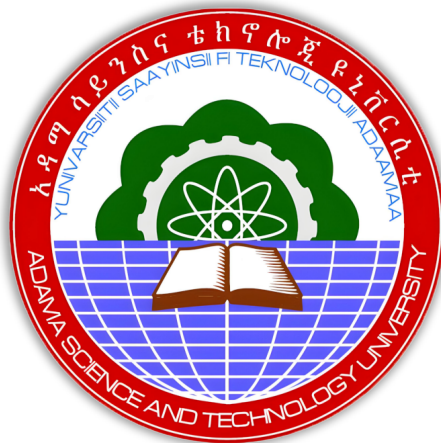


**Antibacterial, Radical Scavenging Activity Evaluation, and
Phytochemical Analysis of Root Extracts of *Cordia africana*
from Adama, Oromia Regional State of Ethiopia**



Sofonyas Kidaneweld Teferi

**A Thesis Submitted to
The Department of Applied Biology
College of Applied Natural Science**

**Presented in Partial Fulfillment of the Requirement for the
Degree of Master of Science in Applied Biology (Applied
Microbiology)**

**School of Post-Graduate Studies
Adama Science and Technology University**

April 2025

Adama, Ethiopia

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**Advisor(s): Teshome Geremew (Ph.D.) Assoc. Prof.
Aman Dekebo (Ph.D.) Prof.**

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**April 2025
Adama, Ethiopia**

DECLARATION

I hereby declare that this Master Thesis entitled “**Antibacterial, Radical Scavenging Activity Evaluation, and Phytochemical Analysis of Root Extracts of *Cordia africana* from Adama, Oromia Regional State of Ethiopia**” is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Sofonyas Kidaneweld Teferi

Name of student

Signature

Date

RECOMMENDATION

We, the advisor(s) of this thesis, hereby certify that we have read the revised version of the thesis entitled “**Antibacterial, Radical Scavenging Activity Evaluation, and Phytochemical Analysis of Root Extracts of *Cordia africana* from Adama, Oromia Regional State of Ethiopia**” prepared under our guidance by **Sofonyas Kidaneweld Teferi** submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Microbiology. Therefore, we recommend the submission of a revised version of the thesis to the department following the applicable procedures.

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APPROVAL PAGE

We, the advisors of the thesis entitled “**Antibacterial, Radical Scavenging Activity Evaluation, and Phytochemical Analysis of Root Extracts of *Cordia africana* from Adama, Oromia Regional State of Ethiopia**” and developed by **Sofonyas Kidaneweld Teferi**, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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We, the undersigned, members of the Board of Examiners of the thesis by **Sofonyas Kidaneweld Teferi**, have read and evaluated the thesis entitled “**Antibacterial, Radical Scavenging Activity Evaluation, and Phytochemical Analysis of Root Extracts of *Cordia africana* from Adama, Oromia Regional State of Ethiopia**” and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Microbiology.

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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the School of Postgraduate Studies (SPGS) through the Department Graduate Council (DGC) and College Graduate Committee (CGC).

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Date

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LIST OF ACRONYMS AND ABBREVIATIONS

ATCC	American-Type Culture Collections
AsA	Ascorbic Acid
CFU	Colony Forming Unit
DPPH	2,2-Diphenyl-1-Picrylhydrazine
EGCG	Epigallocatechin Gallate
EO	Essential Oil
FAS-II	Fatty Acid Synthase II
FRAP	Ferric Reducing Antioxidant Power
FTIR	Fourier-Transform Infrared Spectroscopy
GC	Gas Chromatography
HPLC	High-Performance Liquid Chromatography
IC₅₀	Half Maximal Inhibitory Concentration
LC	Liquid Chromatography
MBC	Minimum Bactericidal Concentration
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
NCTC	National Collection of Type Cultures
NMR	Nuclear Magnetic Resonance
ORAC	Oxygen Radical Absorbance Capacity
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
TLC	Thin-Layer Chromatography
UV	Ultraviolet
WHO	World Health Organization
2,4-DTBP	2,4-Di-Tert-Butylphenol
%RSA	Percent Radical Scavenging Activity

ABSTRACT

Microbial drug resistance is a worldwide health threat, and new bioactive molecules must be discovered to deal with resistant pathogens. Plants have been a great source of antimicrobial molecules and are full of promise for discovering new drugs. Although traditional healers in Ethiopia have been using the root of Cordia africana, no study has been done; as a result, the study aims to assess the biological activity and chemical composition of root extracts of C. africana. Root samples of the plant were extracted successively with Dichloromethane: Ethanol (1:1), Ethanol, and Methanol using successive (maceration) techniques. Antibacterial activity was assessed using the standard disc diffusion method, and a DPPH assay was used to analyze the antioxidant activity. Phytochemical screening of the extracts was conducted following the standard procedures. Hydro-distillation was employed to extract essential oils, and their composition was analyzed using GC-MS. The Dichloromethane: Ethanol (1:1) extract was subjected to column chromatography to isolate compounds, and structural elucidations of the isolated compounds were done using FTIR and NMR. The phytochemical analysis reveals the presence of alkaloids, flavonoids, Phenol, steroids, and tannins across all three extracts. The GC-MS analysis of essential oils of the plant roots reveals the presence of 29 compounds, and dodecyl alcohol, 2,4-DTBP, and cyclohexadecane were the major constituents. The fractionation process generated a total of 122 fractions, which were further repacked to yield 154 fractions, of which two compounds were isolated. The antimicrobial activity revealed that MeOH extract, and essential oil showed the highest inhibition against S. pyogenes with inhibition values of 18.50 ± 1.50 and 16.33 ± 1.53 mm, respectively. The essential oil showed the highest radical scavenging activity with an IC_{50} value of $1.61 \mu\text{g/mL}$, followed by MeOH extract ($5.52 \mu\text{g/mL}$). Thus, the findings of this study showed that the roots of C. africana have a medicinal potential. Further investigations are needed to discover their full potential and implement them in the fight against multi-drug-resistant pathogens.

Keywords: African Cordia, *Cordia africana*, Phytochemical, Root extract

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the study

Cordia africana (Figure 1B), known as “Wanza” in Amharic, “Wad-deessa” in Afan Oromo, and “Ahwi” in Tigrigna (Geresu et al., 2022), is a small to medium-sized tree of the family Boraginaceae and genus *Cordia*. The genus *Cordia* consists of about 250 species in all continents’ tropical and subtropical regions; ten are found in Ethiopia. *C. africana* is a widespread tree in tropical Africa (Nigussie et al., 2021) with multiple purposes; it is used for firewood, timber (furniture, beehives, boxes, mortars, church, and drums), food (fruit), medicine (bark, roots), fodder (leaves), bee forage, soil conservation, ornamental, and shade (Nigussie et al., 2021).

The World Health Organization estimates that around 80% of the population in Africa uses traditional medicines, and about 85% of conventional medicine involves plant extracts (Asrie et al., 2016). *Cordia* (Boraginaceae) contains flavonoids, polyphenols, anti-androgenic triterpenoids, saponins, sesquiterpenes, and cromenes. Also, due to the presence of heteroterpenoids, naphthoquinones, and naphthoxirene *Cordia* species, they have been reported to have Antifungal and larvicidal properties (Nigussie et al., 2021). Different ethnopharmacological studies have shown that various parts of *C. africana* have traditionally been used as medicines. In Burkina Faso, *C. africana* is used for its appetite suppressant (fruit) and antibacterial activity (Yismaw et al., 2020).

In Ethiopia, *C. africana* is the most preferred multipurpose medicinal plant (Seid & Tsegay, 2011; Teklay et al., 2013), and its use for the treatment of liver disease (leaves) (Figure 1A), gastric ulcer (seeds) (Figure 1C), ascariasis, rabies eye disease, wound dressing (stem bark), and acute febrile illness has been reported (Yismaw et al., 2020). Surveys on ethnopharmacology in several regions of Ethiopia revealed the widespread usage of *C. africana* against malaria. In the Jimma Zone, traditional healers use dried powder from the leaf to treat malaria and its related symptoms. It has been shown that *C. africana* extracts have *in vitro* anti-plasmodial activities against *Plasmodium falciparum* strains (Wondafrash et al., 2019).

It is also reported that the root of *C. africana* has been used traditionally to treat amoebiasis, trachoma, stomach aches, and diarrhea (Giday et al., 2007); however, the claimed traditional usage of the root of *C. africana* is lacking in antimicrobial, antioxidant activity evaluation, and phytochemical analysis reports, which can validate the significance of this study. This study aims to assess the antimicrobial, antioxidant activity evaluation, and phytochemical analysis of *C. africana* root extract.

1.2. Statement of the problem

The biological activities of *C. africana* roots, particularly their antimicrobial and antioxidant potentials, remain largely unverified despite their extensive use in Ethiopian traditional medicine for treating infections and other ailments (Alemayehu et al., 2016). Antimicrobial resistance is a pressing global and local challenge, with pathogens evolving to avoid conventional antibiotics, leading to increased morbidity and mortality in Ethiopia, where infectious diseases are a significant public health burden (Mickymaray, 2019). While ethnobotanical surveys suggest that *C. africana* roots may combat microbial infections, scientific studies validating these properties are scarce. Similarly, oxidative stress contributes to various health conditions, including chronic diseases on the rise in Ethiopia, yet the antioxidant potential of these roots lacks scientific support.

Furthermore, the phytochemical composition of *C. africana* root extracts, which contribute to their biological activities, remains poorly characterized. Secondary metabolites such as alkaloids, flavonoids, and terpenoids are known to have antimicrobial and antioxidant effects in plants (Isa et al., 2016). However, specific compounds in *C. africana* roots and their contributions have not been studied in the Ethiopian context.

In Ethiopia, where access to modern antibiotics is often limited and traditional medicine is widely practiced, the need for validated natural antimicrobial agents is critical (Giday et al., 2007). This study aims to address these deficiencies by evaluating the antimicrobial and antioxidant activities of *C. africana* root extracts using microbiological assays and identifying their key phytochemicals, providing a scientific foundation for their traditional use.

1.3. Objectives of the study

1.3.1. General objective

- ❖ To evaluate the antibacterial and antioxidant activities and analyze phytochemicals of root extracts of *C. africana* collected from Adama city, Oromia Regional State of Ethiopia.

1.3.2. Specific objectives

- ❖ To successively extract the plant roots using maceration with DCM:EtOH (1:1), EtOH, MeOH, and essential oils using hydro-distillation.
- ❖ To assess the antibacterial activity of essential oil, compounds, and extracts of roots of *C. africana* against test strains.
- ❖ To assess the radical scavenging activity of essential oil, isolated compounds, and extracts using the DPPH assay.
- ❖ To conduct a phytochemical analysis of the extracts.
- ❖ To analyze the chemical composition of essential oils using GC-MS.
- ❖ To isolate compounds through column chromatography.
- ❖ To characterize the structures of isolated compounds using FTIR and NMR.

1.4. Significance of the study

This study is essential as it checks how well *C. africana* root extracts are crucial in fighting multi-drug-resistant microorganisms, which is a growing problem because they don't respond to usual medicines, especially in Ethiopia, where infections are common and modern drugs are hard to get. The results offer a natural way to stop these microorganisms, which could save lives. By identifying the natural chemicals in the roots, the study explained what makes them valuable, giving scientists a solid base from which to work. It also tied Ethiopia's traditional use of this plant to real science, proving that old remedies have value today.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Medicinal plants

A medicinal plant is any of several plants that offer medicinal characteristics. These plants are a rich source of chemicals that can be employed in medication development (Jamshidi-Kia et al., 2018). Ten percent of all vascular plants worldwide are employed as medical plants, with an estimated 350,000 to almost half a million species. Plants have been utilized in medicine since ancient times and are still used now (Salmerón-Manzano et al., 2020). Plants were grown as medications roughly 60000 years ago, according to evidence. Scripts on medicinal plants stretch back about 5000 years in India, China, and Egypt, and at least 2500 years in Greece and Central Asia (Jamshidi-Kia et al., 2018).

Different varieties of seeds, roots, leaves, fruits, skin, flowers, and even the entire plant can be utilized as medicinal herbs. Most medicinal plants' active chemicals have direct or indirect therapeutic effects and are employed as medicinal medicines. Until the 18th century, many plants' therapeutic characteristics, effects on the human body, and therapy methods were known, but the active ingredient remained unknown (Salmerón-Manzano et al., 2020). Traditional plant species have medicinal properties due to phytochemical components that have a particular pharmacological action on the human body. Tannins, Alkaloids, Saponins, Cardiac Glycosides, Steroids, Terpenoids, Flavonoids, Phlobatannins, Anthraquinones, and Reducing Sugars are the principal phytochemical components found in medicinal plants (Talema, 2020).

According to WHO, most developing-country populations rely on traditional medicines and natural plant products for primary health care due to the modern medical system's inadequate coverage, a lack of pharmaceuticals, and exorbitant costs for modern drugs (Zerabruk & Yirga, 2012). Traditional practices and activities have a long history in many places in Ethiopia (Talema, 2020). The country has a diverse spectrum of potentially beneficial medicinal plants, including *C. africana* (Yirgu et al., 2019).

2.2. *Cordia africana*

C. africana (Figure 1B) is a small to medium-sized tree belonging to the family Boraginaceae and the genus *Cordia*. Boraginaceae, which has roughly 100 genera and 2000 species, is distinguished by having flowers in helicoid cymes and coarsely hairy herbage. With an altitude range of 900 to 2400 m and an annual rainfall range of 700 to 2000 mm, *C. africana* is suitable for suitably warm locations (Nigussie et al., 2021). Africa is where *C. africana* originates. In tropical Africa, it is widely prevalent. It is moving from Guinea in the east to Eritrea, Ethiopia, and Kenya in the north to Angola, Zimbabwe, and northern South Africa in the south. Additionally, it can be found in Saudi Arabia and Yemen, and many tropical nations have planted it (Guyassa et al., 2013). Ten species of the roughly 250 species in the genus *Cordia* are found in Ethiopia's tropical and subtropical regions (Nigussie et al., 2021).



Figure 1: Picture of *C. africana* (Picture by Sofonyas Kidaneweld, Adama Science and Technology University, Ethiopia, 2023). (A) leaf and flowers of *C. africana*; (B) areal parts of *C. africana*; (C) leaf and seeds of *C. africana*

2.2.1. Ethnobotanical use

Bee foraging, fuel, fodder (leaves), food (fruit), ornamental uses, shade, soil conservation, and the manufacturing of wood (for furniture, beehives, boxes, mortars, churches, and drums) are all examples of uses for *C. africana*. According to some sources, the plant's bark, leaves, and fruit have traditionally been used to treat conditions like migraines, fractured bones, wounds, gastritis, and constipation (Nigussie et al., 2021). In East Africa, *C. africana* is used for general open wound healing, the treatment of schistosomiasis, skin problems, and

jaundice, as well as a stimulating tonic for fatigue, pain, headaches, and exhaustion. *C. africana* is widely used in traditional African medicine to treat inflammatory-related conditions and infectious diseases (Zemichael et al., 2019).

One of the primary sources of traditional remedies used to treat various ailments in humans and domestic animals is *C. africana*. Many parts of the plant are utilized in conventional medicine. A fractured bone is tied with fresh, juicy bark; this splint is periodically replaced with a new one until the bone has healed. The root is used to treat bilharzia, while the bark is used to treat venereal illnesses (Jansen, 1981). In another trial, sterile branches are consumed to treat issues with nighttime urination. Ash is used as skin and mucosae therapy, while wood and roots are utilized as vermifuges. The root is used to treat amoebiasis, the root and root bark are used to treat stomachaches and diarrhea, and the leaves and root are used to treat liver problems (Giday et al., 2007). To cure burns and wounds, the leaves are ashes mixed with butter.

Drinking the juice of the cursed leaf helps with tonsillitis, general body aches, diarrhea, and eye infections. It is also applied topically to cure eye infections (Teklay et al., 2013). Traditional uses of *C. africana* include the treatment of coughs, wounds, and toothaches. The mature fruits are sold in the local markets in Northern Ethiopia and Sudan. They can make sweet drinks that replace milk, sweets, sweetmeats, stimulants, and alcoholic beverages, as well as edible mature fruits (Nigussie et al., 2021).

C. africana is the most popular multipurpose medicinal plant in Ethiopia (Seid & Tsegay, 2011; Teklay et al., 2013), and it has been documented to be used for rabies, wound dressing, ascariasis, eye disease, gastric ulcer, liver disease, and eye disease (leaves) (Zemichael et al., 2019). Surveys on ethnopharmacology in several Ethiopian locations showed that *C. africana* was widely used to treat malaria. Traditional healers in Jimma Zone also treat malaria and its symptoms with dried leaf powder. Additionally, it has been demonstrated that *C. africana* extracts had *in vitro* anti-plasmodial properties against strains of *Plasmodium falciparum* (Wondafrash et al., 2019). The root of *C. africana* has also reportedly been used traditionally to treat amoebiasis, trachoma, stomach aches, and diarrhea (Giday et al., 2007). These activities are attributed to their secondary metabolites, which include flavonoids, triterpenes, tannins, alkaloids, and fatty acids, which were isolated from various plant parts of the *Cordia* species and have a wide range of bioactivities (Nigussie et al., 2021; Zemichael et al., 2019).

2.2.2. Antimicrobial activity

The scientific community is increasingly concerned about the prevalence of microbes developing antibiotic resistance. Numerous researchers are studying plants worldwide to find potential antibacterial chemicals. Several secondary plant chemicals have numerous biological actions that are currently understood. Alkaloids, Flavonoids/essential oils, Tannins, Terpenes, and Quinones may all contribute to plants' antimicrobial properties (Zemichael et al., 2019).

Many phytoconstituents from *C. africana* are effective antibacterial agents against various microorganisms. Various bacteria, including *B. cereus*, *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. aureus*, and *S. Typhimurium*, as well as three fast-growing *Mycobacterium* species strains (*M. aurum*, *M. fortuitum*, and *M. smegmatis*), were tested against extracts from the bark and leaves of *C. africana*. The plant extract was discovered to be particularly effective against *E. coli*, *S. typhimurium*, and fast-growing *Mycobacterium* species (*M. aurum*, *M. fortuitum*, and *M. smegmatis*), with minimum inhibitory doses of 16 g/mL in each case. When the extracts' inhibition zones were compared to those of industry standards like ciprofloxacin, it was discovered that both plant extracts had significant antimicrobial activities against all microorganisms. Both plant extracts were active against *M. fortuitum*, with MIC values of 512 g/mL in both cases. Despite having MIC values of 1024 g/mL in both cases compared to other standards like ciprofloxacin and rifampicin, extract from the leaves of *C. africana* proved effective against *M. aurum* and *M. smegmatis*. Studies revealed that utilizing the outcome approach, the *C. africana* leaf and fruit extract significantly outperformed the control treatment regarding growth inhibition zones against the two bacteria (*E. coli* and *S. aureus*) (Nigussie et al., 2021).

The study conducted by El et al. (2015) on *A. niger* (ATCC 9763), *B. subtilis* (NCTC 8236), *C. albicans* (ATCC 7596), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *S. aureus* (ATCC 25923), showed that the leaves of *C. africana* extract exhibited effects against most of the tested organisms with zones of inhibition ranging from (14-30 mm). The most significant inhibition was against *A. niger* which is (30 mm). The stem of *C. africana* extracts exhibited effects against most of the tested organisms with zones of inhibition ranging from (14-20 mm). The most significant inhibition was against *C. albicans* (20 mm). The bark of *C. africana* extracts exhibited effects against most of the tested organisms, with zones of inhibition ranging from (11-18 mm). The most significant inhibition was against *B. subtilis*

(18 mm). The bark of *C. africana* extracts exhibited effects against most of the tested organisms, with zones of inhibition ranging from (12-22mm) and the most significant inhibition was against *S. aureus*, which is (22 mm).

2.2.3. Antimalarial activity

The liver and intra-erythrocytic forms of the parasite, which may co-exist in the same host, are all resistant to all antimalarial medications. As a result, when treating an existing infection, it may be necessary to use many medications to eradicate the parasite infestation. Additionally, efforts to create a successful blood-stage vaccination have not been very successful, partly due to antigenic diversity and a lack of knowledge of the host immune system's protective mechanisms. Increased medication resistance brought about by the genomic flexibility of the mosquito and the plasmodium parasite has given the issue new dimensions, necessitating investment in developing more effective malaria prevention strategies (Zerabruk & Yirga, 2012). Because some current antimalarial medications (such as artemisinin and quinine) are derived from plants, traditional medicinal herbs could be a potential source of new medicines (Getnet et al., 2016).

Previous research has shown that the four-day suppression test, curative and chemoprophylactic tests were used to investigate the in vivo antimalarial effects of different doses of *C. africana* leaf extracts and solvent fractions. The four-day suppression (51.19%), curative (57.14%), and prophylactic (46.48%) tests revealed that the plant extract significantly reduced parasitemia levels. The n-butanol fraction showed the most significant chemo suppression (55.62%) at 400 mg/kg, followed by the chloroform fraction (45.04%) at the same dose (Wondafrash et al., 2019).

2.2.4. Antioxidant activity

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and the capability of a biological system to detoxify the reactive intermediates quickly or to heal the harm caused (Birben et al., 2012). Because of regular cellular metabolism, living things produce reactive oxygen and nitrogen species which are involved in physiological and cellular processes like gene expression, cellular growth, and infection resistance, as well as acting as stimulants for biochemical processes inside the cell at low to moderate concentrations (Dröge, 2002). At high concentrations, ROS and RNS cause unusual chemical modifications in cellular components

like lipids, proteins, and DNA, which can disrupt the standard cellular and molecular mechanisms involved in the development of cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, and frailty. Both ROS and RNS mediate effects at the molecular level through transcription factors like nuclear factor-kappa B and activator protein-1 (Zemichael et al., 2019).

A DPPH assay technique is used to study the antioxidant activity of plant extracts. Antioxidants are substances that can stop or slow the oxidation of other molecules by scavenging free radicals, chelating free catalytic metals, acting as electron donors, preventing the formation of peroxides, stopping the auto-oxidative chain reaction, and reducing localized O₂ concentrations. The unstable free radical DPPH readily accepts an electron or hydrogen and stabilizes (Brewer, 2011). DPPH is a rich purple color in methanol solution with a maximum absorbance of 517 nm. Due to the scavenging of free radicals in the presence of an antioxidant, the deep purple color turned yellow.

Numerous investigations have revealed the antioxidant activity of various components of *C. africana*. According to a study by Alhadi et al. (2015), the antioxidant activity of the extract of methanol's leaves, stem, bark, and fruit was 80, 88, 74, and 37%, respectively. A chloroform leaf and stem extract also showed antioxidant activity of 79 and 78%, respectively. The extract of water leaves and stems gave 82 and 89% antioxidant activity, while the extract of ethyl acetate leaves and stems gave 95 and 91% antioxidant activity, respectively. This suggests that the extracts are effective in treating pathological conditions that are associated with free radicals. Ethanolic extracts of *C. africana* leaves had a 68% suppression of the DPPH radical, according to a study by Zemichael et al. (2019).

2.2.5. Phytochemicals of *C. africana*

Certain chemical compounds, known as phytochemicals or phytocomponents, are found in traditional medicinal plants and support different physiological and biochemical processes in the human body. These non-nutritive compounds treat infectious diseases and have anti-disease characteristics (Mickymaray et al., 2016). Today, hundreds of medicinal plants are cultivated worldwide to produce bioactive compounds essential for modern medicine and pharmaceuticals. Advances in phytochemical techniques have led to numerous active plant-derived compounds, many of which are now integral to modern medical systems. Phytochemicals are classified based on their metabolic function into primary metabolites-such as carbohydrates, proteins, lipids, and chlorophyll-and secondary metabolites, which

include alkaloids, flavonoids, saponins, tannins, phenolic compounds, and more (Talema, 2020). Though present in lower concentrations than primary components, secondary metabolites are responsible for most of a medicinal plant's pharmacological activity, particularly its antimicrobial properties (Pandian et al., 2006).

Among secondary metabolites, the classes with the most potent antimicrobial effects include flavonoids (flavones, flavonols, flavanols, isoflavones, anthocyanidins), phenolic acids (hydroxybenzoic and hydroxycinnamic acids), stilbenes, lignans, quinones, tannins, and coumarins (simple coumarins, furanocoumarins, pyranocoumarins) (Mickymaray, 2019). These compounds exhibit diverse mechanisms of antimicrobial action, such as disrupting microbial membranes, impairing cellular metabolism, inhibiting biofilm formation and bacterial capsule production, reducing microbial toxin secretion, and attenuating bacterial virulence by regulating quorum sensing (Mickymaray, 2019).

Although reports on the phytochemistry of *C. africana* are limited, various classes of compounds, including Fatty Alcohols, Isoflavonoids, Phenolic Compounds, Phytosterols, Polyphenols, Quinones, and Triterpenoids, have been identified from different parts and extracts of the plant. A study by Sabry et al. (2022) on the methylene chloride extract of *C. africana* bark led to the isolation of two major compounds: the triterpenoid α -amyrin (**1**) and the sterol β -sitosterol (**2**). Additionally, the n-butanol extract yielded two polyphenolic compounds, rosmarinic acid (**3**) and methyl-rosmarinate (**4**) (Figure 2).

