**JIMMA UNIVERSITY**

**SCHOOL OF GRADUATE STUDIES**

**COLLEGE OF NATURAL SCIENCES**

**DEPARTMENT OF CHEMISTRY**

**M.Sc. THESIS**

**ON**

# PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL EVALUATIONOF THE ROOT BARK OF *DODONAEA ANGUSTIFOLIA*

**JANUARY, 2021**

**JIMMA, ETHIOPIA**

# PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL EVALUATIONOF THE ROOT BARK OF *DODONAEA ANGUSTIFOLIA*

**A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES JIMMA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN CHEMISTRY (ORGANIC)**

**BY**

**TEMESGEN MITIKU**

**ADVISORS**

**NEGERA ABDISSA (PhD)**

**TSEGAYE GIRMA (PhD)**

**JANUARY, 2021**

**JIMMA, ETHIOPIA**

## DECLARATION

The data used in this thesis is the results of my investigation and I, hereby declare that, this thesis is my original work and has not been presented for award of any degree or diploma in any university.

_____

Temesgen Mitiku (RM0221/11)

**JANUARY, 2021**

**JIMMA, ETHIOPIA**

## Acknowledgment

I sincerely appreciate the almighty God for his graces, strength, sustenance and above all, his faithfulness and love from the beginning of my academic life up to this master's level. His benevolence has made me excel and successful in all my academic pursuits. First and foremost, I am extremely grateful to my supervisors, Dr. Negera Abdissa and Dr. Tsegaye Girma for their invaluable advice, continuous support, and patience during my masters' study. Their immense knowledge and plentiful experience have encouraged me in all the time of my academic research and daily life. Finally, I would like to express my gratitude to my wife and my parents. Without their tremendous understanding and encouragement in the past few years, it would be impossible for me to complete my study. It is also my great pleasure to thank Department of Chemistry, College of Natural Science, Jimma University for material support for this research work and Department of Biology laboratory technician's for their cooperation in this research work.

## Table of content Page

## Lists of tables

## Lists of figures

## List of Appendix

## List of Abbreviations/Acronyms

| | |
|---|---|
| NMR | Nuclear Magnetic Resonance |
| TLC | Thin Layer Chromatography |
| DMSO | Dimethyl Sulfoxide |
| HIV | Human Immune Virus |
| AMR | Antimicrobial Resistance |
| DEPT | Distortions Enhancement by Polarization Transfer |
| MIC | Minimum Inhibitory Concentrations |
| ATCC | American Type Culture Collection |
| MHA | Muller-Hinton Agar |
| CC | Column Chromatography |
| UV | Ultraviolet |
| HMBC | Heteronuclear Multiple Bond Correlation |
| HSQC | Heteronuclear Single Quantum Coherence |
| COSY | Correlation Spectroscopy |
| NOESY | Nuclear Overhauser Effect Spectroscopy |

## Abstract

Medicinal plants are considered as good resources of ingredients which can be used in drug development. Plant derived drugs are also considered as safe and with little side effects when compared to synthetic drugs. Thus, the aim of this study was to isolate and characterize compounds from the root bark of *Dodonaea angustofolia* and evaluate its antimicrobial activities. The air-dried root bark of the plant material was sequentially extracted with petroleum ether, chloroform, acetone and methanol by maceration at room temperature and gave the crude extracts amounting to 6.5 g, 12 g, 8 g and 36 g respectively. The phytochemical analysis of the extracts revealed the presence of alkaloids, phenols, tannins, saponins, flavonoids and sterols**.** The chloroform and acetone extract was combined as they have displayed similar TLC profile and subjected to silica gel column chromatography (CC) for fractionation and purification of the compounds. The column was eluted with petroleum ether with increasing polarity of ethyl acetate and has resulted six compounds DA1, DA2, DA3, DA4, DA5 and DA6. However, characterizations of only two compounds were performed. The structures of the isolated compounds were performed by NMR ($^{1}$HNMR, $^{13}$CNMR, and DEPT-135) spectroscopic data and comparison with reported literature data. The isolated compounds were characterized as (3β, 22E)-Stigmasta-5, 22-dien-3-ol (DA1) and *β*-Stigmasteryl linoleate (DA3). Both compounds were isolated for the first time from the study plant species. The antibacterial and antifungal activity of the crude extract and isolated compounds were evaluated against four bacterial strains (*Staphylococcus aureus*, *Bacillus cereus*, *and Salmonella Typhimurium* and *Escherichia coli*) and a fungal strain (*Candida albicans*) using agar disk diffusion method. The extracts showed zone of inhibition ranging 7.5-12.75 mm, with the acetone extract exhibited the highest activity against both bacteria and fungi strain. Therefore, the acetone extract was further evaluation for minimum inhibitory concentration and displayed 62.5, 15.6, 15.6, 31.3 and 15.6 mg/ mL against, *E.coli, S. typhi, B. cereus, S. aureus* and *C. albican respectively*. The isolated compounds have weak antibacterial activity but compound DA5 has good antifungal activity. Therefore, the presence of this phytochemicals in this plant root bark provides scientific support for the ethno medicinal use of the plant

Keywords: Medicinal plant, *Dodonaea angustifolia,* antimicrobial activity, stigmasterol *β*-Stigmasteryl linoleate

# 1. Introduction

## 1.1 Background of the study

Medicinal plants are considered as rich resources of ingredients which can be used in drug development. The interest on demand for more plant derived drugs, which sometimes are considered as safe when compared to synthetic drugs, is increasing rapidly [1]. While numerous studies have validated the traditional use of medicinal plants by investigating the phytochemicals present in active extracts, many plants have not yet been scientifically studied to provide reports on their phytochemical constituents and their potential medicinal applications [2].

Plants have always played a central role in traditional systems of medicine for the prevention and treatment of disease around the world [3]. Naturally derived compounds have made considerable contributions to human health and well-being and have been a source of inspiration for novel drug development. They may become the base for the development of a medicine, a natural blueprint for the development of new drugs. The first generation of plant drugs were usually simple botanicals employed in more or less their crude form. Second-generation plant drugs were purified chemical compounds, with an identified structure that had been isolated from plant extracts [4].

Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer-based molecular modeling design and many drugs are made by synthetic chemistry, none of them can replaced the important role of natural products in drug discovery and development as most of the core structures or scaffolds for synthetic chemicals are based upon natural products [5].

The emergence and spread of drug-resistant pathogens that have acquired new resistance mechanisms, leading to antimicrobial resistance, continues to threaten our ability to treat common infections. In recent years this becomes serious challenge for clinicians and a global health crisis [6]. Multi-drug resistance in both Gram-negative and Gram -positive bacteria often leads to untreatable infections using conventional antibiotics, and even last-resort antibiotics are losing their power [7]. The development of new antimicrobial naturalcompounds is one of the main strategies used to eliminate this problem.

The first commercial pure natural product introduced for therapeutic use is morphine(1) marketed by Merck in 1826, and the first semi-synthetic pure drug aspirin, based on a natural product salicin isolated from *Salix alba*, was introduced by Bayer in 1899. During the last decades few plant derived drugs have been launched include Arteether (2), endoperoxide sesquiterpene lactone (3) and semisynthetic natural product derived from *Artemisia annua* used in malarial treatment, Nitisinone (4) derived from natural product Leptospermone (*Callistemon citrinus*) is used in treatment of antityrosinaemia, galantamine (5) is a natural alkaloid (obtained from *Galanthus nivalis*) for Alzhemer's, apomorphine (6) is a semisynthetic compound derived from morphine (*Papaver somniferum*) used in Parkinson's disease, Dronabinol (7) and Cannabidiol (8) obtained from cannabis plant (*Cannabis sativa*) and Capsaicin active compound from *Capsicum annuum* are used as pain relievers[8,9].

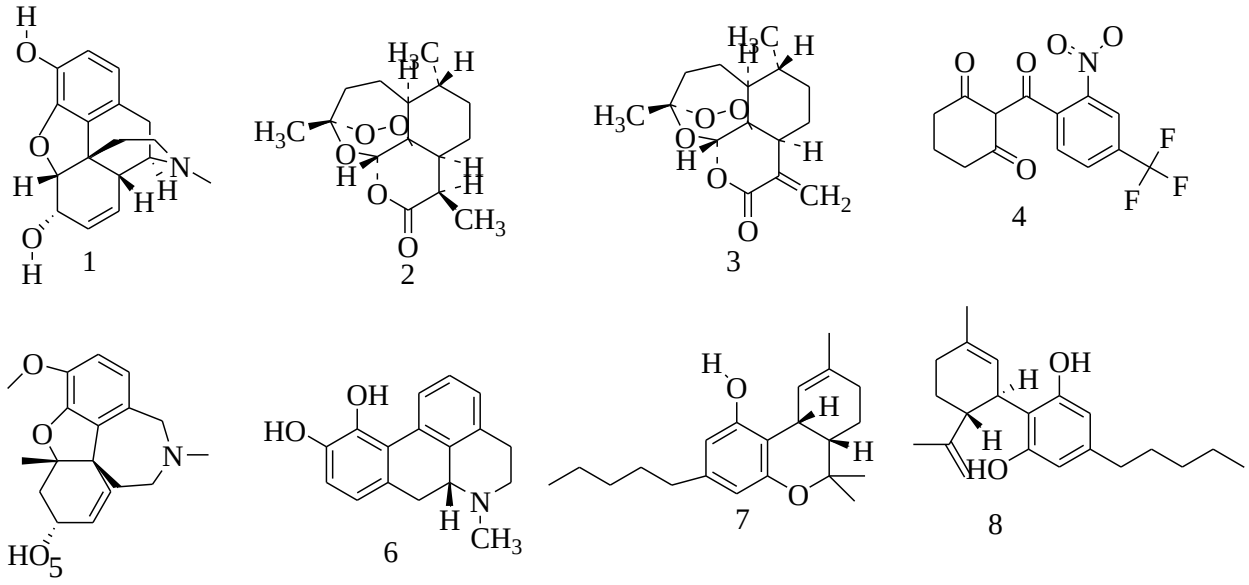



Figure 1.Structure of drugs derived from plants

The diverse tradition of Ethiopia is a prosperous source of traditional medicines, many of which are plant origin. Thousands of the identified medicinal plant species are reported in the Ethiopian Flora, however, many others are not yet identified and about 300 of these species are frequently mentioned in many sources [10]. *Dodonaea angustifolia (Sapindaceae)* commonly referred to as Sand Olive, 'Kitkita' (Amharic),'Itacha'(Afan Oromoo) is a medicinal plant used in folk medicine to treat various ailments in Ethiopia. Previous pharmacological reports indicated that

the plant has antibacterial [11], antifungal [12], antidiabetic [13], anti-inflammatory [14] and antidiarreal properties [15].

The traditional use of this plant to treat various ailments persuaded to undertake isolation, characterization of phytochemical and evaluate its antibacterial and antifungal activity of the crude extracts and isolated compound from the root bark of *Dodonaea angustifolia.*

### 1.2 Statement of the problem

Across the globe, the emergence of antimicrobial resistance (AMR) is threatening the effective and successful treatment of infectious diseases. Numbers of human pathogens have inevitably evolved to become resistant to various currently available drugs causing considerable mortality and morbidity worldwide. It is apparent that novel antibiotics are urgently warranted to combat these life-threatening pathogens [16]. Improving the quality, not just the quantity of medication will require public and professional education towards rational use of antibiotics [17]. Understanding the key molecular mechanisms involved in the screening of bioactive small molecule compounds has become a major challenge for drug discovery scientists. The reduction of efficacy and the increase of toxicity of synthetic drugs is further aggravating the problem. This has led researchers to look towards herbal drugs for a solution, as they are now known to play a crucial role in the development of effective therapeutics [18].There should be a focus on search for bioactive molecules from plants, which could serve as antimicrobial agent. *D.angustifolia* is one of the most known medicinal plants used traditionally for various treatments [19]. However, the phytochemicals information and bioactivity of the molecules from the root barks of *D.angustifolia* has not been sufficiently reported so far. Therefore this research is mainly focused on isolation, of the compounds from roots of *D.angustifolia* and evaluation of its antimicrobial activity.

## 1.3 Objective of the study

### 1.3.1 General objective

- The main aim of this research was to investigate phytochemical constituents of the root bark of *D.angustofolia* and evaluate the antimicrobial activity of the extracts and the isolates.

### 1.3.2 Specific objective

- To extract the root bark of *D.angustifolia* with petroleum ether, chloroform, acetone and methanol.
- To isolate compounds from the root barks of *D.angustifolia* using column chromatographic technique.
- To elucidate structures of the isolated compounds, using spectroscopic techniques such as NMR ($^{1}$H NMR, $^{13}$C NMR and DEPT-135 NMR).
- To evaluate in vitro antimicrobial activity of the crude extracts and isolated compounds against four bacterial strains (*S. aureus, E. coli,S.typhi* and *B. cereus)* and a fungal strains (*Candida albican*).

