



WOLLEGA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

**PHYTOCHEMICAL SCREEINING, ANTIBACTERIAL ACTIVITY
EVALUATION, ACID AND SAPONIFICATION VALUE
DETERMINATION OF STEM BARK OIL EXTRACTS OF *CLAUSENA
ANISATA (RUTACEAE)***

M Sc. Research Thesis

By

MIRESA TOLANI BORENA

MAY, 2025

NEKEMTE, ETHIOPIA



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A Thesis submitted to the College of Natural and Computational Science in partial Fulfillment of
the Requirements for the Degree of Master of Science (MSc) in Organic Chemistry

By
MIRESA TOLANI BORENA

Advisors: GetahunTadese Gurmessa (PhD)

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Nekemte, Ethiopia



WOLLEGA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
P.O. Box: 395, Nekemte, Ethiopia

APPROVAL SHEET FOR SUBMITTING RESEARCH THESIS

As members of the Board of Examining of the Final MSc. Thesis open defense,
We certify that We have read and evaluated the thesis prepared by **Miresa Tolani**
under the title “**Phytochemical Screening, Antibacterial Activity Evaluation,
Acid And Saponification Value Determination of Stem Bark oil Extracts Of
*Clausena Anisata (Rutaceae)***” and recommend that the thesis be accepted as fulfilling the
requirement for the Degree of Master of Science in Organic Chemistry.

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Internal Examiner	Signature	Date
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Thesis Approved By:		
Deparment of PGC	Signature	Date
Dean of College	Signature	Date
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I hereby certify that all the correction and recommendation suggested by the board of examiners are incorporated into the final thesis “ Phytochemical Screening, Antibacterial Activity Evaluation, Acid And Saponification Value Determination of Stem Bark oil Extracts Of <i>Clausena Anisata (Rutaceae)</i> By <u>Miresa Tolani.</u>		
Dean of SGS	Signature	Date

DECLARATION

I declare hereby tha this thesis entitled “**Phytochemical Screeining, Antibacterial Activity Evaluation, Acid And Saponification Value Determination of Stem Bark oil Extracts Of *Clausena Anisata (Rutaceae)*** is the true reflection of my student’s research and accepted in partial fulfillment of the requirement for the award of the Degree of Master of Sceince in Organic Chemistry by the School of Graduate studies, Wollega University through College of Natural and Computational Sciences. This research was done by **Miresa Tolani** under my supervision and guidance. The part has not been submitted for a degree in any other institution of higher education.

Getahun Tadesse (PhD)

Adviser

signature

Date

STATEMENT OF THE AUTHER

I **Mr. Miresa Tolani Borena** hereby declare and affirm that the thesis entitled **“Phytochemical Screeining, Antibacterial Activity Evaluation, Acid And Saponification Value Determination of Stem Bark oil Extracts Of *Clausena Anisata (Rutaceae)*”** is my work conducted under the supervision of Dr. Getahun Tadesse at Wollega University. I have followed all the ethical principles of scholarship in the preparation, plant material collection, phytochemical screening, antibacterial evaluation, acid value,saponification value determination and completion of this thesis. All scholarly matter that is included in the thesis has been given recognition through citation. I have adequately cited and reference all the original sources. I also declare that I have followed all principles of academic honesty and integrity and I have not misused, fabricated or untrue any idea or data or fact or sources in my submission. This thesis is submitted in partial fulfillment of the requirement for a degree from the post Graduate studies at Wollega University. I further declare that this thesis has not been submitted to any other institution anywhere for the award of any academic degree, diploma or certificate. I understand that any violation of the above will be cause for disciplinary action by the University and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken needed.

Name: Miresa Tolani Borena

Signature_____

Date._____

Department: Chemistry

College: Natural and Computational Sciences

BIOGRAPHICAL SKETCH OF THE AUTHOR

Mr. Miresa Tolani was born on October 20, 1988, in Bako Tibe Woreda, West Shoa Zone, Oromia regional state. He attended his primary education (grade 1_6) at Sombo Disasa primary school, Grade 7 & 8 at Bako elementary school and secondary education (Grade 9 -12) at Bako high school. In 2008 he joined University of Bahir Dar and received his first degree (BSc) in chemistry in 2010. He had been teaching Gedo secondary school in West Shoa Zone, Chaliya Woreda since 2010. He joined Wollega University in summer program in 2019 and has been attending his MSc. in Organic chemistry.

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ABBREVIATIONS AND ACRONYMS

AV	:	Acid value
CC	:	Column Chromatography
DMSO	:	Dimethyl Sulfoxide
FAO	:	Food and Agriculture Organization of the United Nations
GC-MS	:	Gas chromatography- Mass spectrometr
IR	:	Infra-Red
MHA	:	Mueller Hinton Agar
MPNS	:	Medicinal Plant Name Services
NMR	:	Nuclear Magnetic Resonance
RF	:	Retention Factor
SV	:	Saponification value
TLC	:	Thin Layer Chromatography
UV	:	Ultra Violet
WHO	:	World Health Organization

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ABSTRACT

Plants are the source of diversified class of secondary metabolites that have been used as antimicrobial agents since long time. *Clausena anisata* is one of such medicinal plant used to treat various ailments in most African traditional medicine. *Clausena anisata* (Willd) widely used by traditional healers to manage several ailments such as chronic cough, tuberculosis and lung ulceration in some Africa society. *Clausena anisata* also used to kill pests such as mosquitoes, flies and weevil. To attain the proposed objective the stem bark of *Clausena anisata* was selected based on its traditional claims. The stem bark of the plant was collected from the study area and extracted with two different solvents (chloroform and methanol) sequentially by maceration methods. From 500g of chloroform extraction 2.58% crude was obtained and 0.98% was obtained from 487.11g of methanol extraction. The crude extract of both solvents were subjected to phytochemical screening and both crude extraction revealed the presence of alkaloids, flavonoids, steroids, terpenoids, tannins and saponins. But concentration of saponins was high in methanol crude extract. The obtained crude extracts were evaluated for their antibacterial activities against two gram positive and two gram negative bacteria. Both chloroform and methanol extracts have shown antibacterial activities at different concentration against the selected bacteria. The chloroform stem bark extracts has shown greater growing inhibition zone in the range of (6.54-11.85 mm) against *Staphylococcus enteric* and lower against *Other coagulase negative staphylococcus* 10.03mm at 100mg/mL. The methanol extracts has shown lower inhibition against theselected bacteria strain. The chloroform extract was selected for isolation based on its yield, antibacterial activity and clearly identified spots (better spots) shown on TLC, then the extract were further fractionated using column chromatography. From chloroform extract wo fraction with signle TLC profile was identified and lebeled as CA-1 and CA-2 and the calculated R_F values of the identified spots were 0.44 in 8:2 solvent ratio and 0.26 in 7:3 solvent ratio resepectively. The plant have potential for soap prepatation.

Keywords: phytochemical investigation, antibacterial activity, extraction, saponification value acid value, *Rutaceae*, *Clausena anisata*, stem bark.

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the study

The use of plants as source of medicine is the oldest system of health care known to humanity that has been used in all cultures throughout history. Our ancestors used some of plants in their daily lives, and early in the history of the human race they learned through trial and error that certain plants could be used to support wellbeing. Herbal preparation usually made from roots, flowers, barks, leaves and their extracts were the only effective remedies available to the ancestors (Wondimu, *et al.*, 2007)

Even today, human beings depend highly on plants for treatment and maintenance of their health. This is because natural drugs are more effective, have less side effects and relatively low cost when compared with synthetic drugs (Raj, 2011). Recently, the consumption and demand of herbal products increased in developed countries partly due to lack of satisfaction and complete cure with conventional medicines, while in developing countries it is due to lack of medical doctors, shortage and unaffordable prices (Abiy, 2018).

The World Health Organization (WHO, 2013) estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants and 80% of the world population uses medicinal plants as remedies since new drugs are often not affordable. However, only an estimated 5% to 15% of the 250,000 species of higher terrestrial plants in existence have been chemically and pharmacologically investigated in systematic fashion (Pieters and Vlietinck, 2005). This is the evidence that shows much of world plant species remain unstudied and there is a great potential for future and investigation of medicinal plants remains a research area for many chemists for the last two decades to come up with affordable, effective, and best quality drugs. In addition to their direct use for primary health care, natural products are also important for development of new drugs. Particularly now a days, some of the previously available conventional drugs becomes less efficient as antidrug resistance microbes evolve at high rate and developing novel antimicrobial drugs has got attention (Abiy, 2018).

Another advantage of natural products is their synergistic effect which enhance “multi-drugs and multi-targets” mode of action as multiple phytochemical components exist in combination (El-Sakhawy, *et al.*, 2015).

For the wider coverage of primary health care, traditional medicines have been in focus and traditional healers using remedies made from plants play an important role in health treatment of millions of people in Africa (Elujoba, *et al.*, 2005). It was estimated that around 80% of the population in Africa use traditional medicines and about 85 % of traditional medicine involves use of plant extracts. In addition to health treatments, medicinal plants have strategic and economic importance in African continent.

Like other countries of the Africa, most of the populations in Ethiopia have relied on a system of traditional medicine for health care. Traditional medicine is defined as the sum total of knowledge, skill, and practices based on theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO 2013). The early practice of the ethno-medicinal uses involves squeezing of leaves, roots, stem, flowers and barks of plants and the application of the liquids coming out from them on wounds, cuts, ulcers, skin and soft tissue of infectious site and drinking as solution (Taura, *et al.*, 2014).

The demand for medicinal plants is always there due to their use in traditional medicine, pharmaceutical industries, cosmetic and agricultural sectors. Therefore, scientific study is required to investigate the chemical profile and also evaluate their biological significances of medicinal plants. In this study, phytochemical screening and antimicrobial activities of organic extracts from stem bark of *Clausena anisata* was evaluated to see the correlation between the traditional claim and scientific findings related to this plant.

1.2. Statement of the problem

People value plants because of plant supply man with food, medicine, dyes and other more benefits. Before the introduction of chemical medicines, man relied on the healing properties of plants. *Clausena anisata* is among the traditional plant that has been used traditionally for different medicinal purposes including antidiabetic, antihypertensive, anti-inflammatory, gastrointestinal disorders, anticonvulsant and treatment of some mental disorders (Okunade and

Olaife, 1987). The traditional medicinal value of various morphological parts of *Clausena anisata* have been reported to be useful as effective remedies against parasitic infections, especially flatworm infestations, such as taeniasis and schistosomiasis, in eye complaints, influenza and other respiratory ailments, (Hamza, *et al.*, 2006; Ojewole, 2002). Because of wide spread traditional use of different parts of this plant to treat different types of ailments, more scientific investigation will be required on different parts of the plant to support the traditional claim particularly in Ethiopian context. And also, the phytochemical constituents of the *Clausena anisata* may vary within the same species depending on the geographical distribution of the plant. Hence this study was intended to conduct further isolation and characterization of secondary metabolites from stem bark extract of *Clausena anisata* to test its antibacterial activities and documents the uses of this plant.

