Antibacterial Activity of the Extracts of the Leaves and Stem Barks of *Calpurnia aurea* Against Selected Human Pathogenic Bacteria

By

Zaru Abise

A Thesis Submitted to the Department of Applied Biology School of Applied Natural Science in Partial Fulfillment of the Requirements for Master of Science Degree in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

October, 2020

Adama, Ethiopia

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DECLARATION

I declare that the work which is being presented in this research entitled as **"Antibacterial Activity of the Extracts of the Leaves and Stem Barks of *Calpurnia aurea* Against Selected Human Pathogenic Bacteria"** in partial fulfillment of the requirement for the award of the degree of MSc in biotechnology. It was authentic record of my original work carried out from October 2019 to September, 2020 under the advisor of Dr.Mulugeta Kebede, Department of Applied Biology and Dr.Yadessa Melaku,Department of Applied Chemistry, School of Applied Natural Science, Adama Science and Technology University .The matter embodied in this has not been submitted by other person or me for the award of any other degree. All relevant resources of information used in this body have been duly acknowledged.

Zaru Abise

Signature: _____

This thesis has been submitted for examination with my approval as a University advisor and co-advisor.

Advisor: Dr.Mulugeta Kebede

Signature: _____

Co-advisor:Dr.Yadessa Melaku

Signature: _____

APPROVAL PAGE

We the undersigned, members of the board of Examiners of the final open defense by Zaru Abise Muda have read and evaluated this thesis entitled "**Antibacterial Activity of the Extracts of the Leaves and Stem Barks of *Calpurnia aurea* Against Selected Human Pathogenic Bacteria"** and examined the candidate. This is therefore to certify that the thesis has been accepted in the partial fulfillment of the requirement of the degree of Master of Science in Applied Biology.

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LIST OF ACRONYMS AND ABBREVIATIONS

^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H –NMR	Proton Nuclear Magnetic Resonance
ANOVA	Analysis of Variance
CC	Column Chromatography
DEPT	Distortion less Enhancement by Polarization Transfer
DMSO	Dimethyl Sulphoide
MBC	Minimal Bactericidal Concentration
MeOH	Methanol
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
RF	Retention Factor
TLC	Thin Layer Chromatography
TMS	Tetra Methyl Silane
UV-Vis	Ultraviolet Visible

ABSTRACT

Natural products have been a source of medicinal agents since thousands of years and remarkable number of modern drugs has been derived from natural sources. *C.aurea*, known as cheekata (in Afaan Oromo), is traditionally been used to treat various diseases such as, wound, diarrhea fever, rabies and cancer. The main objective of this study was to evaluate the extracts of the leaves and stem barks of the isolate bioactive compounds from *C.aurea* for antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Salamonella tphyimurium, Bacillis subtilis* and *Shigella dysenteriae* .The leaves and stem barks of *C.aurea* were extracted using n- hexane, ethyl acetate and methanol for 72h on maceration at room temperature to give yields of 13.96%, 18.24%, and 36.49%, from leaves and 7.21%, 10.36% and 13.74% from stem barks yields respectively. Phytochemical screening conducted on leaves and stem barks of *C. aurea* showed the presence of alkaloid, tannins, flavonoids, saponins, terpenoids and phenols from leaves and absence of stereoid from leaves. The methanol extracts after silica gel column chromatography led to the isolation of three compounds namely compound **1**, compound **2** and compound **3**.The structures of these compounds were established using spectroscopic methods ^{1}H NMR, ^{13}CNMR, DEPT-135 and UV-Vis. Antibacterial activity was evaluated against *Escherichia coli, Staphylococcus aureus, Salamonella tphyimurium, Bacillis subtilis* and *Shigella dysenteriae* at 5 mg/mL and 10 mg/mL concentrations using agar disc diffusion and micro dilution method. The results indicate methanol extract of *C. aurea leaves* and its three isolated compounds show strongest antibacterial activity, higher inhibition zones of antibacterial activity of methanol crude extracts against *Escherichia coli* (20±1.15) and *Salamonella tphyimurium(*17.66±0.88) were observed as compared to standared gentamyicin (24±1.73 and 26±1.15 mm respectively). The MIC and MBC were determined for plant crude extracts that showed antibacterial activity on disc diffusion; by using broth micro dilution method. The isolated compounds also have showed strong Antibacterial activity. This was useful to manage infection diseases caused by pathogenic microorganisms.

Keywords: Antibacterial activity, *Calpurnia aurea*, phytochemical screening,Tiyo woreda

1. INTRODUCTION

1.1. Background

Plants have nutritional, medicinal and ecological values (Adedapo *et al.*, 2008). Medicinal plant has not only played the vital role in providing healing, but has also contributed to discovery of pharmaceutically active substances in plants body (Kassaye *et al*., 2006). Natural products are important sources for biologically active drugs. There has been an increasing interest in the medicinal plants as natural products in different part of the world. Medicinal plants are used for treatments of infectious diseases which are caused by different pathogenic microorganisms and are becoming worldwide threats. Among these microorganisms, bacteria are the most and very hazardous microorganisms in present time. As the result of those characteristics treatment option should be changed to tackle these difficulties. Infectious diseases are one of the major problems in developing as well as developed countries.

Traditional medicinal plants are widely used to treat the microbial diseases due to the in rich source of antimicrobial activity and less cost (Hemalatha *et al*., 2013). The increase of microbial resistance to antibiotics created several problems. The severity of multidrug resistance issue is aggravated by the fact that there are less new antibiotics being discovered. The scientific and medical community should turn their efforts to find alternatives source for antibiotics. One of the most common approaches is the search for the easiest and the safest antibiotics to human and others animals are medicinal plants. The present study permitted the evaluation of antibacterial activity against some selected bacterial strains.

The medicinal value of these plants depends on bioactive phytochemical activities in the plant body. Phyotochemicals are bioactive of plant origin. They are regarded as secondary metabolites, because the plants that manufacture them may have little need for them. Phytochemicals are naturally synthesized in parts of the plant body such as, bark root, leaves, stem, flower, fruits and seeds that are part of the plant body may contain active components (Carlet *et al.*, 2012). Herbs are used in many domains, including medicine, nutrition, flavoring, beverage, dyeing, repellents, fragrance, and cosmetics (Letchamo and Storck, 2006)). Many species have been recognized to have medicinal properties and beneficial impacts in health, example, antioxidant activity, anti-inflammatory and antimicrobial, anti-carcinogenic potential and anti-mutagenic effects (Ekor, 2014).

It has been estimated that up to 90% of the population in the developing countries rely on the use of medicinal plant in one way of another to meet their primary health care need (WHO, 2002). More than 50,000 plant species are used for medicinal purpose world wide of which almost 13% are flowering plants, containing active chemicals (shipman *et al*.,2002). Traditional medicines used to affect singularly or combination to treat, diagnose and prevent illness and maintain well-being (Ogwal,1994). Majority of Ethiopian still use traditional medicine for treating various health problems since medicinal plant has lower cost compared to modern medical attention (Ahmad *et al.*,1999). Ethno medicinal survey in Ethiopia and other part of the world indicated that the genus *Calpurnia* has been traditionally used for many therapeutic purposes. *C.aurea* is a species of flowering shrub/tree growing up to 7m tall in the family Fabaceae. It grows widely in tropical Africa Guinea, Nigeria, Ethiopia, Angola, Malawi, and Zambia, usually at the edge of semi-humid forests at relatively high altitudes (Jansen, 1981).

In Ethiopia *C. aurea* is commonly known as ceekata (Afaan Oromo). Preparation made from different part of plant is used to treat various diseases in the traditional Ethiopia medicine: leprosy, wound, diarrhea, fever, eye disease, rabies and tumor/cancer, among other (Fullas, 2001). In modern medicine, plants are used as sources of direct therapeutic agents, as models for new synthetic compounds, and as a taxonomic marker for discovery of new compounds (Asadi *et al*., 2013). They serve as a raw materials base for the elaboration of more complex semi synthetic chemical compounds. The synthesis of bioactive compounds is chemically difficult, because of their complex structure and high cost.

Wide variations in medicinal quality and content in Phytopharmaceutical preparations have been observed. These are influenced mainly by cultivation period, season of collection, plant-to-plant variability in the medicinal content, adulteration of medicinal preparations with misidentified plant species, a lack of adequate methods for the production and standardization of the crop, a lack of understanding of the unique plant physiology or efficacy with human consumption and consumer fraud (Okwu,2004). Diarrhoeal disease is a leading cause of mortality and morbidity, especially among children in developing countries resulting in a major health care problem (Havagiray *et al* .,2004).

1.2. Statement of the problem

The increase in resistance to many commercially produced synthetic antimicrobial agents by microorganisms has been increasing with time, hence the need for alternative antimicrobial agents (Gray *et al*., 2013). Most of the bacterial pathogens have shown the capability of developing resistance to some of commercially available antimicrobial agent. Genomic studies on various microorganisms indicate that the prolonged use of antimicrobials and antibiotics makes these microorganisms more resistant to them. This has led to the search for new, cheap and effective antimicrobial agent that can combat the increasing, resistance by the pathogenic microbes.

Medicinal plants play a role in the treatment of various human and livestock ailments in rural Ethiopia; but, needs further scientific evidence of their traditional medication. To the best of our knowledge, a comprehensive biological study, and isolation of bioactive compounds of leaves and stem barks extracts of *C.aurea* had not been reported so far particularly in Ethiopia. But the local people in the country use this plant for various ethno medicinal purposes without having proper scientific evidence with regard to the chemical composition and biological uses of the plant. The plant was selected for this study on the basis of ethno medical information by which local communities use them and their genera are rich source of variety classes of secondary metabolites with different pharmacological properties. Therefore, the study is aimed at antimicrobial activity and isolation of bioactive compounds of leaves and stem barks extracts of *C.aurea* to support the existing indigenous knowledge on the medicinal use of the plant with scientific evidence.

2. OBJECTIVES

2.1. General Objective

To extract, isolate and characterize bioactive compounds from the leaves and stem bark extracts of *C. aurea* for antibacterial activities test against disease causing bacterial pathogens.

2.2. Specific Objectives

- To extract leaves and stem barks of *C.aurea* using n-hexane, Ethyl acetate and methanol solvents.
- To fractionate compounds from crude extracts of the leaves and stem barks of *C.aurea* using Column chromatography.
- To perform the antibacterial activities of the crude extracts and their fractionated compounds from the leaves and stem barks of *C.aurea*, using agar disc diffusion method and broth micro dilution method, against, *E.coli, S. aureus, S. tphyimurium,B. subtilis and S. dysenteriae.*
- To characterize the structures of the fractionated compounds using melting point, UV-Vis and NMR (^{1}H-NMR,^{13}C-NMR and DEPT-135).

3. LITERATURE REVIEW

3.1. Traditional Medicinal plant

3.1.1.Calpurnia aurea and its suitable geographical Location

Calpurnia aurea is a Southern African tree belonging to the family Fabaceae, occurring along the coastal regions from the south-eastern Cape northwards and inland to the central Transvaal, with an isolated population in eastern Zimbabwe. The Fabaceae family includes 32 genera and more than 170 species of tree and brushes of pan tropical distribution. *C.aurea* is a member of the subfamily Papilionoideae of the family fabaceae. The plant commonly used in traditional medicine to treat diverse medical conditions and parasitic infestation, in both humans and animals (Hutchings, 1996). It is a small, multi-stemmed and occurring widespread in bush land and grassland in sub-Saharan Africa and India. In southern Ethiopia, it is called cheka by the Borana people it is often found in overgrazed areas and is easily cultivated. It is characterized by its contents of better substances, mostly responsible for its pharmaceutical properties (Coates, 1983).

3.1.2 Genus *Calpurnia*

The genus *Calpurnia* widely distributed in tropical Africa and Asia (Table 1). The genus comprises about seven species of which *C. aurea,C. floribunda* and *C. intrusa* are the most common species (Roberts, 1994). In Ethiopia, *C. aurea, and Calpurnia floribunda are found, of which C.aurea* is well known.