## 1.4 Significance of the study

The study focused on the isolation and characterization of bioactive molecules from *D. angustifolia* root bark. *D.angustifolia* is one of the most known medicinal plants used traditionally for various treatments. However, the phytochemicals information and bioactivity of the molecules from the root barks of *D. angustifolia* has not been sufficiently reported so far. Therefore, this research is mainly focused on isolation, of the compounds from roots of *D. angustifolia* and evaluation of antimicrobial activity. Overall, the study would be expected to have the following significances.

- Provide the chemical profile of root bark of *D. angustifolia.*
- Use this finding for further phytochemical studies on the plant species.
- Forecasting medicinal application of *D. angustifolia* and initiate chemists for further study on the plant.

# 2. Review of Related Literature

## 2.1 Botanical Information

### 2.1.1 The Family *Sapindaceae*

The *Sapindaceae* are a family of flowering plants in the order Spindale's known as the soapberry family. The genus of *Dodonaea* is classified in the family of *Sapindaceae*. The name *Sapindaceae* is derived from the word "sapo" meaning soap. Saponin is a glycoside which forms a soapy substance in leaves, roots and seeds of this family. It comprises between 140-150 genera and between 1,500-2,230 species, typically distributed in tropical and strongly represented in the American, African and Asian and also in Japan and widespread in Australia.Members of the family are mostly trees and shrubs.The East African genera of *Sapindaceae* belong to the following sub-families and tribes:Subfamily *Dodonaea* with three tribes *Dodonaeae* (*Dodonaea*), *Doraloxyleae* (*Filicium)* and *Harpullieae (Majidea)* and the subfamily/genus *Sapindoideae* (with eight tribes *Cupanieae (Aporriza), Nephelieae* (*Stadmania*), *Schleichereae (Macphersonia), Melicocceae (Tristropsis), Lepisantheae* (*Lepisanlhes*), *Sapindeae* (*Deinbollia*), *Thouinieae (Allophylus*) and *Paullinieae (Cardiospermum)* [20].

### 2.1.2 The genus *Dodonaea*

The genus *Dodonaea* belongs to the *Sapindaceae* family. The common name applied to *Dodonaea* is "Hop Bushes", because of its colorful fruits resembling the fruits used in brewing. *Dodonaea* is predominantly an Australian genus which is well distributed in all states. There are more than 70 species and 60 of them are endemic to Australia. Most are small shrubs 1-2 m in height. *Dodonaea* may be found in many habitats (e.g rocky ridges, forest, riverine communities, and woodland and shrub land) however, they are not found in alpine communities, or in dense vegetation communities such as heathland and rainforest.

Some of the species in *Dodonaea* are *Dodonaea adenophora , Dodonaea amblyophylla, Dodonaea aptera* (Western Australia), *Dodonaea baueriEnd, Dodonaea bilobaJ.G.West, Dodonaea boroniifoliaG.Don, Dodonaea bursariifoliaF.Muell, Dodonaea caespitosa Diels, Dodonaea camfieldii Maiden & Betche, Dodonaea ceratocarpa, Dodonaea concinnaBenth, Dodonaea coriacea, Dodonaea divaricataBenth, Dodonaea ericifolia, Dodonaea ericoidesMiq, DodonaeafalcataJ, Dodonaea viscosa* and *Dodonaea viscosa subsp Angustifolia* however, in

east Africa the genus *Dodonaea* is represented by two species D. *angustifolia* and *D. viscosa* [20].

### 2.1.3 *Dodonaea angustifolia*

*D.angustifolia* is commonly referred to as Sand Olive in English, *kitkita* in Amharic and *Itacha* in Afan Oromo. It is a shrub or small tree with narrow shiny pale green leaves with a distinctive small winged fruit resembling those of *Combretum* species. The natural distribution includes the Americas, the Asia-Pacific region, the Arabian Peninsula (pers. obs.) and from tropical to southern Africa [21].Its habitat varies from arid, semi-desert regions to the margins of evergreen forests in high rainfall areas [22].The plant can withstand fires and it reproduces itself from seed "very freely, even in dry rocky localities. It grows on dry rocky slopes between 1500 and 2100 m throughout Ethiopia and is the only shrub, which grows on copper-rich soils [23].

Figure 2.Picture of *D. angustifolia*(Taken by Temesgen; 16, Nov, 2019 from study area, Merewa)

## 2.2 Ethino-medicinal uses of the genus *Dodonaea*

*Dodonaea's species* has many medicinal properties and has been used by native peoples from all regions where it is found. It is traditional medicine worldwide, administered orally or as poultice to treat great variety of ailments. The stems and leaves are used to treat scabies, bone fracture, tapeworm; root to treat trachoma and tooth ache, and seeds fresh fruit is eaten to treat malaria [30].

Table 1.Summary of different species under the genus *Dodonaea* and their medicinal uses

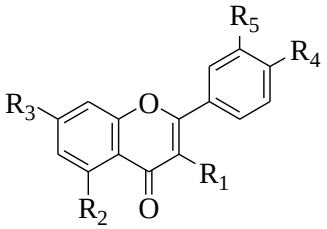
| Species | Plant part | Uses | Reference |
|---|---|---|---|
| *D.viscosa* | Leaves | Antipruritic in skin rashes, Sore throat,Hemorrhoids,AnaestheticVermuge,Dermatitis,Skinlesions,Fever,Malria | [24,25] |
| | Root | Irregular menstruation, Breast cancer, skin cancer | [24,26] |
| *D.viscosa var. angustifolia* | Leaves | Hypotension and antispasmodic properties | [27] |
| *D.lanceolata* | Leaves | Pain and snake bite | [32] |
| *D.madagascariensis* | Leaves | Hypotension and antispasmodic properties | [31] |
| *D.polyzyga* | Leaves | Alleviate symptoms of colds and flu | [28] |
| *D.triquetra* | Root | Wounds and toothache | [30] |

## 2.3 Phytochemical constituents of genus *Dodonaea*

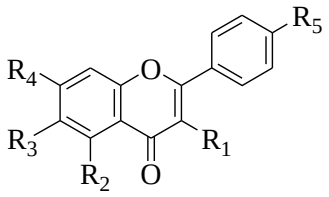
Phytochemical study of the genus of *Dodonaea* revealed that Tannins, Saponins, Flavonoids and Terpenoids are the most commonly reported secondary metabolites from the genus [31].List of compounds so far reported from *Dodonaea* are given in Table 2

Table 2.Compounds so far reported from *Dodonaea* species

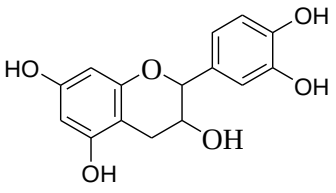
| Compound | Compound name | Plant source | Ref |
|---|---|---|---|
| Flavonoid | | | |
| Flavonol | 5,7,4'- Trihydroxyflavonol(9) | *D.viscosa* | |
| | 5,7,2'.4'-Tetrahydroxyflavonol (10) | *D.viscosa* | |
| | 5,7,4'-trihydroxy-2'- methoxyflavonol(11) | *D.viscosa* | [20] |
| Flavone | 3, 5-Dihydroxy-4,′7-dimethoxyflavone(12) | *D.angustifolia* | [32] |
| | 3,6-Dimethoxy-5,7,4′-trihydroxyflavone(13) | *D. viscosa* | [33] |
| | 3,5,4'-Trihydroxy-7-methoxy flavone(14) | *D.viscosa* | [34] |
| | 5,7-dihydroxy-2-(4-hydroxy-3-(3-methylbut-2-en-1-yl) phenyl)-3-methoxy-4H-chromen-4-one(15) | *D. polyandra* | [35] |
| | 5,6,7-Trihydroxy-3,4′-dimethoxyflavone(16) | *D.angustifolia* | [36] |
| | 5,6-Dihydroxy-3,4′,7-trimethoxyflavone(17) | *D. viscosa* | [37] |
| | 5,7,4′-Trihydroxy-3,6-dimethoxyflavone(18) | *D.angustifolia* | [36] |
| | 5-Hydroxy-3,4′,7-trimethoxyflavone(19) | *D.viscosa* | [38] |
| | 5-Hydroxy-3,6,4′,7-tetramethoxyflavone(20) | *D.viscosa* | [39] |
| Diterpinois | Dodonic acid(21) | *D.angustifolia* | [32] |
| | Dodonic acid(21) | *D. viscosa* | [40] |
| | Dodonolide(22) | *D.viscosa* | [40] |
| | (−)-6α-Hydroxy-5α, 8α, 9α,10α-cleroda-3,13-dien-15,16-olid-18-oic acid(23) | *D.viscosa* | [39] |
| | 13, 14-Dihydroxy-15,16-dimethoxy-(−)-6α-hydroxy-5α,8α,9α,10α-cleroda-3-en-18-oic acid(24) | *D.viscosa* | [39] |
| | 13, 17-Epoxy-13-methyl-15-oxo-labda-7-ene(25) | *D. polyandra* | [41] |
| | 13-Methyl-17-oxolabda-7,13-Z-diene-15-oic acid(26) | *D. polyandra* | [41] |
| | 15, 16,-Epoxy-19-hydroxy-1,3,13-(16),14-cleroda-tetraen-18-oic acid(27) | *D. viscosa* | [42] |
| | 17-Hydroxy-13-methyllabda-7, 13-Z-diene-15-oic acid(28) | *D. polyandra* | [41] |



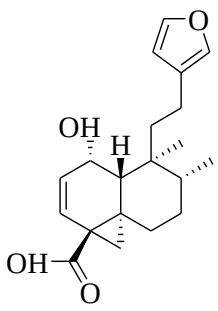
| No | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|
| 9 | OH | OH | OH | OH | H |
| 10 | OH | OH | OH | OH | OH |
| 11 | OH | OH | OH | OH | $OCH_3$ |
| 12 | OH | OH | $OCH_3$ | $OCH_3$ | H |
| 14 | OH | OH | $OCH_3$ | OH | H |
| 15 | H | OH | OH | OH | H |
| 29 | H | OH | OH | H | H |



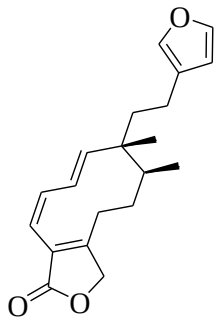
| No | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|
| 13 | $OCH_3$ | OH | OCH3 | OH | OH |
| 16 | $OCH_3$ | OH | OH | OH | $OCH_3$ |
| 17 | $OCH_3$ | OH | OH | $OCH_3$ | $OCH_3$ |
| 18 | $OCH_3$ | OH | $OCH_3$ | OH | OH |
| 19 | $OCH_3$ | OH | H | $OCH_3$ | $OCH_3$ |
| 20 | OH | OH | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| 30 | $OCH_3$ | OH | $OCH_3$ | OH | $OCH_3$ |



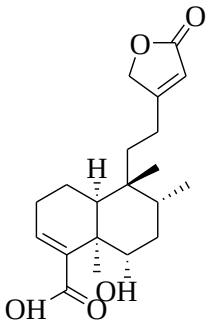
31



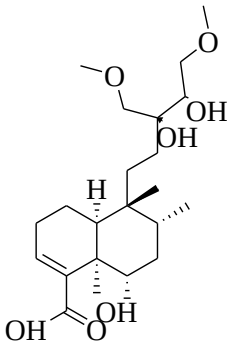
21



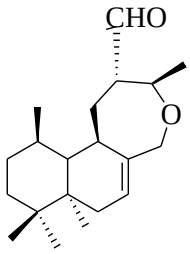
22



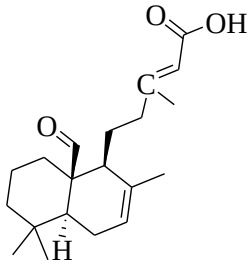
23



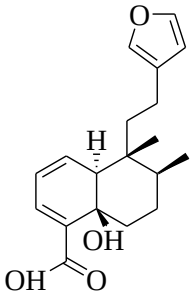
24



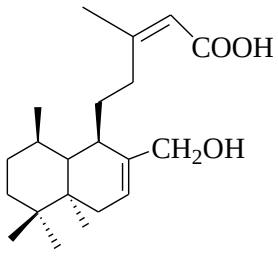
25



26



27



28

Figure 3.Some of the compounds reported from *dodonaea* species

## 2.4 Pharmacological uses of genus *Dodonaea*

### 2.4.1 Anti-plasmodial activity

*D.angustifolia* is used in Ethiopian traditional medicine to treat malaria. For example,the ethyl acetate soluble portion of the 80% aqueous MeOH extract of the leaves of *D. angustifolia* significantly suppressed parasitaemia in *Plasmodium berghei* infected mice (80.28% at 150 mg/kg).From this extract three active compounds, namely;5,7-Dihydroxyflavanone (29),5,7,4'trihydrodimethoxyflavone(18),5,6,7- trihydroxy-3,4'- dimethoxyflavone(17) were identified, and exhibited significant percent suppression of parasitaemia by 81% at 40mg/kg, 80% at 50 mg/kg and 70 % at 40 mg/kg, respectively [67].Aqueous hydro alcoholic and n-butanolcrude extract of *D.angustifolia* show anti- plasmodium[43,45,46].