1.3. Objectives of the study

1.3.1. General Objective

- ❖ To analysis phytochemical profile, evaluation of antibacterial activities and soapnificatin and acid value determantions of stem bark oil extracts of *Clausena anisata*.

1.3.2. Specific Objectives

- To extracts organic compound from stem bark of *Clausena anisata* by maceration method using chloroform and methanol.
- To analysis phytochemical profiles of the extracts of *Clausena anisata* using standard methods.
- To evaluate antibacterial activities of the crude extracts of *Clausena anisata* by agar disk diffusion method.
- To fractionate extract using column chromatography.
- To determine the acid and soapnification values of the oil from chloroform extract.

1.4. Significance of the study

The study investigated the phytochemical composition and antibacterial activity of *Clausena anisata's* stem bark extracts, aiming to assess its biological significance. To validate the traditional medicinal use of the plant through *in vitro* screening against pathogenic microorganisms and provides scientific insights to support its use against bacterial infections. Additionally, the findings will aid in identifying the phytochemical profile presenting in the stem bark of the plant and documenting its significance for future studies.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. General Overview of Medicinal Plants

A medicinal plant is any plant traditionally used in order to prevent, relieve or cure various diseases or produce physiological action on human body and alter pathological process or any plant used as a principal source of modern drugs or their raw materials. They have been used starting from ancient time and remain as the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Kaur, *et al.*, 2015).

Plants are sources of large amount of drugs comprising to different groups such as antispasmodics, emetics, anti-cancer, antitubercular, antimicrobials, antiviral, antioxidant, anti-inflammatory, etc. Plants have the medicinal value because of chemical substances called phytochemicals that produce a definite physiological action on the human body (Akin moladun, *et al.*, 2007). Plants are rich in active compounds or secondary metabolites such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids which are present in their organs such as leaves, flowers, bark, seeds, fruits, root, etc. Plants contain thousands of constituents and are valuable sources of new and biologically active molecules possessing antimicrobial properties (Das, *et al.*, 2010). So many of them have been investigated by a number of researchers worldwide to clarify their main active ingredients.

Medicinal plants with reliable therapeutic effects are valuable for modern systems of herbal and natural drug discovery. Plants could serve as a direct source of bioactive or therapeutic agents, and these bioactive ingredients act as a raw material for the development of more complex semi synthetic chemical compounds. Isolated compounds of medicinal plants can lead to the discovery of new drugs, and finally, plants can be used as bioactive markers (Ali, *et al.* 2020).

2.2. Medicinal Plants in Ethiopia

Ethiopia is a country with various types of climatic, topographic, soil features and different altitudes which makes the country possesses a wider range of potentially useful medicinal plants compared to many other parts of the world (Yeneayehu and Girma, 2017). Traditional medicines

play a significant role in the health care of the majority of the people in developing countries, including Ethiopia and have continued to be the most affordable and easy accessible source of treatment in primary health care system.

Traditional medicinal practices are common in Ethiopia in which about 80% of human population and 90% of livestock in the country use plant based traditional medicine by indigenous knowledge as their major primary health care system (Yeneayehu and Girma, 2017). It has been reported that approximately 800 species of the medicinal plants grown in Ethiopia are used for treating about 300 medical conditions. However, in Ethiopia investigation of medicinal plants is found to be insignificant compared to the wealth of Ethiopian ethno medicinal plants (Abebe, 2016).

2.3. Plant *Clausena anisata*

Clausena anisata belongs to the genus *Clausena* in the *Rutaceae* (or *Citrus*) family. The Medicinal Plant Names Services (MPNS) gives 34 scientific synonyms for this plant species (<http://mpns.kew.org/mpns-portal/>), which include *Clausena dentata* (Willd.) M.Roem., *Clausena inaequalis* (DC.) Benth. and *Amyris anisata* Willd. The *Clausena* genus comprises 15 species, and is distributed in Africa, southern Asia, Australia, and the Pacific Islands (Tchinda, 2011). The most distinctive morphological character of the genus that separates it from the species of other related genera is the gynophore, which is a large, well-developed, hourglass-shaped structure supporting the ovary (Swingle and Reece, 1967). *Clausena anisata* is the only representative of the *Clausena* genus in tropical Africa (Schmelzer, 2001). It is widespread across the continent occurring in forests and forest margins, riverine thickets and bushed from Guinea and Sierra Leone eastwards to Ethiopia and the Sudan and southward down to the Cape in South Africa, only avoiding the driest regions. *Clausena anisata* is also found in tropical and South east Asia, growing in India, Nepal and Sri Lanka and extending as far as Queensland in north-eastern Australia and some Pacific islands.

It is cultivated for its oil in Malaysia, Indonesia and the Philippines (Axtell and Fairman, 1992; Tchinda, 2011). The oil is used as a medicinal flavour and in the Philippino local brandy "Anisdos" (Axtell and Fairman, 1992). *Clausena anisata* is a deciduous shrub or small tree with pinnate compound leaves which are densely dotted with glands and have a strong aniseed like

scent, when crushed. The flowers are small and white with orange-yellow stamens. The inflorescence forms a branched axillary spray (Hyde and Wursten, 2011).

2.3.1. Taxonomy of *Clausena anisata*

Table 1 Taxonomic Classification *Clausena anisata*

Kingdom	<i>Plante</i>
Order	<i>Roids</i>
Family	<i>Rutaceae</i>
Genus	<i>Clausena</i>
Species	<i>Clausena anisata</i>

Binomial Naame: *anisata*(willd).Hook.fex.Benth

Local names: Ulumaa'ii or Ulumaayii(AfaanOromoo)

2.3.2. Botanical Background of *Clausena anisata*

The plant is a tropical shrub or tree up to 10m high is growing in and on the margins of evergreen forests. The leaves are pinnately compound with 10 to 17 alternate or sub-opposite leaflets and a terminal leaflet. The leaves are densely dotted with glands and have a strong scent when crushed. The scent has been likened to aniseed and opinions vary on its pleasantness. Inflorescence, a branched axillary spray; flowers are small but attractive, white with orange-yellow stamens. The plant is native Africa, mainly in West Africa and North Africa. In Mozambique, it is locally known as “Horse wood” (Burkill, 1966). *Clausena anisata* (*Rutaceae*) is indigenous to Southern Africa where it is commonly known as ‘isifudu’ in Zulu. It usually grows in areas with high rainfall, such as evergreen forests and woodlands, and along streams and rivers (Coates Palgrave, *et al.*, 2000).



Figure 1:Picture of *Clasuenta anisata* plant taken from Gurra forest, Chaliya district, west shoa zone, Oromia regional state (Photo taken by MiresaTolani in Jun 2023).

2.4. Traditional use of *Clausena anisata*

The leaves of *Clausena anisata* are used for the treatment of malaria (Uwaifo, 1984), worm infections, rheumatism, wounds (Hutchings, *et al.*, 1996; Okunade, 1987), skin infections including boils, ringworm, oral thrush and eczema (Hamza, *et al.*, 2006).

The leaves and roots are also used to treat dysentery fever, toothache and arthritis (Adesina and Adewunmi, 1985; Ayensu, 1978; Mshana, *et al.*, 2000). A mouth wash made from the boiled roots and leaves is used to alleviate toothache and to treat oral infections (Adesina and Adewunmi, 1985; Ayensu, 1978). The roots and stem barks are used against herpes zoster (Mshana, *et al.*, 2000).

The leaf, root and stem of *Clausena anisata* have been reported as effectual remedy against flatworm infestations, like taeniasis and schistosomiasis (Hutchings, *et al.* 1996). It has also been reported that *Clausena anisata* was used in the treatment of oral candidiasis and fungal skin diseases by Tanzanian traditional healers (Hamza, *et al.*, 2006). The use of *Clausena anisata* to treat epilepsy and convulsions by traditional healers in Temeke district of Daressalam (Tanzania) has also been reported (Moshi, *et al.*, 2005). The plant has also been reported as a remedy for broad array of other ailments such as insanity, dementia, infertility, impotence, leprosy, syphilis,

gonorrhea, gingivitis, headaches, respiratory and cardiovascular disorders, constipation and gastroenteritis (Hutchings, *et al.*, 1996).

‘Agbo’ is a concoction made of *Clausena anisata*, *Azadirachthaindica* and *Afraeglepaniculata* which is used as an antimalaria preparation in Nigeria (Uwaifo, 1984). A root preparation of *Clausena anisata* was given as an enema or bathing lotion to treat inconsequential health conditions and to mitigate early signs of pyrexia or to avert the incidence of pyrexia in children. Furthermore, decoction of *Clausena anisata* root is taken at half a wine glass twice a day to remedy cardiovascular disorders and halitosis (Pujol, 1990). Asthmatic conditions are also treated with the roots (Mshana, *et al.*, 2000). A decoction made from the roots is drunk by children to manage convulsions and taken by expectant mothers as a tonic (Ngadjui, *et al.*, 1989). The roots also find use in the treatment of rheumatism and abdominal pain in children (Adesina and Adewunmi, 1985; Ayensu, 1978)

2.5. Phytochemicals reported from *Clausena anisata*

Phytochemicals are secondary metabolites that are biologically active, naturally occurring chemical compounds in plants. They protect plants and human from diseases, environmental hazards, pathogenic attack and accumulate in different parts of a plant such as leaf, stem, stem bark, root, root bark and so on. They include chemical compounds like alkaloids, flavonoids, phenolics, steroids, tannins, coumarin and terpenoids. The content and composition of these phytochemical compounds vary with parts of a plant used for medicinal value and also from plant to plant (Kawo, *et al.*, 2009).

Scientific studies showed that the plant *Clausena anisata* contains a number of coumarins, such as chalepin, osthole, coumarins, scopoleptin, xanthoxyletin, and heliottin (Duncan, *et al.*, 1999). Also, the presence of carbazole alkaloids, such as clausenol and clausamine, has shown to exhibit antimicrobial properties (Chakraborty, *et al.*, 1995). Furthermore, many terpenoid hydrocarbons, sesquiterpenoids and fatty acids have been isolated from the pericarps, roots and leaves of *Clausena anisata* (Reisch, *et al.*, 1985).

