**WOLLEGA UNIVERSITY**

**SCHOOL OF GRADUATE STUDIES**

**MSC RESEARCH PROPOSAL**

# ISOLATION OF SECONDARY METABOLITES FROM STEM BARK OF *MILLETTIA FERRUGENIA* FOR ANTIBACTERIAL TEST

**BY**

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## APPROVAL SHEET FOR SUBMITTING RESEARCH PROPOSAL

Isolation of secondary metabolites from stem bark of millettia ferrugenia for antibacterial test

Submitted by:

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## Abbreviation and Acronym

| | | |
|---|---|---|
| 13C NMR | : | Carbon 13 Nuclear Magnetic Resonance |
| 1H NMR | : | Proton Nuclear Magnetic Resonance |
| CC | : | Column Chromatography |
| COSY | : | Correlation Spectroscopy |
| DEPT | : | Distortionless Enhancement by Polarization Transfer |
| DMSO | : | Dimethyl sulfoxide |
| FT-IR | : | Fourier Transform Infrared Spectroscopy |
| HIV | : | Human Immunodeficiency Virus |
| HMBC | : | Heteronuclear Multiple-Bond Correlation |
| HSQC | : | Heteronuclear Single Quantum Correlation |
| IR | : | Infrared |
| TLC | : | Thin Layer Chromatography |
| UV | : | Ultra Violet |
| WHO | : | World Health Organization |

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## Abstract

The wide spread of disease-causing bacteria is contributed to the global health burden and kills millions of people in developing countries including Ethiopia. The major problem of disease-causing bacteria is resistance to the available drugs. It has been reported that plants are the good sources of antimicrobial compounds that are either used directly or synthetically modified. This study was interested to investigate phytochemicals for antibacterial and antioxidant activity evaluation from medicinal plant that is used in traditional way. For this purpose, *Millettia ferrugenia,* the medicinal plant that have been used in Ethiopia societies to treat different diseases like asthma, inflammation, malaria, snake bites, earache, bacterial infection of nails and infectious micro organism such as bacteria and a worm was selected. To attain the proposed objective the stem bark of *Millettia ferrugenia* was selected based on the traditional claims. The stem bark of the plant will be collect from the study area and extract with different organic solvents from less polar *n*-hexane to more polar methanol by cold maceration methods. The obtained crude extract will be evaluated for their antibacterial activities against four bacterial strains (*Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*). The extract with good antibacterial activity will be further characterized by using UV, IR and NMR spectroscopic methods.

**Key words**: millettia ferrugenia, phytochemical, antibacterial activity, stem bark.

# 1. INTRODUCTION

## 1.1. Background of the study

Medicinal plants have been used since ancient time for human healthcare and still remain the most widely used medication system in developing and least developed nations. Medicinal plant is defined by many authors as any plant that contains substances or precursors that can be used for therapeutic purpose, synthesis of useful drugs, suppressing, preventing, or curing many forms of diseases both for human being and their domestic animals.

The majority (80%) of Ethiopian people depends on traditional medicine for their health care, and more than 95% of traditional medicinal preparations made from plant origin (Ashenif, 2017). Medicinal plants have been used since ancient time for human health care and still remain the most widely used medication in developing and least developed nations. Ethiopia has a long history of using plants as traditional medicine, but knowledge about the extent and characteristics of traditional healing practices is constrained and it has been ignored in the national health care system (Deribe et al., 2006). Plants have a long history of use as traditional medicines and have become an outstanding and reliable source for an essential role in health care which are considered fewer side effects (Abolfazl, 2014). Over centuries of research, several medicinal plants have been proved to be effective in the treatment of wide range of simple to complex diseases (Iwu, 2014).

Medicinal plants contain many special substances and chemicals (micro components) having healing activities used for medicinal purpose achieving curing different diseases (Bajpai and Agarwal, 2015). The world Health organization estimates that 8o% of the people in developing countries of the world rely on traditional medicinal plants (WHO, 1978) for their primary health care needs, and about 85% of traditional medicine involves the use of plant extract. The reliance of people on medicinal plant has been for reasons of cost-effectiveness, acceptability, biomedical benefits and accessibility (Srivastava, 2000). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and other phenol compounds (Edeoga et. al., 2005).