Table1.*Calpurnia*species and their occurrence

Species of the genus *Calpurnia*	Occurrence	Reference
C. aurea	Ethiopian, Asia, Angola, Cameroon,	*Compton, 1976*
C. woodii	Eritrea	Brummitt,1967
C. sericea	Uganda and Tanzania	*Dale&Green, 1961*
C. reflexus	Kenya	Baker, 1871
C. intrusa	Guinea	*Dyer, 1975*
C. floribunda	Ethiopian	Uddin *et. al., 2015*
C. glabarta	Asia	Keya *et al.*, 2017

3.1.3. Part of *Calpurnia aurea* traditionally used for mediation

Plant-based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc. Medicinal properties of plants unique to particular plant species or groups are consistent with the concept that combination of secondary products in a particular plant is taxonomically distinct (Latha,2009).

3.2. *Calpurnia aurea* Economic Importance

3.2.1. Ethno botanical Uses of genus *Calpurnia*

Different species of the genus *Calpurnia* are used as a traditional medicine to cure many diseases worldwide. The root of *C.aurea* has been used in traditional medicine to treat dysentery and malaria (Farooq *et al., 2008*). The root barks of *C.aurea* mixed with milk were used by people living in northern Ethiopia for treating dysentery. The use of plants in religious ceremonies as well as for magic and medicinal purposes is very common and widely distributed in Ethiopia (Endashaw, 2007).The fruit powder mixed with honey and fermented for seven days is taken orally to treat bullad(weight loss, fever, itching, diarrhea), fruit powder mixed with milk is taken orally for three days for hemorrhoids, root powder mixed with honey is taken orally for mushuro (weight loss, dysentery and fever), juice of leave taken orally in the morning for dysentery and dressing with inner bark past mixed with butter or oil for chiffea (Eczema) (Tilahu *et al., 2007*).

Furthermore, the dry root is smoked and inhaled and the flower of *Calpurnia aurea* grounded, boiled and fumigated to treat Evil eye. *C. aurea* has a number of therapeutic applications like treating scabies and external parasites as an antidysentery agent, wound healing effect and treatment of Gonorrhea (Lulekal *et al*., 2008). In South Africa, *calpurni*a leaves and powdered roots are used to destroy lice and to relieve itche as anticancer and ant-malaria and treatment of Trypanosomiasis (Tamiru *et al., 2013*). Among others the leaves and roots of *C. aurea* are mixed with small amount of water and applied to treat wound (Mesfin, 1986).

3.3. Characterization of Calpurnia aurea and its Techniques for structural Elucidation

3.3.1. Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance Spectroscopy gives physical, chemical and biological properties of matter. Chemists to study chemical structure using simple one-dimensional techniques routinely use NMR spectroscopy, this techniques are used to probe molecular dynamics in solutions and solid state NMR spectroscopy is used to determine the molecular structure of solids,^{13}C- NMR is used to identify the types of carbon are present in the compound and 1H- NMR is used to find out types of hydrogen are present in the compound and to find out how the hydrogen atoms are connected (Raaman,2006).

3.4. Pharmacological active compounds and its biological activity

3.4.1. Bioactive compounds

3.4.1.1. Flavonoids

Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. Flavonoids have been reported to exert wide range of biological activities. These includes: anti-inflammatory, antibacterial, antiviral, antiallergic, cytotoxic, anti-tumour, treatment of neurodegenerative diseases, vasodilatory action (Tsuchiya,2010).

3.4.1.2. Saponins

Saponins have been ascribed a number of pharmacological action, the important ones being permeabilizing of the cell membrane,lowering serum cholesterol, stimulation of luteinizing hormone release leading to obortifacient properties, immune modulatory potential via cytokine interplay, cytostatic and cytotoxic effect on malignant tumor cell, adjuvant properties for vaccines as immune stimulatory complexes and synergistic enhancement of the toxicity of immune toxins (Fuchs *et al.*,2009).

3.4.1.3. Phenolics

Phenolics have been considered powerful antioxidants in vitro and proved to be more potent antioxidants than Vitamin C and E and carotenoids (Rice-Evans *et al.*,1995). The inverse relationship between fruit and vegetable intake and the risk of oxidative stress associated diseases such as cardiovascular diseases, cancer or osteoporosis has been partially ascribed to phenolics (Scalbert *et al.*,2005). It has been proposed that the antioxidant properties of phenolic compounds

can be mediated by the following mechanisms (1) scavenging radical species such as ROS suppressing formation by inhibiting some enzymes or chelating trace metals involved in free radical production; (3) up regulating or protecting antioxidant defense (Cotelle .2001).

3.4.1.4. Terpenoids

Terpenoids composed of isoprenoid units constitute one of the largest groups of natural products accounting for more than 40000 individual compounds, with several new compounds being discovered every year (Withers and Keasling, 2007). The diverse array of terpenoid structures and functions has provoked increased interest in their commercial use. Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer, and also to have antimicrobial, antifungal, anti parasitic, antiviral, anti-allergenic,anti spasmodic, anti hyperglycemic, anti inflammatory, and immune modulatory properties (Rabi and Bishayee 2009). In addition, terpenoids can be used as protective substances in storing agriculture products as they are known to have insecticidal properties (Theis and Lerdau, 2003).

3.4.1.5. Alkaloids

In some cases, alkaloids obtained from plants may cause serious illness, injury or even death. The manner of poisoning with plants can be divided into unintentional ingestion of plant material, intentional ingestion of plant material, and ingestion of abused plant material (Beyer *et al.*,2009).

3.4.2. Biological Activities of C. Aurea Plant

3.4.2.1. Antibacterial Activity

Root extracts of *C. aurea* have been used in South Africa to treat maggot-infested wounds and in Ethiopia to treat scabies. In western Ethiopia, the juice of crushed leaves and seeds is used for tick control (Regassa, 2000). The Borana people of northern Kenya and southern Ethiopia soak leaves of *C. aurea* in cold water to treat louse infestations in humans and calves and. The plant is also used to treat stomach disorders, amoebic dysentery and eye diseases. In southwestern Ethiopia, the leaves of *C.aurea,* mixed with other plant species, are crushed and squeezed to obtain a juice, which is applied through the auricular route for 2 days to treat earache in humans (Yineger *et al*.,2008). In the same area, the plant is traditionally used to treat rheumatism antibacterial and antioxidant activity of *C. aurea* have been reported and the plant has been used to treat bacterial dermatitis (Tadeg *et al.*, 2005). It has also been used as a natural pesticide to improve grain storage (Blum and Bekele**,** 2002).

The main pharmacologically active compounds of *C.aurea* may be the alkaloid calpurmenin and its (2′-pyrrolecarboxylic acid) ester. The alkaloids virgiline and lupanine, as well as their carboxylic esters, have also been recorded. As part of a larger ethno veterinary survey to assess plants traditionally used for tick control in southern Ethiopia, investigated the attractant/repellent and acaricidal effects of *C. aurea* extracts (Zorloni, 2007).

3.4.2.2. Antioxidant Activity

The term antioxidant‘refers to the activity of numerous vitamins, minerals and other phyto chemicals to protect against the damage caused by reactive oxygen species (ROS)(Dejian *et al*.,2005).By their ability to react with and damage many structures in the body, ROS are involved in various physiological processes and diseases such as ageing, cancer, diabetes and atherosclerosis(Killedar *et al*.,2013).Dietary antioxidants, including polyphenolic compounds, vitamins E and C, and carotenoids, are believed to be the effective nutrients in the prevention of these oxidative stress related diseases. Ethanol extract of the root sample of *C. aurea* has shown better antioxidant activity when compared to the standard reference(ascorbic acid, which was 86.88%).The ethanol root extract has shown 81.63% of antioxidant activity at100µg/mL as compared to other (chloroform, methanol, and n-hexane i.e., 71.72%, 36.40% and 26.14%, respectively) solvents root extract (Gnanasekaran *et al*., 2015).

3.4.2.3. Anticancer Activity

Anticancer activity of the isoflavonoids isolated from *C.aurea* leaves have preventive anticancer activity and that the methylated isoflavones have much greater anticancer activity than those without methyl groups (Walle *et al.*, 2007). The isoflavones with methoxy groups appear to have more beneficial qualities than their non-methylated counterparts and have been shown to be more bio available and biologically stable than the hydroxylated isoflavones (Fouche *et al*., 2008). Although the isolated isoflavones is not as active as the control, etoposide, it is worth noting that they do show moderate activity against the cancer cell lines tested.

3.4.2.4. Antidiarrhoeal effects

The 80% methanol extract of *Calpurnia aurea* leaves was found to be effective in a dose dependent Manner against castor oil induced diarrhoea on experimental mice at all tested doses. At the dose of 400 mg/kg body weight, the extract produced a significant decrease in the severity of diarrhoea in

terms of reduction in the rate of defecation and consistency of faeces in albino mice(Shemsu Umer *et al*.,2013).

3.5. Factor affects plant growth and its bioactive compounds

Plant growth and geographic distribution are greatly affected by the environment. If any environmental factor is less than ideal, it limits a plant's growth, Either directly or indirectly, most plant problems are caused by environmental stress, Environmental factors that affect plant growth include light, temperature, water, humidity, and nutrition (Bertein,2018). The content of bioactive compounds varies significantly due to many internal and external factors, including plant organs, phenological stage, genetic profile, environmental a biotic and biotic factors, such as growing site, light, temperature, radiation, soil drought and salinity, pathogens, and herbivores attack (Alam,2015).

3.6. General Feature of Test Microorganisms

3.6.1. *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive, non-spore forming spherical bacterium that belongs to the *Staphylococcus* genus. The Staphylococcus genus is subdivided into 32 species and sub species. *S. aureus* produces staphylococcal enterotoxin (SE) and is responsible for almost all staphylococcal food poisoning (FDA, 2012). *S. aureus* is commonly found in the environment (soil, water and air) and is also found in the nose and on the skin of humans. The growth and survival of S. *aureus* is dependent on a number of environmental factors such as temperature, water activity, pH, the presence of oxygen and composition of the food.

The temperature range for growth of *S. aureus* is 7–48°C, with an optimum of 37°C,*S. aureus* is resistant to freezing and survives well in food stored below -20°C; however, viability is reduced at temperatures of -10 to 0°C.*S. aureus* is readily killed during pasteurization or cooking. its growth is occurring over the pH range of 4.0–10.0, with an optimum of 6–7 (ICMSF 1996). *S. aureus* is uniquely resistant to adverse conditions such as low, high salt content and osmotic stress (Montville and Matthews, 2008). *S. aureus* is a poor competitor, but its ability to grow under osmotic and pH stress means that it is capable of thriving in a wide variety of foods, including cured meats that do not support the growth of other food borne pathogens. It is a facultative anaerobe so can grow under

both aerobic and anaerobic conditions. However, growth occurs at a much slower rate under anaerobic conditions (Stewart, 2003).

3.6.2. *Bacillus subtilis*

Bacillus subtitlis is gram-positive model bacterium in biofilm research, is able to form morphologically complex and exceptionally robust colony-type biofilm (Branda *et al*., 2001). This bacterium is considered to be largely non-pathogenic, although it has been linked to food spoilage (Thompson *et al*., 1993). *B. subtilis* commonly resides in association with plant roots in soil as well as within the human oral cavity (Chen *et al*., 2013).

And gastrointestinal tract further contributing to its ability to withstand severe environmental conditions is its ability to sporulate, particularly by forming dormant end spores that are embedded within the biofilm, in terms of biofilm forming capacity, *B. subtilis* exhibits a multitude of morphological phenotypes from thin, flat microstructures to large, spatially heterogenic colony-type biofilms (Bridier *et al*., 2011). *B. subtilis* biofilm formation on solid lysogeny broth (LB) medium was shown by to occur when the growth medium is supplemented with glycerol and manganese (Shemesh *et al*., 2013). The biofilm-promoting was demonstrated by the appearance of a robust biofilm phenotype alongside increased extracellular matrix production and biofilm-associated sporulation(Vlamakis *et al.,*2008.provided evidence that the structuring of *B. subtilis* biofilms is achieved by hetero geneous dierentiation of cells within the colonies into motile, matrix-producing, and sporulating cells. The specific phenotype of a *B. subtilis* colony-type biofilm is determined by multiple environ mental factors including growth medium, temperature, presence of cofactors, oxygen availability (Hong *et al*., 2009).

