PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITIES OF STEM BARKS OF *EMBELIA SCHIMPERI.*

BY

BABE GUYASA DINSA

A THESIS SUBMITTED TO

THE PROGRAM OF CHEMISTRY

SCHOOL OF APPLIED NATURAL SCIENCE

PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER'S IN CHEMISTRY

OFFICE OF GRADUATE STUDIES

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

ADAMA

SEPTEMBER, 2017

PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITIES OF STEM BARKS OF *EMBELIA SCHIMPERI.*

BY

BABE GUYASA DINSA

ADVISOR MILKYAS ENDALE (PhD)

CO-ADVISOR YADESSA MELAKU(phD)

A THESIS SUBMITTED TO

THE PROGRAM OF CHEMISTRY

SCHOOL OF APPLIED NATURAL SCIENCE

PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER'S IN CHEMISTRY

OFFICE OF GRADUATE STUDIES

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

ADAMA

SEPTEMBER, 2017

DECLARATION

I hereby declare that this MSc thesis Dissertation is my original work and has not been pre sented for a degree in any other university, and all sources of material used for this thesis/ dissertation have been duly acknowledged.

Name: - Babe Guyasa Dinsa

Signature:-_____

This MSc Thesis dissertation has been submitted for examination with my approval as thesis advisors/ dissertation Supervisors.

Name:- Milkyas Endale(phD)

Signature:- _____

Date of submission_____

APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defense by Babe Guyasa Dinsa have read and evaluated his/her thesis entitled "on phytochemical investigation and antibacterial study of Stem Barks of *Embelia schimperi* " and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirement of the Degree of MSC in chemistry

Milkyas Endale (phD)		02/10/2017
Supervisors /Advisors	Signature	Date
Yadessa Melaku (phD)		02/10/2017
Co-Advisor	Signature	
Tegene Desalegn (PhD)		02/10/2017
Chairperson	Signature	Date
H/Michael Tesso (PhD)		02/10/2017
Internal Examiner	Signature	Date
Daniel Bisrat (PhD)		02/10/2017
External Examiner	Signature	Date

ACKNOWLEDGMENTS

Above all, I bow my head to the Omnipotent, Omnipresent and Omniscient Almighty God,for giving me grace, life, joy, peace, potency and the capability to complete this program. Secondly, I offer my deepest gratitude to my advisor Dr. Milkyas Endale for his guidance, encouragement, supervision, valuable support and an amiable working environment as well as technical and professional discussions during the project. I am also grateful to my co-advisor Dr. Yadessa Melaku for his valuable comments and interesting discussions throughout project. Dr. H/Michael Tesso is also duly acknowledged for his discussion during laboratory work. I am also grateful to Applied Chemistry Program for offering the PG opportunity and the head, Mr. Solomon Girmay, is also acknowledged for his cooperation during the laboratory work.

This work wouldn't be realized without access to NMR and for this I thank Department of Chemistry, Addis Ababa University for access to the instruments. I would like to extend my deepest gratefulness to my brother Mr. Lamessa Rajo who assisted me during selection of plant material. Last but not least, my special thanks go to all friends who have helped me in one way or another whenever and wherever their assistance was required.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
CC	Column Chromatography
COSY	Correlated Spectroscopy
DEPT-135	Distortion less Enhancement by Polarization Transfer
FT-IR	Fourier Transform Infra-Red Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
RF	Retention Factor
TMS	Tetramethylsilane
TLC	Thin layer chromatography
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Medicinal plants have been used over the years for treating a variety of diseases caused by protozoan, bacteria, fungi, viruses and helminthes. Embelia schemperi is one of these medicinal plants used traditionally for treatment of intestinal tape worm, dysmenorrheal, bacterial and fungal infections. Phytochemical screening test of the dichloromethane/methanol (1:1) and methanol extracts revealed the presence of phenols, alkaloids, tannins, and flavonoids where as terpenoids, glycoside and phytosterols were absent. Silical gel column chromatographic separation of the extract afforded two compounds (3,5,7,3',4' pentahydroxy flavan and 3,5,7,4' tetrahydroxy flavan). The structures were determined as using spectroscopic techniques (UV-Vis, IR, 1D and 2D NMR) and compared with reported data from literature. The crude extracts and isolated pure compounds were tested for their antibacterial activity by Agar-disc diffusion methods. Compound 1 exhibited 15mm zones of inhibition, against S. aureus which was comparable to that of gentamicin (15mm). The present study supports the traditional medicinal use of the plant for the treatment of infectious diseases.

Keywords: E.schimperi Phytochemical investigation, antibacticterial activity, flavans.

1. INTRODUCTION

1.1 Background

Plants have been used from ancient times to attempt cures for diseases, to relive physical suffering and also utilized as sources of spices, dyes, poisons and drugs. Ancient peoples all had acquired some knowledge of medicinal plants. Often times these primitive attempts at medicine were based on superstition and speculation. The chemical compounds responsible for these activities are often the secondary metabolites of plants or natural products as they are usually referred to. The World Health Organization (WHO) defines traditional medicine as health practices, ap proaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being [1].

Medicinal plant extracts and phytochemical constituents present in the plant tissues with well-known antimicrobial properties play an important role in promoting human health and are non-toxic to the human body [2].Plant extracts work in synergy with synthetic antibiotics against drug resistant bacteria [3].They can be defensive substances such as phytoalexins and phytoanticipins, anti-feed ants, attractants [4]. No one knows exactly how many different medicinal plants are used in the world today, but we do know that medicinal plants are enormously important in both traditional and modern medicine**.** Ethno botany, the study of traditional plant use, is a field of growing interest to research scientists and pharmaceutical companies looking to develop new and more effective drugs. It has been estimated that over 40% of medicines have their origins in these active natural products [5].

In general, The study of natural products offers challenging problems of stereo specific synthesis of compounds with a fascinating diversity of structures to synthetic organic chemists. These molecules of life have attracted the attention and efforts of the foremost organic chemists because of their economic importance in industry, their value in medicine and phytochemical studies of medicinal plants are of great importance in developing drugs, study of chemotaxonomy and plant biodiversity as well as in documenting knowledge. Enhancing the knowledge of biological and pharmacological effects of plant constituent and determination of the structures of the active principles may help in sustaining the use of these products [6].

1.2. Natural Products in Drug Discovery

The contribution of natural products to the development of medicine could be demonstrated by the amount of plant derived drugs being used [7]. Medicinal agents used by humans since ancient times provided the starting point for the development of our current arsenal drugs. Higher plants have been the source of medicinal agents since earliest times, and today they continue to play dominant roles in the primary health care of about 80 % of the world's population due to cultural acceptability, it's attributed efficacy against certain types of diseases and economic affordability as compared to modern medicine [1] .Research in to the chemical and biological properties of natural products over the past two centuries has not only yielded drugs for the treatment of human ailments, but has provided the stimulus for the development of modern synthetic organic chemistry, and the emergence of medicinal chemistry as a major route for the discovery of the novel and more effective therapeutic agents [8].

By 1882, more than 50 different herbs were commonly used to make medicines. The active ingredients of these plants were isolated from the herbs, berries, leaves, roots, barks and stems used in traditional medicine. Of the 119 plant derived drugs commonly in use, 74% were discovered as a result of chemical studies directed at the isolation of the active constituents of plants used in traditional medicine [9]. Different plant parts such as fruits, seeds, barks or roots of *Embelia schimperi* (Vatke) are traditionally used as an antibacterial and anthelminthic remedy.Phytochemistry research is the backbone of herbal industry and directly or indirectly responsible for both failure and success of herbal drugs. For promoting the use of herbals in modern medicine, phytochemistry should be investigated for:-isolation, purification and characterization of new phyto constituents, use of newly isolated phytoconstituent for the synthetic design of analogues with either improved therapeutic activity or reduced toxicity and conservation of phytoconstituents into medicinally important drugs. Scientists still search the world for plants, bark, and the oceans for flora and fauna that might yield of the problem.

The genus *Embelia* is the source of large number of compounds and exhibit diverse biological activities. *E. schimperi* is one of the medicinal plants within the genus present at various geographical locations with a wide traditional use in Ethiopia. Nevertheless, there are limited

studies conducted in Ethiopia. The use of plants for the treatment of various diseases is universal and has been practiced by many people for many years. It has not only continued to be used for primary health care in developing countries, but has also been used in countries where conventional medicine is predominant in the national health care system [1]. A plant's medicinal value is due to the presence in its tissues of some chemical substance(s) that produce a physiological action on the body. Worldwide, there are several thousand plants that have been used and are still being used for medical purposes. Many of these are restricted in use by native people who have long resided in any given area [10]

(a) (b)

Fig.1.(a) Photo of *E. schimperi* plant taken by Babe Guyasa in Horo Guduru Wellaga Zone Jarte woreda, Sombo kumi kebele, on December 8, 2016. **(b)** photo of powdered stem barks of *E.schimperi*

1.3 Justification of the Study

This thesis has a practical and experimental result of a scientific relevance due to this they are very important. This research would like to make an addition to the work in the future due to the *Embelia schimperi* plant contains many polar chemical constituents that are not isolated by this work and the plant is highly effective traditional medicine to treat bacterial and fungal infections. Scientific literature is scarce on the isolation of all chemical constituents of the plant to promote this traditional medicine to modern medicine and documenting of knowledge for drug industry; this thesis tries to fill some of this gap.

1.4 Statement of the Problem

There are many plant species that are traditionally used for the treatment of different diseases like inflammation, asthma and infectious microorganisms such a worms and bacteria. *E. schimperi* is one of the medicinal plants found at various geographical locations and their presumptive folklore use prescribed for bacterial and fungal infections. Considering its wide traditional use, to the best of our knowledge, there are very few studies conducted on the species with in Ethiopia. Thus, this project is intended to fill some of this gap.

1.5 Significance of the Study

The studies on antimicrobial activities of the genus are still scarce, and there is need for further investigations along this line to strengthen the pharmacological study profile, evaluate the effectiveness of the plant against disease causing agents and their ultimate utilization in drug development. Clinical observations on traditional remedies are possible and useful. Some herbal remedies may be safe and effective for the treatment of microbial infection. Nevertheless, better evidence from randomized clinical trials and cytoxicity assay is required before herbal remedies can be recommended on a large scale. In order to prioritize remedies, there is need for preliminary clinical observational studies based on existing data from the ethno botanical and ethno pharmacological study of traditional Ethiopian plants. This research can provide an evidence base for the traditional use of the plants by identifying their chemical constituents of the plant through biological screening. This study also aims to engender further pharmacological studies on the active ingredients of the plant.

1.6 Objectives of the Study

1.6.1, General objective of the study

To extract, isolate and characterize the chemical constituents from the stem bark of *E. schimpri* plant and evaluate their antibacterial activities.

1.6.2, Specific objectives of the study

- To successively extract stem barks of the plant using dichloromethane/methanol (1:1) and methanol solvents.
- To conduct phytochemical screening test of the crude extracts
- To isolate compounds from the crude extracts of the stem bark of the plant.
- To characterize structures of the isolated compounds using different spectroscopic technique (UV, IR ,1D and 2D NMR)
- To investigate the antibacterial activity of the crude extracts as well as the isolated compounds against *Staphylococcus aureus, Escherichia coli, Protens mirabilis and Klebsolap pneumonia* and compared with standard drug.

2. LITERATURE REVIEW

2.1 General Description of Medicinal Plants

Traditional healers use medicinal plants for a variety of illness caused by protozoan, bacteria, fungi, viruses and helminthes. Traditional knowledge to solve health problems of mankind and animals exists in all countries of the world. The use of medicinal plant as the source of remedies for the treatment of many diseases dates back to prehistory and people of all continents have this old tradition. The use of plants as medicines is not restricted to one particular region or continent. Plants as medicines are capitalized in Africa, Greek, Islamic, Chinese, Indian and Western systems of medicines. This indicates the consensus among the nations upon the use of plants as medicines [10].

The first official recognition of traditional medicine as important participant in primary health care was expressed in the World Health Organization's Primary Health Care Declaration of Alma Ata in 1978. Traditional medicine according to the declaration is the sum total of all the knowledge and practices used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation verbal writing [11]. Medicinal plant typically contains mixtures of different compounds that may individually or in combination improve health. In previous studies, many plant extracts have been shown to Possess combinatory antimicrobial activity, including improving antibiotic's efficacy against *S. aureus* [12].The importance of medicinal plants lays not only on their chemotherapeutic effect but also in their role as a source of model compounds for drug development. In addition to plant constituents being used directly as therapeutic agents, they can be utilized as starting material for drug synthesis [13].

2.2. Botanical Discriptions of *E. schimberi*

Embelia is a genus of climbing shrubs in the family *Myrsinaceae*; there are about 130 species which occur in tropical and subtropical areas across a wide range including Africa and from eastern Asia to the Pacific Islands as well as Australia [14]. The plant can grow to a maximum height of about 7 m [15]. The plant is a highland family of trees and/or shrubs that are established all over the world. In Kenya, the species are found in the West and South of Mt. Kenya,

Ngong'Hills, Kakamega forests and Western slopes of Mau Ranges around Kericho District [16].

Embelia schimperi Vatke locally called Enkoko in Amharic and Hanku in Afan Oromo is climbing shrub which reaches the height of 2-13 m [17]. The fruit, 5-8 mm in diameter, is orange yellow, reddish green to red in color when ripen. Each fruit often has one seed that has a diameter of 4.5-7 mm. It is brown in irregular orange markings when ripen [18]. *E. schimperi* seeds found to possess antibiotic and anti-tubercular properties. They are also useful against fevers and chest and skin diseases [19]. Decoction of the leaves is used as a blood purifier Believed to eliminate adult *Taenia saginata*, the beef tape worm [20]. This result indicates that the crushed seeds of *E. schimperi* taken orally by the Masai people indeed have an anthelmintic effect against human intestinal tapeworms [21]. This plant widely used in traditional medicine as an anthelmintic and anti-microbial mostly known for anti-bacterial and anthelmintic properties.