The antimicrobial value of medicinal plants relies on some chemical substances produced by this plant. The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic effects of the phytochemicals in them. (Adesokan, 2008). A large proportion of the population of developing countries, use traditional medicine alone or in combination with Western drugs to treat a wide variety of health ailments ( Gidey, Y. 2010 ). With the renewed interest from Western countries in herbal remedies, and the increasingly urgent need to develop new effective drugs, traditionally used medicinal plants have recently given the attention of the pharmaceutical and scientific communities (Taylor et al, 2010). Various researchers have been discovering new biologically active compounds from natural products for the most successful source of potential drug leads (Newman, 2012). Medicinal plants are 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 (Ncube et al., 2008).

## 1.2. Statement of the problem

Bacterial infection diseases are highly prevalent and contribute largely to the global disease burdens and kill millions of people in which this burden is serious in developing countries including Ethiopia (Borkotoky, 2013).

The major contributor is the drug resistance, which has become a major clinical and public health problem in the world today. It has led to higher treatment costs, increased morbidity and mortality, and in some cases, permanent loss of specific drug therapies. Moreover, due to less availability, accessibility and affordability of modern drugs coupled with its high cost and adverse effects have worsen the situation; the investigation of safe, more effective, affordable and readily available anti-bacterial agent is necessary to combat or minimize the current situation.

Therefore, a continued investigation of medicinal plants and screening against their biological activities is still needed today specifically on those plants which have recognized traditional uses. It is necessary to investigate plant extract in order to validate their medicinal use and to identify the active constituents which may use in drug discovery. A number of researchers reported biological activity of the crude extracts from different parts of *Millettia ferrugenia* as well as the pure isolation of compounds. However, until today, no phytochemical studies have

been carried out to identify the active metabolites from the stem bark of *Millettia ferrugenia* in Ethiopia. In contrast to its wide use of traditional practices, the phytochemical studies on *Millettia ferrugenia* are rare and much is still unknown about its chemically active constituents due to its limited distribution. Thus, in this project phytochemical analysis of plant constituents will be carried out from its stem bark. Considering its wide traditional use, to the best of our knowledge, there are very few studies conducted on this genus with in Ethiopia. Thus, this project is intended to fill this gap.

## 1.3 Objectives of the study

### 1.3.1. General objective of the study:

The main objective of this study is to isolates secondary phytochemical compounds from stem bark of *Millettia ferrugenia,* and evaluate their antibacterial activities of the extracts.

### 1.3.2. Specific objectives of the study

1. To extract compounds from stem bark of the *Millettia ferruginea* using *n*-hexane, ethyl acetate and methanol solvents, successively.

2. to conduct phytochemical screening of the crude extract by using standard procedures.

3. To isolate and characterize compounds from the crude extracts of the stem bark of the *Millettia ferrugenia* Using chromatographic and spectroscopic methods.

4. To investigate the ant bacterial activity of the crude extracts as well as isolated compounds by disc diffusion method.

## 1.4 Significance of the study

The study on the stem bark extracts of *Millettia Ferruginea may* help to arrive at new natural products. This study can also provide important information about the phytochemical components of *Millettia Ferruginea* stem bark extract. In addition, the study can provide some information for researchers who are interested to conduct further studies on this plant. Therefore, the findings of the work would help to isolate the promising compound from stem bark of *Millettia ferrugenia* which can be used as guide compound in the discovery of antimicrobial agents. This research can provide an evidence base for the traditional use of the plants by identifying their chemical constituents of the plant and validate through biological screening.

