PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITY OF ROOT EXTRACTS OF *RUMEX NEPALENSIS SPRENG*

BY: **DESSALEGN WAKO KUMSA**

A THESIS SUBMITTED TO THE DEPARTMENT OF APPLIED CHEMISTRY

SCHOOL OF APPLIED NATURAL SCIENCE

OFFICE OF GRADUATE STUDIES

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MAJOR ADVISOR: Dr. HAILEMICHAEL TESSO (PH.D)

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APPROVAL SHEET

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

SCHOOL OF APPLIED NATURAL SCIENCE

PROGRAM OF APPLIED CHEMISTRY

The under signed here by certifying I have read and recommended to school of applied natural science graduated studies for the acceptance of the thesis entitled. "PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITY OF ROOT EXTRACTS OF *RUMEX NEPALENSIS SPRENG*"

In partial fulfillment of requirement for the degree of Master of Science in Chemistry

Submitted by:

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APPROVAL OF BOARD OF EXMINERS

We, the undersigned, member of the board of examiners of the final open defense by **Dessalegn Wako** have read and evaluated his thesis entitled "Phytochemical investigation and isolation of root extracts of *rumex nepalensis spreng*" 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 of Science in Chemistry.

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Declaration

This thesis is my original work and has not been presented for any other degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

Name: Dessalegn Wako Signature_____ Date_____

Name of Adviser: Hailemichael Tesso(phD) Signature_____ Date_____

Dedication

This thesis manuscript is dedicated to all my family especially to my beloved Mather, who was eager to see my successes.

Acknowledgement

I am grateful to God for giving me strength to carry on with this study, without which I would not have come this far. My sincere gratitude goes to my adviser Dr.Hailemichael Tesso for their immeasurable advice, remarkable commitment to supervise my work, limitless encouragement and patience throughout this research and writing of thesis. I would like to express my warm thanks to Addisalem Abebe who extends his tremendous support, encouragement and running NMR and IR Spectroscopic data for this research work. I also owe a deep sense of gratitude to my family who shouldered alone the burdens of family responsibility, which enable me to concentrate on my study, and this research always it remains with me live her and my brother Alemayehu Wako whole hearted cooperation support and best wishes throughout my study. I would also like to express my deepest appreciation to my sister Chaltu Wako for the encouragement and advice they gave me during my stay in the university. I am also grateful to all academic staff and technical assistant of chemistry department of Adama Science and Technology University. I would like to express my deepest gratitude and heartfelt thanks to Masgabu Tafasa to collect the medicinal plant for me.

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List of abbreviation

CC	Column chromatography
DMSO	Dimethyl sulfoxide
DEPT-135.	Distortion less enhancement polarization transfer.
IR	Infra-red
MHA	Mueller Hinton Agar
MHz	Mega Hertz.
NMR	Nuclear magnetic resonance
1D-NMR	One Dimensional-Nuclear magnetic resonance
TLC	Thin Layer chromatography.
R_f	Retention factor
UV	Ultra-violate
d	Doublet
s	Singlet
J	Coupling constant
t	Triplate
TMS	Tetra methyl silicate
EtOAc	Ethyl acetate
MEOH	Methanol
n-hexane	Normal hexane
R	*Rumex*
ATCC	American culture collection

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ABSTRACT

Rumex nepalensis spreng belongs to polygonaceae family. Locally in Afan Oromo it is known as"Timiji" and in Amharic"Tult". It is used traditionally for the treatment of various ailments including pain, inflammation, bleeding, constipation, head ache, fever and stomach ulcer. Therefore, the present study was aimed to investigate the phytochemical constituents, isolating compounds and evaluating for antibacterial activities on the solvent extracts of the root of R.nepalensis spreng. The powdered and dried roots of R.nepalensis spreng was successively extracted with equal volume of n-hexane, ethyl acetate and methanol by maceration methods in increasing polarity. The solvents were evaporated using rotary evaporator to afford n-hexane (1g), ethyl acetate (6.9g) and methanol (70g) crude extracts respectively. Structure elucidation was done by employing some physical properties, spectroscopic methods and comparing with literature data reported for the same compounds. For each of the crude extracts, the preliminary phytochemical screening was carried out by chemical methods to analyze the presence of phytochemical constituents namely: tannins, saponins, terpenoids, flavonoids, glycosides, anthraquinones, steroids, and phenols but Alkaloids were absent in all extracts. Silica gel column chromatographic separation of the ethyl acetate extract afforded Oleic acid (16), 4''-acetoxy-3',4'-diethoxy epicatechin-3-O-gallate compound(17) and 4''-acetoxy-*4'-ethoxy epicatechin-3-O-gallate compound(18) closes to flavan derivatives. The crude extracts and isolated compounds were screened for in vitro antibacterial activity against strains of two gram positive (Staphylococcus aureus and Bacillus subtilus) and two gram negative bacterium (Escherichia coli and Pseudomonas aeruginosa). Among the extract of the root, methanol extract exhibited the highest inhibition zone against Pseudomonas aeruginosa with inhibition zone of (17.2 ± 2.12mm) whereas* EtOAc was *the second with inhibition zone of (13.5 ± 1.73mm) against S.aures and least inhibition zone was observed for n- hexane extract against E.coli and p.aeruginosa with inhibition zone ineffective to that of the +ve control antibiotic chlorampheni col(30 µg/mL), with zone of inhibition of 18mm, respectively, at concentration of 20µg/mL for each extract and isolated compound were used for the test.*

***Keywords**: Traditional herbal medicines, R.nepalensis, isolation of compounds, structure elucidation, phytochemical, antibacterial, oleic acid, and epicatechin-3-O-gallate.*

1. INTRODUCTION

1.1. Back Ground of the Study.

Medicinal plants have a long history of use in most communities throughout the world. It has been confirmed by WHO that herbal medicines serve the health needs about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries. In Africa, the use of traditional medicine has persisted over the years and the last few decades have witnessed an upsurge of interest in traditional medicine and other alternative forms of health care in the developing and developed countries [1]. In Ethiopia traditional medical practitioners use different types of medicine such as minerals, plants and animal products. Plants have been used as a source of traditional medicine in Ethiopia from the time immemorial to combat different ailments and human sufferings. Due to its long period of practice and existence, traditional medicine has become an integral part of the culture of Ethiopian people [2].

The traditional medicines are prepared in various dosage forms such as liquid, powder and prescribed in a non-formulated form [3]. The complete phytochemical investigations of medicinal plants should be carried out, because of these secondary metabolites are responsibly for medicinal activity of the plant. Numbers of plants was screened for secondary metabolites for their medicinal values [4]. In order to promote herbal drugs, there is an urgent need to evaluate the therapeutic potentials of the drugs as per WHO guidelines and mentioned that 30% of the worldwide sales of drugs are based on natural products [5]. Traditional indigenous medicines are limited to small tribal and geographical areas called "Little Traditions" are an excellent repository of knowledge about medicinal properties of botanical sources [6]. The bioactive extract should be standardized on the basis of phytochemical compounds [7]. Phytochemical screenings of medicinal plants are very important in identifying new sources of therapeutically and industrially important compounds. The current issues of phytochemical properties of medicinal plants are: to improve the health status of people and also to use in pharmaceutical products of commercial importance. Semi syntheses of these natural products have also provided potent leads for the pharmaceutical industry [8]. There are several reports on the antibacterial activity of different herbal extracts in different regions of the world [9]. Many previous studies conducted in Ethiopia have shown the antibacterial activities of many indigenous plants used in

traditional medicine. Plant extracts has great potential as antibacterial activity against micro-organisms [10]. The increasing use of plant extracts in the food, cosmetic, and pharmacological industries suggests that extract active compounds for a systematic study of medicinal plants are very important[11].Plants belonging in the family of polygonaceae are known to produce a number of biologically important secondary metabolites such as: steroids, Anthraquinones,naphtalenes,stilbenoides, flavonoids glycosides, leucoanthocyanidins, and phanolic acid. The aerial parts, leaves and roots of the plants are used in traditional medicine for the treatments of several health disorders such as infection, diarrhea, constipation, liver and gallbladder disorders and inflammation and as an antihypertensive. *R.nepalensis spreng* is *polygonaceae* family commonly known as "Timiji" in Afan Oromo and "Tult" in Amaric. The previous studies revealed that, the biological activities of the root of *R.nepalensis spreng* has a wide range of activities including purgative, antipyretic, antibacterial, antifungal, anti-inflammatory and psychopharmacological activities [12-15].

Traditionally, the leaf and root parts of *R.nepalensis spreng* extracts are applied to cure: skin sores, colic, syphilitic ulcers, scabies and reducing body pain [16, 17]. The leaf juice in water is applied to stop bleeding during accidents [18].On the other hand, a tea spoon of leaf powder of *R. nepalensis spreng* boiled in 150 ml of water has been traditionally used to treat ascariasis, abdominal pain and gastric disorders in East Wollega, Ethiopia [19]. Rubbing the affected part of the body by ringworm has been traditionally treated with *R. nepalensis spreng* leaf extracts in Oromia Region, Ethiopia [20]. The medicinal values of these plants are well-known for the treatment of various diseases due to the presence of phytochemical constituents of *rumex nepalensis spreng* [21]. In general, *Rumex nepalensis spreng* is one of the potential medicinal plants in Ethiopia. However, up to now there is no research report on phytochemical investigation, isolation, characterization and antibacterial activities of this plant species in Ethiopian. Thus this work is believed to fill the gap.

1.5 Statement of the problem

More than 80% of the people used traditional medicine to treat various systems based on indigenous knowledge [12]. Indigenous communities have for a long time incorporated the use of traditional medicines, mainly from plant sources in the cure or lessening of impact of common ailments. In Ethiopia, quite a number of plants used in folklore medicine have been identified

and application of their crude extracts documented. A lot of medicinal plants are available for the treatment of various diseases especially diarrhoea which is a major problem in the country [22]. However, little information is known about the phytochemistry of the active ingredients of these plants. The combined effects of the health-related challenges due to the rapidly growing population, emergence of antibacterial resistant pathogenic strains to frequently used commercial drugs such as penicillin, increased side effects, and the failure of modern medicine to provide effective treatment, high cost of new drugs and emerging diseases, research into natural products with antibacterial activity is being pursued in earnest. Systematic pharmacological studies of the genus *Rumex* have been done to some extent. However, quite a number of the species, including *Rumex nepalensis spreng*, call for further screening to determine the active principles, their efficacy and mechanism of their action. Moreover, little information is available on the phytochemistry of *R.nepalensis spreng*. This study sought to isolate, elucidate structures and determine bioactivity of both crude and pure secondary metabolites in the root extract of *R.nepalensis spreng*.

1.8 Significance of the study

Medicinal plants were used for primary health care for the population living in developing countries. Many of the drugs which are in use today were discovered through their ethnobotanical route. Moreover, pathogen resistance to existing drugs coupled with increased side effects, lack of curative treatment for several chronic diseases, high cost of drugs and emerging diseases is very common in today's world [23]. This makes it necessary to research on medicinal plants in order to obtain more and potent pharmacological agents owing to the fact that natural products and their related moieties have historically been incredible as a source of therapeutic agents [24].Bacterial infections still pose a problem in Ethiopia especially in rural areas where medicinal plants are mostly used as part of healthcare system. *Rumex nepalensis* species used by communities for traditional therapy of these infections have not been fully investigated phytochemically for antibacterial agents. The findings of this study were expected to provide information on the phytochemistry of the main bioactive chemical constituents in the root of *R.nepalensis spreng* that can be evaluated for the treatment of antibacterial diseases.

1.4. Objective of the Study.

1.4.1. General objective.

- To investigate phytochemical constituents and evaluating antibacterial activities of root extracts of *R. nepalensis spreng.*

1.4.2. Specific objectives.

1. To extract the root of the *R.nepalensis spreng* successively using n-hexane, EtOAc and MeOH.
2. To carry out phytochemical screening from the roots of *R. nepalensis spreng* crude extracts.
3. To isolate compounds from extracts of root *R.nepalensis spreng* by using CC and TLC.
4. To characterize the isolated active compound from the root of *R. nepalensis spreng* by using, IR, NMR, DEPT-135.
5. To determine the antibacterial effect of the crude extract and isolated compound of the roots o*f R. nepalensis spreng* against *Staphylococcus aureus, Escherichia coli, Bacillus subtillus and Pseudomonas aeruginosa.*

2. LITERATURE REVIEW

2.1. Medicinal plants uses, toxicity and their antimicrobial activities.

Crop plants manufacture the primary products such as carbohydrate, protein and lipid which are the sources of food for animals whereas medicinal plants produce secondary metabolites include alkaloids, tannins, flavonoids, steroids, terpenoids and phenylpropanoids which have medicinal values in human health care system[25]. Essential oils and cosmetics are also derived from the secondary products of plant in the form of alkaloids, terpenoids and flavaonoids. These plants contain mixtures of different chemical compounds which can be used to treat diseases and improve health [26].The use of natural products mainly from plants and animals in human health is as ancient as human civilization [27], but the discovery of modern drugs, vaccine and antibiotics decline the interest of traditional remedies and left them to be the only options for poor who could not afford the price the approved drugs in pharmaceutical market.