In Ethiopia, the bark from *E. schimperi* are combined with other species, such an *Albizia anthelmintica, Guizotia abyssinica, Glinus lotoides*, and *Hagenia abyssinica,* mixed with water and taken as a disinfectant which suggests that the pharmacological effect of this plant can be increased by interaction with other plant ingredients . The plant was screened for alkyl benzoquinones that the family is known to accumulate up to 5-16 %, w/w which was investigated for larvicidal activity against *Aedes aegypti[22]*.

2.3. Phytochemistry of the Genus Embelia

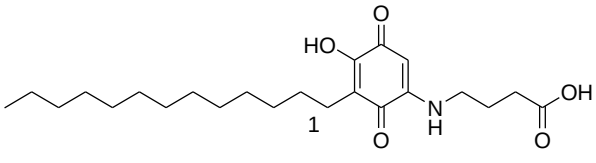
The EtOH extract of the roots of *Embelia ribes* led to isolation of a nitrogen-containing 3-alkyl-1,4-benzoquinonederivative,*N*-(3-carboxylpropyl)-5-amino-2-hydroxy-3-tridecyl-1,4- benzoquinone (1**),**and a gomphilactone derivative,5,6-dihydroxy-7-tridecyl-3-[4-tridecyl-3-hydroxy-5-oxo-2(5*H*)-furylidene]-2-oxo-3(2*H*)-benzofuran **(**2), as well as the common plant metabolites sitosterol and daucosterol [23]. Chromatographic separation of an ethyl acetate extract of dried ground stem bark of *E. schimperi* led to the isolation of a new compound identified as 2,5-dihydroxy-3-methyl-1,4-benzoquinone (3) which was screened for in *vitro* antimicrobial activity against clinical strains of *Salmonella spp., Protsseusspp., Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Cryptococcus neoformans, Shigella dysentriae and Staphylococcus aureus*. Compound (4) as exhibited comparable antibacterial activity to that of the control

antibiotic gentamicin, with zone of inhibition of 20 and 21 mm diameter for compound 4 and that of standard, respectively [24]

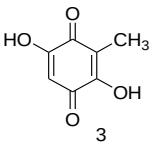
Chromatographic analysis of air-dried berries of *E. schimperi* from cold ethyl acetate led to the isolation of methylvilangin **(**5**)** and Biembelin **(**6**).** Methyl vilangin was found to be lethal against 2nd instars larvae of *Aedes aegypti* (yellow fever vector) by first stopping the process of meta-morphosis from the 2nd instars stage to the other stages and finally causing mortality to the larvae [25].An investigation of MeOH extract of *E. schimperi* stem led to the isolation of a new resor-cinol derivative, 5-(7'Z-pentadecenyl) resorcinol (7), along with the known compounds lupeol (8) and β-sitosterol glucoside (9). Compound 5 exhibited moderate in *vitro* cytotoxic activity against human Hela cell line [26].

Fractionation of the methanolic extract of *E. schimperi* leaves led to the isolation of novel flavonol glycosides (10) characterized as isorhamnetin 3-O-β-galactoysyl (1→4)-β-galactoside and quercetin 3-O-[α-rhamnosyl (1→2)] [α-rhamnosyl (1→4)]-α-rhamnoside together with known compounds quercetin, myricetin, quercetin 3-O-α-rhamnoside, quercetin 3-O-β-glucoside, quercetin 3-O-rutinoside, myricetin 3-O-β-xyloside, isorhamentin 3-O-β-glucoside and myricetin 3-O-β-glucoside [27]. Five oleanane-type pentacyclic triterpenoids were isolated from chloroform extract of the stem bark of *E. schimperi* of which **t**hree of them have a methyleneoxy bridge and two compounds, embelinone and schimperinone, were reported for the first time from the plant. With promising antibacterial properties against the gram-positive bac-teria strain *Rhodococcus* sp. [28].

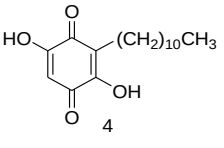
Some compounds isolated from the *embelia* genus plant



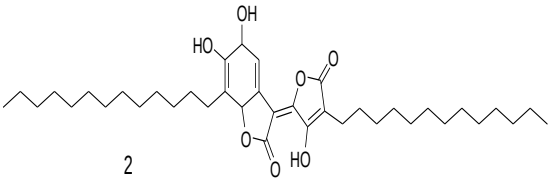
1



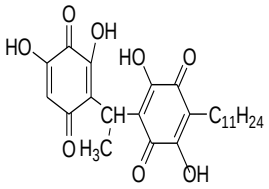
3



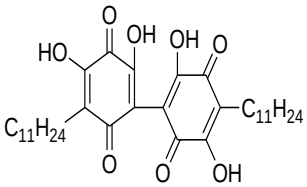
4



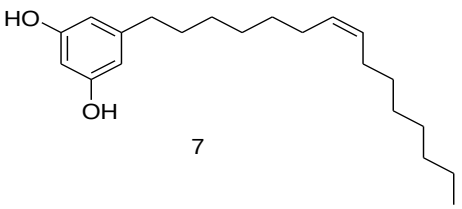
2



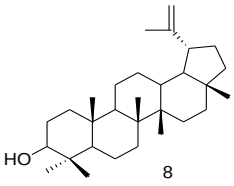
5



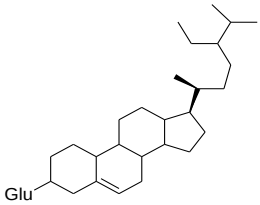
6



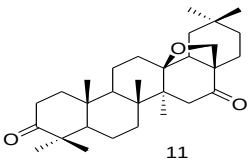
7



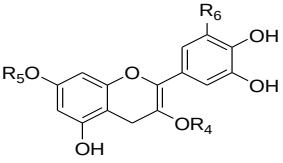
8



9



11



10

1. R4 = rhamnosyl (1-3)-galactoside, R5 = H, R6 = OH
2. R4 = R6 = H, R5 = galactosyl (1-4)-galactoside
3. R4 = glucosyl (1-2)-glucoside, R5 = glucosyl(1-4)-rhamnoside, R6 = OH

2.4 Biological Activity of *E.schimperi*.

Staphylococcus aureus is one of the most serious human pathogens, responsible for dangerous community- and hospital-acquired infections. Most of its strains are resistant to β-lactams as well as to other classes of antibiotics, such as tetracycline's [29]. Although mortality and morbidity associated with *S. aureus* infections in less-developed countries far exceed those occurring in developed ones, staphylococcal diseases in low-income countries are still perceived as trivial in comparison with other infections, such as malaria [30]. Even though common antibiotics can still manage this pathogen, many of them are no longer effective against majority of staphylococcal strains (such as penicillin or tetracycline). Spread of antibiotic resistant strains now has become an important public health problem worldwide [31].

Thus, it is crucial to use new strategies to overcome complications in the treatment of staphylococcal infections caused by drug-resistant strains, such as antimicrobial effect of plant-derived products and commonly used therapeutics [32].*E.schimperi* play an important role in the traditional treatment of many diseases including bacterial infections. It is well-known that indigenous healers use remedies prepared from this plant species to treat, for example, bacterial infection, intestinal tapeworm, and skin diseases. This traditional medical knowledge is useful not only for community healthcare but also for future drug development [33].

Bacterial infections in less-developed countries are traditionally treated by remedies prepared from medicinal plants. In spite of many years of research into yellow fever drugs, the disease still remains the leading killer disease mainly in Africa. The yellow fever parasite has lately been found to be resistant to most conventional drugs in the market today. In view of this worrying trend, *Embelia schimperi* is a plant belonging to the family *Myrsinaceae*; known for antibacterial and anthelmintic properties [22]. Therefore, in the present study we evaluated pure compound and methanol extract prepared from stem barks *E. schimperi* and the main constituents of the extracts were identified by column chromatography assay, for their *in vitro* anti *staphylococcal* effect with representatives of typical antibiotic classes associated with *staphylococcal* resistance.

3. MATERIALS AND METHODS

3.1 Apparatus and Equipments

NMR spectrum were measured on a Bruker Avance 400 MHz. The ultraviolet and visible (UV-Vis) spectrum was taken on Spectronic Genesys^{TM} 2PC UV-Vis scanning spectrometer. Infrared (IR) absorptions were measured on a Perkin Elmer System FT-IR spectrometer as KBr pellets in the range of 4000-400 cm^{-1}. Analytical thin layer chromatograms were run on readymade 0.25 mm thick layer of Merck silica gel 60 F_{254} coated on aluminum foil. The spots on the chromatograms were detected in 2%vanillin in H_2SO_4. Silica gel with fluorescent indicator 254nm on aluminum cards with layer thickness 0.1 mm used for TLC. Column chromatography were conducted using column packed with Merck silica gel 60, particle size 0.063–0.200 mm (70-230 mesh ASTM), test tube, water bath, refrigerators, stand, beaker, round bottom flask, gaze jar, spatula, stirrer, cotton, analytical balance, capillary, oven, ruler, pencil, pen, reagent bottle, pipette, Rota vapor, funnel, spatula, electronic balance, dropper, mortar, and pestle.

3.2 Chemicals and Reagents

n-hexane, ethyl acetate, dichloromethane, methanol, iodine, chloroform, , ethanol, distilled water, dimethyl sulfoxide–d_6 (DMSO-d_6), sulfuric acid, ferric chloride, KBr, TMS as an internal standard, silica gel, 2,4-dinitrophenylhydrazine, HCl, ammonia solution, Mayer's reagent, Dragendroff's reagent, ferrous sulphate, Fehling's solution, acetic acid anhydride, soap, distilled water, $HgCl_2$, KI, NaOH, $Bi(NO_3)_3$, and benzene.

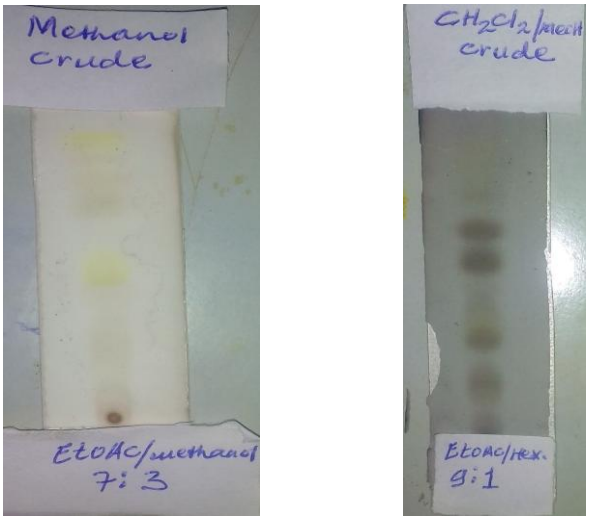
3.3 Plant Material Collection and Identification

The stem barks of *Embelia schimperi* plant was collected from Oromia region, Horo Guduru Wellaga Zone in Jarte woreda Sombo kumi kebele, which is 381 km west of Addis Abeba on December, 08, 2016. The plant was identified by botanist Shambel Alemu Herbarium in Addis Abeba University. The barks were dried in an open air protected from direct exposure to sun light. The dried plant barks was finely grounded and ready for extraction.

3.4 Experimental Procedures

3.4.1, Solvent extraction at room temperature.

The collected plant material was cut in to smaller pieces and dried at room temperature until it becomes well dried. Air dried stem bark powder (500g) of *E.schemberi* was first soaked with dichloromethane/methanol (1:1) for 72 h at room temperature. The mixture was filtered using what man filter paper No.1 (150mm) and the filtrate was concentrated under reduced pressure and temperature of 40℃ using rotary evaporator and afforded 32.5g (6.5%) deep dark crude extract. The marc was further extracted with methanol after soaking for 72 h at room temperature. Then the extract was filtered using what man filter paper No.1 (150mm) and concentrated in rotary evaporator and afforded (20.6 g) deep blue black crude extract. TLC analysis of crude extract showed better TLC profile preferred for further work.



a b

Fig 2 (a) TLC profile of methanol crude extract (b) TLC profile of CH_2Cl_2/ MeOH crude extract of the plant. This shows that the stem barks of *E.schimberi* contains polar compounds.

3.4.2 Qualitative analysis of the chemical constituents of crude extracts

There was a scarce in the previous phytochemical report on the stem bark of the plant. To the best of our knowledge phytochemical screening of the stem bark of *E.schemberi* was carried out to analyze the presence of compounds namely: Saponins, Flavonoids, Phenols, Alkaloids, Tannins, Terpenoids, and Glycosides.

Test for Saponins: To 0.5 g of each crude extract (dichloromethane/ methanol and methanol) 5mL of distilled water was added and shaken and then heated to boil. Frothing (appearance of creamy miss of small bubbles) appears.

Test for Flavonoids: To 0.5 g portion of (dichloromethane/methanol and methanol) crude extract 10mL of ethyl acetate was added and heated with a steam bath for 3 min. The mixture was filtered and 4mL of the filtrate was shaken with 1 mL of dilute ammonia solution and formation of intense yellow color.

Test for Phenols: 0.5 g of each of crude extracts (dichloromethane/methanol and methanol) extracts were put in a different test tube and treated with a few drops of 2% of $FeCl_3$; bluish green or black coloration was formed.

Test for Alkaloids: 0.5 g of Extracts dissolved individually in dilute Hydrochloric acid and filtered.

Dragendroff's Test: The filtrate was treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide) forms a red precipitate.

Test for Tannins: 0.5 g of each crude extract was boiled in 10mL of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added to give brownish green or a blue-black coloration.

