Phytochemical, Antibacterial and Antioxidant Studies of the Leaves Extracts of *Plantago lanceolata*

BY: - Firew Derebew

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 of Science (MSc) in Chemistry

Office of Graduate Studies

Adama Science and Technology University

Adama

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Advisor: Yadessa Melaku (PhD)

Co-advisor: Milkyas Endale (PhD)

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APPOROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defense by Firew Derebew have read and evaluated his thesis entitled "Phytochemical, Antimicrobial and Antioxidant Studies of the Leaves Extract of *Plantago lanceolat*"" 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 Master's of science (MSc) in chemistry

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ADVISOR'S APPROVAL SHEET

To: chemistry department

Subject: Thesis Submission

This is to certify that the thesis entitled "Phytochemical, Antimicrobial and Antioxidant Studies of the Leaves Extract of *Plantago lanceolata"* submitted in partial fulfillment of the requirements for the degree of Master's in science, the Graduate program of the department of chemistry, and has been carried out by Firew Derebew Id. No GSS/0214/05, under our advise. Therefore, we recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the department.

Name of major Advisor: Yadessa Melaku (PhD) Signature …….Date……

Name of Co-Advisor: Milkyas Endale (PhD) Signature ……Date……

DECLARATION

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

Name: Firew Derebew

Signature: ……..

This MSc has been submitted for examination with our approval as thesis advisor/co-advisor

Name of Advisor: Yadessa Melaku (PhD) Name of Co-Advisor: Milkyas Endale (PhD)

Signature: …….. Signature: ……..

Date of submission: ……..

ACKNOWLEDGEMENT

This study, was not unlike any other study, could not have come to fruition without the help of numerous dedicated individuals. Words would appealing much express my feeling of gratitude for all his sincere, valuable guidance, provision of constructive suggestion throughout the research work, honest and enormous devotion for the achievement of this study to my advisor Dr. Yadessa Melaku , and appreciation is expressed for which I am extremely grateful to my co-advisors Dr. Milkyas Endale

I am grateful to Mr. Solomon Gimay, a head of Chemistry Program of Adama Science and Technology University, for his administrative support, for providing laboratory service for this research work and all the laboratory staff for their cooperation. My thank goes to Oromia Public Health Research Capacity Building and Quality Assurance for allowing me to conduct antibacterial test

I am forever grateful to my wife Seble Debelo for her prescience and values paved the way for a privileged education and unconditional support at each turn of my life, for her pray, constant encouragement, love and care, without which this was quite impossible. I wish to be grateful to all my family members, particularly to my brother Tibebu Derebew and my friends Gudeta Terfa and Tesema Abera for their well understanding and support throughout the study period. I am extremely grateful to Eshetu Dirriba and Robi Wami for their tolerance to take my responsibility at school during my research stay at ASTU. Last but not least, I would like to pass my deepest gratitude to my parents, brothers and friends for their encouragement throughout my study.

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ACRONYMS AND ABBREVIATIONS

ASTU	Adama Science and Technology University
^{13}C-NMR	Carbon -13 Nuclear Magnetic Resonance
CC	Column Chromatography
DEPT	Distorsionless Enhancement by Polarization Transfer
EMEA	European Medicine Agency
FT-IR	Fourier Transform Infra-Red
HPLC	High performance liquid chromatography
MHA	Muller Hinton Agar
^{1}H- NMR	Proton Nuclear Magnetic Resonance
Rf	Retention Factor
TLC	Thin Layer Chromatography
TM	Traditional medicine
UV	Ultra Violet

ABSTRACT

Plantago lanceolata (Plantaginaceae) is a perennial cosmopolitan species traditionally used for blood clotting and healing of wound. In view of its medicinal uses, the powdered leaves were successively extracted with n-hexane, ethyl acetate and methanol to give1.55, 2.16, and 8.2%, respectively. Phytochemical screenings results revealed the presence of flavonoids, phenols, tannins and glycosides in the extracts of leaves of Plantago lanceolanta. The methanol extract after silica gel column chromatography has led to the isolation of two compounds, namely, 6''-O-ethyl-4'''-acetyl verbascoside and verbascoside. The structures were elucidated using spectroscopic methods such as NMR and IR. The EtOAc and methanol extracts, and pure compounds isolated from the methanol extract were evaluated in vitro for antibacterial activities by using the disc diffusion method against Staphylococcus aureus, Escherichia coli, Klebsiella pneumania and Proteus miabilis .The maximum inhibition zone (20 mm) was shown by 6''-O-ethyl-4'''-acetyl verbascoside against S. aureus which is significant compared with Ciprofloxacin showing the significance of this plant against bacteria. The radical scavenging activity of the methanol and ethyl acetate extract, and the isolated 6''-O-ethyl-4'''-acetyl verbascoside and verbascoside compounds were 64.2%, 79.2, 87.7, and 83.9 respectively. Both the isolated compounds displayed powerful radical scavenging activity indicating the potential of this plant as pharmacology.

Key words: Plantago, P. lanceolata, verbascoside, Antioxidant, Antibacteria, inhibition zone

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the Study

The study of natural products has been and continues to be a major deriving force in the development of the fields of organic and medicinal chemistry. A knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of such economic materials as tannins, oils, gums and precursors for the synthesis of complex chemical substances. Such phytochemical screening of various plants is reported by many workers [1-3]. At present, the demand for these medicinal plants is increasing due to their use in traditional medicine, pharmaceutical industries, cosmetic fields and agribusiness, and for the quality of their essential oil [4]. Although several drugs have been developed based on natural product chemistry, Natural products are expected to play an important role as one of the major sources of new drugs in the years to come because of their incomparable structural diversity and their "drug like'' properties, i.e. their ability to be absorbed and metabolized [5]. Clinical microbiologists have two reasons to be interested in the topic of antimicrobials from plant extracts. First, it is likely that these phytochemicals will find their way into the arsenal of anti-microbial drugs prescribed by physicians, of which several are already being tested on humans. It is reported that on average, two or three antibiotics derived from microorganisms are launched each year [6]. Secondly, the public is becoming increasingly aware of the problem of over prescription and miss use of traditional antibiotics.

Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine. The majority of the population in the developing countries still relies on herbal preparations to help enhance health. This is also true of Ethiopia where the majority of the population depends on plants for their healing action in various health problems. Plants and animals have been utilized as TM throughout history [7]. Plant based medicines are used worldwide and have been recognized as medicines by many countries [8]. These medicines

were initially used in crude forms such as tinctures, teas, poultices, powders and other herbal formulations and some are still commercially available in these forms [9, 10].

Traditional medicinal practices are common in Ethiopia in which about 80% of the population in the country use plant based TM by indigenous knowledge as their major primary health care system [11]. The ways TM are used as diverse as the different cultures existing in the country. Traditional medicine healing practice is not only concerned with curing of diseases but also with the protection and promotion of human physical, spiritual, social, mental and material well-being [12]. Furthermore, studies related to herbal medicines can help to stimulate confidence in traditional medicine and enhance appreciation of herbal medicines among local communities [13].

Traditional remedies for wound healing also have a wide usage among the people living in the rural areas. Several medicinal plants have been reported to be used for the treatment of wounds and ulcers [14]. Among these plants, *Plantago* species were reported to be used as wound healing agent with its severe, haemostatic and antimicrobial properties. Those *Plantago* is a genus of about 265 species of small, inconspicuous plants commonly called plantains. Previous studies have shown that *Plantago* species have analgesic, anti-inflammatory, antimicrobial, antioxidant, hepatoprtective activities, and cytotoxic effect on the cancer cells [15-17].

The aerial parts of *Plantago lanceolata* have been reported to possess ant-inflammatory, antibacterial, diuretic, anti asthmatic and wound healing potential [18, 19]. *Plantago* is an important medicinal plant which has different compounds such as phenolic compounds (caffeic acid derivatives), flavonoids, alkaloids, terpenoids, anti oxidants, anti inflammatory agents. *Plantago lanceolata* (ribwort plantain) belonging to the genus *Plantago* and family Plantaginaceae is a perennial cosmopolitan species which shows high ecological plasticity, being found naturally in grassy areas on roadsides, in pastures and in crops as weeds. *P. lanceolata* is an adaptable perennial weed, indigenous to Britain but now naturalized all over the world. This plant is locally termed as “gurteb” in Amharic and “kortobe” in Afan Oromo

Despite the traditional use of this plant against various diseases, to the best of our knowledge there is limited report on the chemical constituents, antibacterial and antioxidant studies of the

leaves extract of this plant. Hence, the current study was undertaken primarily to isolates and characterizes compounds from the leaves of *P. lanceolata* plant. Furthermore, the antibacterial and antioxidant studies of the leaves extract and isolated pure compounds were also incorporated herein.

1.2 Statement of the Problem

In Ethiopia various plants are commonly used for the disease treatment by traditional healers and local community. The acceptance of traditional medicine as an alternative form of health care and the development of bacterial resistance to the available synthetic drug have led to investigate active antibacterial drug from natural products. In Ethiopia, there are several indigenous and endemic medicinal plants used against bacteria. Hence this study is intended to find out active antibacterial compound from the leaves of *Plantago lanceolata.* Furthermore an attempt will be made to isolate compound with pronounceable antioxidant

1.3 Significance of the study

The study serves as a steppingstone for researchers who are paying attention to carry out additional studies in correlated areas. Moreover the compounds to be isolated may add a new compound to the natural product database. The information obtained both from phytochemical as well as antibacterial aspect will help to support the traditional use of the plant to treat various infectious diseases.

1.4 Justification of the study

World Health Organization (WHO) estimates that one-third of the global population still lacks regular access to essential drugs, and that in the poorest parts of Africa and Asia, this figure rises to over 50% [20]. According to the WHO report, more than 80% of the people in Africa depend on traditional medicine [21]. Besides, the origins of more than 50% of modern clinical drugs are natural products [22]. In Ethiopia the use of herbs to treat different types of ailment is a wide spread practice [23]. *P. lanceolata is* used as a poultice to treat burns, to cure wounds and injuries and prevent pricking sensation. It relieves stomach pain and cures anemia [24]. In spite of the wide array of uses of this species as a remedy against diseases, there is

limited scientific report on the chemical constituents, antibacterial and antioxidant studies of the leaves of *P. lanceolata* growing in Ethiopia.

1.5 Objectives

1.5.1 General Objective

To conduct the phytochemical investigation, isolation of compounds, antibacterial and antioxidant activities of extracts of the leaves of *P. lanceolata*

1.5.2 Specific Objectives

The specific objectives of this study were to:

1. successively extract the leaves of *P. lanceolata* using n-hexane, ethyl acetate and methanol
2. to conduct phytochemical screening of the crude extracts
3. isolate compounds from the methanol extract of the leaves of *P. lanceolata* using chromatographic techniques
4. characterize the isolated compounds employing spectroscopic methods including ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and IR
5. assess the antibacterial activities of the extracts and isolated compounds against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumania* and *Proteus miabilis* and
6. evaluate the radical scavenging activities of the extracts and isolated compounds using DPPH

CHAPTER TWO

2. REVIEW OF LITRATURE

2.1. Natural Products with Antimicrobial Agents

Antimicrobial agents are chemical or biological substances used to kill or prevent the growth of microorganisms. A great number of these agents already exist and their actions on microorganisms are due to the presence of certain substances in plants. Medicinal plants are rich source of wide variety of secondary metabolites belonging to chemical classes such as sterols, alkaloids, glycosides, saponins, flavonoids, tannins, and carbohydrates are generally superior in their anti-microbial activities [25]. The study of natural products involves isolation in a pure form of these compounds and investigation of their structure, formation, use, and purpose in the organism. Natural products have been the greatest source of successful antibiotic compounds throughout history. Over the years, 80% of the bioactive, natural products isolated have been antibiotic compounds, and this trend is continuing [26]. One of the most important areas of application of natural products is in the treatment of human and veterinary ailments. Currently, at least 119 chemical substances derived from 90 plant species can be considered important drugs that are in use in one or more countries[27].