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

## 2. Literature Review

### 2.1 Plant Description and Taxonomic Classification of *Millettiaferruginea*

*Millettiaferruginea* (sotallo in Afan Oromo) belongs to the family Leguminosae (fabaceae) and belongs to Papilionoideae subfamily. It is an indigenous plant species found only in Ethiopia. The family Leguminosae is the third largest family of flowering plants with 730 genera and over 200 species, most of which are shrubs and trees found in both temperate and tropical regions. *Millettiaferruginea* is umbrella-shaped or flattened at top, and grows up to a height of 25-35m (Karunamoorthi et.al., 2009, Kothai and Befirdu, 2012).In Ethiopia, two sub species of *Millettia* are found namely, *Millettia ferruginea* and *M. darassana* (Thulin, 1983). The distribution of Millettia *ferruginea* is between 1,000 - 2,500 m above sea level (MacLachlan, 2002), commonly found in the northern part of the country (Thulin, 1983) with multipurpose tree as all parts of *Millettia ferruginea* (fruit, leaves, stems and root) possess traditional medicinal action to treat amoeba, wound healing, earache and nails infection, tooth aches and gonorrhea (Belachew, 1993; Fisseha et al., 2009) or skin infection (mujele) having antimicrobial and insecticidal activity (Eyob et.al., 2010; Choudhury et.al., 2016). Despite its seed oil is used as nutritive source for human food rich in amino acids (Berhanu and Amare, 2013), this seed extract has a toxic effect on adult ticks in vitro (Choudhury and Yoseph, 2015), while leaves, flowers and twigs of *Millettia ferruginea* are important feed resource for ruminants animals(Takele et.al., 2014). *Millettia ferruginea* has been investigated extensively in documents recommending different plant parts extracts application against skin infection, tooth ache, *STD (gonorrhea)* or storage insect pests (Marick et.al., 2017). *Millettia ferruginea* Oil is clearer brown yellow in colour and less viscous (Catarelli et al., 1993).In addition to its high protein content, *Millettia ferrugenia* seed contains a high concentration of minerals, especially phosphorus, potassium, magnesium sodium and calcium (Andualem & Gessesse, 2010). It has a potential to supply sufficient amount of minerals for consumers and microbial media for microorganisms (Andualem & Gessesse, 2014). The seeds of these species are used as insecticides, pesticides and in folk medicine.

Figure 3: Image of *Millettiaferruginea*

Taxonomy of *Millettiaferrugenia*

Kingdom: plantae

Family : Leguminoseae

Class : Angiosperms

Afan Oromo : Sootallo

Species : ferruginea

Genus : Millettia

Binomial name : *Millittia ferruginea*

## 2.2 Traditional uses of Millettia ferrugenia

*Millittia ferruginea*(seed, leaves, stem barks and root) possess traditional medicinal action to treat amoeba, earache and nails infection, tooth aches and gonorrhea (Belachew, 1993; Fisseha et al., 2009) or skin infection (mujele) having antimicrobial and insecticidal activity (Eyob et al., 2010; Choudhury et al.,2016).Its leaf powder is also used to treat wounds, treatments of insects bites and its bark juice is used to treat wounds by applying it once a day for time of a period of 4-5 days( Kamble *et al*2010*).Millettia ferrugenia* is traditionally used for dressing mujele an infection caused by an insect present in the soil(Giday, 2007). *Millettiaferrugenia* plant is used traditionally for the treatment of bacterial and malarial infections (Khalid et al., 1986).

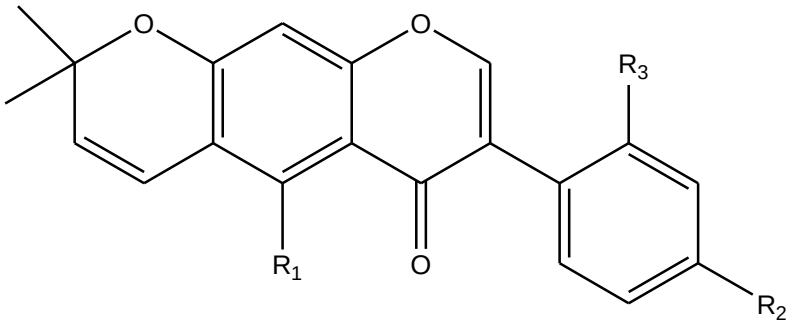
## 2.3. Phytochemicals from Genus Millettia and Compounds isolated from Millettia ferrugenia

Phytochemical are naturally occurring in leaves, fruits, vegetables and roots of these plants, and used for defense mechanism and protect the plants from various diseases. The genus *Millettia* is a rich source of different classes of flavonoids including isoflavonoids and also consists of minor compound such as coumarins, terpenoids and alkaloids (Asomaning et al, 1999). Isoflavones constitute the largest group of natural isoflavonoids. It has been mostly found in the subfamily Papilionoideae of the Fabaceae family. However, the common emphasis of the fact that isoflavonoids are characteristic metabolites of leguminous plants sometimes leads to overlooking that the presence of isoflavonoids in other families. Previous studies of extracts of *Millettia* species have led to the isolation of flavones, flavanones, chalcones, rotenoids and isoflavones (Dewik, 1994).