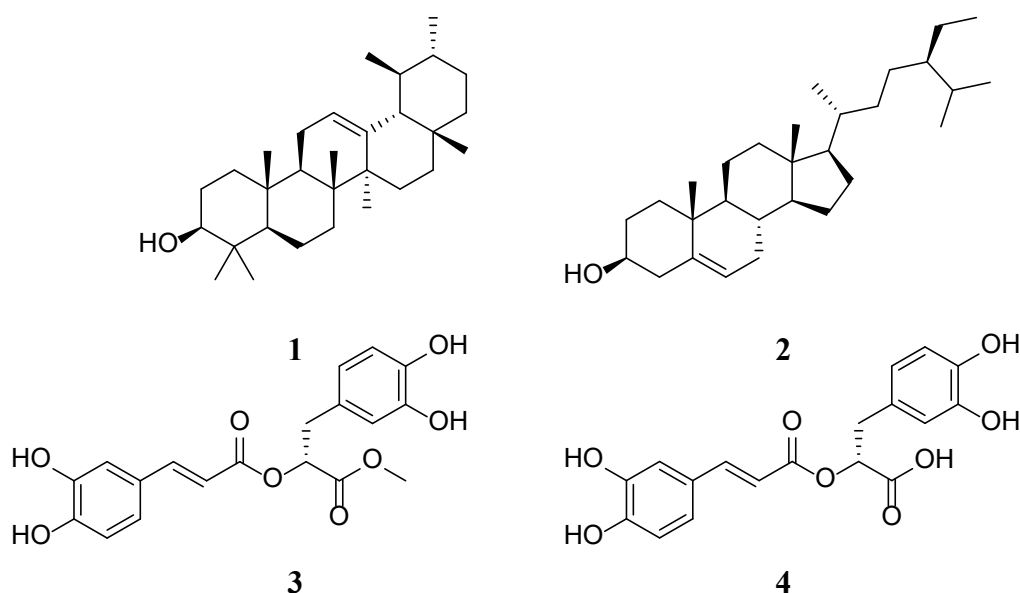


Figure 2: Chemical structures of Compound **1-4** reported from methylene chloride and n-butanol bark extract of *C. africana*

Similarly, Kamau et al. (2019) investigated the root bark of *C. africana* using a methanol (MeOH) and dichloromethane (CH₂Cl₂) (1:1) mixture, leading to the isolation of three triterpenoids: oleanolic acid (**5**), 3- β -lup-20(29)-en-3-ol (**6**), and stigmast-5,22-dien-3 β -ol (**7**). Additionally, the study identified a hydroquinone derivative ((*Z*)-2-(3-hydroxy-7-methylocta-2,6-dien-1-yl) benzene-1,4-diol (**8**)), a benzaldehyde derivative (4-hydroxy-3-methoxy-benzaldehyde (**9**)), and an isoflavone (7-hydroxy-4'-methoxyisoflavone (**10**)) (Figure 3).

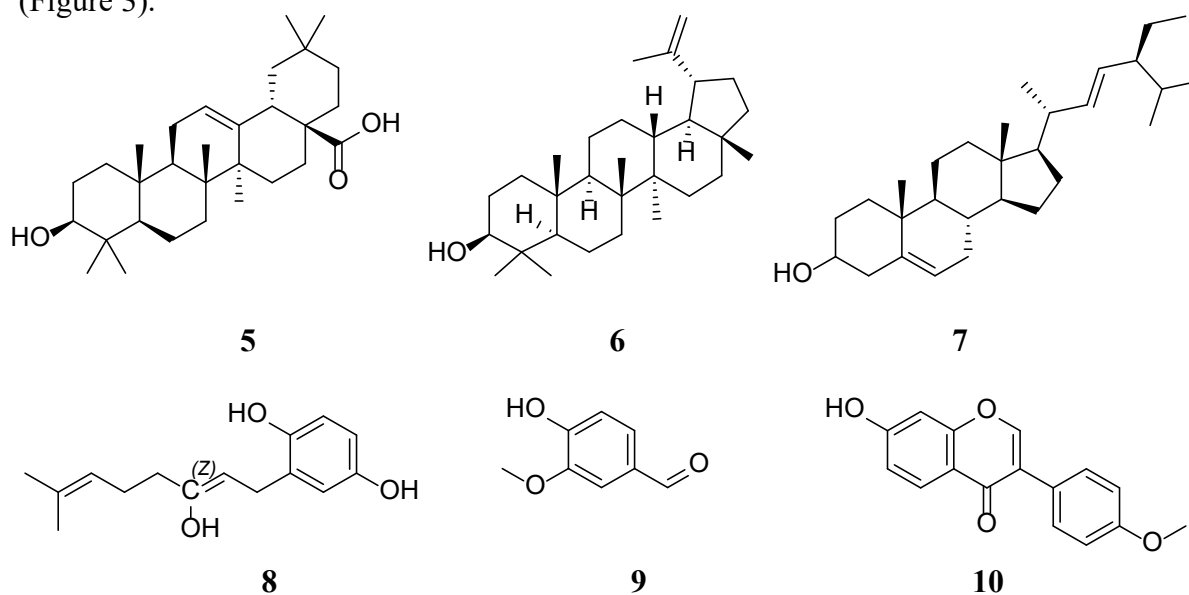


Figure 3: Chemical structures of Compound **5-10** reported from MeOH: CH₂Cl₂(1:1) root bark extract of *C. africana*

Further phytochemical analysis of the stem bark extract resulted in the re-isolation of oleanolic acid (**5**) and 3- β -lup-20(29)-en-3-ol (**6**), along with the identification of a terpenoid benzoquinone, ubiquinone-8 (**11**), and a long-chain fatty alcohol, 1-octacosanol (**12**) (Figure 4).

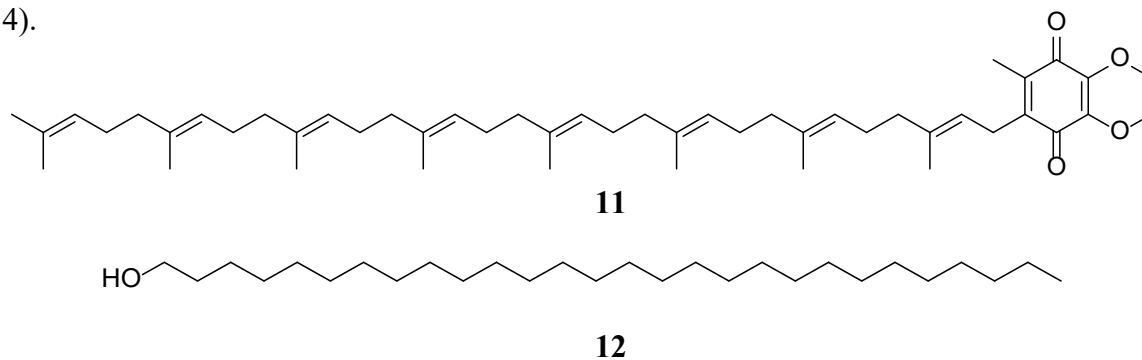


Figure 4: Chemical structures of Compound **11** and **12** reported from MeOH: CH₂Cl₂(1:1) stem bark extract of *C. africana*

2.3. Mechanism of actions of extracts of *C. africana*

In vitro tests have shown that medicinal plants create infinite secondary metabolites with antibacterial solid action. These tiny molecular weight antibiotics produced by plants can be divided into two groups: phytoalexins, which are typically anti-oxidative and are created de novo by plants in response to microbial infection, and phytoanticipins, which are engaged in microbial inhibitory activities (Sukalingam et al., 2017). Alkaloids, phenolic chemicals, and terpenes are the three major classes into which plant antimicrobial secondary metabolites are typically divided. According to numerous studies, plant extracts and their active compounds have the potential to promote cell wall disruption and lysis, induce the production of reactive oxygen species, prevent the formation of biofilms, prevent the construction of cell walls, prevent microbial DNA replication, prevent the synthesis of energy, and prevent the transmission of bacterial toxins to the host. Additionally, these substances might inhibit the development of antibacterial resistance and operate synergistically with antibiotics to finally kill harmful organisms.

2.3.1. Cell wall disruption and lysis

The hydroxyl functional group (-OH) of phenolic compounds, a class of aromatic rings, is thought to be poisonous to microbes, though increasing hydroxylation reactions cause the lysis of microbial cells. Quinones also include two ketone molecules in their aromatic rings, which allows for the formation of an irreversible combination with nucleophilic amino acids and enhances their antibacterial characteristics. These possible aromatic compounds typically aim for membrane-bound polypeptides, enzymes, adhesins on microbial cell surfaces, and ultimately lysis of the bacteria. Flavonoids are hydroxylated phenolic compounds that can damage microbial membranes and form complexes with bacterial cell walls (Ganesan & Xu, 2017b)

Quercetin, rutin, naringenin, sophora flavanone, tiliroside, and 2, 4, 6-trihydroxy-30-methyl chalcone are highly active flavonoids that increased membrane permeability and decreased lipid bilayer thickness, which supported the leakage of intracellular protein and ions in *S. aureus* and *S. mutans*. These substances aid in the synergistic interaction between tetracycline and ampicillin (Mickymaray, 2019). The other active flavonoids, including acacetin, apigenin, morin, and rhamnetin, weaken bacterial cell walls by causing the lipid bilayer to become disorganized and disoriented, which ultimately encourages vesicle leakage

(Matijašević et al., 2016). It was discovered that the synthetic flavonoid lipophilic 3-arylidene is particularly effective against *S. aureus*, *S. epidermidis*, and *E. faecalis* because it disrupts biofilm, which affects the integrity of the bacterial cell wall (Mickymaray, 2019). Tannins are polymeric phenolic materials that can inhibit the activity of membrane transporter systems, enzymes, and adhesins in microorganisms (Ganesan & Xu, 2017a). Terpenes are chemical molecules that break down microbial membranes and include isoprene subunits (Guimarães et al., 2019). The exterior membrane of several Gram-negative bacteria is destroyed by thymol, eugenol, cinnamaldehyde, carvone, and carvacrol, which releases LPS and increases permeability (Mickymaray, 2019).

2.3.2. Inhibition of biofilm formation

The main characteristics of bacteria that form biofilms are typically 100-1000 times more resistant to antibiotics than their typical planktonic forms (Kahaliw et al., 2017). Interestingly, several researchers have detailed how flavonoids drive multicellular bacterial composites to aggregate and then suppress bacterial growth following aggregate formation, indicating that flavonoids are effective antibiofilm substances. *S. aureus* and *S. mutans* aggregate into pseudo multicellular structures when exposed to bioactive flavonoids such as galanin, isovitexin, EGCG, and 3-O-octanoyl-epicatechin as well as 5, 7, and 40-trihydroxy flavanol (Ganesan & Xu, 2017). Quorum sensing, a significant regulator of biofilm development, involves cell signaling molecules termed autoinducers found in *E. coli*, *V. cholerae*, and *S. typhi* (Vikram et al., 2010). Interestingly, enteroaggregative biofilm development in *P. aeruginosa* and *E. coli* is inhibited by the potent cell-cell signaling antagonists apigenin, kaempferol, quercetin, and naringenin in a concentration-dependent manner (Ouyang et al., 2016). The development of *E. coli* biofilms is inhibited by hydrophilic flavonoids such as 6-aminoflavone, 6-hydroxyflavone, apigenin, chrysin, daidzein, genistein, auronol, and phloretin. Phloretin also decreased the expression of the curli genes (*csgA*, *csgB*) and toxin genes in *E. coli*, preventing the development of fimbriae and biofilm (Mickymaray, 2019).

2.3.3. Inhibition of cell wall construction

The bacterial cell wall controls osmoregulation, respiration, transport mechanism, and lipid production. Membrane integrity is critical for executing these processes, and its disruption can directly or indirectly cause metabolic malfunction, eventually leading to bacterial

mortality. Catechins attract membrane lipid bilayers in the following ways. Catechins make hydrogen bonds with polar head groups of lipids at the membrane edge, attracting them. Epicatechin and epigallocatechin gallate modify phospholipids, causing structural alterations in the cell membrane. Furthermore, these catechins block or inactivate intracellular and extracellular enzyme production (Reygaert, 2014).

Antimicrobial medicines that inhibit enzymes in fatty acid production are an appealing target for preventing bacterial growth. In particular, the primary enzyme fatty acid synthase II (FAS-II) is crucial as an antibacterial medication. It has been proven that the antioxidants quercetin, apigenin, and sakuranetin prevent *Helicobacter pylori* from producing 3-hydroxy acyl-ACP dehydrase. Inhibitors of fatty acid production enzymes include quercetin, kaempferol, 4, 20, 40-trihydroxy chalcone, fisetin, morin, myricetin, baicalein, luteolin, EGCG, butein, and isoliquiritigenin (Mickymaray et al., 2016). Baicalein, EGCG, galanin, kaempferide, DL-cycloserine, quercetin, apigenin, and kaempferide-3-O-glucoside disrupt the bacterial cell wall by inhibiting the formation of peptidoglycan, a crucial element of the cell wall (Zielinska et al., 2019).

2.4. Techniques for isolating and purifying bioactive molecules from plants

The extraction of medicinal plants was carried out using various techniques, including maceration, infusion, decoction, percolation, digesting, Soxhlet extraction, superficial extraction, ultrasound-assisted extraction, and microwave-assisted extraction. The secondary metabolites were also separated from one another and purified using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), paper chromatography (PC), and gas chromatography (GC). The plant material type, the solvents, pH, temperature, and the solvent-to-sample ratio all play a role in selecting the best extraction technique (Abubakar & Haque, 2020). Nowadays, the rate of bioassay-guided fractionation has been significantly enhanced by the development of precision instruments such as high-performance liquid chromatography (HPLC/MS), liquid chromatography-mass spectrometry (LC/MS), magnetic field and nuclear magnetic resonance (NMR), a recent breakthrough for the categorization of compounds that are extremely limited in quantity in their organisms of origin (Jamshidi-Kia et al., 2018).

2.5. Antioxidant activity test

Blois first described the 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging experiment in 1958. Numerous researchers then slightly updated Blois' original description, and it is one of the most used antioxidant assays for plant samples. The stable free radical DPPH reacts with substances that can give off a hydrogen atom. This approach relies on the DPPH being scavenged by adding an antioxidant or radical species that makes the DPPH solution less colored. The subsequent decrease in absorption at 515 nm is used to gauge the antioxidant activity (Krishnaiah et al., 2011).

Ferric Reducing Antioxidant Power (FRAP) assay and Oxygen Radical Absorbance Capacity (ORAC) assay are other primary antioxidant assays, aside from the DPPH assay. The FRAP assay measures the reducing power of antioxidants by evaluating their ability to reduce Fe^{3+} to Fe^{2+} . The sample is mixed with a FRAP reagent containing ferric ions, and the reduction of Fe^{3+} to Fe^{2+} is monitored by measuring the absorbance at 593 nm when antioxidants in the sample reduce Fe^{3+} ions, a blue-colored complex forms. This method estimates the antioxidant's ability to donate electrons, which is closely associated with its capacity to counter oxidative stress (Benzie & Strain, 1996).

On the other hand, the ORAC assay measures the antioxidant's ability to neutralize peroxy radicals, which are common in biological systems. The assay typically involves incubating a fluorescent probe with the sample and generating peroxy radicals. Antioxidants in the sample prevent the decay of fluorescence caused by the radicals. The extent to which the fluorescence decay is inhibited is measured and used to calculate the antioxidant capacity of the sample. The ORAC assay is particularly useful for evaluating the antioxidant capacity in a more biologically relevant context, as it simulates oxidative stress scenarios in living organisms (Ou et al., 2001).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Description of the study area

Adama City (Figure 5), located at an elevation of 1,712 m above sea level, between 8° 32' 24" latitude and 39° 16' 12" longitude, is around 13,366.5 acres, approximately 99 km from Addis Ababa, Ethiopia's capital. The yearly lowest and maximum temperatures in the study area are 13 and 27 °C, respectively. The annual average rainfall ranges from 837 to 1005.7 mm, and the climate varies due to the significant altitude variance (Koricho et al., 2022). The city is rich in medicinal plants, with around 131 species of medicinal plants belonging to 109 genera and 54 families (Kefalew et al., 2023).

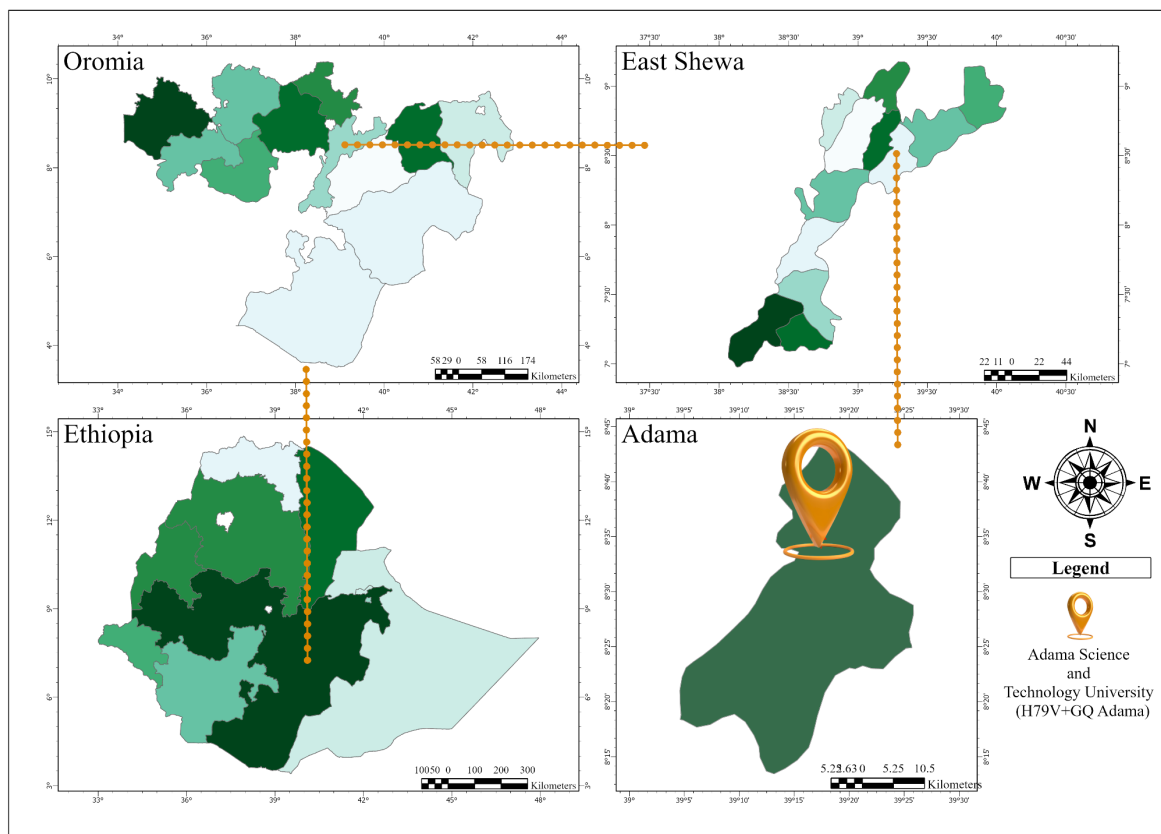


Figure 5: Sample collection area (Drawn by Sofonyas Kidaneweld, ArcGIS Pro v.3.0.0)

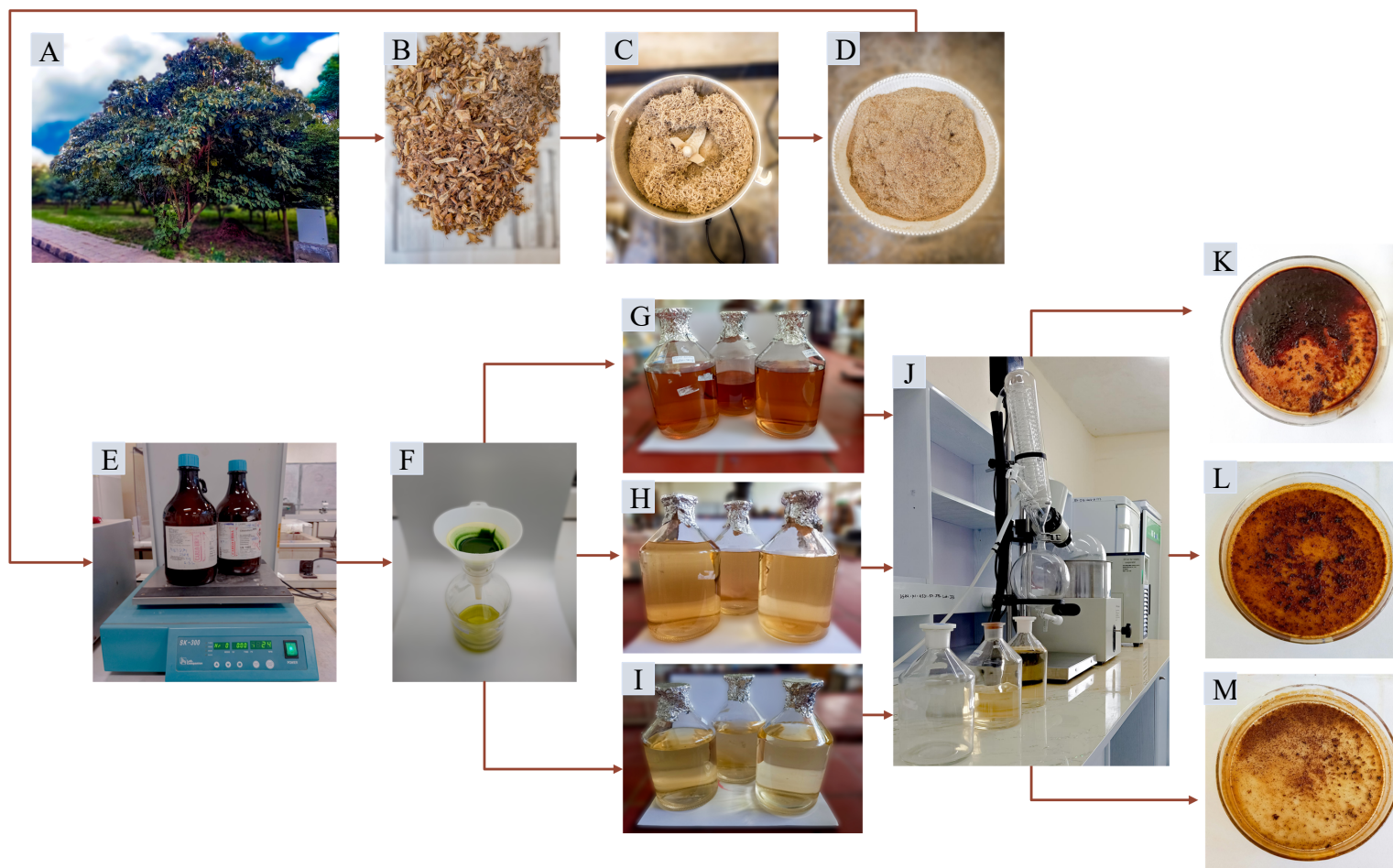
3.2. Sample Collection

Roots of *C. africana* were collected from Adama Science and Technology University in Adama City in February 2023 from three locations and different growth stages. The samples were brought to the Microbiology Lab at Adama Science and Technology University, Adama, Ethiopia. A voucher specimen was collected and submitted for taxonomic identification. A plant taxonomist performed taxonomic identification in the National Herbarium of Ethiopia, Department of Biology, Addis Ababa University, Ethiopia, and a voucher specimen (SK-002) was deposited there.