The stem bark and roots of *Clausena anisata* were extracted with methanol. Each extract was subjected to column chromatography and TLC to yield three carbazole alkaloids and two peptide derivatives (Figure 2) identified as: girinimbine (1), murrayamine-A (2), ekeberginine(3),

aurantiamide acetate (4) and N-benzoylphenylalaninyl-N-benzoylphenylalaninate (5) by spectroscopic methods and direct comparison of spectral data to those published in the literature. A mixture of sitosterol (6) and stigmasterol (7) was also isolated from both stem and root bark.

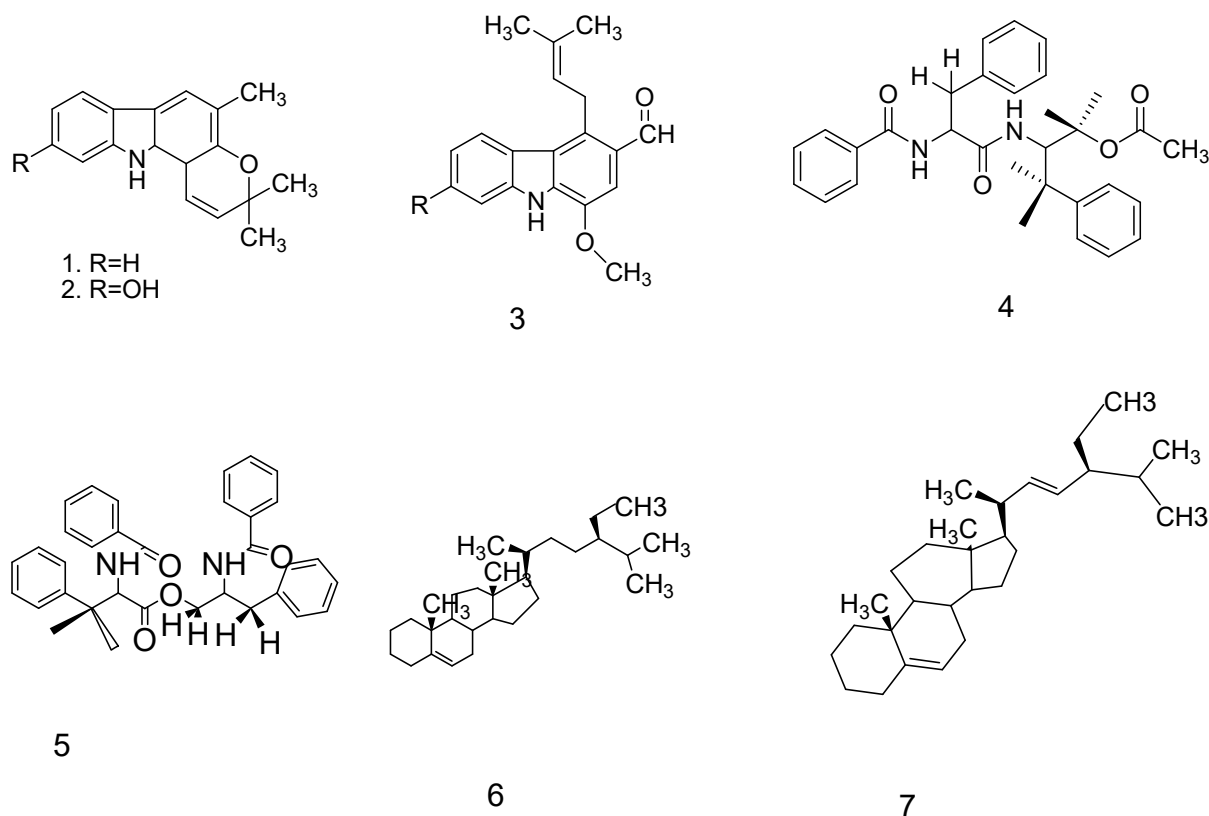
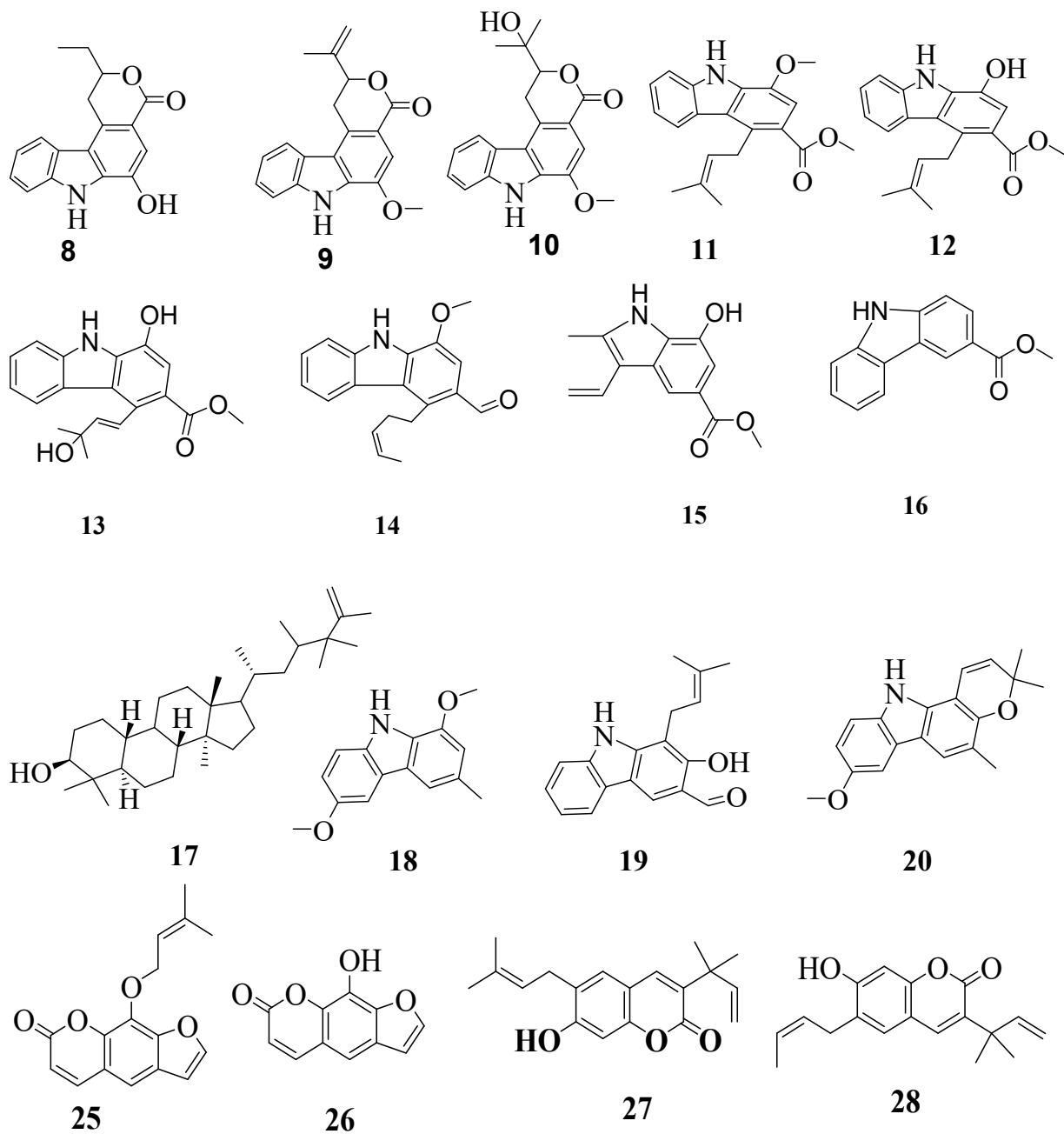


Figure 2: The structures of seven chemical constituents isolated from stem bark and root *Clausena anisata*.

Other chemical structures of some compounds isolated from different part of *Clausna anisata* are: Clausamine A(8), Clausamine B(9), Clausamine C(10), Clausamine D(11), Clausine E(12), Clausamine F(13), Ekeberginine(14), Clausine G(15), methyl carbazole-3-carboxylate(16), Clausenol(17), Clausenine(18), Heptaphyline(19), Girinimbine(20), Murrayamine(21), Chalepin(22), Osthel(23), Xanthoxyletin(24), Imperatorin(25), Xanthotoxol(26), unidentified compound with chromene skeleton(27), Gravelliferone(28), Excavatin(29), Phellopterin(30), Bergapten(31), Isooxpeucedanin(32), Seselin(33), 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester(34), Trans-4-hydroxy-N-methylproline(35).



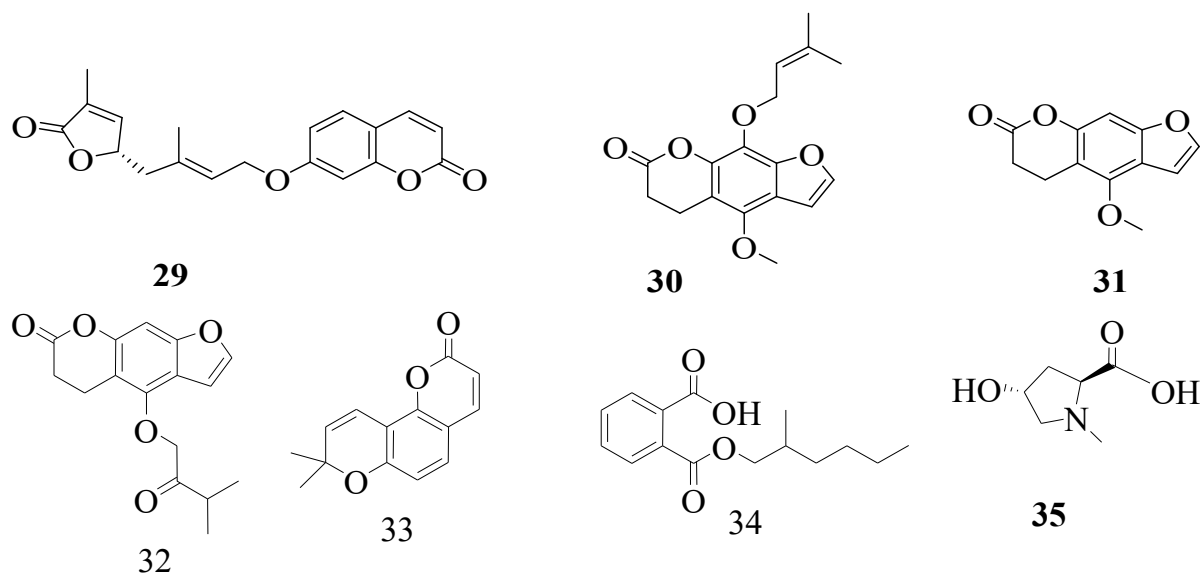


Figure 3 Chemical structure of some compounds isolated from different part of *Clausena anisata*.

