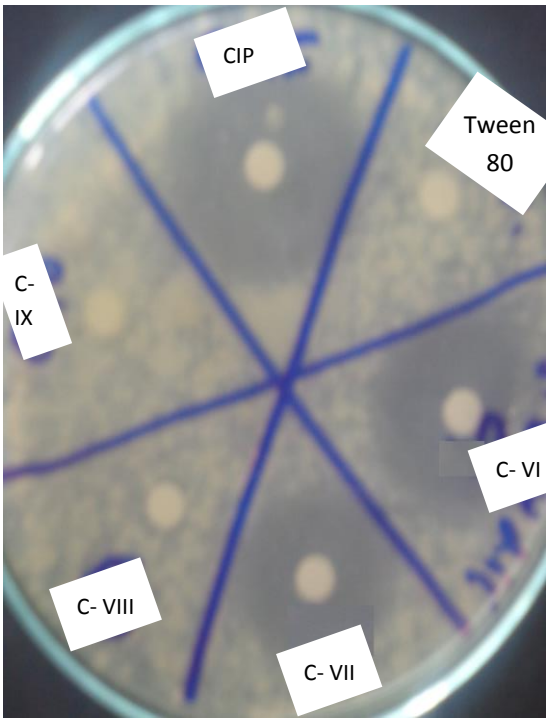
d) *Staphylococcus aureus*



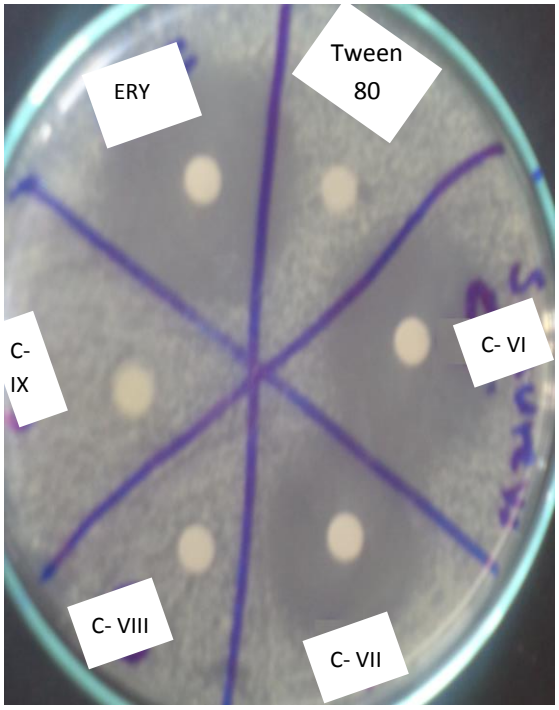
e) *Escherichia coli*



f) *Salmonella typhi*



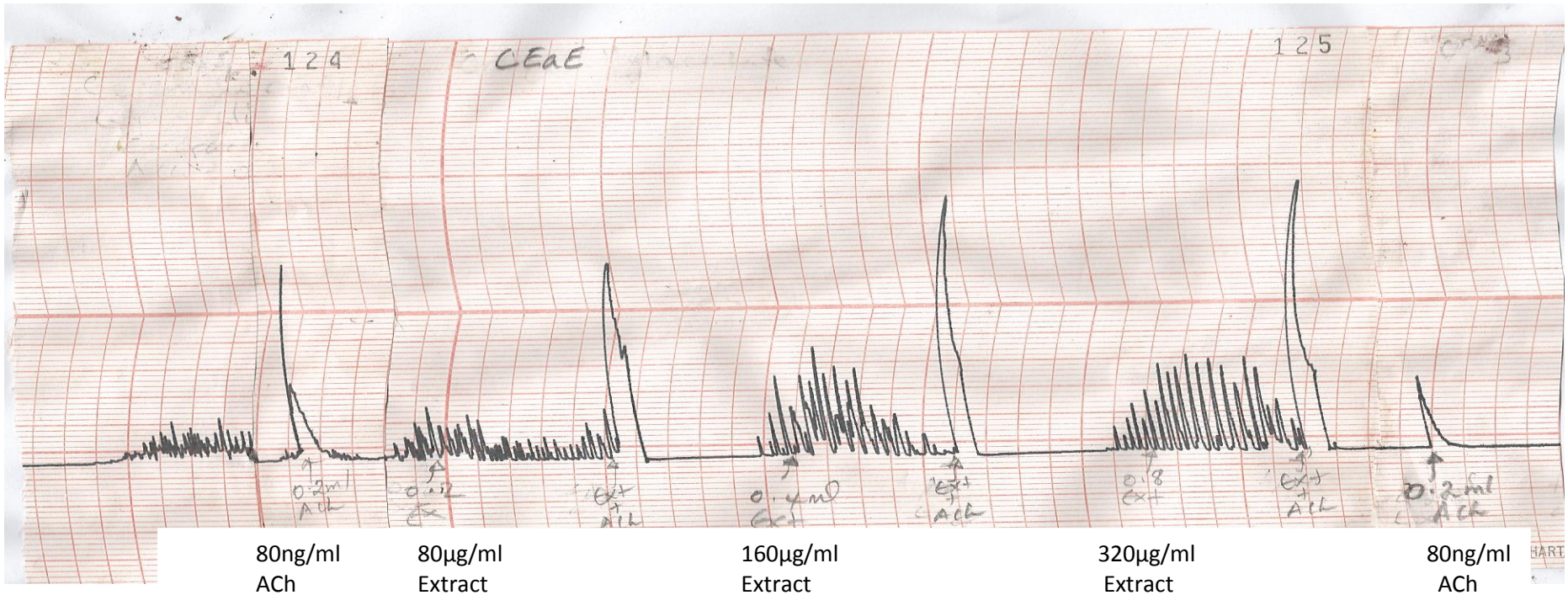
g) *Shigella boydii*