Test for Terpenoids: 0.5 g of each (dichlormethane/ methanol and methanol) crude powder was separately dissolved in 5mL of methanol. 2 mL of the extract was treated with 1 mL of 2, 4- dinitrophenyl hydrazine dissolved in 100mL of 2M HCl. Which results in disappearance of yellow-orange color.

Detection of Phytosterols

Salkowski's Test: 0.5g the extracts was treated with a few drop of chloroform and filtered. The filtrates was treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Results in dis appearance of golden yellow color.

Test for Glycosides: 0.5 g of (dichlorormethane/methanol and methanol) extract was hydrolyzed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test: The extracts was treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. This results in the absence of rose-pink color in the ammonical layer.

The phytochemical screening test showed absence of terpenoids glycosides and phytosterols in both extracts where as saponins, phenols, alkaloids, tannins, and flavonoids are present.

3.4.3, Isolation of compounds from dichloromethane/methanol crude extract.

Silica gel column chromatography was conducted for separation of the chemical constituents using increasing gradient of ethyl acetate in n-hexane as eluent. A silica gel (200g) was mixed with *n*-hexane and the slurry was used to pack the column. The crude CH_2Cl_2/MeOH (1:1) extract (20g) was adsorbed on 20g of silica gel and applied on column. Elution was carried out with increasing gradient of ethyl acetate in n-hexane. Fractions that showed the same $R_{f\ value}$ and the same characteristic color on TLC were combined and further purified. The column was then eluted with increasing polarity of solvent system. A total of 38 fractions each with 100mL were collected according to the following table:

Table 1: Silica gel colmn chromatography fractionation of CH_2Cl_2/MeOH(1:1)extract

Solvent system	Ratio	Fractions	Solvent system	Ratio	Fractions
n-hexane.	100x2	F_1&F_2	n-hexane/EtoAc	5:5	
n-hexane/EtOAc	9.9:0.1	F_3	EtOAc/n-hexane	5.5:4.5	F_{20}
"	9.8:0.2	F_4	EtOAc/hexane	6:4	F_{21}
	9.7:0.3	F_5	"	6.5:3.5	F_{22}
"	9.6:0.4	F_6	"	7:3	F_{23}
"	9.5:0.5	F_7	"	8:2	F_{24}
"	9.4:0.6	F_8	"	9:1	F_{25}
"	9.3:0.7	F_9	EtOAc	100	F_{26}
"	9.2:0.8	F_{10}	EtOAc/methanol	9:1	F_{27}
"	9.1:0.9	F_{11}	"	8:2	F_{28}
"	9:1	F_{12}	"	7:3	F_{29}
"	8.5:1.5	F_{13}	"	6:4	F_{30}
"	8:2	F_{14}	"	5:5	F_{31}
"	7.5:2.5	F_{15}	Methnol/EtOAc	6:4	F_{32}
"	7:3	F_{16}	"	7:3	F_{33}
"	6.5:3.5	F_{17}	"	8:2	F_{34}
"	6:4	F_{18}	"	9:1	F_{35}
"	5.5;4.5	F_{19}	Methanol	100X3	F_{36}

3.4.4, Purification of compounds from CH_2Cl_2/MeOH .

TLC analysis of the fractions was done and some the fractions that have the same TLC profile were combined. The elutes were concentrated under reduced pressure to dryness and 33 fractions were obtained. Out of 33 fractions collected, fraction F_{28} was showed single spot on TLC plate. Fractions F_{25} and F_{26} showed the same Rf value (0.65) and the same characteristic color

on TLC were not pure and combined together for further purification. Fraction 14 showed a single spot and kept separately. These fractions (F_{25} + F_{26}) were further purified, to give one compound named as F_{03}. Fraction 28 analyzed with EtOAc/MeOH (9:1) showed a single spot and characterized as compound 2.

a b

Fig 3 (a) TLC profile of methanol crude fraction (b) TLC profile of CH_2Cl_2/ MeOH crude fraction.

Table 2: Fractions that was collected from the CH_2Cl_2/MeOH extract

No	Solvent	ratio	Fraction	Yield of each fraction(g)	TLC profile
1	Hexane/EtoAc	8:2	F_{14}	7.6g	One spot
2	EtOAc/hexane	9:1	F_{25}	38g	One spot with impurity
3	EtOAc	100	F_{26}	22.6g	One spot with impurity
4	EtOAc/MeOH	8:2	F_{28}	30.2g	One spot

Fraction 14 showed a single spot on TLC but dried with small yield. Fractions (25 and 26) analyzed with EtOAc/n-hexane (7:3) showed the same R_f (0.65) value and the same characteristic color on TLC were combined (60.6 g) and subjected to small column chromatography packed with silica gel / n-hexane for purification and eluted with EtOAc/n-hexane(7:3)v/v isocratic ally. Based on TLC analysis, 7:3 EtOAc/n-hexane was the most important elution ratio to purify the impurity from the compound.

Table 3: Silica gel purification of fractions using (7:3) v/v EtOAc/n-hexane solvent system.

no	Solvent	ratio	Fraction	Yield of each fraction(g)	TLC profile
1	EtOAc/hexane	(7:3)x2	F_1	10.6	One spot with impurity
2	EtOAc/hexane	(7:3)x3	F_2	15.7	One spot and impurity
3	EtOAc/hexane	(7:3)x4	F_3	34.3	One spot

Finally, F_3 was the second compound that was obtained from the CH_2Cl_2/MeOH crude extract through isocratic elution of the fraction on small column chromatography which was coded as compound 3.

3.4.5, Isolation of compounds from methanol crude extract.

A crude methanol extract of 15 g was adsorbed on 15g of silica gel and applied on silica gel column chromatography (silica gel 200 g). The column was eluted using the following solvent system in table 2. Fraction 27 eluted using solvent system 9:1 EtOAc/MeOH solvent system showed a single spot. Fractionations of methanol extract on column chromatography afforded 1 compound (F_{27}) and characterized as compound 1. Compound 1 analyzed with EtOAc/n-hexane (9:1) is a single spot Rf value 0.52. Visualization of the chromatogram was achieved by 2%vannilin-H_2SO_4 or Iodine then observation was carried out on hot plate.

Table 4: Silica gel colmn chromatography fractionation of Methanol extract

Solvent system	Ratio	Fractions	Solvent system	Ratio	Fractions
n-hexane.	100x2	F_1&F_2	n-hexane/EtoAc	5:5	F_{20}
n-hexane/EtOAc	9.9:0.1	F_3	EtOAc/n-hexane	5.5:4.5	F_{21}
“	9.8:0.2	F_4	EtOAc/hexane	6:4	F_{22}
“	9.7:0.3	F_5	‘”	7:3	F_{23}
“	9.6:0.4	F_6	“	8:2	F_{24}
“	9.5:0.5	F_7	“	9:1	F_{25}
“	9.4:0.6	F_8	EtOAc	100	F_{26}
“	9.3:0.7	F_9	EtOAc/methanol	9:1	F_{27}
“	9.2:0.8	F_{10}	“	8:2	F_{28}
“	9.1:0.9	F_{11}	“	7:3	F_{29}
“	9:1-8.5:1.5	F_{12}+F_{13}	“	6:4	F_{30}
“	8:2	F_{14}	“	5:5	F_{31}
“	7.5:2.5	F_{15}	Methnol/EtOAc	6:4	F_{32}
“	7:3	F_{16}	“	7:3	F_{33}
“	6.5:3.5	F_{17}	“	8:2	F_{34}
“	6:4	F_{18}	“	9:1	F_{35}
“	5.5;4.5	F_{19}	Methanol	100X3	F_{36}

3.5. Antibacterial Assays.

3.5.1, Preparation of discs containing extracts

The same concentrations of 20µg/mL were prepared from the extract, isolated pure compound and the standard. The concentration was incorporated into sterile agar –disc diffusion and was dried at 37^{o}C. The agar disc was weighed carefully for confirming exact amount of the extract and isolated pure compound being incorporated (compared to pre-weighed blank discs).

3.5.2, Bacterial culture

Escherichia coli and *Protens mirabilis* which were isolated from stool specimens in the clinic were identified according to routine cultural properties and biochemical tests. Four strains of each was included in the study. A few colonies from the overnight culture of Eosin Methylene Blue (EMB) agar was transferred into approximately 4-5mL Tripticase soy broth (TSB) medium. The broth was incubated at 37^0 c for 3-4 hours and the turbidity of suspension was adjusted to that a0.5McFarland barium sulfate standard. The standard suspension was used for both qualitative and quantitative antibacterial assays.

3.5.3, Bacterial susceptibility testing

Standardized inoculums (20µg/mL) were introduced on to the surface of sterile agar plates, and a sterile glass spreader was used for even distribution of the inoculums. A sterile Agar- disc diffusion previously soaked in a known concentration of extract or pure compound (20µg/mL per disc) was carefully placed at the centre of the labeled seeded plate. The same procedure was used for all the MRSA strains used. The plates were incubated aerobically at 37°C and examined for zones of inhibition after 24 hr. The inhibition zones were measured with a ruler and compared with the control disc (disc containing only physiological saline).

3.5.4, Antibacterial testing

The antibacterial activities of both the extract and pure compound of the plant species were determined using agar disc diffusion method. The tests were conducted at one concentration levels (20µg/mL) in both crude extracts and in case of isolated compound. The zone of inhibitions reported in all cases includes the diameter of the wells. Strains of human pathogen microorganisms used in this study were as follows. Three gram-negative bacteria; *Klebsolapneumonia, Protensmirabilis ,Escherichia coli* and one Gram-positive bacteria, *Staphylococcus aureus* chosen based on their clinical and pharmacological importance. The bacterial strains obtained from oromia public health research capacity building and quality assurance laboratory center, were used for evaluating antibacterial activity. The bacterial stock cultures were incubated for 24 hours at 37°C on nutrient agar medium (oromia public health research laboratory, Adama), respectively, following refrigeration storage at 4°C. The bacterial strains were grown in Mueller-

Hinton agar (MHA) plates at 37°C (the bacteria were grown in the nutrient broth at 37°C and maintained on nutrient agar slants at 4°c.

The agar was melted (50^{o}C) and the microorganism cultures were then added aseptically to the agar medium at 40 ^{o}C in plates and poured into sterile Petri dishes to give a solid plate. All these experiments were performed in duplicate. The plates were incubated for 24–48 hr, at 37 ^{o}C for bacteria. The inhibition zones produced by the plant extracts were compared with the inhibition zones produced by commercial standard antibiotics: The minimal inhibitory concentration (MIC) was applied to the methanol extract and pure compound that had proved to be highly effective against microorganisms by the agar-disc diffusion method. One dilution (20µg/mL) of *E.shimperi* extract, pure compound and standard drugs were prepared in methanol using nutrient agar tubes. Mueller-Hinton sterile agar plates were seeded with indicator bacterial strains (108 cfu) and allowed to stay at 37°C for 3 hours. Control experiments were carried out under similar condition by using gentamicin for antibacterial activity as standard drugs. The zones of growth inhibition around the disks were measured after 18 to 24 hours of in incubation at 37°C for bacteria .The sensitivities of the microorganism species to the plant extract and isolated pure compound were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks, and values <6 mm were considered as not active against microorganisms.

4. RESULTS AND DISCUSSION

4.1 Extraction Yields

Stem barks of *E. schimperi* (500 g) were subjected to successive extraction with dichloromethane/methanol (1:1) and methanol to afford crude extracts with 6.5%(w/w) and 4.1% respectively.

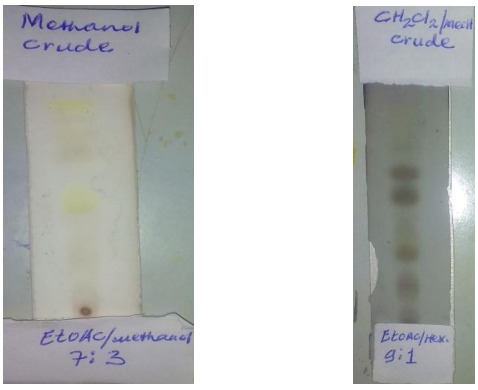
Table 5: **percent yield of the crude extracts.**

Mass of the plant macerated in (g)	Solvent used for maceration	Percent of the crude extract collected	Mass of the plant
500	CH_2Cl_2/MeOH	6.5%	32.5g
467.5	MeOH	4.1%	20.6g

Based on TLC analysis and yield both extracts were selected for further isolation. Chromatographic purification of the dichloromethane/ methanol and methanol extracts gave compounds coded as 1 and 2.The structures of these compounds have been elucidated on the basis of the following spectroscopic evidences.

4.2 TLC Profile of the Extract of the Stem Barks of *E.schimperi*

The CH_2Cl_2/MeOH and methanol extracts of the stem barks of *E.schimperi* were analyzed with TLC and the profile is shown in Fig 2.



(a) (b)

Fig 4 (a) TLC profile of methanol extract (b) TLC profile of dichloromethane methanol extract of the plant. As shown in fig a and b the CH_2Cl_2/MeOH and MeOH extracts display well resolved spots and hence further analyzed for their chemical constituent using chromatography.

4.3 Phytochemical Screening Test Results

Table 6: Phytochemical screening test results.

Class of secondary metabolites	MeOH extract of stem bark of *Embelia schemberi*	MeOH/CH_2CL_2 extract of stem bark of *Embelia schemberi*
Saponins	+	+
Terpenoids	-	-
Phytosterols	-	-
Flavonoides	+	+
Alkaloides	+	+
Phenols	+	+
Glycosides	-	-
Tannins	+	+

\+ = the presence of phytochemical constituents

\- = the absence of phytochemical constituent

The phytochemical screening test results showed absence of terpenoids glycosides and phytosterols in both extract where as saponins, phenols, alkaloids, tannins, and flavonoids are present.