3.3. Preparation of plant extract

The roots of *C. africana* collected were washed repeatedly with tap water and then with deionized water to remove contaminants from the surface. Then, they were chopped into small pieces and air-dried at room temperature for three weeks (Scheme 1B). The dried roots were ground into a fine powder using a grinder (Electrical grinder, M.M2114) (Scheme 1D). Then, 400 g of the powdered sample was consecutively extracted using a cold maceration procedure with 4L (1:10) of Dichloromethane (DCM) (CH_2Cl_2) (LOBA CHEMIE PVT.LTD., H36388213, India): 97% Ethanol (EtOH) (1:1) followed by 97% EtOH and finally with 99.8% Methanol (MeOH) (Alpha Chemika, MA321, India) with continuous shaking using a basic shaker (JEIO TECH, SK-300, Korea) for three days at room temperature (Scheme 1E). After every three days, the extracts were filtered using Whatman No. 1 filter paper (Whatman International Ltd, Cat3001652, England) (Scheme 1F), and the filtrates were concentrated using a rotary evaporator (DW-RE-3000, China) at 40 °C (Scheme 1J). Then, the extracts were air-dried at room temperature, and their yield was calculated using equation (1). Finally, the extracts were stored in a refrigerator (LG, GL-D322RPJL/2014, India) until further use (Asrie et al., 2016).

$$\text{Yield \%} = \frac{\text{Weight of extract obtained}}{\text{Weight of plant sample}} \times 100 \quad (1)$$

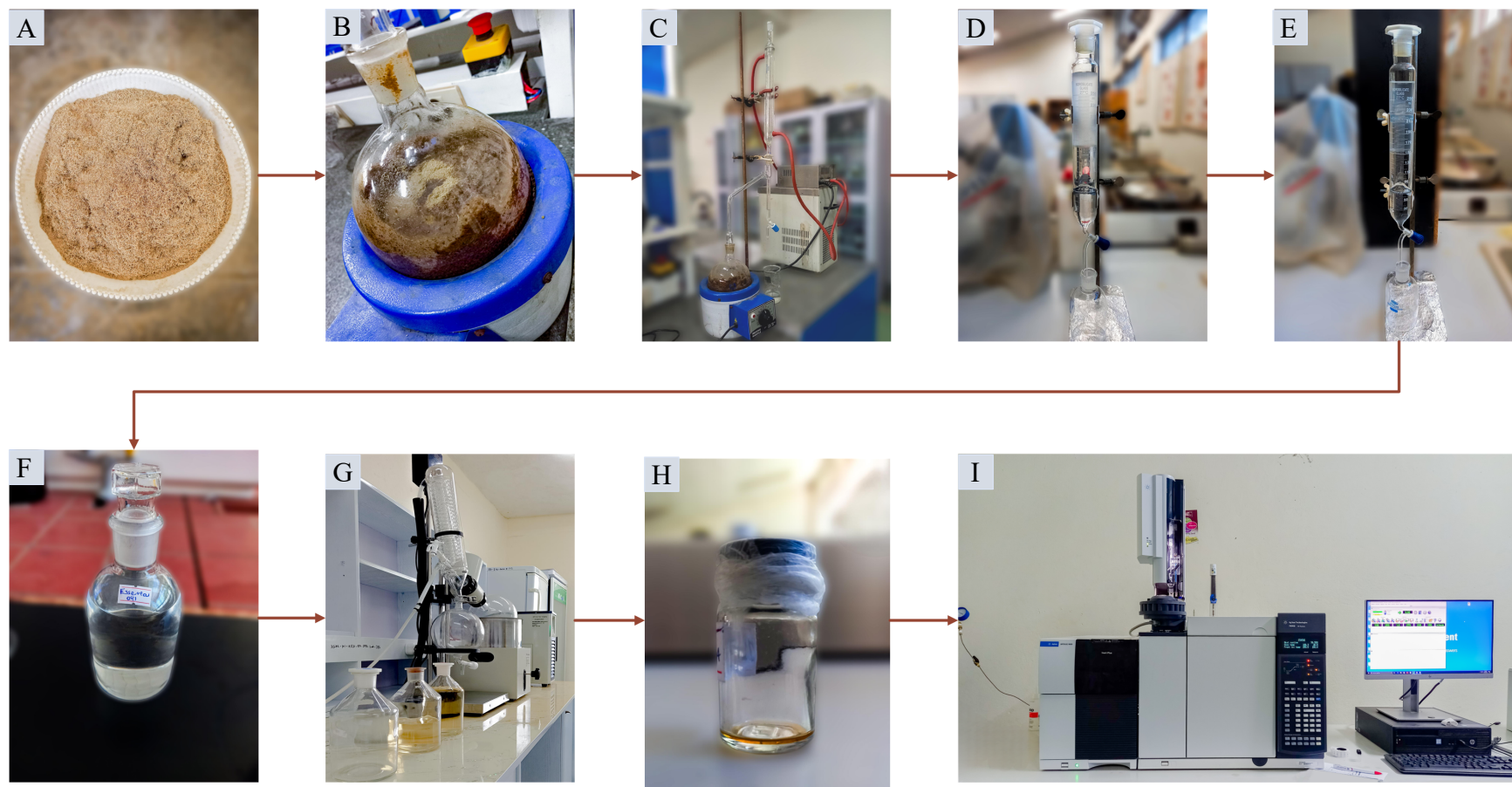


Scheme 1: Extraction of extracts. (A) tree of *C. africana*, (B) air drying collected root samples, (C) crushing airdried samples using electrical grinder, (D) powdered roots *C. africana*, (E) maceration of powdered roots of *C. africana* using organic solvents, (F) filtration of the macerated sample using Whatman No. 1 filter paper, (G) DCM:EtOH (1:1) extract, (H) EtOH extract, (I) MeOH extract, (J) concentrating the filtrates using a rotary evaporator, (K) extract of DCM:EtOH (1:1), (L) extract of EtOH, (M) extract of MeOH

3.4.Extraction of essential oil via hydro-distillation

300 g of plant material (Scheme 2A) was mixed with 3000 mL of distilled water (DH₂O) in a round bottom flask and subjected to hydro distillation for 4 hr. using a Clevenger apparatus (Aarson Scientific Works, India) (Scheme 2C) at atmospheric pressure. It was then subjected to liquid-liquid extraction using an equal volume of chloroform (Alpha Chemika, CM179, India) (Scheme 2E) for two rounds; then the chloroform layer was collected from the separatory funnel, and 5 g of anhydrous sodium sulfate (Na₂SO₄) (SAMIR TECH-CHEM PVT, LTD., 07J0850, India) was added to it to dry out the water left in the solution and was filtered using Whatman No. 1 filter paper (Whatman International Ltd, Cat3001652, England). Finally, the mixture was concentrated using a rotary evaporator (DW-RE-3000, China) (Scheme 2G), and the oil (Scheme 2H) was kept in a refrigerator (LG, GL-D322RPJL/2014, India) at 4°C until GC-MS analysis (Aydeniz & Guneser, 2024). The extraction yield of the EOs was determined using equation (2).

$$\text{Extraction yield (\%)} = \frac{\text{Mass of concentrated oil}}{\text{A dry mass of plant powder used}} \times 100 \quad (2)$$



Scheme 2: Essential oil extraction and analysis. (A) powdered root sample, (B) boiling the powder with DH₂O in hot plate, (C) hydro distillation using Clevenger apparatus, (D) liquid-liquid extraction of EO using chloroform, (E) optimum dissolution of the EO in chloroform after 24 hr., (F) EO in chloroform layer collected, (G) concentrating the essential oil using rotary evaporator, (H) extracted essential oil, (I) subjecting the essential oil to GC-MS analysis

3.5. Antibacterial activity

3.5.1. Inoculum preparation

American-type culture collections (ATCCs) pathogenic bacterial strains, *Escherichia coli* (ATCC-25922), *Pseudomonas aeruginosa* (ATCC-27853), *Staphylococcus aureus* (ATCC-25923), and *Streptococcus pyogenes* (ATCC-19615), were acquired from Oromia regional laboratory, Adama, Ethiopia and transported aseptically to Microbiology Lab, Adama Science and Technology University in an icebox and stored at 4°C in a refrigerator (LG, GL-D322RPJL/2014, India). The standard bacterial strains obtained then were sub-cultured in Muller Hinton Broth (MHB) (HIMEDIA, M173-500G, India) (Scheme 3A), which was prepared as per the guideline of the company and was sterilized at 121°C 0.11Mpa for 15 min (Zonkia, LX-B75L, China). Bacterial cells were adjusted to a concentration of 1.5×10^8 CFU/mL by adding 0.8% NaCl solution while monitoring the absorbance (625nm) until it reached an absorbance between 0.08 to 0.10 which is an absorbance of 0.5 McFarland standard, which was prepared by mixing 0.05 mL of 1% barium chloride (BaCl_2) solution with 9.95 mL of 1% sulfuric acid (H_2SO_4) based on the method reported by Calvo et al., (2011).

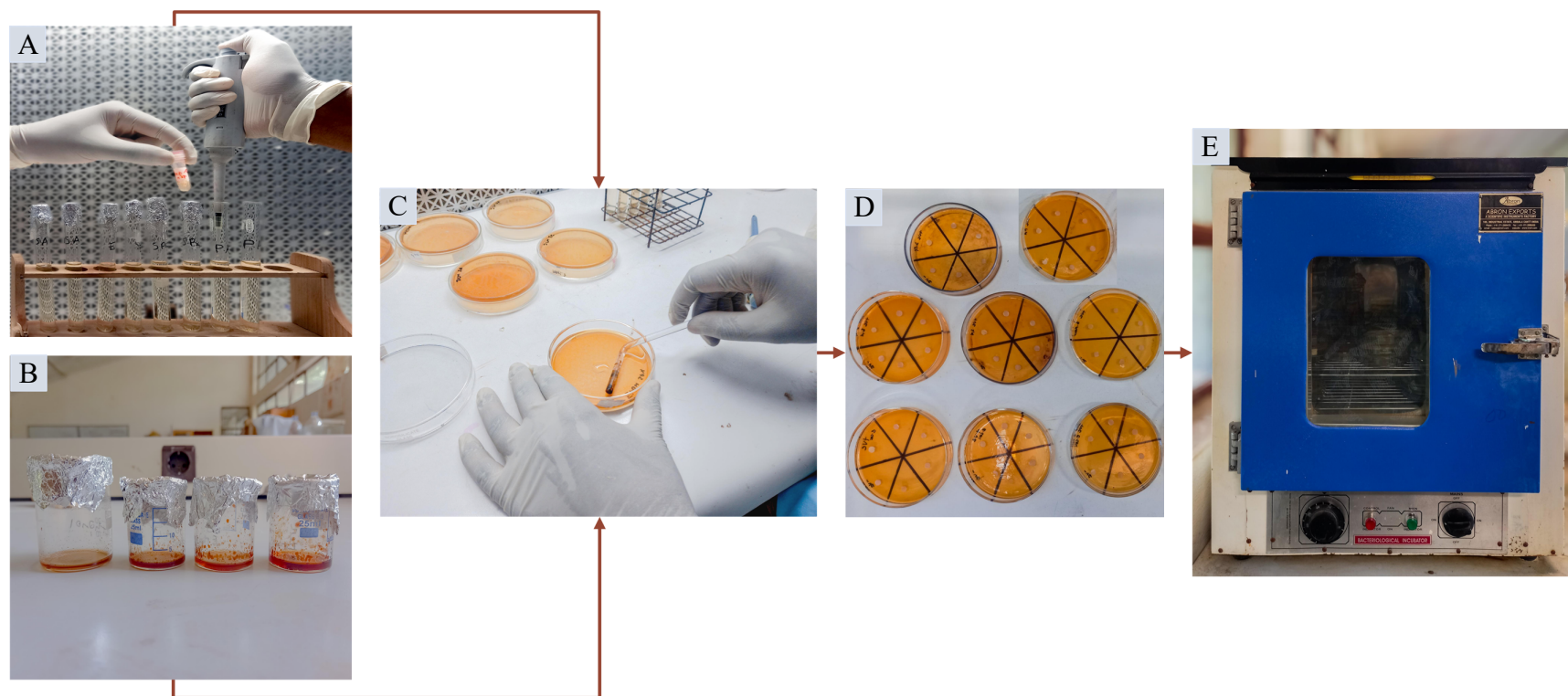
3.5.2. Paper Disk Diffusion Assay

Muller Hinton Agar (MHA) (HIMEDIA, M173-500G, India) was prepared as per the placed guideline of the manufacturer by considering 25 mL media per petri dish (HOVERLABS, BORO 3.3, India) to create a uniform thickness of around 3 mm in width and Whatman No.1 filter paper (Whatman International Ltd, Cat3001652, England) discs (6 mm in diameter) were also made using a puncher (Kangaro, DP-600, India) and was sterilized at 121°C 0.11Mpa for 15 min (Zonkia, LX-B75L, China).

A stock solution of the extracts was prepared by weighing 0.4 g of the extract using an electronic balance (SHIMADZU, AUW220D, Japan) and mixing it with 2ml of 4% extra pure AR Dimethyl Sulphoxide (DMSO) ($\text{C}_2\text{H}_6\text{SO}$) (SLR, 28580, India), prepared by dissolving 4 mL DMSO in 100 mL DH_2O and serially diluted to produce a concentration of 200, 100, 50, and 25 mg/mL (Scheme 3B). At the same time, 40mg of the isolated compounds were mixed with 2 ml of 4% DMSO (SLR, 28580, India) and serially diluted to produce a concentration of 20, 10, 5, and 2.5 mg/mL. Meanwhile, 20 mg of the extracted essential oil was serially diluted similarly to create concentrations of 10, 5, 2.5, and 1.25

mg/mL. 0.25 mg/mL Ciprofloxacin was prepared by powdering 0.5 mg of commercially available tablets and mixing with 2 mL 4% DMSO (SLR, 28580, India), used as a positive control for the test bacterial strains, respectively. At the same time, 4% DMSO-soaked filter paper disks were used as a negative control.

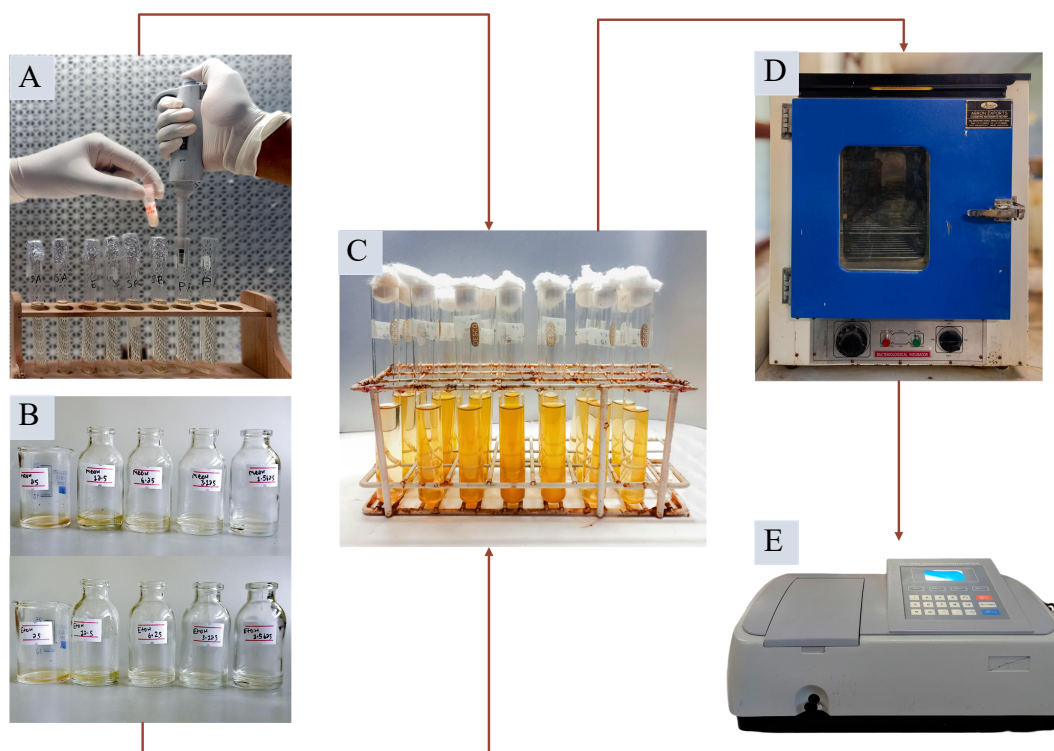
One hundred microliters of standardized inoculum of the bacterial strains were inoculated onto the agar surface in the Petri dishes and spread evenly over the agar surface using a sterilized glass rod (Scheme 3C). Then, 1 mL from each extract dilution was impregnated on the discs and left for 30 min for a thorough absorption of the solutions by the paper discs. The back side of the plates was then segmented into six equal parts using a permanent marker, and the disks were placed at a place labeled for each concentration and left for 30 min for diffusion in the Laminar airflow workstation (NUAIRE, NU-201-330E, USA) (Scheme 3D). The plates were incubated at 37°C for 24 hr. in the bacteriological incubator (ABRON Instruments, India) (Scheme 3E) (Hudzicki, 2012). Using a ruler, the antibacterial activity was assessed by measuring the extracts' zone of inhibition (mm). The entire tests were triplicates, and the findings were reported as mean $\pm$ standard deviation using statistical analysis software (SPSS, V.27.0.1 IF026).



Scheme 3: Disc diffusion assay. (A) subculturing test strains in MHB, (B) preparing different concentrations of the extracts, essential oil, and isolated compounds, (C) spreading the sub-cultured strains adjusted to a McFarland standard on a solidified MHA, (D) placing discs with samples onto the MHA covered with test strains at their respective concentrations and left for 30 min for the samples to diffuse, (E) incubating the Petri dishes for 24 hr.

3.6.Determination of Minimum Inhibitory Concentration (MIC)

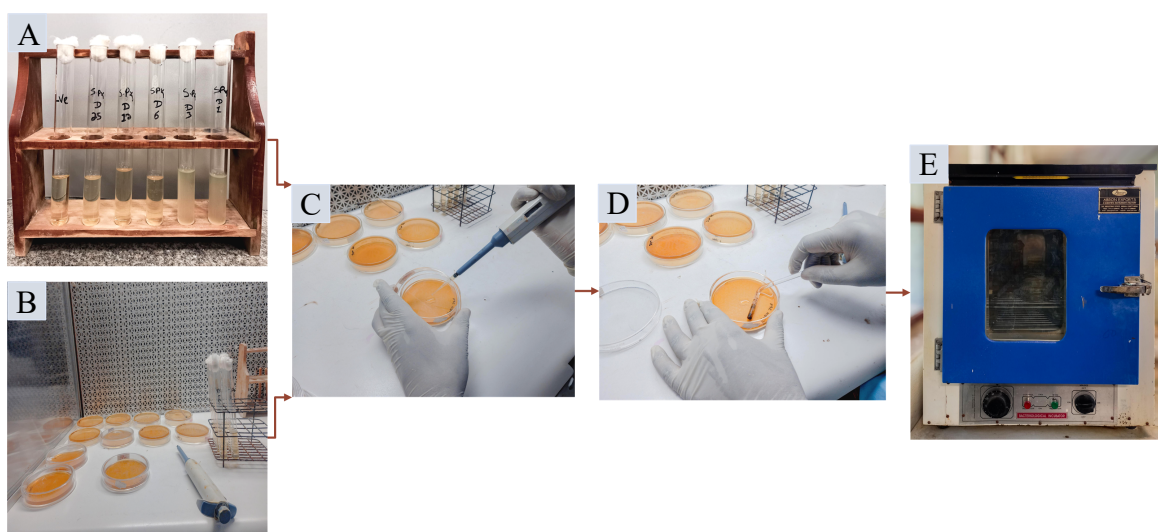
MHB (HIMEDIA, M173-500G, India) was prepared per the manufacturer's guidelines by considering 5mL MHB per test tube. It was sterilized at 121°C 0.11Mpa for 15 min (Zonkia, LX-B75L, China). A stock solution of 25 mg/2mL (12.5 mg/mL) was prepared. Two-fold serial dilution was made to obtain concentrations of 6.25, 3.125, and 1.5625 mg/mL for the extract (Scheme 4B). At the same time, the already prepared concentrations were used for the essential oil (10, 5, 2.5, and 1.25 mg/mL) and isolated compounds (20, 10, 5, and 2.5 mg/mL). Five milliliters of broth were added to each test tube, and 100 μ L of the standardized inoculum for each test strain was added to the respective tubes. Then, 1 mL of the already prepared concentrations were added to their respective tubes (Scheme 4C) and were incubated (ABRON Instruments, India) at 37°C for 24 hr. (Scheme 4D). Finally, the absorbance (600 nm) was measured (SCITEK, SP-UV6000, China) (Scheme 4E), and the ones close to the control were considered MIC (Hemeg et al., 2020).



Scheme 4: Determination of minimum inhibitory concentration. (A) subculturing test strains in MHB, (B) preparing different concentrations of the extracts, essential oil, and isolated compounds, (C) placing 100 μ L of the standardized strains and 1 mL of prepared samples into 5mL MHB, (D) incubating the test tube for 24 hr., (E) measuring the absorbance of each tube after incubation

3.7. Determination of Minimum Bactericidal Concentrations (MBC)

From tubes that had an absorbance close to the control and higher (Scheme 5A), 10 μ L was taken and spread onto MHA (HIMEDIA, M173-500G, India) using a sterilized glass rod (Scheme 5D) and were incubated at 37°C for 24 hr. (Scheme 5E). After incubation, the number of colonies on each agar plate was counted. The MBC is the lowest concentration of the antimicrobial agent, which results in a reduction of at least 99.9% in CFU/mL (Rodríguez-Melcón et al., 2022).



Scheme 5: Determination of minimum bactericidal concentration. (A) test tubes having an absorbance reading close to the control and higher, (B) solidified MHA, (C) placing 10 μ L from each tube and placing it onto the solidified MHA, (D) spreading the samples onto the surface MHA, (E) incubating the petri-dishes for 24 hr.

3.8. Phytochemical screening

A qualitative analysis was conducted to detect the presence of different phytochemicals in the extracts. 60 mg of each of the three extracts was dissolved in 6 mL methanol to obtain the concentration of 10 mg/mL; then, from each stock solution, 1 mL was taken and placed into 18 test tubes designated for each extract and to be tested phytochemicals.

3.8.1. Test for alkaloids (Wagner's test)

The plant extract (10 mg/mL) was mixed with 1 mL of 1% hydrochloric acid (HCl). The mixture was heated, six drops of Wagner's reagent were added, prepared by dissolving 2 g of potassium iodide (KI) and 1.27 g of iodine (I₂) in 100 mL of distilled water, and gently

shaken and were observed for the formation of brownish-red precipitate, which indicates the presence of alkaloids (Dahanayake et al., 2019).

3.8.2. Test for flavonoids (Shinoda Test)

A piece of magnesium (Mg) was added to the plant extract (10 mg/mL), followed by three drops of concentrated hydrochloric acid (HCl). The appearance of an orange, pink, or red color indicates the presence of flavonoids (Deshmukh & Theng, 2018).

3.8.3. Test for phenol (Ferric Chloride Test)

Three drops of neutral ferric chloride (FeCl_3) (5%) solution, prepared by adding diluted sodium hydroxide to ferric chloride until a permanent brown precipitate formed and then filtered, was added to the plant extract (10 mg/mL). The change of color to violet, blue, green, or purple coloration indicates the presence of phenol (Godlewska et al., 2023).

3.8.4. Test for saponins (Foam Test)

The plant extract (10 mg/mL) was mixed with 10 mL of distilled water, shaken vigorously for 5 minutes, and allowed the mixture to stand for 30 min. Stable foam (honeycomb froth) was observed at the surface. A positive result indicates the presence of saponins (Kancherla et al., 2019).

3.8.5. Test for steroid (Salkowski's Test)

The plant extract (10 mg/mL) was mixed with 5 mL of chloroform and 1 mL of concentrated sulfuric acid and carefully added along the sides of the test tube. A reddish-brown color in the lower layer indicates the presence of steroids (Dubale et al., 2023).

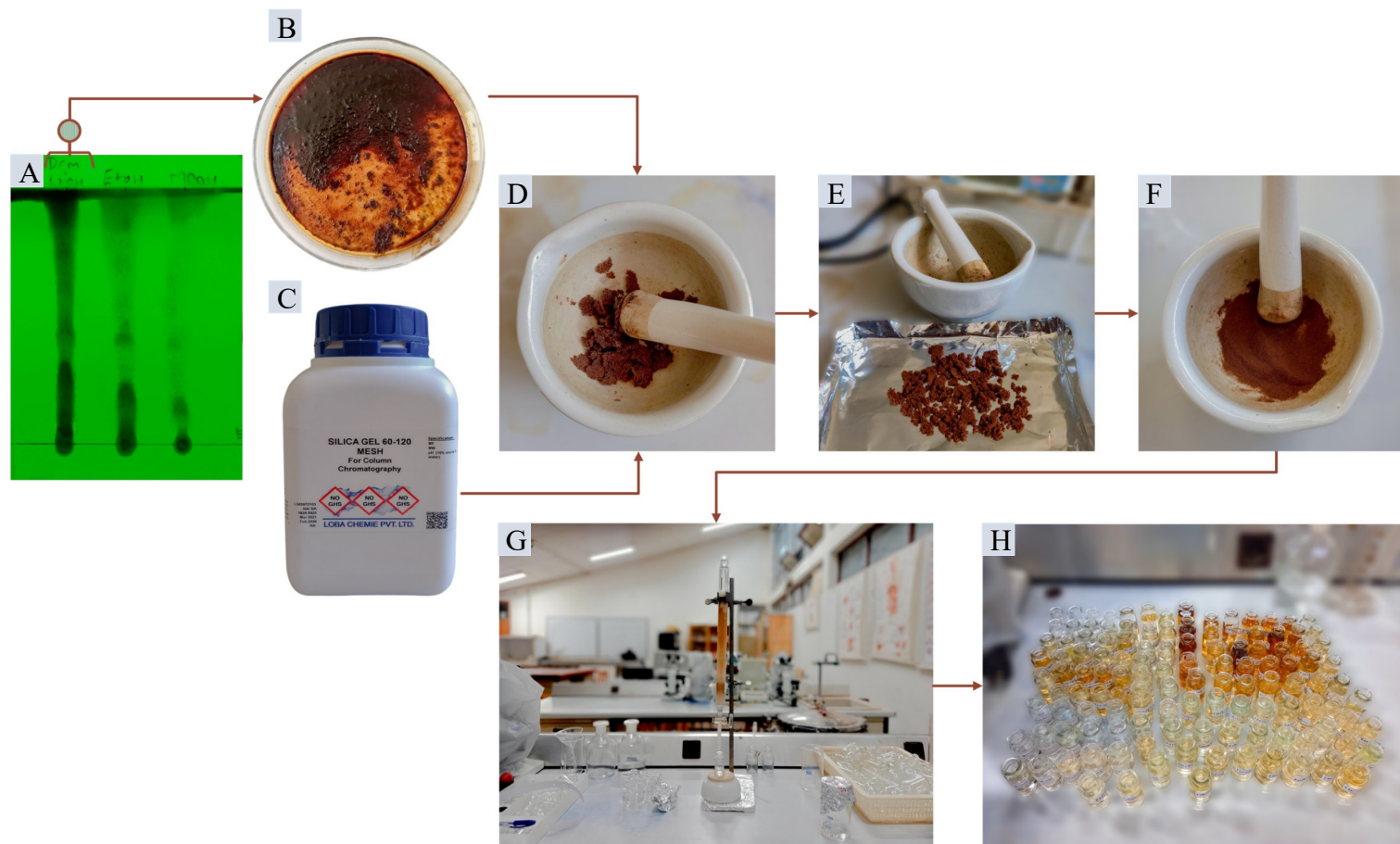
3.8.6. Test for tannins (Ferric Chloride Test)

Four drops of 10% ferric chloride (FeCl_3) solution were added to the plant extract (10 mg/mL). A blackish-blue color indicates the presence of gallic tannins, while a green-blackish color suggests the presence of catechol tannins (Auwal et al., 2014b).

3.9. Chromatographic Separation of Extracts

DCM:EtOH (1:1) extract, one of the active extracts against the test organisms, was chosen for column chromatography due to its antibacterial properties and better separation during thin-layer chromatography (TLC) (Scheme 6A). To generate a loading sample, The DCM:EtOH (1:1) extract (Scheme 6B) (5.0 g) meshed with an equal mass of silica (Scheme 6C) (5 g of silica, 60-120 mesh) (LOBA CHEMIE PVT.LTD., L345972103, India) and ground until it formed a powder (Scheme 6F). The sample was then subjected to silica gel column chromatography (Scheme 6G) (200 g, 60-120 mesh) (LOBA CHEMIE PVT.LTD., L345972103, India), which was run using increasing gradient elution. The column was first eluted with Petroleum ether (C_6H_{14}) (PE) (Alpha Chemika, EP237, India) as the mobile phase 100%, and the polarity of the mobile phase was gradually raised by adding 5% increments of 99.8% ethyl acetate ($C_4H_8O_2$) (EtOAc) (Alpha Chemika, EY684, India). After obtaining 100% EtOAc, the polarity was gradually increased by adding 5% Methanol (CH_3OH) (MeOH) (Alpha Chemika, MA321, India) until 30% MeOH was reached. Finally, MeOH in Chloroform ($CHCl_3$) (Alpha Chemika, CM179, India) elution was carried out until 50:50% and was collected in a drug vial of 15mL each (Scheme 6H) (Gebrehiwot et al., 2024).