3.6.3. *Shigella dysentery*

Shigella dysentery is a gram-negative, non-motile bacillus belonging to the Enterobacteriaceae family. There are four species of *Shigellae*: *S. dysenteriae, S. flexneri, S. boydii and S. sonnei* (designated as serogroups A, B, C and D respectively (Schroeder, and Hilbi, 2008). The first three species include several 19 serotypes. Acquired immunity to Shigella is serotype-specific. While S. boydii and *S. dysenteriae* usually cause a relatively mild illness (watery or bloody diarrhea only), *S. flexneri* and S. sonnei are chiefly responsible for endemic and epidemic shigellosis respectively) in developing countries, with high transmission rates and significant case fatality rates (Ali, 2014).

3.6.4.*Escherichia coli*

Escherichia coli, is a gram-negative, facultative anaerobic, rod-shaped, coli form bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms, gram-negative bacterium, usually motile by peritrichous flagella. Most strains of *E. coli* are not harmful but they are part of the healthful bacterial flora in the human gut (Jain *et al*., 2013).However, some types can cause illness in humans, including diarrhea, abdominal pain, fever, and sometimes vomiting. *E.coli* is, produces trusted Source a toxin known as Shiga. It is one of the most power full trusted Source toxins, and it can cause an intestinal infection (Teresa *et al.*,2013). *Escherichia coli* are the most common cause of acute urinary tract infections as well as urinary tract sepsis (Steven *et al*.,2014). *E.coli* may also cause acute enteritis in humans as well as animals and is a general cause of traveler's diarrhea, a dysentery-like disease affecting humans, and hemorrhagic colitis often referred to as 'bloody diarrhea'.

3.6.5. *Salmonella typhimurum*

Salmonella typhimurum is gram-negative, rod-shaped bacillus that can cause salmonella on a diarrheal illness in humans, *Salmonella* bacterium typically live in animal and human intestines and are shed through feces. (Muyembe *et al.*,2006). Humans become infected most frequently through contaminated water or food. Salmonella infection (salmonellosis) is a common bacterial disease that affects the intestinal tract. Gram-negative bacteria usually have a cell wall composed of a thin layer of peptidoglycan, covered by a membrane (Luby *et al*., 2015).

4. MATERIALS AND METHODS

4.1. Description of plant sample collection Area

The sample for this study was collected from East Arsi Zone, Tiyo woreda, Abditu kabale, Oromia region state, Ethiopia which is 175 kilometers far from Addis Ababa. Tiyo woreda is geographically located at latitude of 7˚32'0" - 7˚54'0" N and longitude of 38˚59'30" - 39˚21'30" E (Figure.1) and an elevation between 2500 and 3560 m.a.s.l. The East Arsi zone is characterized by dissected plateaus and mountains associated with hills and valleys. In general, area is characterized with varied topographic features that resulted for the existence of micro climatic variation and endowment of different natural vegetation resources that contributes for good agricultural development. Agro ecologically, the area is dominantly highland consisting, hills and some mountains with gorges and plain areas. The vegetation includes bush and shrubs covering small areas and scattered trees such as eucalyptus and acacia. The soil type is dominantly sandy loam with pocket areas of clay loam. The area has bimodal characteristics with two rainy seasons, the long rainy season (mid-June to mid-September) and the short rainy season (from mid-February to mid-May). The average annual rainfall of the area ranges from 700 to 900mm. (Central statistical Authority, 2007).

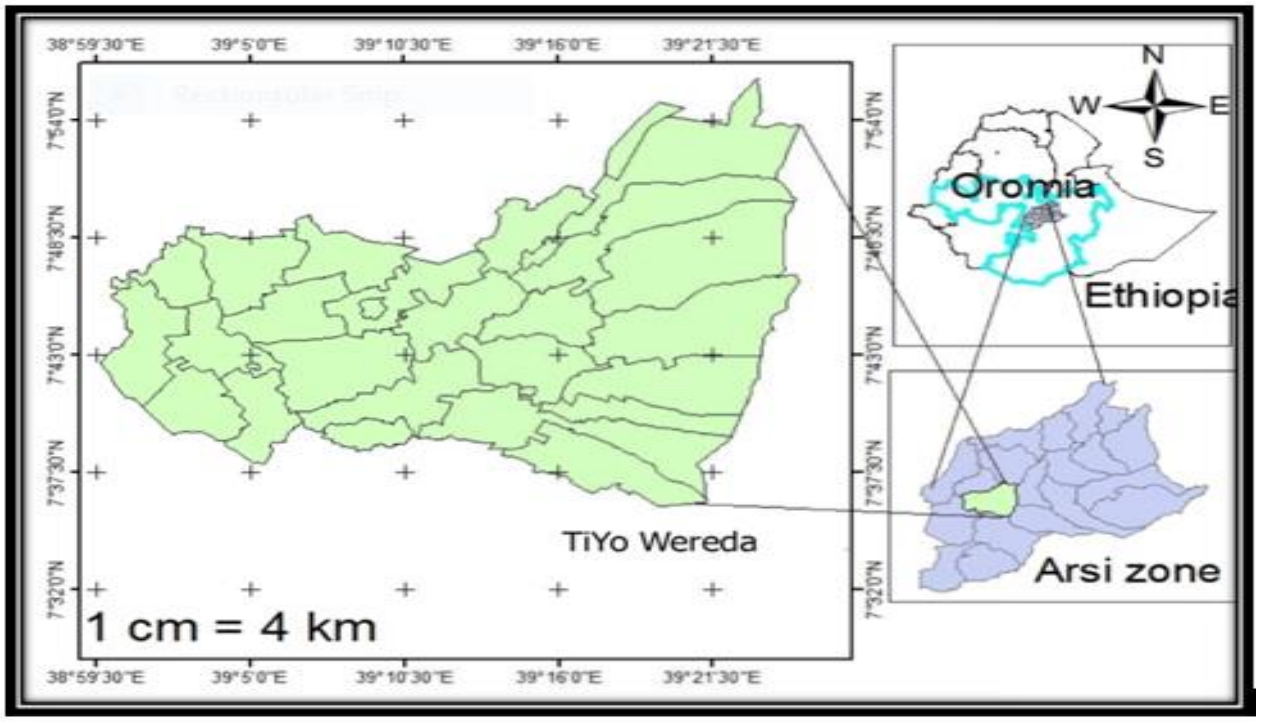


Figure 1. The map of study area

4.2. Collection of experimental plant and Identification

Both leaves (2kg) and stem barks (2kg) of *C. aurea* were collected from Tiyo woreda, Arsi Zone, Oromia regional state, Ethiopia. The fresh samples were packed in plastic bags and transported into the laboratory in the month of January 2020.The plant materials were identified by National Herbarium of Addis Ababa University. (Figure.2).

Figure 2. Picture of *C.aurea* (Picture taken by Zaru Abise on 20/04/2019)

4.3. Preparation of experimental Plant for Extraction

The sequential extraction method described by Ncube *et al.*, (2008), was applied with slight modification as follows. The plant samples were washed thoroughly under running tap water to remove dirt, dust and insects and air dried at room temperature without exposing it to direct sun light in shaded area so as to bring down the large moisture content for about 3 weeks.

The dried plant materials were then ground using a mechanical grinder to form fine powder material of the plant. About 300 g powdered leaves and 300 g of powdered stem barks of *C. Aurea* was gained after the plant material were ground. Then the powdered leaves (300 g) of *C.aurea* were

soaked in1.5L of hexane for 72 hours on an orbital shaker to ensure thorough extraction. It was then filtered using Whatman filter paper no .1, then concentrated using rotary evaporator and weighed in microbiology and molecular laboratory of Adama Science and Technology University. The marc was soaked in 1.5L of ethyl acetate for 72 hours with occasional swirling to ensure thorough extraction then Filtered, concentrated using rotary evaporator, air dried and weighed. The marc after extraction with ethyl acetate was finally soaked in 1.5L methanol for 72 hours with occasional swirling. Filtered, concentrated and weighed. Sequential extraction procedures as above were repeated with the 300 g of stem bark of *C.aurea.* The extracts were stored in the freezer at 4^{o}c until test for antimicrobial activities.

4.4. Phytochemical screening

4.4.1. Test for alkaloids (Wagner's test):

The extract was diluted in an acidic solution of HCl. one milliliter extract was mixed in a test tube with 3 drops of Wagner's reagent prepared be forehand. The formation of A reddish brown precipitate indicates the presence of alkaloids (Puri and Kumar, 2019).

4.4.2. Test for Saponins (Froth Test):

The Extract of 0.5gwere diluted with distilled water of 5 ml and this was shaken in a graduated cylinder for 15minutes.Formation of 1cm layer of foam indicates the presence of saponins (Solanki *et al*., 2019).

4.4.3. Test for Tannins (gelatin test):

The extract of 0.5g was boiled in 10 mL of distilled water in test tube and filtrated. To the filtrate, few drops of 0.1% of $FeCl_3$ was added to give a brownish green color which confirms the presence of tannins (Yadav *et al*., 2017).

4.4.4. Test for Flavonoids (Alkaline Reagent):

To 0.5g of crude extract 10 mL of ethyl acetate was added and heated for 3minute using steam bath. The mixture was filtered, and the filtrate was mixed with 1mL of dilute ammonia solution. Formation of intense yellow color ratifies the presence of flavonoids (Khan *et al*., 2016).

4.4.5. Test for Terpenoids (salkowski test):

The crude extract of 0.5g was mixed with 2mLof chloroform and 3mLconcentrated $H_2SO_4$was carefully added to form a layer. A reddish-brown coloration of the interface was formed to show the presence of terpenoids (Chowdhury *et al.,* 2013).

4.4.6. Test for Phenols (Ferric chloride test):

To 0.5g of crude extract few drops of 2% of $FeCl_3$was added and the formation of black color indicates the presences of phenols (Alebiosu and Yusuf, 2015).

4.4.7. Test for Steroids:

Steroids was conducted following the methods of Alhadi *et al*., (2015) the extract (1 mL) was dissolved in 10 ml of chloroform and equal volume of concentrated H_2SO_4 was added. The upper layer turns red and H_2SO_4 layer showed yellow with green flurescence. This indicates the presence of steroids.

4.5. Preparation of stock solutions

The crude extract of leave and stem barks was dissolved in DMSO(Dimethyl Sulphoide) in two separate vials to furnish 10mg/mL and 5mg/mL. This was repeated for the fractionated compounds of *C.aurea*.

4.6. Preparation of Dried Filter paper discs

Each of the discs was cut from what man N0.1 filter paper with an approximate diameter of 6 mm using a puncher. The prepared filter paper disc were sterilized by autoclaving at 121^oC for15 minutes and impregnated with 20microliter of 10mg/ml crude extract and 20 microliter of 5 mg/ml concentration of fractionated extract of experimental plant. With the help of sterilized forceps, each disc was recovered from the extract and separately placed in sterilized Petri dish and dried to remove the solvent completely in a hot air oven at 40 ^{0}c for 60 minutes before being applied onto the agar surface in a plate.

4.7. Test Microorganisms

The reference strains of human pathogenic bacteria were representing gram negatives (*E.coli* ATCC 2785, *S.dysenteries ATCC* 27853 and *S.typhriumum* ATCC 13062) and gram positive *(S.aureus* ATCC 6538 and *B. subtilis* ATCC 29213) were obtained from Adama regional microbiology

laboratory. Pure cultures of the strains were sub cultured and prepared for in vitro antimicrobial activity test using Muller Hinton agar.

4.8. McFarland Standard preparation

Preparation of McFarland, 0.5Mcfardland turbidity standard were prepared by mixing 1% of sulfuric acid and 1% of barium chloride to be obtained solution with specific optical densities. To prepare a 0.5Mcfardland standard, 2 mL of 1%$BaCl_2$ and 2 mL of 1%H_2SO_4 solution and stirred to maintain a suspension, Well isolate colonies and same type morphology were pick by cotton swap to inoculate test tube contain 2mL of 1%$BaCl_2$ and 2mL of 1%H_2SO_4 solution. A 0.5McFard land turbidity standard provides an optical density comparable to the density of a bacterial suspension $1.5x10^8$ colony forming unity (CFU/ml) (McFarland, 1907).

4.9. Preparation and components of luria Bertani

To prepare 1000 mL of Luria bertani10 g of tryptone 15g of nutrient agar,5 g of sodium chloride, and 5 g of yeast extract were weighted and added in to conical flask.1000mL of distilled water was added and mixed. To dissolve all ingredients completely, boiling for 10 minutes, and was sterilized by autoclaving for 15 minute and used for subculturing of the test microorganisms.