2.2 The Family Plantaginaceae

Plantaginaceae can be treated as a cosmopolitan family consisting of three related genera, i.e. *Bougueria decne, Littorella bergius* and *Plantago L.* and 265 species, almost all of them plantain (*Plantago*) [28, 29]. It is instead a monogeneric family containing only the genus *Plantago* [30].

2.3 The Genus *Plantago*

The genus name *Plantago* descends from the classical Latin name *Plantago* which in classical Latin meant some *Plantago* species, including *Plantago major* and *Plantago media*. In Latin the name was formed from the classical Latin word *planta* = "sole of the foot". The name was so formed in Latin because the leaves of these species grow out near flat at ground level. The suffix-*ago* in Latin means "a sort of". The genus *Plantago* comprises 265 species distributed all

over the world [31]. *Plantago* is an adaptable perennial weed, indigenous to Britain but now naturalized all over the world. Among the species in the genus, *Plantago africana, Plantago arenaria* - Branched Plantain, *Plantago lanceolata* - Ribwort Plantain, *Plantago asiatica, Plantago major* and many more [32].

2.3.1 Ethnobotanical use of the genus *Plantago*

The World Health Organization has approved the use of *plantago* in the following cases:

- laxative: for treating constipation, temporary constipation due to illness or pregnancy, irritable bowel syndrome, constipation caused by duodenal ulcer or diverticulitis; hemorrhoids stool softener after surgery in patients with anorectal [33, 34]
- to treat hypercholesterolemia as an adjunct to diet to reduce the risk of coronary heart disease
- to reduce the blood sugar that is increased after a meal [34]

Some of the traditionally applications of *Plantago lanceolata* are:

- Grind a bunch of fresh *Plantago lanceolata* twice a day and use it as a poultice to treat burns, it also cures wounds and injuries and improves inflammation
- Mix a spoon of *Plantago lanceolata* with barberry juice and drink it every morning and evening ; it will remove the blood in the urine which is due to the weakness of the liver

2.3.2 Biological activities of the genus *Plantago*

Species of the genus *Plantago* (commonly known as plantain) have been used for medicinal purposes and have also been tested for inhibitory activity against viruses, microbes, and insects [35]. For example, *P. intermedia* extracts were inhibitory to *Escherichia coli* [36], extracts of *P. Major* were active against certain viruses [37, 38] and a *P. lanceolata* line with high levels of iridoid glycosides enhanced resistance to the insect *Spodoptera exigua and* the fungus *Diaporthe adunca* [39]. Studies have also examined activity of several *Plantago spp.* against plant-parasitic nematodes. *Plantago major* reduced damage caused by *Xiphinema indexon grapes* [40] and extracts were nematicidal to *X. americanumand, X. index* [41, 42] and controlled *Ditylenchus dipsacion garlic* [43]. *Plantago asiatica* extracts had a negative but reversible effect on activity of *Meloidogyne javanica* and were lethal to *Pratylenchus*

vulnus [44]. *Plantago lanceolata* plants reduced population densities of *Mesocriconema xenoplax* on peach seedlings, but also suppressed seedling growth [45].

2.4 The Species *Plantago lanceolata*

P. lanceolata is one of the species that belongs to Plantaginceae families. The species of *P. lanceolata* occurs in different parts of Ethiopia. This plant is locally termed as "gurteb" in Amharic, "kortobe" in Afan Oromo and has got application for many skin disorders in Ethiopian traditional medicine. Medicinally, it is astringents, emollients, expectorants, cough, gastrointestinal disorder, diarrhoea, diuretics, antibacterial and antiviral [46, 47]. The leaves are long and narrow, smooth, and dark green. It is perennial herb growing on slopes and sand hills near the sea. Generally, plantain leaves are applied externally to heal sores and wounds; against furuncles, against insect and snake bites [48, 49] and internally as expectorant, anti tussive, emollient, anti-inflammatory, astringent, antimicrobial, bronchitis, laryngotracheal catarrh, diarrhoea [50, 51].

Figure 1: *Plantago lanceolata* picture taken by Firew Derebew from Mugher, January 2017

2.4.1 Phytochemistry of *Plantago lanceolata*

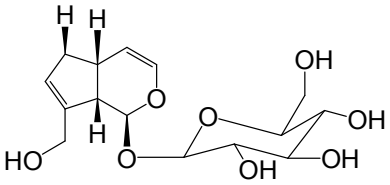
According to the iridoid fingerprint, *Plantago* species are classified as follows: species containing mainly aucubin (**1**) and their derivatives (*P. major*, *P. cornuti*, *P.gentianoides*) and species containing aucubin and catalpol (**2**) (*P. lanceolata*, *P. altissima*, *P. argentea*, *P. lagopus*, *P. atrata*). The iridoid content depends on the maturity of the leaves. Young leaves

contain up to 9%, while in the older ones iridoids are present only in traces [52]. Chemical variation within and between individuals was studied and it was found that young and intermediate age leaves have the highest content of catalpol while aucubin (biosynthetic precursor of catalpol) is predominant in the leaves of intermediate age and less in the mature ones [53]. *P. lanceolata* leaves also contain polysaccharides with L-arabinose (**3**), D-galactose(**4**), D-glucose(**5**), D-mannose (**6**), L-mannose (**7**), D-galacturonic acid(**8**), D-glucuronic acid(**9**), and minor proportions of L-fructose(**10**) [54].

Polyphenols: Acteoside (verbascoside) (**11**) is the main phenyletaonidic compound in *P. lanceolata*. Other compounds are lavandulyfolioside (**12**) and plantamajoside(**13**) [55].
Based on the differences in polyphenolic compounds (iridoids, flavonoids, phenyletanoids) of different *Plantago* species, a classification in two groups was proposed: one group with *P. asiatica* and *P. major* as main representatives and the other one with *P.depressa* and *P. lanceolata* [51]. The herbal substance also contains 6.5% tannins, phenolic carboxylic acids including chlorogenic-(**14**) p-hydroxybenzoic-(**15**), protocatechuic(**16**) and gentisinic-(**17**)[56], among others. It was showed that cultivated ribwort plantain has a higher polyphenol composition, both in terms of quality (various phenolic compounds with antioxidant character) and quantitative (1000 mg/100 g) than of wild specimens [57].

Flavonoids: class includes luteolinas (**18**) and apigenin (**19**) well as their derivatives with the main compounds apigenin-6, 8-di-C-glucoside (**20**), luteolin-7-O-glucoside (**21**) as well as apigenin-7-O-glucoside (**22**) and 7-O-glucuronide (**23**) [58].

Volatile oil: The volatile compound proportion corresponds to 0.05%, 0.03% and 0.001% of fresh weight for fruits, leaves and scapes, respectively. Thirty-five and twenty-six components were identified from fruits and leaves, respectively, while scapes contained only seven volatile components as it was showed by GC/MS. The major constituents of fruits were oct-1-en-3-ol(**24**) (24.9%), hexahydrofarnesyl acetone(**25**) (15.7%), vanillic acid (**26**) (9.8%) and neophytadienes (>10%); leaves contained mainly oct-1-en-3-ol(**27**), 6-(3-hydroxy-1-butenyl)-1,5,5-Trimethyl-7-oxabicyclo[4,1,0]heptan-3-ol(**28**) (6.9%) and benzoic acid(**29**) (6.3%)[59].



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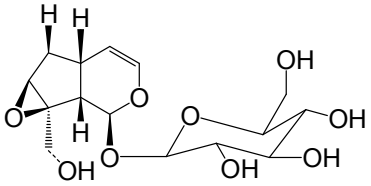

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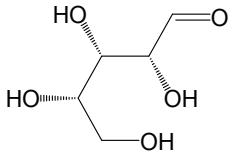

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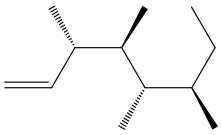

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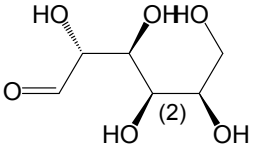

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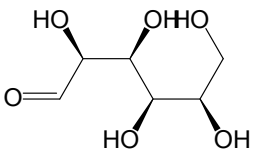

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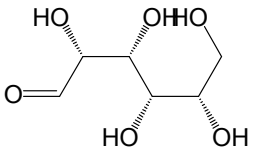

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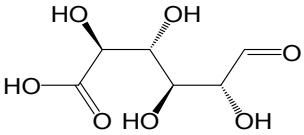

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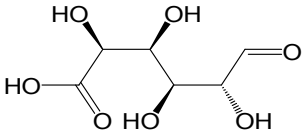

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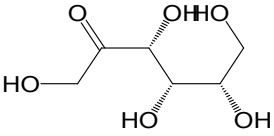

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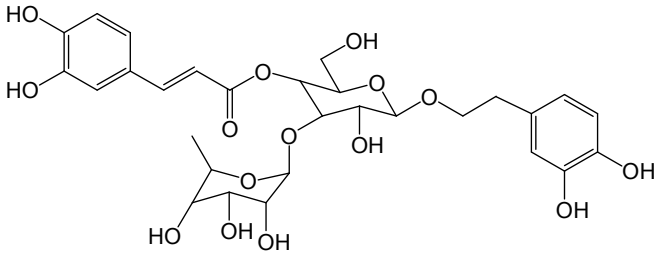

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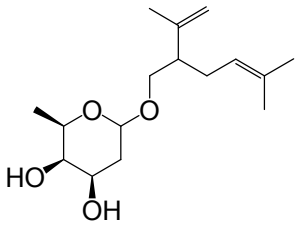

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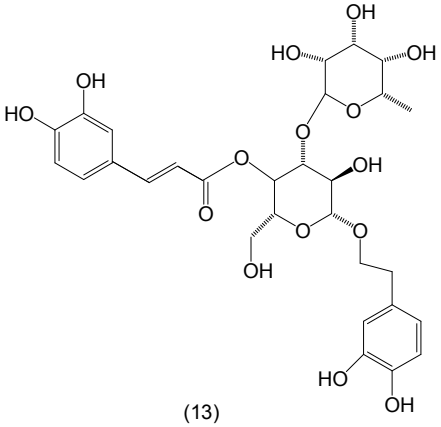

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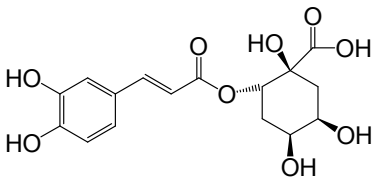

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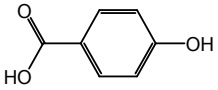