However, today the demand for traditional medicines gets popularity and become mandatory to investigate the scientific basis of herbal remedies both in developing and industrialized countries [28]. Similarly in the last 20 to 30 years, the analyses of secondary metabolites of the plants have shown remarkable progresses. Thus medicinal plants are important in modern medicine in one of the following ways: directly as a drug in pure or extract form, they can be the bases for synthetic drug; the ingredients can be used as tool for the development of new drug [29]. Some others have anti-plasmodia, antibacterial, antifungal properties and cytotoxicity [30]. They have also a critical role in the development of human cultures and tradition in the world [31].

Medicinal plants not only have medicinal and nutritional values but also they are toxic to the normal physiology of the animals. Plants therefore, produce secondary metabolites as defense against animals, parasites, bacteria, and viruses. These secondary metabolites are produced for the purpose of the plants themselves [32]. Before new drugs are approved, toxicological studies on the drugs are very essential experiments in animals such as mice and rat. As a result, toxicological studies help to make a decision whether the new drug is approved for clinical use or not [33].Therefore, it is important to have variety of toxicological investigations on drugs under the study before exposing the prospected drugs to humans [34].The curative potential of medicinal plant can be due to the presence of complex composition of the secondary metabolites which can be derived from the bark, leaves, flowers, roots, fruits and seeds of the plants [35].The

ingredients of these medicinal plants are synergic which mean the secondary metabolites of the plants interacts each other. As a result the secondary metabolites can complement or neutralize their negative effects in animals [36]

2.2 The *polygonaceae* Family

The *Polygonaceae* commonly known as the knot wood or smartweed family, are a family of flowering plants and comprise about 1200 species containing 50 genera. Among which largest ones are *Eriogonum* (2410 species), Rumex (200 species), Cocoloba (120 species) and *Persicaria* (100 species) etc. This family is wildly distributed in North Temperature Zone although can be found worldwide. Various medicinal uses have been attributed to this family, like for asthma, bronchitis, cough, diarrhea, dysentery, eczema, earache, inflammatory conditions, jaundice, kidney disease, leprosy, paralysis, toothache, ulcerative colitis, intestinal parasites and others [37].

2.3 The genus *Rumex*

Rumex nepalensis spreng, which belongs to the family *polygonaceae,* is a perennial herb, up to 2m tall, the leaf of the plant is usually sagittate, and inflorescence and much branched. The rhizomes are used to refine butter and give it a rich yellow color. The family polygonaceae comprises some 50 genera and 200 species largely concentrated in temperate areas of the northern hemisphere and tropical-subtropical mountains. Some of the species which are found in Europe,Asia,Africa and North America are *Rumex nepalensis, Rumex crispus, Rumex vesicarius, Rumex abyssinicus, Rumex nervosus, R.obtusifolius, R.palustaries, R.ecklonianus, R.hydrolapathum, R.scutatus, R.altissimus, R.stenophyllus, R.arifolius, R.patientia, R.confertus, R.sanguineus, R.brownii, R.pulcher,R.acetosa, R.conglumeratus R.acetosella, R.maritimus, R.alpinus, R.palustris and R.obtusifolius* [38].

2.3.1 Ethno botanical information of the *Rumex nepalensis spreng*.

Rumex nepalensis Spreng is an erect and perennial herb which measures about 2 m tall and it is called ‘Timiji in Afan Oromo and “Tult” in the local language Amharic. It bears petioleted leaf with various shapes ranging from lanceolate to elliptic. *R.nepalensis spreng* is perennial ascending herbs having tap roots and erect stems which is 50-100 cm tall. Its leaves structure is basal and petiole is 4-10 cm wide, Leaves entire, lower ones long-stalked, oblong, ovate, petiole, base widely or narrowly cordite. Flowers are bisexual, in whorls forming long, nearly leafless

racemes, reddish or green [39]. The genus *Rumex* consisted of more than 200 species and is widely distributed in the worldwide including Europe, Asia, African and American countries [40].*R. nepalensis spreng* is distributed in the altitude range of 900–4000 m above sea level on moist and dry slopes in plains throughout India [41].It also grows in altitude range of 1200-3900 meter above sea level and distributed throughout Africa and in most parts of Ethiopia like Tigray, Amhara, Oromia and Southern parts of Ethiopia [42].

2.3.2 Biological activities of the Genus *Rumex*.

Rumex, a genus of *polygonaceae* family, is very important prevalent worldwide. There are about 200 species of this genus, many of which are beneficial and used traditionally for medicinal purposes [43]. Root, seed, leaf, fresh plant juice, aerial parts etc. are the parts generally used. Different species of rumex genus contain various types of biological activities e.g. anti-inflammatory, antioxidant, cytotoxic, anti-fertility, antibacterial, purgative, anti-diarrhoeal, antifungal, antipyretic, antiviral activities [12, 44-48]. Traditionally one species of *rumex* genus named *Rumex abyssinicus*, has been used for hypertension and pain relief. This plant also possesses antibacterial and diuretic properties [45].

The methanolic extract of this species of rumex has many biological effects including antioxidant, anti-nociceptive, anti-diarrhoeal, and cytotoxic potential [49, 50]. *Rumex hymenosepalus* contains Leucodelphinidin and Leucopelargonidin which are antitumor us substituent's. Neopodin, a substance found in *Rumex japonicas* has inhibitory effect of osteoclast [51]. *Rumex nepalensis* Spreng is another species that is widely used for various actions like antifungal, antibacterial, purgative. This species has moderate cytotoxicity and high phytotoxic activity [52].The chloroform and ethyl acetate of the root extracts of *R. nepalensisspreng* has anti-inflammatory, cycloxygenase (COX)-2, COX-1 inhibitory and free radical scavenging effects on mouse model and demonstrated a significant reduction in ear edema [14].

2.3.3 Ethno Pharmacological Information of Genus *Rumex*.

The medicinal values of root of *R.nepalensis spreng* used for the treatment of: Pain, inflammation, bleeding, constipation, head ache, fevers and stomach ulcer. The medicinal value of the leaf of *R.nepalensis* in China, Africa and Ethiopia were revealed that: To cure skin sores the leaf infusion is give incollic and applied syphilitic ulcer. To treat scabies, aqueous extract used wash reducing body pain [15, 16]. The roots and leaves of *R. nepalenis spreng* are used for

dental care and the leaf juice in water is applied to stop bleeding during accidents [20]. On the other hand, a tea spoon of leaf powder of *R. nepalensis spreng* boiled in 150 ml of water has been traditionally used to treat ascariasis, abdominal pain and gastric disorders in Gondar district, Ethiopia [19]. Rubbing the affected part of the body by ringworm has been traditionally treated with *R. nepalensis spreng* leaf extracts in Tigray Region, Ethiopia [21]. *Rumex abyssinicus* (*Polygonacea*e) is one of the potential plants used for the treatment of various diseases caused by micro-organisms. *R.hastatus* is used for the treatment of caught, headache fevers (53).

2.3.4 Phytochemistry of *Rumexspecies*.

Phytochemicals are a large group of plant-derived compounds which are responsible for the protection disease and obtained from fruits, vegetables, beans, cereals and plant-based beverages [54]. Phytochemical can be either primary or secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents whereas; terpenoid, alkaloids anthraquinones, stilbenoid, naphthalenes and phenolic acid compound are secondary compounds [55]. Phytochemicals are not vitamins rather, they are physiologically active compounds which are produced in secondary metabolism and they in relatively small amounts, but they have significant health potentials.

Flavonoids are important group of polyphenols which are found in fruits, vegetables, coffee, tea and wine. Flavinoids include anthocyanins, flavanols, flavones, isoflavones, anthocyanins, quercetin, kaempferol and catechin are believed to be used in antioxidant activity as hydrogen donating free radical scavengers, anti-inflammatory properties and maintain a healthy heart and urinary tract [56].Flavonoids deactivate carcinogens by inhibiting the expression of mutated genes and the activity of enzymes that promote carcinogenesis by promoting detoxification of xenobiotics[57].

Alkaloids are largest group of secondary chemical constituents which are made up of mainly ammonia compounds and synthesized from amino acid. Alkaloids exist in solid such as atropine or stimulants caffeine, nicotine, codeine, atropine, morphine, ergotamine, cocaine, nicotine and ephedrine. Alkaloids are intensely bitter and function in the defense of plants against herbivores and pathogens. They are widely exploited as pharmaceuticals, stimulants, narcotics and poisons

due to their potent biological activities. Alkaloids have pharmacological applied as anesthetics and central nervous system stimulants [58].

Terpenes are hydrocarbons of chemically diverse groups of natural products and are lipid-soluble compounds. Terpenoids include hydrocarbons of plant origin of general formula $(C_5H_8)_n$ and are classified as mono, di, tri and sesquiterpenoids depending on the number of carbon atoms. Terpenes include perillyl alcohol, limonene and carnosol. These help cells from becoming cancerous, slow cancer cell growth, strengthen immune function, limit production of cancer-related hormones, fight viruses and work as antioxidant. Among the pharmaceuticals, the anticancer drug taxol and the anti-malarial drug artimesinin are two of the most renowned terpene-based drugs. The application of traditional medicine against diseases has a very long history in Ethiopia. Traditional medicines mostly obtained from natural products especially from medicinal plants are not only concerned to curing diseases but also applied to physical, spiritual, social, mental and material well being of human beings [59].

Anthraquinones are derivatives of phenolic and glycosidic compounds. They are solely derived from anthracene giving variable oxidized derivatives such as anthrones and anthranols [60]. Other derivatives such as chrysophanol, aloe-emodin, rhein, salinos poramide, luteolin and emodin have in common a double hydroxylation at positions C-1 and C-8. To test for free anthraquinones, powdered plant material is mixed with organic solvent and filtered, and an aqueous base, e.g. NaOH or NH_4OH solution, is added to it.

2.3.5. Review of the chemistry of some the *Rumex species*.

Important chemical constituents of rumex include anthraquinones, naphthalenes, tannins, flavonoids, phenolic acids, stilbenoids, triterpenes, carotenoids,etc .Several species of rumex have important medicinal properties and they have been the subjected of several pharmalogical investigation. The most important medicinal compounds in this species are anthroaquinone .The monomeric anthraquinones extracted from rumex species such as physcion (1), Aloe-emodin (2), Alizarin (3), Chrysophanol (4) are from species of *Rumexs*.

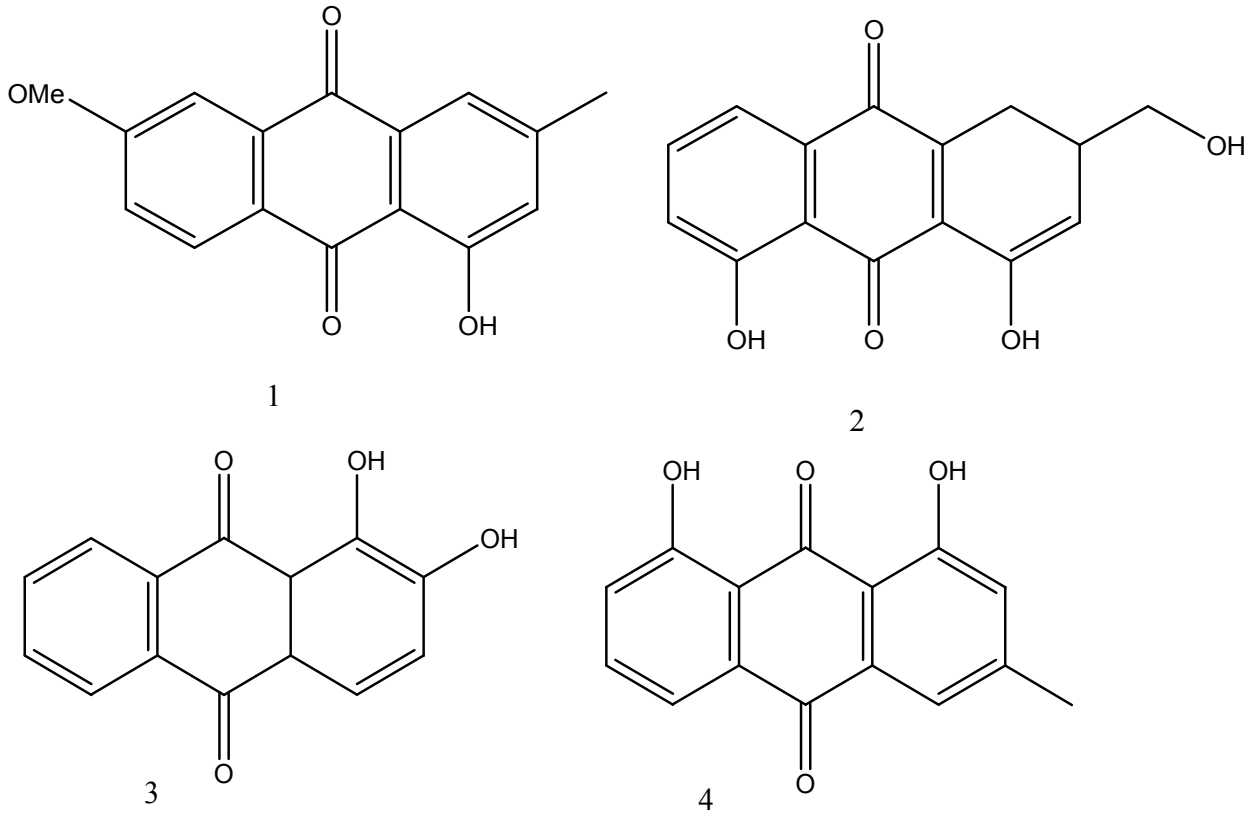
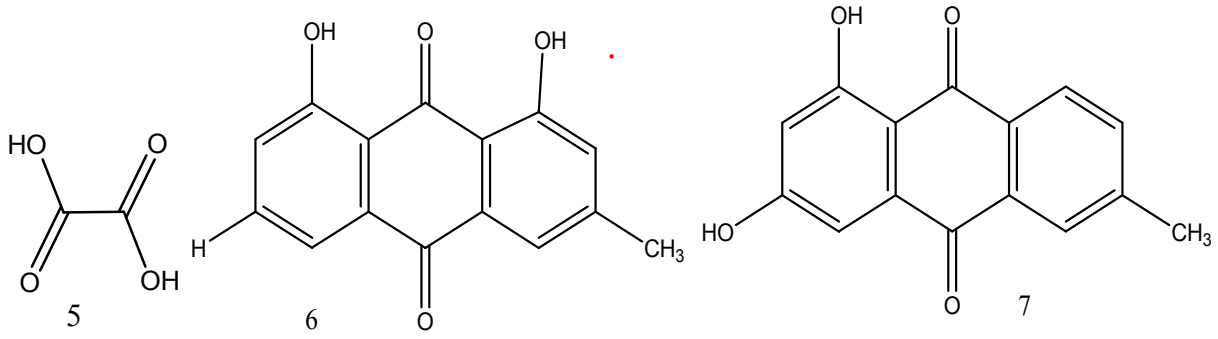
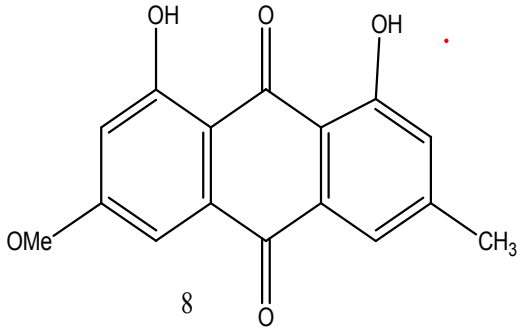