4.10. Antimicrobial Activity test of plant Extracts

4.10.1. Agar Disc Diffusion Assay

The antibacterial activity test of crude extracts and fractionated compounds of experimental plant against two gram positive (*S.aureus* and *B.subtilis* and three gram negative bacteria (*E.coli, S.dysenteriae and S.typhimurim)* were carried out by disc diffusion method. The Sterile Muller Hinton agar mediumswas prepared for each organism as follows.

Twenty milliliter of sterile MHA (maintained at 45-50^0C in a molten state), poured into sterilized Petri dishes. After solidifying Muller Hinton agar, 0.1ml of test organism were spread on MHA plate. Then the filter paper discs were placed on the surface of the agar plate at equal distance from each other and 15 mm from edge of the plate as described by (Clutter buck*et al*., 2007). Each disc was pressed down to ensure complete contact with the agar surface and each plate was inverted and placed in an incubator set at 37 ^{0}c for 24 hours. The antimicrobials susceptibility were evaluated after 24 hours by measuring zone of inhibition using plastic ruler in mm and the result were

compared with the standard drug Gentamycin as a reference standard and sterile saline discs were placed as negative control.

4.10.2. Minimum Inhibitory Concentration (MIC)

The MIC and MBC were determined for plant crude extracts that showed antibacterial activity on disc diffusion, by using broth micro dilution method proposed by (Novy *et al.,* 2015) with minor modifications. Briefly, 10mL of Mueller-Hinton Broth plus different concentrations of plant extracts were prepared and transferred to each micro plate well to obtain dilutions of the active extract, ranging from10 to 0.63mg/mL. Then100 micro litter of a fresh culture of test bacterial suspension prepared by comparing with Mc-Farland turbidity standard, 0.5McFarland standard of test organisms were added. Micro plates were incubated at 37 ^{0}C for 24 hrs. MIC was defined as the lowest concentration of the extracts that restricted the visible growth of microorganism tested were.

4.10.3. The Minimal Bactericidal Concentration (MBC)

To determine MBC 10mL from each well that showed no visible growth was reinoculated on extracts free MHA plates; then the plates were reincubated at 37 ^{0}C for 24h, the absence of visible growth of bacterial colony after 24 hours indicated bactericidal activity (Ncube *et al.,* 2008).

4.11. Isolation of Bioactive Compounds

4.11.1. Column Chromatography Technique

The leaves and stems barks of *C.aurea* were successively extracted on maceration with n-hexane, EtOAc and MeOH. The most active methanol extract of *C.aurea* was subjected to silica gel column chromatography (garmany). Briefly, silica gel 140g was mixed with 200 mL of n-hexane to form a homogenous suspension/ slurry and stirred using a glass strring rod to remove bubbles. The silica gel slurry was then poured in to a glass column. The methanol extract (10g) was adsorbed on equal amount of silica gel (mesh size 60-120) and subjected to column chromatography using hexane: EtOA: MeOH using increasing gradient of ethyl acetate in n-hexane and methanol in ethyl acetate as eluent to afford 84 fractions, each 100mL was collected. The column was first eluted with n-hexane as the mobile phase with the polarity increasing by 5% increments of ethyl acetate. After getting to100% ethyl acetate, the polarity was further increased by 5% increments of methanol in ethyl acetate, each 100 mL was collected.

A total of 84 fractions were collected. Fraction that showed similar Retention factor values and the same characteristic color on Thin Layer Chromatography were combined and concentrated at 40^0c under reduced pressure. Fractions 65:35,60:40, 55:45 and 50:50 eluted using methanol in ethyl acetate as eluent showed a single spot on TLC (EtOAc/methanol,3:7, Retention factor value 0.66,30 mg) was labeled as compound **1.**

Fraction 80:20 eluted using ethyl acetate in n- hexane as eluent showed a single spot on TLC (EtOAc/n- hexane,7:3, Retention factor value 0.5,25 mg) was labeled as compound **2** and Fractions 85:15,80:20 and75:25 eluted using methanol in ethyl acetate as eluent showed a single spot on TLC(EtOAc/methanol,4:6, Retention factor value 0.41,30 mg) was labeled as compound **3** and finally subjected to UV, NMR and DEPT-135 spectroscopic studies.

4.11.2. Structural elucidation of fractionated compounds

All solvents used are analytical grade. Column chromatography was performed using silica gel (60-120) mesh) Merk, and the ^{1}H and ^{13}C- NMR spectra of the compounds were recorded on Bruker advanced 400 and 101 MHz NMR spectrophotometer using Chloroform-*d* and Methanol *d4* as the solvent and TMS as an internal standard and the values are expressed in δ ppm. UV/Vis spectrums were recorded in range of 200-500 nm using methanol and chloroform solvents as blank. 1mg of each of the isolated compounds were dissolved in 10 ml of methanol solvent, then the absorbance of the compounds at some wavelength were recorded on double UV/Visible spectrophoto meter. Melting points of the isolated compounds were measured by using a melting point apparatus (Thiele tube).

4.12. Data analysis

The experimental data were analyzed using the Statistical Package for Social Sciences (SPSS) (version 20.0) software and excel Microsoft Word and the results were presented using graphs and tables in form of mean ±SEM (standard error of the mean). After NMR analysis using chamdraw software for draw of structure. The statistical difference of the mean zone of inhibition of the extracts for individual bacterium was carried out by employing one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test at a significance level of $p<0.05$.

5. RESULTS

5.1. Yields of extracts

Percentage Yield = Individual Yield * 100

Total Yield obtained

The highest percentage yields (36.49%) was obtained from leaves by methanol which was followed by ethyl acetate extract (18.24%) and the lowest yield was from stem barks extract by n- hexane (7.2%) (Figure. 4, appendix 1).

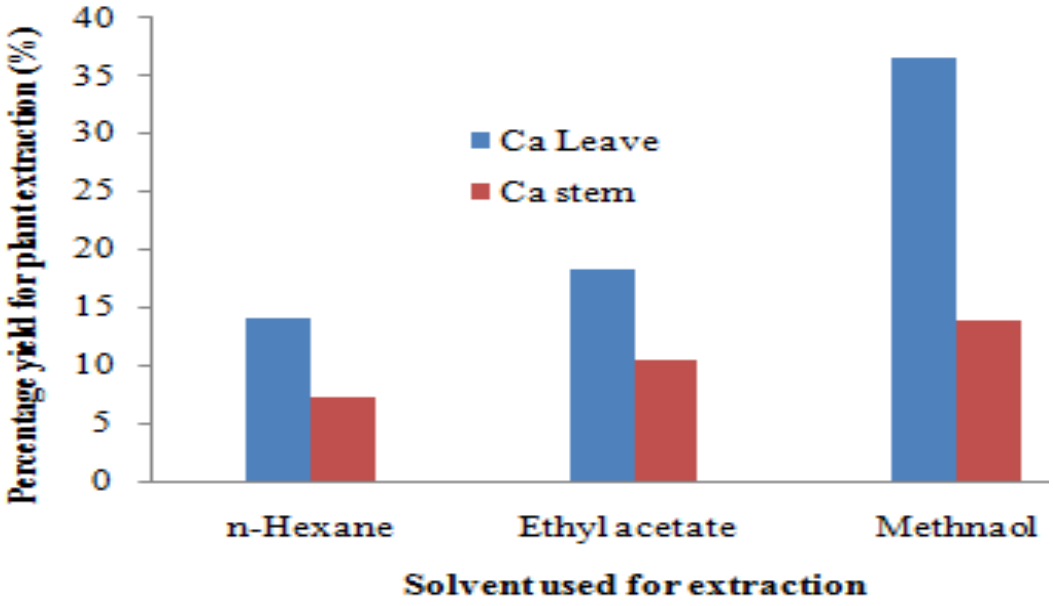


Figure 3. The yield of *C. aurea* from leaves and stem barks extracts by n-hexane, ethyl acetate and Methanol.

5.2. Phytochemical screening

The phytochemicals screening test results showed that leaves extract of *C.aurea* was rich in alkaloids, flavonoids, saponins,phenols, tannins and terpenoids, but, only Saponnins and Phenols were obtained from the stem barks (Table.2, Figure.5).

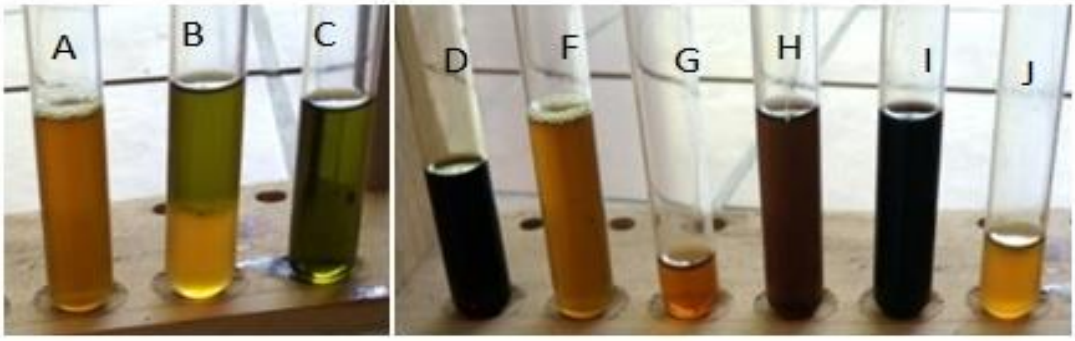


Figure 4. Showing phytochemical test results for different plant compounds.

Aand F,saponnin for stem and leave show positive,

D and I, phenols for stem and leaves positive result

H, steroid for both negative result

B, Terpenoid,C, Tannins,G,Alkaloid and J,flavonoid, for leaves positive result

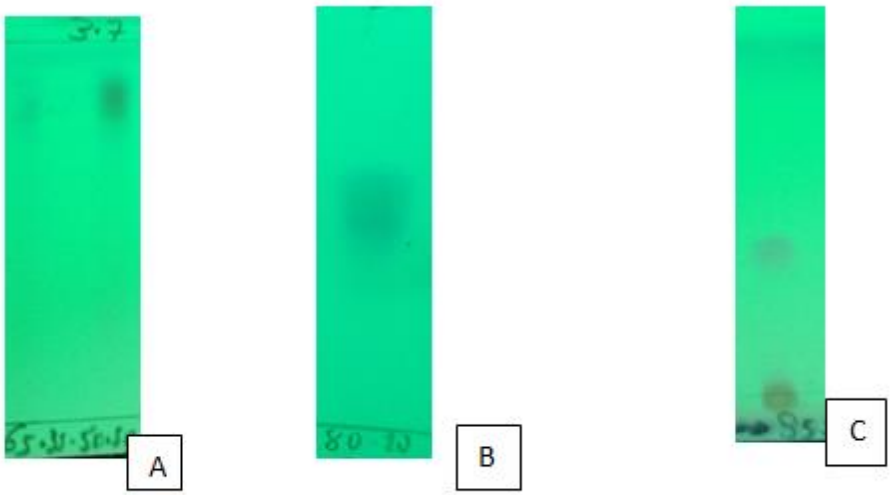
Table 2. Phytochemical screening results of experimental plants

Phytochemical screening	**Test**	**Leave extract**	**Stembarks extract**
		MeOH 100%	**MeOH 100%**
Alkaloids	Puri and Kumar, 2019	+	-
Saponnins	Solanki*et al*., 2019	+	+
Tannins	Yadav*et al*., 2017	+	-
Flavonoids	Khan *et al*., 2016	+	-
Terpenoids	Chowdhury *et al.,* 2013	+	-
Phenol	Alebiosu and Yusuf, 2015	+	+
Steroid	Alhadi*et al*., 2015	-	-

\+ indicates presence and - indicates absence

5.3. TLC profile of the three isolated bioactive compounds

After extraction all extracts obtained from the two parts of experimental plant were analyzed with TLC and tested for antimicrobial activity, based on their activity the three bioactive compounds were selected (Figure 6).



Figures 5 . The TLC profiles of the three isolated compounds from *C. aurea* leaves. (A) Compound **1**, (B) Compound **2** and (C) Compound **3**. Black dot on TLC shows visible spots.