Each fraction obtained from column chromatography was concentrated using a rotary evaporator (DW-RE-3000, China) before being subjected to thin-layer chromatography. Short and long ultraviolet light (DRAWELL, UV4AC6/2, China) and a mixture of 4-hydroxy-3-methoxybenzaldehyde (vanillin) ($C_8H_8O_3$) (SRL, 7730079, India) and sulfuric acid (H_2SO_4) reagent were used to visualize the TLC spots (Mawire et al., 2021). Similar fractions with identical R_f values in the same solvent system were mixed and repacked similarly. Finally, the pure compounds were sent to Amrita School Bangalore, India, for the NMR analysis.



Scheme 6: Fractionation of DCM:EtOH (1:1) extract. (A) TLC of the extracts at 60(EtOAc):40(Pet ether) solvent system, (B) DCM:EtOH (1:1) extract, (C) silica gel 60-120 mesh, (D) the extract meshed with silica gel using mortar and pestle, (E) airdrying the meshed sample at room temperature in the shade, (F) powdering the airdried meshed sample using mortar and pestle, (G) carrying out column chromatography, (H) fractions collected in a vial.

3.10. GC-MS analysis

The extracted essential oil was first dissolved in hexane and then filtered using Whatman No. 1 filter paper (Whatman International Ltd, Cat3001652, England); then, 1 µl was drawn for GC-MS analysis. GC-MS (Agilent Technologies, 7890B Gc system, USA) (Scheme 2I) equipped with a spitless programmable temperature injector and capillary column (30 m length, 250 µm Internal diameter, 0.25 µm Film thickness), which uses helium (He) as a carrier gas (1 mL/min) was employed for the study, the GC oven temperature was programmed as follows: initial 333 min. at 50 °C, 10 °C/min. to 200 °C, then 6 °C/min. to 280 °C and held for 5 min. The total run time was 36 minutes (Abdelrahim et al., 2016). Mass spectra were recorded in electron-impact mode, with an ionization energy of 70 eV and a scanning range of 33-550 m/z. The standard n-alkane was analyzed under the same GC-MS conditions (Appendix 7) as the sample to ensure an accurate comparison of retention times, and the Kovats Retention Indices for the compounds identified in the root essential oil of *C. africana* were calculated using equation (3) (Kováts, 1958).

$$I_X = 100 \times \left[n + \frac{\log(t_{R(X)}) - \log(t_{R(n)})}{\log(t_{R(n+1)}) - \log(t_{R(n)})} \right] \quad (3)$$

Where:

I_X : Kovats Index of the compound X

$t_{R(X)}$: Retention time of the compound X

$t_{R(n)}$: Retention time of the n-alkane with n carbon atoms (eluting just before X)

$t_{R(n+1)}$: Retention time of the n-alkane with $n + 1$ carbon atoms (eluting just after X)

n : Number of carbon atoms in the n-alkane eluting before X

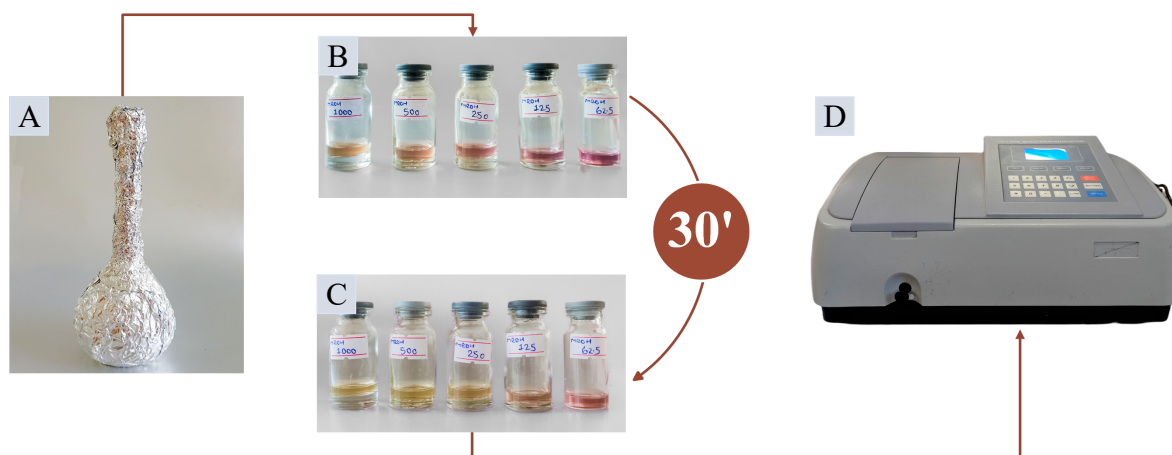
The components of the EOs were identified from the resulting chromatograms based on their elution duration, retention indices, and mass fragmentation patterns and by comparison with the spectrum data to the spectra of the standard n-alkane and NIST11 GC-MS library database. The relative percentage quantity of each chemical was estimated by comparing its average peak area to the overall area in Ms-Excell (Microsoft® Excel® for Microsoft 365 MSO (Version 2409 Build 16.0.18025.20030) 64-bit)

3.11. Antioxidant activity

Different concentrations of the essential oil, isolated compounds, and plant extracts were prepared by producing a stock solution by mixing 0.004 g of the samples with 2 mL of MeOH (Alpha Chemika, MA321, India) and two-fold serially diluted to produce concentrations of 2000, 1000, 500, 250, and 125 µg/mL. Ascorbic acid concentrations were also made similarly as a positive control. At the same time, 0.01 g of DPPH (Calbiochem, CAS 1898-66-4, USA) was dissolved in 25 mL MeOH (Alpha Chemika, MA321, India) to produce a 0.1 mM stock solution. Its absorbance was measured at 517 nm (1.0 ± 0.1) (SCITEK, SP-UV6000, China), and the container was wrapped in aluminum foil to protect it from light and was stored in a refrigerator until use (Scheme 7A).

In a vial, 1 mL of DPPH solution (0.1 mM) was mixed with 1 mL of each sample solution at different concentrations (Scheme 7B), which now have the concentration of 1000, 500, 250, 125, and 62.5 µg/mL and placed in the dark at room temperature for about 30 min. (Scheme 7C). Finally, they were transferred into a cuvette. Finally, their absorbance and the absorbance of a sample-free DPPH in MeOH, which was used as a negative control, were measured at 517 nm using a spectrophotometer (SCITEK, SP-UV6000, China) in triplicate (Scheme 7D). The percentage of DPPH radical scavenging activity was calculated using equation (4), and the results were presented as in mean $\pm$ SD. IC₅₀ of the samples was calculated using logarithmic regressions by Plotting a % scavenging activity against sample concentration in OriginPro (OriginLab Corporation, V.2024, USA) (Sannigrahi et al., 2010) (Appendix 3A-F).

$$\% \text{ DPPH scavenging} = \frac{\text{Control absorbance} - \text{extract absorbance}}{\text{Control absorbance}} \times 100 \quad (4)$$



Scheme 7: DPPH scavenging assay. (A) DPPH solution, (B) mixing different concentrations of the samples with 1 mL DPPH solution, (C) visual observation of color change of the samples after 30 min in the dark, (D) measuring the absorbance of each concentration at 517 nm.

3.12. Statistical analysis

The collected data for the antibacterial activity were analyzed using SPSS (IBM corporation, v.27.0.1.0, USA), and all results were presented as mean $\pm$ standard deviation, *P* values less than 0.05 are considered statistically significant. OriginPro (OriginLab Corporation, V.2024, USA) was used to present the results in a graphical form, to calculate the IC₅₀ value, and to analyze FTIR results, while Ms-Excell (Microsoft® Excel® for Microsoft 365 MSO (Version 2409 Build 16.0.18025.20030) 64-bit) was used to analyze GC-MS data. ArcGIS Pro (Esri, inc., v.3.0.0, USA) was used to plot the study area map. ChemDraw (Revvity Signals Software, Inc., v.23.1.1.3, USA) was used to draw chemical structures.

CHAPTER FOUR

4. RESULTS AND DISCUSSIONS

4.1. Yield of extracts

The air-dried roots of *C. africana* macerated in DCM:EtOH (1:1), EtOH, and MeOH provided deep brown (Scheme 1K), reddish brown (Scheme 1L) and light brown extracts (Scheme 1M).

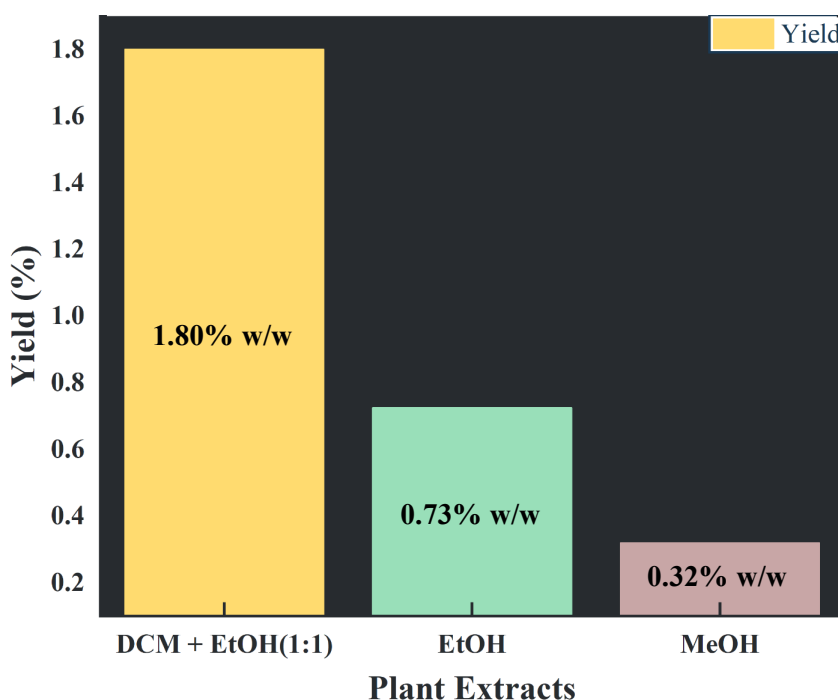


Figure 6: Yield of root extracts of *C. africana*

The 0.73% w/w yield of the EtOH extract (Figure 6) is in a close agreement with the study reported by Zemichael et al. (2019), who obtained a 0.438 g yield (0.44% w/w) from 100 g of *C. africana* leaves macerated in ethanol, representing the highest yield in their study. In contrast, Wondafrash et al. (2019) used 80% methanol to macerate 1200 g of coarsely powdered *C. africana* leaves, resulting in a much higher actual yield of 144.72 g (12.06% w/w). Similarly, Yismaw et al. (2020) achieved a yield of 425 g (26.56% w/w) by macerating 1.6 kg of fine seed powder of *C. africana* in 80% methanol.

The variation in extraction yields of *C. africana* can be attributed to several factors. Different plant parts (e.g., leaves, seeds, roots) contain varying concentrations of phytochemicals, with seeds and leaves typically having higher levels of secondary metabolites like phenolics and oils than roots. Solvent polarity also plays a crucial role, as polar solvents like methanol and ethanol extract a broader range of compounds, resulting in higher yields. In contrast, non-polar solvents like petroleum ether and chloroform are more selective and yield less. Particle size, such as fine powder versus coarsely ground material, also affects yield and the surface area exposed to solvents. Environmental factors, including altitude, soil, and climate, along with the plant's maturity and harvest time, further contribute to variations in metabolite production and extraction efficiency (Azwanida, 2015).

4.2. Phytochemical screening

The *in vitro* phytochemical analysis of extracts from the roots of *C. africana* revealed the presence of alkaloids, flavonoids, phenols, steroids, and tannins consistently across all three extracts while saponin was only detected in the DCM:EtOH (1:1) extract showing their absence in the EtOH and MeOH extracts (Table 1).

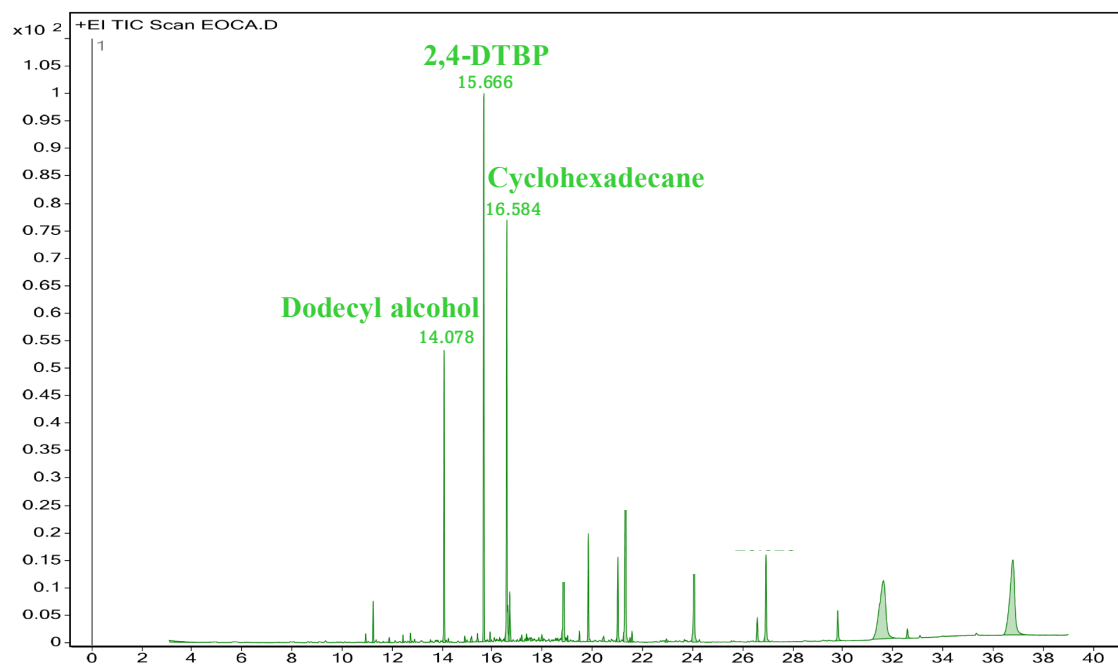
To support these findings, a phytochemical study conducted by Ganesan et al. (2015) on the aerial parts (leaves, stems, and fruits) of *C. africana* collected from the Jimma University Garden in Southwest Ethiopia provides relevant insights. The study employed various solvents, including benzene, carbon tetrachloride (CCl₄), hexane, and aqueous extracts, to analyze the phytochemical content. The results indicated the presence of alkaloids, flavonoids, and tannins across the extracts. Phenols were detected in all three plant parts, while saponins were explicitly found only in the leaf extract when CCl₄ was used as the solvent. However, in the current study, saponins were not detected in the EtOH and MeOH extracts, potentially due to differences in the extraction methodology, such as the solvents used. Interestingly, steroids were identified in the aqueous extracts in the study by Ganesan et al. (2015), whereas the current study detected steroids in all three extracts. These variations in phytochemical profiles can likely be attributed to several factors, including the specific plant parts analyzed, the choice of solvents for extraction, and the growth altitude of the plant (Mohammadi Bazargani et al., 2021).

Table 1: Phytochemical profile of roots extracts of *C. africana*

Phytochemicals	Plant Extracts		
	DCM:EtOH (1:1)	EtOH	MeOH
Alkaloid	✓	✓	✓
Flavonoid	✓	✓	✓
Phenol	✓	✓	✓
Saponin	✓	✗	✗
Steroid	✓	✓	✓
Tannin	✓	✓	✓

4.3. Essential oils profile and GC-MS analysis of roots of *C. africana*

The powdered roots (300 g) of *C. africana* yielded an EO of 20.2 mg (0.67% w/w) with yellowish color and distinctive odor. The GC-MS analysis (Figure 7) of EO of roots of *C. africana* showed a total of 29 components, of which 24 compounds accounted for 74.11% of the total composition (Table 2); the rest was occupied by hentriacontane (25.89%), which might be a contaminant as its spectrum is not observed in the chromatogram at the RT (31.629).

**Figure 7:** Gas chromatogram of root essential oil of *C. africana*

The majority of the reported compounds were from the chemical class of aromatics (25.99%), followed by alkanes/alkenes (20.97%), carboxylic acids & esters (13%), alcohols (12.63%), terpenoids/other cyclic compounds (1.05%) and aldehydes/ketones (0.47%). Dodecyl alcohol (11.43%) (**15**, Figure 8, Appendix 6), 2,4-DTBP (23.83%) (**20**, Figure 8, Appendix 4), and Cyclohexadecane (19.95%) (**22**, Figure 8, Appendix 5) were among the significant compounds accounting for 55.21% of the total composition of the reported compounds.

Dodecyl alcohol, also known as 1-dodecanol, is a long-chain fatty alcohol that has been found to exhibit bactericidal activity against *S. aureus*, *E. coli*, and *Bacillus subtilis* (Togashi et al., 2007). The antibacterial activity of dodecyl alcohol is related to the length of its aliphatic carbon chain, with longer chains generally exhibiting higher membrane damage activity (Pala et al., 2024).

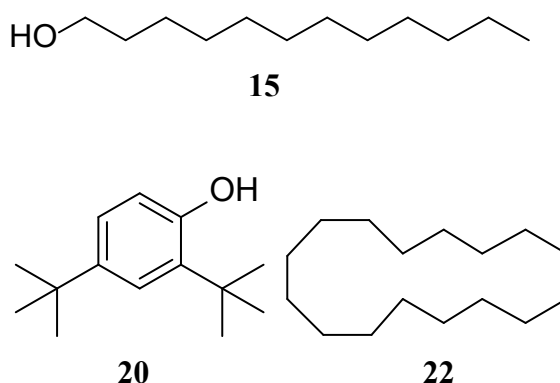
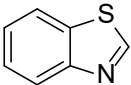
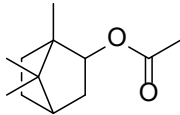
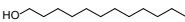
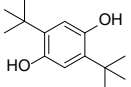
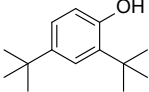


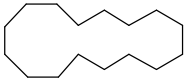
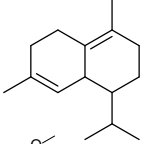
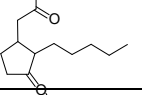
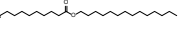
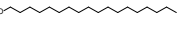
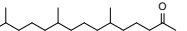
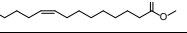
Figure 8: Chemical structure of the three major compounds identified from GC-MS analysis of essential oil of *C. africana* roots

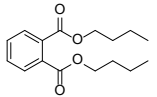
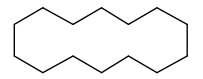
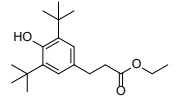
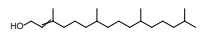
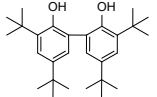
2,4-DTBP, also known as 2,4-di-tert-butylphenol, is a phenolic compound exhibiting a broad spectrum of antimicrobial activity against various microorganisms and significant antioxidant properties. It has been found to inhibit the growth of bacteria such as *Serratia marcescens*, *P. aeruginosa*, and *S. aureus*. It has also exhibited antiviral activity against coxsackievirus B-3 and herpes virus type 2 (Zhao et al., 2020). The antimicrobial activity of 2,4-DTBP is attributed to its ability to modulate the secreted extracellular polymeric substances (EPS) of bacteria, which can facilitate the disruption of biofilms and enhance the efficacy of conventional antibiotics.

The chemical composition of the EO of *C. africana* shared two similar compounds, phytol and hexahydrofarnesyl acetone, with previously reported study by Ashmawy et al. (2021). The study identified a total of 80 constituents of EOs from the leaves of *C. africana*, of which β -caryophyllene (32.00%), α -caryophyllene (6.82%), germacrene D (10.12%), δ -cadinene (7.20%) and Phytol (5.27%) were the significant compounds accounting for 61.41% (Ashmawy et al., 2021). The majority of compounds in the report differed from this study's findings. This is because the EOs extracted from different parts of the plant and chemical compositions also rely on certain environmental factors, such as soil type, climate, harvesting time, and growth altitude (Khan et al., 2023). To the best of our knowledge, the chemical composition of EOs from *C. africana* roots is reported herein for the first time.

Table 2: Essential oil composition of roots of *C. africana*

No	RT	Compound Name	Structure	Molecular formula	Composition (%)	Kovat Index (KI)	Reported Kovat index	Reference
13	10.942	Nonyl alcohol		C ₉ H ₂₀ O	0.404	1171	1171	(R. P. Adams, 2007)
14	11.883	BT		C ₇ H ₅ NS	0.273	1230	1218	(Tellez et al., 2001)
15	12.432	Decyl alcohol		C ₁₀ H ₂₂ O	0.287	1262	1272	(R. P. Adams, 2007)
16	12.732	Isobornyl acetate		C ₁₂ H ₂₀ O ₂	0.471	1280	1291	(Priestap et al., 2003)
17	14.078	Dodecyl alcohol		C ₁₂ H ₂₆ O	11.428	1372	1473	(Marques et al., 2000)
18	14.904	Viridiflorol		C ₁₅ H ₂₆ O	0.305	1460	1590	(Rodrigues et al., 2013)
19	15.175	DBHQ		C ₁₄ H ₂₂ O ₂	0.38	1480	1512	(NIST, 2023)
20	15.666	2,4-DTBP		C ₁₄ H ₂₂ O	23.834	1518	1502	(Yu & Zhang, 2010)

21	15.909	Calamenene, cis c		C ₁₅ H ₂₂	0.463	1536	1538	(Cui et al., 2011)
22	16.584	Cyclohexadecane		C ₁₆ H ₃₂	19.947	1588	1580	(NIST, 2023)
23	16.7	DEP		C ₁₂ H ₁₄ O ₄	2.375	1598	1546	(Özel et al., 2006)
24	17.173	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene		C ₁₅ H ₂₄	0.308	1704	1506	(bin Ahmad & bin Jantan, 2003)
25	17.37	MDJ		C ₁₃ H ₂₂ O ₃	0.264	1721	1650	(R. P. Adams, 2007)
26	17.549	α-Atlantone (Z)		C ₁₅ H ₂₂ O	0.276	1735		
27	17.982	Undec-10-ynoic acid, tetradecyl ester		C ₂₅ H ₄₆ O ₂	0.334	1772		
28	19.01	Stearyl Alcohol		C ₁₈ H ₃₈ O	0.246	1858	2080	(Morteza-Semnani et al., 2006)
29	19.478	Hexahydrofarnesyl acetone		C ₁₈ H ₃₆ O	0.47	1898	1833	(Gohari et al., 2006)
30	19.836	Phthalic acid, hept-4-yl isobutyl ester		C ₁₉ H ₂₈ O ₄	4.71	1934		
31	20.448	Methyl myristoleate		C ₁₆ H ₃₀ O ₂	0.422	1979		

32	21.014	DBP		$C_{16}H_{22}O_4$	4.373	1963	1594	(Kotowska et al., 2012)
33	21.505	Cyclotetradecane P391		$C_{14}H_{28}$	0.25	2000	1669	(Robledo & Arzuffi, 2004)
34	21.58	Ethyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoate		$C_{19}H_{30}O_3$	0.519	2007		
35	22.954	Phytol		$C_{20}H_{40}O$	0.269	2190	2096	(Lawal & Oyedeji, 2009)
36	26.581	4,4'-Bis(2,6-di-tert-butylphenol)		$C_{28}H_{42}O_2$	1.505	2370		

4.4. Fractionation of DCM:EtOH (1:1) extract of roots of *C. africana*

The fractionation process of the DCM:EtOH (1:1) extract from the roots of *C. africana* yielded a total of 122 fractions (Appendix 12), each collected in 15 mL portions. Each fraction was then subjected to TLC for purity assessment. No single pure compound was identified at this stage. Consequently, fractions with similar R_f values were combined, producing a total extract weighing 79.1 mg. This pooled extract was further subjected to an additional fractionation process using column chromatography, producing 154 fractions (Appendix 12), each collected in 15 mL portions. Again, all fractions were analyzed via TLC, and fractions with similar R_f values were combined. Notably, fractions F₄₅-F₅₀ (Compound **32** and **37**) (Figure 9A) exhibited a single spot under UV light at 254 nm but not under 365 nm. While F₆₉-F₇₃ (Compound **38** and **39**) have not showed a spot under UV light upon treatment with vanillin solution, by immersing the TLC into a beaker having the solution, a single spot was observed.

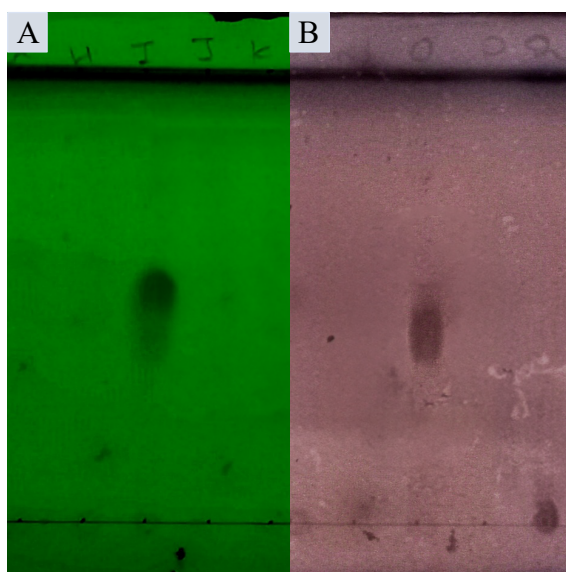


Figure 9: Thin layer chromatogram of isolated compounds. (A) TLC of Compound **32** and **37** under 254 nm UV light, (B) TLC of Compound **38** and **39** after being treated with vanillin and placed on a hotplate

4.4.1. Structure elucidation of isolated compounds

4.4.1.1. Mixture of Compound 32 and 37

Compound **32** and **37** (Figure 10) a co-eluted mixture, approximately 4:3 ratio of **32** to **37** based on the integration of proton signals in its ^1H NMR (400 MHz, CDCl_3) signals, was brown in coloration and amorphous in texture. The ^1H NMR spectrum (Appendix 10A) highlights an aromatic system with signals at δ 7.73 (m, 2H) and δ 7.54 (m, 2H), integrating to four protons in an AA'BB' pattern, suggesting a 1,2-disubstituted benzene ring with deshielding from electron-withdrawing ester substituents affecting the ortho and meta positions, while a triplet at δ 4.28 (t, $J=6.7$ Hz, 4H) indicates four equivalent $-\text{OCH}_2-$ protons characteristic of ester linkages, complemented by aliphatic signals at δ 1.76-1.49 (m, 4H), δ 1.45-1.15 (m, 20H), δ 0.88 (t, $J=6.8$ Hz, 6H), and δ 0.86 (t, $J=7.0$ Hz, 6H), pointing to butyl chains (Table 3); in the same spectrum, signals at δ 6.97 (d, $J=8.3$ Hz, 2H) and δ 6.88 (d, $J=8.3$ Hz, 2H) suggest a para-substituted aromatic system, with a singlet at δ 3.96 (s, 3H) indicating a methoxy group and a broad singlet at δ 4.69 (s, 1H) corresponding to an exchangeable hydroxyl proton, consistent with a phenolic structure (Table 5).