### 2.4.2 Antibacterial activity

The ethanol crude extract and n-hexane, dichloromethane, ethyl acetate, n-butanol and aqueous fractions of *D.viscosa* were analyzed for antibacterial potential against four gram-positive bacteria*: Bacillus subtilis, Bacillus cereus, Micrococcus luteus, Staphylococcus aureus*, and three gram-negative bacteria: *Escherichia coli, Salmonella typhi, and Pseudomonas aeruginosa*. Preliminary screening showed inhibition against *Staphylococcus aureus, Micrococcus luteus, Escherichia coli and Pseudomonas aeruginosa.* The thin layer chromatograms of the fractions were then subjected to contact bioautography, which showed inhibition zone at different Rf values against *Bacillus subtilis, Micrococcus luteus, Escherichia coli, Salmonella typhi* and *Pseudomonas aeruginosa,* indicating the presence of antibacterial components. The MIC of each fraction was determined through a 96-well micro-titer plate method [65]. The dichloromethane extract of *D.viscosa Jacq.Var angustifolia* dicholomethane leads to isolation of 5,7,4'-trihydroxy-3,6-dimethoxyflavone(18),5-hydroxy-3,7,4'-trimethoxyflavone(19) ,3,5,7-trihydroxy-4'-methoxyflavone(30),2-(3,4-dihydroxyphenyl)chroman-3,5,7-triol,(31) pure compounds. The chloroform crude extract had also antibacterial effect [44].

### 2.4.3 Antioxidant activity

A methanolic extract of *D.viscosa* was dissolved in distilled water and sequentially partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The antioxidant potential of all these fractions and remaining aqueous fraction was evaluated and the results revealed that the ethyl

acetate soluble fraction exhibited the highest percent inhibition (81.14 ±1.38 %) DPPH radical at (at a concentration of 60 µg /mL) with $IC_{50}$ value of 33.95±0.58 µg /mL, compared to butylated hydroxytoluene (BHT), having an IC50 of 12.54±0.89 µg /mL-1[63]. The methanol extract of *D. viscosa* led to the isolation of 3, 3,'4,'5, 7-pentahydroxyflavane (32) with the crude extract exhibiting an antioxidant activity [47].

### 2.4.4Anti-diabetic activity

The crude extract of *D.viscosa* was tested for anti-diabetic activity, by glucose tolerance test in normal rats and alloxan induced diabetic rats. Aqueous ethanol and butanol extracts showed significant protection and lowered the blood glucose levels to normal in glucose tolerance test. In alloxan induced diabetic rats, the maximum reduction in blood glucose was observed after 3hrs at a dose level of 250 mg/kg of body weight [62].

### 2.4.5 Antifungal activity

Antifungal activity of crude extract of *D.viscosa var.angustifolia* were evaluated using 41 strains of *C.albicans,*20 from HIV-positive patients, 20 from HIV-negative subjects and *C.albicans* ATCC 90028. Acetone extract of *D.viscosa var.angustifolia,* chlorhexidine gluconate and triclosan were measured using a microtitre double dilution technique, and the time taken to kill 99.5% of the strains was determined. Plant extract, chlorhexidine gluconate and triclosan were 6.25–25, 0.008–0.16 and 0.0022–0.009 mg/ml respectively. Plant extract killed all the test strains within 30s and chlorhexidine 40% of the isolates from HIV-positive patients and 20% of strains from HIV-negative subjects in 1 min. During the same time triclosan killed 55%and 35% of isolates from HIV-positive and HIV-negative patients. *D.viscosa var. angustifolia* has antifungal properties and is more effective than commercially available mouth rinses [64].

### 2.4.6 Anti-inflammatory

The powdered leaves of D. *viscosa* were extracted with hydro alcohol and n-hexane successively. The anti-inflammatory activity was studied using carrageenan-induced rat paw edema at the dose of (100 and 200 mg/kg) of hydro alcoholic and n-hexane extract respectively. The hydro alcoholic extract of *D. viscosa* exhibited significant anti-inflammatory activity at the dose of 100 & 200 mg/kg than the n-hexane extract. Indomethacin (10 mg/kg) had exhibited significant anti-inflammatory activity [66].

## 2.5 Challenges and recent progress of infectious disease

Infectious diseases are disorders caused by organisms such as bacteria, viruses, fungi or parasites. Many organisms live in and on our bodies. They're normally harmless or even helpful. But under certain conditions, some organisms may cause disease. Infectious diseases form one of the greatest global challenges in medicine in our time. A large number of new infectious diseases emerged: major threats to the world like AIDS and SARS, but also less threatening infections, like those caused *by Campylobacter spp, Borrelia spp, Bartonella henselae, Clostridium difficile*, Hanta viruses, not only new infectious agents, but also known microorganisms have appeared that acquired new virulence or new antimicrobial resistance [68].

Infectious tropical diseases cause a huge toll in terms of mortality and morbidity, as well as a large economic burden to the countries concerned. These diseases predominantly affect the world's poorest people. Unfortunately, current drugs are inadequate for the majority of these diseases, and there is an urgent need for new treatments. There are a numbers of challenges arise to treat tropical disease using the available drugs such as, lack of understanding of the detailed biology of many of the pathogens, physicochemical properties of a molecule and drug resistance of pathogens[69].

# 3. Materials and Methods

## 3.1 Chemicals

All chemicals and reagents used for this study were petroleum ether, chloroform, methanol, acetone and ethyl acetate. All the chemicals were analytical grade. Silica gel 60-120 mesh size, distilled water, iodine for detection of spots on TLC, dimethyl sulfoxide (DMSO). Deuterated chloroform was used for recording NMR spectra. Mueller Hinton agar and saline solution were used as a culture media antimicrobial test. Gentamycin and clotrimazole was used as standard antibacterial and antifungal respectively.

## 3.2 Apparatus

Apparatus that were used for this study includes rotary evaporator (Labo Rota 4000, Heidolph Instrument),Autoclave, Incubator TLC plates, round bottom flask (50, 100 and 250 mL), measuring cylinders, Erlenmeyer flasks, mortar and pestle, filter papers, weighing balances, oven for drying purpose, Melting point apparatus (Griffin 590), glass columns for CC (500mm) and UV-254 and 365 nm chamber (UV-Tec) for detection of spots on TLC were used for the study. Spectral recording was done using Bruker 500 MHz advance NMR spectrometer with TMS as internal standard.

## 3.3 Plant collection and identification

The plant materials, *D.angustofolia* vernacular name 'Itacha' (Afan Oromo),'Kitkita' (Amharic)was collected from their natural habitat, uncultivated land in Jimma zone South western Ethiopia, Welda district, and Merewa Kebele in April, 2020.The collected plant material was identified and voucher specimen has been deposited with voucher No.TM01 at Addis Ababa University, Herbarium. The root bark of the plant was collected and allowed to air dry under shade at room temperature. After being well dried, the plant materials was grinded to suitable size to improve the subsequent extraction by rendering the sample more homogenous, increasing the surface area, and facilitating the penetration of solvent into the cells.

### 3.4 Extraction and Isolation

#### 3.4.1 Extraction

Part of the powdered plant material (0.85 kg) was sequentially extracted with petroleum ether, chloroform, acetone and methanol using maceration technique with occasional shaking at 25°C for 72 hrs. The extracts were filtered and concentrated using rotary evaporator under reduced pressure at 40°C. The resulting semidried extracts were further dried and weighed. The resulting semisolid extracts were stored in desiccators.

#### 3.4.2 Isolation

TLC analyses were carried out on each crude extract, and those with similar TLC profile were combined adsorbed on silica gel and applied to column chromatography over silica gel. The column was initially eluted with 100 % petroleum ether and then with mixture of petroleum ether and increasing amount of ethyl acetate (up to 100% ethyl acetate).

A total of 65 fractions each with 150 ml were collected and pooled together according to their TLC profile to afford nine combined fractions. A fractions from 23-27 eluted with petroleum ether to ethyl acetate (with the ratio of 87:13,80:20,78:22, 75:25, 70:30) showed a yellow color and it was partitioned into petroleum ether soluble and insoluble portion. The petroleum ether insoluble portion was dissolved in chloroform and applied to Sephadex LH-20 for further purification to provide white crystalline solid (51 mg) with $R_f$ 0.45 PE/EtOAc (9:1) assigned a code DA1. Fraction obtained from 45-65 (1.2 g) was subjected to column chromatography of mini column filled with silica gel then eluted with petroleum ether in increasing amounts of ethyl acetate gradient. Based on TLC profile, fractions that have the same R*f* value were combined together and fraction 46, 47 and 48 afford a compound DA5 (123 mg R*f* PE/EtOAc (6:4)) eluted with 70% EtOAc with brownish color. Compound DA3 was isolated from crude extract of petroleum ether. 0.6 g of the extract was dissolved in petroleum ether then applied to sephadex LH-20 for purification using MeOH:$CHCl_3$ (1:1). Total of 6 fraction were collected and fraction 5 and 6 afford compound DA3(31 mg yellowish) with R*f* of PE/EtOAc (9.9:0.1).

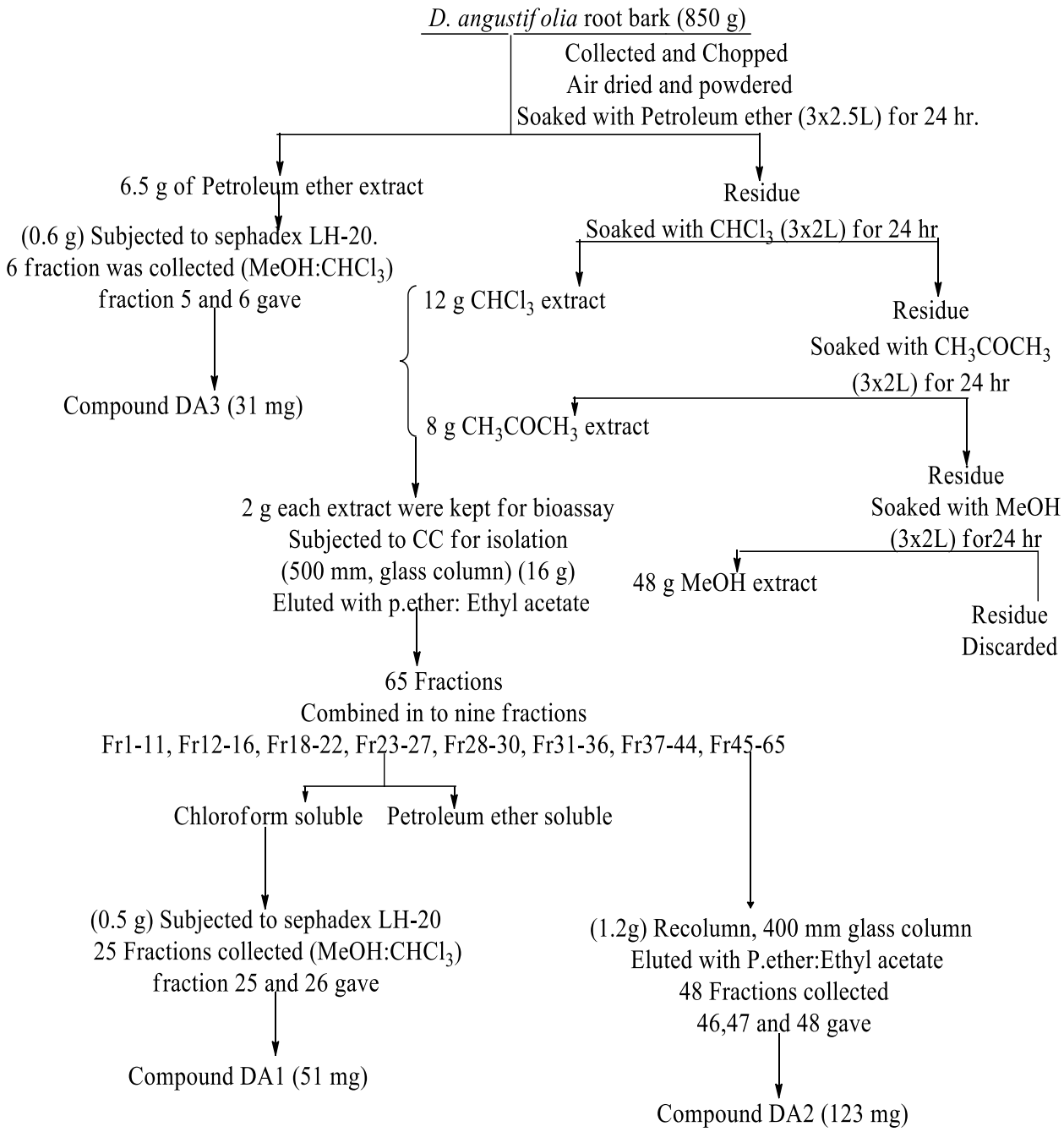



Figure 4. Schematic flow chart of the extraction and isolation

Likewise, based on their TLC resemblances again three combined fractions Fr1-11 (1.3 g), Fr 31-36 (2.3 g), Fr 37-44 (2.5 g) were subjected to CC separately for further purification afford compound DA2 (yellow PE/EtOAc 9:1),DA4 (greenish PE/EtOAc 8:2) and DA6 (brawn PE/EtOAc 7:3) respectively.