h) *Staphylococcus aureus*

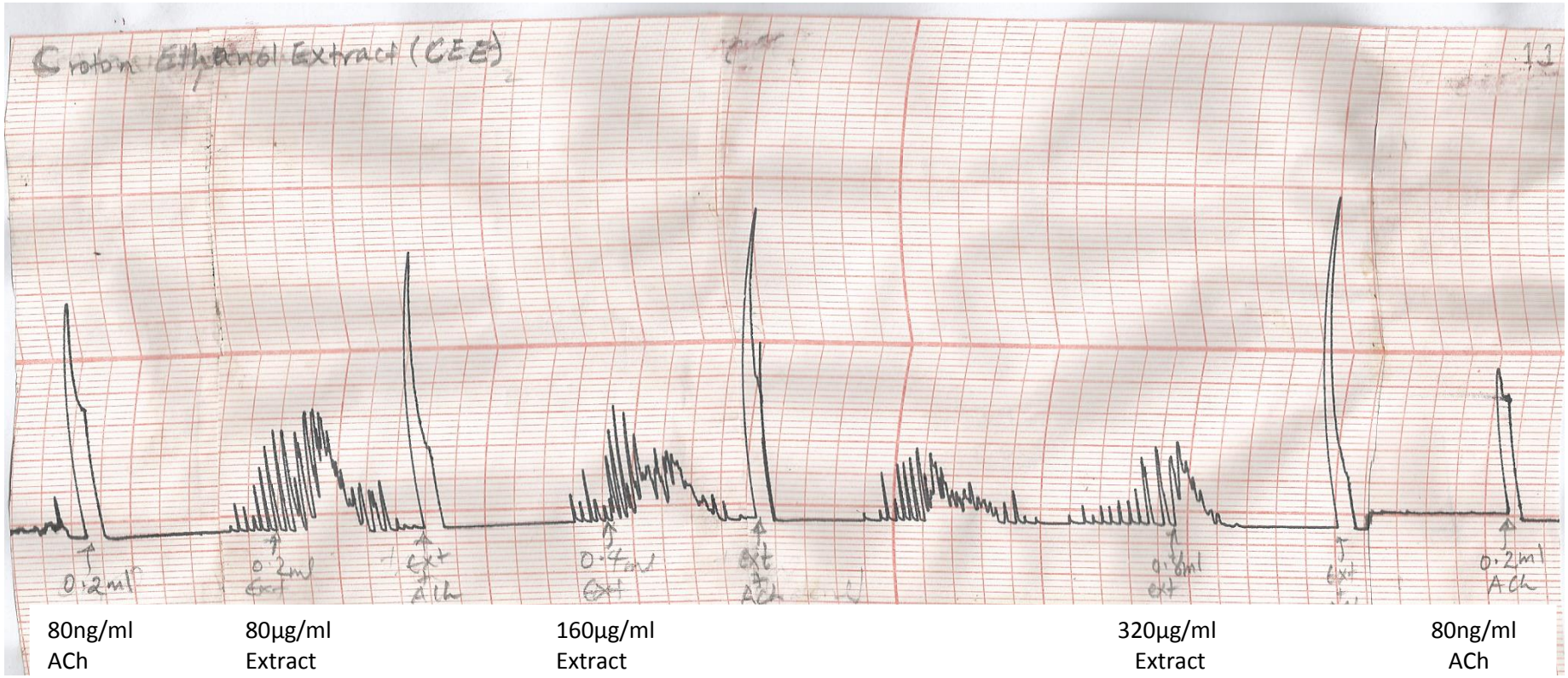
Appendix E: Polygraph records for the effect of crude extracts of *C. macrostachyus* leaves on acetylcholine (ACh) induced guinea pig ileum contraction