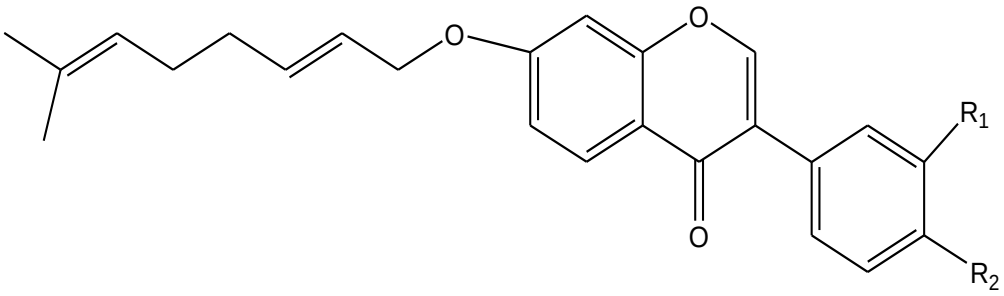
Isoflavones form the largest group of natural isoflavonoids. Among the isoflavonoids of this genus, isoflavones are the most predominant secondary metabolites (Dewik, et al 1999). Carren (2017) several isoflavonoids have been reported from different *Millettia* species (table 1). The majority of these isoflavonoids contain one or two phenyl group(s) as a side chain, either in acyclic or in cyclic forms, or as a combination of the two forms.

**Table1. Selected compounds from *Millettia* species**

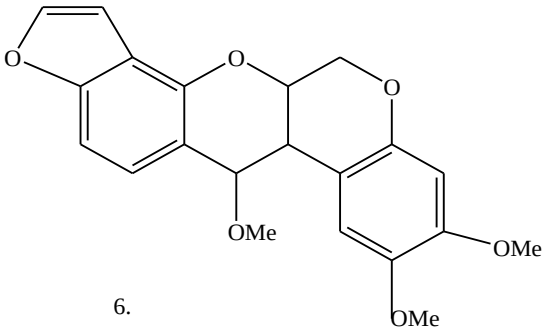
| Compound | Plant species | Plant part | Reference |
|---|---|---|---|
| Isoflavone | M. thonningi | Pods, Root bark, root wood | Harvey,2008 |
| Alpinum isoflavonone (1) | | | |
| 2-methoxyalpinum isoflavone(2) | | | |
| 5-methoxy-4-o-(3- methyl-2-butenyl) (3) | | Stem bark Root bark | Rakholi-ya,2013 |
| 7-O-Geranylpseudobaptigenin (4) | M. dura, M. grifoniana | Stem bark Root bark | Carren,2014 |
| 7-O-Geranyl formanonetin(5) | | | |
| 3',4',6'-Trimethoxy-2",2"- dimethylpyra-no(5",6")isoflavone (6) | M. ichthyochtona | Leaves | Kamperdick, 1998 |
| Rotenoid | M. duchenseni | Twigs | François et al, 2008 |
| 12-Deoxo-12α- methoxylelliptone (7) | | | |
| 12α-Hydroxyelliptone (8) | | | |
| Elliptone (9) | | | |
| Chalcone | | | |
| 3,4- methylenedioxy- 2',4'Dimethoxychalcone (10) | M. Leucantha | Stem bark | Phrutivora et al |
| 4'-O-Geranylisoliquiritegenin (11) | M.ferruginea darassana, M.usaramensis | root bark, stem bark | Dagne et al,2008 |
| 4'-Hydroxylonchocarpin (12) | M. dura M. pachycarpa | Stem bark, seeds | Sritularak et.al ,2006 |
| 2'- hydroxy-3, 4-dimethoxy-(2", 3": 4', 3')-furanochalcone (13) | M.erythrocalyx | Seed pods | Ye, 2012 |