The ^{13}C NMR spectrum (Appendix 10B) supports these features with aromatic carbons at δ 132.46, δ 130.17, and δ 129.87, aligning with the 1,2-disubstituted benzene core of the ester, and ester carbonyls at δ 167.88, with $-\text{OCH}_2-$ carbons at δ 65.73, alongside aliphatic carbons at δ 39.03, δ 30.72-22.83, δ 14.26, and δ 12.12, confirming the butyl chains (Table 4), while the phenolic component's aromatic carbons are likely overlapped around 130 ppm (potentially including quaternary carbons expected at $\sim$ 150-155 ppm) (Table 6), with a methoxy carbon expected at $\sim$ 55 ppm missing, possibly due to low intensity or overlap. The FTIR spectrum (Appendix 8) further supports this mixture with a strong $\text{C}=\text{O}$ stretch at $\sim$ 1720 cm^{-1} and C-O stretches at $\sim$ 1100-1300 cm^{-1} , confirming the ester functionality, while a broad 3000-3300 cm^{-1} O-H stretch reflects the hydroxyl group of the phenolic component, alongside aromatic and aliphatic C-H stretches at 2800-3000 cm^{-1} , consistent with the mixed aromatic ester and phenolic framework of the co-eluted sample.

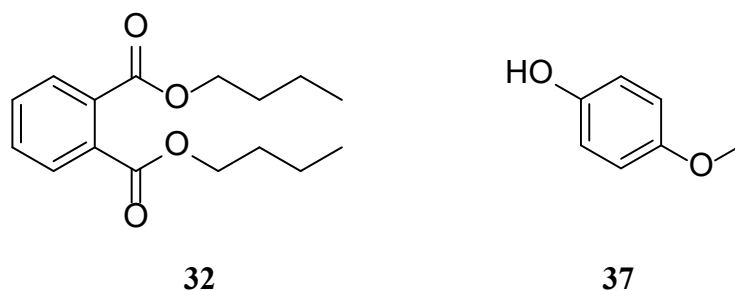


Figure 10: Proposed structure of Compound **32** and **37**

Table 3: ^1H NMR data of Compound **32** in CDCl_3

Chemical Shift (δ , ppm)	Multiplicity (Integration)	Assignment
7.72	dd, $J = 5.7, 3.3$ Hz (2H)	Aromatic (H-3, H-6)
7.53	dd, $J = 5.7, 3.3$ Hz (2H)	Aromatic (H-4, H-5)
4.31	t, $J = 6.7$ Hz (4H)	$-\text{OCH}_2-$ (butyl chain)
1.76-1.53	m (4H)	$-\text{CH}_2-$ (next to $-\text{OCH}_2-$)
1.45-1.31	m (4H)	$-\text{CH}_2-$ (next to $-\text{CH}_3$)
0.92-0.77	m (6H)	$-\text{CH}_3$

Table 4: ^{13}C NMR data of Compound **32** in CDCl_3

Chemical Shift (δ , ppm)	Assignment
167-170	$\text{C}=\text{O}$ (ester carbonyl)
130.92	Quaternary aromatic (C-1, C-2)
128.85	Aromatic (C-3, C-6)
128.85	Aromatic (C-4, C-5)
65.59	$-\text{OCH}_2-$ (butyl chain)
30.93	$-\text{CH}_2-$ (next to $-\text{OCH}_2-$)
19.19	$-\text{CH}_2-$ (next to $-\text{CH}_3$)

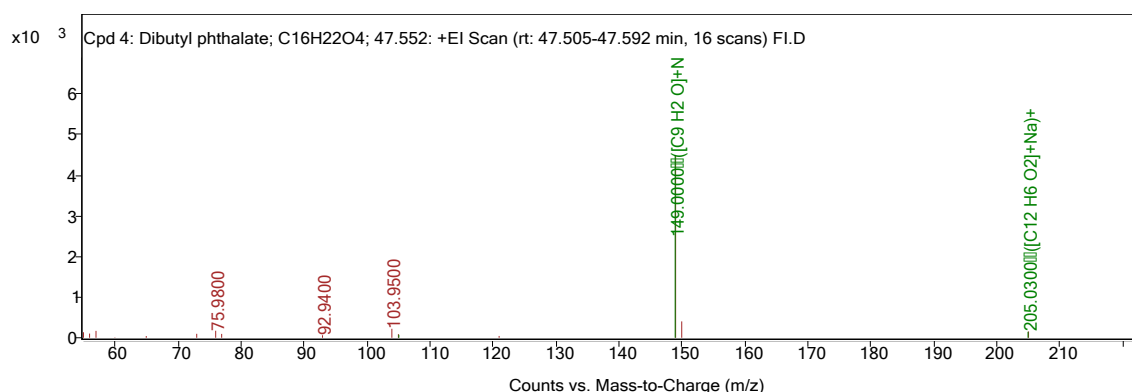
Table 5: ^1H NMR data of Compound **37** in CDCl_3

Chemical Shift (δ , ppm)	Multiplicity (Integration)	Assignment
6.97	d, $J = 8.3$ Hz (2H)	Aromatic (H-2, H-6)
6.88	d, $J = 8.3$ Hz (2H)	Aromatic (H-3, H-5)
4.69	s (1H)	$-\text{OH}$
3.96	s (3H)	$-\text{OCH}_3$

Table 6: ^{13}C NMR data of Compound **37** in CDCl_3

Chemical Shift (δ , ppm)	Assignment
153-155	C-4 (attached to $-\text{OCH}_3$)
150-153	C-1 (attached to $-\text{OH}$)
115.5-116	C-2, C-6 (ortho to $-\text{OCH}_3$)
114-115.5	C-3, C-5 (ortho to $-\text{OH}$)
55-56	$-\text{OCH}_3$

The data generated matches a previous report for dibutyl phthalate (Wang et al., 2010) (Table 7). The compound (dibutyl phthalate) was also identified from the GC-MS analysis of the essential oil *C. africana* (**32**, Table 2). Also, the isolated compound and its structural information were supported by mass fragmentation patterns (Figure 11).

**Figure 11:** Mass fragmentation pattern of Compound **32****Table 7:** ^1H (400 MHz), ^{13}C (100 MHz) NMR spectral data of Compound **32**, and ^1H , ^{13}C NMR values of dibutyl phthalate in literature.

C. No.	Compound 32	Dibutyl Phthalate (Wang et al., 2010)		
	¹ H NMR	¹³ C NMR	¹ H NMR	¹³ C NMR
Aromatic Carbons				
1,2	-	132.46	-	132.5
3,6	7.73 (dd, J=5.7, 3.3 Hz)	130.17	7.70 (dd, J=7.5, 1.5 Hz)	130.0
4,5	7.53 (dd, J=5.7, 3.3 Hz)	129.87	7.50 (dd, J=7.5, 1.5 Hz)	128.5
Carbonyl Carbons				
7,8	-	167.88	-	167.5

Butyl Chains				
9,9'	4.31 (t, J=6.7 Hz)	65.73	4.20 (m)	65.0
10,10'	1.76-1.53 (m)	30.93	1.70 (m)	30.0-23.0
11,11'	1.45-1.31 (m)	19.19	1.30-1.60 (m)	30.0-23.0
12,12'	0.92-0.77 (m)	14.12	1.30-1.60 (m)	14.0

Table 8: ^1H (400 MHz), ^{13}C (100 MHz) NMR spectral data of Compound **37**, and ^1H , ^{13}C NMR values of 4-Methoxyphenol in literature.

C. No.	Compound 37		4-Methoxyphenol (Kim et al., 2014)	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
Aromatic Carbons				
1	-	150-153	-	150.0
2,6	6.97 (d, J=8.3 Hz)	115.5-116	6.80 (d, J=9.0 Hz)	115.5
3,5	6.88 (d, J=8.3 Hz)	114-115.5	6.75 (d, J=9.0 Hz)	114.5
4	-	153-155	-	153.5
Methoxy Group				
7	3.96 (s)	55-56	3.75 (s)	55.8

4.4.1.2. Mixture of Compound **38** and **39**

Compound **38** and **39** (Figure 12) a co-eluted mixture, approximately 1:2 ratio of **38** to **39** based on the integration of proton signals in its ^1H NMR (400 MHz, CDCl_3) signals, was brown in coloration and amorphous in texture. The ^1H NMR spectrum (Appendix 11A) highlights an aromatic system with signals at δ 7.73 (m, 2H) and δ 7.54 (m, 2H), integrating to four protons in an AA'BB' pattern, suggesting a 1,2-disubstituted benzene ring with deshielding from electron-withdrawing ester substituents affecting the ortho and meta positions, while a triplet at δ 4.28 (t, J=6.7 Hz, 4H) indicates four equivalent $-\text{OCH}_2-$ protons characteristic of ester linkages, complemented by aliphatic signals at δ 1.76-1.49 (m, 4H), δ 1.49-1.15 (m, 20H), δ 0.88 (t, J=6.8 Hz, 6H), and δ 0.86 (t, J=7.0 Hz, 6H), pointing to branched 2-ethylhexyl chains (Table 9); in the same spectrum, an olefinic signal at δ 5.26 (t, J=3.5 Hz, 1H) suggests a double bond within a polycyclic structure, with an anomeric proton at δ 4.36 (d, J=7.5 Hz, 1H) indicating a β -glycosidic linkage, supported by sugar protons at

δ 3.18-3.30 (m, 4H), δ 3.62 (dd, $J=11.0$, 5.0 Hz, 1H), and δ 3.82 (dd, $J=11.0$, 2.0 Hz, 1H), and methyl singlets at δ 0.76-1.13 (e.g., δ 0.76, 0.91, 0.93, 0.96, 0.99, 1.12, 1.13, s, 3H each), consistent with a triterpenoid framework (Table 11).

The ^{13}C NMR spectrum (Appendix 11B) supports these features with aromatic carbons at δ 132.46, δ 130.17, and δ 129.87, aligning with the 1,2-disubstituted benzene core of the ester, and ester carbonyls at δ 167.88, with $-\text{OCH}_2-$ carbons at δ 65.73, alongside aliphatic carbons at δ 39.03, δ 30.72-22.83, δ 14.26, and δ 12.12, confirming the 2-ethylhexyl chains (Table 10), while the triterpenoid component shows olefinic carbons at δ 122.2 and δ 144.1, an anomeric carbon at δ 105.2, a glycosylated carbon at δ 88.1, sugar carbons at δ 75.1, 78.6, 71.2, 78.1, and 62.1, a carboxylic acid carbon at δ 180.6, and methyl carbons at δ 28.6, 16.7, 15.2, 17.1, 26.6, 33.1, and 23.6, with other aliphatic carbons spanning δ 15.2-55.3 (Table 12). The FTIR spectrum (Appendix 9) further supports this mixture with a strong $\text{C}=\text{O}$ stretch at $\sim 1700\text{-}1800\text{ cm}^{-1}$ (δ 167.88, 180.6), confirming the ester and carboxylic acid carbonyls, while a broad $3000\text{-}3300\text{ cm}^{-1}$ O-H stretch reflects the hydroxyl groups of the sugar and carboxylic acid, alongside C-O stretches at $\sim 1100\text{-}1300\text{ cm}^{-1}$ (δ 4.36, 105.2), aromatic and olefinic bends at $700\text{-}1000\text{ cm}^{-1}$ (δ 7.54, 7.73, 129.87-132.46; δ 5.26, 122.2, 144.1), and aliphatic C-H stretches at $2800\text{-}3000\text{ cm}^{-1}$, consistent with the mixed aromatic ester and triterpenoid saponin framework of the co-eluted sample.

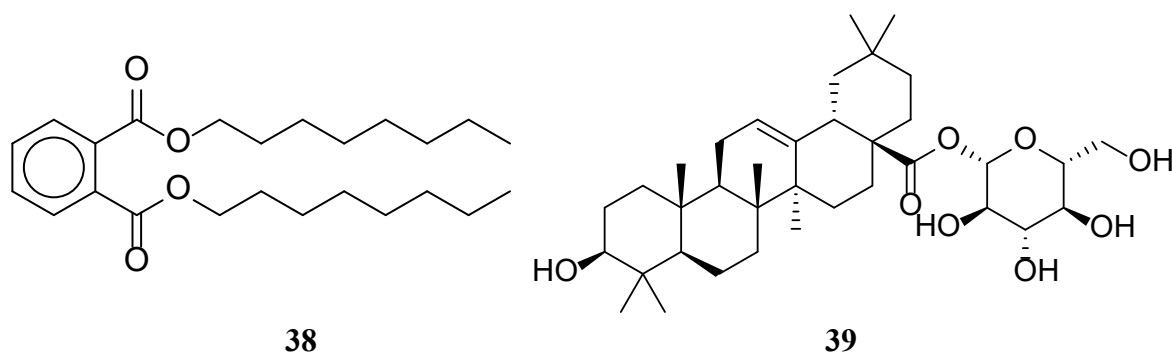


Figure 12: Proposed structure of Compound **38** and **39**

Table 9: ¹H NMR data of Compound **38** in CDCl₃

Chemical Shift (δ , ppm)	Multiplicity (Integration)	Assignment
7.73-7.52	m (4H)	Aromatic protons (H-3, H-4, H-5, H-6 on the benzene ring)
4.28	t, J = 6.7 Hz (4H)	-CH ₂ - of octyl ester groups (-O-CH ₂ -CH ₂ -(CH ₂) ₅ -CH ₃)
1.76-1.49	m (4H)	-CH ₂ - (second methylene in octyl chains, -O-CH ₂ -CH ₂ -(CH ₂) ₅ -CH ₃)
1.49-1.15	m (20H)	-CH ₂ - (third to seventh methylenes in octyl chains, -O-CH ₂ -CH ₂ -(CH ₂) ₅ -CH ₃)
1.03-0.74	m (6H)	-CH ₃ of octyl chains (-O-CH ₂ -CH ₂ -(CH ₂) ₅ -CH ₃)

Table 10: ¹³C NMR data of Compound **38** in CDCl₃

Chemical Shift (δ , ppm)	Assignment
167.88	Carbonyl carbons (C=O of ester groups)
132.46	Aromatic carbons (C-1, C-2, ipso to ester groups)
131.06	Aromatic carbon (C-4)
130.17	Aromatic carbon (C-5)
130.05	Aromatic carbon (C-6)
129.87	Aromatic carbon (C-3)
67.72	-CH ₂ - of octyl ester groups (-O-CH ₂ -CH ₂ -(CH ₂) ₅ -CH ₃)
30.72	-CH ₂ - (second methylene in octyl chains)
29.84, 29.47, 29.30, 29.84	-CH ₂ - (third to sixth methylenes in octyl chains)
22.83	-CH ₂ - (seventh methylene in octyl chains)
14.26	-CH ₃ of octyl chains

Table 11: ¹H NMR data of Compound **39** in CDCl₃

Chemical Shift (δ , ppm)	Multiplicity (Integration)	Assignment
5.37-5.26	m (1H)	Olefinic proton (H-12, C=C in oleanolic acid skeleton)
4.49-4.29	m (5H)	Sugar protons (anomeric H-1' of glucose ~4.4 ppm, H-2' to H-5' ~3.5-4.5 ppm)
3.8-3.5	m (2H)	-CH ₂ - of glucose C-6' (-CH ₂ OH)
2.37-2.20	m (3H)	Aliphatic protons near functional groups (H-18, H-9 in oleanolic acid)
2.01	qd, J = 15.9, 9.4 Hz (4H)	Aliphatic -CH ₂ - protons (H-1, H-2 near C-3 glycosidic linkage)
1.76-1.49	m (3H)	Aliphatic -CH ₂ - and -CH- protons in the triterpenoid skeleton
1.49-1.15	m (21H)	Aliphatic -CH ₂ - and -CH- protons (bulk of triterpenoid skeleton)
1.03-0.74	m (8H)	Methyl protons (-CH ₃ groups, C-23, C-24, C-25, C-26, C-27, C-29, C-30)

Table 12: ^{13}C NMR data of Compound **39** in CDCl_3

Chemical Shift (δ , ppm)	Assignment
178.0	Carboxylic acid carbon (C-28, -COOH)
140.42	Olefinic carbon (C-13, C=C in oleanolic acid)
122.32	Olefinic carbon (C-12, C=C in oleanolic acid)
104.08	Anomeric carbon (C-1' of β -D-glucose)
79.72	Sugar carbon (C-3' of glucose)
77.48	Sugar carbon (C-5' of glucose)
76.84	Sugar carbon (C-2' of glucose)
74.11	Sugar carbon (C-4' of glucose)
63.33	Sugar carbon (C-6' of glucose, -CH ₂ OH)
56.90	Quaternary carbon (C-17 in oleanolic acid)
50.32	Methine carbon (C-9)
45.98	Quaternary carbon (C-14)
42.47	Quaternary carbon (C-8)
39.90	Methine carbon (C-18)
37.41	Methylene carbon (C-1)
34.09	Methylene carbon (C-7)
32.07	Methylene carbon (C-22)
29.84	Methylene carbon (C-21)
23.22	Methyl carbon (C-23)
19.96	Methyl carbon (C-24)
12.12	Methyl carbon (C-25)

Table 13: ^1H (400 MHz), ^{13}C (100 MHz) NMR spectral data of Compound **38**, and ^1H , ^{13}C NMR values of Dioctyl Phthalate in literature.

C. No.	Compound 38		Dioctyl Phthalate (Sasikala et al., 2017)	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
Aromatic Carbons				
1,2	-	132.46	-	132.5
3,6	7.73 (m)	130.17	7.70 (dd, J=7.5, 1.5 Hz)	130.0
4,5	7.54 (m)	129.87	7.50 (dd, J=7.5, 1.5 Hz)	128.5
Carbonyl Carbons				
7,8	-	167.88	-	167.5
Octyl Chains				
9,9'	4.28 (t, J=6.7 Hz)	65.73	4.20 (m)	65.0
10,10'	1.75 (m)	39.03	1.70 (m)	38.5
11-15, 11'-15'	1.49-1.15 (m), 1.76-1.49 (m)	30.72-22.83	1.30-1.60 (m)	30.0-23.0
16,16'	0.88 (t, J=6.8 Hz)	14.26	0.90 (t, J=7.0 Hz)	14.0
17,17'	0.86 (t, J=7.0 Hz)	12.12	0.85 (t, J=7.0 Hz)	11.0

Table 14: ^1H (400 MHz), ^{13}C (100 MHz) NMR spectral data of Compound **39**, and ^1H , ^{13}C NMR values of Oleanolic Acid 3-O- β -D-Glucoside in literature.

C. No.	Compound 39		Oleanolic Acid 3-O- β -D-Glucoside (Tanaka et al., 2018)	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
1	-	38.7	-	38.5
2	-	26.2	-	26.0
3	-	88.1	-	88.0
4	-	39.2	-	39.0
5	-	55.3	-	55.0
6	-	18.2	-	18.0
7	-	33.7	-	33.5
8	-	40.1	-	40.0
9	-	48.2	-	48.0
10	-	37.6	-	37.5
11	-	23.1	-	23.0
12	5.26 (t, J=3.5 Hz)	122.2	5.25 (t, J=3.5 Hz)	122.0
13	-	144.1	-	144.0
14	-	42.6	-	42.5
15	-	28.1	-	28.0
16	-	23.6	-	23.5
17	-	46.2	-	46.0
18	-	41.7	-	41.5
19	-	46.6	-	46.5
20	-	30.7	-	30.5
21	-	34.6	-	34.5
22	-	33.2	-	33.0
23	1.12 (s)	28.6	1.10 (s)	28.5
24	0.96 (s)	16.7	0.95 (s)	16.5
25	0.76 (s)	15.2	0.75 (s)	15.0
26	0.99 (s)	17.1	0.98 (s)	17.0
27	1.13 (s)	26.6	1.12 (s)	26.5
28	-	180.6	-	180.5
29	0.91 (s)	33.1	0.90 (s)	33.0
30	0.93 (s)	23.6	0.92 (s)	23.5
Glucose Moiety				
1'	4.36 (d, J=7.5 Hz)	105.2	3.15-3.35 (m)	75.0
2'	3.18 (m)	75.1	3.15-3.35 (m)	78.5
3'	3.28 (m)	78.6	3.15-3.35 (m)	71.0
4'	3.22 (m)	71.2	3.15-3.35 (m)	78.0
5'	3.30 (m)	78.1	3.60 (dd, J=11.0, 5.0	62.0
			Hz), 3.80 (dd, J=11.0, 2.0 Hz)	
6'	3.62 (dd, J=11.0, 5.0 Hz), 3.82 (dd, J=11.0, 2.0 Hz)	62.1	3.15-3.35 (m)	75.0

4.5. Antibacterial activity

4.5.1. Antibacterial activity of extracts of roots of *C. africana*

Extracts of *C. africana* exhibited antibacterial activity against the tested strains. Among the extracts, the methanolic (MeOH) extract at a concentration of 200 mg/ml demonstrated the highest inhibitory activity, with zones of inhibition recorded as follows: *S. pyogenes* (18.50 ± 1.50 mm) (Figure 13D), *S. aureus* (18.00 ± 1.00 mm), *E. coli* (17.50 ± 0.50 mm), and *P. aeruginosa* (17.00 ± 1.00 mm). Notably, the MeOH extract at 200 mg/ml exhibited antibacterial activity against *S. pyogenes* (18.50 ± 1.50 mm) comparable to the positive control, ciprofloxacin (0.25 mg/ml), which showed an inhibition zone of 19.00 ± 1.00 mm. Similarly, an extract prepared using DCM:EtOH (1:1) also displayed significant activity against *E. coli* (14.00 ± 3.00 mm), which was comparable to the positive control ciprofloxacin (15.50 ± 1.50 mm) (Table 15).

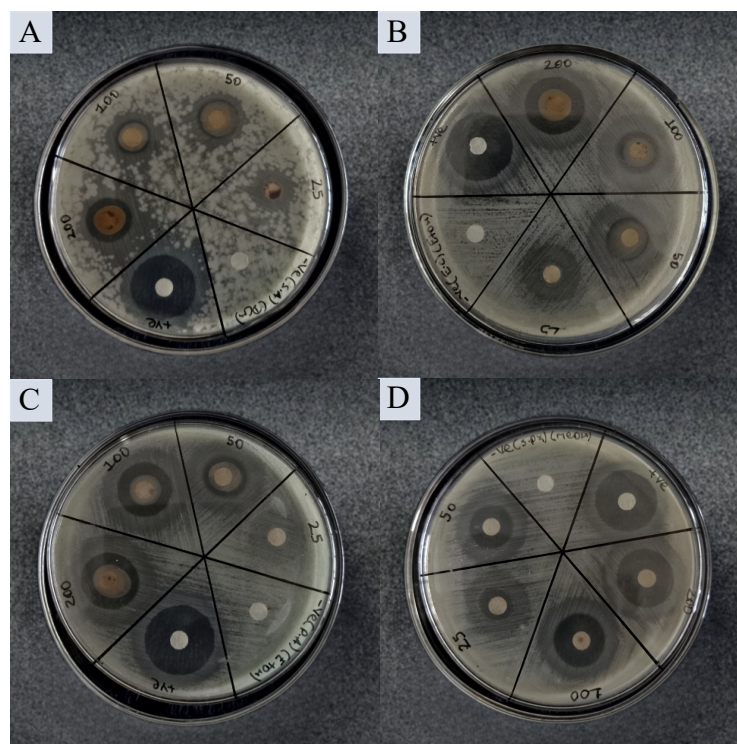


Figure 13: Antibacterial activity of extracts from *C. africana* roots. (A) DCM:EtOH (1:1) extract against *S. aureus*; EtOH extract against (B) *E. coli* and (C) *P. aeruginosa*; (D) MeOH extract against *S. pyogenes*

All extracts demonstrated dose-dependent antibacterial activity, with higher concentrations (200 mg/ml) consistently resulting in more significant inhibition across all tested strains ($p < 0.05$). However, there were no statistically significant differences in the antibacterial activity of the various extracts against the test strains ($p > 0.05$) (Appendix 13-19). The antibacterial activities observed are attributed to bioactive phytochemicals, including alkaloids, flavonoids, and tannins (Table 1). These compounds possess unique chemical structures that enable them to interact with microbial cells through various mechanisms, including cell wall disruption, enzyme inhibition, and membrane disruption (Othman et al., 2019).

Table 15: Antibacterial activity (inhibition zone, mean $\pm$ SD in mm) of extracts against test strains.