E-1: The effect of CEaE on ACh induced guinea pig ileum contraction at different organ bath concentrations



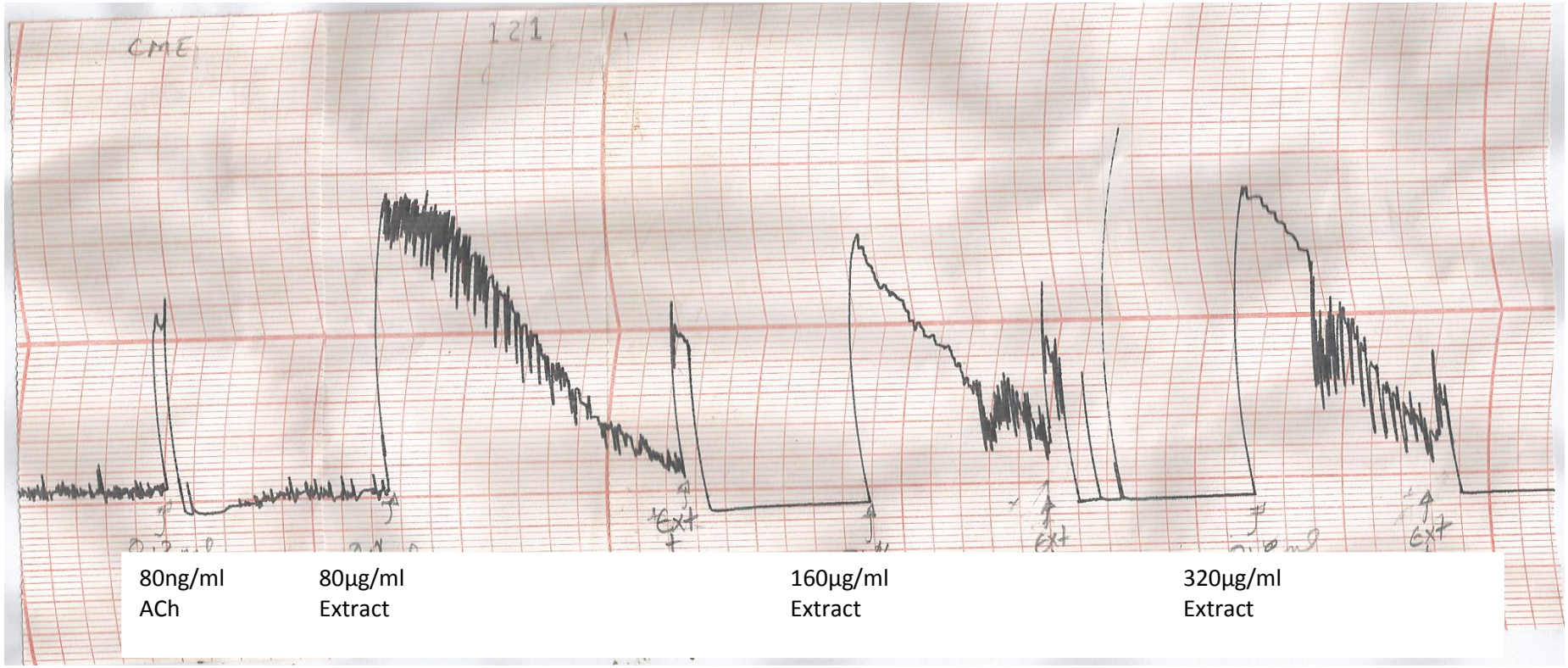
80ng/ml ACh | 80µg/ml Extract | 160µg/ml Extract | 320µg/ml Extract | 80ng/ml ACh