1. $R_1$=OH R2=OH $R_3$=H
2. $R_1$=OH R2=OCH3 $R_3$=H
3. $R_1$=$OCH_3$ R2= O-Preny $R_3$=H




4. $R_1$=-$OCH_2O$- $R_2$=-$OCH_2O$-
5. $R_1$=H $R_2$=-$OCH_3$

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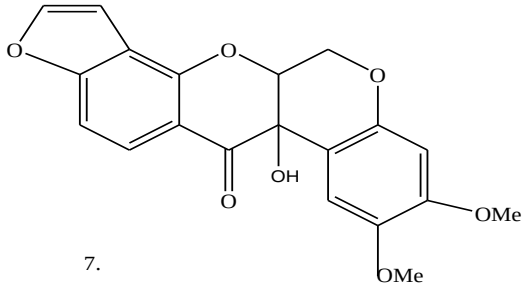


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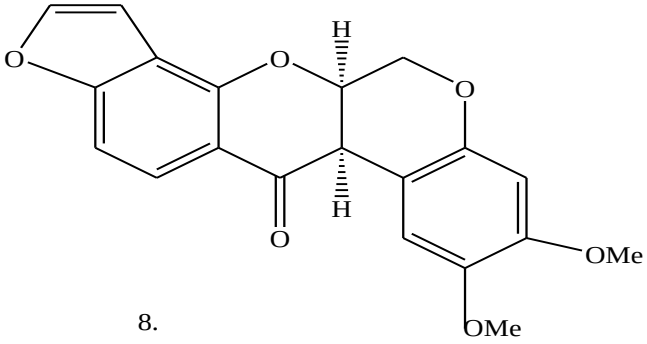


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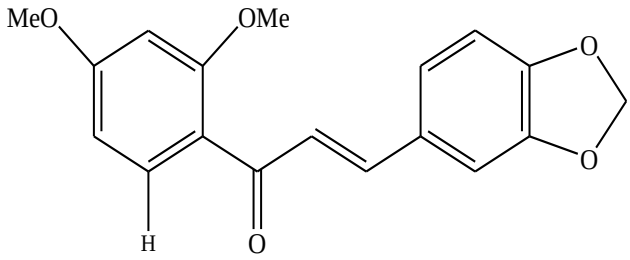


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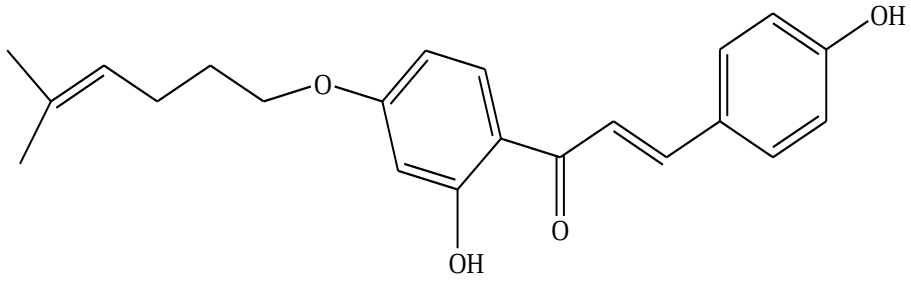


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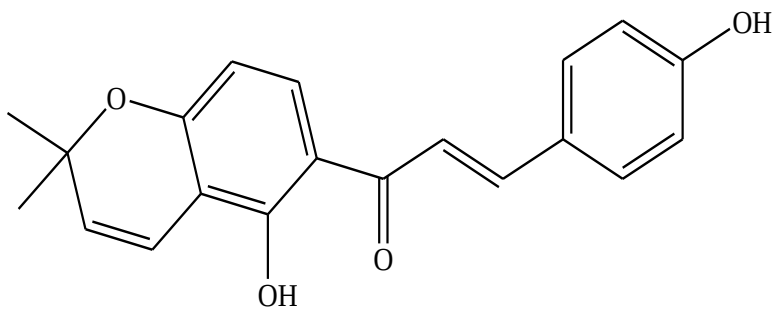


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Figure: 4. Structure of some reported isolated compound from *Millettia* species

# 3. Materials and Methods

## 3.1. Study area and place

Most of the experiments such as Solvent extraction, phytochemical screening, chromatographic separation, and UV-Vis analysis, will be done at Wollega University, post graduate Chemistry laboratory. Then antimicrobial assay test will be done at department of Biology (Plant Pathology) research laboratory. IR and NMR spectroscopic experiments of the isolated compounds will be done at Addis Ababa University. The experiment work will conduct from January 2022 to June 2022.