Figure 1.Chemical constituent of *Rumex* species

2.3.5.1. *Rumex abyssinicus*

Some of the previously isolated and reported chemical constituents from R.abyssinicus are Oxalic acid (5), Chrysophanic acid (6), emodine(7), physcion(8).and catechin(9)





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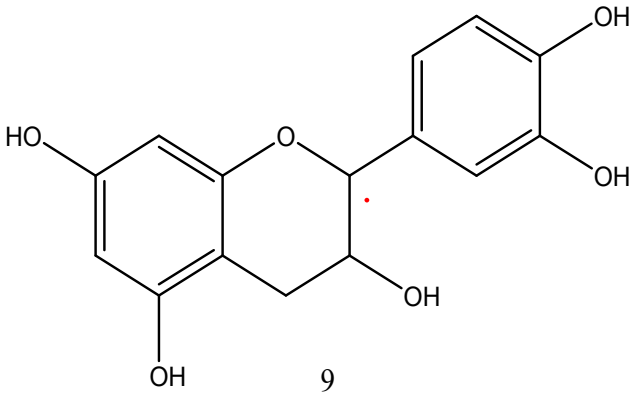

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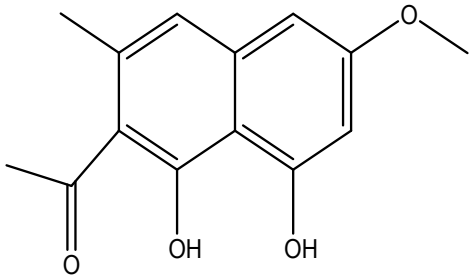
Figure 2.Chemical constituent of *R.abyssinicus*.

2.3.5.2. Reported Chemical Constituent of *Rumex nepalensis Spreng*.

The presence of new secoanthraquinone glucosides, nepalensides and some known compounds such as rumexoside, torachrysone(10), , orientaloside, orcinol glucoside, aloesin, lyoniresinol 3*α*-O-*β*-D-glucopyranoside, (-)-epicatechin-3-O-gallate(11), (3,5-dimethoxy-4-hydroxyphenol)-1-O-β- D-(6-O-galloyl) glucose, and (-)-epicatechin gallete have been previously reported in the root of *R. nepalensis*. Determination of chrysophanol-8-O-*β*-D-glucopyranoside and nepodin were detected as major constituents of anthraquinone and naphthalene derivatives in *R. nepalensis spreng* roots by HPLC.

The root of *R. nepalensis spreng* is purgative, also used against venereal diseases and bilharziasis. According to the previous investigation root contains chrysophanol(4),stilben(14) Physcion(1), *β*-sitostero(12), Leupeol(13), and its glucoside, Orientalone, emodin(7), 1-O-*β*-D-glucopyransylmusizin, 3-methoxy-5,6-methylenedioxy benzaldehyde(15) and chrysophanol-8-O-*β*-D-galactopyranoside have been previously reported in the roots of *R. nepalensis spreng* [61].



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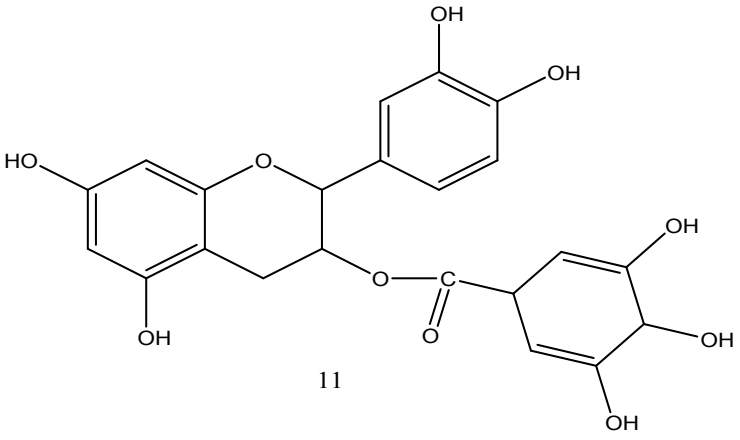

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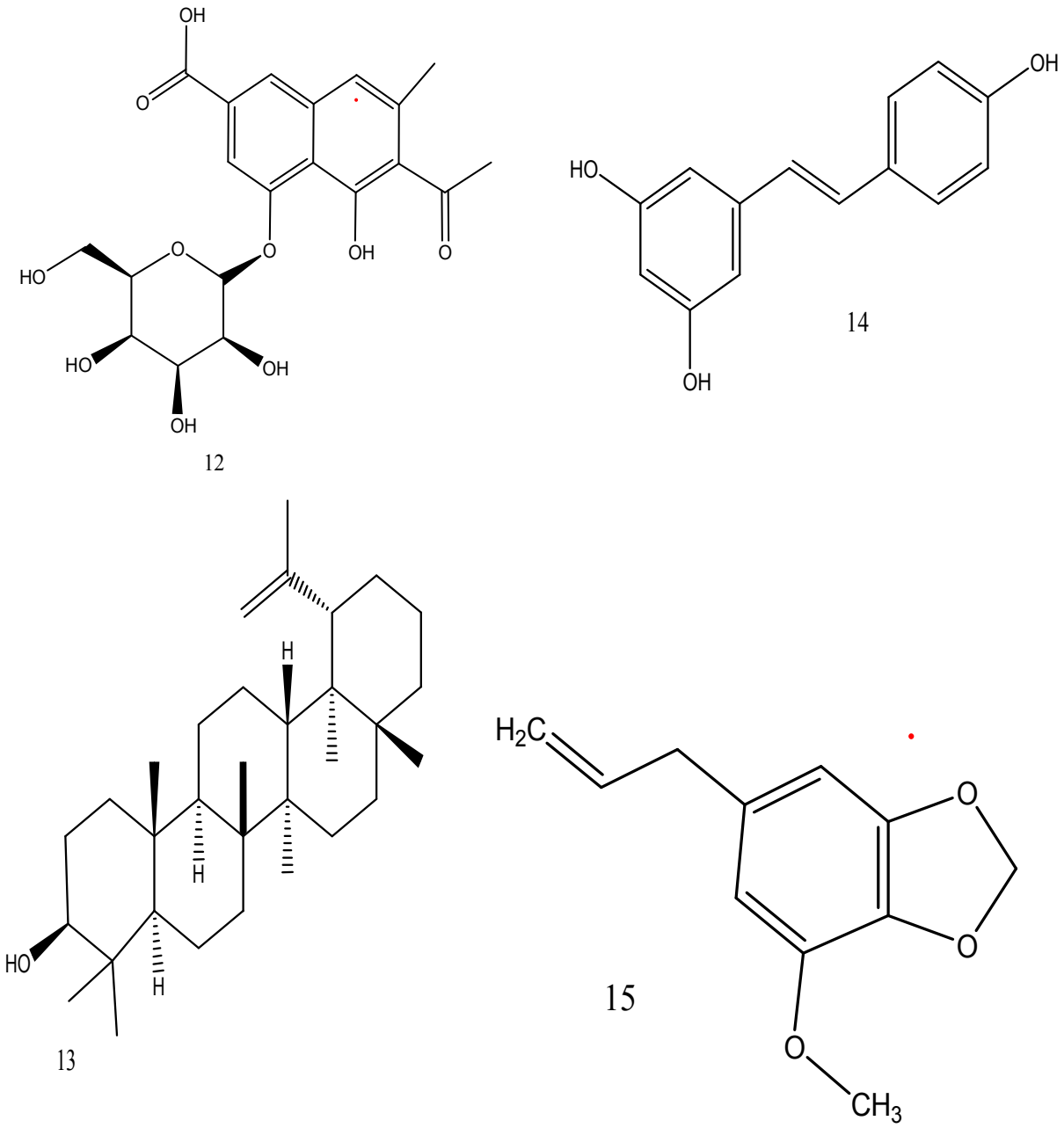


Figure 3.Chemical constituents of *R.nepalensis*.

3. MATERIALS AND METHODS.

3.1. Plant materials.

The fresh roots of the *Rumex nepalensis (polygonaceae* family) (Figure 4 a and b**)** were collected from Arjo Woreda, East Wollega, Oromia region, Ethiopia, which is 379 km far from West of Addis Ababa, this is located near Nekemte in November 2017.The plant material was authenticated by botanist Shambal Alamu, and a Voucher specimen (Voucher No: GW 009) was deposited in the National Herbarium, Ethiopia, Department of Botany/Biology, Addis Ababa university, Addis Ababa. The collected roots of the plant was thoroughly washed using tap water to remove dirtiness, air dried in the shade and stored at the laboratory of Arjo preparatory school for chemical investigation.

(A) (B)

Figure 4.Aerial part (A) and roots (B) of *R.nepalensis* taken by Dessalegn.

3.2. Materials and Instrument.

Column chromatography was performed using silica gel (200-400 mesh) Merck. Analytical thin layer chromatography to used was a 0.2 mm thick layer of silica gel GF254 (Merck) coated on aluminum plate. NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz, IR and UV-light. The materials that used in this study were: TLC plate, silica gel, TLC chamber, Rotary evaporator, reagent bottles, capillary tube, round bottom flasks, beakers, digital melting point apparatus, funnels, vials, glass wares, refrigerator, gravity filtration apparatus, What man No.1 filter papers, grinder, drying oven, and measuring cylinders.

3.3. Chemicals.

The chemicals and reagents which were used in this study include:99% *n*-hexane (Loba Chem. Pvt,Ltd.,India), 99.8%chloroform (Loba Chem. Pvt,Ltd.,India), 99.5%ethanol, 97%ethyl acetate(ATICO medical PTV.,Ltd,India),97% methanol(Loba chemPvt,Ltd., India), methyl chloride, ferric chloride, NaCl/KBr, HCl, acetone, distilled water, HNO_3,.Vanillin, NaOH and H_2SO_4.

3.4. Preparation of the Powder

The air dried roots of *Rumex nepalensis spreng* sample were ground using the grinder and the amount of powder yield (400 g) .The resulting powder were packed in polyethylene bag to avoid it from entrance of air, moisture, and also to prevent it from any other mixing of surrounding materials until the experiment was done and were stored in the refrigerator.