2.6. Biological activity of *Clausena anisata*

The investigation of anti-inflammatory and antipyretic activities of the ethanolic leaves extract of *Clausena anisata* and found out that the extract administered intraperitoneally (i.p) at 39–117 mg/kg, dose dependently reduced inflammation and pyrexia in different experimental animal models. The mean lethal dose (LD₅₀) of the ethanolic leaves extract was 393.7 ± 25.64 mg/kg (Okokon, *et al.*, 2012).

The ethanolic roots extract of *Clausena anisata* was shown to demonstrate significant dose-dependent hypoglycemic activity in fasted streptozotocin treated and fasted normal diabetic rats at a dose of 100-800 mg/kg (Ojewole, 2002). A comparative study on the anti-epileptic activity of the root, stem and leaf of *Clausena anisata* revealed that the ethanol extract of the *Clausena anisata* possessed anti-epileptic activity with 33.33 % anti-convulsant effect (Kenechukwu, *et al.*, 2012)

The stem bark of *Clausena anisata* has been found to possess antimicrobial property (Chakraborty, *et al.*, 1995), while root and stem bark exhibiting hypoglycaemic property (Ojewole, 2002).

CHAPTER 3

3.MATERIAL AND METHODS

3.1.Study area

Experiments such as extraction of plant materials, phytochemical screening and Chromatographic separation was done at Department of Chemistry of Wollega University; Antibacterial assay test was done at Department of veterinary medicine laboratory of Wollega University.

3.2.Instruments and apparatus

Instruments and apparatus used in this study were mortar and pestle, Whatmann no.1 filter paper, TLC plate (pre coated), UV lamp, vacuum oven, separatory funnel, rotary evaporator, TLC chambers, glass column chromatography (borosilicate glass 3.3 ,34/35), beam balance and electronic balance (FA224), measuring cylinder, hot plate and burrette, conical flask, and reflux during saponification value analysis.

3.3.Chemicals and Reagents

The chemicals and reagents used in the experiment were analytical grades of solvents such as *n*-Hexane (99%), chloroform (99.8 %), ethylacetate (99.5%) and methanol (99%), sodium hydroxide, distilled water, hydrochloric acid, sulphuric acid, ferric chloride, ammonia, sodium hydrogen carbonate, potassium hydroxide, Dragendorff's reagent, DMSO, acetic anhydride, neutralized ethanol, silica gel, MH agar and chloramphenicol (30 µg).

3.4.Experimental Methods

3.4.1.Plant material collection and preparation

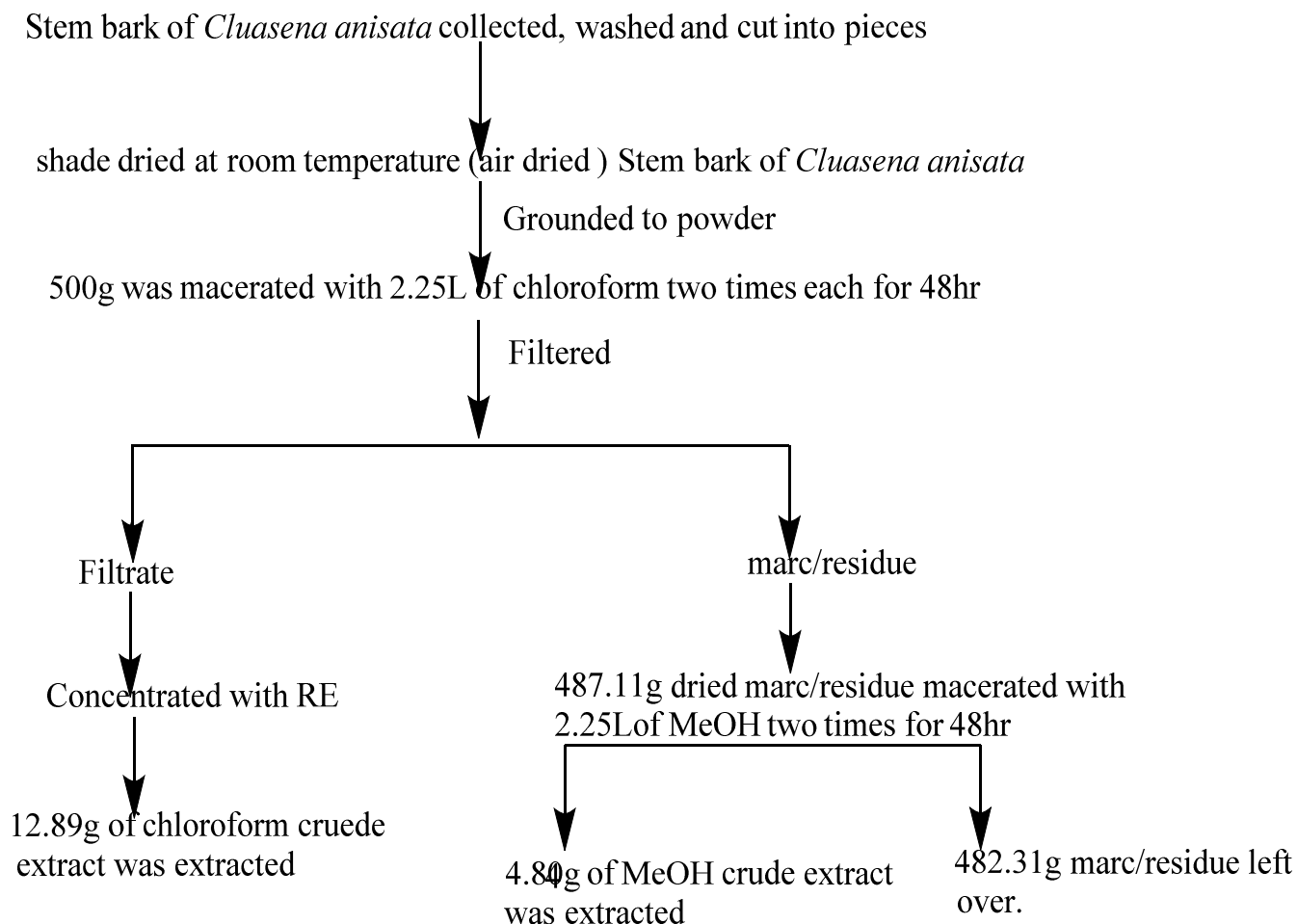
The fresh stem bark of *Clausena anisata* was collected from Gurra forest of Caliya district, west showa Zone Oromia region in June 2023. The plant was identified and authenticated by Dr. Fikadu Gurmesa the botanist at Wollega University department of biology and the voucher name was given (MT01/2023). The fresh stem bark of the *Clausena anisata* collected was washed with tap water. The washed stem bark of *Clausena anisata* were separately cut into small pieces and

air dried under shadow at room temperature. The air dried sample was grinded into powder using a mortar and pestle before being subjected to solvent extraction.

3.4.2.Solvent Extraction

About 500g of air dried stem bark of *Clausena anisata* was sequentially extracted by maceration extraction method using chloroform and methanol to obtain chloroform and methanol crude extracts.

First the powdered stem bark sample was extracted with 1.5 L of chloroform for 48 hours and then filtered using cotton followed by Whatman No.1 filter paper. Further the marc was extracted for another extra 48 hours, (2X48 hours) by following the same steps. All filtrate from the chloroform extract was concentrated using rotavapor at 30-35°C and combined together, lastly marcs were air dried and their masses were recorded. The extract was kept in fume were air dried, weighed and then the residue (marc) was extracted by methanol in similar way with chloroform for 96 hours (twice for 48 hours). Extracts of stem bark of *Clausena anisata* were collected and filtered using filter paper. Then the extracts were concentrated on rotary evaporator under reduced pressure, at 40°C and kept in fume hood until other analysis had carried out. The process is outlined in the following schume 1.



Scheme 1: General extraction procedure for the stem bark

3.5. Phytochemical analysis

Analyzing the presence and absence of phytochemicals, such as alkaloids, flavonoids, saponins, steroids, terpenoids and tannins in plant extract is an important part to target metabolites that are required for further analysis. Therefore in this study phytochemical analysis was conducted on the stem bark of *Clausena anisata* to screen phytochemicals. For the phytochemical analysis, one gram (1 g) of each crude extract (chloroform and methanol) were taken and dissolved in 25ml of respective solvents in different beakers. Then the stock solution was used for further phytochemical analysis by using the following standard methods.

3.5.1.Detection of alkaloids (Wagner's Test)

From stock solution of stem bark extracts of each solvent (1.0ml of each chloroform and methanol extract) was treated with Wagner's reagent (1.30g of iodine and 2.0g of potassium iodide was dissolved in 100ml volumetric flask with distilled water) and brown (reddish brown) precipitation was observed in both extracts which indicate the presence of alkaloids (Mathewos, *et al.*, 2015)

3.5.2.Detection of flavonoids (Alkaline Reagent Test)

From stock solution of stem bark extracts of each solvent (1.0 ml of each chloroform and methanol extract) was treated with alkaline reagent with 10% NaOH solution. Yellow fluorescence color observed in both extracts which indicate the presence of flavonoids (Sahira and Catrine, 2015).

3.5.3.Detection of steroids

From stock solution of stem bark extracts of each solvent (1.0 ml of each chloroform and methanol extract) was dissolved in 1.0ml of chloroform. A 2.0 ml of concentrated sulphuric acid (H_2SO_4) was slowly added then two lower layers with upper reddish-brown color formed for both extract which indicates the presence of steroids (Poongothai, *et al.*, 2011).

3.5.4.Detection of tannins

From stock solution of stem bark extracts of each solvent (1.0 ml of each chloroform and methanol extract) was treated with 2.0 ml of distilled water and boiled in a test tube and three drops of 0.1 % of $FeCl_3$ was added. The appearance blue black or brownish in both extracts indicates the presence of tannins (Akinpelu, *et al.*, 2008).

3.5.5.Detection of terpenoids (Salkowski Test)

From stock solution of stem bark extracts of each solvent (1.0 ml of each chloroform and methanol extract) was added with 0.4 ml of chloroform. A 0.6 ml of concentrated H_2SO_4 was added, two layers formed. The formation of reddish brown coloration in both extracts was observed which indicates the presence of terpenoids (Wadood, *et al.*, 2013).