5.4. Antibacterial activity of crude extract and isolated compounds of *C.aurea*

5.4.1. Agar disc diffusion assay

The methanol crude extracts of *C.aurea* leaves showed antibacterial activity aganist all tested bacteria except, *Bacillus subtilis* at 5mg/mL and 10mg/mL concentrations and gentamycin at a concentration of 1mg/ml fully inhibited the growth of all the bacterial strains except *S. dysenteriae*. But the stem barks extract did not shown any activity against all tested bacteria by three extracted solvents (Table 3).

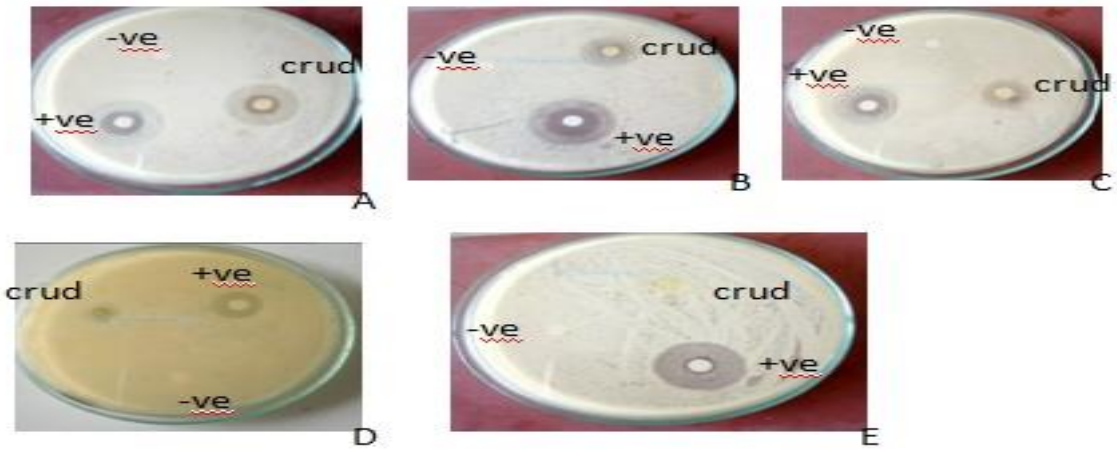


Figure 6. Agar disc diffusion assay of crude extract of *Calpurnia aurea* against *E.coli*, *S.typhimurium, S. dysenteriae, S. aureus* and *B. subtilis* at 10mg/ml concentrations.(A), *E.coli*,(B), *S.typhimurium*,(C) *S.dysenteriae*, (D) (*S.aureus* (E) *B.subtilis* and (+ve) positive control gentamyicin, but for (Sd) positive control was Cefoxitin ,(-ve) negative control.

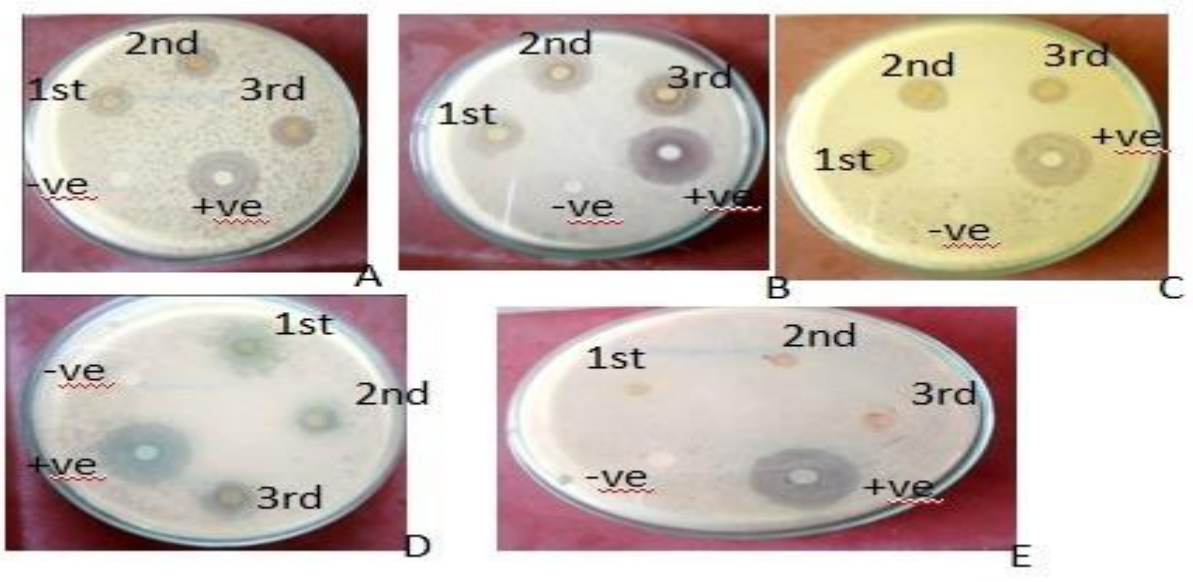


Figure 7.Effect of the three isolated compounds against *E.coli, S.typhimurium, S.dysenteries, S.aureus* and *B. subtilis* at 5mg/ml concentrations. (A),*S.typhimurium* (B), *E.coli,* (C) (*S.aureus,* (D) *S. dysenteriae,* (E) *B.subtilis* and (+ve) positive control gentamycin, but for (Sd) positive control was Cefoxitin,(-ve) negative control,(1^{st}) compound **1**, (2^{nd}) compound **2**,(3^{rd}) compound **3** and circle shows diameter of inhibition zone.

Table 3. Agar disc diffusion assay of leaves and stem barks crude extract at 10mg/ml against *E. coli*, *S.aureus B.subtilis, S.typhimurim* and *S.dysenteriae*.

Plant parts	extracted by	Inhibition zone(mm) ±SEM				
		Test microorganism				
		E.coli	***S.aureus***	***B. subtilis***	***S.typhimurim***	***S.dysenteriae***
C.A leave	n-hexane	NI	NI	NI	NI	NI
	Ethyl acetate	7.66 ± 0.78	6.33± 0.57	NI	7± 1.00	NI
	Methanol	20 ± 1.15	7.66± 1.20	NI	17.66 ± 0.88	11 ± 1.52
C.A stem barks	n-hexane	NI	NI	NI	NI	NI
	Ethyl acetate	NI	NI	NI	NI	NI
	Methanol	6.66 ± 0.32	NI	NI	6 0±.52	NI
	Gentamycin	24 ± 1.73	19.3± 0.33	22 ± 1.52	26±1.15	NI
	Cefoxitin	-	-	-	-	20.66 ± 0.81
	Saline	0	0	0	0	0

Values are means ±SEM (standard error of mean). NI: no inhibition zone and C.A (*Calpurnia aurea).*

The methanol crude extracts of *C.aurea leaves s*howed highest degrees of antibacterial activity against gram negative and the largest zone of inhibition were observed form ethanol crude extracts of leaves with inhibition zone of 20±1.15 for *E.coli,*17.66± 0.88for *S. typhimurium*. *E.coli,* and *S. typhimurium* were found to be susceptible to methanol extracts since the zone of inhibition diameters were higher than others. But, *B. subtilis* was resistant to all extracts; the hexane extracts showed no antibacterial activity towards both gram positive and gram-negative bacteria on both extracts (Figure 7 and Table 3).

Table 4. Effect of methanol crude extract of *C. aurea* leaves and fractionated compounds at 5mg/ml against *E.coli S.aureus B. subtilis, S.typhimurim and S.dysenteriae*.

sample(5mg/mL)		Inhibition zone(mm) ± SEM				
		Test microorganism				
		E.coli	***S.aureus***	***B.subtilis***	***S.typhimurim***	***S.dysenteriae***
C.A leaves	Methanol	19.3±0.33	7.66±1.20	NI	16± 1.15	9.34± 1.45
Isolated compounds	Compound **1**	14.330±.88	12 ±0.57	NI	12 ±1.15	13 ±.0.57
	Compound **2**	16 ±1.15	10.66±0.67	NI	14.66±0.78	10±1.15
	Compound **3**	15.33±0.88	9.34 ±1.45	NI	11 ±2.02	11.33±0.87
	Gentamycin	24±1.73	19.3±0.33	22±1.52	26±1.15	NI
	Cefoxitin	-	-	-	-	20.66 ± 0.81
	Saline	0	0	0	0	0

Values are means ±SEM (standard error of mean). NI: no inhibition zone

The purified compounds from methanol extracts of *C.aurea* leaves have also different activities against *E.coli S.aureus, S.typhimurim and S.dysenteriae* at 5mg/ml concentration. Compound **2** showed strong antibacterial activity (16 ±1.15 and 14.66±0.78 mm) against *E.coli and S.typhimurium* compared to Gentamycin **(**24±1.73 and 26±1.15) respectively.Compound **3** showed strong activity(15.33±0.88mm) against *E.coli* compared to Gentamycin **(**24±1.73) and Compound **1** and **3** showed strong antibacterial activity*against S.dysenteriae* (13 ±.0.57 and 11.33±0.87compared to Cefoxitin (20.66±0.81) respectively(Figure 8 and Table 4).

4.4.2. Determination of MIC and MBC

As indicated in Table 5, the leaves extract of methanol had the maximum MIC and MBC of 10mg/ml (against *S.aureus* and *S. dysenteriae) and* the minimum MIC and MBC value was 1.25 mg/ml against *E.coli* and *S.typhimurim*.

Table 5. The MIC and MBC in (mg/ ml) of *C.aurea* methanol crude extract against *E.coli S.aureus, S.typhimurim and S.dysenteriae* at different concentrations

Bacteria tested	**Methanol extract of different concentration (mg/mL)**	
	MIC	MBC
S.typhimurim	1.25	1.25
S.aureus	10	10
E.coli	1.25	1.25
S.dysenteriae	10	10

MIC= Minimum Inhibitory Concentration, MBC= minimum bactericidal concentration

5.5. Characterization of isolated Bioactive Compounds

Among the extracts obtained from the two part of *C.aurea*, the most bioactive extract was the methanol extract of *C.aurea* leaves (Table 3).The air dried leaves of *C.aurea* were extracted separately with n-hexane, ethyl acetate and methanol at room temperature. The methanol extracts were subjected to column chromatography on silica gel, because of yielding six secondary metabolites, shown a higher extract yields and active against tested selected bacterial pathogen than those extracted by hexane and ethyl acetate. Bioactivity guided fractionation was performed using column chromatography technique and thin layer chromatography (TLC) to isolate bioactive compounds for antibacterial activity. Each fractions were analysed with TLC and those having similar RF value were combined. Among the fractions collected, Fraction 65:35,60:40,55:45 and 50:50 ratio of ethyl acetate and methanol was afforded (compounds **1**), fraction80:20 ratio of hexane and ethyl acetate afforded (compounds **2**) and Fraction 85:15,80:20 and 75:25 ratio of ethyl acetate and methanol was afforded (compounds **3**).

5.5.1 Compound 1

Compound **1** (40 mg) was isolated as a black solid from the methanol extract of the leaves of *C. aurea*. It melted at 165 ºC. TLC on a 0.25 mm thick layer of silica gel on aluminum plate showed a spot at Rf value of 0.66 using as methanol:ethyl acetate (--) as eluent. UV-Vis spectrum showed maximum absorption (λmax) at 203nm, 269nm and 340nm for compound 1 (Appendix 8).

Table 6. ^{1}H-NMR, ^{13}C- NMR and DEPT-135 of compound **1**

C. No	^{1}H-NMR	^{13}C-NMR	DEPT-135
1	4.29	82.7	CH
2	4.27	72.3	CH
3	4.11	71.6	CH
4	4.10	70.8	CH
5	3.96	70.1	CH
6	3.64	59.1	CH
7	3.95	71.1	CH

The proton decoupled ^{13}C-NMR spectrum (101 MHz, $CDCl_3$) of compound **1** (Table 6 and Appendix 3), analyzed with the aid of DEPT-135 spectrum (Table 6 and Appendix 4), showed the presence of seven well resolved carbon resonances. All of which are turned out to be methine carbons. The signals at δ59.1, 70.1, 70.8, 71.1, 71.6, 72.3 and 82.7 are due to C-6, C-5, C-4, C-7, C-3, C-2, and C-1, respectively. The signal due to C-1 appears downfield since the carbon bears two electronegative atoms viz oxygen and carbon. This compound was isolated for the first time from leave of *C.aurea.* Based on the spectral data, the structure of compound 1 is suggested as depicted in (Figure 9).