### 3.5 Characterization of isolated compounds

Column chromatographic separation of the petroleum ether, acetone and chloroform extracts of root barks of *D. angustofolia* has resulted in the isolation of a total of six compounds. The purity of the isolated compounds was monitored by TLC analysis. However, only two compounds (DA1 and DA3) were analyzed for structural determination. Structural elucidation of these compounds were performed by NMR ($^{1}$HNMR, $^{13}$CNMR and DEPT-135) spectroscopic and comparison with reported literature data. The Melting point was determined using a Griffin 590 melting point apparatus equipped with a thermometer.

### 3.6 Antibacterial activity of the plant extracts

#### 3.6.1 Test micro-organisms

Two Gram-positive (*Staphylococcus aureus* (ATCC25923) and *Bacillus cereus* (ATCC) and two Gram-negative (*Salmonella Typhimurium* (ATCC-13311) and *Escherichia coli* (ATCC-25922)) as well as one fungal strains *Candida albicans* (ATCC-14053) were used for the evaluation of antimicrobial activities of the extracts and isolated compounds. The standard cultures of all experimental bacteria and fungi strains were obtained from, Biology Department, Jimma University.

#### 3.6.2 Inoculum Preparation

The microorganisms were separately cultured on sterilized Muller-Hinton Agar (MHA) at 37°C for 24 hours by using streak plate method. Then, well-isolated overnight-cultured colonies of the same morphological type were selected from the cultured media. Each colony was touched with a flamed wire-loop and the growth was transferred into a sterilized test tube containing 5 ml sterile normal saline solution. The test tubes that contain the bacterial suspension were vortexes to be mixed well uniformly. The bacterial suspension was adjusted with 0.5 McFarland turbidity standards. The adjustment and comparison of turbidity of inoculum tubes were performed by visually observing them with naked eye against a 0.5 McFarland turbidity equivalence standard with white background and contrasting blue lines in the presence of adequate light.

### 3.6.3 Antibacterial and antifungal activity

The study on antimicrobial activity extracts and isolated compound were carried using the agar disk diffusion method. Zones of inhibition were measured in mm as a diameter in areas where no growth of the bacteria was visible [48].

Bacteria suspension ($1.0 \times 10^8$CFU/ml) was then inoculated to standard Petri dishes which were formerly prepared using Muller-Hinton Agar according to manufacturer procedure. Tests were performed in duplicate. Small volumes of bacterial suspensions were swabbed to each MHA plate and then evenly seeded and streaked by means of sterile cotton swab on the agar plate surface. This procedure was repeated by streaking two or more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculums and, finally, the rim of the agar was swabbed. The test solutions were prepared by dissolving 250 mg of plant extracts into 1ml of DMSO to get final concentrations of 250 mg/mL. Sterile filter paper discs (6 mm) were soaked with stock solution of the extract then placed over the seeded plates at equidistance. The plates were then inverted and incubated at 37 ℃ for 24 hr.

Bioassay for antifungal activities was the same method described for bacteria adopted except the medium (potato dextrose agar)and incubation time (3 days). For each bacterial and fungal strain, the standard gentamicin and clotrimazole were taken respectively and DMSO as the negative control.

### 3.6.4 Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the microbial growth after 24 h. of incubation. The most effective plant extracts which exhibiting a strong antibacterial activity at 250 mg/ml was manipulated to determine their MIC using disk diffusion method and evaluate their efficiency. Different concentrations of the effective plant extract (250, 12.5, 62.5, 31.3, 15.6, 7.8 mg/ml) were prepared by serial two-fold dilution method separately by dissolving 250 mg in 1 ml of acetone, sterilized through Millipore filter and loaded their requisite amount over sterilized filter paper discs (6 mm in diameter). Mueller-Hilton agar was poured into sterile Petri dishes and seeded with bacterial suspensions of the pathogenic strains. The loaded filter paper discs with different concentrations of the effective plant extract were placed on the top of the Mueller-Hilton agar plates then incubated at 37 C for 24 h. The inhibition zones were measured by ruler and recorded against the concentrations of the effective plant extracts.

### 3.7 Qualitative phytochemical analysis

The qualitative analyses of secondary metabolites were carried out following standard methods.

#### 3.7.1 Test for Saponins

About 1 ml of the extract was dilute separately with 20 ml of distilled water and was shaking in a graduated cylinder for 15 minutes. A 1 cm layer of foam was indicates the presence of saponin [49].

#### 3.7.2 Test for Tannins and Phenolic compounds

Ferric chloride test: 1 ml of the extract was treated with few ml of 5% neutral ferric chloride. A dark blue or bluish black color product was show the presence of tannins or phenolic compounds [49].

#### 3.7.3 Test for Alkaloids

Wagner's reagent: Few drops of Wagner's reagent (solution of iodine in potassium iodide) were added to each filtrate and observed for the formation of reddish brown precipitate which may indicate the presence of alkaloids [50].

#### 3.7.4 Tests for Flavonoids

Alkaline reagent Test: Extract sample is treated with a few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids [50].

#### 3.7.5 Test for Quinones

1ml of the extracts was treated with 5ml of HCl. Formation of yellow color precipitate indicates the presence of Quinone [51].

#### 3.7.6 Test for Sterols

*Salkowski test*: The extract sample is dissolved in chloroform and equal volume of concentrated sulphuric acid is added. Bluish red, cherry red and purple color indicates presence of sterols[52].

## 4. Resultand Discussion

### 4.1 Percentage yield of crude extracts

Air dried root bark of *D. angustofolia* was sequentially extracted with petroleum ether, chloroform, acetone and methanol. The percentage yields of the extracts were calculated as follows (Table 3).

$$yield = \frac{weight\ of\ the\ crude\ extract\ (g)}{weight\ of\ the\ sample\ used\ (g)}\ x\ 100$$

Table 3. Percentage yield of crude extracts

| Solvents | Mass of crude extract (g) | Yield of extract (%) |
|---|---|---|
| Petroleum ether | 6.5g | 0.8 |
| Chloroform | 12g | 1.4 |
| Acetone | 8g | 0.9 |
| Methanol | 48g | 5.6 |

Relatively higher yield of extract (5.6%, 48 g) was obtained with methanol as solvent and the least with Petroleum ether (0.8%, 6.5 g).

### 4.2 Preliminary phytochemical screening

The crude extracts obtained from the root bark of *D. angustofolia* subjected to phytochemical screening tests and the results summarized in Table 4.

Table 4. Phytochemical screening from root barks of *D.angustofolia*

| Phytochemicals | Petroleum ether | Chloroform | Acetone | Methanol |
|---|---|---|---|---|
| Alkaloids | - | - | - | + |
| Flavonoids | - | ++ | ++ | - |
| Phenols | - | ++ | - | - |
| Tannins | - | - | + | ++ |
| Saponins | - | ++ | + | ++ |
| Quinone | - | - | - | - |
| Sterols | ++ | ++ | ++ | - |

Key present (+) and absent (-)

### 4.3 Characterization of the Isolated compounds

#### 4.3.1 Characterization of compound DA1

Compound DA1was isolated as a white crystalline solid with melting point 169-172 °C. The $^{1}$H-NMR spectrums (Table 5 & Appendix 4, 5 and 6) showed the presence of three olefinic protons at $\delta_H$ 5.37 *d* (H, 5.2Hz), 5.17 *ddd* (H, 8.5, 15.1Hz) and 5.04 *dd* (H, 8.3, 15.2Hz) corresponding of a tri-substituted and a di-substituted olefinic groups and were assigned to H-6, H-22 and H-23 respectively. It also showed multiplet signal at 3.56 ppm corresponding to oxymethine proton, whose position and multiplicity corresponds to the H-3 of the steroid nucleus [53].The presence of six methyl groups at $\delta_H$ 0.72 *s*, 0.82 *t* (6.6 Hz), 0.87 *d* (6.2Hz), 0.87 *d* (7.0 Hz), 0.90 *s* and 1 .04 *d* (7.3Hz) were evident that the compound is steroid with six methyl groups as in case of stigmasterol [54].

The $^{13}$C NMR spectrum (Table 5)showed twenty-nine carbon signals with the following functionalities: four 122.2 (C-6), 129.8 (C-23), 138.8 (C-22), 141.2 (C-5) olifinic; six methyl carbons $\delta_C$ 12.5 (C-18), 12.8 (C-29), 9.5 (C-27), 19.9 (C-19), 12.5 (C-18), 21.7 (C-26), a carbioxy carbon $\delta_C$ 72.3 (C-3); seven methine carbons 32.4 (C-8), 32.4 (C-25), 50.6 (C-9), 40.9 (C-20), 51.7 (C-24), 56.0 (C-17), 56.4 (C-14); three quaternary carbon atoms 141.2 (C-5), 36.9 (C-10), 42.3 (C-13) which were confirmed from corresponding DEPT-135 spectrum. Dept-135 spectrum further showed the presence nine methylene carbons $\delta_C$ 21.7 (C-21), 24.6 (C-15), 25.9 (C-28), 30.2 (C-16), 31.1 (C-2), 32.3 (C-7), 37.7 (C-1), 39.5 (C-12), 42.7 (C-4)).

The observed melting point (169-172°C) was also comparable with the reported melting point (167-169°C) for stigmasterol [53]. Based on these spectroscopic data and comparison with reported literature data, compound DA1 was found to be stigmasterol [54].

Table 5.$^{1}$H(500 MHz) and $^{13}$C NMR Spectroscopic Data for compound DA1

| Position | Observed for DA1 ($CDCl_3$) | | | Reported ( $CDCl_3$) [54] | | |
|---|---|---|---|---|---|---|
| | $\delta_H$(*m*, J) | $\delta_C$ | (*m*, J) | $\delta_C$ | DEPT DA1 | Remarks |
| 1 | | 37. 7 | | 37.2 | 37.71 | $CH_2$ |
| 2 | | 32. 1 | | 31.6 | 32.08 | $CH_2$ |
| 3 | 3.56 | 72. 3 | 3.53 | 71.8 | | CH |
| 4 | | 42. 7 | | 42.3 | 42.72 | $CH_2$ |
| 5 | | 141. 2 | | 140.7 | - | C |
| 6 | 5.37 (H,*d* J=5.2Hz) | 122. 2 | 5.35 (H, *d*, J=4.7 Hz) | 121.7 | | CH |
| 7 | | 32. 3 | | 31.9 | 32.34 | $CH_2$ |
| 8 | | 32. 4 | | 31.9 | | CH |
| 9 | | 50. 6 | | 50.1 | | CH |
| 10 | | 36. 9 | | 36.5 | - | C |
| 11 | | 21. 5 | | 21.0 | 21.53 | $CH_2$ |
| 12 | | 39. 5 | | 39.7 | 39.54 | $CH_2$ |
| 13 | | 42. 3 | | 42.2 | - | C |
| 14 | | 56. 4 | | 56.8 | | CH |
| 15 | | 24. 6 | | 24.4 | 24.64 | $CH_2$ |
| 16 | | 30. 2 | | 28.9 | 30.16 | $CH_2$ |
| 17 | | 56. 0 | | 55.9 | | CH |
| 18 | 0.72*s* (Me) | 12. 5 | 0.70 *s* (Me) | 12.0 | | $CH_3$ |
| 19 | 0.90*s* (Me) | 19.8 | 1.01 *s* (Me) | 19.4 | | $CH_3$ |
| 20 | | 40.9 | | 40.5 | | CH |
| 21 | 1 .04*d*(7.3 Hz ,Me) | 21. 6 | 1.02 *d* (6.8 Hz) Me | 21.1 | | $CH_3$ |
| 22 | 5.17 *ddd*(8.5,15.1Hz) | 138. 7 | 5.15 *dd* (8.4,15.1 Hz) | 138.3 | | CH |
| 23 | 5.04 *dd*(8.3,15.2Hz*)* | 129. 7 | 5.02*dd* (8.4,15.1 Hz) | 129.2 | | CH |
| 24 | | 51. 7 | | 51.2 | | CH |
| 25 | | 32. 4 | | 31.9 | | CH |
| 26 | 0.87 *d* (7.0 Hz, Me) | 21. 7 | 0.84 d (6.4 Hz, Me) | 21.2 | | $CH_3$ |
| 27 | 0.87 *d* (6.2Hz, Me) | 19. 5 | 0.83 d (6.1 Hz, Me) | 18.9 | | $CH_3$ |
| 28 | | 25. 9 | | 25.4 | | $CH_2$ |
| 29 | 0.82*t*(6.6 Hz, Me) | 12. 7 | 0.80 t (6.0 Hz, Me) | 12.3 | | $CH_3$ |

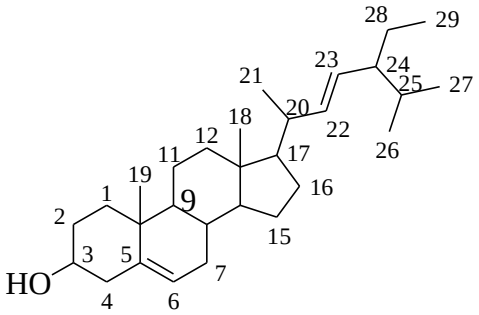



Figure 5.The proposed structure of compound (3*β*, 22E)-Stigmasta-5, 22-dien-3-ol

### 4.3.2Characterization of compound DA3

Compound DA3 was isolated as yellow semi solid. The $^{1}$H-NMR spectrum (Table 6) of DA3 exhibited similar spectral features with that of DA1. The presence of three olefinic resonances at $\delta_H$ 5.36 *m* (5H),5.16 *dd* (H, 8.6,15.0Hz) and 5.03 *dd* (H, 8.6,15.1Hz) for olifinic protons and an oxygenated aliphatic carbon at $\delta_H$ 3.53 as well as cluster of resonances up field shifted signals for seven methyl groups at $\delta_H$ 0.71 *s* ( 3H), 0.82 *t* (7.20 Hz 3H),0.87 *d* (6.26 Hz, 3H), 0.88 *d* (7.02 Hz, 3H), 0.99*s* (3H) and 1.03 *d* (8.8 Hz, 3H) indicates presence of protons of aliphatic methyl's which suggests steroidal nucleus [54,55].