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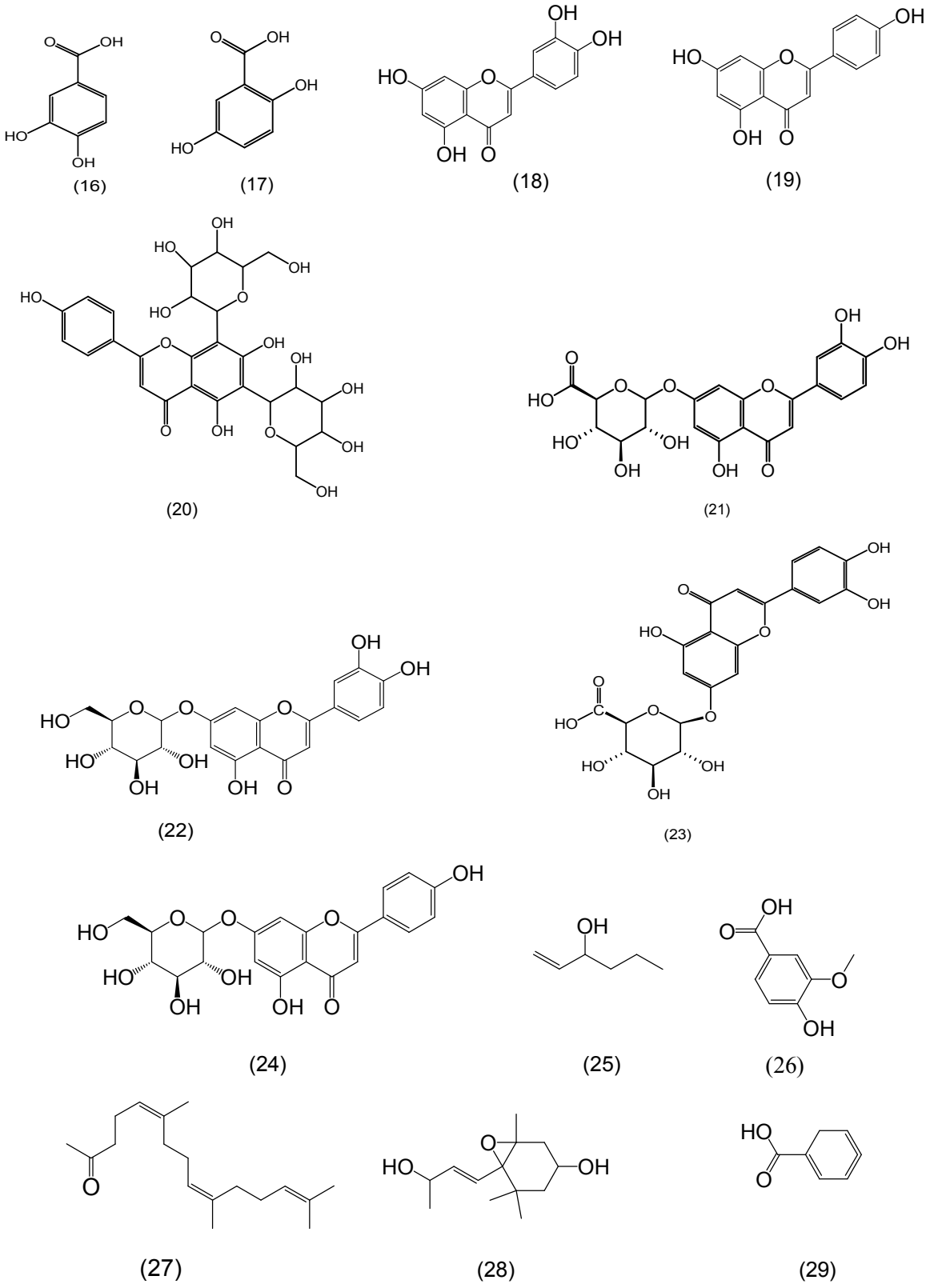


Figure 2: Previously reported compounds from the *Plantago lanceolata*

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Plant Material Collection and Preparation

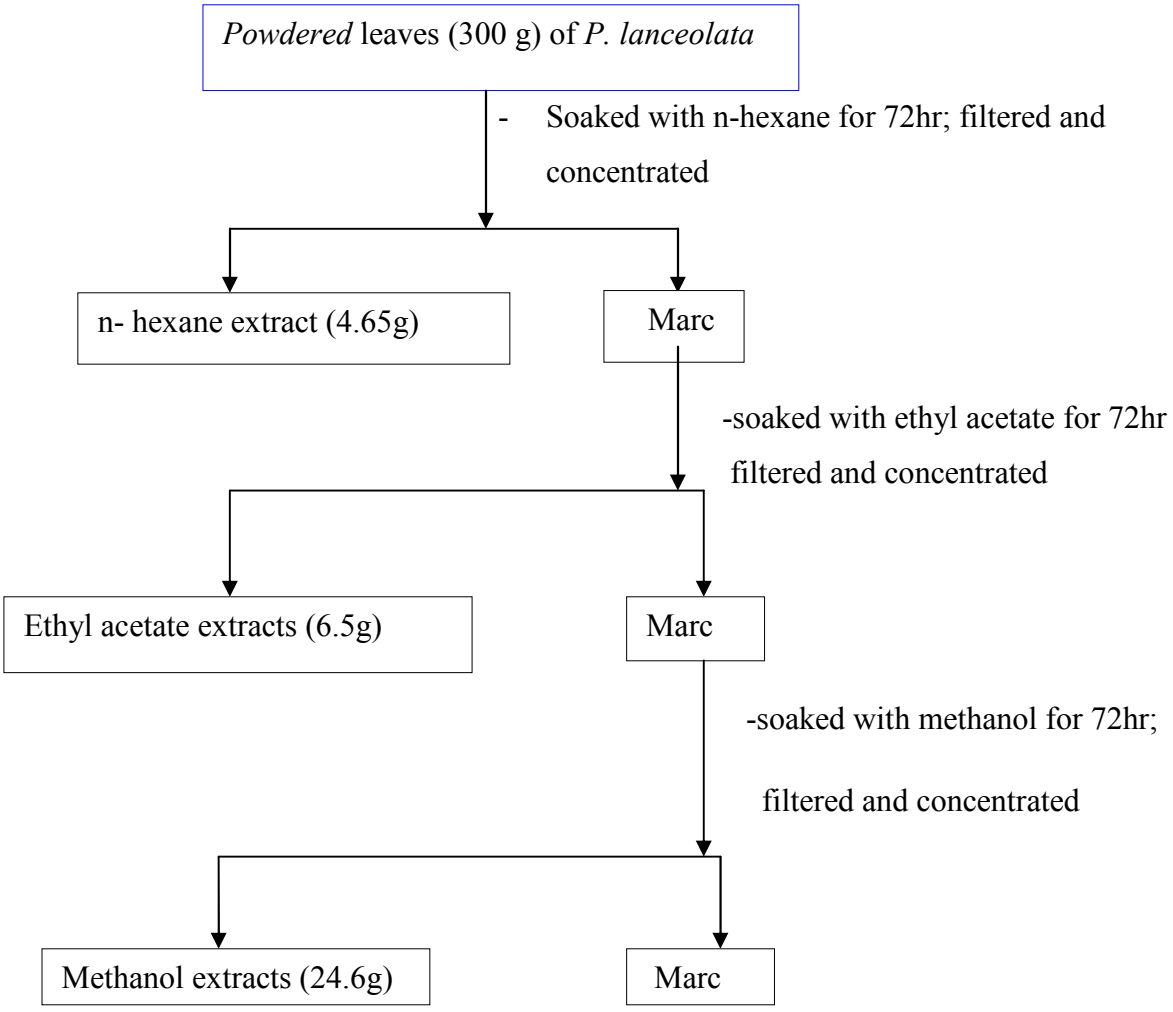
Fresh leaves of *P. lanceolata* were collected in November, 2017 from Mugher town, Adea Berga Woreda, west shoa zone, Oromia, Ethiopia. The plant was authenticated by a botanist Shambel Alemu at the Biology Department of Addis Ababa University and specimen stored (Voucher no: F 001/2016) in the National Herbarium of Addis Ababa University, Addis Ababa, Ethiopia. The leaves were washed with water without squeezing to remove trash and dust particles and then air-dried at room temperature (26°C) for one week. The air-dried leaves were chopped into small pieces and finally grounded using a mortar and weighed.

3.2. Instruments, Apparatus and Chemicals

The FT-IR samples were prepared using spectral grade KBr and made into pellets and measured on Spectrum 65-FT-IR (Perkin Elmer) in the range between 4000 cm^{-1} and 400 cm^{-1} in Addis Ababa University. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectra were recorded on a bruker avance 400 MHz NMR spectrometer in the Addis Ababa University using Acetone-d_6 as solvent and TMS as the internal standard. Rotary evaporator was used to remove solvents or used to concentrate the filtrates. The absorbance was recorded by using $T80^{+}$ UV/VIS spectrometer. TLC analyses were carried out on TLC plate 0.2 mm thick layer coated on aluminum sheet and pre-coated Silica gel plates (Merck, Kiesel gel 60 F254, 0.2mm). Compounds on TLC were detected using UV lamp (254 and 365 nm) and iodine; while CC was performed using silica gel (250-400 mesh, Merck) in ASTU Organic Chemistry laboratory. TLC chamber, rotary evaporator, condenser, grinder, beakers, reagent bottles, TLC plate, capillary tubes, round bottom flasks, digital electronic balance, measuring cylinder, ruler, pencil, glass rod, spatula, Aluminum foil, Whitman no1 filter paper, separator funnel, tong, test tube, funnel, and conical flasks were used in the present study. The solvents used in extraction and CC including: n-hexane, ethyl acetate and methanol were pure HPLC grades. Distilled water, dilute ammonia, $FeCl_3$, HCl, Fehling's solutions, were used in the study.

3.3. Extraction

Air dried leaves powder (300 g) of *P. lanceolata* was first soaked in n-hexane (1.5 L) for 72 hr at room temperature. The mixtures were filtered and concentrated under reduced pressure at a temperature of 40^{o}C using rotary evaporator to afford 4.65 g bright green crude extract. The marc was then extracted with ethyl acetate (1.5 L), after soaking for 72 hr at room temperature. Then filtered and concentrated in rotary evaporator to furnish 6.5 g deep green crude extract. Finally the marc remaining was extracted using methanol following similar procedure and afforded 24.6 g of dark green crude extract. The outline of the extraction procedure was shown in Scheme 1.



Scheme 1: Successive extractions of leaves of *P. lanceolata*

3.4. Isolation and Purification of Compounds

The methanol extract (13.6 g) was adsorbed on silica gel and fractionated over silica gel (150 g) column chromatography using n-hexane for packing. It was eluted with n-hexane, ethyl acetate and methanol of increasing polarities to furnish 27 fractions with the whole procedure shown in Table 1.

Table 1: Isolation of compounds from the methanol extract of the leaves of *P. lanceolata*

Solvents	Ratios of solvent	Volume (ml)	Code of fraction	Solvents	Ratios of solvent	Volume (ml)	Code of fraction
n-hexane:ethyl acetate	10:0	100	F_1	Ethyl acetate: methanol	10:0	100	F_{11}
"	9:1	100	F_2	"	9:1	100	F_{12}
"	4:1	100	F_3	"	4:1	100	F_{13}
"	7:3	100	F_4	"	7:3	100	F_{14}
"	3:2	100	F_5	"	3:2	100	F_{15}
"	1:1	100	F_6	"	1:1	100	F_{16}
"	2:3	100	F_7	"	2:3	100	F_{17}
"	3:7	100	F_8	"	3:7	100	F_{18}
"	1:4	100	F_9	"	1:4	100	F_{19}
"	1:9	100	F_{10}	"	1:9	100	F_{20}
"	0:10	100	F_{11}	"	0:10	7x100	$F_{21\text{-}27}$

Fraction 17 (0.5 g) was shown to have two spots on TLC which was rechromatographed over silica gel using ethyl acetate and methanol as eluent by increasing polarity to furnish twelve fractions. Fraction 10 (106 mg) was found to have one spot on TLC and coded as FD-2-1A. See Table 2 for the whole fractionation procedure.

Table 2: Solvent system used for purification of fraction F17

Fractions	Solvents	Ratios of solvent	Volume (ml)	Code of fraction
1	ethyl acetate: methanol	10:-0	10	Fs_1
2	"	9:1	10	Fs_2
3	"	8:2	10	Fs_3
4	"	7:3	20	Fs_4, Fs_5
5	"	6:4	10	Fs_6
6	"	5:5	10	Fs_7
7	:	5:5	10	Fs_8
8	:	4:6	10	Fs_9
9	"	3:7	10	Fs_{10}
10	"	2:8	10	Fs_{11}
11	"	1:9	10	Fs_{12}
12	"	0:10	10	Fs_{13}

Fractions 22 and 23 (2.35 g) were combined and fractionated using silica gel (40 g) column chromatography using methanol as eluent to give five fractions. Fraction 3 (66 mg, FD-2-4A) was single spot in iodine and hence analyzed with NMR.

3.5. Phytochemical Screening of Leaves Extracts of *P. lanceolata*

The preliminary phytochemical screening for the leaves of *P. lanceolata* was carried out to analyze the presence of compounds namely: saponins, flavonoids, phenols, tannins, terpenoids, and glycosides.

Test for saponins:

To 0.5 g of each crude extract (n-hexane, ethyl acetate and methanol) 5mL of distilled water was added and shaken and then heated to boil. Frothing did not observed [60].

Test for flavonoids:

To 0.5 g portion of (n-hexane, ethyl acetate 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 1mL of dilute ammonia solution and a yellow coloration was observed [61].