3.5. Extraction techniques and isolation of compounds.

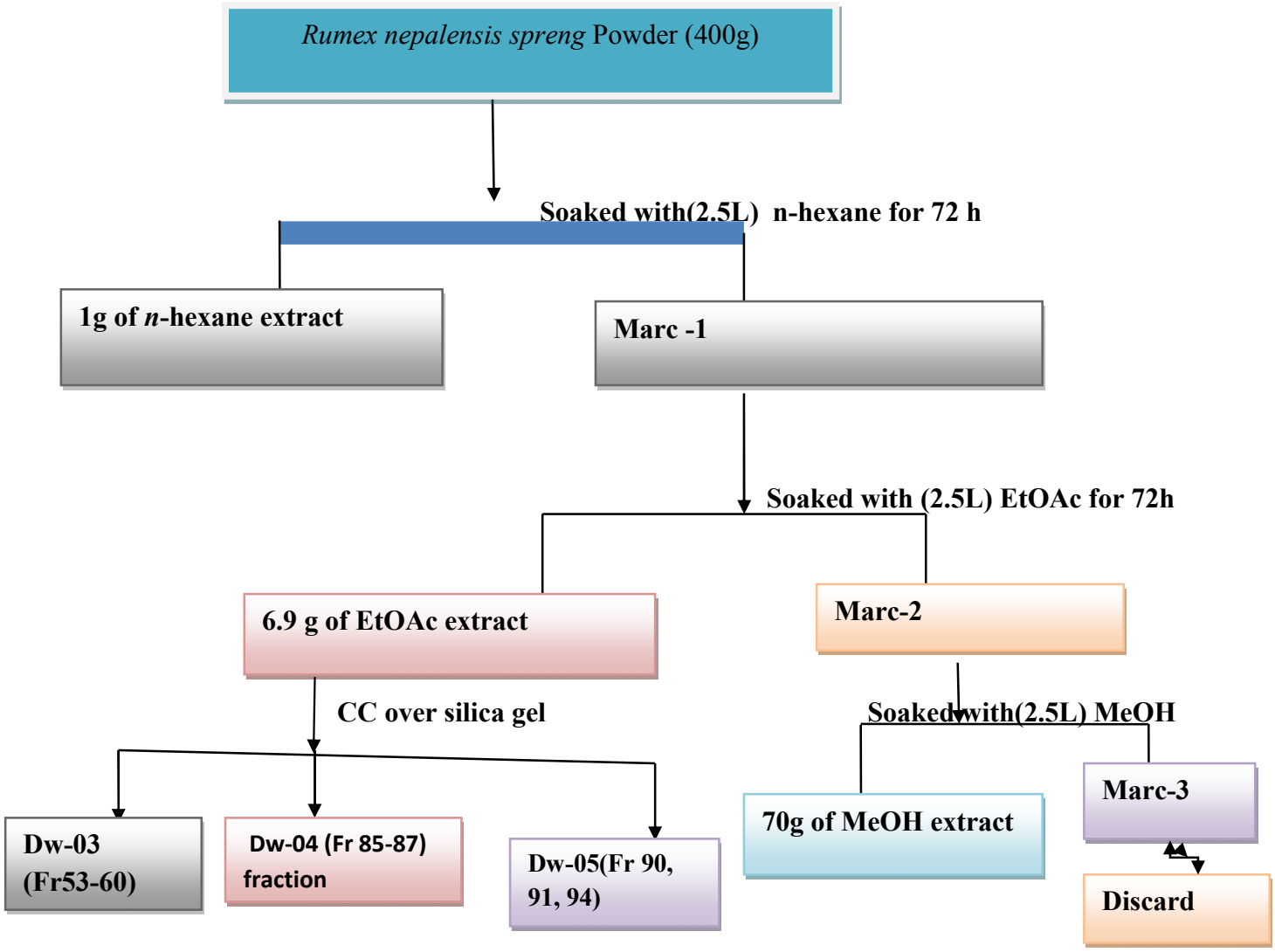
The root of *R.nepalensis spreng* was chopped into small pieces and air dried at room temperature for 21 days. The dry root was ground into fine powder using an electric grinder. The powdered plant material (400g) was sequentially extracted with equal volume of (2.5L) *n* –hexane, EtOAc and MeOH soaked for 72 h at room temperature with continuous shaking by maceration method. The mixture of each solvent were filtered using filter paper(what man No 1,what man Ltd.,England),the filtrates were concentrated under reduced pressure and temperature of 40°C using rotary evaporator and afforded (1g) of yellow color *n*-hexane,(6.9g) of reddish brown of EtOAc and (70g) of MeOH crude extract. The resulting semidried extract of each solvent was weighed and stored in refrigerator below 4°C until used for antibacterial assay and TLC analysis.

The most active crude extract (Ethyl acetate extract) was subjected to silica gel (200-400 mesh) column chromatography. Briefly, silica gel (150 g) was mixed with *n*-hexane to form a homogenous suspension and stirred using a glass-stirring rod to remove bubbles. The silica gel slurry was then poured into a glass column. The sample to be loaded on the column was prepared by dissolving (6.9 g) of the ethyl acetate extract adsorbed on (6.9g) silica gel and mixed by stirring with a glass rod. The mixture was allowed to dry at room temperature. The dried silica extract mixture was layered on the column layer bed. The column was eluted by using 100% of *n*-hexane, and then with increasing polarity of EtOAc in hexane was used as eluting during

chromatographic separation. A total of 100 fractions were collected, each fraction was monitored by TLC and concentrated under reduced pressure to dryness. Fractions that showed similar R_f values and the same characteristic color on TLC were combined. Fr 53,55,57,58 and 60 were combined together gave compound Dw-03, Fr 85, 86 and 87 were combined together gave compound Dw-04 and Fr 90, 91, 94 were collected together gave compound Dw-05(Table1).

Table: 1.Fraction collected from EtOAc extract and coding system

No	Code	Fraction number	Solvent system	Ratio	TLC test	Color
1	Dw-01	Fr40- 46	EtOAc:*n*- hexane	1:9	Single spot	Yellow
2	Dw-02	Fr47 – 50	EtOAc: *n*-hexane	2:8	Single spot	Yellow
3	Dw-03	Fr53,55,57,58 and60	EtOAc:*n* -hexane	2:8	Single spot	Yellow
4	Dw-04	Fr85,86 and 87	EtOAc:*n* -hexane	9:1	Single spot	brown color
5	Dw-05	Fr90, 91 and94	EtOAc: MeOH	9:1	Single spot	brown color



Scheme 1 Flow chart of Extraction of root of *R.nepalensis spreng*

3.6. Preliminary phytochemical screening on the crude extracts of the roots of *R.nepalensis.*

The preliminary phytochemical screening for each of the crude extracts of the roots of *R.nepalensis spreng* was carried out according to the methods to analyze the presence of phytochemical constituents namely: tannins, saponins, alkaloids, terpenoids, flavonoids, glycosides, anthraquinones, steroids and phenol

3.7.1. Test of Alkaloides

To identify presence of alkaloids in the give active fractions, 4mL of 1% HCl was added to 250mg of plant extract and then it was warmed and filtered. 6 drops of Mayor's reagents added

to 1ml filtrate. Creamish precipitate/orange precipitate indicated the presence of respective alkaloids.

3.6.2. Test of Flavonoides.

Alkaline reagent test was testing the presence and absence of flavonoids. For this test few drops of NaOH is added to extract them an intense yellow color formed. Few drops of dilute acetic acid should be again added to yellow color mixture and allow for a colorless change that indicates the presence of flavonoids. Flavonoids were detected on TLC stained with the $AlCl_3$ reagent in which a positive result was indicated by the observation of pink spots visualized in vanillin-sulphuric acid.

3.6.3. Test of Glycoside.

About 0.5g of plant extracts were hydrolyzed with HCl and neutralized with NaOH solution and treated with a few drops of Feling's solution .The formation of red color indicates the presence of glycosids.

3.6.4. Test of Anthraquinones/ Borntrager's test

About 0.5g of plants powder was boiled with 10%HCl in water for few minutes. The mixture were filtered and shake with equal volume of 10% of ammonia solution .A pink, red or violate color in the aqueous layer, after shaking indicates the presence of free anthraquinones .

3.6.5. Test of steroid and triterpenoids.

Salkolwsk's test was used for triterpenoids test. For this, 200mg extracts should be treated with chloroform and a few drops of concentrated H_2SO_4 was added, and allowed to stand for some time. The formation of red color in upper layer indicates the presence of sterol and formation of yellow color at the lower layer indicates the presence of triterpenoids.

3.6.6. Test of Tannins.

About 200mg of plant extracts were treated with a few drops of 0.1% ferric chlorides and observed for blue or black color confirmed the presence of tannins.

3.6.7. Test of Saponins**.**

About 200mg of powdered sample was mixed with 5ml of dilute water and shaken vigorously for a stable persistent broth. Formation of foam indicates the presence of saponins.

3.6.8. Test of poly phenol

100mg of extract in the test tube was treated with 3% ferric chloride. The brown color of solution shows the presence of phenol.

3.7. Antibacterial screening tests.

3.7.1. Preparation of test solution and Bacterial strains for preliminary activity

The test solution was prepared by dissolving 50 mg from each crude extract in 1 mL of dimethyl sulfoxide (DMSO) to achieve final stock concentration of 50 mg/mL solution of the test sample. The activity of the plant extracts was tested against two gram-negative bacterial strains: *Pseudomonas aeruginosa* and *Escherichia coli* (ATCC 25922) and Two gram- positive bacterial strains: (*Staphylococcus aureus ATCC25923* and *Bacillus subtilus ATCC6633*) both of which were obtained from the Oromia Public Health Research Laboratory, Adama. The root of *R.nepalensis spreng* is used locally for the treatment of infection (commonly caused by *P.aeruginosa*) and diarrhea (caused by *E.coli*). The bacterial stock cultures were incubated for 24 hr at 37°C on nutrient agar medium [62]. The most active extract was selected for fractionation and further analysis.

3.7.2. Preparation of fresh inoculums for bioactive test crude extract

The antibacterial activity test was done using agar well method standard procedures [62]. Muller Hinton Agar culture media were used for growing of organisms. The culture media were boiled in distilled water to dissolve the media and autoclaved at 121°C for 50 min, and poured into sterile Petri dishes. After the culture media was solidified, organisms were uniformly seeded with it. Four well-isolated colonies of the same morphological type were selected from an agar plate culture and the top of each colony was touched with a loop, and the growth was transferred into a tube containing 4.5 mL of a suitable nutrient broth medium.

The broth culture was incubated at 37°C until it achieves or exceeds the turbidity of the 0.5 McFarland standards for 8 hours. The turbidity of the actively growing broth culture was adjusted with sterile saline solution to obtain turbidity optically comparable to that of the 0.5 McFarland standards which was resulted in a suspension containing approximately 108 CFU/mL for different strains. To ensure even distribution of inoculums, the plate was rotated approximately 60^0 each time and finally rim of the agar was swabbed. Then, the seeded media

were allowed to dry at room temperature for 30 minutes. On each plate, wells were made with a 6 mm diameter sterilized cork borer and labeled with numbers corresponding to the extract placed. Then, 100 µL volumes of both crude extract and solvent fractions at concentration of 20µg/mL were introduced in to the wells using micropipette.

DMSO for negative control was impregnated using No.1 What man filter paper disc (diameter 6 mm) with the help of micropipette. Positive control using chloramphenicol (30µg/disc) was assayed simultaneously. Plates were left for 10 minutes till the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 hours. After overnight incubation, the plates were observed for the zone of inhibition and the diameter of the inhibition zone was measured using ruler and mean was recorded.

4. RESULTS AND DISCUSSION

4.1. Extraction yield

The extraction yield was a measured the solvent efficiency to extract specific components from the original material. The *n*-hexane extract yields a yellow oil (1.009 g), the EtOAc extract yields a reddish brown crude extract (6.9 g) and the methanol extract yields a reddish brown crude extract (70g). The percentage yields of crude extract in respective solvent were listed in (Table 2). The MeOH extract yielded the maximum While, the *n*-hexane extract result was a minimum yield.

$$\% \text{ yield} = \frac{Weight\ of\ crude\ extract}{Weight\ of\ the\ sample} \text{x}100$$

Weight of sample taken = 400 g dried roots of *Rumex nepalelensis spreng*

Table: 2. Percent yield of each crude extract at room temperature and Maceration methods

No	Crude extract	Mass of crude extract	Yield in %
1	n-hexane extract	1g	0.25%
2	EtOAc extract	6.9g	1.725%
3	MeOH extract	70g	17.5%

In general as shown in (Table 2) the yields of EtOAc and methanol extract are higher than *n*-hexane extracts. Therefore, in root part of the plant was found to be rich with polar constituents. For the effectiveness of extracting technique, the results showed that the yields of the extract were better when extraction was done under maceration.

4.2. Phythochemical screening test for Rumex nepalensis spreng

The phytochemical screening test on the root of *R.nepalensis spreng* are the presence of Phenols, Anthraquinone, Flavonoid, Glycosides, Triterpenoid, Tannins, and Saponin. Hexane crude extracts of roots of *Rumex nepalensis spreng* showed the presence of non polar compound as a major class of compounds. However, the absences of polar compounds were observed. This is due to the non-polar *n*-hexane extract dissolves non-polar compounds. Methanol and EtOAc extracts showed the presence of flavonoids, tannins, glycosides, Anthraquinone, saponins and phenols among major class of compounds.

However in the crude extract of root of *Rumex nepalensis spreng* Alkaloid was not present in *n*-hexane, EtOAc and MeOH extractions (Table 3). This study suggests that phytochemical constituents in the root extracts of the plant which might have high level of medicinal values. This could be responsible for curing various ailments and possess antibacterial which leads to the isolation of polar compounds for the versatile medicinal properties of plant.

Table: 3. Phytochemical Constituents of different crude extracts of roots of *Rumex nepalensis spreng*

No	Phytochemicals	Reagent	*n*-hexane extract	EtOAC extract	MeOH extract
1	Alkaloids	Mayer reagent test	-	-	-
2	Phenol		-	+	+
3	Anthraquinone	Borntrager's test	-	+	+
4	Glycosides	Fehling's test	-	+	+
5	Flavonides	Ferric chloride test	-	+	+
6	Triterpenoides	Burchardt test	-	+	+
7	Tannins	Braemer's test	-	+	+
8	Saponins	Foam test	-	+	+

EtOAc-Ethyl acetat

MeOH-Methanol

n-hexane-normal hexane

+ → phytochemical present

- → Phytochemical absent

Therefore, the phytochemical constituent testing result reveals that the presence of seven phytochemical constituents in the plant extract. 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.