E-2: The effect of CEE on ACh induced guinea pig ileum contraction at different organ bath concentrations



80ng/ml ACh	80µg/ml Extract	160µg/ml Extract	320µg/ml Extract	80ng/ml ACh

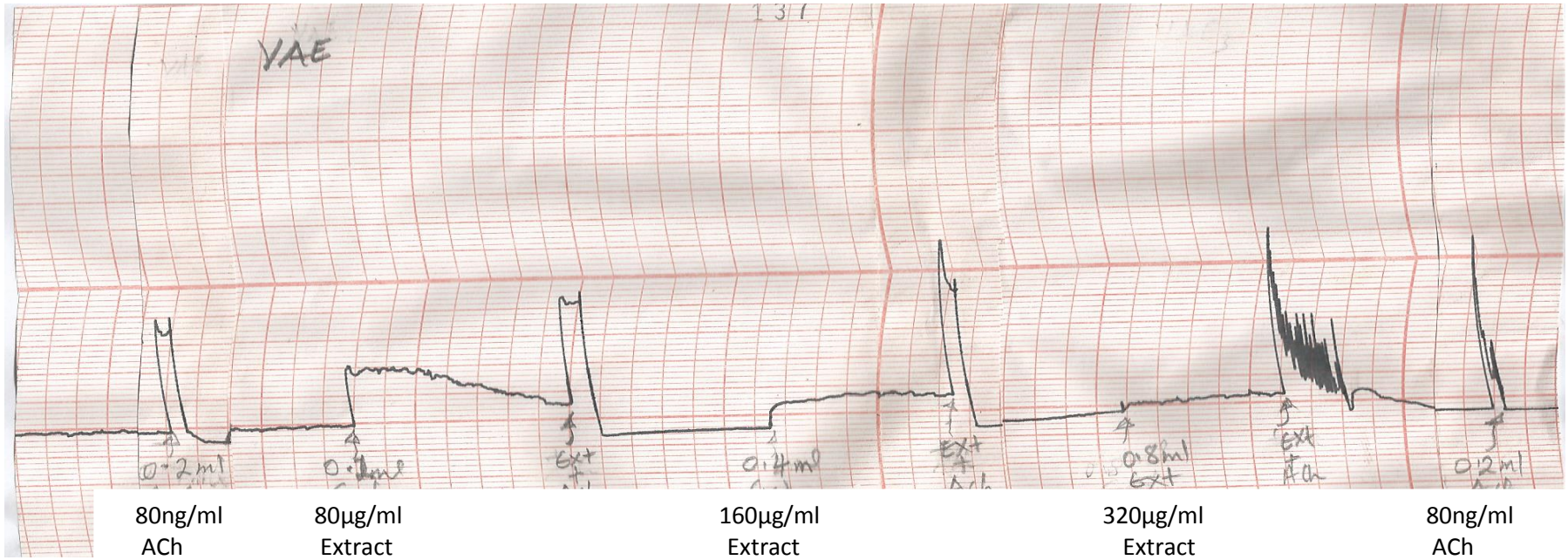
E-3: The effect of CME on ACh induced guinea pig ileum contraction at different organ bath concentrations