4.4 Characterization of Compound 1.

Compound 1 was a colorless crystalline substance that showed Rf value of 0.52 in solvent ratio of 9:1EtOAc/n-hexane. Its melting point was found to be 173^0c -176^0c. In the UV spectrum at λ_{max} (inEtOH) (Appendix 1) revealed absorption maxima at 282nm suggesting the presence of Π to Π* transition due to the presence of aromatic ring chromophore. In the IR (KBr disk)(Appendex 2) spectrum showed broad vibration at 3436 cm^{-1} due to the presence of alcohols (OH), sharp absorption at 1644 attributed to aromatic benzene ring, and strong absorption band at 1255 cm^{-1} due to C-O stretching.

The ^{1}H NMR δ_H (400 MHz, DMSO-d_6) spectrum (Appendix 3 and Table 7) revealed the presence of proton signals at δ_H 5.7 (1H, d, *J* = 1.2Hz, H-6) and 5.89 (1H, d, *J* = 1.2 Hz, H-8) suggest the presence of two meta coupled aromatic protons that belong to a tetra substituted phenyl ring. The presence of signals with ABX multiplicity pattern at δ_H 6.88 (1H, dd, *J* = 8.1, 1.2 Hz, H-6') and 6.73 (1H, dd, *J* = 2.1, 8.1 Hz, H-2', 5) suggest a tri substituted phenyl ring. Signals at δ_H 4.73 (1H, d, H-2, J= 2.2), 4.01 (1H, m, H-3) suggest the presence of two oxygenated methine

Whereas signals peak at δ_H 2.67(1H, dd, *J* = 16.5, 4.5 Hz, H-4) and at δ_H 2.47 (1H, dd, *J* = 16.5, 4.5 Hz, H-4) suggest the presence of diasterotopic methylene protons.The above ^{1}H NMR pattern suggest that the compound have flavan skeleton with two aromatic protons (H-6,8) on ring A and three aromatic protons (H-2', 5', 6) in ring B and devoid of carbonyl group at C-4 of ring C (also supported by ^{13}C NMR).

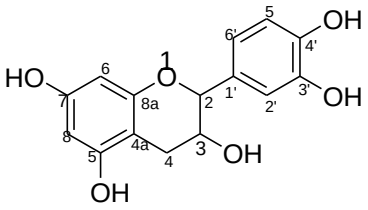
The ^{13}C NMR spectrum of compound 1 revealed a total of fifteen carbon signals. Two oxygenated sp^2 quaternary carbons at δ_C 144.7 (C-3) and 144.6 (C-4) suggesting vicinal substititution pattern in agreement with the ABX multiplicity pattern of ring C where the methines appear at 118.5 (C-6') and 115.1 (C-2', 5'). In addition, the presence of two sp^2 oxygenated quaternary carbons at 156.7 (C-5) and 156.6 (C-7) along with two upfeild carbons chemical shifts i.e. 95.3 (C-6) and 94.5 (C-8) suggest that ring A have 5,7dioxygenated substitution pattern. The follow-

ing quaternary carbons are also clearly evident at $\delta_{C,}$ 99.0 (C-4a), δ_C: 131.1 (C-1') and 156.2 (8a). Signals at 78.4 (C-2) and 65.2 (C-3) are clearly evident due to the presence of sp^3 oxygenated methines C-2 and C-3 of ring B. Moreover, the presence of one methylene (also supported by DEPT-135 pointing down) at 28.6 (C-4) is in good agreement with spectral data of the compound flavan skeleton.

In agreement with the observation from 1D, the COSY spectra showed a correlation between protons at 4.01 (H-3) and 4.73 (H-2) in agreement with the substitution pattern in ring C. Aromatic proton at δ_H 5.73 (H-6) showed HMBC correlations with C-5,7, 8 at δ_C 156.8, 156.3 and 94.5 where as proton at δ_H 5.89 (H-7) showed HMBC correlations with C-6, 7, 8a, 4a at δ_C 95.3, 156.3, 156.2 and 99.0 in agreement with the substitution pattern in ring A. The methylene proton at δ_C 2.67(H-4) showed HMBC correlations with the δ_C 78.4 (C-2), 65.2 (C-3), 156.2 (8a) and 99.0 (4a) in agreement with the substitution pattern in ring C. The HMBC correlations between the proton at δ_H 6.88 (H-6') and δ_H 6.67 (H-5') with that of δ_C 78.4 (C-2) and with the two oxygenated sp^2 vicinal quaternary carbons at δ_C 144.6 (C-4') and 144.7 (C-3') further support the ABX multiplicity pattern of ring B where the two hydroxyl groups are located at C-3',4' positions. Thus, based on the above, the structure of the compound was found to be 3,5,7,3', 4'-penta hy droxy flavan.

Table 7 : 1H-NMR, 13C-NMR AND DEPT-135 spectral data of compound 1 and literature data in DMSO-d6

No of C	1H-NMR	1H-NMR [34]	13C-NMR	13C-NMR[34]	DEPT-135
1’	--	--	131.1	131.1	cq
6	5.73 (1H,d,J=1,2 Hz’)	6.88 (1H, dd,J= 8.1, 2.1Hz)	118.4	118.4	CH
8	5.89 (1H,d, J=1.2Hz,)	6.67 (1H,dd, J=8.1, 2.1Hz)	115.2 115.4	115.2 115.4	CH CH
	-	-	-	-	-
4a	-	-	99	99	-
6’	6.88 (1H, dd,J= 8.1, 2.1Hz)	5.73 (1H,d,J=1,2 Hz’)	95.6	95.6	CH
5’ 2’	6.67 (1H,d J=8.1, 2.1Hz)	5.89 (1H,d, J=1.2Hz,)	94.6	94.6	CH
2	4.73(1H,d,H-2)	4.73(1H,s,H-2)	78.5	78.5	CH
3	4.01(1H,m,J=2.1HZ, H-8)	4.01(1H,m,J=2.1HZ, H-8)	65.4	65.4	CH
4	2.67(2H,dd,J=16.5,4.5,HZ,H-4	2.67(2H,dd,J=16.5,4.5,HZ,H-4	28.7	28.7	CH_2
4’	-	-	144.9	144.9	cq
3’	-	-	144.9	144.9	cq
8a	-	-	156.2	156.2	cq
7	-	-	156.7	156.7	cq
5	-	-	156.9	156.9	cq



3,5,7,3',4'-pentahydroxyflavan.

Fig 5:Chemical structure of compound 1.

3,5,7,3’4’-pentahydroxyflavan possess antioxidant and anti bacterial activities that provide protection against harmful free-radicals and bacterial infection. The compound have been known to reduce the risk of certain types of cancer,coronary heart disease (CHD),cardiovascular disease

(CVD), stroke, athero sclerosis, and other degenerative disease associated with oxidative stress[34]. Therefore the presence of this flavan adds one positive attributes to this plant.

4.5 Characterization of compound 2

Compound 2 is a brownish powder with Rf value of 0.75 in solvent ratio of 9:1EtOAc/MeOH . UV spectrum (EtOH) (Appendix 9) revealed absorption maxima at 282nm suggesting the presence of Π to Π* transition due to the presence of aromatic ring chromophore. the IR (KBr disk)(Appendex 10) spectrum showed broad vibration at 3254 cm^{-1} due to the presence of hydroxyl group (OH), sharp absorption at 2933due to saturated group C-H stretching of methylene , 1599 attributed to aromatic benzene ring, and strong absorption band at 1132 cm^{-1} due to C-O stretching, sharp absorption at 1509cm^{-} due to saturated group C-H stretching.

The ^{1}H NMR δ_H (400 MHz, DMSO-d_6) spectrum (Appendix 12 and Table 9) revealed the presence of proton signals at δ_H 5.73 (1H, d, *J* = 2.1Hz, H-6) and 5.89 (1H, d, *J* = 1.2 Hz, H-8) suggest the presence of two meta coupled aromatic protons that belong to a tetra substituted phenyl ring. The presence of signals at δ_H 6.88 (2H, dd, *J* = 8.1, 1.2 Hz, H-2',6') and 6.73 (2H, d, *J* = 2.1, 8.1 Hz, H-3', 5) suggest a disubstituted phenyl ring. Signals at δ_H 4.73 (1H, d, H-2, J= 2.2), suggest the presence of oxygenated methine, signals, at δ_H 4.01 (1H, m, H-3) suggest the presence of methine whereas signals peak at δ_H 2.67(2H, d, *J* = 16.5, 4.5 Hz, H-4) suggest the presence of methylene protons. The above ^{1}H NMR pattern suggest that the compound have flavan skeleton with two aromatic protons (H-6,8) on ring A and four aromatic protons (H-2', 3',5', 6) in ring B and devoid of carbonyl group at C-4 of ring C (also supported by ^{13}C NMR).

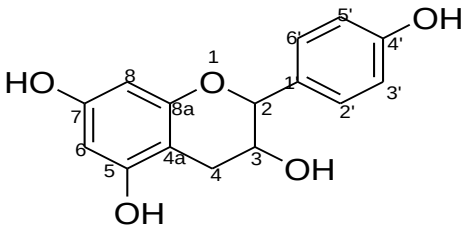
The ^{13}C NMR spectrum of compound 2 revealed; one oxygenated sp^2 quaternary carbons at δ_C 144.7 (C-4'), where the methines appear at 118.5 (C-2',6') and 115.1 (C-3', 5'). In addition, the presence of two sp^2 oxygenated quaternary carbons at 156.7 (C-5) and 156.6 (C-7) along with two upfeild carbons chemical shifts i.e. 95.3 (C-6) and 94.5 (C-8) suggest that ring A have 5, 8a-dioxygenated substitution pattern. The following quaternary carbons are also clearly evident at δ_C, 99.0 (C-4a), δ_C: 131.1 (C-1') and 156.2 (8a). Signals at 78.5(C-2) is due to the presence of sp^3 methine of ringB and 65.2 (C-3) is clearly evident due to the presence of sp^3 oxygenated methines of ring B. signals at,28.6 is due to the presence of sp^3 (C-4) is in good agreement with

spectral data. Thus, based on the above, the structure of the compound was found to be 3,5,7, 4'-tetrahydroxy flavan.

Table 8: ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral data of compound 2 in DMSO-d_6

C. No	^{1}H-NMR	^{13}C-NMR	DEPT-135	C. No	^{1}H-NMR	^{13}C-NMR	DEPT-135
1’	-	131.1	cq	3	4.0(1H,m,J=2.1HZ,H-8)	65.2	CH
6	5.8(1H,d,J=2.1HZ,H-8)	95.3	CH	4	2.6(2H,ddJ=16.5,4.5,HZ,H-4	28.6	CH_2
8	5.7(1H,d,J=2.1HZ,H-6)	94.5	CH	4’	-	144.7	cq
2’ 6’	6.8(2H,dd,J=1.2HZ,H-2’,6’)	118.4	CH	5	-	156.8	cq
4a	-	99	cq	8a	-	156.2	cq
5’, 3’	6.6((2H,dd,J=8.1HZ,H-3’,5’)	115.3	CH	7	-	156.5	cq
2	4.7(1H,d,H-2)	78.5	CH				

Cq =quaternary carbon



3,5,7,4' tetrahydroxyflavan.

Fig 6:Chemical structure of tetrahydroxy flavan.

4.6 Anti bacterial Test Results

The anti bacterial activity of the extract and pure compound of *E.shimperi* were studied in the same concentrations (20µg/mL) against four pathogenic bacterial strains, one Gram-positive *Staphylococcus aureus* and three Gram-negative *Escherichia coli, Klebsola pneumonia, Protens*

mirabilis. These strains have been selected for the basis of their application purpose of further formulation study. Antibacterial potential of crude extract and isolated pure compound were assessed in terms of zone of inhibition of bacterial growth. The results of the antibacterial activities are presented in Table 10.

Table 9: Zone of bacterial growth inhibition (mm) for crude extract and isolated pure compounds from stem bark of *E.schemberi.*

		Types of bacteria with mean inhibition diameter (mm)			
Sample code	Sample conc. In µg/mL	Staphylococcus aureus	Escherichia coli	Protens mirabilis	Klebsola pneumonia
MeOH crude		N	n	n	n
Compound 2	20	11	13	10	10
Compound 1	20	15	12	6	11
Gentamicin	20µg/mL	15	15	15	15

n≤ 6 is null and n>6 is sensitive.

As shown in table 9; Depending on zone of inhibition diameter antibacterial test of compound 2 and 1 showed sensitivity against a gram positive and gram negative bacteria than the crude extract which was not sensitive to both bacteria. As compared with standard drugs, the results revealed that in the pure compounds for bacterial activity, *E. coli* and *S. aureus* were more sensitive as compared with *Protens mirabilis* and *Klebsola pneumonia* with inhibition zone 13mm(compound 2) and 15mm(compound1), 10mm(compound 2) and 11mm(compound 1) respectively. The growth inhibition zone measured ranged from 7 to 15 mm for all sensitive bacteria. This growth inhibition zone indicate that the isolated pure Compound 1 especially exhibited comparable antibacterial activity against *S. aureus* to that of the standard antibiotic gentamicin, with zone of inhibition diameter 15 and 15mm for compound 1 and that of standard. This result shows that an isolated pure compounds of *E.schemperi* as a good candidate for development of antibacterial drug.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

For decades, medicinal plants has been used and continues to be an alternative approach on treatment for various diseases caused by protozoan, bacteria, fungi, viruses and helminthes**.** *E. schimperi* is one of these medicinal plants within the genus present at various geographical locations with a wide traditional use in Ethiopia. The phytochemical screening results showed presence of saponins, phenols, alkaloids, tannins, and flavonoids where as terpenoids, glycosides and phytosterols were absent in both extracts. After repeated successive solvent extraction and column chromatography different compounds were isolated from the stem bark of the plant. To the best of our knowledge two compounds were isolated for the first time from the plant and characterized as 3,5,8a,3',4'-pentahydroxy flavan and 3,5,8a,4'-tetrahydroxy flavan are reported here for the first time from the plant. Other fractions contain impurity as well as mixed compounds even after combining and attempts to purify. The structures of the compounds were characterized on the basis of spectral data (UV-Vis, IR, 1D and 2D NMR) as well as comparison with the literature data. As demonstrated by plant *in vitro* in this study, there is considerable evidence that the plant fractions have the potential to be developed into agents that can be used as treatment for different diseases caused by bacteria.