4.3. Characterization of the isolated compounds

Silica gel column chromatographic separation of EtOAc root extract of *R.nepalensis* gave three compounds labeled as Dw-03 (16**)**, Dw-04 (17**)** and Dw-05 (18**)**. The three of the compound were characterized using the help of spectroscopic methods (UV, TLC, IR and NMR) spectra. This is the first time these compounds are being reported in this plant. The details of the structure elucidation of compound are discussed in the sub- section below:

4.3.1. Characterization of Compound -16 (Dw-03)

Compound -16(Dw-03)was isolated yellow solid (18mg) with R_f value was determined as 0.45 (20%EtOAc in *n*-hexane).The IR spectrum of compound -16, (Appendix 1a) showed the absorption band at 3417cm^{-1} and 1719cm^{-1} corresponding to the stretching vibration peak of the OH and carbonyl(C=O) group respectively. The appearance of band at 2931cm^{-1} indicates C-H stretching of alkanes, the medium intensity at 1278cm^{-1} indicates C-O stretching of the carbonyl carbon of the carboxylic acid and absorption band at 1642cm^{-1} indicate C=C bonding of unsaturated alkenes.

The ^{1}H-NMR (400MHz, DMSO-d$_6$, Appendix 1b, Table 4) spectrum showed a triplet at δ H 2.51 which was assigned to methylene protons adjacent to carbonyl moiety. The multiplicity at δ H 1.97 was characteristic of allylic protons and was assigned to the two methylene protons at the position of adjacent to the double bond. The multiplet signals δ H 5.33 is attributed for olefinic methylene protons. The intense triplet signal at δ H 0.96 indicated the methyl hydrogen's (H-18) adjacent to methylenes (Appendix 1b and Table 4).

The ^{13}C-NMR spectrum of compound -16 (100Mz, DMSO-d6, Appendix 1c, Table 4) showed 18 signals which indicated 18 carbons in compound. DEPT-135 spectrum of compound -16(Appendix 1d) showed 17 signals with three of the signals at (δ 128.20, 130.16 and 14.37) indicating two olefinic hydrocarbons and one for terminal methyl carbons. The remaining 14 carbons signals are in aliphatic region indicating the open chain natural of the compound. The ^{13}C-NMR spectrum showed the signal of carbonyl at δ C 174.89 and the adjacent methylene carbon at δ C 34.03. The olefinic carbons were observed at δ C 128.20 and 130.162 which were assigned to C-9 and C-10. The other methylenes of hydrocarbon chain resonated between δ C 31.78- 22.58 while the terminal methyl group showed the signal at δ 14.37. Based on the above spectral data and comparison of this data with the literature values the compound -16 is proposed to be same as oleic acid (Figure 5). The summary of ^{1}H-NMR, 13C-NMR data of compound-16 and literature data is presented in (Table 4) [63-65]. This is the first time the oleic acid has been obtained from *Rumex nepalensis spreng*.

Table: 4. ^{1}H-NMR (400MHz, DMSO-d_6), ^{13}C and DEPT-135(100Mz, DMSO-d6 spectral data of Compound 16.

Carbon	13CNMR compound -16(δ C PPm)	1H-NMR for compound -16 (δ Hppm)	Reported 13C NMR of one (δ Cppm)	Reported1H-NMR for compound-16 (δ Hppm) [63-65]
2	34.16	2.51(2H,t,H-2)	34.11	2.34(2H,t,H-2)
3	24.8	1.49(2H,m,H-3)	24.71	1.63(2H,m,H-3)
4	25.68	2.04(2H,m,H-4)	25.71	1.61(2H,m,H-4)
5	27.09	2.04(2H,m,H-5)	27.20	1.61(2H,m,H-5)
6	29.05	2.04(2H,m,H-6)	29.10	1.61(2H,m,H-6)
7	29.1	2.04(2H,m,H-7)	29.17	1.61(2H,m,H-7)
8	31.39	1.97(2H,m,H-8)	31.52	2.02(2H,m,H-8)
9	128.70	5.33(1H,d,H-9 and H-10)	128.07	5.34(1H,d,H-9)
10	130.06	-	130.02	5.34(1H,d,H-10)
11	31.79	2.19(2H,m,H-11)	31.91	2.02(2H,m,H-11)
12	29.22	2,04(2H,m,H-12)	29.52	1.26(2H,m,H-12)
13	29.34	2,04(2H,m,H-13)	29.33	1.26(2H,m,H-13)
14	29.42	2.04(2H,m,H-14)	29.43	1.26(2H,m,H-14)
15	29.50	2.04(2H,m,H-15)	29.52	1.26(2H,m,H-15)
16	29.54	2.19(2H,m,H-16)	29.59	1.26(2H,m,H-16)
17	22.8	2.19(2H,m,H-17)	22.67	1.26(2H,m,H-17)
18	14.3	0.96(3H,t,H-18)	14.11	0.88(3H,t,H-18)
C=O	174.89		175.23	

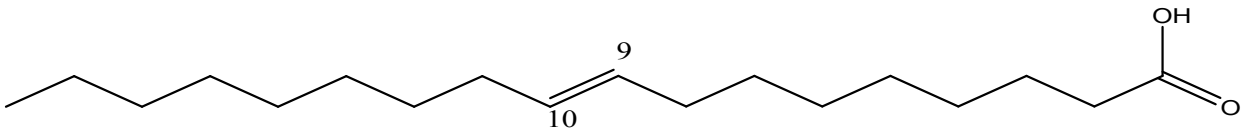


Figure.5. The proposed structure of compound 16 (Dw-03)

4.3.2. Characterization of Compound- 17 (Dw-04)

Compound- 17 (Dw-04) was obtained as solid powder isolated from EtOAc extract. On analytical TLC, the compound had an R_f of 0.55 in (90% EtOAc: *n*- hexane). When the TLC chromatogram was sprayed with vanillin-sulphuric acid, it turned to reddish brown color, suggested that the compound was phenolic.

IR (KBr disc, Appendix 2a) spectrum showed broad vibration at 3418cm^{-1} attributed to hydroxyl moiety (OH) sharp absorption at 1633cm^{-1} attributed to aromatic benzene ring, strong absorption band at 2922cm^{-1} due to C-H stretching of saturated moiety, absorption band at 1709cm^{-1} due to C=O stretching indicates the presence of carbonyl group of conjugated ester, and absorption band at 1236cm^{-1} due to C-O stretching. A band at 2852cm^{-1}to 2879cm^{-1} C-H stretching for CH_2O. Based on extensive analysis and interpretation of the spectroscopic data (IR and NMR) of the compound, a compound with an isoflavonoid skeleton, with a galloyl substituent at C-3 has been arrived at (see Fig.6). The detailed interpretation of the acquired NMR data is as follows.

The ^{1}H-NMR (400MHz**,**DMSO-d_6, Appendix 2b,Table 5) spectrum revealed the presence of proton signals at δ H 5.97 (1H,d,*J*=2,H-8) and 5.94(1H,d,*J*=2,H-6) suggest the presence of two meta coupled aromatic proton that belong to tetra substituted phenyl ring, ring A of the isoflavonoid skeleton. The presence of signal with ABX multiplicity pattern at δ H 6.79 (1H,dd,J=8.4,1.6Hz,H-6') and δ H 6**.**77 **(**1H,dd, *J* =8.4,1.2Hz,H-2' and H-5') is indicative of a tri substituted benzene ring ,ring "B," due to protons at C-2',C-5' and C-6' are overlapping owing to the insignificant different in chemical shift. Signals at δ H 5.86 (1H, d, J=2, H-2) and δ H 5.39 (1H,m,H-3) suggested that the presence of two oxygenated methines. Signals peaks at δ H 3.0 (1H,dd,*J*=17.2,4.4Hz,H-4a) and δH 2.73(1H,dd,*J*=16.4,4Hz,H-4b) suggest the presence of diastereotopic methyelene protons at C-4 .The data also showed that the presence of an ethoxy group at C-3' and C-4' position in aromatic ring, ring "B". The signals at δ H 4.01 (4H, q, *J*=7.2, 6.8Hz, H-7') and δ H 1.18(6H, t, *J*=7.2Hz, H-8') suggested the presence of equivalent oxygenated methylene and methyl proton of an ethoxy group respectively at C-3'and C-4'position on" B" ring.

The galloyl moiety has a para acetoxy group with chemical shift δ H 1.99(3H, s, 4''-acetoxy) on "D" ring. The signal at δ H 6.85 (2H, s, H-2'' and H-6'') suggested that the presence of symmetric proton of methines were observed. The pattern of the above mentioned protons

strongly suggested isoflavonoid skeleton with hydroxyl group at position 5 and 7, ethoxy group at position 3' and 4,' and the galloyl moiety at position C- 3 at ring C. Also they have one acetoxy group at para position on "D" ring.

The proton decoupling of the ^{13}C-NMR (100Mz, DMSO-d_6, Appendix2c, Table 5) spectrum showed well resolved resonance of 28 carbon atoms with their interpretations. The multiplicity of each carbon atom was determined by using DEPT-135 spectrum(Appendix 2d),which showed the presence of two oxygenated sp^2 quaternary carbon was observed at δ C 145.2(C 3') and δ C 145.17(C 4'), suggesting the vicinal substitution on ring "C" in agreement with the ABX multiplicity pattern, whereas methines appears at chemical shift: 68.64 (C-3),76.98 (C-2),94.8 (C-8),96,1(C-6).109.08 (C-2'',6''),114.74 (C-2'),115.0 (C-5') and 118.0 (C-6') . Thirteen signals are quaternary carbons at chemical shift carbon on: 97.7(C-4a),119.70 (C-1''), 129.8 (C-1'), 139.0(C-4''),145.17(C-4'),145.20 (C-3'),145.89 (C-3'',5''),156.1 (C-8a),156.95 (C-7), 157.0 (C-5) and chemical shift of carbon at 165.64 and 170.89 are carbonyl carbon of ester.

The presence of two sp^2 oxygenated quaternary carbon at chemical shift (δ C 156.10 (C-8a) and δ C 157.00 (C-5) along with two up field carbons chemical shift at δ C 96.02 (C-6) and δ C 94.82 (C-8) suggest that ring "A" has 5, 7-dioxygenated substituent pattern. The signal at δ C 76.95 (C-2) and δ C 68.60(C-3) are clearly evident due to the presence of sp3 oxygenated methines C-2 and C-3 ring C. The symmetrical carbon signals were observed for C-2'' and C-6''at δ C 109.09 and C-3'' and C-5'' at δ C 145.89 from the galloyl moiety of the "D" ring. However, the presence of one methylene δ C 26.14 (C-4) on "C" ring and two oxy methylene on ethoxy group moiety on" B" ring respectively (also supported by DEPT-135 pointing down, Appendix 2d) observed at δ C 60.28 (at C-3' and C-4') position are in good agreement with spectral data, whereas methyl signals appeared at δ C 14.25 and 21.1. From all above NMR data and agreed with literature value the following structure proposed for compound -17(Fig 7), which isolated for first time from root of *R.nepalensis spreng* by EtOAc extraction was 4"-acetoxy-3',4'-diethoxy epicatechin-3-O-gallate compound. The summary of ^{1}H-NMR and ^{13}C-NMR data of compound -17 and literature data is presented in (Table 5) [66, 67]. This is the first time that has been obtained from *Rumex nepalensis spreng*.

Table: 5. ^{1}H-NMR (400MHz, DMSO-d_6), ^{13}C and DEPT-135(100Mz, DMSO-d6 spectral data of Compound 17.