Test for phenols:

0.5 g of each of crude extracts (n-hexane, ethyl acetate and methanol) were put in a different test tube and treated with a few drops of 2% of $FeCl_3$; bluish green or black coloration were observed [62].

Test for tannins:

0.5 g of each crude extract was boiled in 10 mL 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 [63].

Test for terpenoids:

0.5 g of each (n-hexane, ethyl acetate and methanol) crude powder was separately dissolved in 5 mL of methanol. 2 mL of the extract was treated with 1 mL of 2, 4-dinitrophenyl hydrazine dissolved in 100 mL of 2M HCl. No any color change was observed [64].

Test for glycosides:

0.5 g of (n-hexane, ethyl acetate and methanol) crude extract was dissolved separately in 5 mL of methanol. 10 mL of 50% HCl was added to 2 mL of each extract in test tubes. The mixtures were heated in a boiling water bath for 30 min. 5 mL of Fehling's solution was added and the mixtures were boiled for 5 min and brick red precipitate were showed [65].

3.6 Antibacterial Activity

The EtOAc and methanol extracts of the leaves and the isolated compounds from the methanol crude were evaluated *in vitro* for antibacterial activity by using the disc diffusion method against one gram positive bacterium *Staphylococcus aureus* (*S. aureus*) and three gram negative bacterium *Escherichia coli* (*E. coli), Klebsiella pneumania (K.pneumonia)* and *Proteus miabilis*

(P. miabilis) that was obtained from Oromia public health research capacity building and quality assurance. The bacterial cultures were inoculated into the Muller Hinton Agar (MHA).

Ciprofloxacin was used as positive control. Approximately, 20 mL of sterile MHA were poured into sterile culture plates and allowed to set wells of about 6 mm in diameter which were punched on the plates. Standard solutions of 1.5 mg/mL concentration of the extracts and isolated compounds were prepared and 10 µL solutions from the concentration were loaded to the discs in different replications. The plates were incubated at 37^{o}C. The antibacterial activity of the plant extracts were evaluated by measuring the zone of inhibition against the test organism after 24 hrs [66].

3.7. Antioxidant Activities

DPPH assay: The free radical scavenging activity of the EtOAc, MeOH extract and isolated compounds were measured by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH.) method [67]. With this method it is possible to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm. As a result of the color changing from purple to yellow the absorbance is decreased when the DPPH radical is scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule [68]. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity [69].

The EtOAc extract was dissolved in four vials containing methanol to give 500, 250, 125 and 62µg/mL. To each 1 mL of the above EtOAc extracts was added each 4 mL of 0.04%DPPH which gave 100, 50, 25 and 12 µg/mL. The resulting solution was placed in an oven at 37^{o}C for 30 minutes and subjected to UV-Vis spectrophotometer to record absorbance at 517 nm. This was repeated for the methanol extracts and isolated compounds. The absorbance of 0.04% DPPH in MeOH solution was found to be 1.06. The percentage DPPH inhibition was calculated according to the following formula [70]

$$\% \text{ of radical scavenging activity} = \frac{\mathrm{Ab}_{\mathrm{standard}} - \mathrm{Ab}_{\mathrm{analayte}}}{\mathrm{Ab}_{\ \mathrm{standard}}} \times 100$$

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1. Determination of Extraction Yield

The extraction yield is a measure of the solvent efficiency to extract specific components from the original material. The percentage yield of crude extract in respective solvent was recorded in Table 3. It could be calculated according to the method as follows.

$$\%yield = \frac{weight\ of\ crude}{weight\ of\ sample}\ x\ 100$$

Table 3: Yield of the crude extracts

Crude extract	Weight of extract in gram	Yield in (%)
n-hexane	4.5	1.55
Ethyl acetate	6.5	2.16
Methanol	24.6	8.2

The methanol yields obtained from the leaves of *Plantago lanceolata* were significantly higher than the hexane and EtOAc extract. This clearly showed as the plant is rich in polar organic compounds.

4.2. Phytochemical screening

The preliminary phytochemical screenings on leaves extract of *P. lanceolata* (n-hexane, ethyl acetate and methanol) were tested in this study. Tannin, flavonoids, glycosides and phenolic compounds were present in the leaves extract of *P. lanceolata* (Table 4). The presence of these secondary metabolites in the leaves is one good attributes of the plant. These metabolites are likely responsible for the medicinal values [71, 72].

Table 4: Phytochemical screening of different crude extracts of leaves of *P. lanceolata*

No	Constituents	n-hexane extract	Ethyl acetate extract	Methanol extract
1	Saponins	-	-	-
2	Tannins	-	+	+
3	Flavonoids	+	+	+
4	Phenols	-	+	+
5	Terpenoids	-	-	-
6	Glycosides	-	+	+

+ = the presence of phytochemical constituents

- = the absence of phytochemical constituents

These secondary metabolites are known to be biologically active and play significant roles in bioactivity of medicinal plants, because the medicinal values of medicinal plant lies in these phytochemical compounds which produce a definite and specific action on the human body. The presence of phenols could confer antibiotic property on the plant as reported by various scholars [73]. Therefore, the phytochemical screening result reveals that the presence of these phytochemical constituents supports the use of *P. lanceolata* plant in folklore medications and it is probable that these phytochemicals are responsible for the healing properties of this plant.

4.3. Structural Elucidation of Isolated Compounds

The methanol extract of the leaves of *P. lanceolata* was subjected to repeated silica gel column chromatography which has led to the isolation of two compounds: FD-2-1A yield (106 mg) and FD-2-4A (66 mg). Herein is the description of characterization of these compounds.

4.3.1. Characterization of FD-2-1A

FD-2-1A was obtained as gel like reddish brown solid from the CC of methanol extract of the leaves of *P. lanceolata*. The compound exhibited Rf value 0.48 in its TLC with ethyl acetate: methanol (8: 2) as a mobile phase (Figure 3).

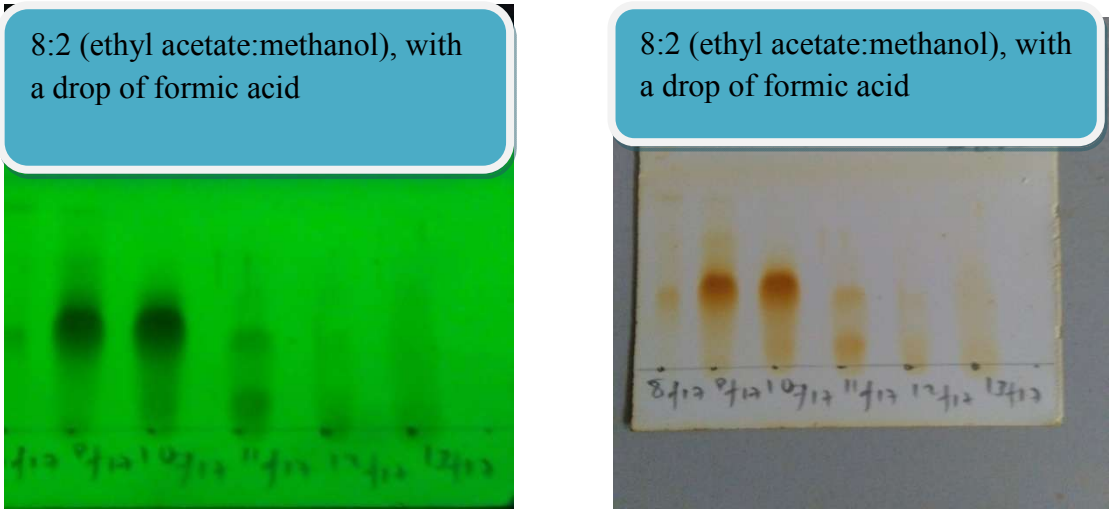


Figure 3: a) TLC of compound FD-2-1A in UV lamp b) TLC of compound FD-2-1A iodine

The FT-IR spectrum of **FD-2-1A** (Appendix 1) displayed absorption bands at δ3429 cm^{-1} which is diagnostics of the presence of hydroxyl stretching. The presence of α,β-unsaturated carbonyl carbon is evident at δ1692 cm^{-1}. The signal characteristic of carbon carbon double bond was observed at δ1604 cm^{-1}. The signal due to methyl stretching was observed at δ2923 cm^{-1}

The ^{1}H NMR spectrum of **FD-2-1A** (Appendix 2) exhibited six aromatic protons (δ H 7.20-6.58 region), two trans-olefinic protons (δ H 6.32 and 7.61, J = 16 and 15.99 Hz respectively), a benzylic methylene at δ H 2.77 (2H, *m*). These data were consistent with the presence of a caffeic acid unit and 3, 4- dihydroxyphenethyl alcohol moiety. In addition, two anomeric proton signals at δ H 4.46 (*d*, J = 7.9 Hz) and 5.31 (*d*, J= 1.6 Hz) were attributed to the β-glucose and α-rhamnose units, respectively, indicating the disaccharide structure of FD-2-1A. The acyl group was positioned at the C-4" position of the glucose unit, on the basis of the deshielding of the H-4" signal (δ H 4.95 *t*, J= 9.4 Hz) of the glucose unit. The methyl group of acetyl attached to the

rhamnose was observed at δ1.98 where as methyl of the ethyl moieties attached to the glucose were observed at δ1.20.

Table 5: ^{1}H NMR data of compound FD-2-1A

Carbon number	^{1}H-NMR δ data	Carbon number	^{1}H-NMR δ data
2	6.78 (1H, *d*, *J*= 1.99)	6”	3.62(2H, *m*)
5	6.74 (1H, *d*, *J*= 7.99)	$\underline{C}H_2CH_3$	3.64(2H, *m*)
6	6.57 (1H, *dd*, J= 10.00)	$CH_2\underline{C}H_3$	1.20(3H, *t*)
7	2.76 (2H, *m*)	1”’	5.31(1H, *d*, *J*= 1.6)
2’	7.21 (1H, *d*, *J*= 1.99)	4”’	4.82 (1H, *br*)
5’	6.89 (1H, *d*, 7.99)	6”’	1.11(3H, d, *J*= 5.99)
6’	7.05 (1H, *dd*, *J*= 9.99)	$\underline{C}H_3CO$	1.97(3H, *s*)
7’	7.62 (1H, *d*, *J*= 16)		
8’	6.33 (1H, *dd*, *J*= 15.99)		
1”	4.46(1H, *d*, *J*= 8.00)		
4”	4.94(1H, *t*, *J*=9.6,9.1)		

The proton decoupled ^{13}C-NMR spectrum (Appendix 3) with the aid of DEPT-135 (Appendix 4) revealed the presence of thirty-three carbon resonances of which eight are quaternary, eighteen methines, four methylenes and three methyl groups. The spectrum showed signal at δ170.3 due to acetyl carbonyl carbon and δ166.4 due to *α,β*-carbonyl carbon. This is supported by the IR spectral data. The presences of four oxygenated aromatic carbons were evident at δ143.4, δ144.8, δ145.5, δ148.3. The signal observed at δ146.3 is ascribed to the *β*-carbon of the *α,β*-unsaturated CO. The spectrum also showed the presence of two sugar moieties at δ 61.4, 68.6, 69.4, 70.7, 71.1, 72.6, 74.8, 75.2, 79.4, 100.9 and 102.8. Among these, the signal characteristics of anomeric carbons were appeared at δ100.9 (C-1”’) and 102.8 (C-1”). The methyl group of acetyl attached to the rhamnose was observed at δ20.0, methylene and methyl of the ethyl moieties attached to the glucose were observed at δ59.8 and δ13.6 respectively. Another diagnostics signal due to

methyl group was observed at δ17.6 suggesting one of the sugar moieties as rhamnose. The NMR spectral data of FD-2-1A was compared and the whole result was presented in Table 6.