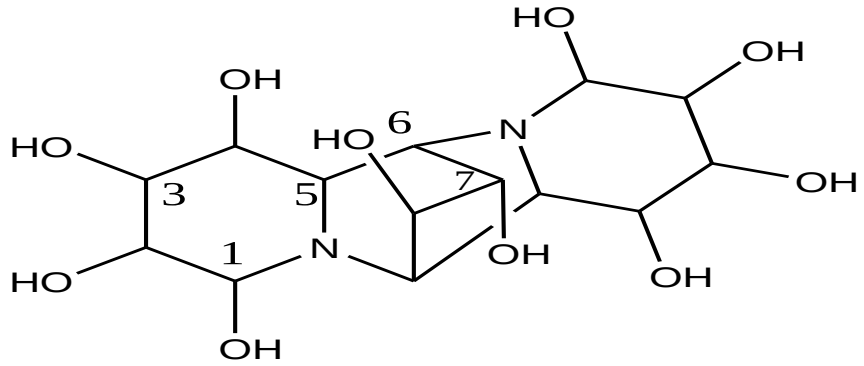


Figure 8: Chemical Structure of compound **1**

(dodecahydro-6,12-ethanodipyrido[1,2-a:1',2'-d]pyrazine-1,2,3,4,7,8,9,10,13,14-decaol)

5.5.2. Compound 2

Compound **2** was isolated as a yellow solid from the methanol extract of the leaves of *C. aurea*. It melted at 178-180^{0}c. TLC on a 0.25 mm thick layer of silica gel on aluminum plate showed a spot at Rf value of 0.5,30 mg using ethyl acetate in n- hexane as eluent. UV-Vis spectrum showed maximum absorption (λmax) at 203nm, 267nm and 340nm for compound 2(Appendix 9). The^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **2** displayed signals in the olefinic/ aromatic, oxygenated aliphatic and non-oxygenated aliphatic carbons (Appendix 5). The signals observed at δ 5.42(7H, m) is diagnostic for the presence of olefinic/aromatic protons. The signals due to protons alpha to carbonyls were observed at δ 2.88.

Table 7.^{1}H-NMR, ^{13}C- NMR and DEPT-135 of Compound **2**.

C. No	^{1}HNMR	^{13}CNMR	DETP-135
1	5.42	130.8	130.8
2	-	129.7	-
3	2.88	129.1	129.1
4	-	127.2	-
5	-	127.0	-
6	4.6	126.7	126.7
7	1.04	126.1	126.1
8	2.39	124.2	124.2
9	0.93	117.0	117.0
10	-	79.8	-
11	-	60.3	-

The ^{13}C-NMR spectrum (101 MHz, $CDCl_3$) (Appendix 6) with the help of DEPT 135 showed signals due to quaternary, methine, methylene and methyl carbons. The most downfield signal at δ173.4 is attributed to ester carbonyl. The signals displayed at δ 130.8, 129.7, 129.1, 127.2, 127.0, 126.7, 126.1, 124.2 and 117.0 are due to the presence of five double bonds. This indicates that compound 2 contains ten signals in the double bond region, one of which is quaternary. The signal at δ 79.8 and 60.3 are due to methine and methylene carbons bearing electronegative oxygen atoms, respectively. The other signals were observed in the aliphatic regions. based on the NMR spectral data, the structure of compound **2** is suggested as shown in Figure 10.

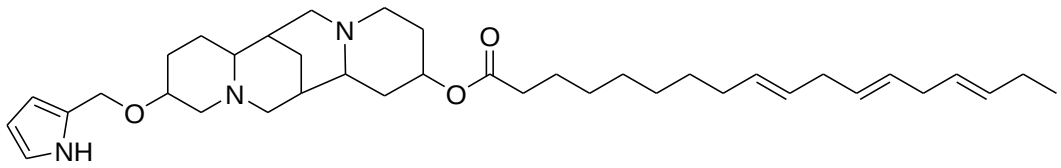


Figure 9: Chemical Structure of compound **2**
(0-((1H-pyrrol-2-yl)methoxy)dodecahydro-2H,6H-7,14-methanodipyrido[1,2-a:1',2'-e]
[1,5]diazocin-2-yl(9E,12E,15E)-octadeca-9,12,15-trienoate)

5.5.3. Compound 3

Compound 3was isolated as a brown solid from the methanol extract of the leaves of *C. aurea*. It melted at 152-154ºC. TLC on a 0.25 mm thick layer of silica gel on aluminum plate showed a spot at Rf value of 0.41,30 mg using methanol in ethyl acetate as eluent. UV-Vis spectrum showed maximum absorption (λmax) at 203nm, 267nm and 343nm for compound 3 (Appendix 10). Close inspection of the NMR spectral data showed as the signals are not well resolved for interpretation. The signals require some more time to mature. Hence it was not interpreted.

6. DISCUSSION

The highest extraction percentage yield was recorded from methanol extraction of *C.aurea* leaves (36.49%)followed by ethyl acetate (18.24%) and the lowest yield was obtained from n-hexane extract of *C.aurea* stem barks (7.21%)(Figure 4) and appendix 1).This indicate that the secondary metabolites present in the leaves of *C.aurea* are mainly polar. In another study slightly lower yield (12.65%) was reported with methanol seed extract of *C.aurea* (Al-Naqeb, 2015). The slight difference might be due to factors like extraction technique, concentration and plant part extracted to be able to greatly affect the yield of extracts.

This study indicated that considerable difference in antibacterial activity between extracts obtained with different solvents. Methanol extracts were more active than other extracts against gram- negative bacteria, *E.coli, S. tphyimurium* and *S. dysenteriae* and no activity was observed by crude extracts and isolated compounds against gram-positive bacteria *B. subtilis*.The Gram-negative bacteria have an outer phospholipid membrane making their cell wall impermeable to lipophilic solutes, but according to this study the crude extracts and isolated compounds were more effective against gram negative than gram positive bacteria. This study provided evidence that methanol extract of *C.aurea* possesses significant antibacterial effect against *E.coli*,*S. tphyimurium* and *S. dysenteriae* and moderate aganist *S. aureus*. This activity may be due to the major components of *C.aurea* which include alkaloids and terpenoids (Budri *et al*., 2015).Similar results were obtained by (Kasimala *et al*. 2014), and these results were also comparable with the result obtained by(Ferrazzano *et al*., 2015).

Where they showed a strong antibacterial activity of *Plantago lanceolata* extract against *S. tphyimurium* and *Shigella* spps. In addition, the results obtained in this study agreed with the result obtained by (Demeke *et al*., 2018), that showed that the extract of *Sida rhombi folia* had higher inhibitory effect against *E.coli*. A study by Nair, (2005) showed that *Rourea minor* leaves possessed a significant antimicrobial activity against the human pathogens *S.aureus, Citrobacter,P. aeruginosa,S. Paratyphoid, Shigella spps and E.coli* .This is in full agreement with our result against *E.coli, S. tphyimurium,S. aureus and S. dysenteriae*.

The bioactivity results were considered evidence of the presence of more compounds in the crude extract of methanol, compared to the ethyl acetate and hexane extracts of *C. aurea*, the probable reason being that the compounds leaves of *C.aurea* were more soluble in methanol as compared to

ethyl acetate and hexane and highest inhibition zone against *E.coli, S.tphyimurium* and *S,dysenteries* were observed. The inhibition activity at different concentration showed different zone of inhibition by purified compounds from methanol extract of *C. aurea* (Figure 8 and Table 4). This purified compound from methanol extract of *C.aurea* used in this study showed no antibacterial activities against *B. subtilis the* reason behind may be this organism was forms dormant end spores that are embedded within the biofilm (Bridier *et al*., 2011) and remains resistant to the administred phytochemicals.

The antibacterial screening findings in terms of zone of inhibition of the study's plant extracts of *C.aurea* against the respective tested bacteria were directly proportional to their values of MIC and MBC, which means having higher inhibition with increase concentration, with the exception of *E.coli* and *Salmonella tphyimurium* which have inhibited their growth at 1.25mg/ mL concentration (Table 5). The MIC results obtained in this study agreed with the result obtained from *C.aurea* fruit extracts by chloroform, and petroleum extracts of the previous study of concentration of 12.5mg/ml up to 100mg/ml aganist *S.aureus* and *E.coli* (Rashmi *et al*., 2010).

Medicinal plants certainly contain various types of minerals and both primary and secondary metabolites and for these, they confer antimicrobial effects (Wang, 2016). The bioactivity of plant extracts significantly varied based on the solvents used for extraction and depends on geographical source, harvest time, storage conditions and drying (Tanver *et al*., 2017).

In this study, phytochemical analysis of the plant extracts showed positive results for several compounds. The plant extracts have shown to contain alkaloids, flavonoids, saponins, phenols, tannins and terpenoids in leaves extract. but in stem bark extracts only, phenols and saponin. The results obtained in this study agreed with the result obtained from *C.aurea* leaves conducted *by*((Shemsu *et al*.,2013) that contained alkaloids, flavonoids, saponins and tannins in leaves. But phenols and terpenoids were obtained from *C.aurea* leaves in this study. The results obtained in this study suggest that identified phytochemical compounds may be the bioactive constituents of this plant indicating that plants are valuable reservoir of bioactive compounds of substantial medicinal advantage.

Compound **1** was obtained as a black solid from methanol extract and isolated from the ratio of ethyl acetate and methanol. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$/MeOD) (Table 6 and

Appendix 2) of compound **1** revealed signals at δ 3.64 (1H) suggesting the presence of methine protons on carbon bearing nitrogen. The other signals observed at δ3.96 (2H), 4.11 (2H) and 4.29 (1H) are evident for the signals of protons on carbon bearing electronegative carbons. This compound was isolated for the first time from leave of *C.aurea.* Compound **2** was isolated as a yellow solid from the methanol extract of the leaves of *C. aurea*. The signals of protons on oxygenated carbons were observed at δ4.6. Diagnostic signal for the presence of linolenic acid as pendent group is due to the presence of triplet signal at δ 1.04 (3H, t). The remaining aliphatic proton signals were observed in the region between δ 2.39 to 0.93 Compound **2** have similar basic structure with O-(2-pyrrolycarbonyl) virgiline, but have different formula and number of carbon from the previously isolated from *calpurnia aurea* (Isao *et al*.,1984).

Compound **3** was isolated as a brown solid from the methanol extract of the leaves of *C. aurea*. TLC on a 0.25 mm thick layer of silica gel on aluminum plate showed a spot at Rf value of 0.41,30 mg using methanol in ethyl acetate as eluent. Close inspection of the NMR spectral data showed as the signals are not well resolved for interpretation. But this compound was clearly observed on TLC and under UV-Vis spectrum so, the signals require some more time to mature. Hence it was not interpreted.

7. CONCLUSION AND RECOMMENDATION

7.1. Conclusion

In this study the following points are drawn:

- ✓ Phytochemical compounds which are Saponnin,Tannin,Flavonoid,Phenol,Alkaloid and Terpenoid were obtained.
- ✓ Methanol was identified as best solvent for *C.aurea* compound extraction.
- ✓ After crude extract fractionation, three compounds are identified using NMR.
- ✓ Antibacterial activities were performed against *C.aurea* different solvent extract and from those methanol extract shown the best inhibition effect against these selected human pathogens.

7.2. Recommendation

Based on the results of this study the following recommendations are suggested:

- ✓ Further study should be performed to synthesize an antimicrobial agent of biologically active compounds from the leaves of *C.aurea*.
- ✓ The compounds that have been characterized in this study exhibited wide range of bioactivity on bacterial growth, but compound **3** did not characterized. So further studies needed to know the structure of this compound.
- ✓ These observations promote further research work with biologically activity on crude extracts as well as isolated compounds for other microorganisms.
- ✓ Additional research efforts are also recommended to conduct structure activity relationships studies to have a better understanding of structural and chemical parameters required to develop related drug lead compounds.