## 3.2 Instruments and apparatus

The following instruments will be utilized to conduct the experiments: oven, spatula, separator funnels, TLC plate (pre-coated), rotary evaporator, beakers (different sizes), UV lamp, TLC chambers, test tubes, fume hoods, hot plates, round bottom flask, Petri dish, measuring cylinder, condenser, micropipettes (different sizes), glass column, electronic balance, Erlenmeyer flask, water bath, NMR spectrometer, and UV-Visible spectrometer. Thin layer chromatography (TLC) made of aluminum plate coated with silica gel for absorbation of samples, filter paper (27 cm) and Rota-Vapor with Vacuum Pump, are used for extraction, filtration and concentration of extracts respectively.

## 3.3 Chemicals and Reagents

Different chemicals and reagents will be used in this study. These include analytical grades of solvents (*n*-hexane, methanol, ethanol, petroleum ether, ethyl acetate, acetone and chloroform), hydrochloric acid, sulphuric acid (for visualized UV inactive spots),ferric chloride, Mayer's reagent, dichloromethane, ammonia, acetic anhydride, silica gel(for column chromatography).Dimethyl sulfoxide(DMSO),used as negative control for antibacterial tests),Mueller-Hinton agar(MHA),standard antibiotic gentamicine (10µg), 1,1-diphenyl-2-picryl hydrazyl (DPPH) ,Ascorbic acid( positive control for anti oxidant test) and distilled water will be used for absorbance.

## 3.4 Experimental methods

### 3.4.1Plant material collection and preparation

Fresh stem bark of *Millettia ferrugenea* will be collected from ,Figa kobera kebele Boji Coqorsa district, West Wollega during the month of January,2022,after it is authenticated by a botanist at biology department of Wollega university. The fresh stem bark of this plant will be washed with tap water and dried in shade at room temperature. The stem bark will be separately cut into small bits, and air dried under shadow for three weeks. After dried, it will be grinded into powder by using electrical grinder before being subjected to solvent extraction. Finally, the powder was weighted and kept for further use.

### 3.4.2 Extraction of plant sample

Three solvents will be used for the Serial exhaustive extraction of stem bark of the plant based on their increasing polarity. These are 2 bottles of *n*-hexane, 1 bottle of chloroform, 1 bottle of methanol and 1 bottle of ethyl acetate. About 600g of the powdered stem bark of *Millettiaferrugenia* will be extracted with the selected extraction solvents each for 72hrs with random shaking. The samples will be soaked three times in n-hexane, chloroform and methanol in round bottom flask and ethyl acetate will used for column packing. Solvents will be removed by rotary evaporator under reduced pressure and fractions will be collected separately. The semisolid mass will be made to dry in vacuum oven at about room temperature and the dried masses will be weighed and the percentage yield will be calculated. The obtained crude extracts will be kept in closed container for preliminary qualitative phytochemical analysis.

### 3.4.3 Phytochemical analysis

The collected plant extracts will be subjected to qualitative phytochemical analysis for identification of various classes of active chemical constituents and will be carried out using standard methods.

#### 3.4.3.1 Test for Steroids:

2 mL of acetic anhydride will be added to 2 mL of extract that is dissolved in methanol and then 2 mL of $H_2SO_4$ will be added into the test tube containing the mixture (Tiwari *et al* ., 2011). Appearance of a blue-green ring indicates the presence of steroids.