Table 6: ^{13}C NMR data of compound FD-2-1A

Carbon number	^{13}C NMR δ data	Type of Carbon	Carbon number	^{13}C NMR δ data	Type of carbon	Carbon number	^{13}C NMR δ data	Type of Carbon
1	130.3	C	4’	145.5	C	6”	61.4	CH_2
2	116.0	CH	5’	115.6	CH	$\underline{C}H_2CH_3$	59.76	CH_2
3	143.4	C	6’	122.1	CH	$CH_2\underline{C}H_3$	13.6	CH_3
4	144.8	C	7’	146.3	CH	1”’	100.9	CH
5	115.1	CH	8’	113.9	CH	2”’	71.3	CH
6	120.2	CH	C=O	166.6	C	3”’	69.4	CH
7	35.3	CH_2	1”	102.8	CH	4”’	74.7	CH
8	71.1	CH_2	2”	75.2	CH	5”’	68.6	CH
1’	126.6	C	3”	79.4	CH	6”’	17.6	CH_3
2’	114.4	CH	4”	70.7	CH	$\underline{C}H_3CO$	20.0	CH_3
3’	148.3	C	5”	72.7	CH	$CH_3\underline{C}O$	170.3	C

The ^{13}C-NMR data were proving the presence of the dihydroxy-*ß*-phenethyl moiety. The attachment of the caffeoyl and the glucose moieties at the C-4” and C-3” carbons respectively is supported by the ^{13}C-NMR. Only signals corresponding to two monosaccharides were found bearing 4”’- acetyl and 6”-O- ethyl group. Based on the evidence, FD-2-1A is identified as 6”-O-ethyl-4”’-acetyl verbascoside whose structure was shown in Figure 4.

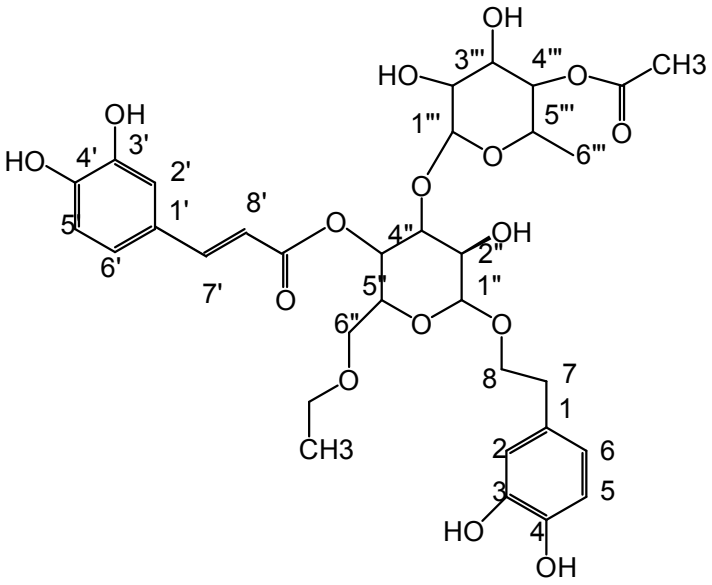


Figure 4: Chemical structure of 6”-O-ethyl-4”’-acetyl verbascoside

FD-2-4A is gel like reddish color obtained from the methanol extract of the leaves of *P. lanceolata*. The TLC result showed spot at Rf value 0.76 using ethyl acetate:methanol (7:3) as a mobile phase (Figure 5). It was visualized after dipping in iodine and under UV lamp.

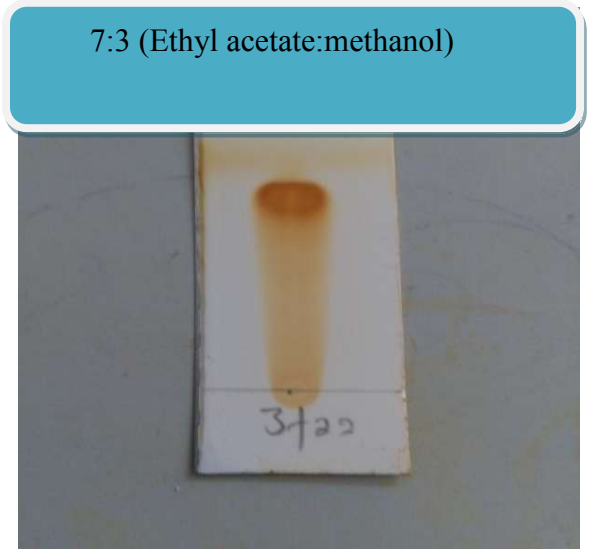


Figure 5: TLC profile of FD-2-4A

The FT-IR spectrum of **FD-2-4A** (Appendix 5) displayed absorption bands at δ3398 cm^{-1} which is diagnostics of the presence of hydroxyl stretching. The presence of α,β-unsaturated carbonyl

carbon is evident at δ1697 cm^{-1}. The signal characteristic of carbon carbon double bond was observed at δ1610 cm^{-1}. The signal due to methyl stretching was observed at δ2926 cm^{-1}

The ^{1}H NMR spectrum of **FD-2-4A** (Appendix 6) exhibited six aromatic protons (δ H 7.20-6.58 region), two trans- olefinic protons (J = 15.99 Hz), a benzylic methylene at δ H 2.77 (2H, *t, J* = 7.39 Hz) and two non-equivalent proton signals at δ H 3.69 m and 4.02 (each 1H, *m*). These data were consistent with the presence of a caffeic acid unit and 3, 4- dihydroxyphenethyl alcohol moiety. In addition, two anomeric proton signals at δH 4.37 (*d*, J = 7.9 Hz) and 5.30 (*d*, J= 1.8 Hz) were attributed to the β-glucose and α-rhamnose units, respectively, indicating the disaccharide structure of FD-2-4A. The acyl group was positioned at the C-4" position of the glucose unit, on the basis of the deshielding of the H-4" signal (δ H 4.92 *t*, J= 9.4 Hz) of the glucose unit.

Table 7: Comparison of ^{1}H-NMR spectral data of compound FD-2-4A with literature report [74]

Position of hydrogen	Chemical shift (δH) FD-2-4A	Literature value	Remark
Aglycone			
2	6.78(1H, *d*, 1.99)	6.70(1H, *d*,1.8)	CH
5	6.74(1H, *d*, 8.0)	6.68(1H, *d*, 8.2)	CH
6	6.58(1H, *dd*, 10)	6.56(1H, *dd,* 8.2,1.8)	CH
7	2.76(2H, *t*, *6.8, 6.4*)	2.79(2H, *m*)	CH_2
8	3.68(1H, *m*), 4.02(1H, *m*)	3.72(1H, *m*), 4.04(1H,*m*)	CH_2
Caffeoyl			
2''	7.20(1H, *d*, 2.0)	7.06(1H, *d*, 1.9)	CH
5''	6.88(1H, *d,* 8.39)	6.78(1H, *d*, 8.2)	CH
6''	7.05(1H, *dd*, 9.99)	6.95(1H, *dd*, 8.2, 1.9)	CH
7''	7.60(1H, *d*, 15.99)	7.59(1H, *d*, 15.8)	CH
8''	6.32(1H, *d,* 16)	6.28(1H, *d*, 15.8)	CH
Glucose			
1'	4.45(1H, *d,* 7.9)	4.38(1H, *d*, 7.8)	CH
4'	4.92(1H, *d*)	4.93(1H, *m*)	CH
6'	3.47(1H, *m*), 3.440(1H,*m*)	3.52(1H,*m*),3.62(1H,*m*)	CH_2
Rhamnose			
1'''	5.30(1H, *d*, 1.,6)	5.19(1H, *d*, 1.4)	CH
2'''	3.94(1H, *br*)	3.92(1H,*m*)	CH
6'''	1.11(3H, *d*, 6.0)	1.09(3H, *d*, 6.0)	CH_3

The proton decoupled ^{13}C-NMR spectrum (Appendix 7) with the aid of DEPT-135 (Appendix 8) revealed the presence of twenty nine carbon resonances of which seven are quaternary, eighteen methines, three methylenes and one methyl groups. The spectrum showed signal at δ166.4 due to $\alpha\beta$ carbonyl carbon. This is supported by the IR spectral data. The presences of four oxygenated

aromatic carbons were evident at δ143.4, 144.9, 145.5, and 148.2. The signal observed at δ146.1 is ascribed to the β-carbon of the $\alpha\beta$-unsaturated CO. The spectrum also showed the presence of two sugar moieties at δ 61.4, 68.7, 69.4, 70.7, 71.1, 72.7, 74.9, 75.3, 79.1, 101.1 and 102.8. Among these, the signal characteristics of anomeric carbons were appeared at δ102.8 (C-1') and 101.1 (C-1'''). Another diagnostics signal representing rhamnose moiety was observed at δ17.6. The NMR spectral data of FD-2-4A was compared and the whole result was presented in Table 8.

Table 8: Comparison of ^{13}C NMR and DEPT-135 data of compound FD-2-4A with literature reports [74]

Carbon number	^{13}C NMR δ data	Reference data	Type of Carbon	Carbon number	^{13}C NMR δ data	Reference data	Type of carbon
1	130.3	131.5	C	8''	114.1	114.5	CH
2	116.1	117.1	CH	C=O	166.4	168.1	C
3	143.4	144.6	C	1'	102.8	102.1	CH
4	144.9	146.1	C	2'	75.3	76.5	CH
5	115.2	116.3	CH	3'	79.1	79.5	CH
6	120.2	121.3	CH	4'	70.7	70.3	CH
7	35.3	36.5	CH_2	5'	74.9	75.8	CH
8	71.3	72.2	CH_2	6'	61.4	62.7	CH_2
1''	126.6	127.5	C	1'''	101.1	101.6	CH
2''	114.4	115.2	CH	2'''	71.1	72.2	CH
3''	148.2	149.9	C	3'''	69.4	70.0	CH
4''	145.5	146.9	C	4'''	72.7	75.4	CH
5''	115.5	116.6	CH	5'''	68.6	67.8	CH
6''	121.9	123.3	CH	6'''	17.7	18.1	CH_3
7''	146.1	148.0	CH				

The ^{13}C-NMR data were comparable with those described by [90] thus proving the presence of the dihydroxy-β-phenethyl moiety. The attachment of the caffeoyl and the outer glucose moieties at the C-4' and C-3' carbons respectively is supported by the ^{13}C-NMR.

Based on the evidence by comparison with literature 3, 4-dihydroxy-ß-phenethyl- O - β -D – glucopyranosyl (1'→3')-4'-O-caffeoyl-β-D–glucopyranoside (varbascoside) whose structure was shown below (Figure 5)

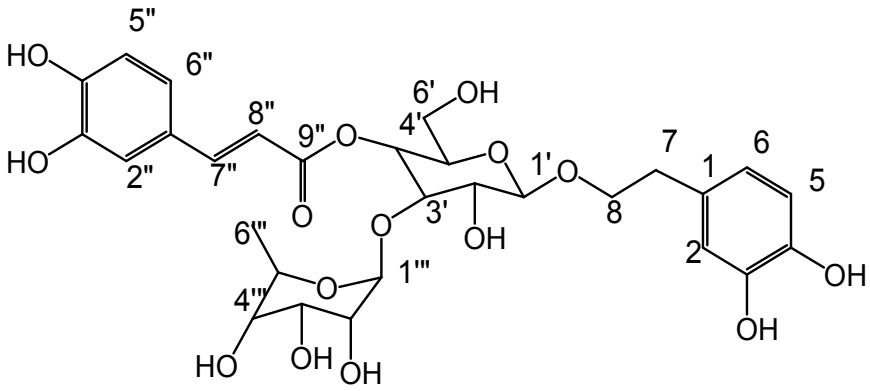


Figure 6: Chemical structure of verbascoside

Verbascoside belong to the group of naturally occurring polyphenols that are known as phenylethanoid glycosides, supporting each one a moiety of glucose esterified by caffeic acid and bonded to an ethylcatechol unity by an ether linkage. The polyphenols often showed virucidal effects in several viral systems by attaching to the proteins and/or host cell surfaces, resulting in reduction or prevention of viral adsorption and RNA polymerase of influenza virus [75].

4.4. Antibacterial Activities of the leaves *P. lanceolata*

Infectious diseases are the leading cause of death worldwide, accounting for nearly one half of all deaths in tropical countries which are also becoming a significant problem in all countries. Microbial infections are great challenge to human health concern and it is even exacerbated by the growing resistance to the conventional drugs [74]. Thus, researchers have resort to find remedy from plants for infectious diseases. Development of new antibacterial agents was among the proposed solution to solve this problem. In this regard plants can be provided a good alternative in search for new chemicals with a wide range of antibacterial and antifungal activities [76]. *P. lanceolata* is traditionally known plant for healing wounds and blood clotting.