Plant extract	Conc. (mg/ml)	Inhibition Value (mm)			
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. pyogenes</i>
DCM:EtOH (1:1)	200	14.00 $\pm$ 3.00 ^{cd}	13.50 $\pm$ 0.50 ^{cdef}	14.50 $\pm$ 1.50 ^{def}	15.00 $\pm$ 2.00 ^{cd}
	100	10.50 $\pm$ 1.50 ^{bc}	11.50 $\pm$ 1.50 ^{bcde}	11.50 $\pm$ 1.50 ^{bcd}	11.50 $\pm$ 0.50 ^{bc}
	50	08.50 $\pm$ 0.50 ^{ab}	10.50 $\pm$ 0.50 ^{bcd}	10.00 $\pm$ 1.00 ^{abc}	10.00 $\pm$ 0.00 ^{ab}
	25	05.00 $\pm$ 0.00 ^a	07.50 $\pm$ 0.50 ^{ab}	07.50 $\pm$ 0.50 ^{ab}	07.00 $\pm$ 0.00 ^a
	Cipro.	15.50 $\pm$ 1.50 ^d	17.00 $\pm$ 2.00 ^f	18.50 $\pm$ 1.50 ^g	18.00 $\pm$ 3.00 ^d
	200	16.00 $\pm$ 1.00 ^d	15.00 $\pm$ 2.00 ^{ef}	17.00 $\pm$ 2.00 ^{efg}	17.00 $\pm$ 0.00 ^d
EtOH	100	15.00 $\pm$ 1.00 ^{cd}	14.00 $\pm$ 1.00 ^{def}	13.50 $\pm$ 0.50 ^{cde}	15.50 $\pm$ 0.50 ^d
	50	11.00 $\pm$ 1.00 ^{bc}	10.50 $\pm$ 0.50 ^{bcd}	11.50 $\pm$ 0.50 ^{bcd}	11.00 $\pm$ 2.00 ^b
	25	05.00 $\pm$ 0.00 ^a	05.50 $\pm$ 0.50 ^a	06.00 $\pm$ 1.00 ^a	06.50 $\pm$ 1.50 ^a
	Cipro.	20.00 $\pm$ 1.00 ^d	16.00 $\pm$ 2.00 ^f	19.00 $\pm$ 3.00 ^g	18.50 $\pm$ 1.50 ^d
	200	17.50 $\pm$ 0.50 ^d	17.00 $\pm$ 1.00 ^f	18.00 $\pm$ 1.00 ^{fg}	18.50 $\pm$ 1.50 ^d
MeOH	100	14.00 $\pm$ 1.00 ^{cd}	14.50 $\pm$ 1.50 ^{def}	15.00 $\pm$ 0.00 ^{defg}	16.00 $\pm$ 0.00 ^d
	50	09.00 $\pm$ 3.00 ^{ab}	09.50 $\pm$ 1.50 ^{abc}	09.00 $\pm$ 2.00 ^{ab}	08.50 $\pm$ 0.50 ^{ab}
	25	08.50 $\pm$ 1.50 ^{ab}	08.50 $\pm$ 3.50 ^{ab}	07.50 $\pm$ 2.50 ^{ab}	07.00 $\pm$ 2.00 ^a
	Cipro.	18.50 $\pm$ 0.50 ^d	18.00 $\pm$ 0.00 ^f	18.50 $\pm$ 0.50 ^g	19.00 $\pm$ 1.00 ^d
	200	18.50 $\pm$ 0.50 ^d	18.00 $\pm$ 0.00 ^f	18.50 $\pm$ 0.50 ^g	19.00 $\pm$ 1.00 ^d

^{abc}; statistical differences according Tukey HSD test, **Cipro.**; ciprofloxacin

El et al. (2015) studied the antimicrobial activity of extracts from various parts of *C. africana*, including bark, fruits, leaves, and stems. The extracts were prepared using different solvents: chloroform, ethyl acetate, methanol (MeOH), water, and petroleum ether (pet ether). These were tested against the bacterial strains *E. coli*, *P. aeruginosa*, and *S. aureus*. The findings revealed that methanolic extracts from the bark and stem exhibited significant antibacterial activity against *S. aureus*, with inhibition zones measuring 18 mm for both. This aligns with the results of the current study, supporting the effectiveness of methanol as a solvent for extracting bioactive compounds targeting *S. aureus*. On the other hand, extracts from the fruits and leaves demonstrated higher activity against *E. coli*, with inhibition zones of 19 mm and 17 mm, respectively.

4.5.2. Antibacterial activity of essential oil and isolated compounds from roots of *C. africana*

Compounds isolated from DCM:EtOH (1:1) extract and the essential oil of *C. africana* roots demonstrated antibacterial activity against all tested strains across all concentrations. Among the samples, the essential oil exhibited the highest activity, particularly against *S. pyogenes*, *S. aureus*, *E. coli*, and *P. aeruginosa* at 10 mg/mL, with inhibition zones of 16.33 ± 1.53 , 16.00 ± 1.00 , 15.67 ± 1.15 , and 15.00 ± 2.00 mm, respectively. Conversely, the lowest activity was observed for mixture of Compound **38** and **39** against *P. aeruginosa* at 2.5 mg/mL (8.33 ± 1.53 mm).

Among all test strains, *S. pyogenes* was the most susceptible to the essential oil (16.33 ± 1.53 mm at 10 mg/mL), mixture of Compound **38** and **39** (14.00 ± 1.00 mm at 20 mg/mL), and mixture of Compound **32** and **37** (13.33 ± 1.53 mm at 20 mg/mL) (Table 16). This indicates that the extracts and essential oil are more effective against Gram-positive bacterial strains than Gram-negative ones, with all activities being dose- and strain-dependent. Higher antibacterial activities were observed at 20 mg/mL concentrations for the isolated compounds and 10 mg/mL for the essential oil.

Table 16: Antibacterial activity (inhibition zone, mean $\pm$ SD in mm) of essential oil and isolated compounds of the root of *C. africana* against test strains.

Plant extract	Conc. (mg/ml)	Inhibition Value (mm)			
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. pyogenes</i>
Compound 32 and 37 mixture	20	13.00 $\pm$ 1.00 ^{abcd}	12.33 $\pm$ 1.53 ^{abcd}	13.50 $\pm$ 1.50 ^{abc}	14.00 $\pm$ 1.00 ^{abc}
	10	12.67 $\pm$ 2.08 ^{abcd}	11.33 $\pm$ 1.53 ^{abcd}	12.00 $\pm$ 1.00 ^{abc}	13.33 $\pm$ 1.53 ^{abc}
	5	12.00 $\pm$ 1.00 ^{abcd}	10.33 $\pm$ 1.53 ^{abc}	11.33 $\pm$ 1.53 ^{ab}	11.67 $\pm$ 2.08 ^{ab}
	2.5	11.33 $\pm$ 1.53 ^{abc}	09.00 $\pm$ 1.00 ^{ab}	10.67 $\pm$ 1.53 ^a	10.33 $\pm$ 1.53 ^a
	Cipro.	19.00 $\pm$ 1.00 ^e	18.67 $\pm$ 1.53 ^e	20.67 $\pm$ 1.53 ^d	21.00 $\pm$ 2.00 ^d
Compound 38 and 39 mixture	20	12.33 $\pm$ 1.53 ^{abcd}	11.33 $\pm$ 1.53 ^{abcd}	13.00 $\pm$ 1.00 ^{abc}	13.33 $\pm$ 1.53 ^{abc}
	10	11.00 $\pm$ 2.00 ^{abc}	10.33 $\pm$ 1.53 ^{abc}	12.33 $\pm$ 1.53 ^{abc}	12.33 $\pm$ 1.53 ^{abc}
	5	10.67 $\pm$ 1.53 ^{ab}	09.00 $\pm$ 2.00 ^{ab}	11.00 $\pm$ 2.00 ^{ab}	11.00 $\pm$ 2.00 ^{ab}
	2.5	09.00 $\pm$ 1.00 ^a	08.33 $\pm$ 1.53 ^a	10.33 $\pm$ 1.53 ^a	10.00 $\pm$ 2.00 ^a
	Cipro.	18.33 $\pm$ 1.53 ^e	18.00 $\pm$ 2.00 ^e	20.00 $\pm$ 2.00 ^d	21.00 $\pm$ 1.00 ^d
EO	10	15.67 $\pm$ 1.15 ^{de}	15.00 $\pm$ 2.00 ^{de}	16.00 $\pm$ 1.00 ^{cd}	16.33 $\pm$ 1.53 ^c
	5	15.00 $\pm$ 2.00 ^{cde}	14.00 $\pm$ 2.00 ^{cde}	15.00 $\pm$ 2.00 ^{bc}	14.67 $\pm$ 1.53 ^{bc}
	2.5	14.33 $\pm$ 1.53 ^{bcd}	13.33 $\pm$ 1.53 ^{bcd}	13.67 $\pm$ 1.53 ^{abc}	14.00 $\pm$ 1.00 ^{abc}
	1.25	13.67 $\pm$ 1.53 ^{bcd}	12.33 $\pm$ 1.53 ^{abcd}	13.00 $\pm$ 1.00 ^{abc}	13.00 $\pm$ 1.00 ^{abc}
	Cipro.	18.67 $\pm$ 1.53 ^e	18.33 $\pm$ 1.53 ^e	19.33 $\pm$ 1.53 ^d	20.33 $\pm$ 1.53 ^d

The antibacterial activity of the essential oil is attributed to its primary component, 2,4-Di-tert-butylphenol (2,4-DTBP) (Scheme 7), which has been reported to exhibit bioactivity against multidrug-resistant bacteria such as *P. aeruginosa* and *S. aureus* (Zhao et al., 2020). On the other hand, the antibacterial activity of the isolated compounds is linked to their chemical structures, which facilitate effective interactions with microbial cells. Phenolic compounds like 4-Methoxyphenol are well-known for their antibacterial properties. The phenolic -OH group can disrupt bacterial cell membranes, denature proteins, and interfere with bacterial enzymes. The electron-donating methoxy group at the para position may enhance the electron density on the ring, potentially increasing the -OH group's ability to interact with bacterial targets (Kim et al., 2014) while the amphiphilic nature of Oleanolic Acid 3-O- β -D-Glucoside-combining a hydrophobic triterpenoid core with a hydrophilic sugar unit-allows it to interact with lipid bilayers, potentially causing membrane permeabilization and cell lysis (Kurek et al., 2012).

4.5.3. Minimum inhibitory and bactericidal concentration of extracts, essential oil, and isolated compounds

In this study, the essential oil had the best antibacterial activity based on its minimal bactericidal concentrations (5-10 mg/mL) and minimal inhibitory concentration (2.5-5 mg/mL). It was the most active against *S. aureus* and *S. pyogenes*, with an MIC value of 2.5 mg/mL and an MBC value of 5 mg/mL, which indicates strong inhibition and bactericidal activity. Similarly, the isolated compounds showed higher activity against *S. aureus* and *S. pyogenes*, with MIC of 5 mg/mL and MBC of 10 mg/mL having the same effectiveness. EtOH and MeOH extracts also had strong antibacterial activity with a minimum inhibitory concentration of 3.125 mg/mL towards *S. aureus* and *S. pyogenes* (Table 17). This indicates that the Gram-positives were highly sensitive to the antibacterial activities of the extracts.

On the other hand, the semi-polar DCM:EtOH (1:1) was compared to the other extracts with MIC and MBC values between 6-25 mg/mL for all the strains. The polar solvents are active in extracting the bioactive compound from the roots of *C. africana*. This is in good agreement with the study done by Miguel et al. (2016) on the antimicrobial activity of essential oil of *Cordia globosa*, which showed a minimum inhibitory concentration of 1.000 and 0.750 mg/mL against *S. aureus* and *P. aeruginosa* similarly indicating Gram-positives are sensitive to the essential oil of both *C. africana* and *C. globosa* than that of the Gram-negatives.

Table 17: MIC and MBC of extracts, essential oil, and isolated compounds against test strains

Extracts	MIC and MBC (mg/mL)							
	<i>E. coli</i>		<i>P. aeruginosa</i>		<i>S. aureus</i>		<i>S. pyogenes</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
DCM:EtOH (1:1)	12.5	25	12.5	25	6.25	12.5	6.25	12.5
EtOH	6.25	12.5	6.25	12.5	3.125	6.25	3.125	6.25
MeOH	6.25	12.5	6.25	12.5	3.125	6.25	3.125	6.25
Compound 32 and 37 mixture	10	20	10	20	5	10	5	10
Compound 38 and 39 mixture	10	20	10	20	5	10	5	10
EO	5	10	5	10	2.5	5	2.5	5

4.6. Antioxidant activity

4.6.1. Antioxidant activity of extracts of roots of *C. africana*

In this study, the *in vitro* antioxidant activity tests showed the extracts exhibited strong scavenging potential ($IC_{50} < 50 \mu\text{g/mL}$). Although the positive control ascorbic acid showed an IC_{50} value of $0.28 \mu\text{g/mL}$, the MeOH extract showed a promising activity with an IC_{50} value of $5.52 \mu\text{g/mL}$, followed by EtOH with an IC_{50} value of $12.71 \mu\text{g/mL}$ and DCM:EtOH (1:1) with an IC_{50} value of $18.09 \mu\text{g/mL}$ (Figure 14, Table 18) indicating as polarity increases; their antioxidant is also increased this is due to the presence of the phytochemicals Phenols, flavonoids, and tannins which are known for their more significant antioxidant activity (Formagio et al., 2014).

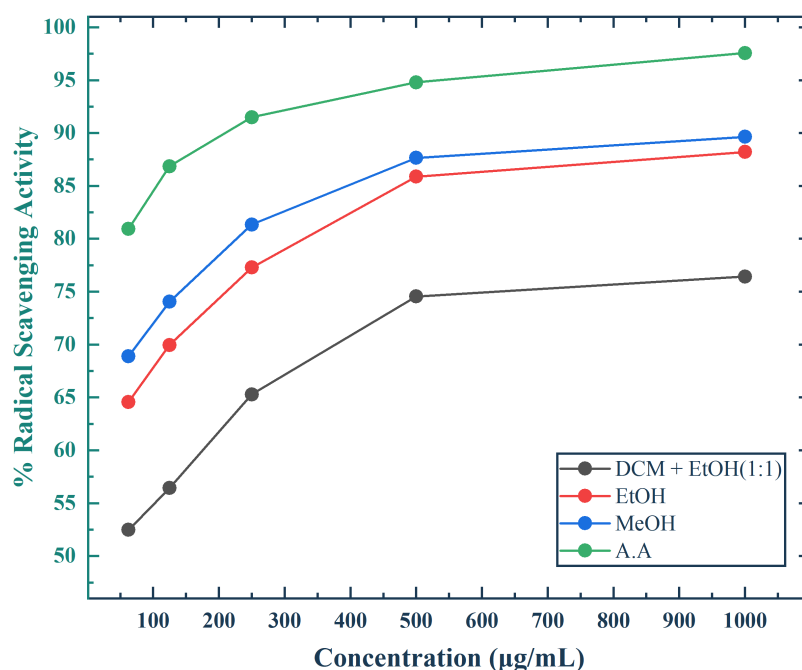


Figure 14: DPPH % radical scavenging activity of extracts against the control ascorbic acid

The results agree with previous findings regarding the antioxidant activities of the plants' methanolic extracts. Isa and his group studied the antioxidant potentials of the methanol and hexane extract of bark of *C. africana* using a DPPH radical scavenging assay whose methanol extract exhibited the highest scavenging activity with IC_{50} value of $6.79 \mu\text{g/mL}$ (Isa et al., 2014). The results also agree with the study done in Sudan, where the methanolic extracts of the leaves and stem of *C. africana* gave an antioxidant activity of 80 and 88 %RSA (Alhadi et al., 2015).

Table 18: DPPH % radical scavenging activity and average absorbance of extracts of *C. africana*

Concentration ($\mu\text{g/mL}$)	-ve control	Extracts						+ve	
		DCM:EtOH (1:1)		EtOH		MeOH		Control	
		Abs.	%RSA	Abs.	%RSA	Abs.	%RSA	Abs.	%RSA
500	1.009	0.146	86.43	0.128	88.21	0.114	89.63	0.034	97.56
250	1.009	0.165	84.55	0.152	85.87	0.134	87.65	0.062	94.79
125	1.009	0.258	75.3	0.238	77.28	0.197	81.34	0.095	91.48
62.5	1.009	0.348	66.44	0.312	69.95	0.271	74.04	0.142	86.86
31.25	1.009	0.388	62.45	0.367	64.56	0.323	68.89	0.201	80.95
IC ₅₀		18.09		12.71		5.52		0.28	

Abs.; absorbance, %RSA; percent radical scavenging activity, -ve; negative, +ve; positive

4.6.2. Antioxidant activity of essential oil and isolated compounds from roots of *C. africana*

In this study, the essential oil and isolated compounds demonstrated antioxidant activity, with the essential oil exhibiting the most significant effect.

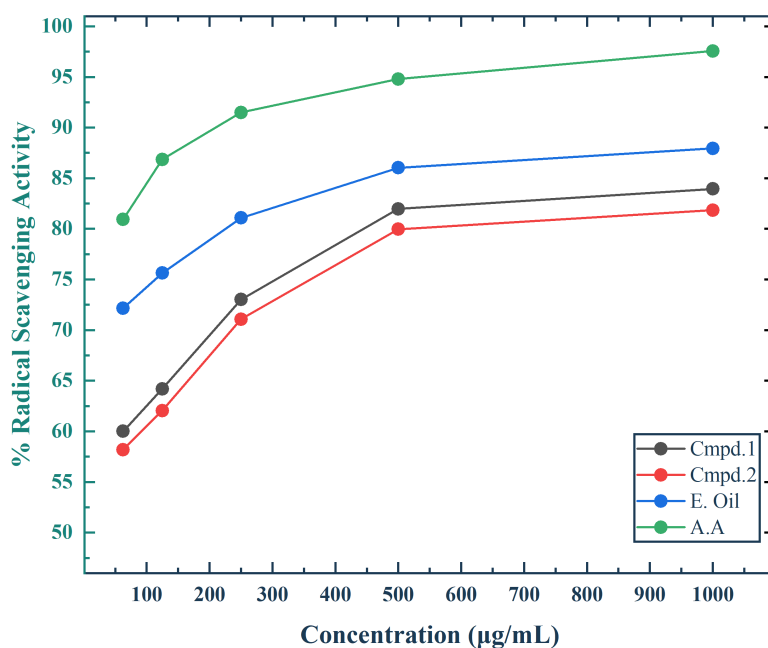


Figure 15: DPPH % radical scavenging activity of the compounds isolated from DCM:EtOH (1:1) extracts and essential oil of roots of *C. africana* against the control ascorbic acid

The essential oil achieved an IC₅₀ value of 1.61 µg/mL, notably close to the positive control, ascorbic acid, with an IC₅₀ value of 0.28 µg/mL (Table 19). Among the isolated compounds, mixture of Compound **32** and **37** showed an IC₅₀ value of 22.94 µg/mL, while mixture of Compound **38** and **39** exhibited a similar antioxidant activity with an IC₅₀ value of 27.95 µg/mL (Figure 15).

The highest antioxidant activity of the essential oil is likely attributed to its primary component, 2,4-DTBP, which has been reported to possess an IC₅₀ value of 8.2 µg/mL (Zhao et al., 2020). Phenolic compounds are well-known antioxidants because the -OH group can donate a hydrogen atom to free radicals, forming a stable phenoxyl radical that is resonance-stabilized by the aromatic ring. The methoxy group at the para position in 4-Methoxyphenol is electron-donating, which can enhance the stability of the phenoxyl radical, thereby increasing its antioxidant capacity (Kim et al., 2014). While The hydroxyl groups on the glucose moiety can donate hydrogen atoms to neutralize reactive oxygen species (ROS), a mechanism supported by the FTIR O-H stretch at 3400 cm⁻¹. Additionally, the carboxylic acid group may contribute to radical stabilization. Oleanolic acid and its glycosides exhibit antioxidant effects due to their phenolic-like behavior and ability to chelate metal ions that catalyze oxidative reactions. The presence of the sugar unit may also enhance solubility in aqueous environments, improving its antioxidant activity in biological systems (Kurek et al., 2012).

Table 19: DPPH % radical scavenging activity and average absorbance of compounds isolated from DCM:EtOH (1:1) extracts and essential oil of *C. africana*

Concentration (µg/mL)	-ve							+ve	
	control	Compound 32 and 37		Compound 38 and 39		EO		Control	
	Abs.	Abs.	%RSA	Abs.	%RSA	Abs.	%RSA	Ascorbic acid	
500	1.009	0.171	83.95	0.192	81.84	0.131	87.95	0.034	97.56
250	1.009	0.191	81.97	0.211	79.96	0.15	86.03	0.062	94.79
125	1.009	0.281	73.02	0.301	71.07	0.2	81.08	0.095	91.48
62.5	1.009	0.37	64.2	0.392	62.05	0.255	75.63	0.142	86.86
31.25	1.009	0.413	60	0.431	58.18	0.29	72.16	0.201	80.95
	IC₅₀	22.94		27.95		1.61		0.28	

EO; essential oil

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

This study investigated biological activities, phytochemistry, and characterization of essential oil and isolated compounds from the roots of *C. africana*. The phytochemical studies indicated the presence of alkaloids, flavonoids, steroids, and tannins, which have been known to exhibit significant antimicrobial and antioxidant activities. The three extracts were tested for their antibacterial activity against the test bacterial strains, and the methanolic extract showed the highest activity, demonstrating inhibition zones of 18.50 ± 1.50 , 18.00 ± 1.00 , 17.50 ± 0.50 , and 17.00 ± 1.00 mm against *S. pyogenes*, *S. aureus*, *E. coli*, and *P. aeruginosa* respectively. The ethanolic extract was also active against the test bacterial strains, especially *S. pyogenes* (17.00 ± 0.00 mm), at 200 mg/mL, indicating polar extracts from the plant could be helpful medicinally for treating infectious diseases. Fractionation of the extract led to the isolation of two compounds not previously reported for their biological activities. However, it showed promising antibacterial activity, with mixture of Compound **32** and **37** exhibiting the highest activity against *S. pyogenes* (14.00 ± 1.00 mm) and showing slightly better antioxidant activity with an IC_{50} of 22.94 μ g/mL compared with mixture of Compound **38** and **39** (27.95 μ g/mL), showing mixture of Compound **32** and **37** possesses better antibacterial and antioxidant activity.

GC-MS analysis of the essential oil showed that it contains 29 compounds, some of which have been reported in the literature as having effective antibacterial and antioxidant activities. The essential oil was highly effective against all bacteria tested with mean inhibition zones of 16.33 ± 1.53 , 16.00 ± 1.00 , 15.67 ± 1.15 , 15.00 ± 2.00 mm, against *S. pyogenes*, *S. aureus*, *E. coli*, and *P. aeruginosa* respectively. It also showed a very high antioxidant activity with an IC_{50} value of 1.61 μ g/mL, compared to the positive control, ascorbic acid (0.28 μ g/mL). Suggesting that the essential oil extracted from the roots of *C. africana* is a potential antibacterial and antioxidant agent.

Generally, the study has supported the medicinal potential of *C. africana* for its traditional use in various therapeutic purposes. The outstanding antibacterial and antioxidant properties of the plant's extracts, isolated compounds, and essential oils indicate its potential as a natural source for developing new antibacterial and antioxidant agents.

5.2. Recommendations

This study demonstrated the antibacterial and antioxidant activities of the extracts, essential oil, and pure compounds of the roots of *C. africana*. However, there is a need for further investigation to maximize the extraction process and explore the plant's bioactive potential in-depth, and as a result, the following recommendations are made.

- ❖ By optimizing the extraction methods and employing solvents not used in this study, more extracts can be obtained, which enhances the recovery of more active compounds and increases the prospect of identifying more active antibacterial and antioxidant compounds.
- ❖ Evaluation of more biological activities on extracts, EOs, and isolated compounds.
- ❖ Based on the TLC profile of the extracts, re-extracting and fractionating the plant material may result in compounds with more active antimicrobial and antioxidant activities. Large amounts of starting plant materials should also be utilized to achieve maximum extract yields, which will allow the isolation of sufficient quantities of bioactive compounds for structural analysis and characterization, as the yield from a root sample is small.
- ❖ Isolation of more bioactive compounds using modern chromatographic techniques such as HPLC and elucidation of their structures using 1D and 2D NMR techniques and MS
- ❖ Performing a Drug-Likeness Test to assess the physicochemical and structural attributes of the purified compounds will help to determine the potential of the isolated compounds as oral drugs from their pharmacokinetic profiles, e.g., absorption, distribution, metabolism, and excretion (ADME).
- ❖ Molecular docking studies are also recommended to explore the interaction between the isolated compounds and their target biological molecules, which will help explain the mechanism of action on the test bacterial strains and contribute to the formulation of new drugs.

Based on these recommendations, future studies can take the bioactivity assay of roots of *C. africana* to another level, such as the possibility of identifying new drugs of pharmaceutical interest with antimicrobial and antioxidant activities.