5.2 Recommendations.

Based on TLC analysis the plant contains several polar chemical constituents which were not isolated in this study because of luck of an advanced instruments and time constraints.

- It is possible to isolate more polar compounds using advanced chromatographic techniques such as RP-HPLC and PTLC techniques.
- Further bioassay guided isolation and characterization work should be done to fully characterize more bioactive constituents of the plant stem bark extracts to identify all active compounds against a variety of bacteria and fungi.
- This plant should be studied more extensively to explore its activity on bacteria species, protozoa and helminthes among others. It is also important to evaluate the crude extract and fractions for antioxidant activities to check their potential as in the $DPPH^{-}$ and $ABTS^{+}$ method. Therefore, much more Phytochemical investigation, anti microbial and antioxidant study should be carried out on the plant in future.

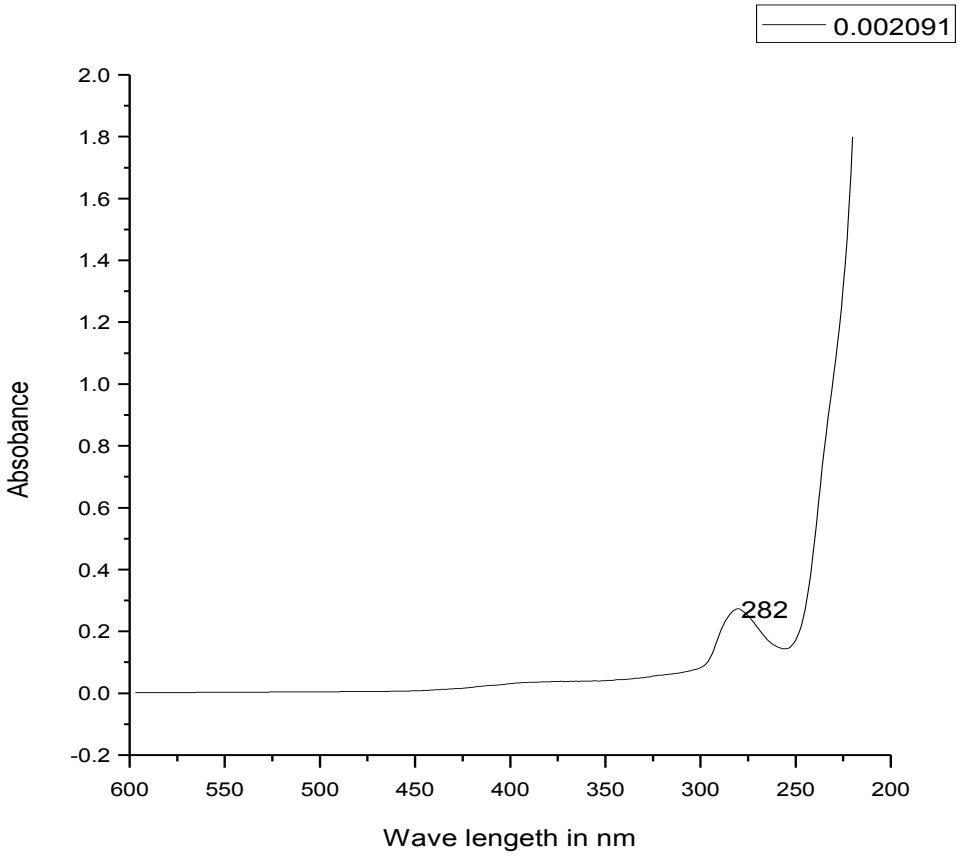
REFERENCES

1. WHO, Traditional medicines, global situation, issues and challenges, Geneva, 2011.
2. Sheeba, E.; (2010) Antibacterial activity of solanum surattense burm. F. Kathmandu University Journal of Science, Engineering and Technology, 6(1), 1-4.
3. Ncube, N. S.; Afolayan, A. J.; Okoh, A. I.; (2008) Assessment techniques of antimicrobial properties of natural compounds of plant origin, current methods and future trends. African Journal of Biotechnology, 7(12), 1797-1806
4. Gupesh P.; Pal S.; Pavani A.; Gadge M.;(2010) Quantitave estimation of the active constituents of Parkiabiglandulosaby using HPTLC and FTIR, International Journal of Pharma and Bio Sciences, (1) ,Pp315-322.
5. James J.; DuberyI.(2009)PentacyclicTriterpenoids from the Medicinal Herb, Centellaasiatica(L.) Urban J. Ethno pharmacol, Molecules, 14), 3922-3941.
6. Lequesne, P.W.; Cooper-Driver, G.A.;Villani,M.; Do,M.N.; (1986) in new Trends in natural product chemistry, proceeding of the 2nd international symposium Pakistan U.S.; Binational workshop, (eds. Atta-Ur-Rahman and Quesene, P.W.) Shamin printing press; Karachi, p.271.
7. Hagos, M.;(1989) Ph.D.; Dissertation ,Uppsala University, Uppsala, P.11.
8. Bak,J.T.;Borris,R.P.;Curete.B.;Cragg.G.M.;Gupta,M.P.;lwu,M.M.;Madulid, D.R.and Tyler,V.E.;(1995) in Natural products Drug Discovery and Development, New Perspectives on international Collaboration. J. Nat. Prod.; 58(9), 1325-1357.
9. Karborne, J.B.; Grayer, R.J.; (1994) in the Flavonoids, Advance in research since 1986 (ed.Harborne,J.B.) Chapman and Hall, London, pp.589-618.
10. Khurram, M.; (2010) Studies on the isolation and characterization of secondary metabolites from Dodonaea viscosaand Quercus balootand their potential as antibacterial agents, PhD, thesis, Quaid-i-azam University, 1-137.
11. Shai, L.; (2007) Characterization of compounds from Curtisia dentate (Cornaceae) active against Candida albicans, PhD, thesis, Pretoria University, South Africa, 1-177.
12. Adwan, G.M hanna, M.; (2008)Synergistic effects of plant extracts and antibiotics on Staphylococcus aureus strains isolated from clinical specimens. Middle-East Journal of Scientific Research, 3(3):134–139.

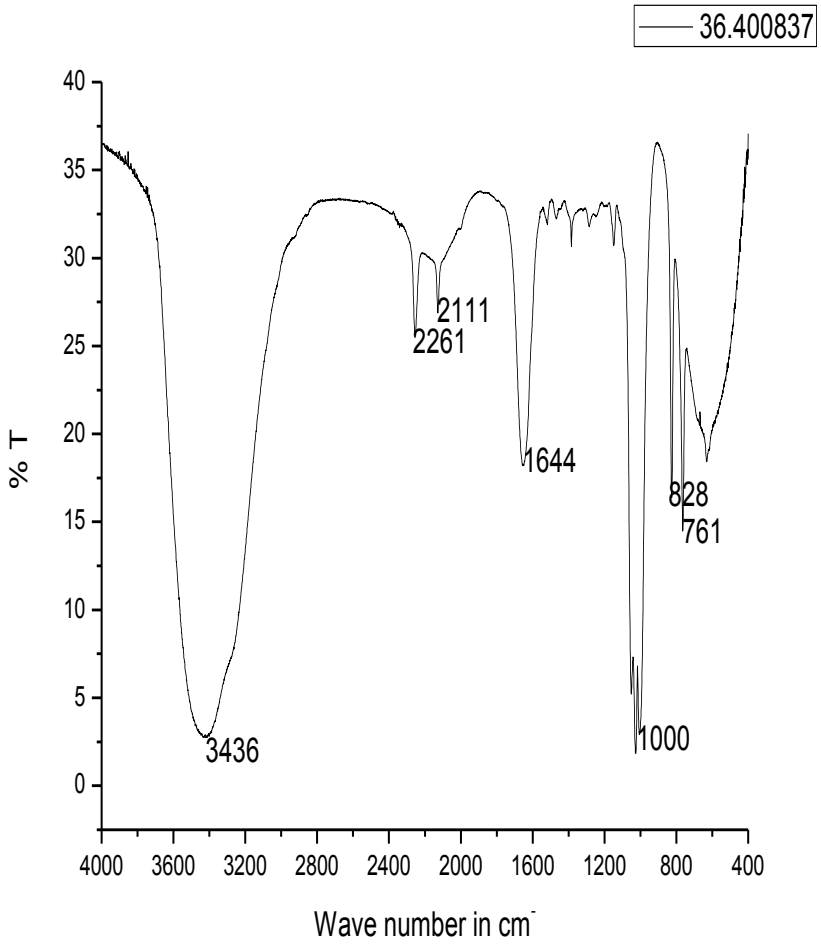
13. Kamboj, A.; Kumar, A.; isolation of stigmasterol and β-sitosterol from petroleum ether extract of aerial parts of ageratum conyzoides, International Journal of Pharmacy and Pharmaceutical Sciences, 2011;(3) 94-96.
14. Muell, F.; (2009) Genus Embelia". PlantNET - New South Wales Flora Online. Royal Botanic Gardens and Domain Trust, Sydney Australia. Retrieved,2009-08-11.
15. Kokwaro, J.O.; (1993) Medicinal Plants of East Africa, 2nd ed. E. A.; Lit. Bureau, Nairobi, p 49 and 164.
16. Bogh, H.O.; Andreassen, J.; and Lemmic, J.; (1996) Anthelmintic usage of extracts of Embelia schimperi from Tanzania. J .; Ethnopharmacol. 50(1):35-42.
17. Midiwo, J.O.; Mwangi RW, Ghebremeskel, Y.; (1995). Insect antifeedant, growth-inhibiting and larvicidal compounds from R.melanophloeos (Myrsinaceae). Insect Sci. Appl. 16(2): 163-166.
18. Warrier, P.K.; Nambiar VPK, Ganapati PM, (2001). Some Important Medicinal Plants of the Western Ghats, India, a Profile, Blackwell Publishers, New Delhi,141-156.
19. Lulekal, E.; Rondevaldova, J.; Bernaskova ,E.; (2014). Antimicrobial activity of traditional medicinal plants from Ankober District, North Shewa Zone, Amhara Region, Ethiopia. Pharmaceutical Biology,52(5):614–620.
20. Zutshi, U.; Johri, RK, Atal CK ,(1989). Possible interaction of potassium embelate: A putative analgesic agent, with opiate receptors. Ind Exp. Biol., 27: 656-657.
21. Midiwo, J.O.; Arot, L.M.; Mbakaya, C.L.;(1988). Bull. Chem. Soc. Ethiop,83.
22. d'Avigdor, E.; Wohlmuth, H.; Asfaw, Z.; Awas, T.; (2014). The current status of knowledge of herbal medicine and medicinal plants in Fiche, Ethiopia. Journal of Ethnobiology and Ethnomedicine. 10(1):38–71. doi: 10.1186/1746-4269-10-38.
23. Pengcheng, Lin,Shuai Li, Sujuan Wang, Yongchun,Yang and Jiangong Shi.(2006).J. Nat.Prod.;69 (11)1629–1632.
24. Ooko, S.A.; Paul, C.K.; ipkemboi P.K.;Festus Kaberia, and Andrew A.O. (2008)Naturforsch. 63 c, Pp 47-50.
25. Kiprono, C.P.; Midiwo J.O.; Kipkemboi1, P.K.; Santino L, (2004) Bull. Chem. Soc. Ethiop. 18(1),Pp 45-49.
26. Blanche, L.N.; Faustine LMD, Michel, F.T.; Hippolyta. W. Guang-Zhi. Z, Ning-Hua. T and Pierre. T, Rec. Nat. Prod. 8:1 (2013) Pp 37- 40.

27. Lawrence, O.A.M.; Ivar. U and Peter. L, (2004)Bull. Chem. Soc. Ethiop, 18(1), 51-57.
28. Alex, K.M.; Paul Kiprono, Sarina, Grinberg and Shmuel Bittner, (2003). Isolation Pentacyclic triterpenoids from *Embelia schimperi*, 573–577.
29. Andremont, A.; Bonten, M.;Kluytmans,(2011) ,J.; Carmeli. Y.; Cars, O.; Harbarth S. Fighting bacterial resistance at the root, need for adapted EMEA guidelines. *The Lancet Infectious Diseases*.; 11(1):6–8. doi: 10.1016/s1473-3099(10)70227-9.
30. Nickerson, E. K.; West T. E.; Day N. P.; Peacock S. J.; (2009). *Staphylococcus aureus* disease and drug resistance in resource-limited countries in south and east Asia. *The Lancet Infectious Diseases*.; 9(2):130–135. doi: 10.1016/s1473-3099(09)70022-2.
31. Kurek ,A.; Grudniak, A. M.; Kraczkiewicz-Dowjat, A., Wolska, K. I.; 2011, New antibacterial therapeutics and strategies. *Polish Journal of Microbiology*.;60(1):3–12.
32. Drago,L.; De Vecchi E.; Nicola, L.; Gismondo, M. R.; (2007). *In vitro* evaluation of antibiotics' combinations for empirical therapy of suspected methicillin resistant *Staphylococcus aureus* severe respiratory infections. *BMC Infectious Diseases*.;7(1, article 111) doi: 10.1186/1471-2334-7-111.
33. Cowan, M. M.; (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*.; 12(4):564–582.
34. Hai-Ming Zhang , Cheng-Fang Wang , Sheng-Min Shen , Gang-Li Wang , Peng Liu , Zi-Mu Liu , Yong-Yan Wang , Shu-Shan Du , Zhi-Long Liu and Zhi-Wei Deng . Antioxidant Phenolic Compounds from Pu-erh Tea.2012; *17*, 14037-14045.