3.5.6.Detection of saponins

From stock solution of stem bark extracts of each solvent (1.0 ml of each chloroform and methanol extract) was added with 5ml of distilled water in a test tube and shaken vigorously for

30 minutes .stable foam was observed after 30 minutes in the methanol extract which indicates the presence of saponins in methanol extract whereas in chloroform extract the foam was not observed after 30 minutes which indicates presence of saponins in chloroform extracts (Mojab, *et al.*, 2003; Sarwars, *et al.*, 2010). But from the two extracts methanol extracts contains more saponins than chloroform extracts.

3.6.Column chromatographic separation of chloroform extract

3.6.1.TLCAnalysis of the chloroform crude extracts

The crude extract were analyzed using precoated TLC plate (silica gel, 254nm and 365nm UVlamp) using *n*-hexane and ethyl acetate. The *n*-hexane and ethyl acetate mixture have showed good resolution and exhaustively assessed by varying the polarity (9:1,..., 1:9), Accordingly,*n*-hexane and ethyl acetate (8:2) has showed well resolved spots for the crude extracts when visualized under UV lamp at 254 and 356 nm. Further to detect the UV inactive spots the TLC was dipped in 10% H₂SO₄and heated on the plate until the spots was visible. Chromatogram of the crude extracts presented on(Appendix 5)

3.6.2.Isolation

3.6.2.1.Fractionation of chloroform extract

The chloroform extract acquired from the stem bark was utilized to conduct this experiment due to the possession of higher resolution on TLC, greater amount of crude extract and relatively good antibacterial activity test results. The chloroform crude extract of *Clausena anisita* (7 g) was dissolved in small amount of chloroform and adsorbed on 20g of silica gel (200-400 mesh). The 7g chloroform extract was placed in a glass column of size 34/35 (60 cm) for fractionation, by gravity elution. About 350ml of 100% *n*-hexane utilized for initial elution and 3.6L mixtures of *n*-hexane and ethyl acetate (EtOAc) in the following ratios (*n*-hex. 9:EtOAc 1), (*n*-hex. 8:EtOAc 2), (*n*-hex. 7:EtOAc 3),(*n*-hex. 6:EtOAc 4). The collected fraction were analyzed by TLC using 10ml of *n*-hexane and EtOAc mixtures as tank solution in the following ratios(*n*-hex. 9:EtOAc 1), (*n*-hex. 8:EtOAc 2), (*n*-hex. 7:EtOAc 3),(*n*-hex. 6:EtOAc4). A total of 72 fractions each 50ml were collected during the column chromatographic separation.

The collected fraction from 1-7 (100% *n*-hexane elution) has no spots and discarded. Fraction from 8-13(*n*-hexane 9: EtOAc 1 elution) has no spot and also discarded. Fraction 14 (*n*-hexane 9: EtOAc 1 elution) has one spot when observed under UV light at 254nm. Up on drying its mass is very small (unmeasurable). Fraction 15 and 16 (*n*-hexane 9: EtOAc1 elution) has two spots with different retention factors (Rf) when observed under UV and also appeared two spot when dipping in 10% H₂SO₄. Fraction 17-20 (*n*-hexane 9: EtOAc 1 elution) has three spot with different retention factor when observed under UV. Fraction 21(*n*-hexane 8: EtOAc 2 elution) has one spot when observed under UV and also appeared single spot when dipping in 10%H₂SO₄. But when solvent ratio of elution changed to 7:3, there was a few impurities seen when observed under UV. Up on drying the impurities washed with ethyl acetate and filtrated then TLC checked under UV shows one spots. Up on drying again it give **9.67mg** and it was then **labeled as CA₁**. The TLC of fraction 22-27 has more than two spots with different Rf values under UV light at 254nm and when dipped in 10% H₂SO₄. The TLC of fraction 28 has showed a single spot under UV lamp and after dipping in 10% H₂SO₄. But up on changing solvent ratio it showed two spots under UV light at 254nm and when dipped in 10% H₂SO₄so the fraction was discarded. The TLC of fractions 29, 30, 31 and 33 was showed two spots under UV light at 254nm and when dipped in 10% H₂SO₄with different Rf so the fractions was discarded. The TLC of fraction 32 has showed a single spot under UV lamp and when dipped in 10% H₂SO₄, but its mass was very small and also has oil nature. The TLC of fraction 34-40 was showed two spots with different Rf values under UV light at 254nm and when dipped in 10% H₂SO₄, so the fractions was discarded. The TLC of 41 and 42 was showed a single spot under UV lamp at 254nm and gave two spots when dipped in 10% H₂SO₄. The TLC of fraction 43-45 has showed a single spot under UV light and also appeared single spot when dipped in 10% H₂SO₄ but up on changing solvent ratio it showed two spots. The TLC of fraction 46-50 has showed a single spot with the same Rf under UV lamp and also appeared single spot when dipping in 10%H₂SO₄. The fraction ware combined together. Upon changing the solvent ratio it has a little impurity. Because of this it is separated into two as hexane soluble and ethyl acetate soluble. The hexane soluble fraction 46-50 has showed a single spot. Up on drying it gave **4.57 mg** and **coded CA₂**. Fraction 46-50 ethyl acetate soluble has showed a little impurity on TLC so it discarded. The TLC of fraction 51-54 has showed a single spot under UV light at 254 nm, but showed two spot when dipped in 10% H₂SO₄. The TLC of fraction 55-65 has no spots under UV light but when

dipping in 10% H₂SO₄ the spot was seen with similar R_f. Upon changing solvent ratio it showed two spots. The TLC of fraction 66-72 has shown no spot under UV light at 254 nm as well as also when dipped in 10% H₂SO₄ and discarded. Finally the fraction that showed single spot on TLC (fraction 21 and fraction 46-50 hexane soluble) were checked with different ratios of developing solvents *n*-hexane: ethyl acetate (9:1, 8:2, 7:3, 6:4 and 5:5) and again they are single spot (figure 2). They were then coded as **CA-1(9.67 mg)** and **CA-2 (4.57 mg)**. The isolated compounds were coded as CA-1 (R_f= 0.44) in 8:2 ratio of solvent system and CA-2 (R_f = 0.26) in the solvent system of 7:3 *n*-hexane: ethyl acetate as shown in (figure 2). Due to financial problem the obtained isolated compounds were not taken for further spectroscopic characterization (like ¹HNMR, ¹³CNMR, DEPT and IR spectroscopy). The following table 2 show number of fractions and ratio of elution solvents

Table 2 The number of fractions and ratio of elution solvents

S.No	Fractions	Ratio of elution solvents (<i>n</i>-hexane:Ethylacetate)	Total solvent volume (mL)
1	F ₁ -F ₇	100% hexane	350ml
2	F ₈ - F ₁₃	9 : 1	300ml
3	F ₁₄	9 : 1	50ml
4	F ₁₅ and F ₁₆	9 : 1	100ml
5	F ₁₇ -F ₂₀	9 : 1	150ml
6	F ₂₁	8 : 2	50ml
7	F ₂₂ – F ₂₅	8 : 2	200ml
8	F ₂₆ and F ₂₇	8 : 2	100ml
9	F ₂₈	8 : 2	50ml
10	F ₂₉ – F ₃₁ and F ₃₃	8 : 2	150ml
11	F ₃₂	8 : 2	50ml
12	F ₃₄	7 : 3	50ml
13	F ₃₅	7 : 3	50ml
14	F ₃₆	7 : 3	50ml
15	F ₃₇ – F ₄₂	7 : 3	300ml
16	F ₄₃ – F ₄₅	7 : 3	150ml
17	F ₄₆ – F ₅₀	7 : 3	250ml
18	F ₅₁ – F ₅₄	7 : 3	200ml
19	F ₅₅ – F ₇₂	6 : 4	900ml

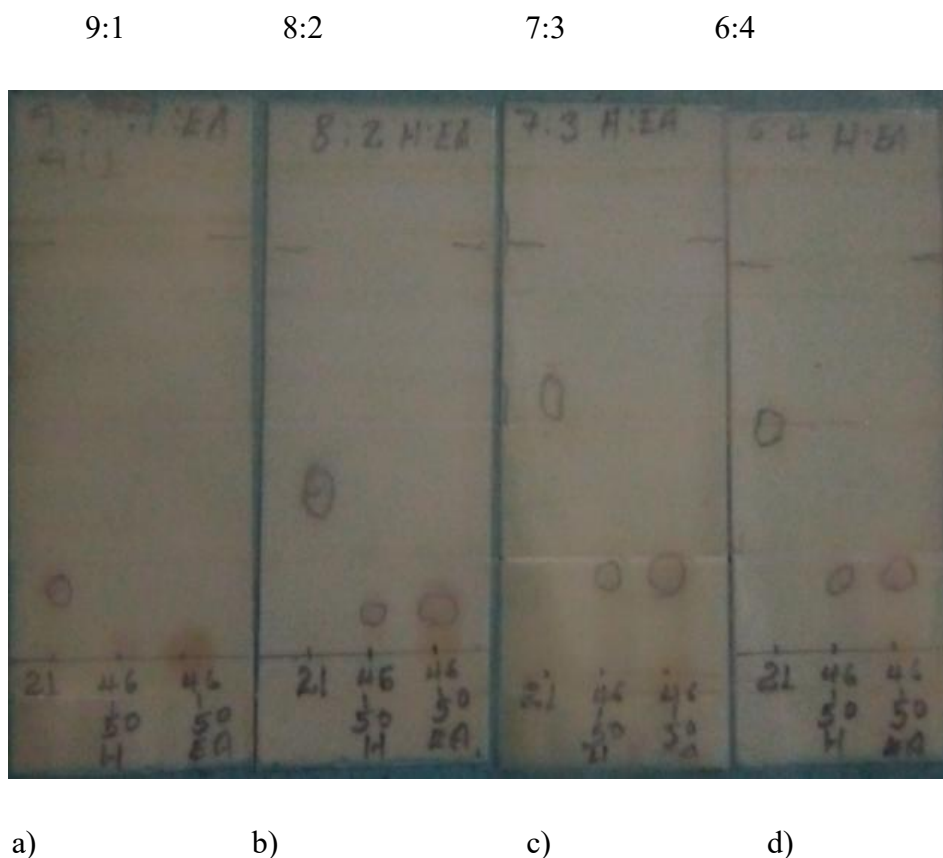


Figure 4:: TLC profiles of fraction collected from chloroform extracts of *Clausena anisata* in different solvent system after sperrying with H₂SO₄ and heating. (a , 9:1, b, 8:2, c, 7:3 and d, 6:4 with n hexane : ethylacetate solvent ratio).

3.7.Antibacterial Test

The antibacterial activity of *Clausena anisata* stem bark extracts was determined by using Agar disk diffusion method.