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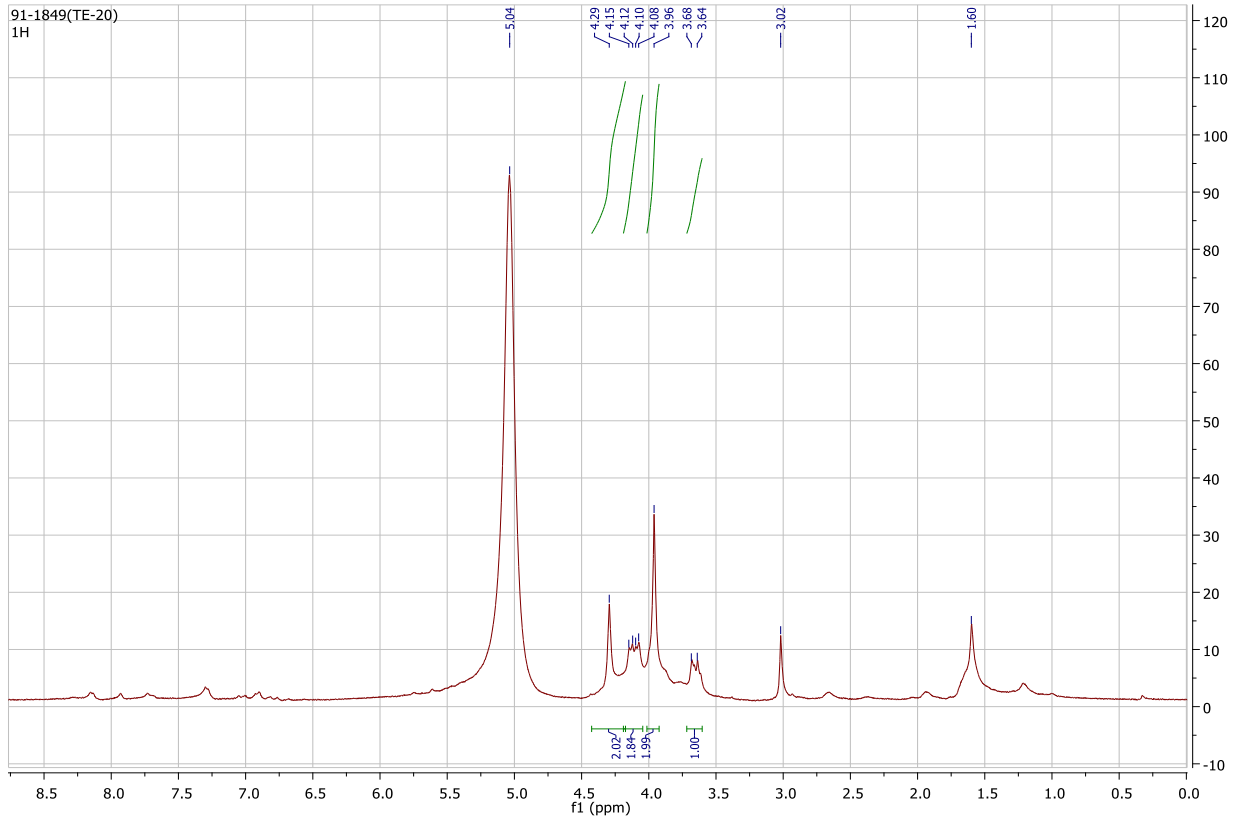
9. APPENDIXES

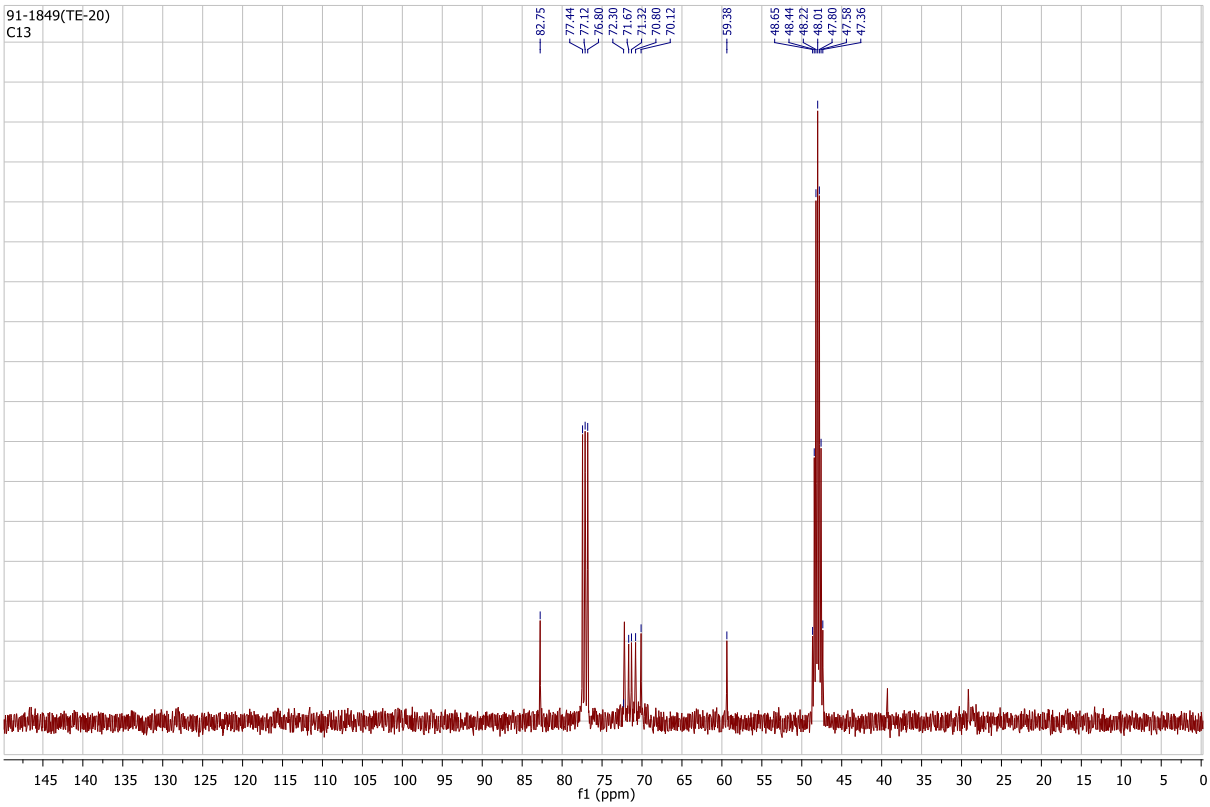
Appendix 1: Masses of extracts obtained and Yields in %

Part of plant extracts	Solvent	Amount(g)	Yield in %
C.aurea leaves	n-hexane	6.2g	13.96%
	ethyl acetate	8.1g	18.24%
	Methanol	16.2g	36.49%
C.aurea stem barks	n- hexane	3.2g	7.21%
	ethyl acetate	4.6g	10.36%
	Methanol	6.1g	13.74%

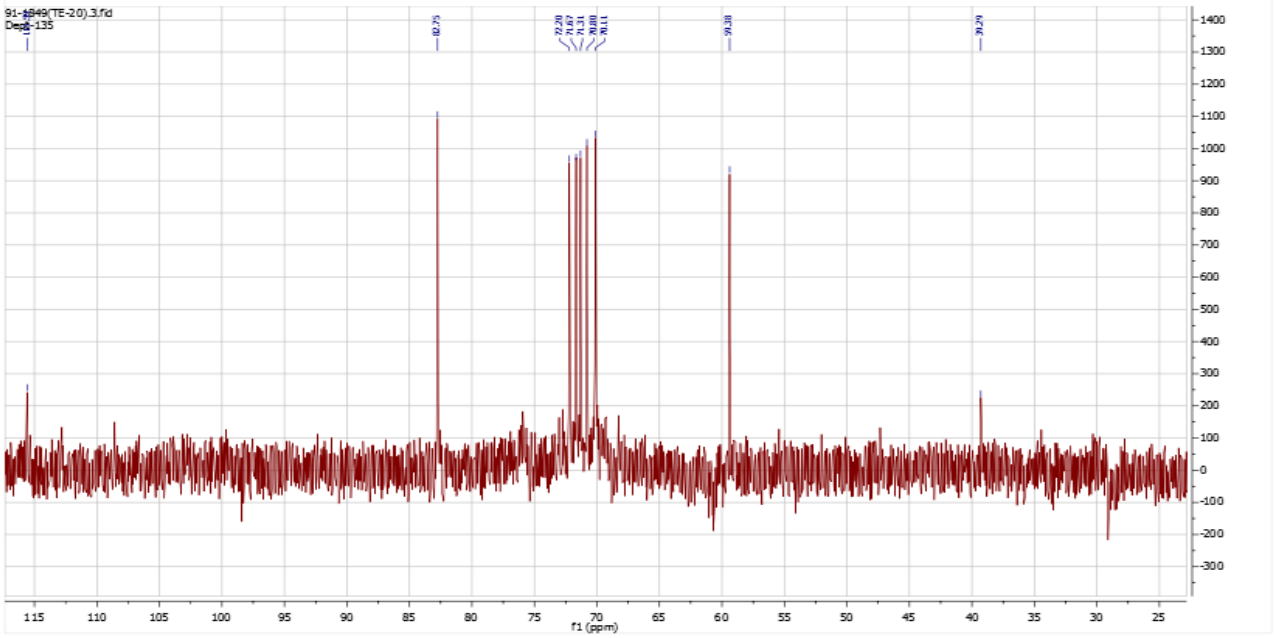
Appendix 2. ^{1}H-NMR Spectrum of compound **1**



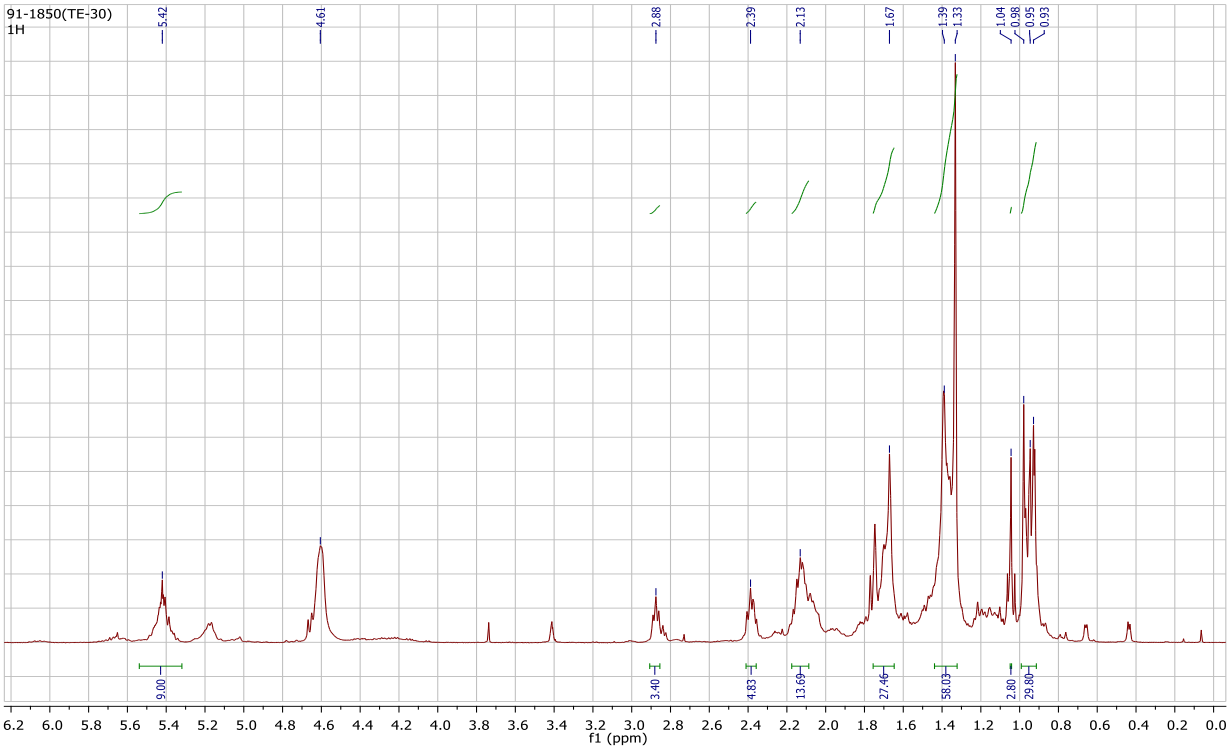
Appendix 3. ^{13}C NMR-NMR Spectrum of compound **1**