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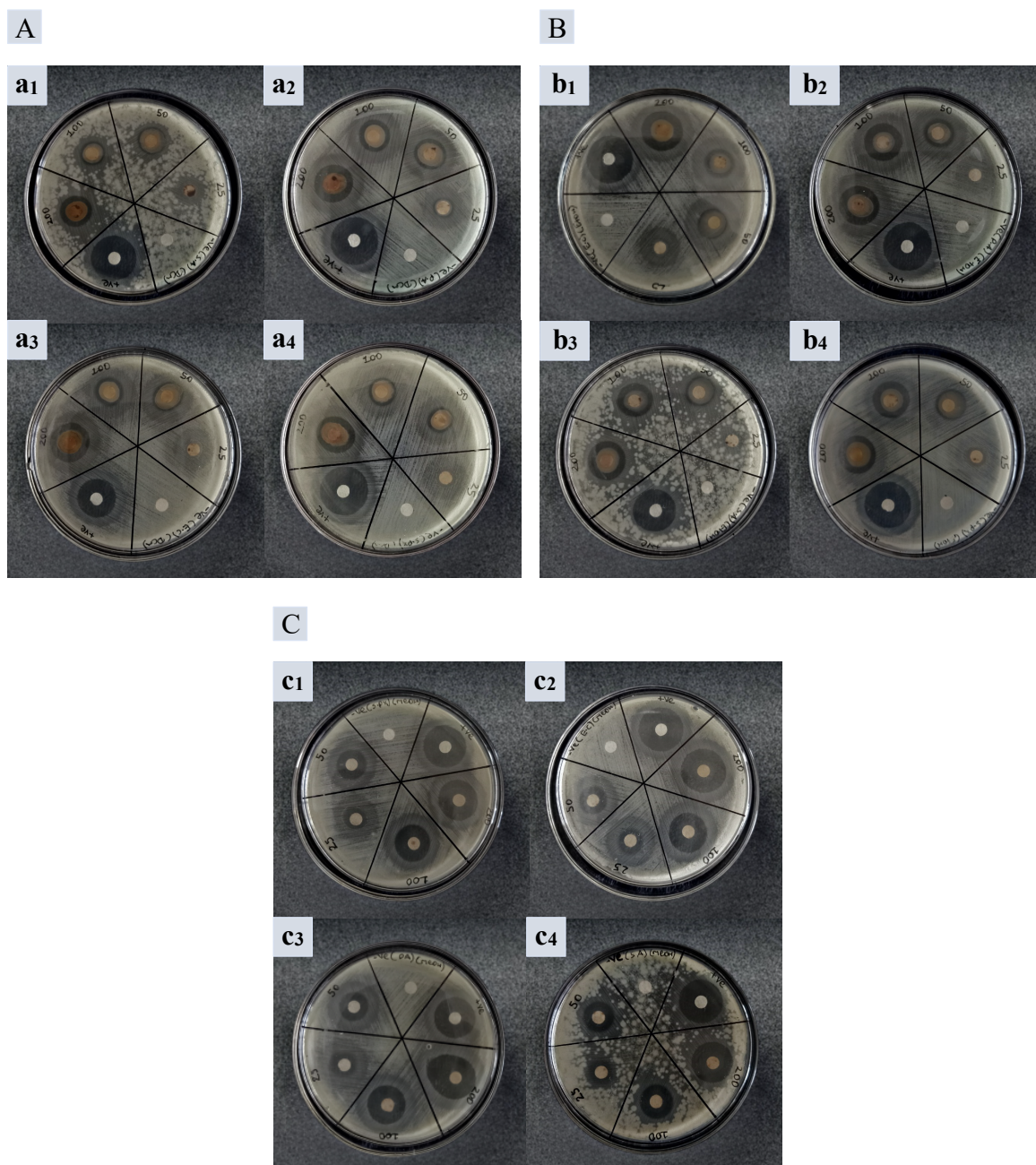
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7. APPENDICES

Appendix 1: Antibacterial activity of extracts against test strains. (A) DCM:EtOH (1:1) extract against; (a₁) *S. aureus*, (a₂) *P. aeruginosa*, (a₃) *E. coli*, (a₄) *S. pyogenes*, (B) EtOH extract against; (b₁) *E. coli*, (b₂) *P. aeruginosa*, (b₃) *S. aureus*, (b₄) *S. pyogenes*, (C) MeOH extract against; (c₁) *S. pyogenes*, (c₂) *E. coli*, (c₃) *P. aeruginosa*, (c₄) *S. aureus*.

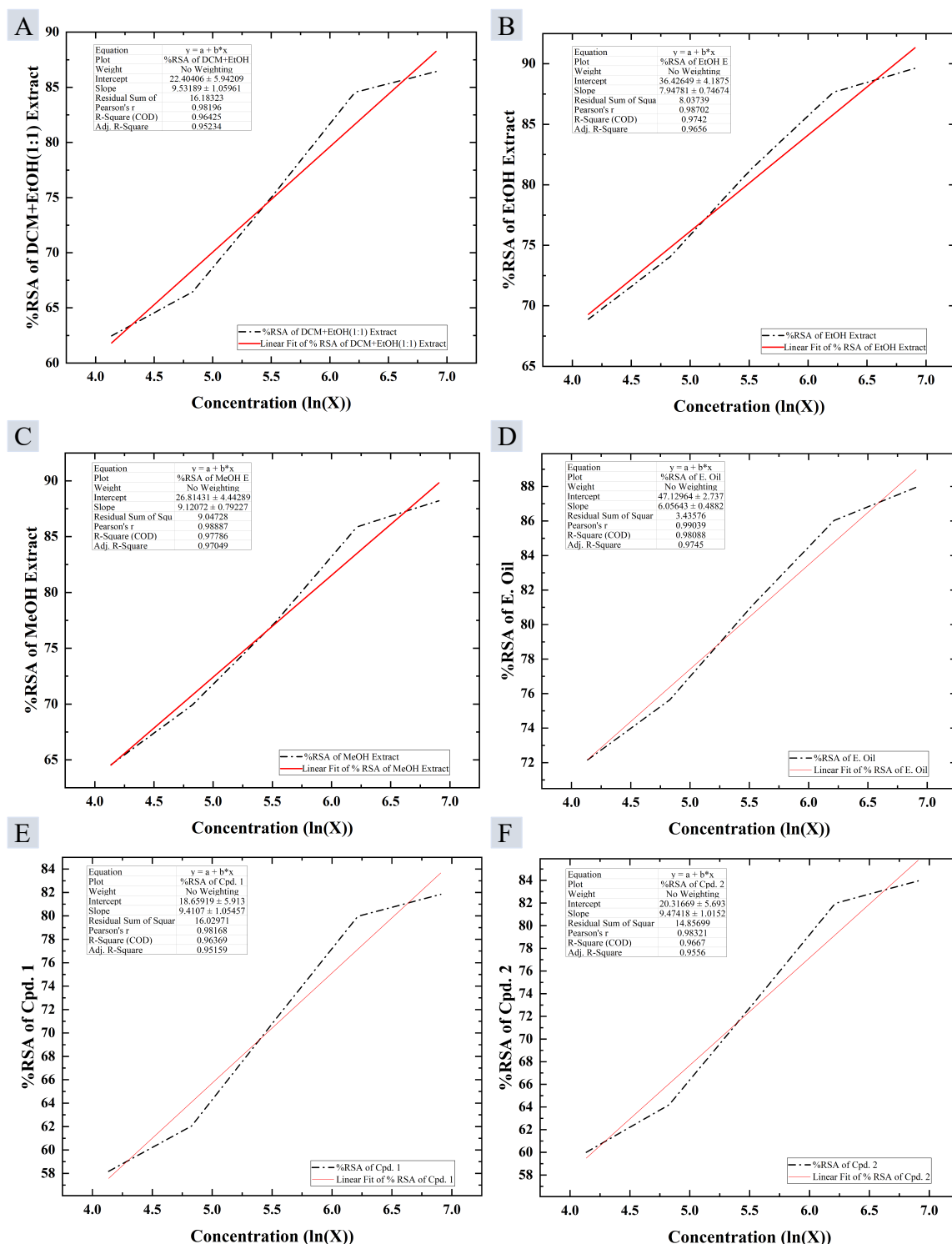


Appendix 2: Phytochemical analysis of extracts. (A) DCM:EtOH (1:1) extract, (B) MeOH extract, (C) EtOH extract

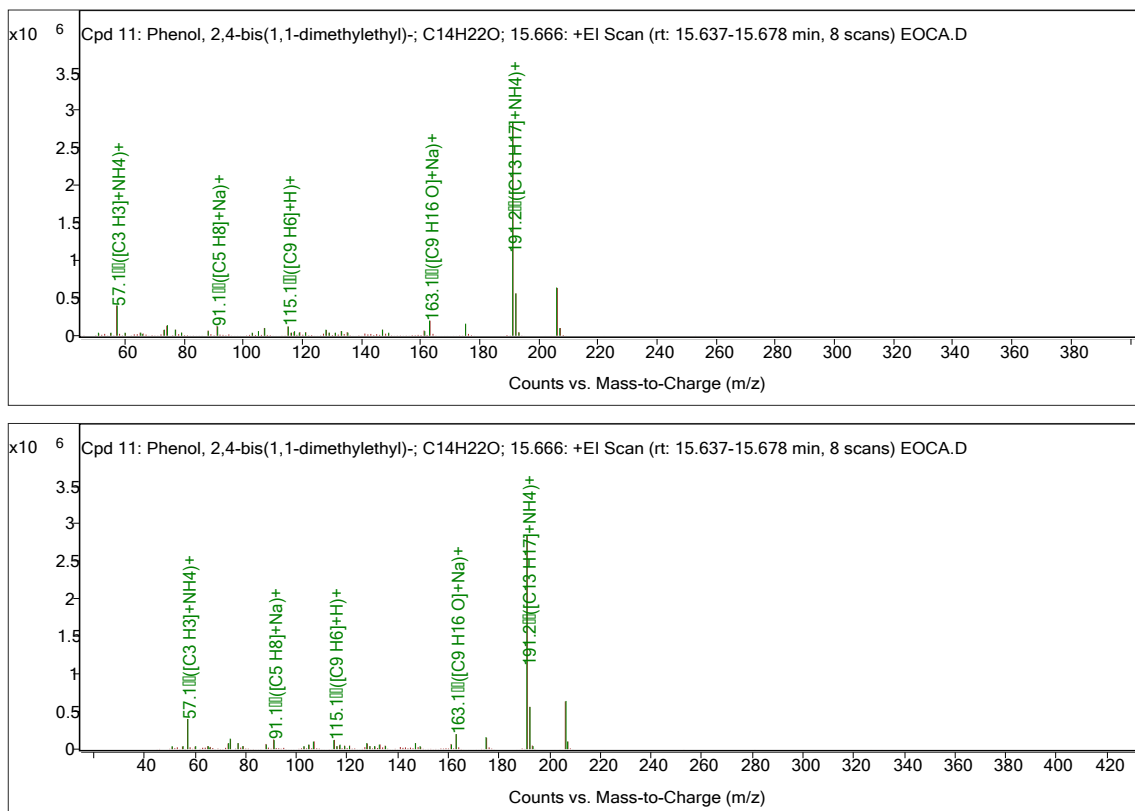


Appendix 3: Linear regression analysis of %RSA to determine IC₅₀ value.

(A) DCM:EtOH (1:1) extract, (B) EtOH extract, (C) MeOH extract, (D) essential oil, (E) mixture of Compound **32** and **37**, (F) mixture of Compound **38** and **39**

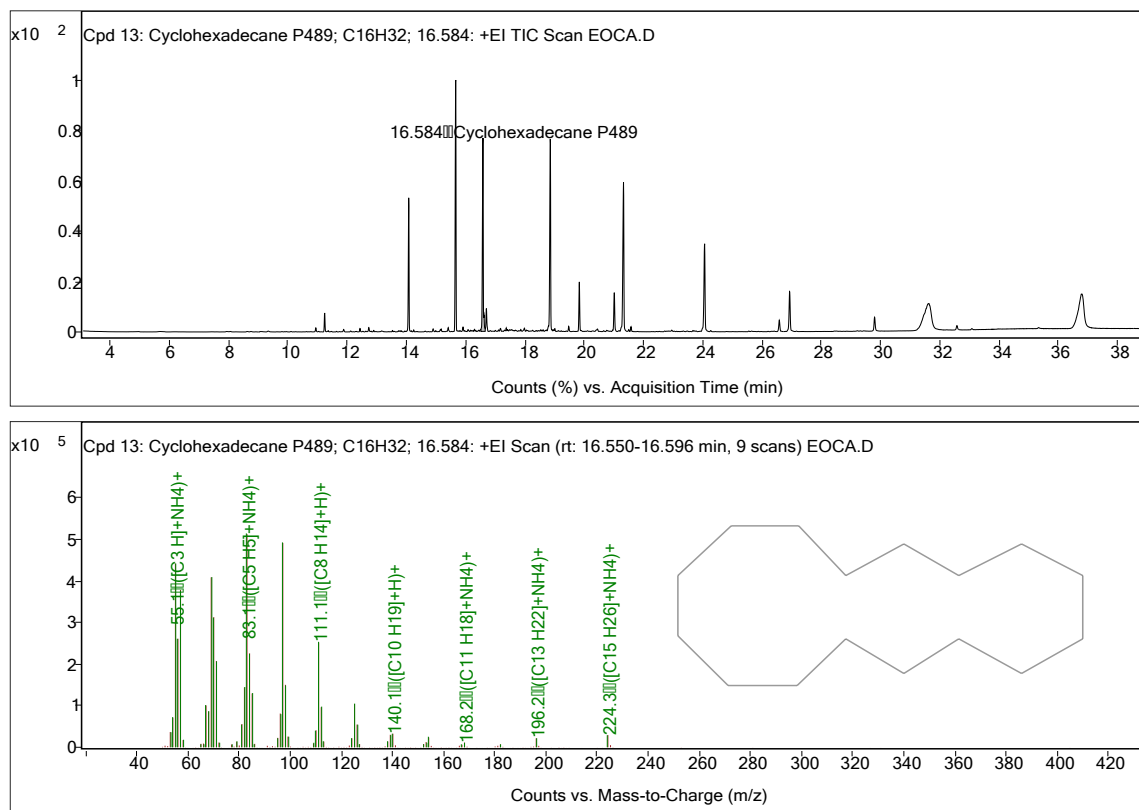


Appendix 4: Mass spectrometry analysis of 2,4-DTBP; TIC and EI scan results



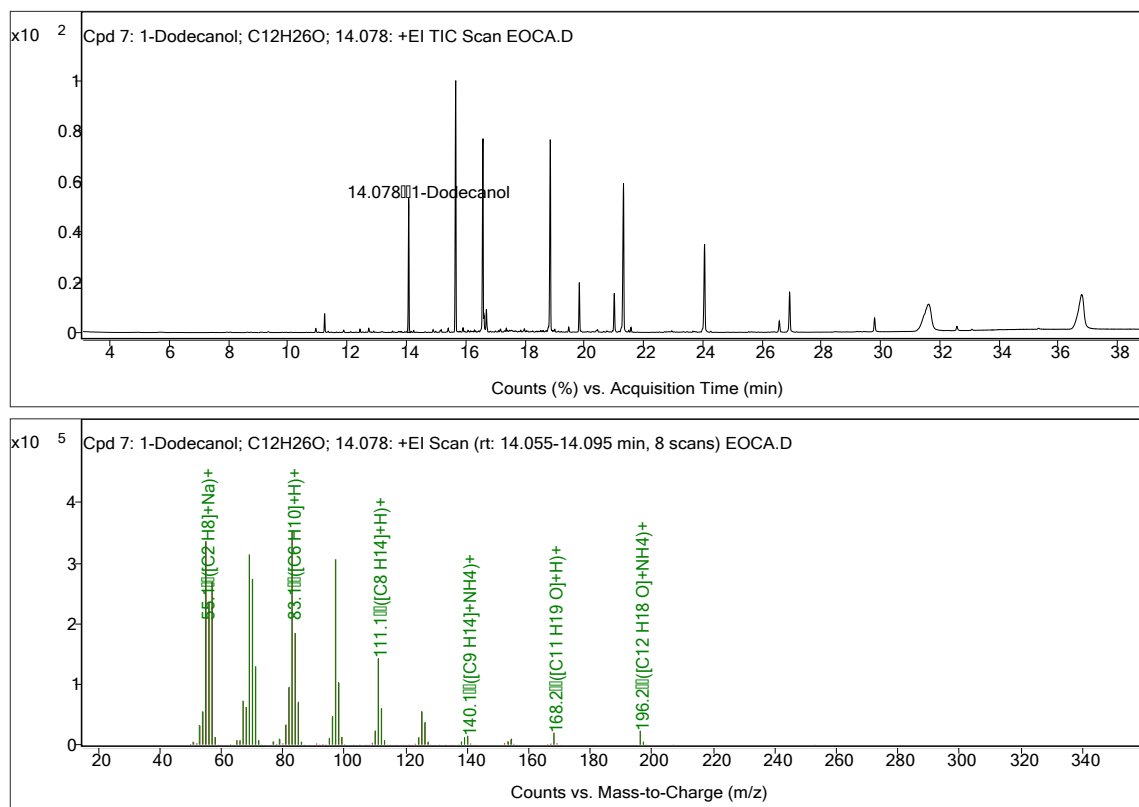
<i>m/z</i>	<i>Calc m/z</i>	Diff(ppm)	<i>z</i>	Abund	Formula	Ion
57.1	39	-316346.77	1	401583.09	C ₃ H ₃	(M+NH ₄) ⁺
74	74.1	270.67		132422.41	C ₄ H ₃	(M+Na) ⁺
91.1	91	-201.51		130241.99	C ₅ H ₈	(M+Na) ⁺
107.1	106	-9422.55	1	103039.49	C ₇ H ₆ O	(M+H) ⁺
115.1	115	-159.48		123674.06	C ₉ H ₆	(M+H) ⁺
163.1	140.1	-140950.48	1	199782.73	C ₉ H ₁₆ O	(M+Na) ⁺
175.1	174.1	-5802.21	1	150524.05	C ₁₂ H ₁₄ O	(M+H) ⁺
191.2	173.1	-94400.69	1	2839315	C ₁₃ H ₁₇	(M+NH ₄) ⁺
192.2	192.2	0.5	1	564296.44	C ₁₃ H ₁₇	(M+NH ₄) ⁺
206.2	188.2	-87418.99	1	635370.88	C ₁₄ H ₂₀	(M+NH ₄) ⁺

Appendix 5: Mass spectrometry analysis of Cyclohexadecane; TIC and EI scan results



<i>m/z</i>	<i>Calc m/z</i>	Diff(ppm)	<i>z</i>	Abund	Formula	Ion
55.1	55	-514.79		441959.88	C ₃ H	(M+NH ₄) ⁺
56.1	56	-366.05		261242.36	C ₃ H ₂	(M+NH ₄) ⁺
57.1	39	-316346.77	1	378498.28	C ₃ H ₃	(M+NH ₄) ⁺
69.1	69.1	-183.86		408872.34	C ₄ H ₃	(M+NH ₄) ⁺
70.1	70.1	-212.25		313196.22	C ₄ H ₄	(M+NH ₄) ⁺
71.1	53	-253923.53	1	207626.05	C ₄ H ₅	(M+NH ₄) ⁺
83.1	83.1	-84.85		512964.16	C ₅ H ₅	(M+NH ₄) ⁺
84.1	84.1	-109.7		226066.36	C ₆ H ₁₁	(M+H) ⁺
97.1	97.1	-14.41		491753.78	C ₆ H ₇	(M+NH ₄) ⁺
111.1	111.1	-51.74		252145	C ₈ H ₁₄	(M+H) ⁺

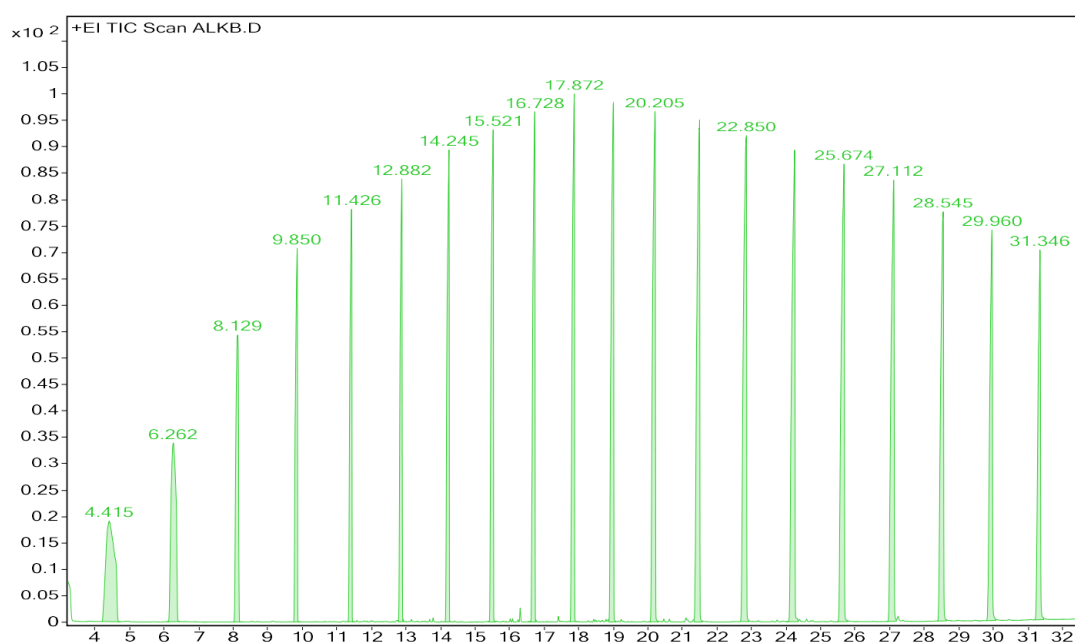
Appendix 6: Mass spectrometry analysis of 1-Dodecanol; TIC and EI scan results



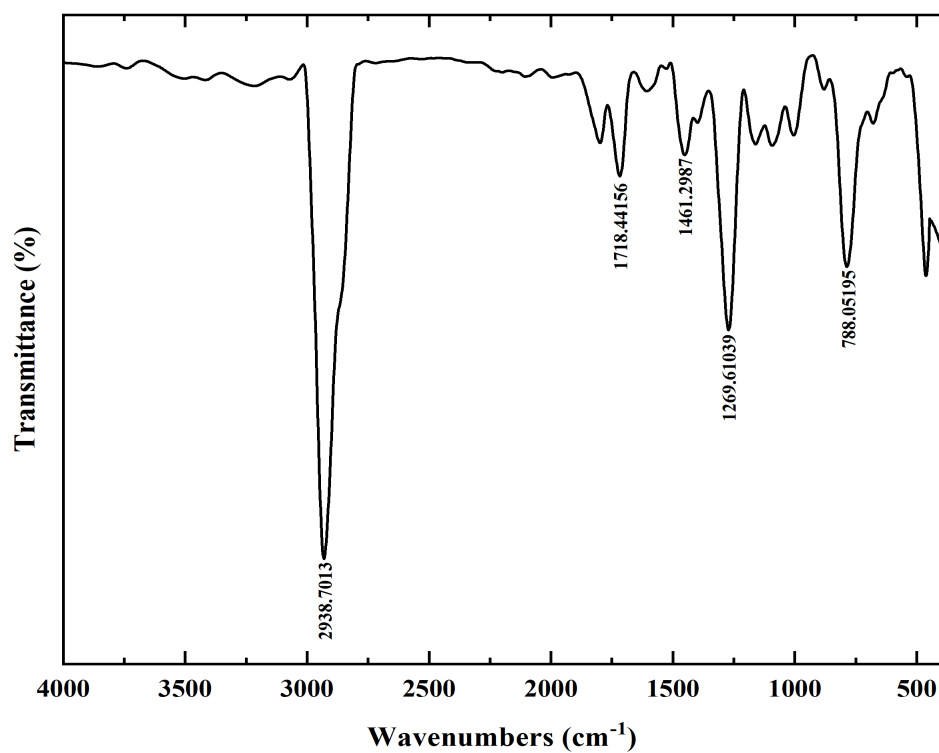
<i>m/z</i>	<i>Calc m/z</i>	Diff(ppm)	<i>z</i>	Abund	Formula	Ion
55.1	55	-333.26		336334.63	C ₂ H ₈	(M+Na) ⁺
56.1	56	-366.05		233950.36	C ₃ H ₂	(M+NH ₄) ⁺
57.1	56.1	-17833.71	1	269321.72	C ₄ H ₈	(M+H) ⁺
69.1	69.1	-183.86		314342.13	C ₄ H ₃	(M+NH ₄) ⁺
70.1	70.1	-212.25		273087.66	C ₅ H ₉	(M+H) ⁺
71.1	48	-324712.28	1	129103.55	C ₄	(M+Na) ⁺
83.1	83.1	35.52		352468.75	C ₆ H ₁₀	(M+H) ⁺
84.1	84.1	9.23		184197.31	C ₅ H ₆	(M+NH ₄) ⁺
97.1	97.1	-14.41		305940.5	C ₆ H ₇	(M+NH ₄) ⁺
111.1	111.1	38.26		142921.59	C ₈ H ₁₄	(M+H) ⁺

Appendix 7: Compound table and Gas chromatogram of Standard n-alkane

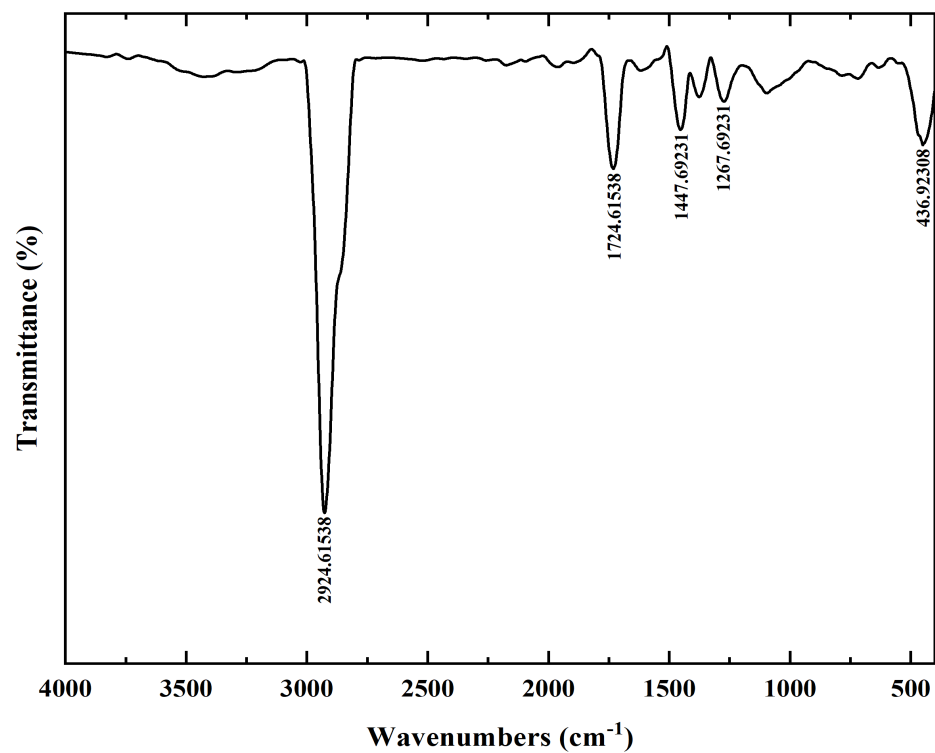
No	RT	Compound Name	Molecular formula
1	4.415	Hexane, 2,4-dimethyl-	C ₈ H ₁₈
2	6.262	Nonane	C ₉ H ₂₀
3	8.129	Decane	C ₁₀ H ₂₂
4	9.85	Undecane P300	C ₁₁ H ₂₄
5	11.426	Dodecane P324	C ₁₂ H ₂₆
6	12.882	Tridecane P358	C ₁₃ H ₂₈
7	14.245	Tetradecane P396	C ₁₄ H ₃₀
8	15.521	Pentadecane	C ₁₅ H ₃₂
9	16.728	Hexadecane P497	C ₁₆ H ₃₄
10	20.205	Nonadecane	C ₁₉ H ₄₀
11	21.499	Eicosane	C ₂₀ H ₄₂
12	22.85	Heneicosane	C ₂₁ H ₄₄
13	24.248	Docosane P973	C ₂₂ H ₄₆
14	25.674	Tricosane P1059	C ₂₃ H ₄₈
15	27.112	Tetracosane	C ₂₄ H ₅₀
16	29.96	Hexacosane P1294	C ₂₆ H ₅₄
17	31.346	Hentriacontane	C ₃₁ H ₆₄