80ng/ml ACh	80µg/ml Extract	160µg/ml Extract	320µg/ml Extract

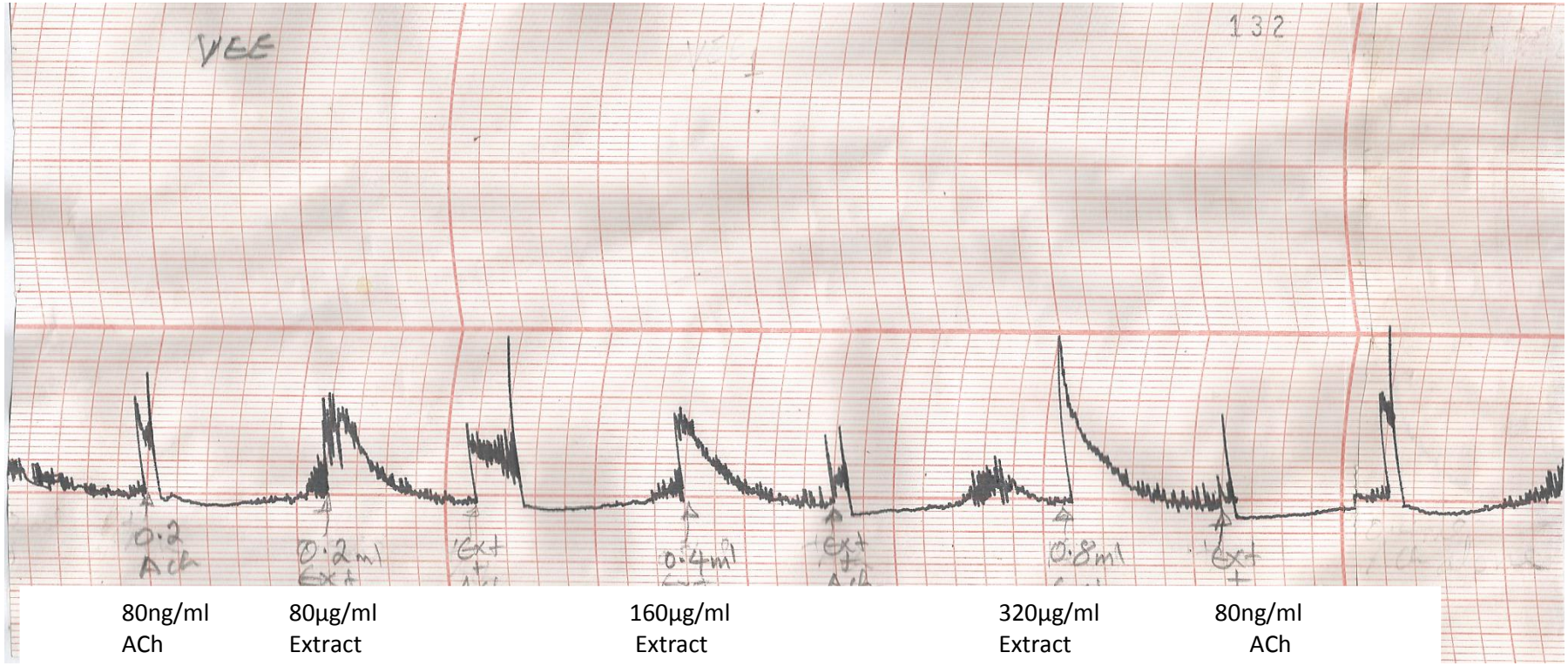
Appendix F: Polygraph records for the effect of crude extracts of *V. galamensis* leaf on acetylcholine (ACh) induced guinea pig ileum contraction

F-1: The effect of VAE on ACh induced guinea pig ileum contraction at different organ bath concentrations