C-position	^{13}C-NMR compound2(δ Cppm)	Reported ^{13}C-NMR compound17	^{1}H-NMR for 17(Dw-04) (δ Hppm)	Reported 1H-NMR For compound 17[66,67]
2	76.95	78.4	5.864(1H,d,J=2,H-2)	5.17(1H,s,H-2)
3	68.60	70.8	5.39(1H,m,H-3)	5.53(1H,s,H-3)
4	26.130	26.4	3.0(1H,dd,J=17.2,4.4Hz,H-4a) 2.73(1H,dd,J=16.4,4Hz,H-4b)	2.99(1H,dd,J=17.3,4.5,H-4a) 2.85(1H,d,J=16.8,H-4b)
4a	97.77	99.3	-	
5	157.00	157.9	-	
6	96.02	96.6	5.87(1H,d,J=1.2Hz,H-6)	5.75(1H,d,J=1.2Mz,H-6)
7	156.95	157.1	-	
8	94.8	95.8	5.97(1H,d,J=1.2Hz,H-8)	5.89(1H,d,J=1.2Mz,H-8)
8a	156.10	156.8	-	
1’	129.86	131.5	-	
2’	114.74	115	6.77(1H,dd,J=8.4,1.2Hz,H-2’)	6.73(1H,d,J=8.4Mz,H-2’)
3’	145.20	145.9	-	
4’	145.17	146	-	
5’	115.04	115	6.77(1H,dd,J=8.4,1.2Hz,H-5’)	6.7(1H,d,J=8Hz,H-5’)
6’	118.04	119.0	6.79(1H,dd,8,1.6Hz,H-6’)	6.88(1H,dd,J=8.1,1.2,H-6’)
1’’	119.72	121.9		
2’’/6’’	109.09	109.09	6.85(2H,s,H-2’’/ 6’’)	6.9(2H,s,H-2’/6’)
3’’/5’’	145.90	149		
4’’	139.02	141		
C=O	166.09	165.9		
C=O	170.9	169.9		
CH_2O	60.28		4.01(4H,q,J=7.2,6.8,Hz,H-7’)	
CH_3	14.25		1.18(6H,t,J=7.2Hz,H-8’)	
CH_3	21.24		1.99(3H,s,acetoxy)	

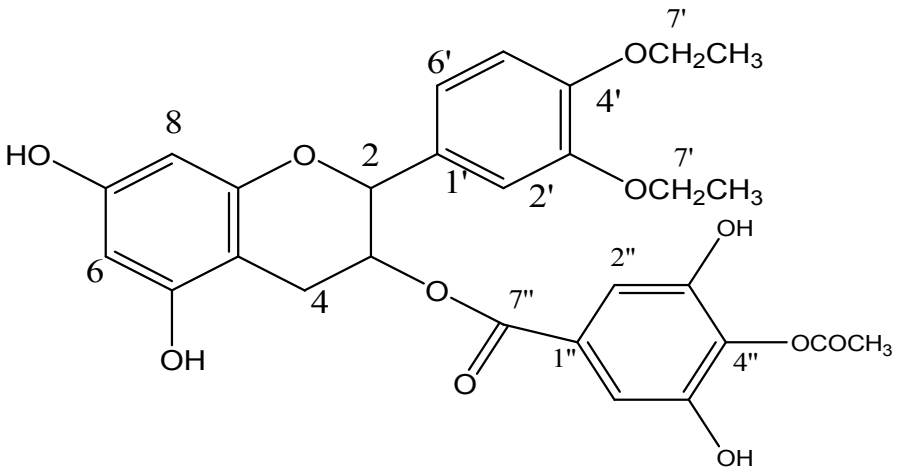


Figure: 6.The proposed structure of compound -17 (Dw-04)

4.3.3. Characterization of Compound- 18(Dw-05)

Compound- 18(Dw-05) was obtained as solid powder isolated from EtOAc extract. On analytical TLC, the compound had an Rf of 0.50 (90% EtOAc in MeOH). When the chromatogram was sprayed with vanillin-sulphuric acid it turned to reddish brown color, suggested that the compound is phenolic.

IR (KBr disc, Appendix 3a) spectra showed broad vibration at 3388cm^{-1} attributed to hydroxyl moiety (OH) sharp absorption at 1618cm^{-1} attributed to aromatic benzene ring, strong absorption band at 2914cm^{-1} due to C-H stretching of saturated moiety, absorption band at 1717cm^{-1} due to C=O stretching indicates the presence of carbonyl group of conjugated ester, and absorption band at 1226cm^{-1} due to C-O stretching. A band at 2852cm^{-1} to 2879cm^{-1} C-H stretching for CH_2O. Based on extensive analysis and interpretation of the spectroscopic data (IR and NMR) of the compound, a structure with an isoflavonoid skeleton, with a galloyl substituent at C-3 has been arrived at (see Fig.7). The detailed interpretation of the acquired spectral data is as follows.

The ^{1}H-NMR δ H (400MHz**,**DMSO-d_6,Appendix 3b,Table 6) , spectrum revealed the presence of proton signals at δ H 5.97 (1H,d,J=2,H-8) and 5.94(1H,d,J=2,H-6) suggest the presence of two meta coupled aromatic proton that belong to tetra substituted phenyl ring, ring A. The presence of signal with ABX multiplicity pattern at δ H 6.79 (1H,dd,J=8.4,1.6Hz,H-6') and δ H 6.67 **(**1H,d, J =8,Hz,H-2' and H-5') is indicative of a tri substituted benzene ring, ring "B," due to protons at C-2'andC-5' are overlapping owing to the same in chemical shift. Signal at δ H 5.86 (1H, d, J=2, H-2) and δH 5.39 (1H, m, H-3) suggested that the presence of two oxygenated

methines. Signals peaks at δ H 3.0 (1H,dd,*J*=17.2,4.4Hz,H-4a) and δ H 2.73(1H,dd, *J*=16.4,4Hz,H-4b) suggest the presence of diastereotopic methyelene protons at C-4 .
The signals at δH 4.01 (2H, q, J=7.2, 6.8Hz, H-7') and δH 1.18(3H, t, J=7.2Hz, H-8') suggested the presence of oxygenated methylene proton and methyl proton on ethoxy group moiety at C-4'position on" B" ring. Signal at δH 1.99(3H, s, 4''-acetoxy) on "D" ring. The galloyl moiety has para acetoxy on "D" ring, the signal at δH 6.85 (2H, s, H-2'' and H-6'') suggested that the presence of symmetric methine were observed. The pattern of the above mentioned protons strongly proposed as isoflavonoid skeleton with hydroxyl group at position 5, 7 and 3,'ethoxy group at position 4,' acetoxy group at position C-4''and the galloyl moiety at position C- 3 at ring C. Also they have one acetoxy group at para position on "D" ring.

The proton decoupling of the ^{13}C-NMR (100Mz, DMSO-d_6, Appendix 3c, and Table 6) spectrum showed well resolved resonance of 26 carbon atoms with their interpretations. The multiplicity of each carbon atom was determined by using DEPT-135 spectrum from the data (Appendix 3d), which showed the presence of two oxygenated sp^2 quaternary carbon was observed at δC 145.20(C 3') and δC 145.17(C 4'),suggesting the vicinal substitution pattern on ring "C" in agreement with the ABX multiplicity pattern.

Whereas methines appears at chemical shift: 68.64 (C-3), 76.98 (C-2), 94.8(C-8), 96.1(C-6), 109.08(C-2'', 6''), 114.74(C-2'), 115.0(C-5) and 118.0(C3). Thirteen signals are quaternary carbons at chemical shift carbon on:97.7(C-4a),119.70(C-1''),129.8(C-1'),139.0(C4''),145.17 (C-4'),145.20(C-3'),145.89(C-3'',5''),156.1(C-8a),156.95(C-7),157.0(C-5) and chemical shift of carbon at (δ C 165.64 and 170.9) carbonyl carbon of ester moiety. The presence of two sp^2 oxygenated quaternary carbon at chemical shift (δ C 156.10 (C-5) and δ C 157.01 (C-7) along with two up field carbons chemical shift at δ C 96.02 (C-6) and δ C 94.82 (C-8) suggest that ring "A" has 5, 7-dioxygenated substituent pattern. The signal at δ C 76.95 (C-2) and δ C 68.60 are clearly evident due to the presence of sp3 oxygenated methines on C-2 and C-3 ring C. Moreover, the presence of one methylene and oxy methylene (also supported by DEPT-135 pointing down, Appendix 2d) observed at δ C 26.14 (C-4) and δ C 60.28(from ethoxy group) are in good agreement with spectral data, whereas methyl signals appeared at δ C 14.25. The symmetry carbon signals were observed for C-2'' and C-6'' at a chemical shift δ C 109.09 and again at chemical shift carbon δ C 145.89 at C-3" and C-5" from galloyal moiety of the "D" ring.

From all above data, the following structure proposed for compound- 18 (Fig 7), which isolated from the root of *R.nepalensis spreng* by EtOAc extraction was 4-acetoxy- 4'-ethoxy epicatechin-3-O-gallate compound. The summary of ^{1}H-NMR and ^{13}C-NMR data of compound -18 and literature data is presented in (Table 6) [66, 67]. This is the first time that has been obtained from *Rumex nepalensis spreng*.

Table: 6. ^{1}H-NMR (400MHz, DMSO-d_6), ^{13}C and DEPT-135(100Mz, DMSO-d6 spectral data of Compound 18.

C-position	13C-NMR of compound-18(δ Cppm)	Reported 13C-NMR Copound18(δ C)	H-NMR for compound18(δ H ppm)	Reported 1H-NMR for Compound-18 [66,67]
2	76.953	78.8	5.84(1H,d,J=2,H-2)	5.17(1H,s,H-2)
3	68.602	70.0	5.38(1H,m,H-3)	5.53(1H,s,H-3)
4	26.130	27.0	2.99(2H,dd,J=17.2,4.4Hz,H-4a) 2.73(2H,dd,J=16.4,4.4Hz,H-4b)	2.99(1H,dd,J=17.3,4.5,H-4a) 2.85(1H,d,J=16.8,,H-4b)
4a	97.77	99.6	-	
5	157.00	157.4	-	
6	96.02	96.7	5.94(1H,d,J=2Hz,H-6)	5.75(1H,d,J=1.2Mz,H-6)
7	156.95	157.1		
8	94.8	95.8	5.95(1H,d,J=2Hz,H-8)	5.89(1H,d,J=1.2Mz,H-8)
8a	156.10	156.8	-	
1'	129.86	131.5	-	
2'	114.74	115	6.67(1H,d,J=8,Hz,H-2')	6.71(1H,d,J=8.Mz)
3'	145.20	145.9	-	
4'	145.17	146	-	
5'	115.04	115.9	6.67(1H,d,J=8Hz,H-5')	6.71(1H,d,J=8Mz)
6'	118.04	119.0	6.79(1H,dd,J=8,2,1.2Hz,H-6')	6.88(1H,dd,J=8.1,1.2,H-6')
1''	119.9	121.6	-	
2''/6''	109.09	110.4	6.85(2H,s,H-2" and 6'')	6.9(2H,s,H-2'/6')
3''/5''	145.89	146.5		
4''	139.09	140		
CH_2O	60.23	-	4.05(2H,q,J=7.2,6.8,7.2Hz,H-7')	
C=O	165.64	165.5		
C=O	170.9	-	-	
CH3	21	-	1.99(3H,s,3'-OCOCH3)	
CH3	14.56	-	1.17(3H,t,J=7.2Hz,H-8')	

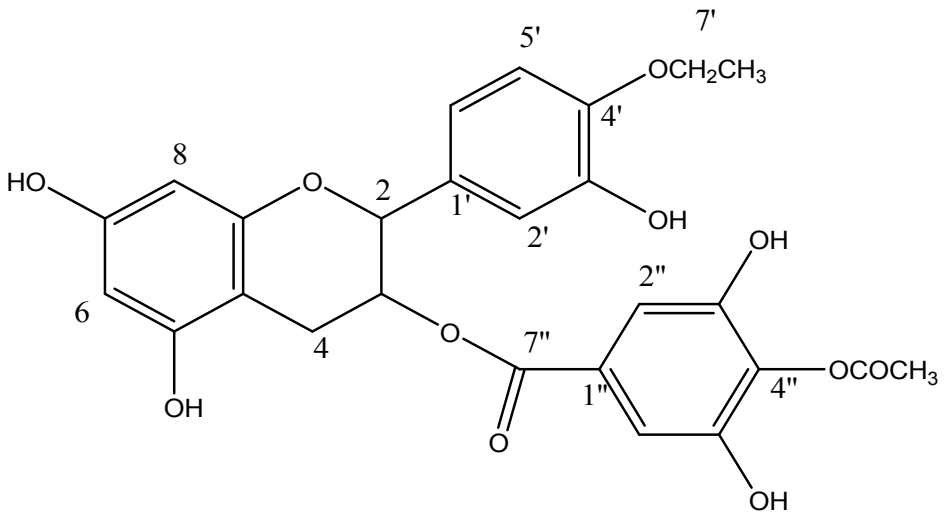


Figure: 7. The proposed structure of compound- 18(Dw-05)

Epicatechin-3-O-gallate compound -17 and 18 were belongs to the flavonoids, a class of metabolites with a wide range of pharmacological properties. It is a polyphenolic compound naturally abundant in *Rumex nepalensis*, green tea, grape seeds and other plants. It is reported to exhibit physiological effects, including antibacterial, antifungal, antiviral, antioxidant, anti-carcenogenic activity, it is also an anti-artherogenic, anti-hypertensive, and anti-tumer agent [68].Additionally, oleic acid can be used a candidate for antibacterial and antifungal activity. This supports the traditional use of *R.nepalensis spreng spreng* to treat diseases of bacterial origin and also as a skin tonic.

4.4. Analysis of Antibacterial Activities of *Rumex nepalensis spreng* root extract

The antibacterial activity was determined by measuring the diameter of zone of inhibition. All crude root extracts and the fraction of *Rumex nepalensis spreng* were investigated for their potential antibacterial activities and the plant was found to have maximum antibacterial activity. The results obtained in the evaluation of the antibacterial activity of the extracts were screened for antibacterial activities (Table 7). The Standard antibiotic chloramphenicol ⟨positive test⟩ showed good inhibitory action on the antibacterial tested and the negative control ⟨DMSO ⟩ has no effect on the inhibiting antibacterial test.

4.4.1. Antibacterial Activity

Two gram- positive (*Staphylococcus aureus ATCC25923* and *Bacillus subtilus ATCC6633*) and two gram negative bacterium (*Escherichia coli ATCC25922* and *Pseudomonas aeruginosa*

ATCC7553) used to evaluate the antibacterial activity of the crude root extracts of *R.nepalensis spreng* at concentration of 50 mg/mL. A 20 µg/mL of each crude extract and the isolated compound were placed at the center of the labeled plate.