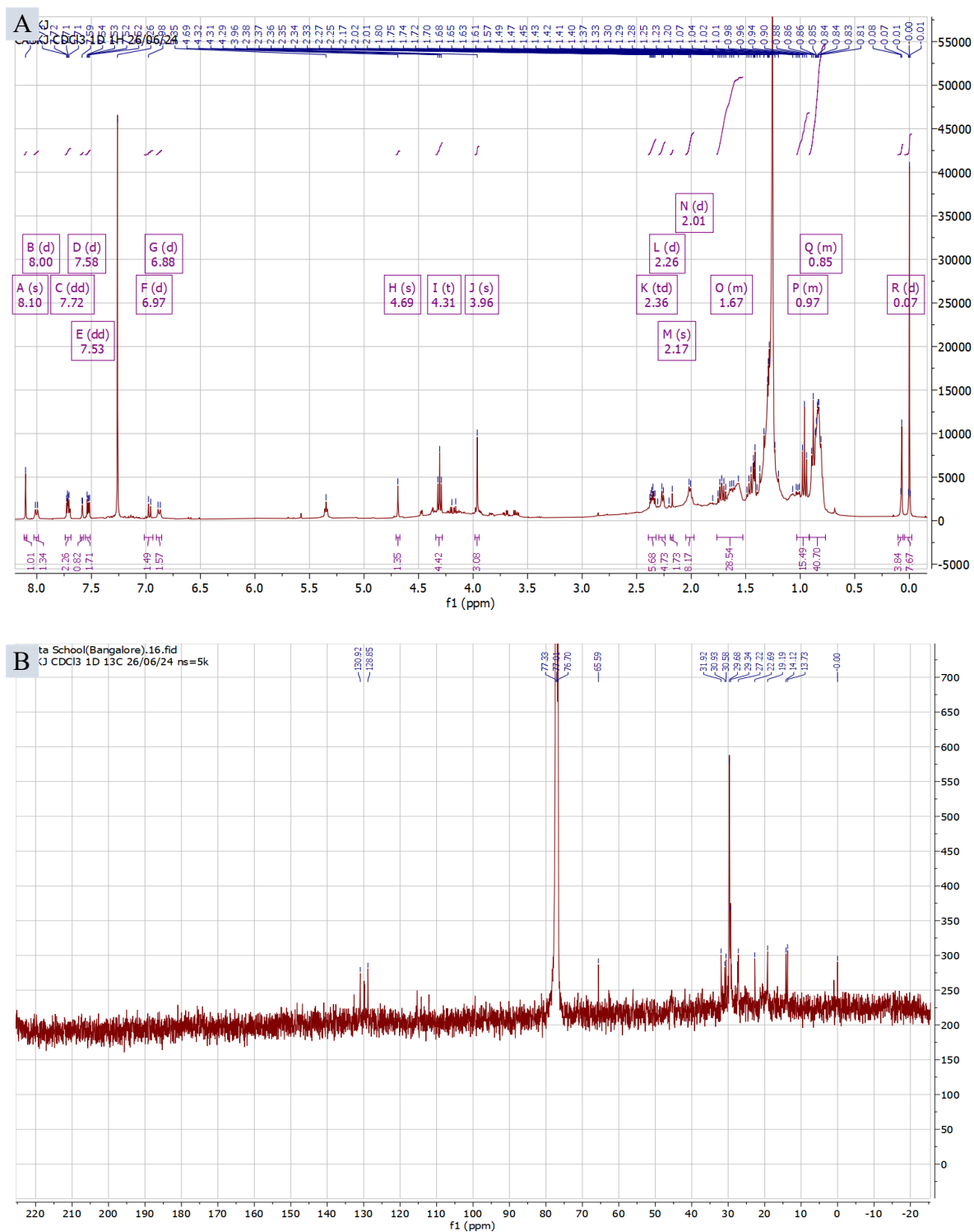
Appendix 8: FTIR spectrum of a mixture of Compound **32** and **37**



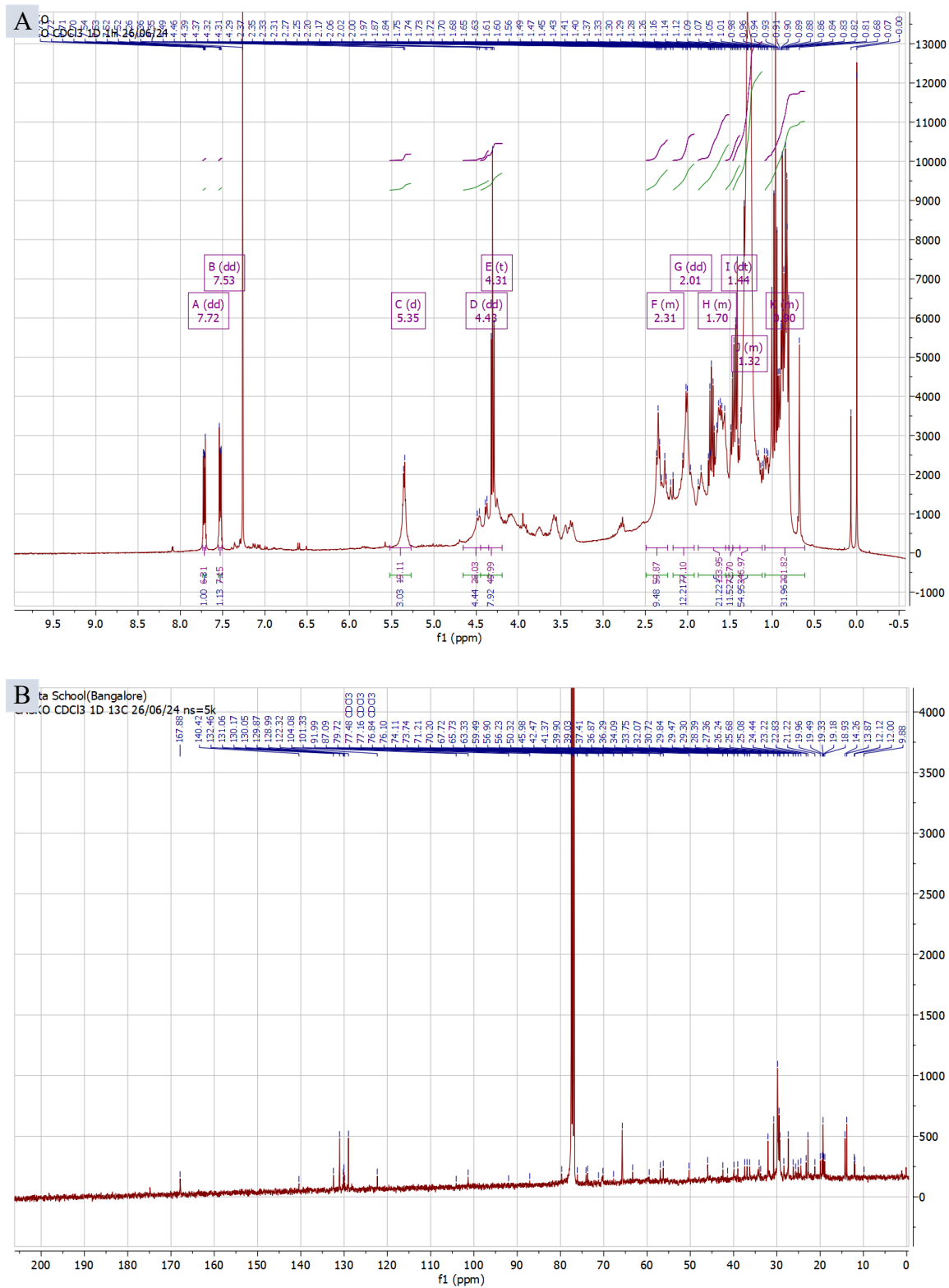
Appendix 9: FTIR spectrum of a mixture of Compound **38** and **39**



Appendix 10: ^1H NMR (400 MHz, CDCl_3) and ^{13}C NMR (101 MHz, CDCl_3) spectra of Compound **32** and **37** mixture.



Appendix 11: ^1H NMR (400 MHz, CDCl_3) and ^{13}C NMR (101 MHz, CDCl_3) spectra of Compound **38** and **39** mixture.



Appendix 12: Fractions collected from column chromatography using different solvent systems and ratios.

Solvent System	Ratio (%)	Fractions	Mixed	Repacking			
				Solvent System	Ratio (%)	Fractions	Mixed
Pet Ether	10:0	F ₁ -F ₂	F ₁ -F ₉ (F _A)	Pet Ether	100:0	F ₁ -F ₆	F ₁ -F ₁₂ (F _A), F ₁₃ -F ₁₄ (F _B)
Pet Ether: EtOAc	90:10	F ₃ -F ₅		Pet Ether: EtOAc	95:5	F ₇ -F ₁₀	
	80:20	F ₆ -F ₁₁			90:10	F ₁₁ -F ₁₄	
	70:30	F ₁₂ -F ₁₇	85:15		F ₁₅ -F ₂₂	F ₁₅ -F ₁₉ (F _C), F ₂₀ -F ₂₂ (F _D)	
	60:40	F ₁₈ -F ₃₁	F ₂₆ -F ₂₇ (F _B), F ₂₈ -F ₃₀ (F _C)		80:20	F ₂₃ -F ₂₉	F ₂₄ -F ₂₈ (F _E)
	50:50	F ₃₂ -F ₄₀	F ₃₇ -F ₄₄ (F _D),		75:25	F ₃₀ -F ₃₆	F ₃₀ -F ₃₂ (F _F), F ₃₃ -F ₃₆ (F _G)
	40:60	F ₄₁ -F ₄₇	F ₄₆ -F ₄₉ (F _E),		70:30	F ₃₇ -F ₄₄	F ₃₈ -F ₄₄ (F _H)
	30:70	F ₄₈ -F ₅₅	F ₅₀ -F ₅₄ (F _F),		65:35	F ₄₅ -F ₅₁	F ₄₅ -F ₅₀ (F _I)
	20:80	F ₅₆ -F ₆₂	F ₅₅ -F ₆₂ (F _G)		60:40	F ₅₂ -F ₆₆	F ₅₁ -F ₅₂ (F _J) F ₅₃ -F ₅₄ (F _K) F ₅₅ -F ₅₆ (F _L) F ₅₇ -F ₆₄ (F _M) F ₆₅ -F ₆₈ (F _N) F ₆₉ -F ₇₃ (F _O) F ₇₄ -F ₇₅ (F _P) F ₇₆ -F ₉₄ (F _Q) F ₉₅ -F ₁₁₃ (F _R) F ₁₁₄ -F ₁₃₂ (F _S) F ₁₃₃ -F ₁₅₀ (F _T)
	10:90	F ₆₃ -F ₇₀			55:45	F ₆₇ -F ₇₄	
EtOAc	10:0	F ₇₁ -F ₈₄		50:50	F ₇₅ -F ₈₁		
EtOAc: MeOH	90:10	F ₈₅ -F ₉₂	F ₉₁ -F ₉₉ (F _H), F ₁₀₀ -F ₁₀₇ (F _I)	Pet Ether: EtOAc	45:55	F ₈₂ -F ₈₈	
	80:20	F ₉₃ -F ₁₀₅			40:60	F ₈₉ -F ₉₅	
	70:30	F ₁₀₆ -F ₁₁₃			35:65	F ₉₆ -F ₁₀₃	
CHCl ₃ : MeOH	50:50	F ₁₁₄ -F ₁₂₂			30:70	F ₁₀₄ -F ₁₁₁	
					25:75	F ₁₁₂ -F ₁₁₉	
					20:80	F ₁₂₀ -F ₁₂₇	
					15:85	F ₁₂₈ -F ₁₃₅	
					10:90	F ₁₃₆ -F ₁₄₃	
					5:95	F ₁₄₄ -F ₁₅₁	
					0:100	F ₁₅₂ -F ₁₅₄	

CHCl₃; chloroform, EtOAc; ethyl acetate, MeOH; methanol **Pet Ether**; petroleum ether

Appendix 13: LSD post-hoc test for inhibition zones of plant extracts and ciprofloxacin against *E. coli*.

Multiple Comparisons

Dependent Variable: Inhibition_zone (mm)

LSD

(I) Plant_Extracts_Against_E.coli	(J) Plant_Extracts_Against_E.coli	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EtoH against <i>E. coli</i>	MeoH against <i>E. coli</i>	-.50000	1.57470	.752	-3.6802	2.6802
	DCM:EtOH (1:1) against <i>E. coli</i>	2.25000	1.57470	.161	-.9302	5.4302
	Cipro against <i>E.coli</i>	-6.25000*	1.70087	.001	-9.6850	-2.8150
MeoH against <i>E. coli</i>	EtoH against <i>E. coli</i>	.50000	1.57470	.752	-2.6802	3.6802
	DCM:EtOH (1:1) against <i>E. coli</i>	2.75000	1.57470	.088	-.4302	5.9302
	Cipro against <i>E.coli</i>	-5.75000*	1.70087	.002	-9.1850	-2.3150
DCM:EtOH (1:1) against <i>E. coli</i>	EtoH against <i>E. coli</i>	-2.25000	1.57470	.161	-5.4302	.9302
	MeoH against <i>E. coli</i>	-2.75000	1.57470	.088	-5.9302	.4302
	Cipro against <i>E.coli</i>	-8.50000*	1.70087	.000	-11.9350	-5.0650
Cipro against <i>E.coli</i>	EtoH against <i>E. coli</i>	6.25000*	1.70087	.001	2.8150	9.6850
	MeoH against <i>E. coli</i>	5.75000*	1.70087	.002	2.3150	9.1850
	DCM:EtOH (1:1) against <i>E. coli</i>	8.50000*	1.70087	.000	5.0650	11.9350

*. The mean difference is significant at the 0.05 level.

Appendix 14: LSD post-hoc test for inhibition zones of plant extracts and ciprofloxacin against *P. aeruginosa*.

Multiple Comparisons

Dependent Variable: Inhibition_zone (mm)

LSD

(I) Plant_Extracts_Against_P. aeruginosa (J) Plant_Extracts_Against_P. aeruginosa		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EtoH against <i>P. aeruginosa</i>	MeoH against <i>P. aeruginosa</i>	-1.12500	1.34355	.407	-3.8384	1.5884
	DCM:EtOH (1:1) against <i>P. aeruginosa</i>	.50000	1.34355	.712	-2.2134	3.2134
	Cipro against <i>P. aeruginosa</i>	-5.75000*	1.45120	.000	-8.6808	-2.8192
MeoH against <i>P. aeruginosa</i>	EtoH against <i>P. aeruginosa</i>	1.12500	1.34355	.407	-1.5884	3.8384
	DCM:EtOH (1:1) against <i>P. aeruginosa</i>	1.62500	1.34355	.233	-1.0884	4.3384
	Cipro against <i>P. aeruginosa</i>	-4.62500*	1.45120	.003	-7.5558	-1.6942
DCM:EtOH (1:1) against <i>P. aeruginosa</i>	EtoH against <i>P. aeruginosa</i>	-.50000	1.34355	.712	-3.2134	2.2134
	MeoH against <i>P. aeruginosa</i>	-1.62500	1.34355	.233	-4.3384	1.0884
	Cipro against <i>P. aeruginosa</i>	-6.25000*	1.45120	.000	-9.1808	-3.3192
Cipro against <i>P. aeruginosa</i>	EtoH against <i>P. aeruginosa</i>	5.75000*	1.45120	.000	2.8192	8.6808
	MeoH against <i>P. aeruginosa</i>	4.62500*	1.45120	.003	1.6942	7.5558
	DCM:EtOH (1:1) against <i>P. aeruginosa</i>	6.25000*	1.45120	.000	3.3192	9.1808

*. The mean difference is significant at the 0.05 level.

Appendix 15: LSD post-hoc test for inhibition zones of plant extracts and ciprofloxacin against *S. aureus*.

Multiple Comparisons

Dependent Variable: Inhibition_zone (mm)

LSD

(I) Plant_Extracts_Against_ <i>S. aureus</i>	(J) Plant_Extracts_Against_ <i>S. aureus</i>	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EtoH against <i>S. aureus</i>	MeoH against <i>S. aureus</i>	-.37500	1.50490	.804	-3.4142	2.6642
	DCM:EtOH (1:1) against <i>S. aureus</i>	1.12500	1.50490	.459	-1.9142	4.1642
	Cipro against <i>S. aureus</i>	-6.66667*	1.62548	.000	-9.9494	-3.3839
MeoH against <i>S. aureus</i>	EtoH against <i>S. aureus</i>	.37500	1.50490	.804	-2.6642	3.4142
	DCM:EtOH (1:1) against <i>S. aureus</i>	1.50000	1.50490	.325	-1.5392	4.5392
	Cipro against <i>S. aureus</i>	-6.29167*	1.62548	.000	-9.5744	-3.0089
DCM:EtOH (1:1) against <i>S. aureus</i>	EtoH against <i>S. aureus</i>	-1.12500	1.50490	.459	-4.1642	1.9142
	MeoH against <i>S. aureus</i>	-1.50000	1.50490	.325	-4.5392	1.5392
	Cipro against <i>S. aureus</i>	-7.79167*	1.62548	.000	-11.0744	-4.5089
Cipro against <i>S. aureus</i>	EtoH against <i>S. aureus</i>	6.66667*	1.62548	.000	3.3839	9.9494
	MeoH against <i>S. aureus</i>	6.29167*	1.62548	.000	3.0089	9.5744
	DCM:EtOH (1:1) against <i>S. aureus</i>	7.79167*	1.62548	.000	4.5089	11.0744

*. The mean difference is significant at the 0.05 level.

Appendix 16: LSD post-hoc test for inhibition zones of plant extracts and ciprofloxacin against *S. pyogenes*.

Multiple Comparisons

Dependent Variable: Inhibition_zone (mm)

LSD

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
(I) Plant_Extracts_Against_S. pyogenes	(J) Plant_Extracts_Against_S. pyogenes				Lower Bound	Upper Bound
EtoH against <i>S. pyogenes</i>	MeoH against <i>S. pyogenes</i>	.00000	1.62058	1.000	-3.2728	3.2728
	DCM:EtOH (1:1) against <i>S. pyogenes</i>	1.62500	1.62058	.322	-1.6478	4.8978
	Cipro against <i>S. pyogenes</i>	-6.00000*	1.75042	.001	-9.5351	-2.4649
MeoH against <i>S. pyogenes</i>	EtoH against <i>S. pyogenes</i>	.00000	1.62058	1.000	-3.2728	3.2728
	DCM:EtOH (1:1) against <i>S. pyogenes</i>	1.62500	1.62058	.322	-1.6478	4.8978
	Cipro against <i>S. pyogenes</i>	-6.00000*	1.75042	.001	-9.5351	-2.4649
DCM:EtOH (1:1) against <i>S. pyogenes</i>	EtoH against <i>S. pyogenes</i>	-1.62500	1.62058	.322	-4.8978	1.6478
	MeoH against <i>S. pyogenes</i>	-1.62500	1.62058	.322	-4.8978	1.6478
	Cipro against <i>S. pyogenes</i>	-7.62500*	1.75042	.000	-11.1601	-4.0899
Cipro against <i>S. pyogenes</i>	EtoH against <i>S. pyogenes</i>	6.00000*	1.75042	.001	2.4649	9.5351
	MeoH against <i>S. pyogenes</i>	6.00000*	1.75042	.001	2.4649	9.5351
	DCM:EtOH (1:1) against <i>S. pyogenes</i>	7.62500*	1.75042	.000	4.0899	11.1601

*. The mean difference is significant at the 0.05 level.

Appendix 17: LSD post-hoc test for inhibition zones of EtOH extract against test strains.

Multiple Comparisons

Dependent Variable: Inhibition_zone (mm)

LSD

(I) EtOH Extract Against Test Strains	(J) EtOH Extract Against Test Strains	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EtOH against <i>E. coli</i>	EtOH against <i>P. aeruginosa</i>	.50000	1.76670	.778	-3.0605	4.0605
	EtOH against <i>S. aureus</i>	-.25000	1.76670	.888	-3.8105	3.3105
	EtOH against <i>S. pyogenes</i>	-.75000	1.76670	.673	-4.3105	2.8105
EtOH against <i>P. aeruginosa</i>	EtOH against <i>E. coli</i>	-.50000	1.76670	.778	-4.0605	3.0605
	EtOH against <i>S. aureus</i>	-.75000	1.76670	.673	-4.3105	2.8105
	EtOH against <i>S. pyogenes</i>	-1.25000	1.76670	.483	-4.8105	2.3105
EtOH against <i>S. aureus</i>	EtOH against <i>E. coli</i>	.25000	1.76670	.888	-3.3105	3.8105
	EtOH against <i>P. aeruginosa</i>	.75000	1.76670	.673	-2.8105	4.3105
	EtOH against <i>S. pyogenes</i>	-.50000	1.76670	.778	-4.0605	3.0605
EtOH against <i>S. pyogenes</i>	EtOH against <i>E. coli</i>	.75000	1.76670	.673	-2.8105	4.3105
	EtOH against <i>P. aeruginosa</i>	1.25000	1.76670	.483	-2.3105	4.8105
	EtOH against <i>S. aureus</i>	.50000	1.76670	.778	-3.0605	4.0605

Appendix 18: LSD post-hoc test for inhibition zones of MeOH extract against test strains.

Multiple Comparisons

Dependent Variable: Inhibition_zone (mm)

LSD

(I) MeOH_Extract_Against_Test_Strains	(J) MeOH_Extract_Against_Test_Strains	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
MeOH against <i>E. coli</i>	MeOH against <i>P. aeruginosa</i>	-.12500	1.86055	.947	-3.8747	3.6247
	MeOH against <i>S. aureus</i>	-.12500	1.86055	.947	-3.8747	3.6247
	MeOH against <i>S. pyogenes</i>	-.25000	1.86055	.894	-3.9997	3.4997
MeOH against <i>P. aeruginosa</i>	MeOH against <i>E. coli</i>	.12500	1.86055	.947	-3.6247	3.8747
	MeOH against <i>S. aureus</i>	.00000	1.86055	1.000	-3.7497	3.7497
	MeOH against <i>S. pyogenes</i>	-.12500	1.86055	.947	-3.8747	3.6247
MeOH against <i>S. aureus</i>	MeOH against <i>E. coli</i>	.12500	1.86055	.947	-3.6247	3.8747
	MeOH against <i>P. aeruginosa</i>	.00000	1.86055	1.000	-3.7497	3.7497
	MeOH against <i>S. pyogenes</i>	-.12500	1.86055	.947	-3.8747	3.6247
MeOH against <i>S. pyogenes</i>	MeOH against <i>E. coli</i>	.25000	1.86055	.894	-3.4997	3.9997
	MeOH against <i>P. aeruginosa</i>	.12500	1.86055	.947	-3.6247	3.8747
	MeOH against <i>S. aureus</i>	.12500	1.86055	.947	-3.6247	3.8747

Appendix 19: LSD post-hoc test for inhibition zones of DCM:EtOH (1:1) extract against test strains.

Multiple Comparisons

Dependent Variable: Inhibition_zone (mm)

LSD

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
(I) DCM:EtOH (1:1)_Extract_Against_Test_Strains	(J) DCM:EtOH (1:1)_Extract_Against_Test_Strains				Lower Bound	Upper Bound
DCM:EtOH (1:1) against <i>E. coli</i>	DCM:EtOH (1:1) against <i>P. aeruginosa</i>	-1.25000	1.24526	.321	-3.7596	1.2596
	DCM:EtOH (1:1) against <i>S. aureus</i>	-1.37500	1.24526	.276	-3.8846	1.1346
	DCM:EtOH (1:1) against <i>S. pyogenes</i>	-1.37500	1.24526	.276	-3.8846	1.1346
DCM:EtOH (1:1) against <i>P. aeruginosa</i>	DCM:EtOH (1:1) against <i>E. coli</i>	1.25000	1.24526	.321	-1.2596	3.7596
	DCM:EtOH (1:1) against <i>S. aureus</i>	-.12500	1.24526	.920	-2.6346	2.3846
	DCM:EtOH (1:1) against <i>S. pyogenes</i>	-.12500	1.24526	.920	-2.6346	2.3846
DCM:EtOH (1:1) against <i>S. aureus</i>	DCM:EtOH (1:1) against <i>E. coli</i>	1.37500	1.24526	.276	-1.1346	3.8846
	DCM:EtOH (1:1) against <i>P. aeruginosa</i>	.12500	1.24526	.920	-2.3846	2.6346
	DCM:EtOH (1:1) against <i>S. pyogenes</i>	.00000	1.24526	1.000	-2.5096	2.5096
DCM:EtOH (1:1) against <i>S. pyogenes</i>	DCM:EtOH (1:1) against <i>E. coli</i>	1.37500	1.24526	.276	-1.1346	3.8846
	DCM:EtOH (1:1) against <i>P. aeruginosa</i>	.12500	1.24526	.920	-2.3846	2.6346
	DCM:EtOH (1:1) against <i>S. aureus</i>	.00000	1.24526	1.000	-2.5096	2.5096

Appendix 20: Experiment activities - visual documentation.