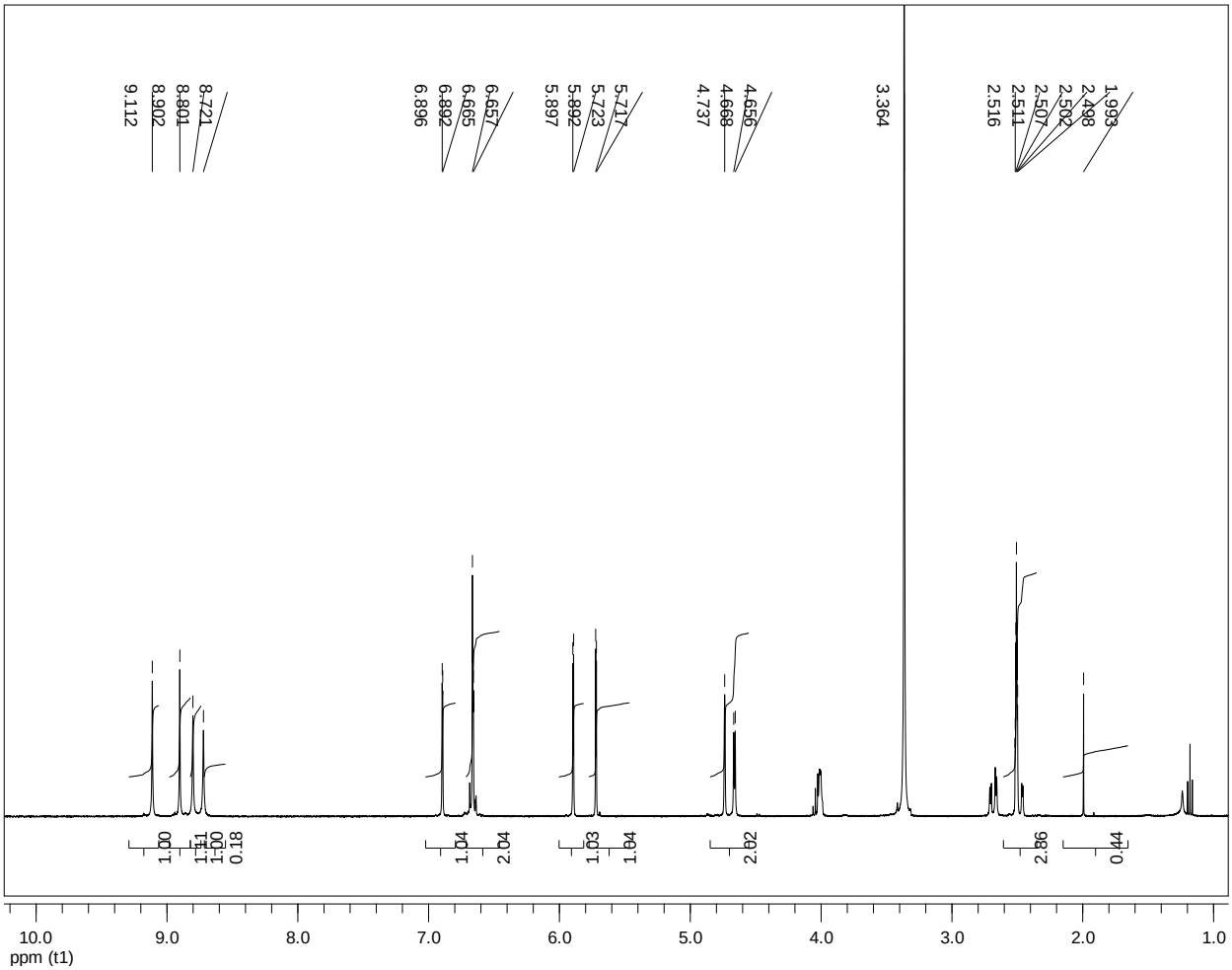
APPENDICIES



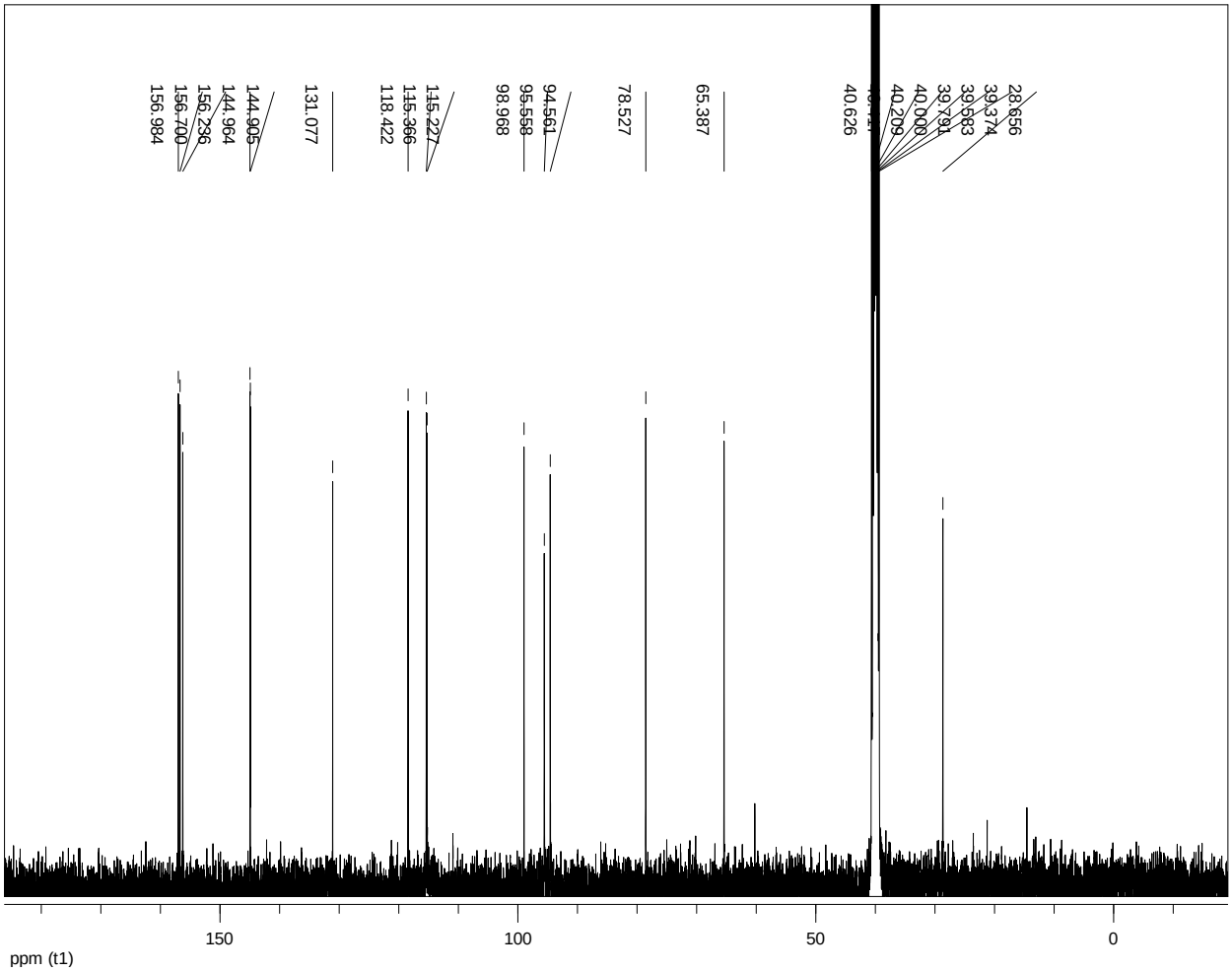
Appendix 1: UV Spectrum of compound 1



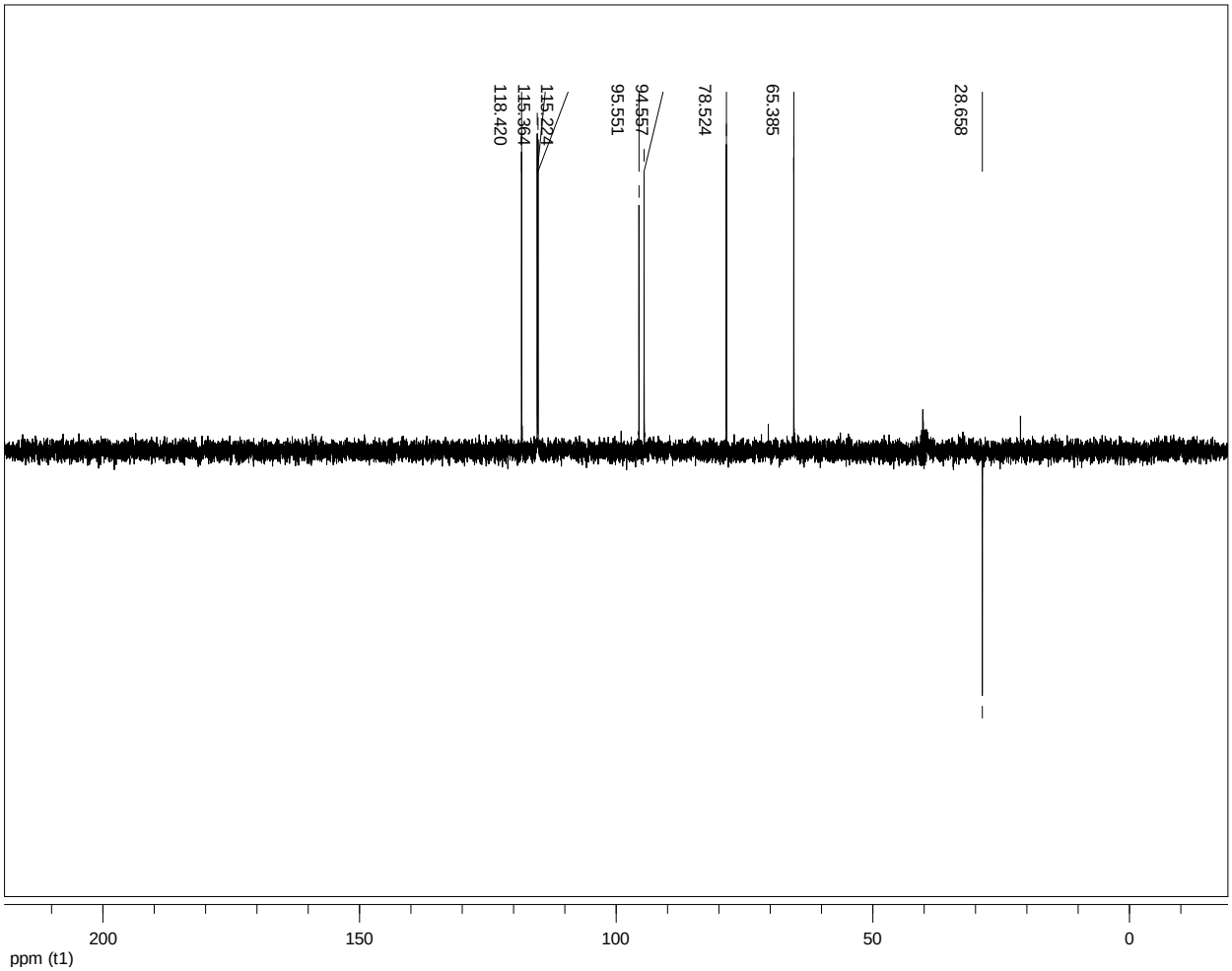
Appendix 2: IR Spectrum of compound 1.



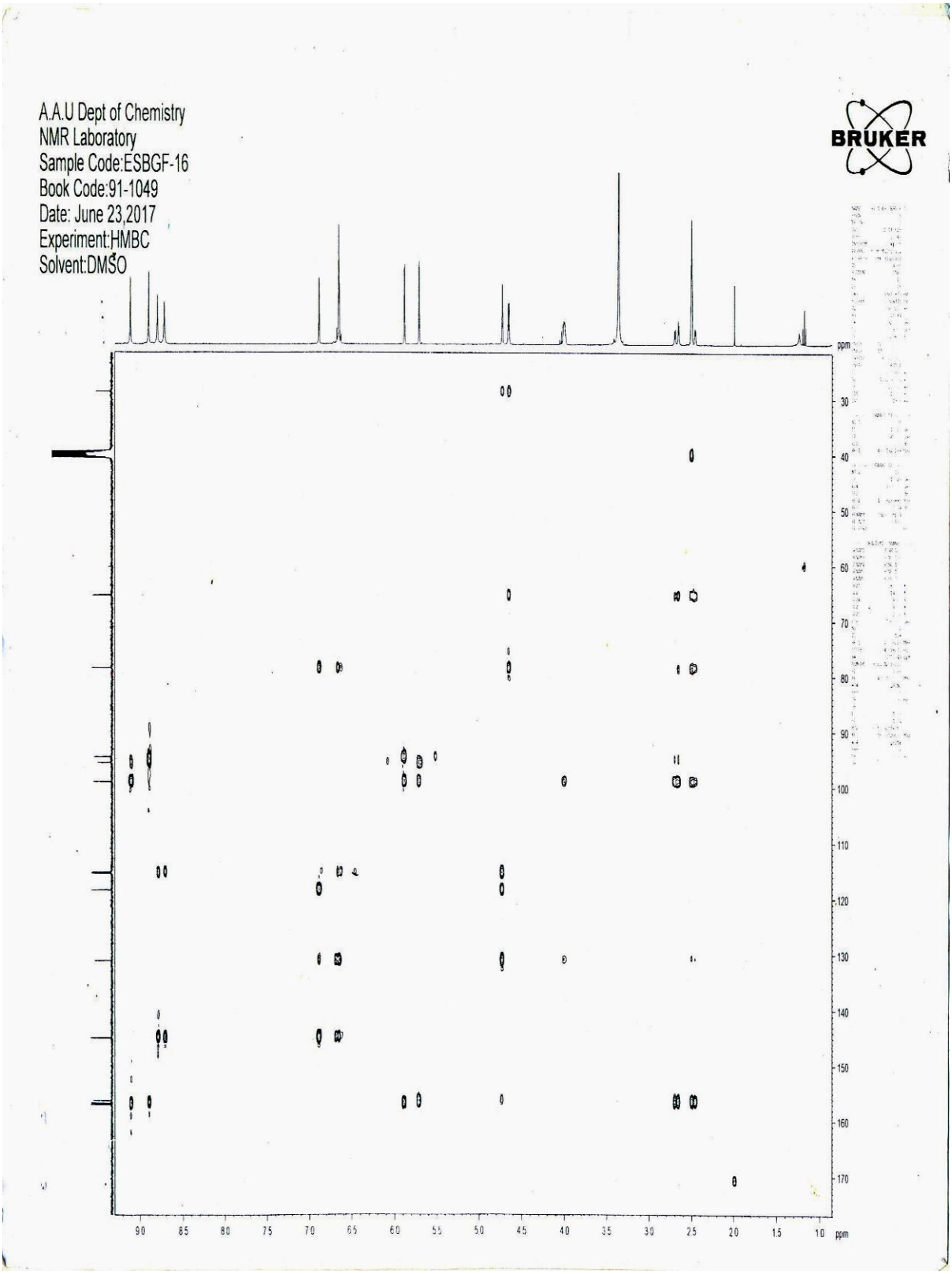
Appendix 3:[1]H-NMR spectrum of compound 1.