The plates were incubated aerobically at 37^{o}c and examined for zones of inhibition. Among the extract of the root, methanol extract exhibited the highest inhibition zone against *p.* aeruginosa with inhibition zone of (17 ± 2.07mm), whereas EtOAc was the second with inhibition zone of ⟨13.5 ± 1.73mm⟩ against *S.aures* and least inhibition zone was observed for *n*-hexane extract against *E.coli and p.* aeruginosa with inhibition zone ineffective. Generally *n*-hexane extract was ineffective against all test organisms. *Bacillus subtillus* were the most susceptible test organism for all extract [69]. *E,coli* was the least susceptible test organism *n*-hexane,EtOAc and fractions extracts. The two fractions were more sensitive *p.* aeruginosa in habitation zone of (12.5±2.1 and 12±1). The antibacterial activity can be attributed to the phytochemical content of the sample extracts. The samples having phytochemical contents were found to be better in inhibiting the growth of bacteria hence were giving zone of clearance of greater diameter.

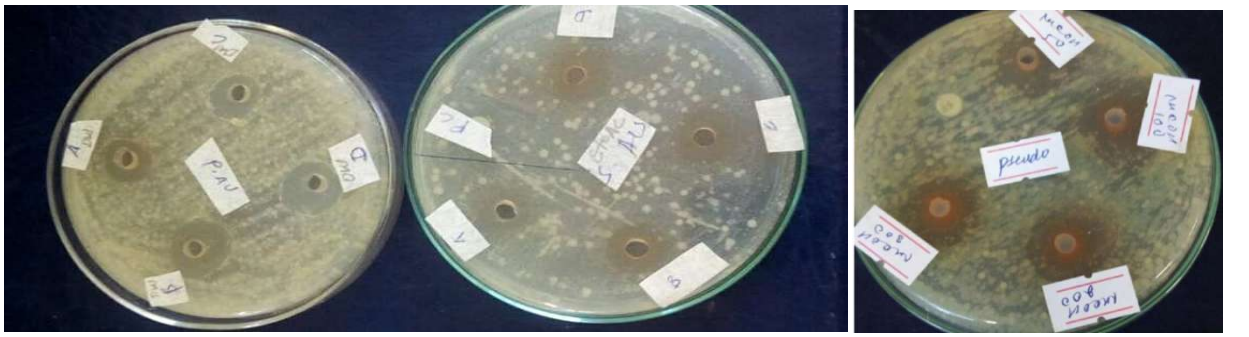
Table: 7. Zone of bacterial growth inhibition (mm) by root extract of *R.nepalensis spreng*

Pathogens	Plant Extract and their fractions(20 µg/mL)						
	Mean bacterial inhibition in mm± SEM						
	n-hexane	EtOAC	MeOH	Compound-16	Compound-17	DMSO (-ve)	Chloramphenicol (+ve control)
S.aureus	8±2.16	13.5±1.73	9±2.16	7±1.41	8±1.41	-	18±0.0
B.subtilus	10.5±1.9	9.5±1.29	16±3.65	6±0.00	6±0.00	-	18±2.30
E.colli	-	6.75±1.51	8.5±3.12	9±1.41	10.5±2.17	-	18±1.15
P.aeruginosa	-	11.25±2.27	17±2.07	12.5±2.12	12±1	-	18±1.15

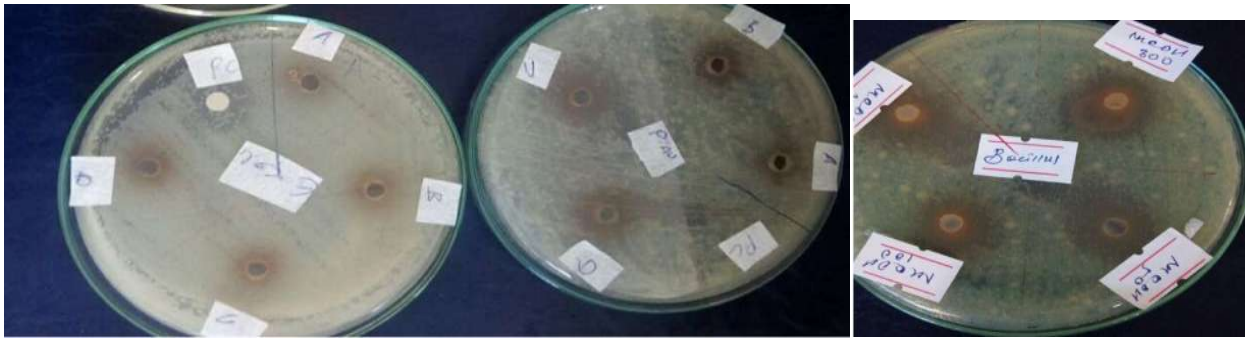
The value represent mean of four replication ± SD.

-ve control (DMSO), +ve control chloramphenicol.

MeOH(methanol extract),EtOAc(Ethyl acetate extract),SEM(standard error of the mean)



P.aeruginosa S.aures P.aeruginosa



E.coli P.aeruginosa B.subtilus

Figure 8 Antibacterial activities on root extract of *rumex nepalensis spreng*

Generally, the antibacterial activity of test plant is shown in (Table 7). The antibacterial efficacy of the solvent extracts namely *n*-hexane, EtOAc and methanol extracts of the plant against selected bacteria showed varied level of inhibition. All the solvent extracts showed very good activity against the test bacteria ranging from 6.0 –17.205 mm. The presence of the secondary metabolites indicated that the antibacterial activity is due to these active compounds present in root part of the test plant. The gram positive bacteria were slightly more susceptible to the extracts and showed greater inhibition zone than the gram negative bacteria [70]. The activities of the three extracts and two fractions of the plant were compared with standard drug chloramphenicol(30µg/disc). As shown in the (Table 7), the results revealed that, the isolated compounds showed promising against S.aureus,B.subtillus,E.coil and P.aeruginosa to that of chloramphenicol with the zone of inhibition diameter 18mm.This results shows the two compound and the genus Rumex are potential candidates that of chloramphenicol(18mm of inhibition zone).

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

In the present study, the dried roots of *Rumex nepalensis spreng* was extracted sequentially with n-hexane (2.5L),EtOAc(2.5L) and methanol(2.5L). The highest crude extract was obtained from methanol mixture extract (17.5%) which followed by EtOAc (1.725%) extract. However, the lowest yield was obtained from *n*-hexane extract (0.25%). Phytochemical compounds such as, flavonoids, saponins, steroids, Anthraquninone, glycosides, phenols and tannins have been tested from root extract of *Rumex nepalensis sprng* plant. Except Alkaloids, all of the components tested were present in EtOAc and methanol extracts.TLC analysis of the crude extract revealed that the compounds extracted are not the only compounds present in the plant material.

The chemical components in EtOAc root extract of *Rumex nepalensis spering* by column chromatographic analysis clearly showed the presence of three compounds. The three compounds are: oleic acid (16), 4"-acetoxy-3', 4'-diethoxy epicatechin-3-O-gallate compound *(17)* and 4''-acetoxy-4'-ethoxy epicatechin-3-O-gallate (18) were isolated for the first time from this plant species. The extract also tested against two gram positive bacteria (*Staphylococcus aureus,bacillus subtillus*), and two gram negative bacteria (*Escherichia coli*, P.aerugnosa). All of the three crude extracts have significant antibacterial activity on most of the bacteria. Whereas, methanol extract had maximum inhibition activity as compared to EtOAc mixture and *n*-hexane.

The methanol extract of roots of *Rumex nepalensis spreng* has shown the maximum antibacterial activity regardless of the solvent system. The isolated compounds showed promising against S.aureus, B.subtillus, E.coli *and* and *P.aerugnosa* to that chloramphenicol with zone of inhibition diameter 18mm compared from the data (Tble7). The antibacterial activities exhibited by various extracts of roots were observed, however, less than the standard drug (chloramphenicol) used. Generally, the result of the current study used to confirm the traditional practice of this medicinal plant for treatments of some antibacterial infection and other diseases**.** These compounds have not been tested previously in the plant roots and might be responsible for the claimed activities by local people. Therefore, the plant root has a high potential for a vast number of bioactive compounds which justified its use for various ailments by traditional practitioners. These finding s have provided scientific basis to the ethno medical usage of the plant.

5.3 Recommendation

- The study has demonstrated that there is need for further investigation and isolation of other compound from *Rumex nepalensis spreng* and other plant family of polygonaceae.
- Additionally, the crude and the pure extract of *R.nepelensis* need to be subjected to further tests on other disease causing bacteria.
- Moreover, there is need to carry out phytochemical studies of the plant species methanolic extract and all other fraction that were not analyzed in this study.

- Furthermore, the isolated compound that showed the highest activity could be subjected to more studies such as cytotoxicity test, in order to be used as antibacterial or templates for the synthesis of drugs used in the treatment of diseases caused by bacteria.

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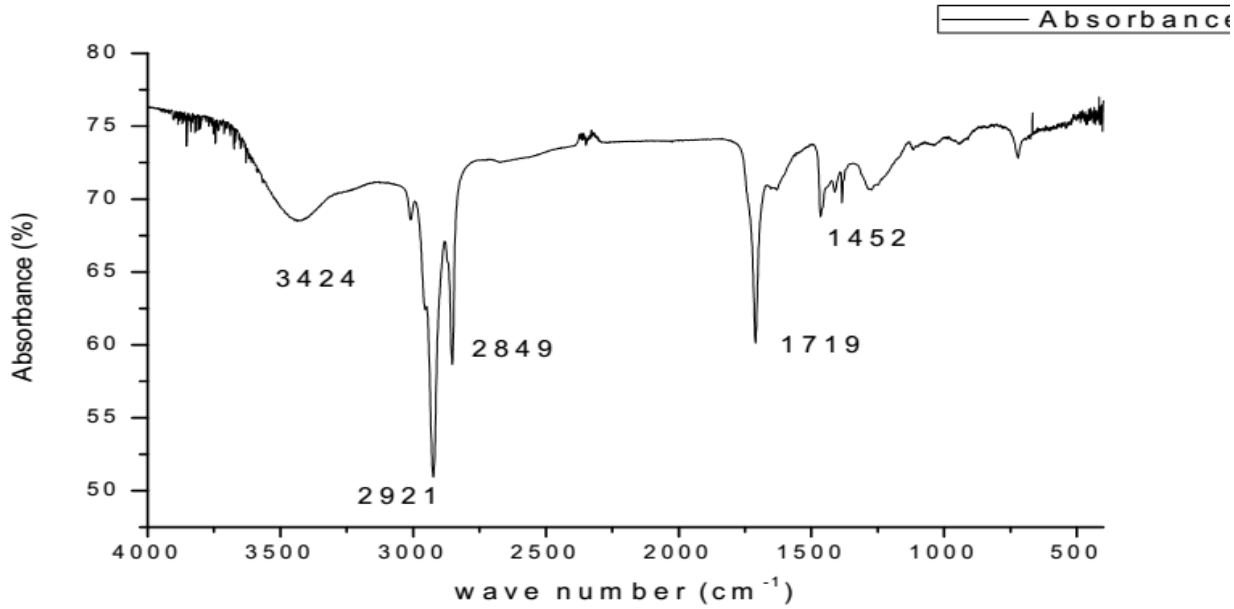
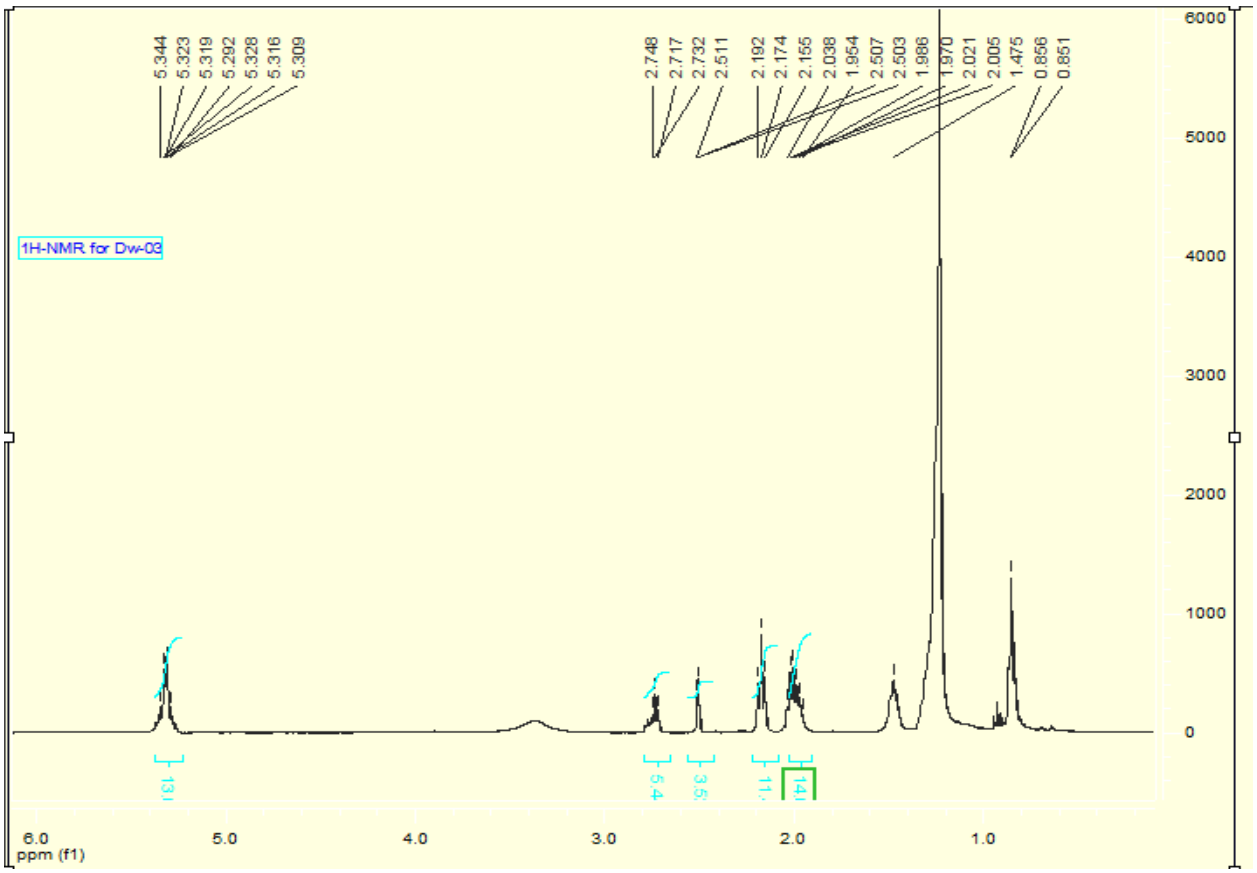
Appendix