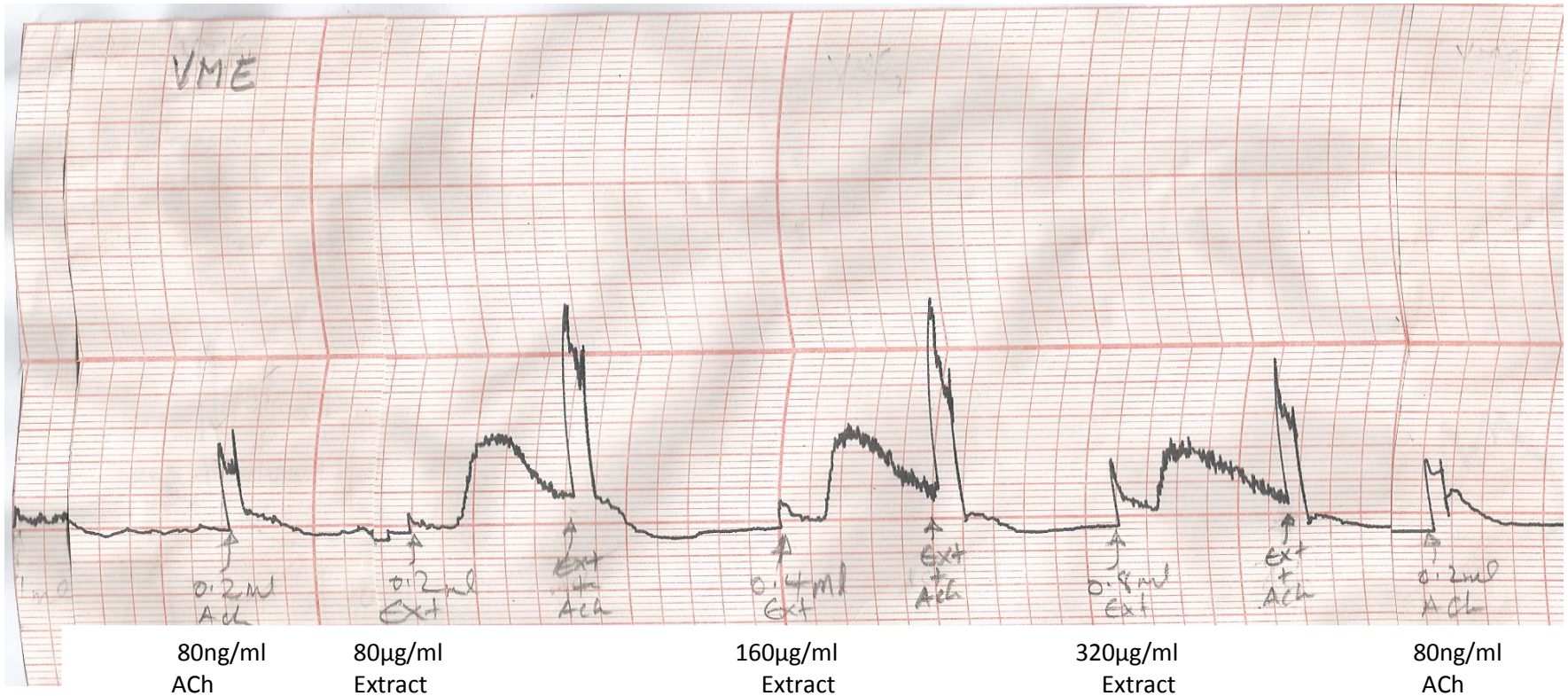
80ng/ml ACh | 80µg/ml Extract | 160µg/ml Extract | 320µg/ml Extract | 80ng/ml ACh

F-2: The effect of VEE on ACh induced guinea pig ileum contraction at different organ bath concentrations



80ng/ml ACh	80µg/ml Extract	160µg/ml Extract	320µg/ml Extract	80ng/ml ACh

F-3: The effect of VME on ACh induced guinea pig ileum contraction at different organ bath concentrations



Appendix G: Results of Acute Toxicity Tests of Crude Extracts of *V. galamensis* and *C. macrostachyus Leaves* on Mice

G1: Observed Physical and Behavioral Changes

Group* n= 6/group	**Doses (mg/kg/b.wt)**	**Signs**				
		Mortality	Hair Erection	Dizziness/ Sleepiness	Over Active	Normal Activity
1 - 6	500	-	-	-	-	+
7 - 12	1000	-	-	-	-	+
13 - 18	1500	-	-	-	-	+
19 - 24	2000	-	-	-	-	+
Control	0.2 ml veh	-	-	-	-	+

*Groups: 1,7,13 and 19 (VAE); 2,8,14 and 20 (VEE), 3,9, 15 and 21 (VME), 4,10, 16 and 22 (CEaE), 5,11, 17 and 23 (CEE) and 6,12, 18 and 24 (CME)

G2: Average weight gain (g) by tested and control mice

Doses (mg/kg/b.wt)	**Average weight gain (g)/Test Groups (n= 6)**						
	VAE	VEE	VME	CEaE	CEE	CME	Control
500	3.8	3.3	3.7	2.9	2.5	3.1	3.4
1000	3	2.9	2.9	2.7	2.8	3.6	3.8
1500	2.8	3	3	3	3.1	3.7	4
2000	2.6	2.5	2.8	2.9	2.4	3	3.5