Antibacterial activities of the crude extracts were evaluated against *staphylococcus aureus*, *other coagulase negative Staphylococcus*, *Escherichia coli* and *staphylococcus enteric*. The bacterial cell suspensions was prepared according to the standard procedure.

The chloroform and methanol extracts of stem bark of *Clausena anisata* were evaluated *in vitro* for antibacterial activity by using the Agar disk diffusion method against two gram positive bacteria (*staphylococcus aureus* and *Other coagulase negative Staphylococcus*) and two gram

negative bacteria (*Escherichia coli* and *staphylococcus enteric*). Muller Hinton Agar (HHA) was used for bacteria growth as medium.

Solvent (chloroform) was used as negative control for chloroform crude extract and 10% dimethyl sulfoxide (DMSO) was used as negative control for methanol crude extract. Chloramphenicol was used as standard (reference) drug (positive control) against bacteria, the test bacteria were obtained from veterinary medicine laboratory of Wollega University.

3.7.1.Preparation of Inoculum

The test bacterial strains were transferred from the stock cultures and streaked on Mueller Hinton Agar (HHA) and incubated for 24 hours. Well separated bacterial colonies were used as inoculums. Bacteria were transferred using bacteriological loop to autoclaved Muller Hinton and mixed by gently swirling the flasks. Then the medium had poured to sterile petridish plates, allowed to solidify and used for the bio test. (Nwinyi, *et al.*,2009)

3.7.2.Preparation of test solution

1000mg of each solvent (chloroform and methanol) stem bark of *Clausena anisata* crude extract was dissolve in 10.00 ml of chloroform for chloroform extract and 10% of DMSO (dimethyl sulfoxide) for methanol extract that used in extraction to make a concentration of 100%(100mg/ml). Dilution of crude extract of this plant took place from its original solution, by taking 10.00mL of each extract and diluting by using chloroform for chloroform crude extract and 10% DMSO for methanol crude extract. Those dilution of extracts were 50%, 25% and 12.5%, (v/v) of the stem bark of *Clausena anisata*. The solutions were used to test antibacterial activities.

3.7.3.Testing for Antibacterial activity

The bacterial inoculums uniformly spread using sterile cotton swab on a sterile Petri dish MH agar. Filter paper discs of about 6 mm diameter were placed in a beaker and sterilized in an autoclave for 10 minutes. Already prepared crude extracts solutions of stem bark extract were then pipette to the sterilized paper.

Discs and reference drug (positive control) were transferred in to petredish. Antibacterial activity evaluated by measuring the inhibition zone (mm) against the studied microorganisms, including disc diameter. Control experiments were carried out under similar conditions using Chloramphenicol for antibacterial activity as a standard drug. The zones of growth inhibition around the disks were measured after 24 hours of incubation at 37°C for bacteria. The inhibition zones was measured with a ruler and compared with the positive control discs (discs containing Chloramphenicol). For chloroform crude extract chloroform were used as negative control and for methanol crude extract 10% DMSO used as negative control during the whole test on bacteria.

The antibacterial activities were evaluated and compared to that of standard drug (Chloramphenicol) by measuring the zone of inhibition against two gram-positive bacteria (*staphylococcus aureus* and *Other coagulase negative Staphylococcus*) and two gram- negative bacteria (*Escherichia coli* and *staphylococcus enteric*)

3.8.Determination of Saponification Value and Acid Value

Saponification Value (SV) refers to the amount of ester that can be hydrolyzed and turned into soap. Saponification value of oils is the number of mg of KOH required to saponify one gram (1gm) of oil or fats. Where as **acid Value (AV)** refers to the amount of free fatty acid in the oil. Acid value is the number of milligrams of KOH required to neutralized the free fatty acids present in one gram (1gm) of oil or fat. From the study it was observed that the stem bark of the plant yeilded greater amount of oils in both chloroform and methanol extract this shows the palnt is reach in oil so SV and AV needed to determined.

3.8.1.Determination of Saponification Value

A 0.65g of oil sample was weighted and added into a volumetric flask. Then 25ml of 0.5N alcoholic KOH was pipette and reflux the mixture using a water condenser on a water path for about 30 minutes for complete saponification. The flask and the condenser ware then cooled but not sufficiently to form a gel, the inside of the condenser was washed down with about 1ml of distilled water. The condenser was disconnected and 1ml of phenolphthalein indicator added.

The solution was titrated with 0.25N HCl until the pink color just disappeared. A blank determination was conducted simultaneously without the oil sample.

$$\text{Saponification value} = \frac{(V_b - V_a) \times \text{Normality of HCl} \times \text{molar weight of KOH}}{\text{Weight of sample (oil) in gram (0.65g)}}$$

Where: V_b = volume in ml of standard HCl required for the blank titration (22.1ml).

V_a = volume of HCl required for the titration of sample or oil (19ml)

M_w = molecular weight of KOH (56.1g/mol) and

N = Normality of HCl (0.25N)

3.8.2. Determination of Acid value

The free fatty acids are estimated by titrating it against KOH using phenolphthalein as indicator. The acid value is mg of KOH required to neutralize the free fatty acids present in one gram of sample. Dissolve 0.65g of oil in 25ml of neutralized ethanol (a mixture of ethanol and ether (1:1 ratio)) and then added 3-4 drops of phenolphthalein indicator and mixed by shaking. Titrate the content against 0.05N KOH solution of a 6ml until a pink color which persisted for fifteen second was obtained.

$$\text{Acid value} = \frac{\text{volume of KOH} \times \text{Normality of KOH} \times \text{molar weight of KOH}}{\text{Weight of sample (oil) in gram}}$$

CHAPTER FOUR

4.RESULTS AND DISCUSSION

The air dried stem bark of *Clausena anisata* was exhaustively extracted with 2.25 L chloroform and 2.25 L methanol respectively. The percentage yield of the crude extracts were calculated and presented in (table 1). The extracts were future subjected to phytochemical screening (table 2), antibacterial activity evaluation (table 3), saponification and acid value determination.

Based on the antibacterial activity, clarity of spots on the TLC and also quantity of the chloroform crude extract, chloloroform crude extract were subjected to column chromatographic separation and resulted two pure isolates compounds. The results of phytochemical screening, antibacterial activity, saponification value, acid value and chromatographic separation were discussed in subsequent section under this chapter.

4.1.Yields of Stem bark of *Clausena anisata* extracts

The yield obtained from chloroform and methanol extracts was described in the below (table 3) was calculated by the following formula.

$$\text{percentage yield} = \frac{\text{mass of crude extract}}{\text{mass of sample}} \times 100$$

Table 3: masses and percentage yield of chloroform and methanol extracts from stem bark of *Clausena anisata*

Solvent used for extraction	Mass of sample in gram	Physical characteristics of the extract	Mass and percentage yield	
			Mass of crude extract in gram	Percentage yield
Chloroform	500	Brown (semi solid)	12.89	2.58
Methanol	487.11	Brown (semi-solid)	4.8	0.98

As presented in this table the chloroform extract yielded 12.89g crude extract and methanol gave 4.8g. From this result the chloroform crude extract is higher in percent than methanol crude extract that shows the plant contains much of medium polar compounds.

4.2. Phytochemical Screening of Crude Extracts of *Clausena anisata*

The phytochemical screening study is intimately related to the needs of finding bio-active chemical constituents from medicinal plant extracts. The chloroform and methanol extracts of stem bark of *Clausena anisata* were qualitatively tested for the presence of phytochemical compounds (secondary metabolite) and the results were presented in the following table below.

Table 4: phytochemical screening test results of stem bark of *Clausena anisata*

Secondary metabolites	Reagents	Crude extracts of the stem bark of <i>C. anisata</i>	
		Chloroform	Methanol
Alkaloids	Wagner's Test	+	+
Flavonoids	Alkaline Reagent Test	+	+
Steroids	Salkowski Test	+	+
Tannins	Ferric chloride test	+	+
Terpenoids	Salkowski Test	+	+
Saponins	Foam test	+	++

Key: + = the presence of phytochemical constituents

++ = the presence of phytochemical constituents in high concentration.

The phytochemical screening tests of both chloroform and methanol crude extracts of stem bark of *Clausena anisata* have showed the presence of secondary metabolites like alkaloids, flavonoids, steroids, tannins, terpenoids and saponins as shown in table 4. The methanol crude extract has higher concentration of saponins than chloroform crude extracts. The image of phytochemical test was given in (Appendix 2). The phytochemical test showed that all alkaloids, flavonoids, steroids, tannins, terpenoids and saponins are present in both chloroform and methanol extracts. However in methanol crude extract, saponin was more concentrated than chloroform crude extracts.

4.3. Analysis of Antibacterial Activities of *Clausena anisata*

The antibacterial activities of stem bark extracts of *Clausena anisata* against two gram positive bacteria (*Staphylococcus aureus* and *Other coagulase negative Staphylococcus*) and two gram

negative bacteria (*Escherichia coli* and *staphylococcus enteric*) for 20µL to 40µL dose levels with respective diameter of zone of inhibitions was presented in table 4.

In all cases the chloramphenicol which was used as a positive control has showed highest zone of inhibitions, while solvents which had been used as a negative control has showed no inhibition zone indicating that the bacterial activities exhibited only due to the plant extracts and the solvent has no effect.

Table 5: antibacterial activities of *Clausena anisata* stem barks crude extracts

Crude extracts, positive and negative control	Concentration of plant extract, positive and negative control	Diameter of zone of growth inhibition (mm)			
		Gram positive bacteria		Gram negative bacteria	
		<i>S. aureus</i>	Other C. negative Staphylococcus	<i>E. Coli</i>	<i>S. Entric</i>
Chloroform (CHCl ₃) extract	100	11.07	10.03	11.85	12.22
	50	8.66	--	10.15	10.59
	25	7.39	--	7.85	6.54
	12.5	6.80	--	--	--
Negative control	Chloroform	--	--	--	--
Methanol (MeOH) extracts	100	7.13	6.39	7.04	7.26
	50	6.6	--	6.25	6.43
	25	--	--	--	--
	12.5	--	--	--	--
Positive control	chloramphenicol(30µg)	25.16	24.68	26.16	24.86
Negative control	10% DMSO	--	--	--	--

Key: (--) indicates extracts that have been not active against bacteria.

The crude stem bark extracts of *Clausena anisata* showed varying degree of response against the tested bacterial species. In all cases the highest dose showed higher inhibition zone than lower dose indicating that the antibacterial activities have exhibited the concentration dependent mode of action.