Furthermore, the $^{13}$C NMR spectrum gave 47 signals; containing seven methyl ($CH_3$) groups, 21 methylene ($CH_2$), seven methine (CH), seven olefinic and four quaternary carbon atoms which is corroborated by DEPT-135 spectrum further indicating the steroidal nature of the compound. The downfield resonances at δ 141.2(C-5), 122.1(C-6), 138.8(C-22), 129.7(C-23) and 130.6(C-6'), 130.4(C-10'), 128.5(C-9'), 128.3(C-7') ppm indicate unsaturation due stigmasterol nucleus and ester bonded substituent. The signals at 19.8 and 12.5correspond to angular carbon atom at C-19 and C-18 respectively [53].The peaks at (44.6and 36. 9ppm) and (141.2and 178.9 ppm) were assignable to saturated and unsaturated quaternary carbon atoms, which are confirmed by absence of corresponding signals from the DEPT-135 spectrum. The resonance at δ72.4(C- 3) is due to C-3 β-hydroxyl group further suggesting the compound as stigmasterol derivative.

From the DEPT-135, a peak at 12.5, 19.8, 21.6, 18.9 and 12.7 ppm are consistent with six methyl carbon atoms. Peaks at 37.7, 32.3, 42.64, 32.4, 20.9, 40.2, 25.2, 29.9 and 25.8 showed methylene carbon atoms. Furthermore, peaks at 72. 4, 32.3, 50.6, 57.3, 56.4, 40.9, 51.7, 31.9, 138.7, 129.7 indicated the presence of sp3 and sp2 carbon atoms.

Hence, from the given data, the structure of the compound possesses sterol skeleton. Therefore, based on spectroscopic data (Table 6 and appendices 7-9) and comparing with the reported literature [54, 55, 56], the compound was identified to be stigmasterol derivative, *β*-stigmasteryl linoleate.

Table 6.$^{1}$H (500 MHz) and 13C NMR Spectroscopic Data for compound DA3

| Position | Observed for DA3 ($CDCl_3$) | | Reported ( $CDCl_3$)[54,55,56,57] | | | |
|---|---|---|---|---|---|---|
| | $\delta_H$ (*m*, J) | $\delta_C$ | $\delta_H$ (*m*, J) | $\delta_C$ | DEPT DA3 | Remarks |
| 1 | | 37. 7 | | 37.2 | 37.70 | $CH_2$ |
| 2 | | 32.3 | | 31.6 | 32.34 | $CH_2$ |
| 3 | 3.53 | 72. 4 | 3.53 | 71.8 | | CH |
| 4 | | 42.6 | | 42.27 | 42.64 | $CH_2$ |
| 5 | | 141. 2 | | 140.7 | - | C |
| 6 | 5.3-5.4 *m*(5H) | 122. 2 | 5.35 *d* (H, 4.7 Hz) | 121.7 | | CH |
| 7 | | 32.4 | | 31.9 | 32.36 | $CH_2$ |
| 8 | | 30.1 | | 31.9 | | CH |
| 9 | | 50. 6 | | 50.1 | | CH |
| 10 | | 36. 9 | | 36.5 | - | C |
| 11 | | 20.9 | | 21.06 | 21.53 | $CH_2$ |
| 12 | | 40.2 | | 39.7 | 39.54 | $CH_2$ |
| 13 | | 44.6 | | 44.56 | - | C |
| 14 | | 57.3 | | 56.8 | | CH |
| 15 | | 25.2 | | 24.4 | 24.64 | $CH_2$ |
| 16 | | 29.3 | | 28.9 | 29.30 | $CH_2$ |
| 17 | | 56.4 | | 55.9 | | CH |
| 18 | 0.71 *s* (Me) | 12. 5 | 0.70 *s* (Me) | 12.04 | | $CH_3$ |
| 19 | 0.99*s* (Me) | 19.8 | 1.01 *s* (Me) | 19.4 | | $CH_3$ |
| 20 | | 40.9 | | 40.5 | | CH |
| 21 | 1 .03*d* (8.8 Hz Me) | 21.5 | 1.02 *d* (6.8 Hz) | 21.09 | | $CH_3$ |
| 22 | 5.16*dd*(8.6,15.0Hz) | 138. 7 | 5.15 *dd* (8.4,15.1 Hz) | 138.3 | | CH |
| 23 | 5.03*dd*(*8.6,15.1*Hz*)* | 129. 7 | 5.02*dd* (8.4,15.1 Hz) | 129.2 | | CH |
| 24 | | 51. 6 | | 51.2 | | CH |
| 25 | | 31.9 | | 31.9 | | CH |
| 26 | 0.88 *d* (7.02 Hz, Me) | 21. 6 | 0.84*d* (6.3 Hz, Me) | 21.2 | | $CH_3$ |
| 27 | 0.87*d* (6.26 Hz, Me) | 19.4 | 0.83 *d* (6.1 Hz, Me) | 18.97 | | $CH_3$ |
| 28 | 1.15 *m* | 25. 8 | 1.15 *m* | 25.4 | | $CH_2$ |
| 29 | 0.82 *t* (7.20 Hz, Me) | 12. 6 | 0.80 *t* (6.0 Hz, Me) | 12.3 | | $CH_3$ |
| (C-1′) | | 179.4 | | 173.25 | | C |
| (C-2′) | 2.33 (*m*) | 34.4 | 2.32 *m* ( H-2′) | 34.72 | | $CH_2$ |
| (C-3′) | 1.67 | 26.1 | 1.67 *m* | 25.6 | | $CH_2$ |
| (C-4′) | 1.27-1.33*m* | 29.7 | 1.28-1.33*m* | 29.7 | | $CH_2$ |
| (C-5′) | 1.27-1.33*m* | 29.6 | 1.28-1.33*m* | 29.6 | | $CH_2$ |

| | | | | | |
|---|---|---|---|---|---|
| (C-6′) | 1.27-1.33*m* | 29.6 | 1.28-1.33*m* | 29.6 | $CH_2$ |
| (C-7′) | 1.27-1.33*m* | 29.5 | 1.28-1.33*m* | 29.5 | $CH_2$ |
| (C-8′) | 2.02-2.08 *m* | 29.3 | 2.02-2.08 *m* | 27.8 | $CH_2$ |
| (C-9′) | 5.3-5.4*m* | 130.2 | 5.3-5.4*m* | 130.2 | CH |
| (C-10′) | 5.3-5.4*m* | 128.5 | 5.3-5.4*m* | 127.9 | CH |
| (C-11′) | 2.78*t* (6 Hz) | 25.9 | 2.79*t* (6 Hz) | 25.0 | $CH_2$ |
| (C-12′) | 5.3-5.4*m* | 128.3 | 5.3-5.4*m* | 127.8 | CH |
| (C-13′) | 5.3-5.4*m* | 130.4 | 5.3-5.4*m* | 130.0 | CH |
| (C-14′) | 2.02-2.08 *m* | 27.6 | 2.02-2.08 *m* | 27.2 | $CH_2$ |
| (C-15′) | 1.27-1.33*m* | 29.4 | 1.27-1.33*m* | 29.4 | $CH_2$ |
| (C-16′) | 1.27-1.33*m* | 31.9 | 1.27-1.33*m* | 31.9 | $CH_2$ |
| (C-17′) | 1.27-1.33*m* | 23.1 | 1.27-1.33*m* | 22.7 | $CH_2$ |
| (C-18′) | 0.83 *m* | 14.1 | 0.83 *m* | 14.1 | $CH_3$ |

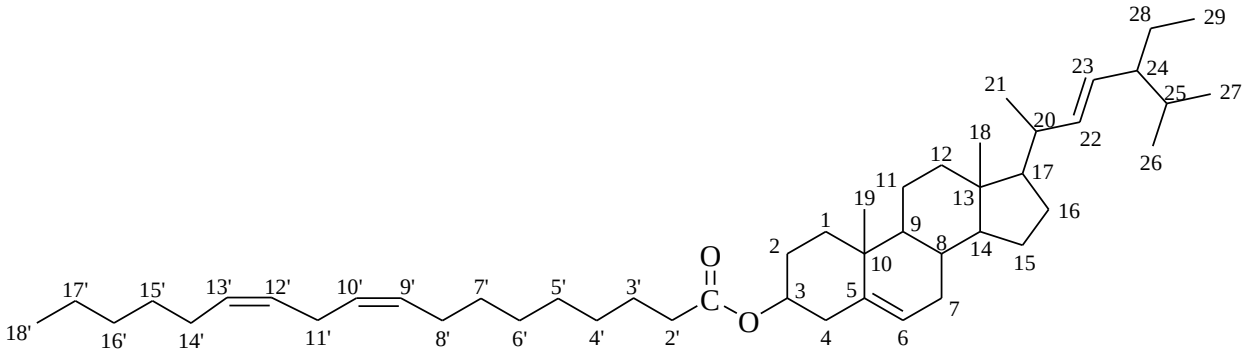



Figure 6. The proposed Structure of compound and $\beta$ –Stigmasteryl linoleate

### 4.4 Antimicrobial activities

The crude extracts and the isolated compounds were in vitro assayed against four bacterial strains (*E.coli, S.aureus, B.cereus, S. typhi* and one Fungi strain *(Candida.albican*). The diameters of zone of inhibition in millimeters are described below (Table **7**).

Table 6.Antimicrobial activity of the extracts and compounds

| Zone of inhibition of the extract *in mm*(Mean± SD mm) | | | | | | | |
|---|---|---|---|---|---|---|---|
| strains | Methanol | Acetone | Chloroform | P.ether | DA5 | Gent/Clotrimazole | DMSO |
| *E.coli* | 10.5 ± 0.5 | 12.7 ±0.75 | 9.5 ±0.50 | 8.5±0.50 | 8.2 ± 0.25 | 22.5 ± 0.50 | NI |
| *S.aureus* | 8.7 ± 0.25 | 12.5 ± 0.50 | 8.5± 0.50 | NI | 7.8 ± 0.50 | 21.5 ± 0 .50 | NI |
| *B.cereus* | 8.7 ± 0.25 | 9.5 ± 0.50 | 7.5 ± 0.50 | 7.5 ± 0.50 | 6.8 ± 0.50 | 21.5 ± 0.50 | NI |
| *S. typhi* | 7.7 ±0.25 | 11.5 ±0.50 | 10.5 ± 0.50 | 10.2±0.25 | 7.2 ± 0.25 | 22.5 ±0.50 | NI |
| *C.albicans* | 8.5 ± 0.50 | 11.5 ± 0.50 | NI | NI | 9 ± 0.50 | 13.5 ± 0.50 | NI |

The antimicrobial activity results (Table 7) showed that all the crude extracts exhibited good in vitro antibacterial and antifungal activities relative to the standard with zones of inhibition greater than 7.5 and 8.5 mm, respectively against the four bacterial and one fungal strains that were used in the experiment. When the zones of inhibitions of the four crude extracts were compared to each other, the antibacterial and antifungal activity of the petroleum ether extract was found to be comparatively lower than that of chloroform, acetone and methanol extracts. Its inhibition zones are in the range of 8.5-9.3 mm (Table 7). On the other hand, the crude extracts of chloroform and methanol were found to show similar antibacterial activities with inhibition zones that are in the ranges of 10.5-7.5 and 10.5-7.75 mm, respectively (Table 7). It was also observed that the acetone extract showing to some extent superior antibacterial and antifungal activities on *E.coli* (12.7 mm), *S.aurues* (12.5 mm), *B.cereus* (7.5 mm), *Candida albican* (11.5 mm) strains. *D.angustofolia* acetone extracts was found to be more substantial suppression on fungi (11.5 and 13.5 mm for extract and clotrimazole respectively) relative to the bacterial strain which also be consistent with previous report on the evaluation of antifungal activities of the crude extracts of *D. viscosa* leaves [58].