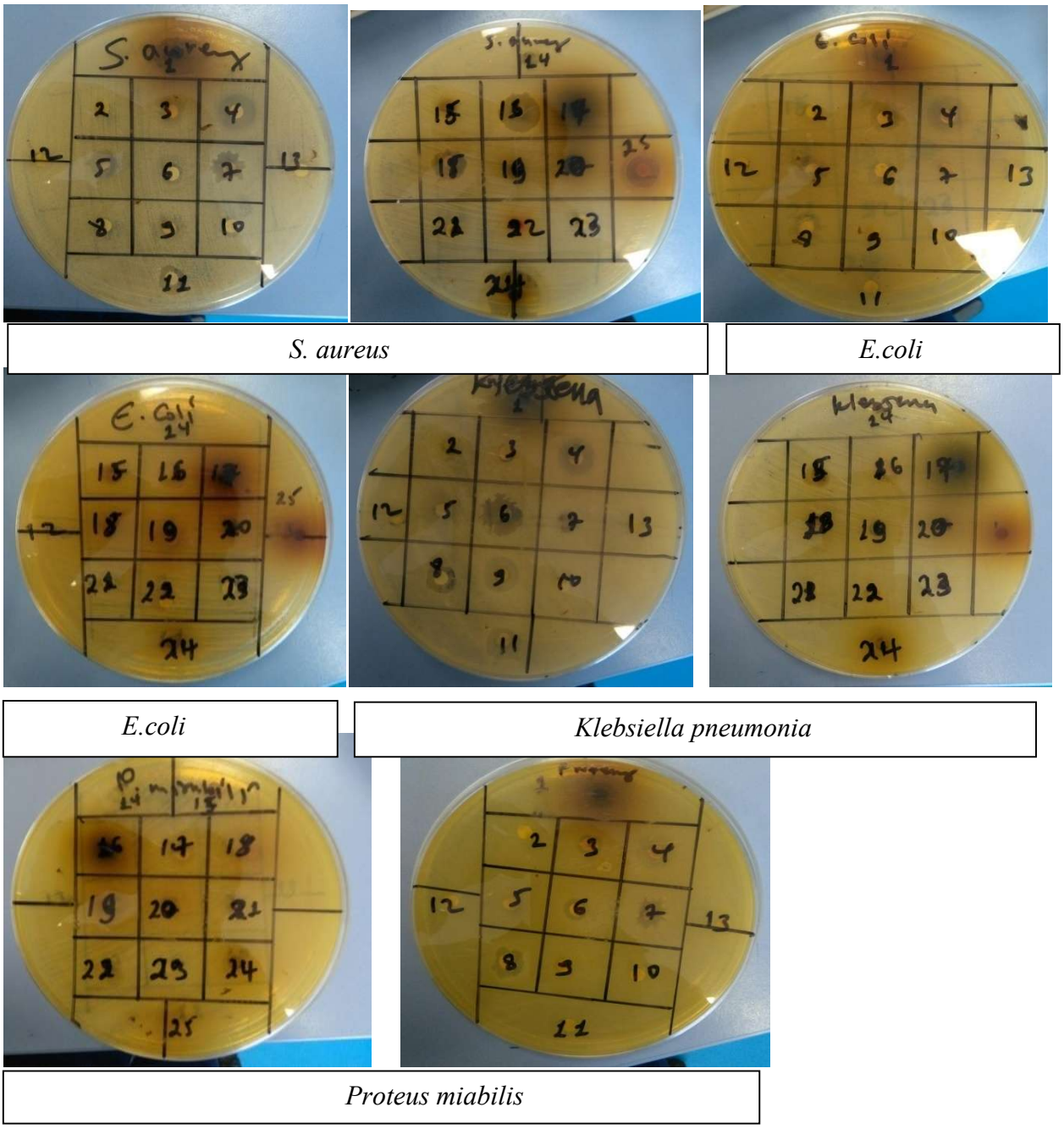


Figure 7: Zone of bacterial growth inhibition (mm).

Key: 1=code FD-2-1A sample 19= code FD-2-4A sample

2=Ethyl acetate crude extract 20= methanol crude extract

Table 9: Zone of bacterial growth inhibition diameter (mm)

Sample	Types of bacteria with mean inhibition diameter (mm)			
	S. aureus	*E. coli*	*K. pneumonia*	*Proteus miabilis*
Ethyl acetate crude extract	-	8	8	-
Methanol crude extract	7	-	-	8
FD-2-1A	20	10	-	12
FD-2-4A	-	13	7	9
Chloroform	-	-	-	-
Ciprofloxacin	23	21	19	24

Key: (-) = resistance and >6mm = sensitive

The antibacterial tests showed that some of the test samples clearly indicate a considerable antibacterial activity against the bacterial species used in the study. In general, FD-2-1A was found to be the most active sample against the tested bacteria except for *K. pneumonia and E. coli*. Close inspection of Table 9 showed that the methanol extract was found to inhibit *S. aureus* and *P. miabilis* compared with EtOAC extrat. On the other hand the EtOAc extract displayed better activity than the MeOH extrat against two strains of bacteria viz *E. coli* and *K. pneumonia*. The isolated compounds were significantly active against *Proteus miabilis* and had inhibition zones of above 6 mm. FD-2-1A in particular had the highest inhibition diameter of 20 mm against *S. aureus* and 12 mm on *Proteus miabilis.* The antibacterial activity displayed by FD-2-1A against *S. aureus* is significant compared with the standard drug. This indicates that FD-2-1A is shown to have significant antibacterial activity against gram positive bacteria.

4.5. Antioxidant activities of the leaves of *P. lanceolata*

The ethyl acetate, methanol extracts and the isolated compounds of *P. lanceolata* were assessed for its radical scavenging activities. The DPPH radical scavenging activity was found to be 64.2%, 79.2, 87.7 and 83.9 at 100µg/mL.

Table 10: % scavenging activity of the extracts and isolated compounds of *P. lanceolata*

concentration	Samples							
	EtOAc extract		MeOH extract		FD-2-1A		FD-2-4A	
	Absorbance	% scavenging activity	absorbance	% scavenging activity	Absorbance	% scavenging activity	absorbance	% scavenging activity
100	0.38	64.2	0.2	79.2	0.1	87.7	0.2	83.9
50	0.45	57.5	0.3	76.4	0.2	83.0	0.3	76.4
25	O.56	47.2	0.3	71.7	0.3	74.5	0.3	67.9
12	0.62	41.5	0.5	57.5	0.4	66.0	0.4	60.4

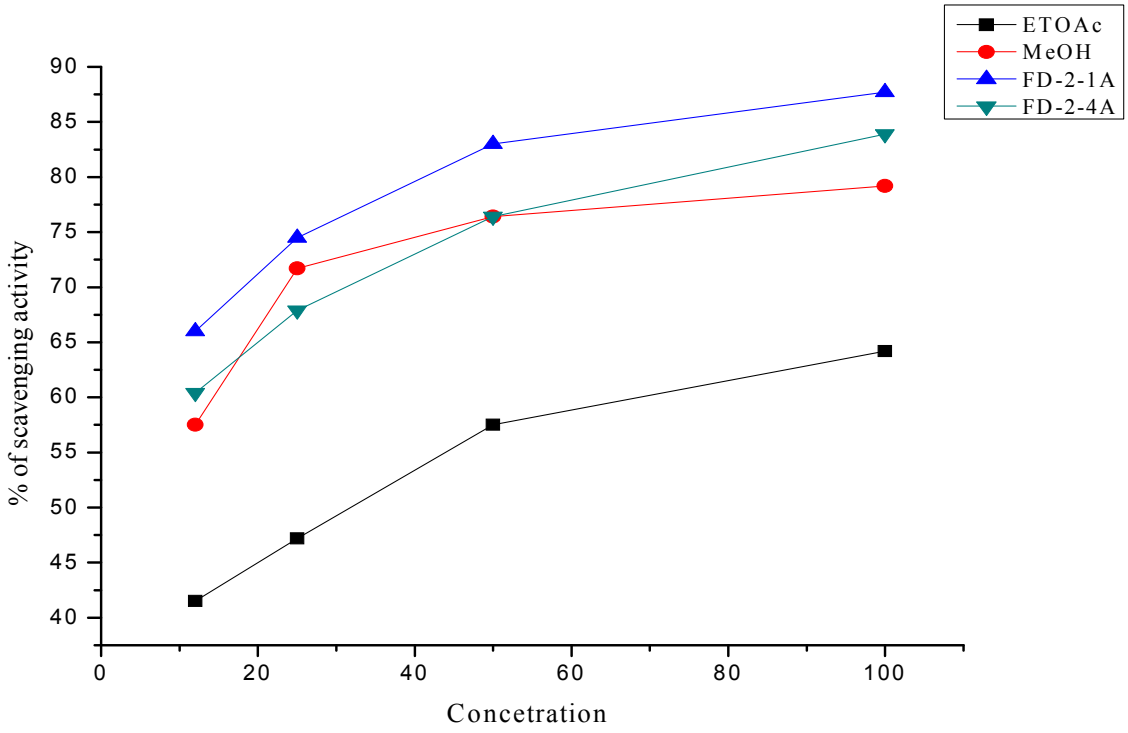


Figure 8: Scavenging activity of the extract and isolated compounds of *P. lanceolata*

The result obtained was found comparable with ascorbic acid which is used as positive control with percent inhibition of radical by 97% at 100µg/mL. The %inhibition of verbascoside was comparable with ascorbic acid. This is likely due to the presence of phenolic hydroxyl groups which after giving hydrogen and an electron furnished a stable phenoxide radical. Therefore the presence of 6”-O-ethyl-4”’-acetyl verbascoside and verbascoside accounts for the radical scavenging activities of the leaves of *P. lanceolata*. Hence the leaves of *P. lanceolata* can be used as a natural antioxidant.

CHAPTER FIVE

5. CONCLUSIONS AND RECOMMENDATIONS

For decades, traditional medicine has been used and continues to be an alternative approach on treatment for various diseases. Currently, the growing interest of consumers in substances of natural origin in association with the increasing concern surrounding potentially harmful infections and disease has directed to a rising interest in the use of plant extracts as functional ingredients in many pharmaceutical products. From the study, the leaves extract was found to contain tannin, flavonoids, glycosides and phenolic compounds. The presence of these important phytochemicals in the plant may be used as a scientific justification of the use of the plant in the traditional treatment against various diseases affecting humans and animals. After silica gel column chromatography, the methanol extract furnished two compounds identified using NMR (^{1}H-NMR, ^{13}C-NMR, DEPT-135) and FT-IR.as 6”-O-ethyl-4”’-acetyl verbascoside and verbascoside. The crude extracts from leaves of *P. lanceolata* showed moderate antibacterial activity. As demonstrated by *in vitro* antibacterial study, there is considerable evidence that 6”-O-ethyl-4”’-acetyl verbascoside had the potential to be developed into agents that can be used as treatment for gram positive bacteria. This supports the ethnomedicinal use of this plant against bacteria.

It is recommended that clinical trials should be carried out to explore the potential of this plant extracts in the treatment of infectious diseases. This can help to reduce the overall burden of diseases using cheaper methods especially among rural poor people worldwide. Further studies should be carried on structure activity relationships and mode of action may be a guide to a better understanding of the relationships between the structure of 6”-O-ethyl-4”’-acetyl verbascoside and its antimicrobial activity. In addition to this the following recommendations were made.