The above results showed the antibacterial activities varied with the bacterial species on which the tests were conducted and the solvents used for extraction. Considering doses, the 100 mg/ml has showed better antibacterial activity and against tested bacterial species, *staphylococcus enteric* (12.22 mm) at 100mg/ml concentration by chloroform crude extracts and 7.26 mm at the same concentration (100mg/ml) by methanol crude extracts, while *Other coagulase negative Staphylococcus* has lowest sensitivity. Thus the present study revealed the antibacterial activities varied with dosage concentration and bacterial species. The variation may be due to difference in quality, quantity and nature of chemical components in the extracts.

4.4.Saponification Value (SV)

The saponification value of *Clausena anisata* crude extracted was 66.88 mg KOH in 0.65g of oil. Since soap is a byproduct of alkaline hydrolysis and was made of potassium or sodium salts of higher fatty acids, saponification is the alkaline hydrolysis of oils or fats.

The basic procedure involves titrating excess potassium hydroxide with a standard volumetric hydrochloric acid solution after boiling a sample under reflux with an ethanolic potassium hydroxide solution. The saponification value is a crucial analytical measure for determining the average molecular weight of the fatty acids in the glycerides that make up oil. The molecular weight of the fatty acids in the glycerides increases with decreasing saponification value and vice versa (Negera,2018)

$$\text{Saponification value} = \frac{(V_b - V_a) \times \text{Normality of HCl} \times \text{molar weight of KOH}}{\text{Weight of sample (oil) in gram (0.65g)}}$$

$$\text{SV} = \frac{(22.1 \text{ ml} - 19 \text{ ml}) \times 0.25 \text{ N} \times 56.1 \text{ g/mol}}{0.65 \text{ g}}$$

$$= 66.88 \text{ mg of KOH needed to saponfy 0.65g oil.}$$

Where: V_b = volume in ml of standard HCl required for the blank titration (22.1ml).

V_a = volume of HCl required for the titration of sample or oil (19ml)

Mw = molecular weight of KOH (56.1g/mol)and

N = Normality of HCl(0.25N)

4.5.Acid value (AV)

The acid value of the extracted oil (0.65g dissolved in 25ml of neutralized ethanol or a mixture of ethanol and ether in 1:1 ratio) (*Clausena anisata*) titrated with 0.05N KOH solution was

26 mg of KOH in 0.65g of oil. The FAO/WHO recommendation states that all edible oils have a maximum allowable level of less than 0.6mg KOH in 1gram of oil.

The acid value is a number that indicates how much potassium hydroxide in mg is needed to neutralize the free acid in 1 g of substance. If the system contains additional acid components like amino acids or acid phosphates, the acid value could be over estimated. Since the acid value is frequently a good indicator of the conversion of triacylglycerol into free fatty acids, it negatively affects the quality of many lipids. The hydrolytic rancidity is quantified by the acid value. It generally indicates whether or not the oil is edible. Because triglycerides are transformed into fatty acids and glycerol as an oil ages, the older the oil, the higher the acid value will be (Negera, 2018)

$$\text{Acid value} = \frac{\text{volume of KOH} \times \text{Normality of KOH} \times \text{molar weight of KOH}}{\text{Weight of sample (oil) in gram}}$$

$$AV = \frac{6 \text{ ml} \times 0.05N \times 56.1 \text{ g/mol}}{0.65 \text{ g}}$$

$$= 26 \text{ mg of KOH is required to neutralize the free fatty acids in 0.65 g of the oil}$$

From the above saponification value and acid value determination we conclude that the plant have potential for soap preparation.

CHAPTER FIVE

5.SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1.Summary

The present study was designated to investigate phytochemical composition, antibacterial activity, saponification and acid value determination of steam bark extract of *Clausena anisata*. The steam bark of *Clausena anisata* was extracted sequentially with chloroform and methanol by maceration techniques. The extract obtained was concentrated by rotary evaporator and stored in fume hood until further analysis. From the extraction processes the highest yield was obtained from chloroform extract. The phytochemical screening test on the stem barks of *Clausena anisata* was revealed the presence of alkaloids, flavonoids steroids, tannins, terpenoids and saponnins. However the concentration of saponnins high in methanol crude extract than chloroform crude extracts.

The antibacterial activity of extract was evaluated by using disc diffusion method. The antibacterial activity of this plant extract was active against selected bacteria (two gram positive bacteria; *staphylococcus aureus* and *Other coagulase negative Staphylococcus* and two gram negative bacteria; *Escherichia coli* and *staphylococcus enteric*). Antimicrobial activity tests showed some antibacterial potency of the extracts when it was compared with standard antibiotics (standard drug; chloramphenicol). The crude extract was more sensitive against *staphylococcus enteric* bacteria and have greater zone inhibition (12.22 mm) at 100mg/ml in case of chloroform crude extracts and (7.26mm) at 100 mg/ml in case of methanol crude extract, where as *Other coagulase negative Staphylococcus* was less sensitive and has low inhibition zone (10.03 mm) at 100mg/ml in case of chloroform extracts and (6.39mm) at 100mg/ml in case of methanol crude extracts. The zone of inhibition was dependingon the concentration of extracts.

The chloroform extract was selected for isolation and column chromatography due to greater amount of crude extract obtained by chloroform extraction and good antibacterial test result. Two single compounds were separated by using *n*-hexane and ethyle acetate solvents as eluent in different ratios. Based on TLC analysis the plant contains several chemical constituents which were not isolated in this study because of financial problem.

5.2.Conclusion

Natural products have been a source of drugs for centuries and continue to be used as an alternative approach on treatment for various diseases caused by protozoa, bacteria, fungi, viruses and helminthes. Currently, the growing interest of consumers in substances of natural origin in association with the increasing concern surrounding potentially harmful infections disease has directed to a rising interest in the use of plant extracts as functional ingredients in many pharmaceutical products. Stem bark of *Clausena anisata* were used locally for treatment of various diseases. Based on this ethno botanical information, this study was designated to perform investigation of phytochemical and antibacterial activity tests and also determination of saponification value and acid value of extracts of stem bark of *Clausena anisata*.

In the present study the phytochemical screening test on the stem bark of *Clausena anisata* was revealed the presence of alkaloids, flavonoids steroids, tannins, terpenoids and saponnins. However, the compositions have varied with solvents used for the extraction. Based on the reported work crude extract of stem bark of *Clausena anisata* has antibacterial property. This could justify the common use of this plant in Ethiopian as traditional medicinal to treat bacteria and other ailments. The TLC profile of chloroform and methanol extract was similar. However, chloroform extract was selected for separation and isolation, due the highest amount of chloroform extract obtained and also chloroform extract has good antibacterial activity.

5.3.Recommendation

From the study it was observed that the stem bark of the plant yeileded greater amount of oils in both chloroform and methanol extract this shows the palnt is reach in oil therefore future study is needed to analysis the oil extracts of the plant using GC-MS and other analytical methods. Also from our preliminary experiment on saponification and acid value determination we observed that the plant have potinatial for soap preparation therefore we recommend further study on this regards.

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Appendix

Appendix 1 picture that shows the process of soaking upto concentrating the extracts.



a) picture during shaking
Macerated stem bark of
C.anisata



b) picture during filtration



c) Picture of filtered solution.



d) Picture taken during concentrating by RE.



e) Crude extract of *C.anisata*

Appendix 2-phytochemical screening of Stem bark extract of *C.anisata*

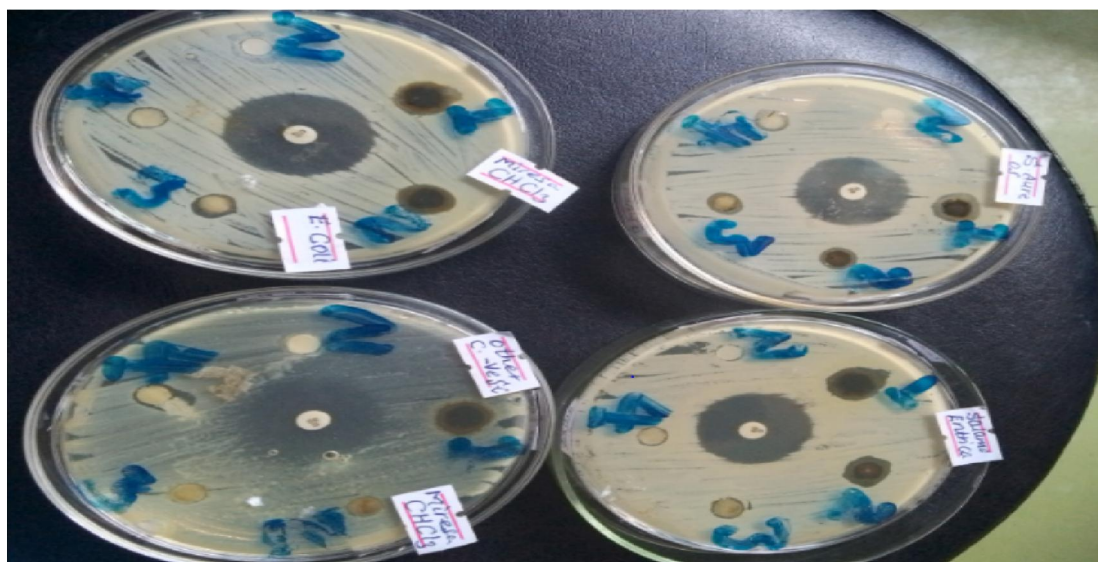


a) phytochemical test of methanol extract of stem bark of *C.anisata*



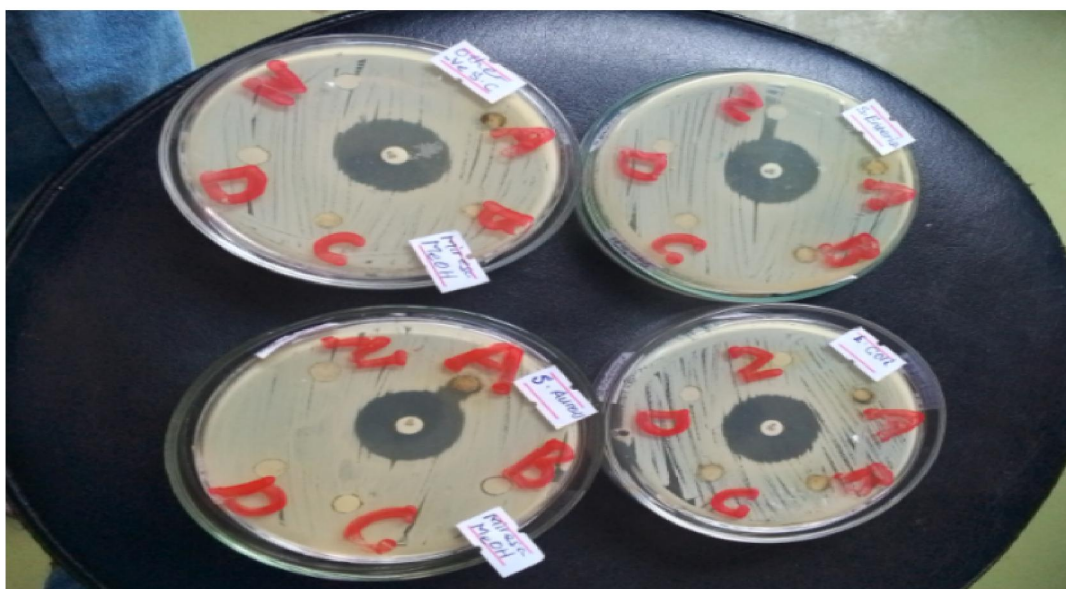
b) phytochemical test of chloroform extract of stem bark of *C. anisata*

Appendix 3-Antibacterial activity test of *Clausena anisata* by: a) chloroform stem bark extract,
c) methanol stem bark extract.