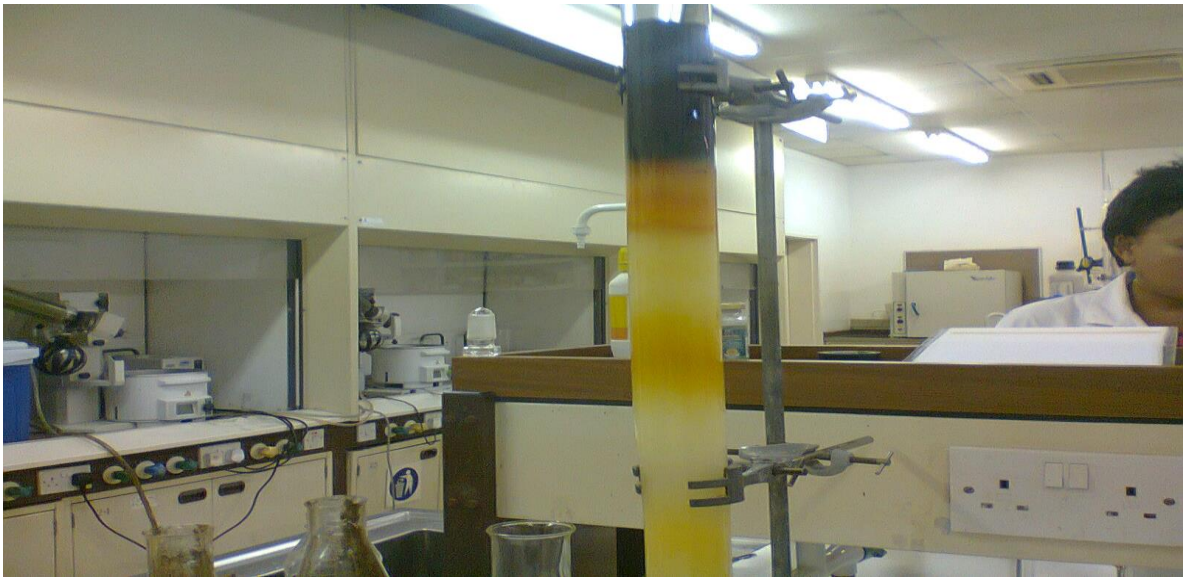
Appendix H: Pictures showing some of the activities of the present research work

H1: Photographs of (a) *V. galamensis* and (b) *C. macrostachyus* taken from the field

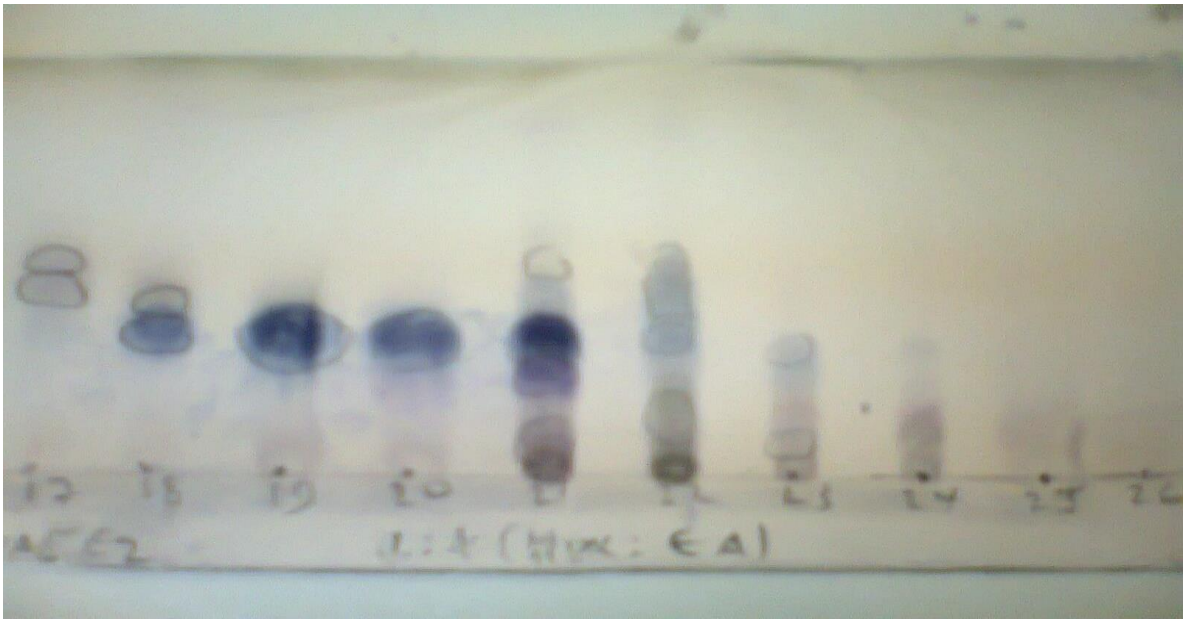
(a)

(b)

H2: Sample set up of column chromatography for fractionation of extracts



H3: Sample photograph for TLC (thin layer chromatography) analysis



H4: Antibacterial Tests

H5: Guinea pig ileum preparation for contractility test

H6: Dosing mice for acute toxicity test