- ✓ It is recommended that further similar studies should be conducted on other parts of the plant such as root, stem, seed, etc.
- ✓ This plant should be studied more extensively to explore its activity on other organisms like other bacteria species, fungi, protozoa and helminthes among others.
- ✓ Toxicity studies of the plant should also be done to determine the safety indices of the extract

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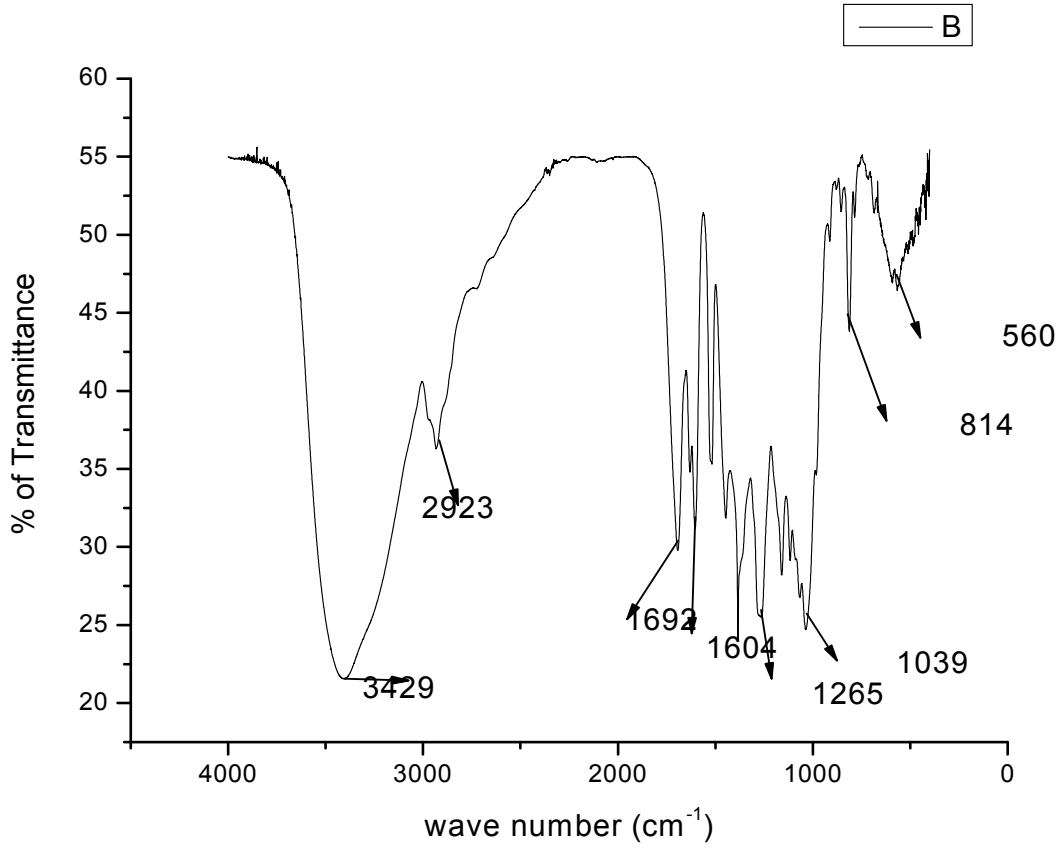
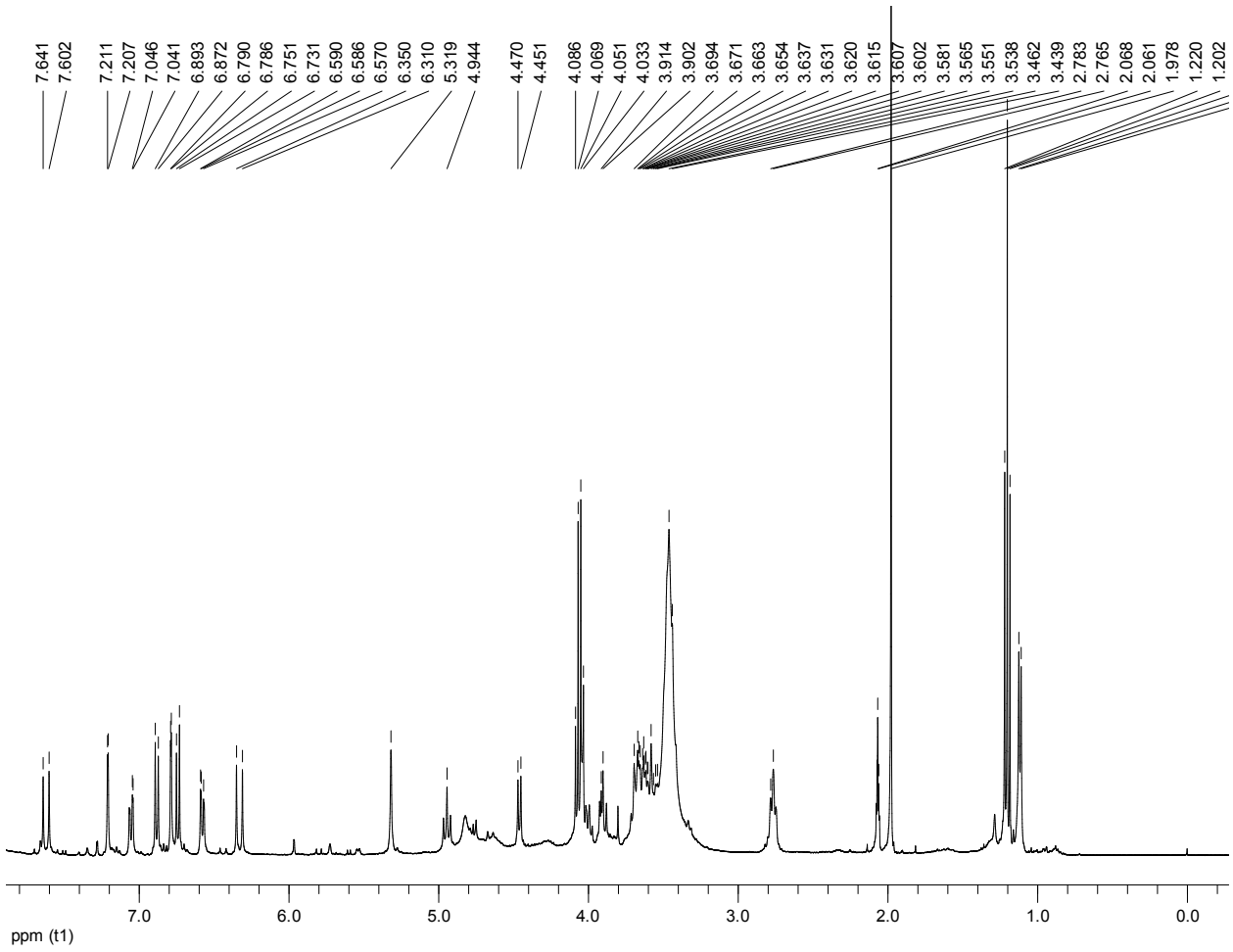
APPENDIX

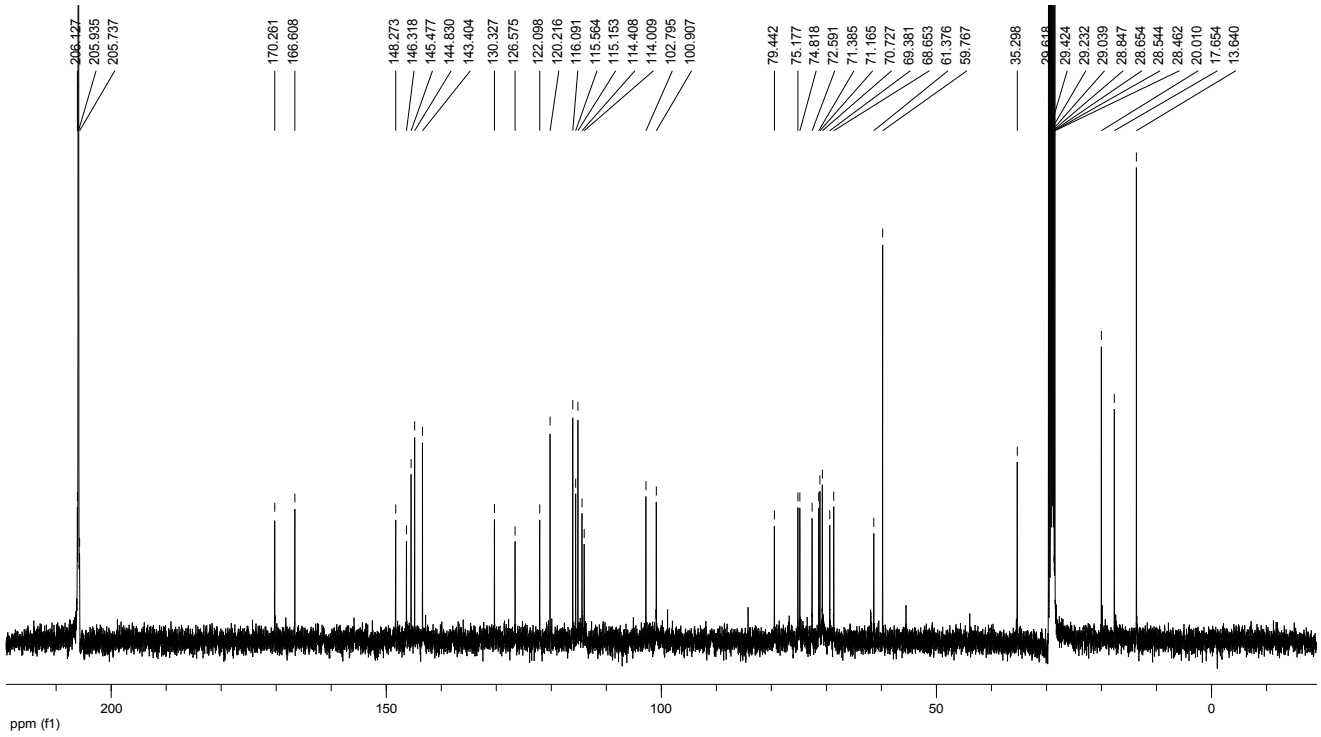
Appendix 1: FT-IR spectrum of FD-2-1A



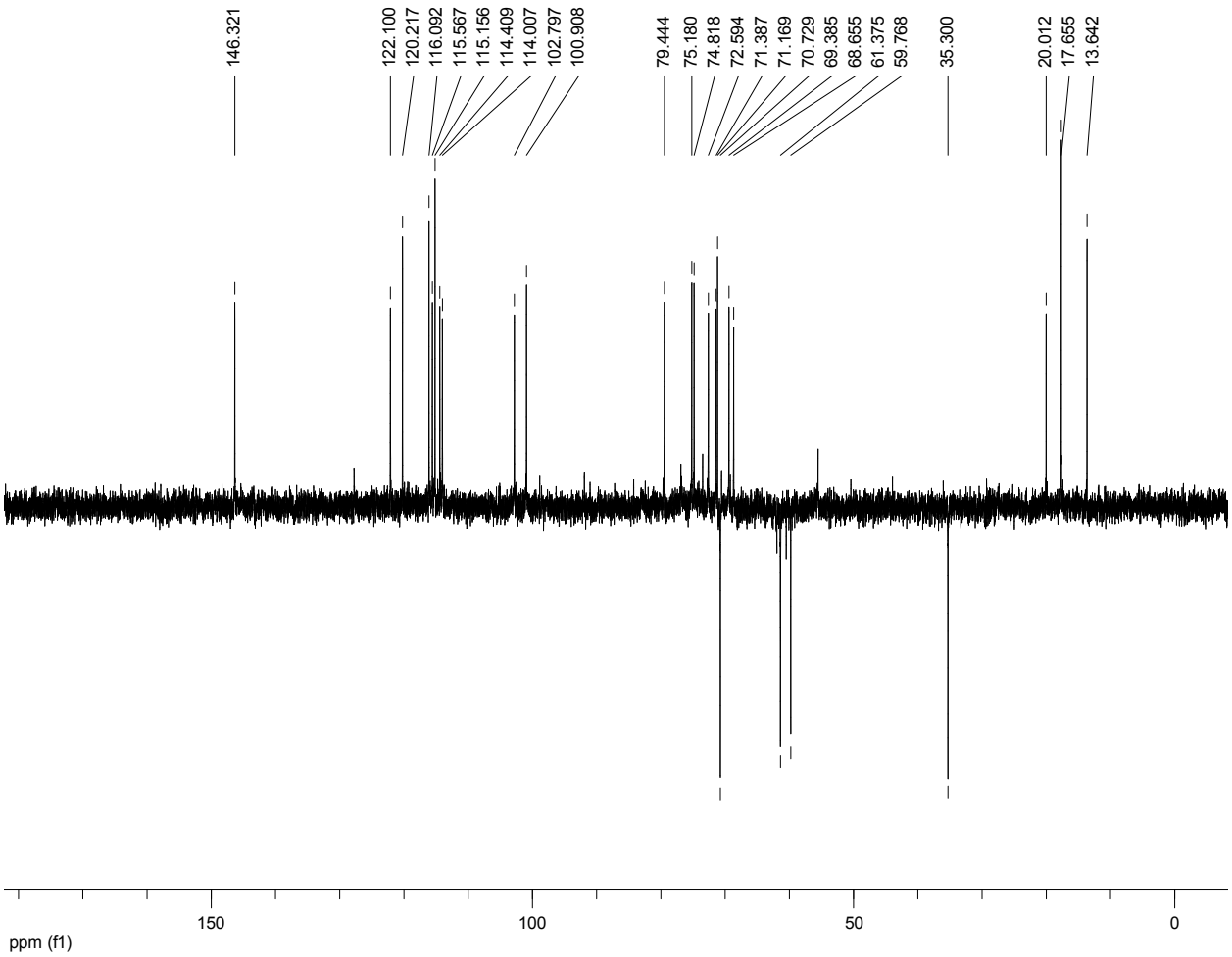
Appendix 2: ^{1}H-NMR spectrum of FD-2-1A