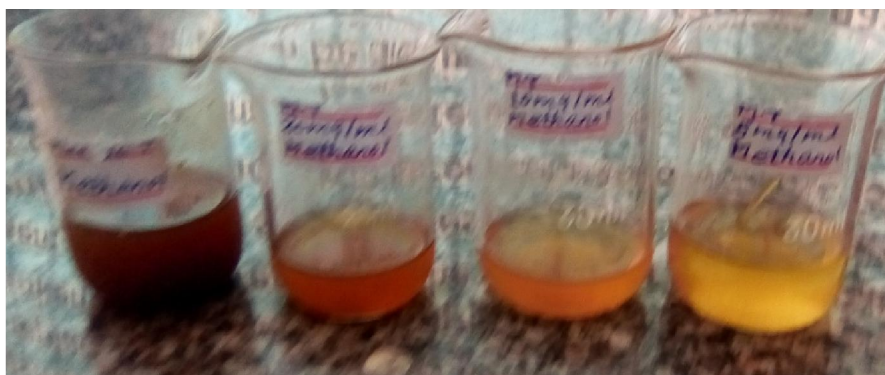
Key: 1 represent 100%, 2 represent 50%, 3 represent 25%, 4 represent 12.5% and N represent negative control

a) antibacterial activity of chloroform extract of *C. anisata*

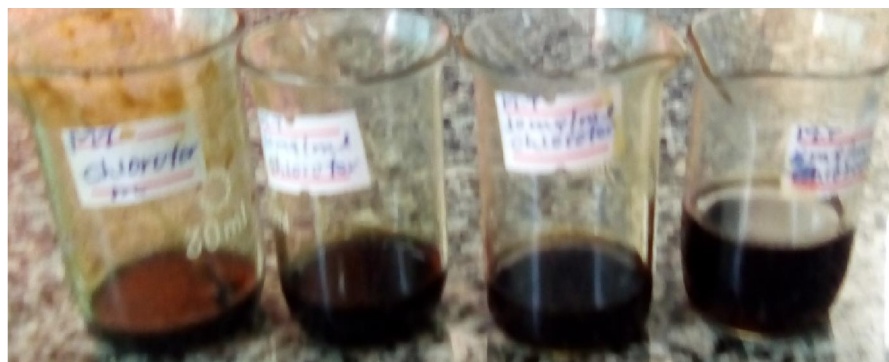


b) antibacterial activity of methanol extract of *C. anisata*

Appendix4 : solution of stem bark extracts of *C. anisata* prepared for antibacterial activity



a) methanol extract

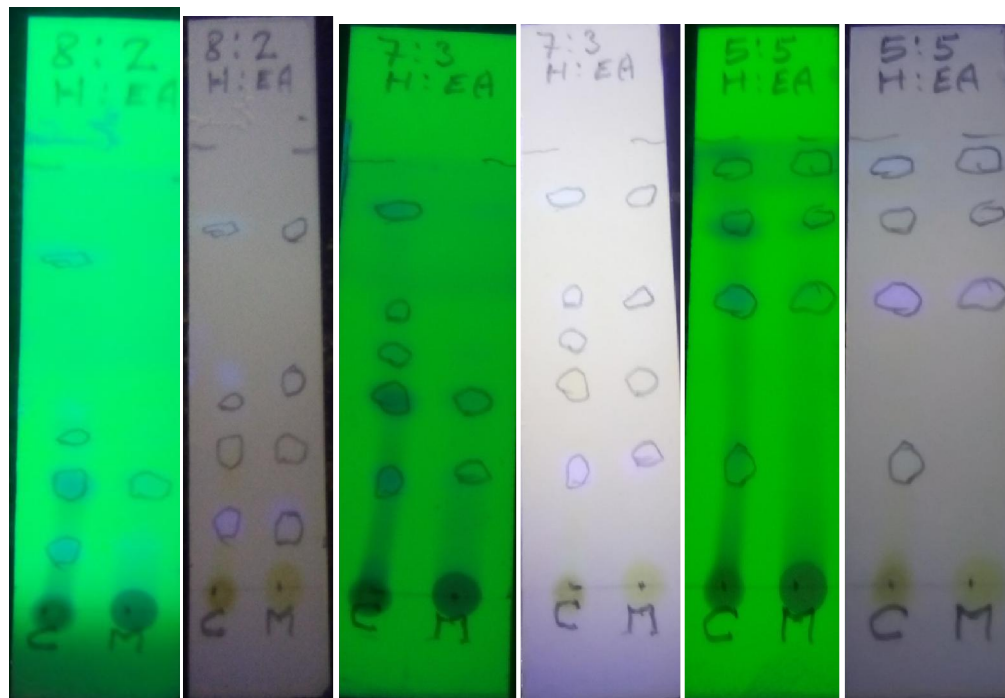


b) chloroform extract



c) both extracts

Appendix 5: TLC profile of chloroform (C) and methanol (M) crude extracts with different solvent ratios.



8:2

8:2

7:3

7:3

5:5

5:5

Appendix 6: Column packing, sample isolated and collected.

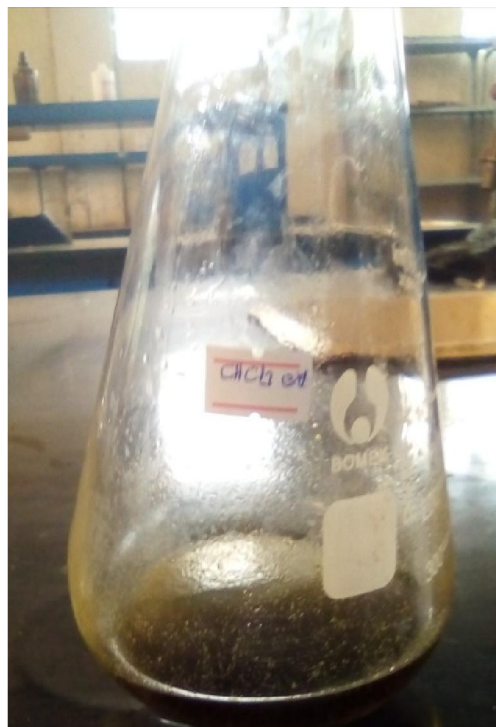


Picture during packing column Chromatography

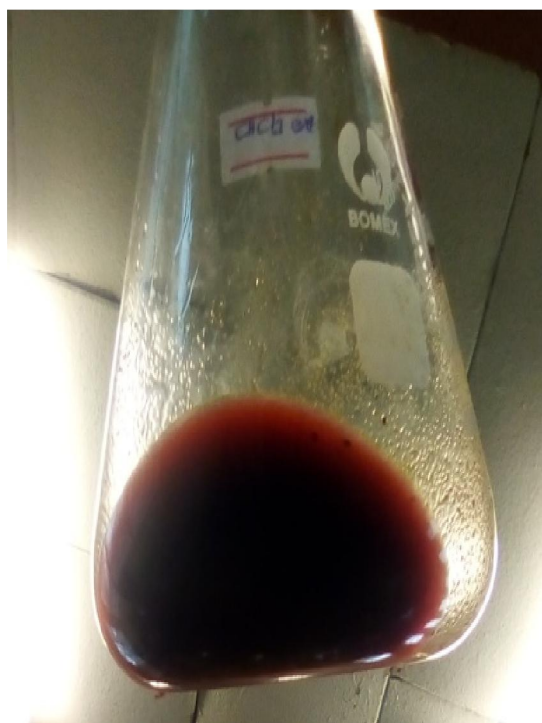


Sample isolated and collected

Appendix 7: color change during acid value determination



Dissolved sample in 25ml of



after titrated with 0.05N KOH, a pink

Neutralized ethanol

color persists for at least 15 seconds.

Appendix 8: saponification value determination process



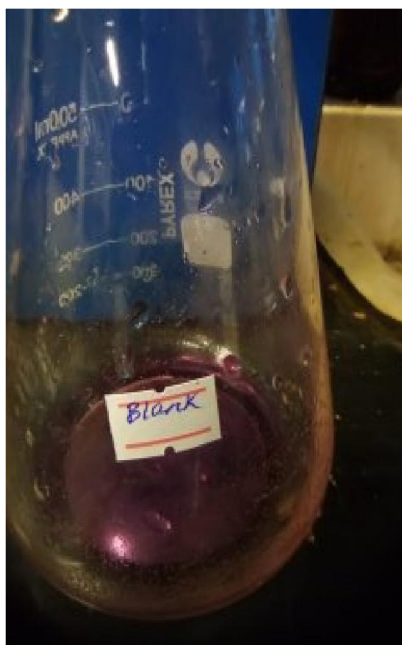
a) Refluxing process

b) color of sample

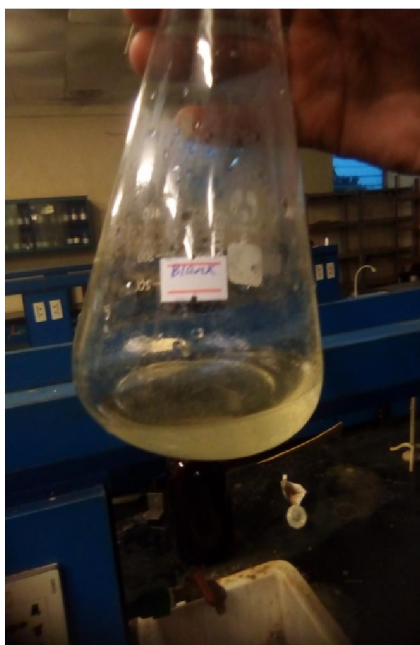
**c) color after addition
of phenolphthalein**

**d) color after
titration**

b) Blank titration:



Before titration



after titration pink color changed to colorless