**3.4.3.2 Test for Terpenoids:**

5mL of extract that is dissolved in methanol will mix with 2 mL of chloroform. Then 3 mL of concentrated $H_2SO_4$ will carefully added into the test tube containing the mixture in order to get a layer (Salkowski test ,Tiwari*etal* ., 2011). Appearance of a reddish brown coloration indicated the presence terpenoides.

**3.4.3.3 Test for saponins:**

0.2 g of crude extract will be dissolved in 5 mL of water in test tube and shacked vigorously for 15 minute (Alebiosu 2015). Formation of 1 cm layer of foam indicates the presence of saponins.

**3.4. 3.4 Tests for Flavonoids:**

3 mL of extract dissolved by methanol will be treated with 3 drops of sodium hydroxide solution (Alkaline Reagent Test). Formation of intense yellow color, which becomes colorless on addition of dilute hydrochloric acid, indicates the presence of flavonoids (Wozniak *et al*., 2015).

**3.4.3.5. Test for tannins:**

0.2 g of crude extract will be boiled in 20 mL of water in a test tube. The solution will be filtered and 4 drops of 0.1% ferric chloride ($FeCl_3$) will added into the test tube (Sheila *et al*., 2016). Appearance of a brownish green or blue-black coloration will confirm the presence of tannins.

**3.4.3.6. Test for Alkaloids (Mayer's Test)**

To 1 mL of extract, 6 drops of Mayer's reagent will be added. The formation of yellowish creamish precipitate indicated the presence of alkaloids (Harbone, 1973)

**3.4.3.7. Test for Phenol (Ferric chloride test):**

2 mL of extracts dissolved by methanol will be treated with 4 drops of ferric chloride solution (Tiwari *et al*., 2011). The formation of green or bluish black color will be observed as indicator of the presence of phenols.

### 3.4.3.8. Test for Glycosides:

2mL of chloroform will be added in 2 mL of extract dissolved by methanol and then 2 mL $H_2SO_4$ will be added carefully and shacked gently (Alebiosu2015). A color change from orange to reddish brown at interface will be observed

### 3.4.4 Isolation and Characterization of compounds

A silica gel (100 g) will be mixed with n-hexane and the slurry method will be used to pack the column. The crude extract will be dissolved in appropriate solvent and adsorbed on silica gel. The concentrated adsorbed extract will be then subjected to the top of packed silica gel column. The column will be then eluted with increasing polarity of solvent systems. The isolated compounds from column chromatography will be further analyzed by IR and characterized using $^{1}H$ NMR, $^{13}C$ NMR and DEPT.

### 3.4.5 Antibacterial test

Bacterial strains: four bacteria (two gram positive): *Bacillus subtilis* and *Staphylococcus aureus* and (two gram negative): *Escherichia coli* and *Pseudomonas aeruginos* will be used for bioactive examinations. Bacterial assay: The crude extract and fraction will be subjected to antibacterial assay by using the disk diffusion technique. The procedures are described below.

### 3.4.5.1. Preparation of inoculums

The test bacterial strains will be transferred from the stock cultures and streaked on Mueller Hinton plates and incubated for 24 hrs at37°C. Well separated bacterial colonies will be then used as inoculums. Bacteria will be transferred using bacteriological loop to autoclaved Mueller Hinton that will be cooled to about 45°C in a water bath and mixed by gently swirling the flasks. The medium will be then poured to sterile Petri plates, allowed to solidify and used for the bio test.

### 3.4.5.2 Preparation of test solutions

The crude extract (0.25 g/ml), fractions (0.1 g/ml) will be dissolved in chloroform and will be used to test antibacterial activities.

### 3.4.5.3. Testing for antibacterial activity

The crude extract and each of the concentrations of the fractions will be then pipette to the sterile paper discs. 20 and 40µl of the samples will be pipetted to the discs in three replica-

tions. Sterilized paper discs will be transferred to Muller Hinton agar plates seeded with bacteria and incubated at 37°C for24hrs. All the tests will be performed in triplicate. The antibacterial activity will be evaluated by measuring the zone of inhibition against the tested organism.