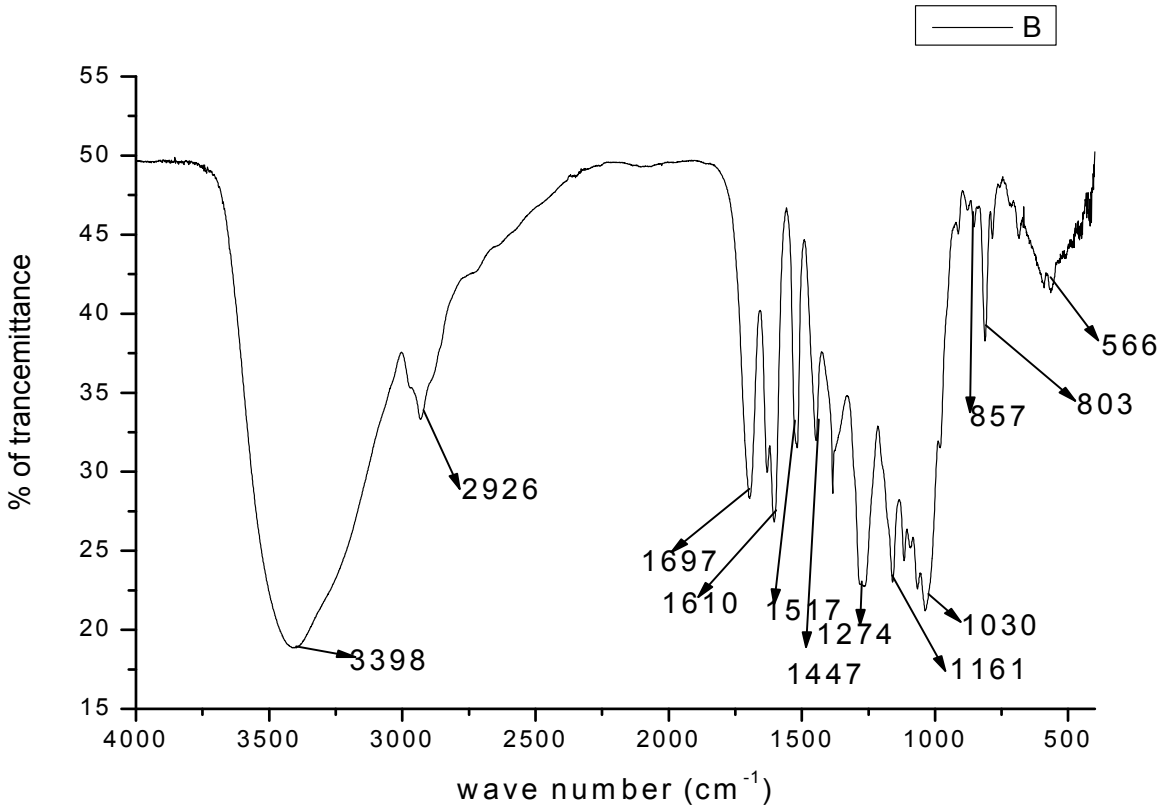
Appendix 3: ^{13}C-NMR spectrum of FD-2-1A



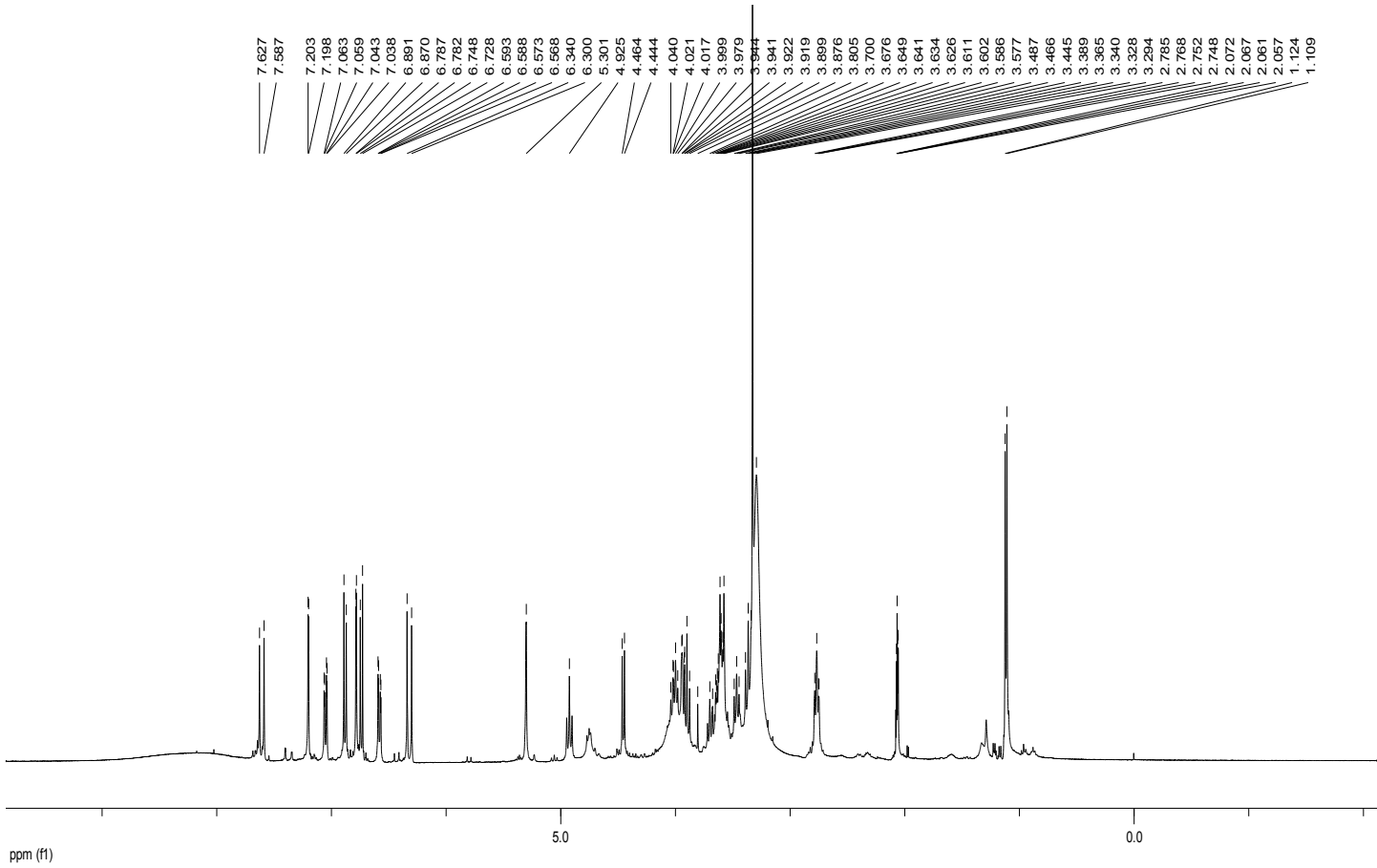
Appendix 4: DEPT-135 spectrum of FD-2-1A



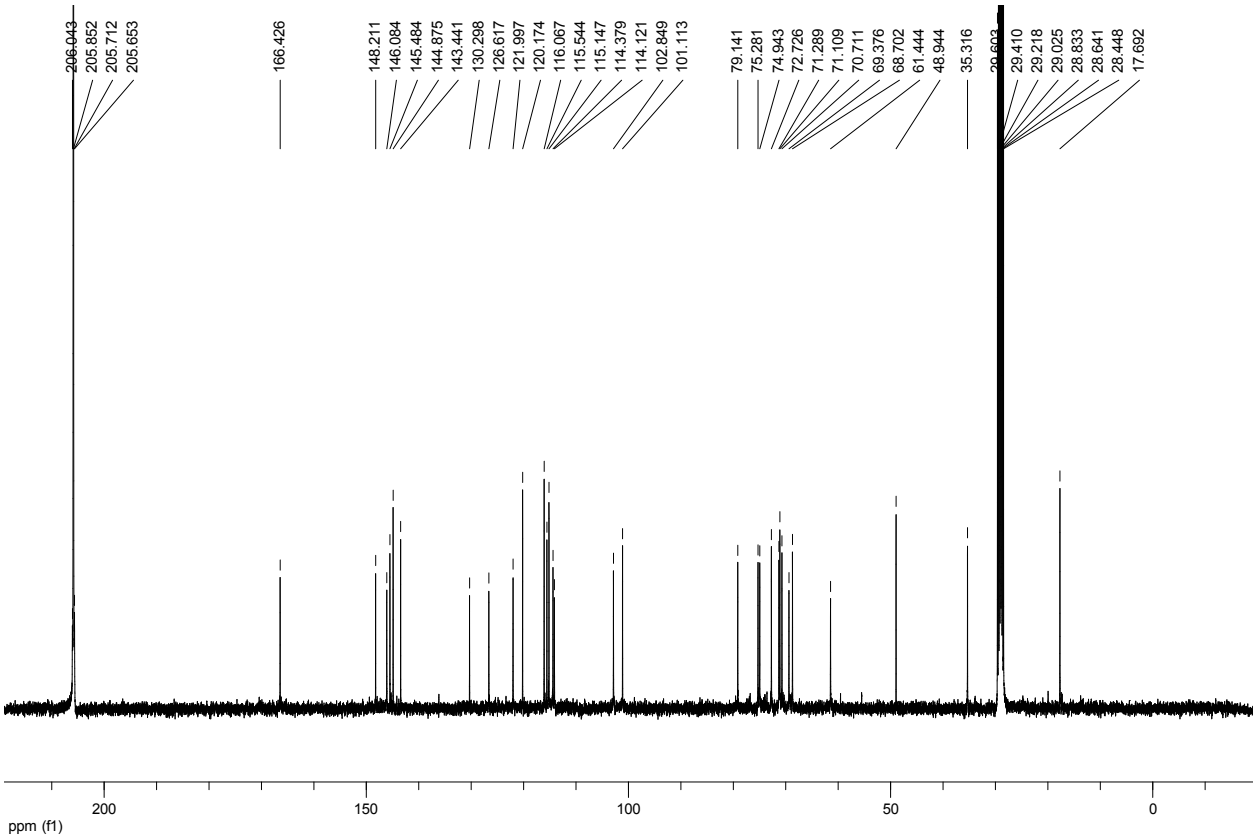
Appendix 5: FT-IR spectrum of FD-2-4A



Appendix 6: ^{1}H-NMR spectrum of FD-2-4A



Appendix 7: ^{13}C –NMR spectrum of FD-2-4A



Appendix 8: DEPT-135 spectrum of FD-2-4A