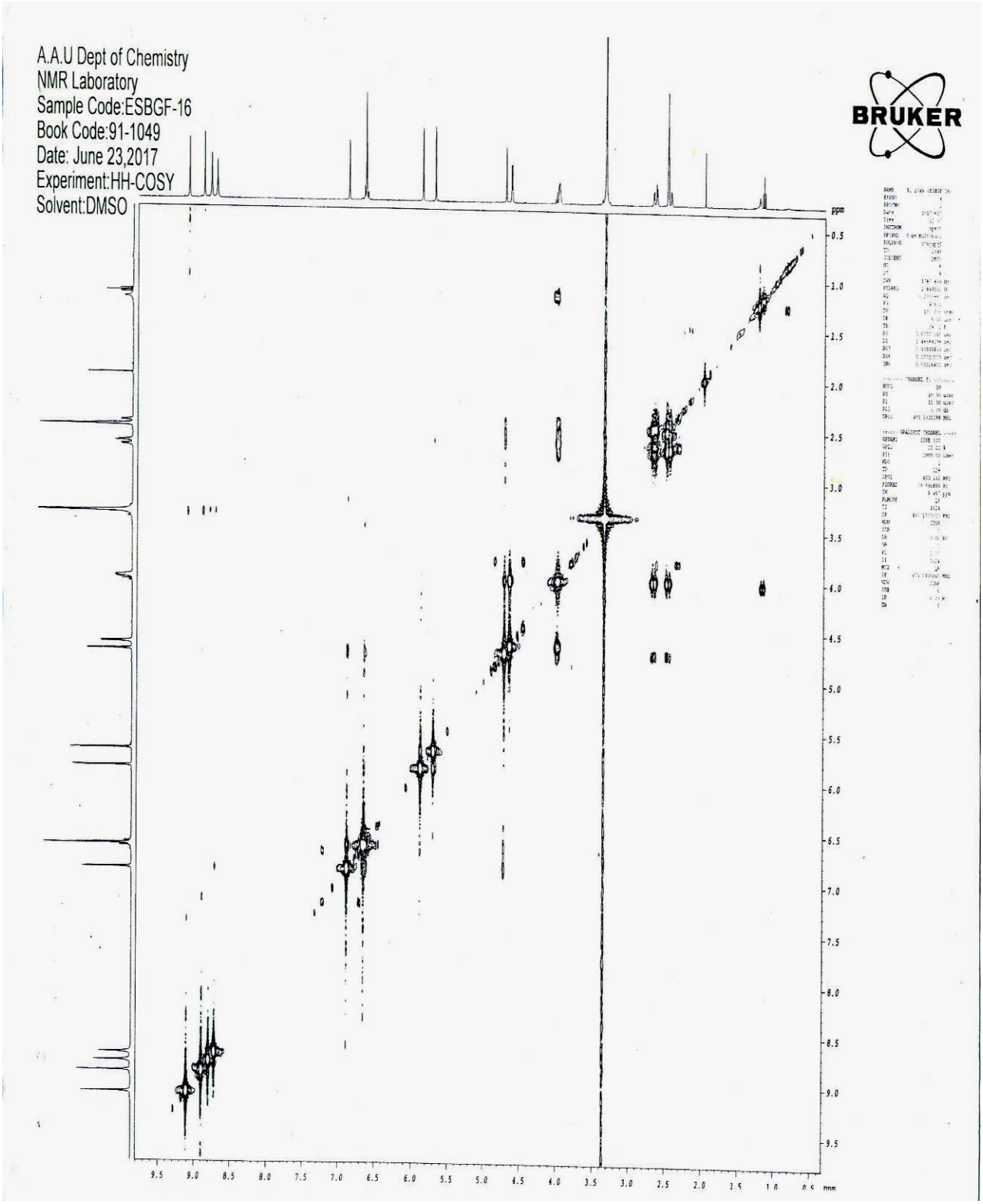
Appendix 4:^{13}C-NMR spectrum of compound 1.



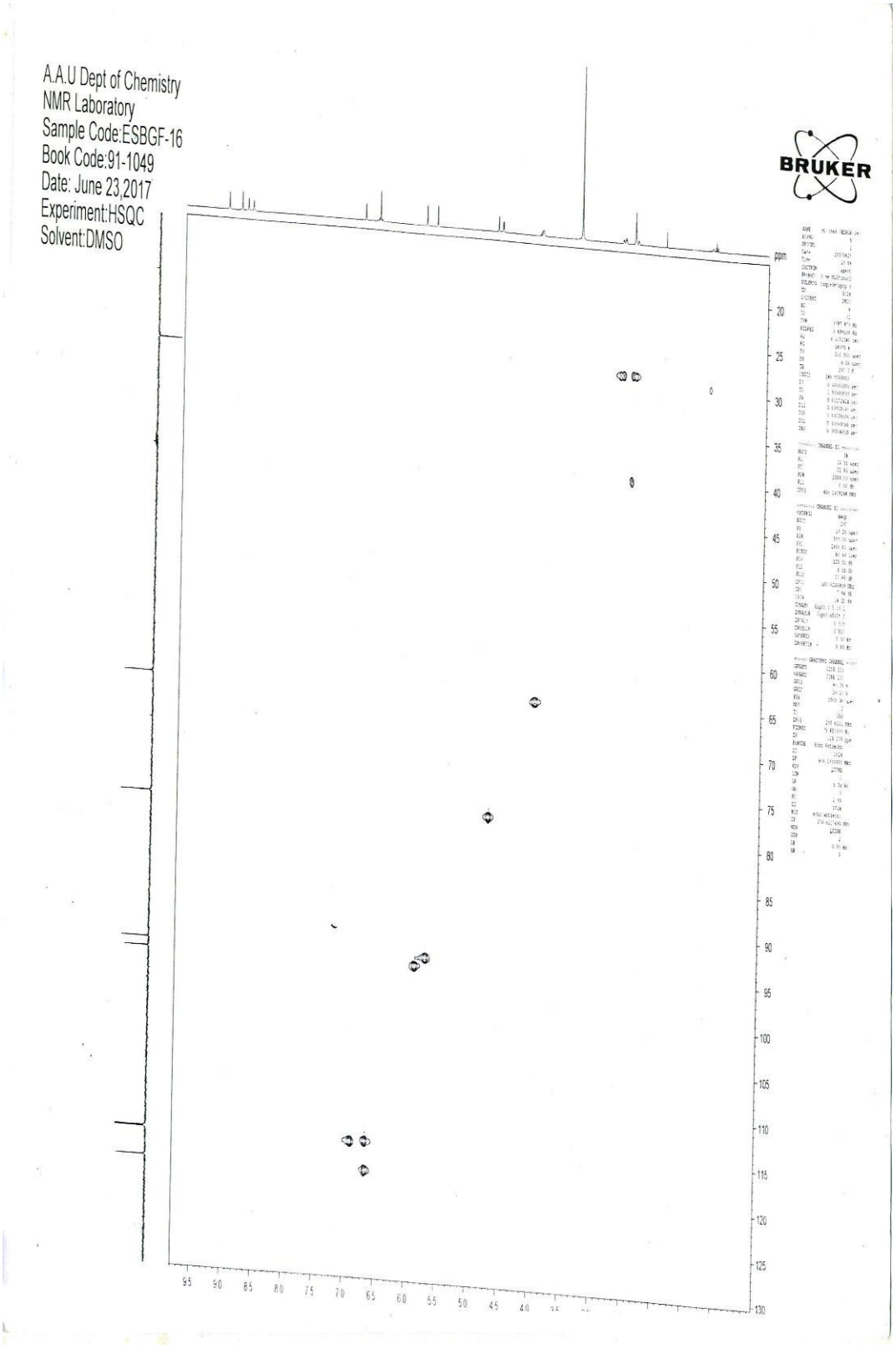
Appendix 5: DEPT spectrum of compound 1.