The inhibition observed for the isolated DA5 compound with zone of inhibition for microorganisms such as *E.coli, S. aureus, B. cereus, S. typhi, C. albicans* were 8.2, 7.8, 6.8, 7.2

mm respectively. Antifungal activity of isolated compound of DA5 has zone of inhibition 9 mm but the rest of compounds haven't been showed any antibacterial and antifungal activity. The crude extract that showed antibacterial and antifungal potential was further assessed for the minimum inhibitory concentration (MIC). Comparatively, the acetone crude extract has displayed significant antibacterial properties on the tested bacteria and fungi. The MIC results are depicted in the following Table 8, Appendix 2).As it can be seen from the data Table 8, the in vitro antibacterial and antifungal activities tests were carried out against four bacterial strains using different concentration of the crude extract.

Table 7.MIC of acetone extract

| Diameter of zone of Growth Inhibition (mm) | | | | | |
|---|---|---|---|---|---|
| Concent.( mg/mL) | *E.coli* | *S.aureus* | *B.cereus* | *S.typhi* | *Candida alb.* |
| 250 | 12.7 | 11.5 | 9.5 | 11.8 | 11.5 |
| 125 | 9 | 8.3 | 8.6 | 11 | 10 |
| 62.5 | 8 | 8 | 8.1 | 10.5 | 9.3 |
| 31.3 | NI | 7.2 | 7.8 | 8 | 8.2 |
| 15.6 | NI | NI | 7 | 7 | 7.5 |
| 7.8 | NI | NI | NI | NI | NI |

Key: NI= No inhibition

Minimal Inhibitory Concentration *E.coli, S. typhi, B. cereus, S. aureus* and *Candida .alb* were 62.5, 31.3, 15.6, 15.6 and 15.6 mg/mL respectively (Table 7, Appendix 2).

# 5. Conclusion and Recommendation

## 5.1 Conclusion

Based on ethno botanical information, phytochemical investigation and antimicrobial activity tests of root bark of *D.angustofolia* were performed and it can be concluded that various phytochemicals are present in the root bark of this plant such as alkaloids, tannins, saponins, flavonoids and steroids. The isolation of the compounds were results two compounds and it has been characterized as (3*β*,22E)-Stigmasta-5,22-dien-3-ol (DA1) and *β*-Stigmasteryl linoleate (DA3) for the first time from the study plant species.

Antimicrobial activity tests showed some antimicrobial potency of the extracts even though it was lower as compared to standard antibiotics. Root bark extract of the plant was active against the five microbial pathogens tested. The acetone crude extract showed the highest degree of antibacterial activity against *Staphylococcus aureus*, and *Escherichia coli* as compared to the other extracts. From the present study we can conclude that this plant contains several phytochemicals, but antimicrobials activities were comparatively lower than that of the standard drug used in the study. The presence of these phytochemicals in this plant root bark enhances their pharmaceutical, therapeutic potentials and provides scientific support for the ethno medicinal use of the plant. The present study indicates the plant root bark is a potential source of bioactive compounds and can be used as source for antibacterial and antifungal agents.

## 5.2 Recommendation

Based on the current finding, the following are recommended on the study:

i. The structural elucidation of isolated compound should be further rely on 2D NMR spectroscopies such as HMBC, HSQC, COSY and NOESY.
ii. Further isolation and characterization of bioactive compounds from methanol extracts should be done.
iii. Study on other bacterial and fungal strains should be included to decide their potential as candidates in development of antimicrobial drugs.
iv. More biological assay such as antioxidant, antiplasmodial and anti-inflammatory activities needs to be conducted on various extracts of the plant so as to establish the traditional use of the plant.

## Reference


[1] Ekor, M. The Growing Use of Herbal Medicines: Issues Relating to Adverse Reactions and Challenges in Monitoring Safety. *Front. Neurol* **2014**, *4* ,1–10.

[2] Egamberdieva, D.; Ovidi, E.; Tiezzi, A.; Craker, L. Phytochemical and Pharmacological Properties of Medicinal Plants from Uzbekistan *J. Med. Act. Plants* **2016**, *5*, 59–75.

[3] De Rycker, M.; Baragaña, B.; Duce, S. L.; Gilbert, I. H. Challenges and Recent Progress in Drug Discovery for Tropical Diseases. *Nature* **2018**, 498–506.

[4] Milner, J. A. Recent Advances on the Nutritional Effects Associated with the Use of Garlic as a Supplement A Historical Perspective on Garlic and Cancer *American Society for Nutritional Sciences* **2001**, 1027–1031.

[5] Iwu, M. M.; Duncan, A. R.; Okunji, C. O. New Antimicrobials of Plant Origin **1999**, 457–462.

[6] Khameneh, B.; Diab, R.; Ghazvini, K.; Fazly Bazzaz, B. S. Breakthroughs in Bacterial Resistance Mechanisms and the Potential Ways to Combat Them. *Microb. Pathog* **2016**, *95*, 32–42.

[7] Chellat, M. F.; Raguž, L.; Riedl, R. Targeting Antibiotic Resistance. *Angew. Chemie - Int. Ed.***2016**, *55* (23), 6600–6626.

[8] Veeresham, C. Natural Products Derived from Plants as a Source of Drugs. *J. Adv. Pharm. Technol. Res.***2012**, *3*, 200–201.

[9] Hazekamp, A.; Simons, R.; Peltenburg-Looman, A.; Sengers, M.; Van Zweden, R.; Verpoorte, R. Preparative Isolation of Cannabinoids from Cannabis Sativa by Centrifugal Partition Chromatography. *J. Liq. Chromatogr. Relat. Technol.***2004**, *27*, 2421–2439.

[10] Gebreyes, T.; Melesse, M. Determination of Informant Consensus Factor and Fidelity Level of Ethnomedicinal Plants Used in Misha Woreda, Hadiya Zone, Southern Ethiopia. *Int. J. Biodivers. Conserv.***2016**, *8*, 351–364.

[11] Teffo, L.S.; Aderogba, M.A.; Eloff, J.N. Antibacterial and antioxidant activities of four

kaempferol methyl ethers isolated from *Dodonaea viscosa* Jacq. var. *angustifolia* leaf extracts. *S. Afr. J. Bot.***2010**, *76*, 25-29.

[12] Pirdada, A.J.; Shaikh,W.;Usmanghani,U.; Mohiuddin, E. Antifungal activity of *Dodonaea viscosa* extract on fungi isolated from superficial skin infection. *Pak. J. Pharm. Sci*., **2010**, *23*, 337-340.

[13] Veerapar, V.P.; Prabhakar, K.R.; Thippeswamy, B.S.; Pansal, P.; Sirinvasan, K.K.; Unikrshanan, M.K. Antidiabetic effect of *D. viscosa* aerial parts in high fructose fedinsulin resistant rats: A mechanism based study. *Indian J. Exp. Biol.*, **2010**, *48*, 800-810.

[14] Huschka, C.; Schmidtke, M.; Abate, A.; Neubert, R.H.H.; Getie, M.; Gebre-Mariam, T.; Rietz, R.; Hohne, C. Evaluation of the antimicrobial and anti-inflammatory activities of the medicinal plants *Dodonaea viscosa*, *Rumex nervosus* and *Rumex abyssinicus*. *Fitoterapia*, **2003**, *74*, 139-143.

[15] Rajamanickam, V.; Rajasekaran, A.; Anandarajagopal, K.; Sridharan, D.; Selvakumar, K.; Rathinaraj, B.S. Antidiarreal activity of *Dodonaea viscosa* root. *IJPBS*, **2010**, *2*, 182-185.

[16] Subramani, R.; Narayanasamy, M.; Feussner, K. D. Plant-Derived Antimicrobials to Fight against Multi-Drug-Resistant Human Pathogens. *Biotech* **2017**, *7* (3), 1–15.

[17] Bisht, R.; Katiyar, A.; Singh, R.; Mittal, P. Antibiotic Resistance - A Global Issue of Concern. *Asian J. Pharm. Clin. Res.***2009**, *2* (2), 34–39.

[18] Anand, U.; Jacobo-Herrera, N.; Altemimi, A.; Lakhssassi, N. A Comprehensive Review on Medicinal Plants as Antimicrobial Therapeutics: Potential Avenues of Biocompatible Drug Discovery. *Metabolites* **2019**, *9* (11), 1–13.

[19] Edet, A.; Olorunfemi, E.; Aniebiet, E.; Isiguzoro, I. African Journal of Pharmacy and Pharmacology Changes in Serum Zinc, Magnesium and Copper in Sickle Cell Patients: A Case Study in Jos, Nigeria. **2015**, *9* (1), 53–59.

[20] Kerubo,L.O. phytochemical investigation of surface exudates of *dodoneaea angustifolia* and senecio rosefflorus for bioactive principles.Ph.D.Thesis, the University of

Nairobi.**2009**.

[21] GhisalbertI, E.L. Ethnopharmacology and phytochemistry of *Dodonaea* species. *Fitoterapia*. **1998**,99-113

[22] Palgrave, K.C. Trees of Southern Africa,Struik Publishers: Cape Town, RSA, **1981**.

[23] Cunningham, P. L.; Joubert, D. F.; Adank, W. *Dodonaea Angustifolia* – an Alien Invasive to the Auas Mountains in Namibia ? *Dinteria* **2004**, 29, 11–18.

[24] Asres, K.; Bucar, F.; Kartnig, T.; Witvrouw, M.; Pannecouque, C.; De Clercq, E. Antiviral Activity against Human Immunodeficiency Virus Type 1 (HIV-1) and Type 2 (HIV-2) of Ethnobotanically Selected Ethiopian Medicinal Plants. *Phyther. Res.***2001**, *15*, 62–69.

[25]. Tesfaye, S.; Belete, A.; Engidawork, E.; Gedif, T.; Asres, K. Ethnobotanical Study of Medicinal Plants Used by Traditional Healers to Treat Cancer-Like Symptoms in Eleven Districts, Ethiopia. *Evidence-based Complement. Altern. Med.***2020**.

[26] Beshah, F.; Hunde, Y.; Getachew, M.; Bachheti, R. K.; Husen, A.; Bachheti, A. Ethnopharmacological, Phytochemistry and Other Potential Applications of *Dodonaea* Genus: A Comprehensive Review. *Curr. Res. Biotechnol.***2020**, *2*, 103–119.

[27] Trotin, F.; Bezanger-Beauquesne, L.;Pinkas, M.;Robelet, A. Research on two plants used in popular medicine in Malagasy: *Cussonia bojeri* Seem. (Araliacea), *Dodonaea madagascariensis* Radik. (Sapindaceae). *Ann. Pharm. Fr.***1972**, 30, 555-560.

[28] Lassak, E. V. ;McCarthy,T.Australian medicinal plants, ;Octopus publishing company:Port Melbourne: Victoria,1990.

[29] Barr,A.Traditional Aboriginal medicines in the Northern territory of Australia.Conservation commission of the Northern territory of Australia:Darwin, 1993.

[30] Dominguez, X. A.; Franco, R.; Cano, C. G.; Chavez C, N.Medicinal plants of Mexico. XL1V. Isolation of 3,6,4'-trimethoxy-5,7-dihydroxyflavone from chapuliztle (*Dodonaea* v/.vcosa var. *angustifolici* jacq.). *Rev. Latinoamer Quim.* **1980**,11, 150-151.

[31] Andrews, J. M. BSAC Standardized Disc Susceptibility Testing Method (Version 5). *J.*

*Antimicrob. Chemother* **2006**, *58*, 511–529.

[32] Omosa,L.K.;Amugune,B.;Ndunda,B.;Milugo,T.K.;Heydenreich,M.;Yenesew, A.;Midiwo, J.O. Antimicrobial flavonoids and diterpenoids from Dodonaea angustifolia. *S.Afr.J.Bot* **2014**, 91, 58–62.

[33] Wabo, H.K.; Chabert, P.;Tane, P.; Noté, O.; Tala, M.F.; Peluso, J.; Muller, C.;Kikuchi, H.;Oshima, Y.; Lobstein, A. Labdane-type diterpenes and flavones from *Dodonaea viscosa*. *Fitoterapia***2012,** 83, 859–863.

[34] Shalaby, N.M.; Abd-alla, H.I.; Hamed, M.A.;Al-ghamdi, S.N.; Jambi, M.S.; Flavones composition and therapeutic potential of *Dodonaea viscosa* against liver fibrosis.*Int. J. Phytomedicine***2012** ,4, 27–39.

[35] Simpson, B.S.;Claudie, D.J.;Smith, N.M.; Gerber, J.P.; Mckinnon, R.A.;Semple, S.J.;Flavonoids from the leaves and stems of *Dodonaea polyandra*: a Northern Kaanju medicinal plant. *Phytochemistry***2011**,72, 1883–1888.

[36] Melaku, Y.; Worku, T.;Tadesse, Y.; Mekonnen, Y.; Schmidt, J.; Arnold, N.; Dagne, E. Antiplasmodial compounds from leaves of *Dodonaea angustifolia*. *Curr.Bioact. Compd.***2017**, 13, 268–273.