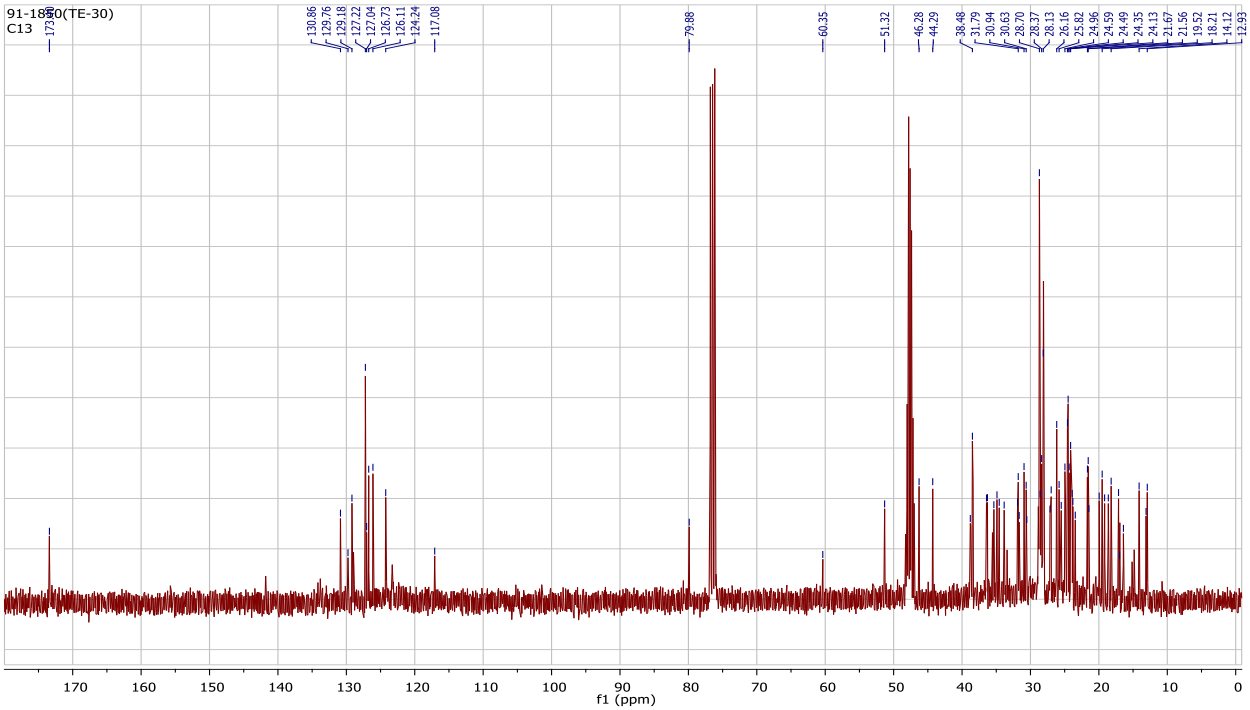
Appendix 4.DEPT-135 NMR Spectrum of compound **1**



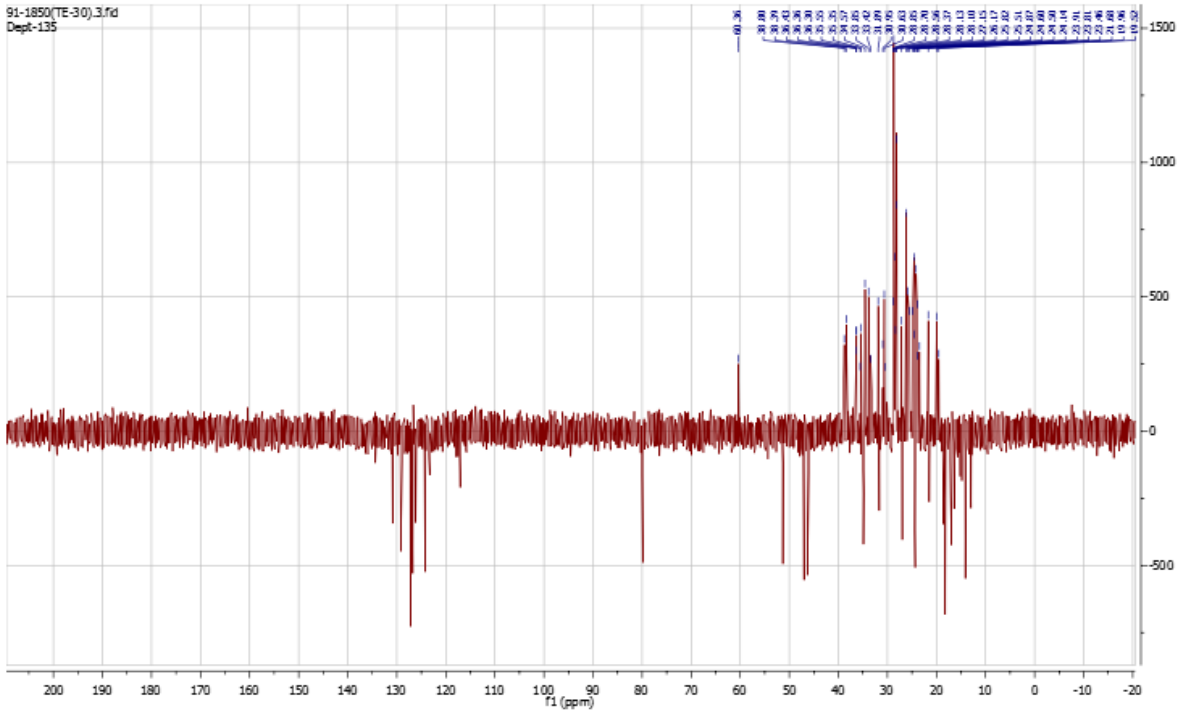
Appendix 5. ^{1}H-NMR Spectrum of compound **2**



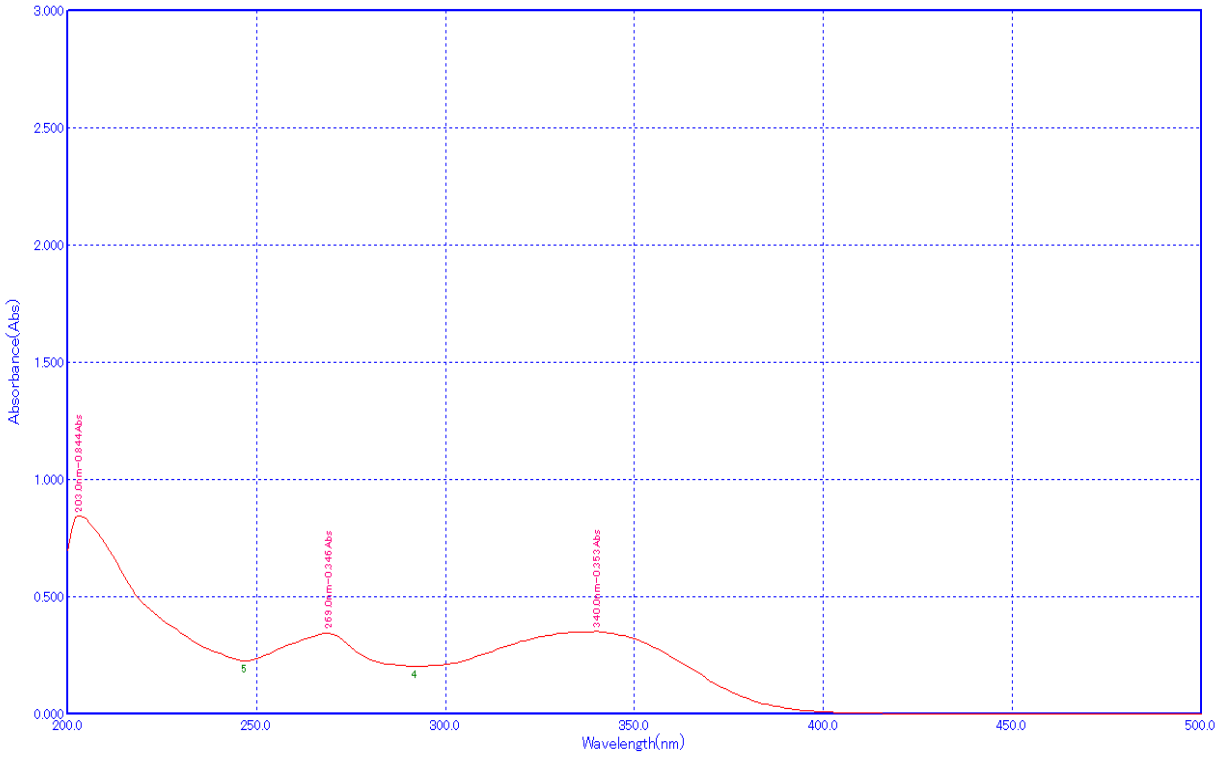
Appendix 6. ^{13}C NMR-NMR Spectrum of compound **2**



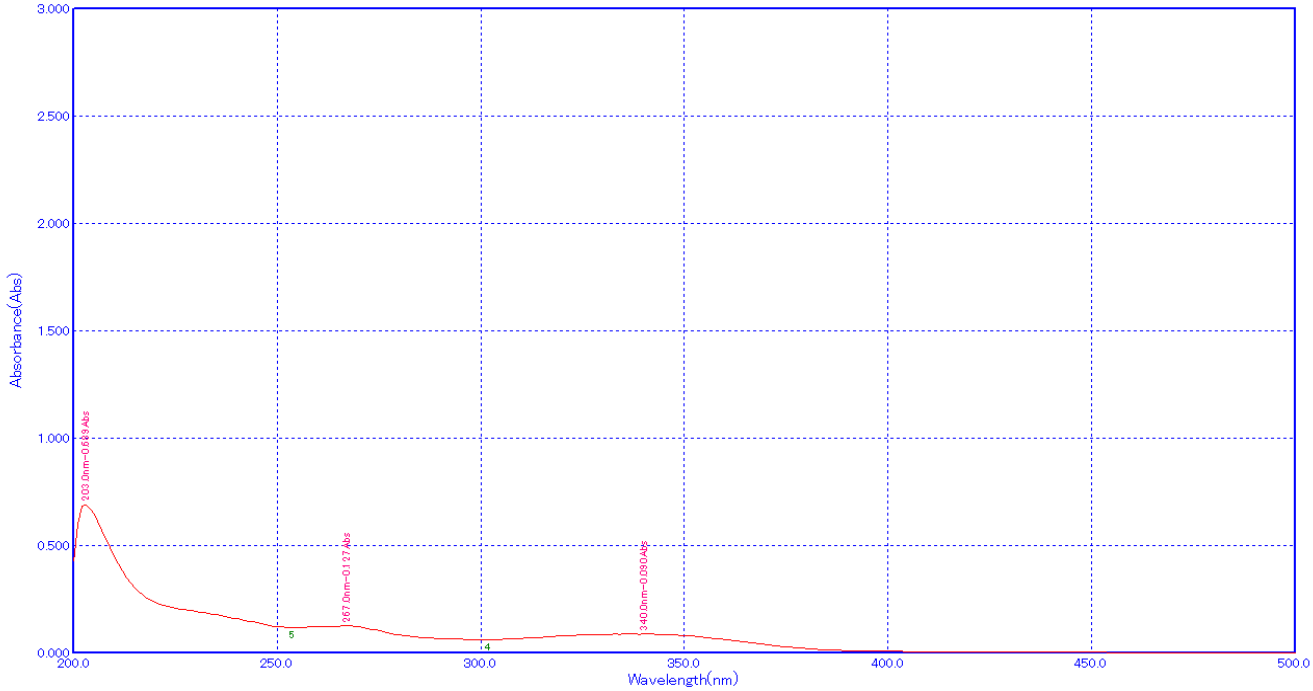
Appendix 7.DEPT-135 NMR-NMR Spectrum of compound **2**



Appendix 8: UV-Visible Spectrum analysis of compound **1**



Appendix 9: UV-Visible Spectrum analysis of compound **2**



Appendix 10: UV-Visible Spectrum analysis of compound **3**