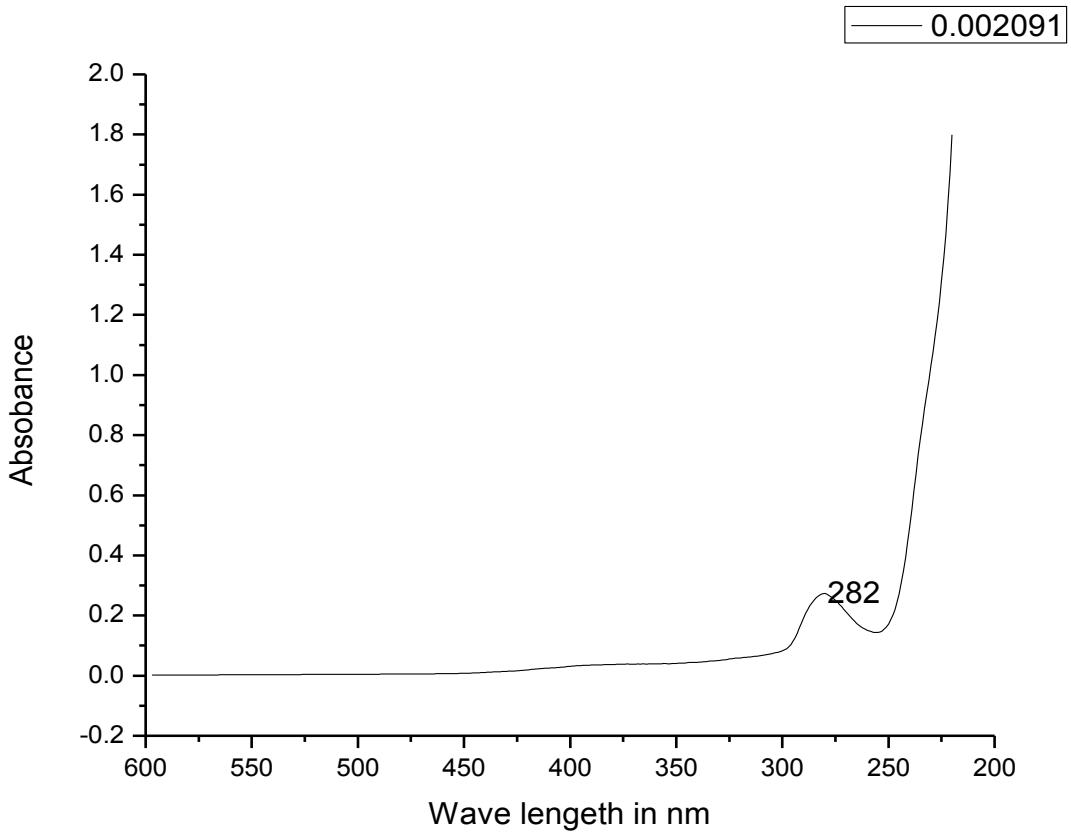
Appendix 6: HMBC spectrum of compound 1



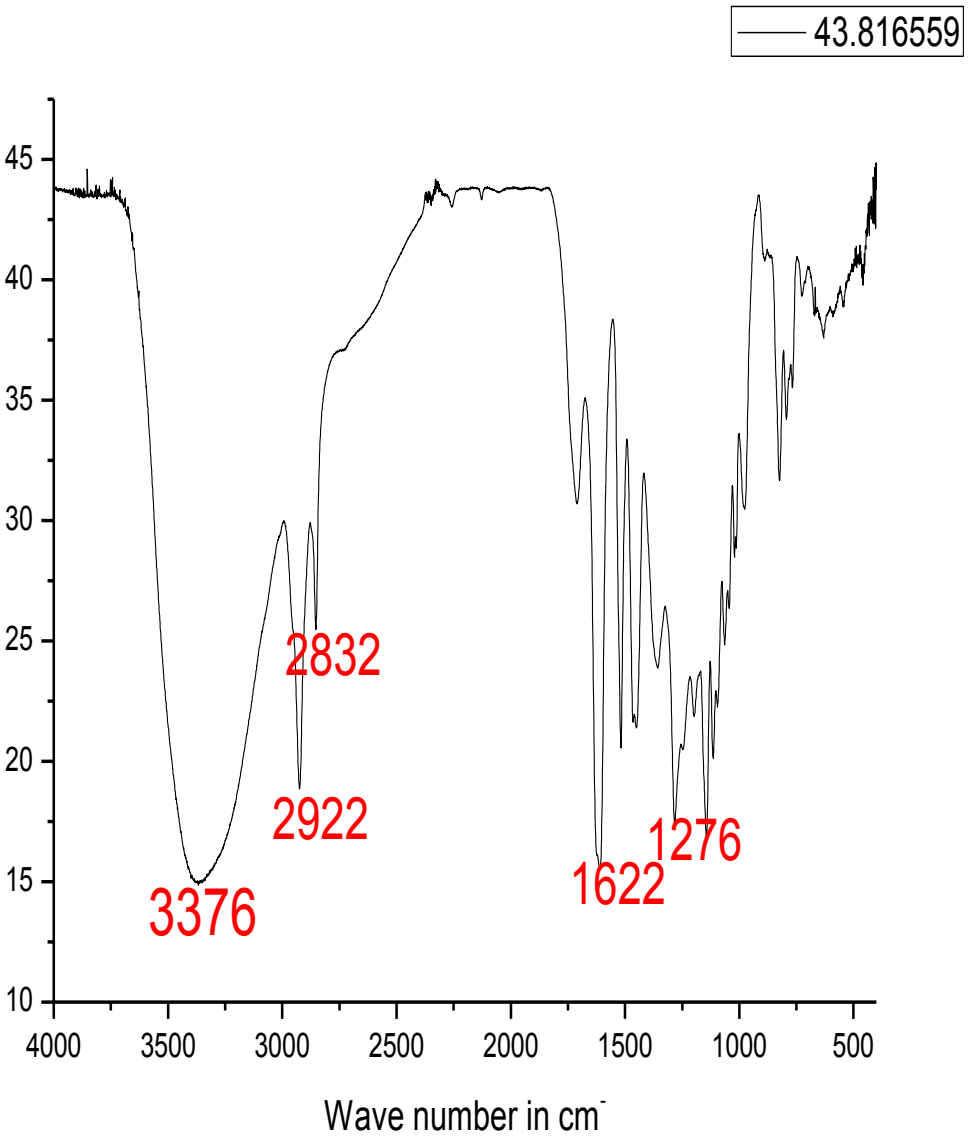
Appendix7: COSY spectrum of compound 1



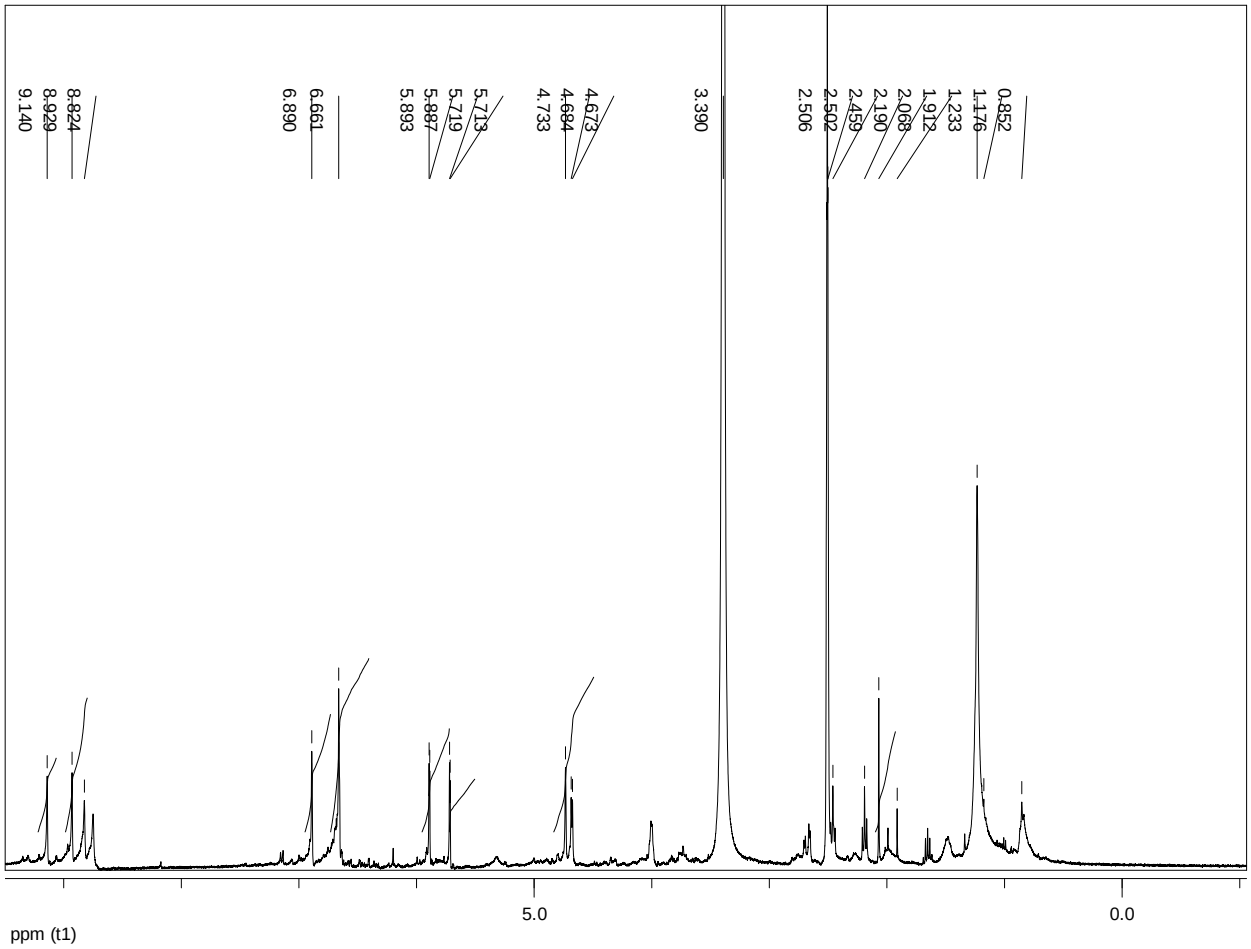
Appendix 8 : HSQC spectrum of compound 1



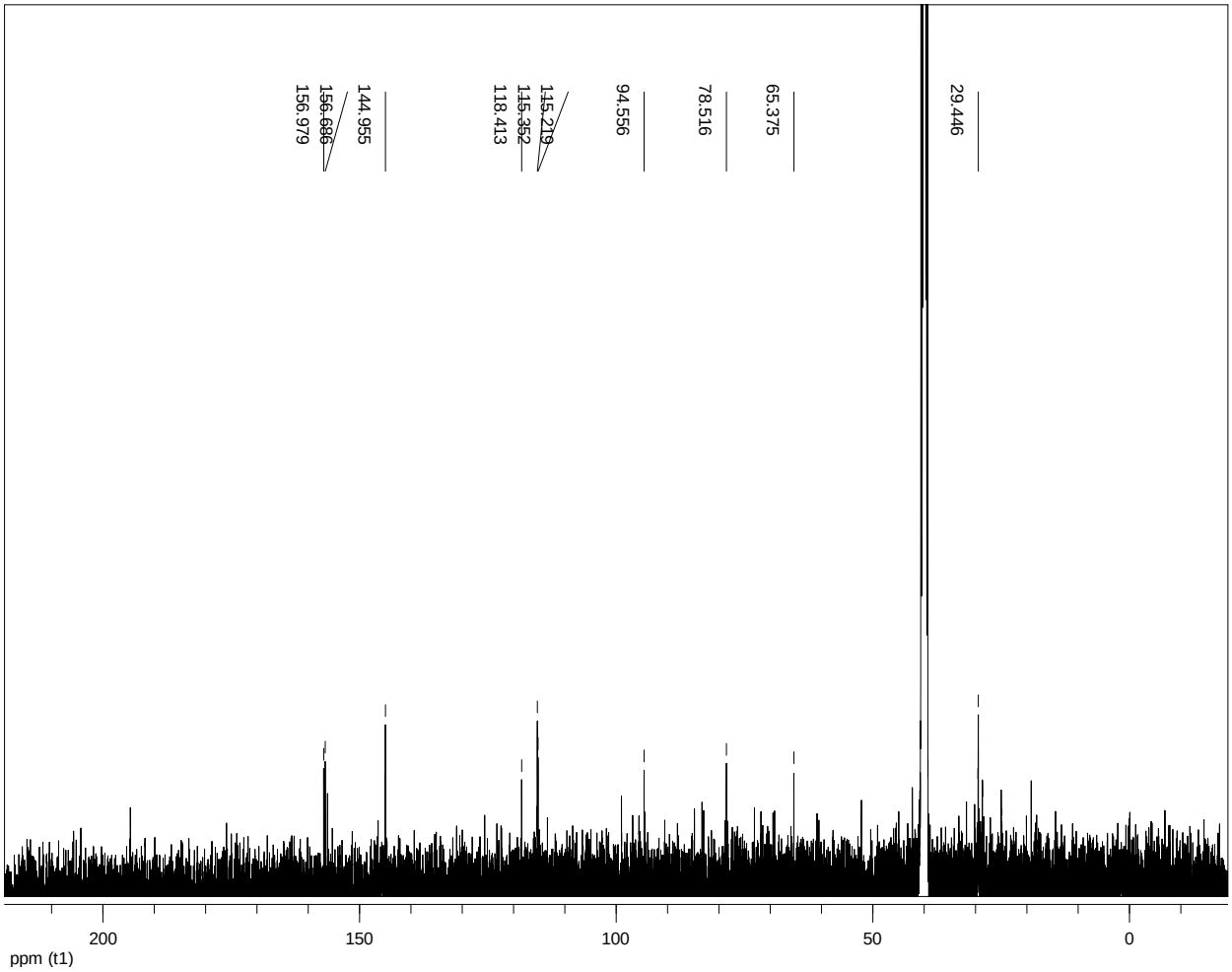
Appendix 9: UV spectrum of compound 2.



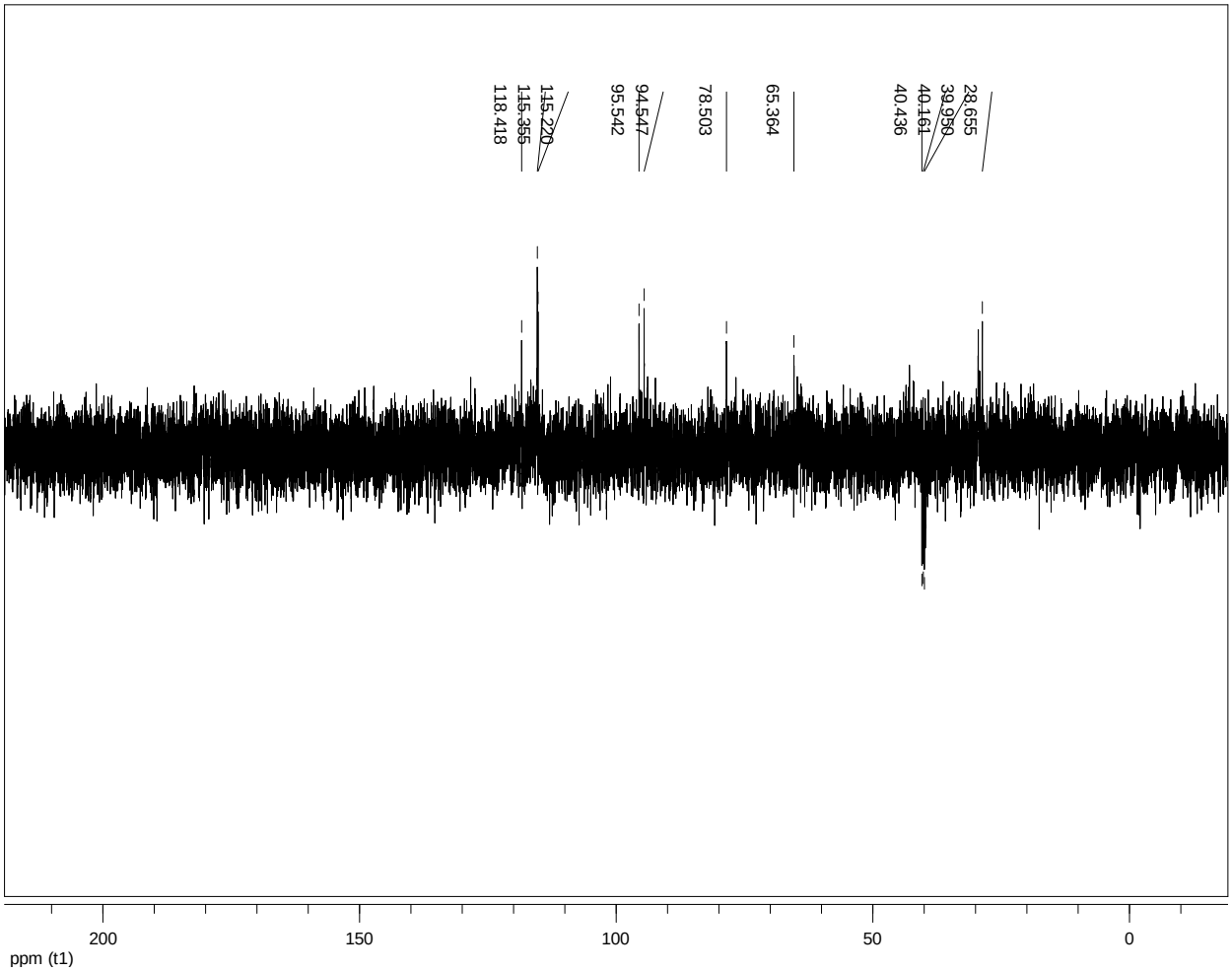
Appendix 10:IR Spectrum of compound 2



Appendix 11 : ^{1} H-spectraum of compound 2



Appendix 12:^{13}C-NMR spectrum of compound 2



Appendix 13:DEPT spectrum of compound 2