### 3.5. Spectroscopic analysis

Isolated pure compounds will be characterized by using Infra-Red, UV-Vis and Nuclear Magnetic Resonance ($^{1}$H-NMR, $^{13}$C-NMR, DEPT and two dimensional COSY, HSQC, and HMBC spectra in deuterated chloroform ($CDCl_3$) and deuterated water ($D_2O$), DMSO or other solvents as necessary with tetra methyl silane as internal standard. Structure determination will be achieved by characterizing structures and comparing the IR and NMR data obtained with that in literature, if available.

## 3.6 Antioxidant Activity

Standard protocol will be used to check the antioxidant activity of the plant (Ohnishi et al., 1994). Standard solutions (140µg/ml, 70µg/ml, 35µg/ml, 17.5µg/ml, 8.75µg/ml and 4.375µg/ml) of the test samples will be prepared in double-distilled methanol. From each concentration, 0.5 ml of the test compound will be added to 3 ml of 0.1mM DPPH that will have been dissolved in methanol. The mixture will be allowed to stand at room temperature for 30 minutes and the absorbance of the remaining DPPH measured at 517 nm. The radical scavenging activity will be measured as the decrease in absorbance due to DPPH radical expressed as a percentage of the control solution (consisting of 0.5 ml of methanol plus 3 ml of 0.1 mm DPPH solution). The activity will then be expressed as EC50, which is the concentration of the test compound required to give a 50% decrease of the absorbance from that of the control solution.

# 4. Time table and Budget Plan

## 4.1 Time schedule

**Table2. Time schedule**

| S/N | Activity | 2021 | | | | | 2022 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Au | Se | Oct | No | De | Jan | Fe | | Ap | Ma | Jun |
| 1 | Title selection | X | | | | | | | | | | |
| 2 | Searching related literature | | X | | | | | | | | | |
| 3 | Proposal preparation | | X | | | | | | | | | |
| 4 | Combining the literature for proposal work | | | X | | | | | | | | |
| 5 | Writing proposal | | | X | | | | | | | | |
| 6 | Proposal submission | | | X | | | | | | | | |
| 7 | Proposal defense | | | | X | | | | | | | |
| 8 | Preparation of plant samples | | | | | X | | | | | | |
| 9 | Extraction of plant samples | | | | | | X | | | | | |
| 10 | Isolation of the bioactive compounds using chromatographic technique | | | | | | X | | | | | |
| 11 | Characterization of isolated compound | | | | | | | X | | | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 12 | Antibacterial test of crude extract and isolated compounds | | | | | | | X | | | | | |
| 13 | Writing the final thesis | | | | | | | X | X | | | | |
| 14 | Submission of the thesis | | | | | | | | | X | X | | |
| 15 | Thesis defense | | | | | | | | | | | X | |

## 4.2 Budget

Table 3.Budget break down

| S/N | Activity or material | numbers | Unit price | Total price | Source |
|---|---|---|---|---|---|
| 1 | Stationary | | | | |
| | Papers | 2 pack | 370 | 740 | Self |
| | Pens and pencil | 3 and 2 respectively | 25 | 125 | Self |
| | Ruler | 1 | 20 | 20 | Self |
| | Binder | 1 | 120 | 120 | Self |
| | CD, | 4 | 30 | 120 | Self |
| | Flash disk | 16 GB | 250 | 250 | Self |
| 2 | Transportation | | | | |
| | During proposal work | 3round | 200 | 600 | Self |
| | During laboratory work | 4 round | 600 | 2400 | Self |
| | During chemical buying | 1 | 3000 | 3000 | Self |
| | For NMR and IR | 1 | 2500 | 2500 | Self |
| 3 | For NMR data at AAU laboratory | $^{1}_{H}$ and $^{13}C$ for samples | 2000 | 2500 | Self2 |
| 4 | Cost for IR characterization | 2 samples | 1000 | 2000 | Self |
| 5 | Cost for chemicals or Solvents | | | | Sponsor/s |
| 6 | Methanol | 2 bottle | 1600 | 3200 | Self |
| | Ethyl acetate | 2 bottle | 2000 | 4000 | Self |
| | Chloroform | 2 bottle | 1600 | 3200 | Self |
| | *n*-hexane | 2 bottle | 3200 | 6400 | Self |
| | Contingency | | 5000 | 5000 | Self |
| 7 | Total | | | 36157 | Self |

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