[37] Uddin, Z., Li, Z.;Song, Y.H.; Kim, J.Y.; Park, K.H. Visconata: a rare flavonol having long chain fatty acid from *Dodonaea viscosa* which inhibits human neutrophil elastase (HNE). *Tetrahedron Lett***2017**, 58, 2507–2511.

[38] Teffo, L.S.; Aderogba, M.A.; Eloff, J.N. Antibacterial and antioxidant activities of four kaempferol methyl ethers isolated from *Dodonaea viscosa* Jacq. var. angustifolia leaf extracts. *S. Afr. J. Bot***2010**, 76, 25–29.

[39] Mostafa, A.E.; El-hela, A.A.; Mohammad, A.I.; Jacob, M.; Cutler, S.J.; Ross, S.A. New secondary metabolites from *Dodonaea viscosa*. *Phytochem. Lett***2014**,8, 10–15.

[40] Niu, H.-M.;Zeng, D.-Q.; Long, C.L.;Peng, Y.-H.; Wang, Y.H.; Luo, J.-F.;Wang, H.S.; Shi, Y.N.; Tang, G.H.;Zhao, F.W. Clerodane diterpenoids and prenylated flavonoids from *Dodonaea viscosa*. *J. Asian Nat. Prod. Res***2010**,12, 7–14.

[41] Simpson,B.S.;Claudie,D.J.;Smith,N.M.;Mckinnon, R.A.; Semple, S.J. Rare, seven membered cyclic ether labdane diterpenoid from *Dodonaea polyandra*.*Phytochemistry*. **2012,**84,141–146.

[42] Muhammad, A.;Tel-çayan, G.; Öztürk, M.; Duru, M.E.; Nadeem, S.; Anis, I.; Ng, S.W.; Shah,M.R.Phytochemicals from *Dodonaea viscosa* and their antioxidant and anticholinesterase activities with structure – activity relationships. *Pharm. Biol***2016**. 54,1649–1655.

[43] https://uses.plantnet-project.org/en/Dodonaea_viscosa_(PROTA)

[44] Anode, S. O.; Abraha, T.; Araya, S. Phytochemical Analysis of *Dodonaea Angustifolia* Plant Extracts Phytochemical Analysis of *Dodonaea Angustifolia* Plant Extracts.*Int. J. Tradit. Herb*.Med**2018**.

[45] Khan.MSY.;Ahmed.Sh.;Jain PC. Chemical investigation of root barks of *Dodonaea viscosa* L. *J. Nat. Prod***1988**,4, 12-13.

[46] Mengiste, B.; Makonnen, E.; Urga, K. Invivo Antimalarial Activity of *Dodonaea Angustifolia* Seed Extracts Against Plasmodium Berghei in Mice Model **2012**,4,*47-63.*

[47] Amelo, W.; Nagpal, P.; Makonnen, E. Antiplasmodial Activity of Solvent Fractions of Methanolic Root Extract of *Dodonaea Angustifolia* in Plasmodium Berghei Infected Mice. *BMC Complement. Altern. Med.***2014,***14.*

[49] Bahera, M.; Karki, R.; Shekar, C. Preliminary Phytochemical Analysis of Leaf and Bark Methanolic Extract of Sesbania Grandiflora. *J. Phytopharm.* **2012**, *1*, 10–20.

[50] Sethupandian, G.; Kokkaiah, I.; Sinthia, G.; Palanichamy, M. Preliminary Phytochemical Screening of Different Solvent Extracts of Leaves and Stem of Crataeva Religiosa Hook & Frost. *Int. J. Bot. Stud.* **2016**, *1* , 21–24.

[51] Hossain, M. A.; AL-Raqmi, K. A. S.; AL-Mijizy, Z. H.; Weli, A. M.; Al-Riyami, Q. Study of Total Phenol, Flavonoids Contents and Phytochemical Screening of Various Leaves Crude Extracts of Locally Grown Thymus Vulgaris. *Asian Pac. J. Trop. Biomed.* **2013**, *3*, 705–710.

[52] Morsy, N. Phytochemical Analysis of Biologically Active Constituents of Medicinal Plants. *Main Gr. Chem.* **2014**, *13* , 7–21.

[53] Haque, M.; Sultana, N.; Abedin, S.; Kabir, S. Stigmasterol, Rengyolone, 2-Phenylethyl β-D-Glucopyranoside and n-Tetradecyl-β-D-Glucopyranoside from the Flowers of Nyctanthes Arbor-Tristis Linn. *Bangladesh J. Sci. Ind. Res.***2019**, *54* (3), 275–282.

[54] Cayme, J. C.; Dep, C. Structure Elucidation of β--Stigmasterol andβ-Sitosterol from Scsbania Urandittorallinn.J Pers. and β-Carotene from Hcliotronium Indicumlinn. By NMR Spectroscopy. **2004**, *20*, 5–12.

[55] Chang, M.; Zhang, T.; Feng, W.; Wang, T.; Liu, R.; Jin, Q.; Wang, X. LWT - Food Science and Technology Preparation of Highly Pure Stigmasteryl Oleate by Enzymatic Esteri Fi Cation of Stigmasterol Enriched from Soybean Phytosterols. **2020**, *128*

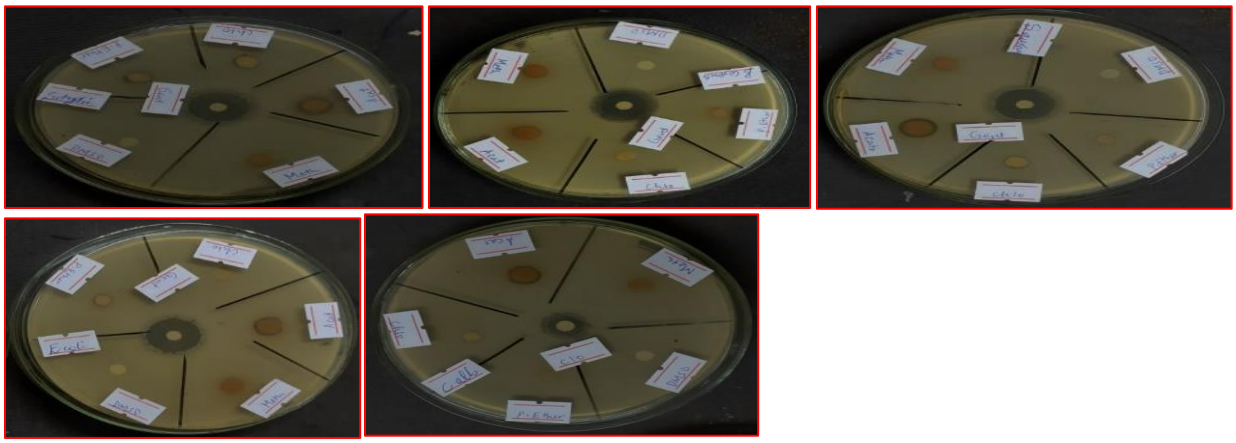
[56] Sajfrtová, M.; Sýkora, J.; Anna, B. LC-NMR Technique in the Analysis of Phytosterols in Natural Extracts. **2013**.

[57] Raczyk, M.; Kmiecik, D.; Przybylski, R.; Rudzin, M. Effect of Fatty Acid Unsaturation on Phytosteryl Ester Degradation **2017**, 701–711.

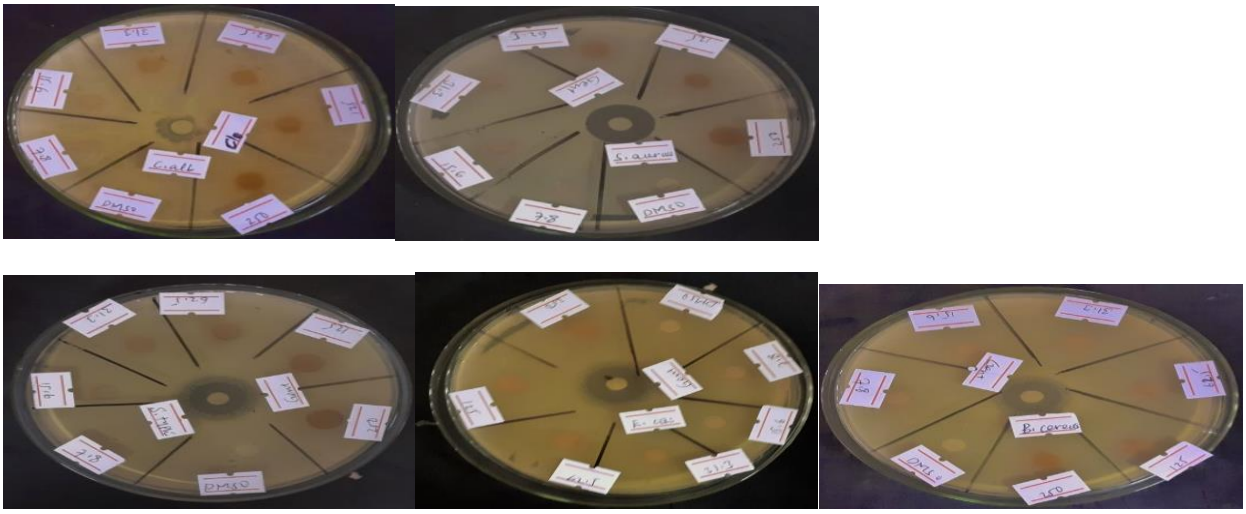
[58] Lee Yean Shan; Tee Chuan Thing; Tan Siow Ping; Khalijah Awang; Najihah Mohd Hashim; Mohd Azlan Nafiah and Kartini Ahmad. Cytotoxic, antibacterial and antioxidant activity of triterpenoids from *Kopsia singapurensis* Ridl.*Journal of Chemical and Pharmaceutical Research* **2014**, 815-822.

# Appendices

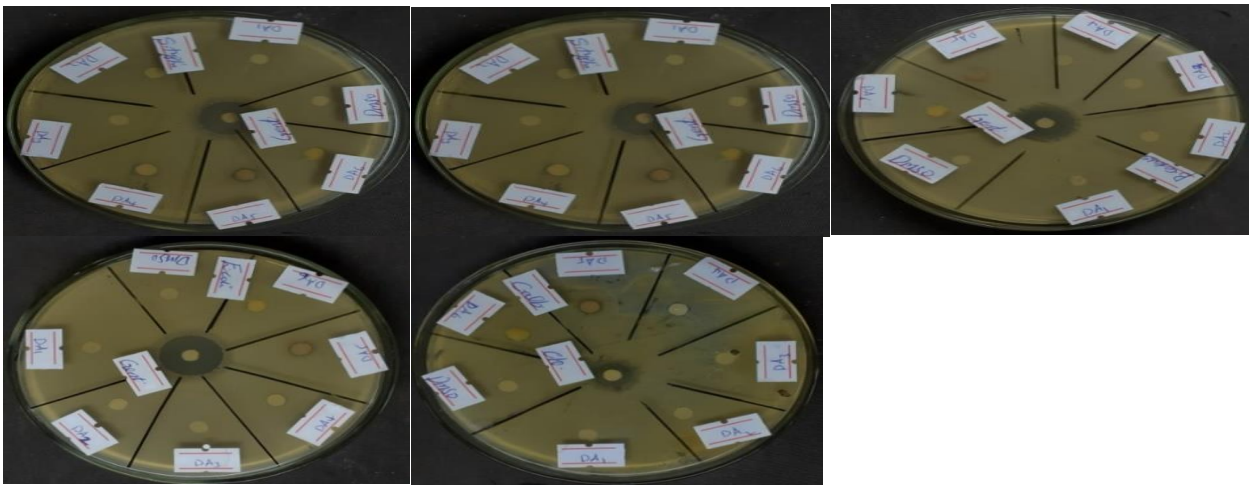
Appendix 1.Bioassays Tests of Crude Extracts zone of growth Inhibition



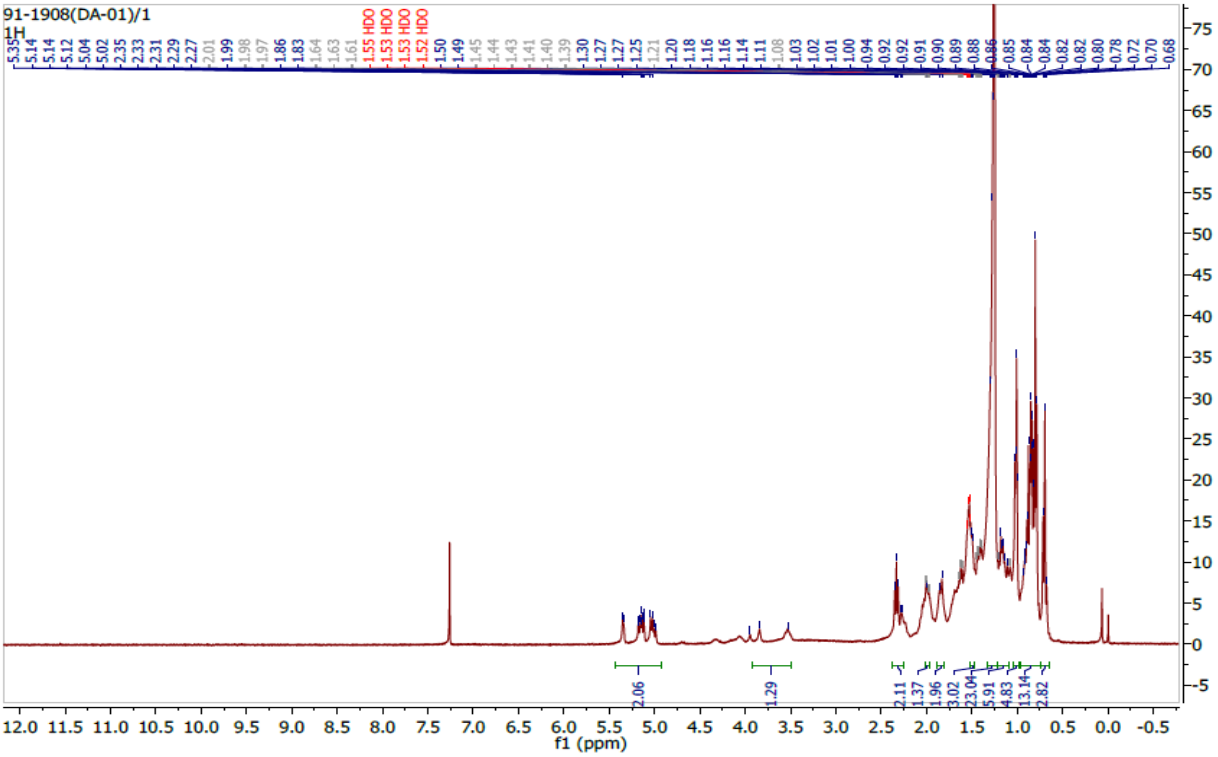
Appendix 2.Bioassays test of different concentrations of acetone extract.



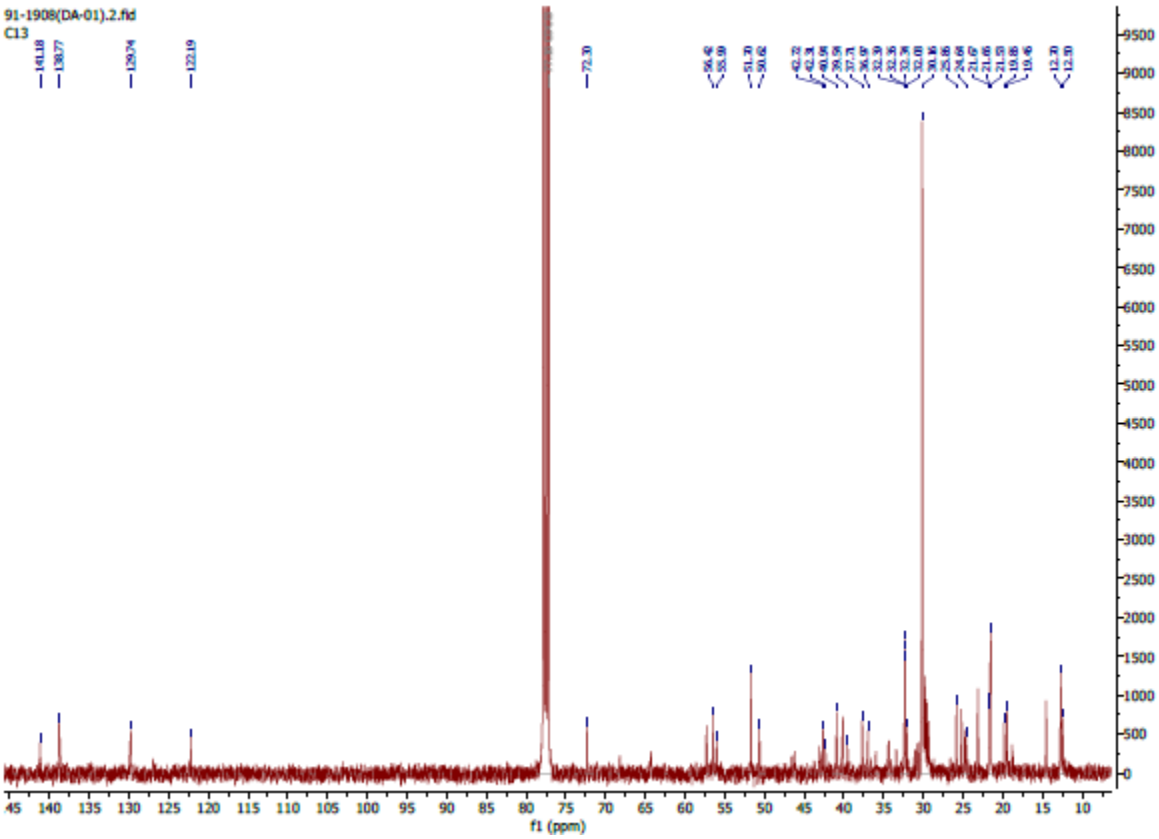
Appendix 3.Bioassays Tests of Isolated Compounds' Zone of Growth Inhibition



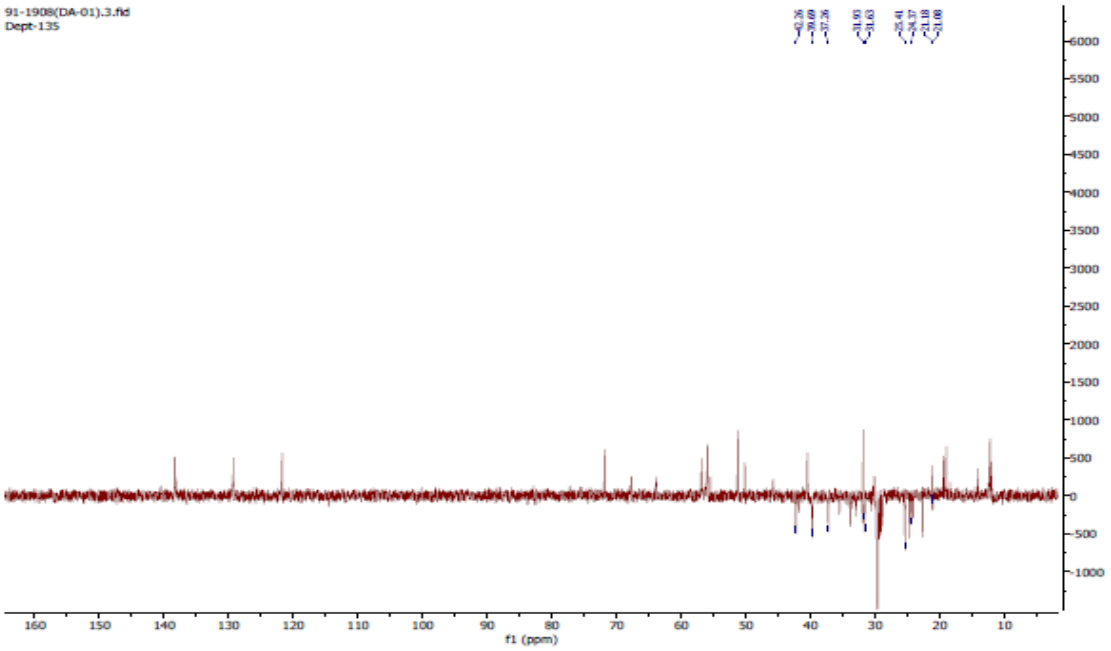
Appendix 4.[1]H-NMR Spectrum of **DA1**in chloroform



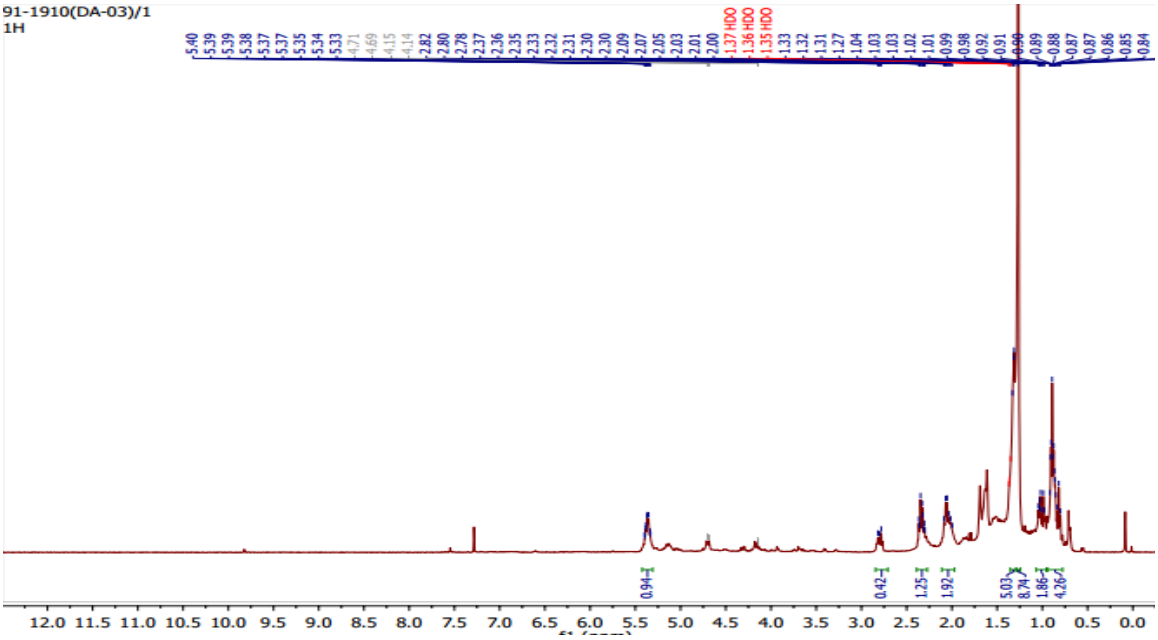
Appendix 5.$^{13}$C-NMR Spectrum ofcompound DA1in chloroform



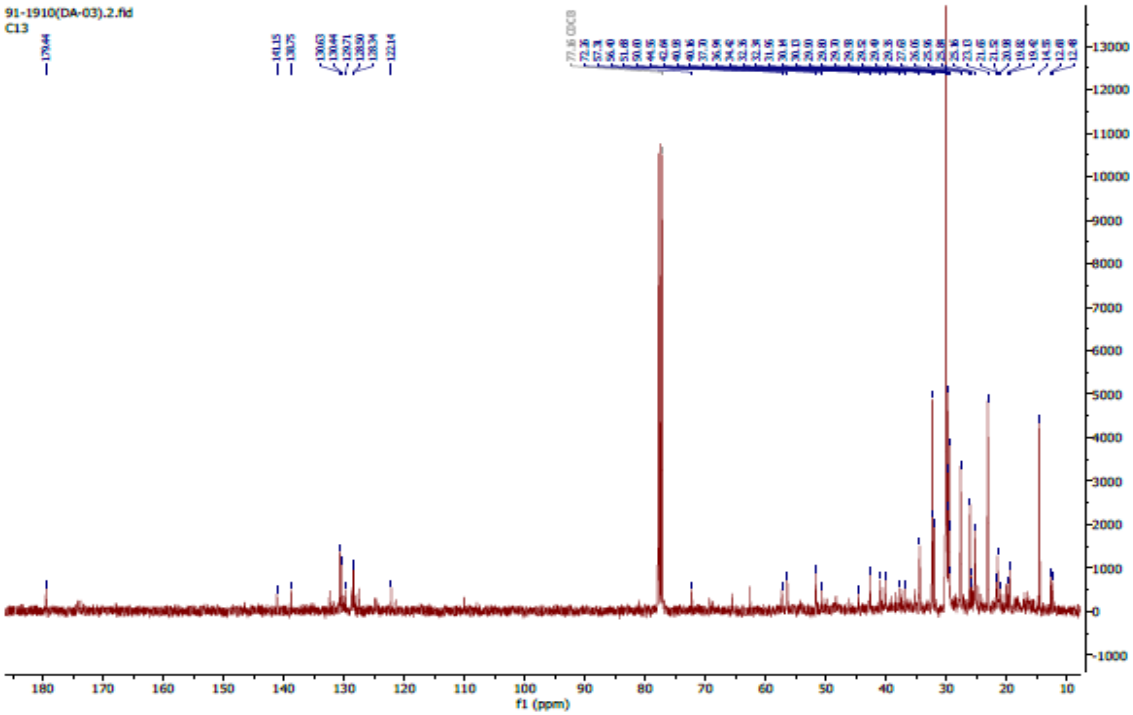

Appendix 6.DEPT Spectrum of compound **DA1**in chloroform



Appendix 7.[1]H-NMR Spectrum of **DA3**in chloroform




Appendix 8.[13]C-NMR Spectrum of compound DA3in chloroform



Appendix 9.DEPT Spectrum of compound **$DA_3$** in chloroform