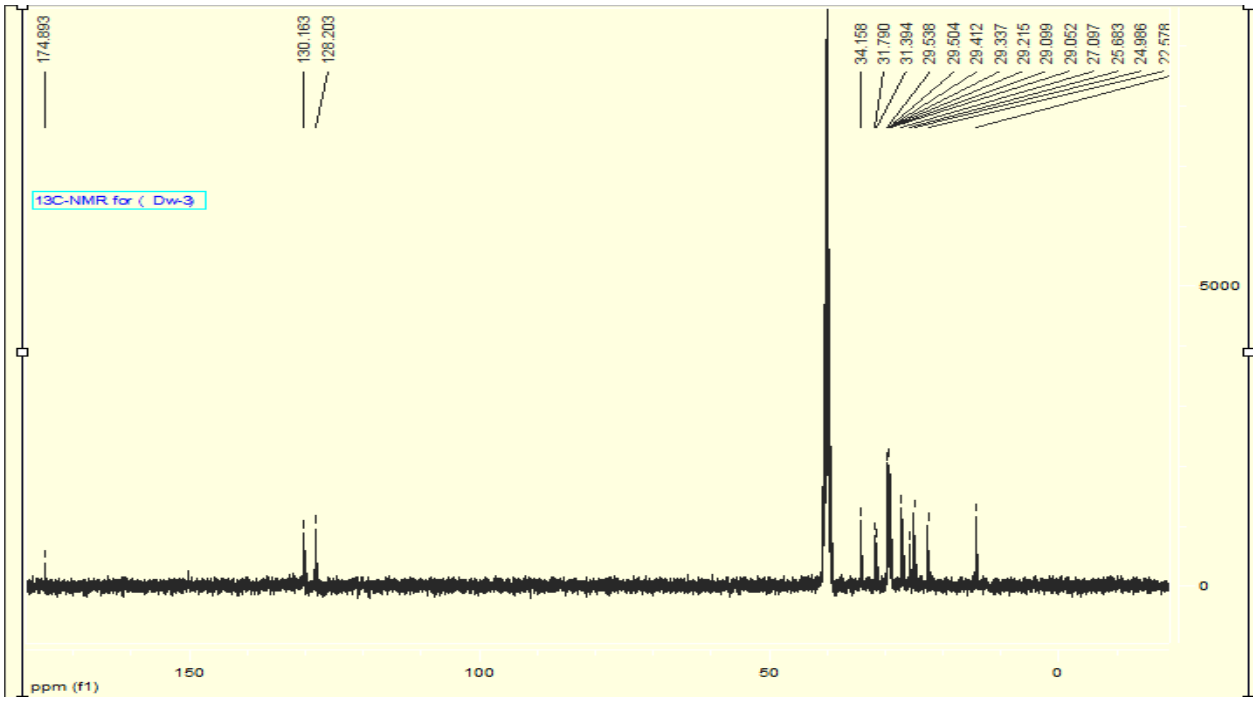
Appendix 1a FT-IR for compound 16(Dw-03).



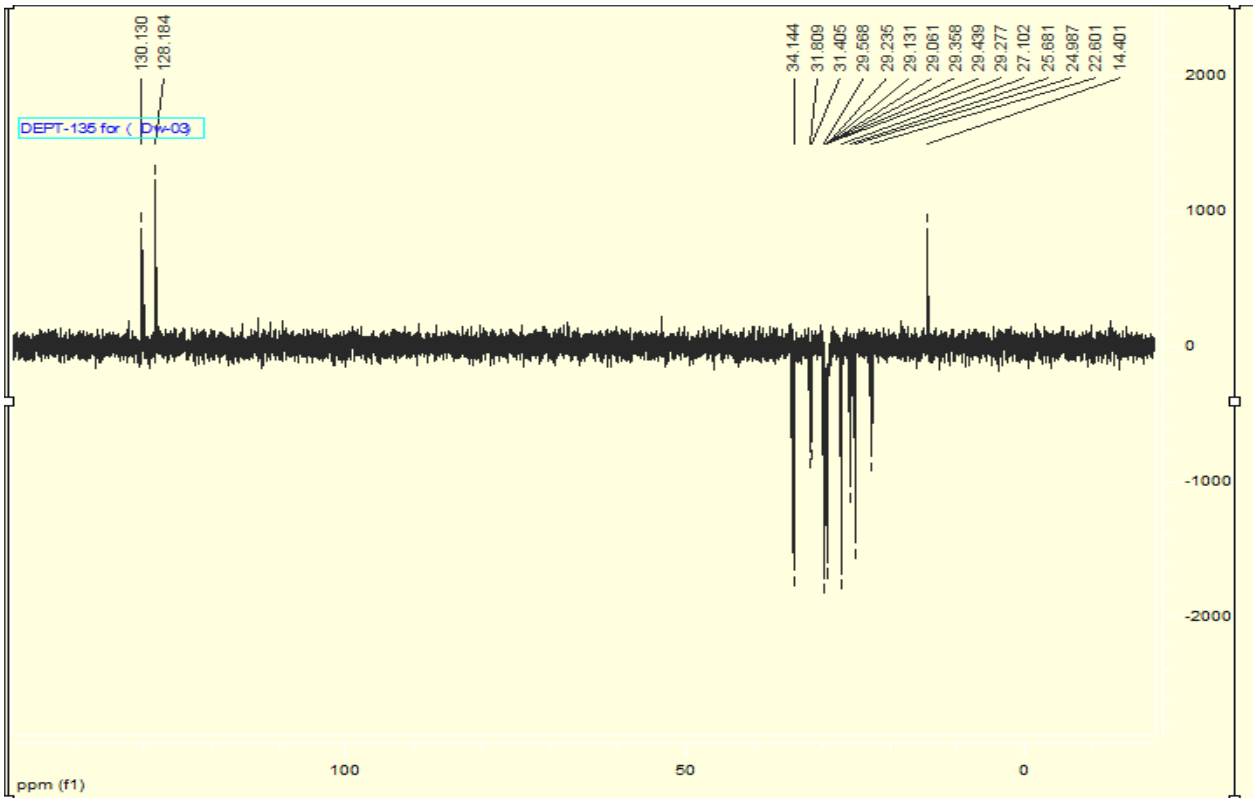
Appendix 1b 1H-NMR for compound 16 (Dw-03)



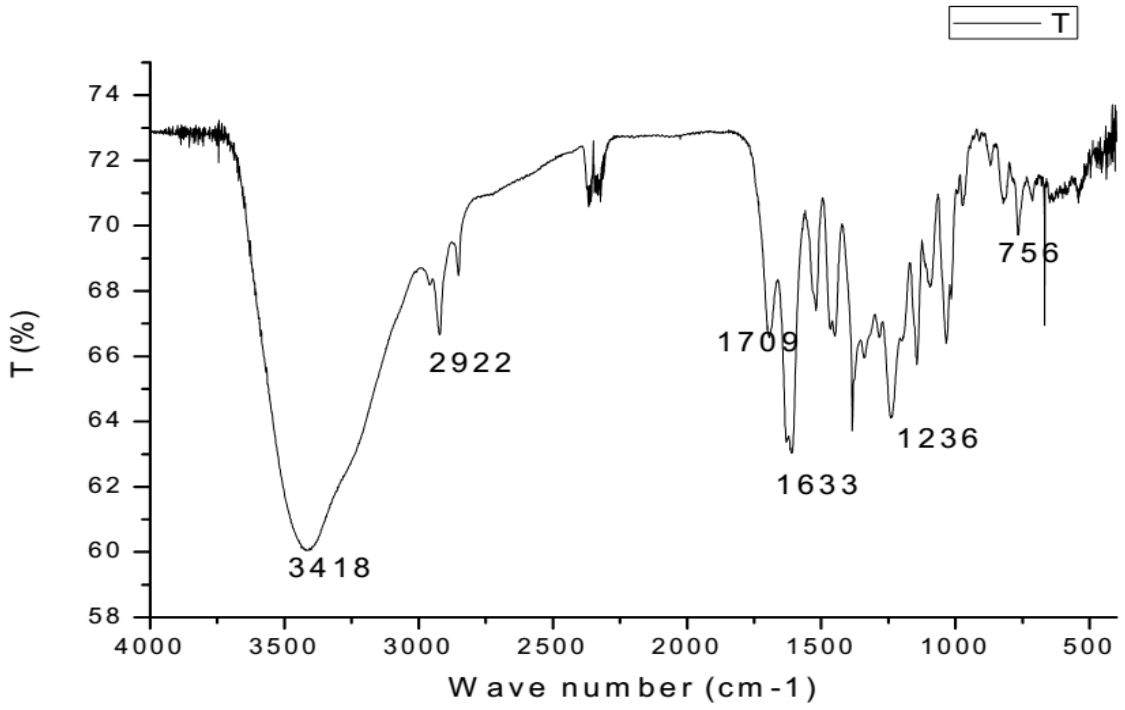
Appendix 1c 13C-NMR for compound 16(Dw-03)



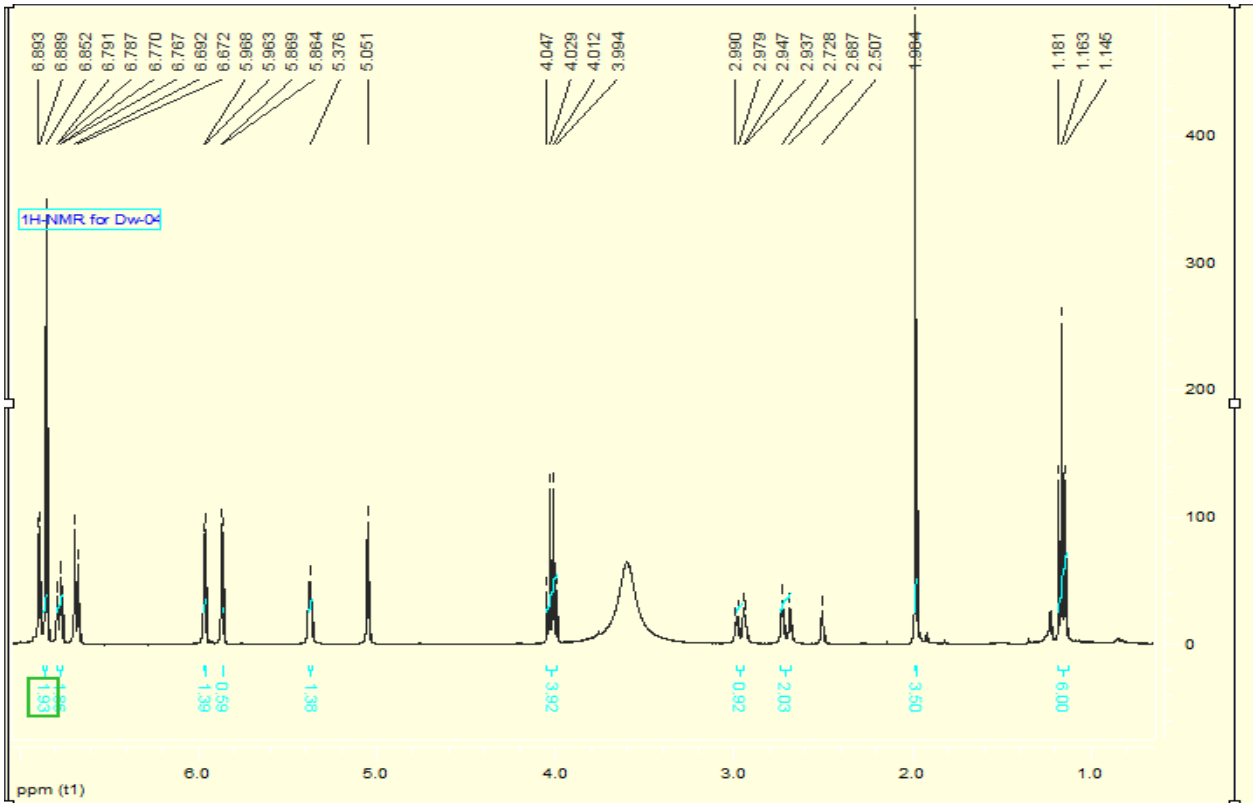
Appendix 1d DEPT-135 for compound 16(Dw-03)



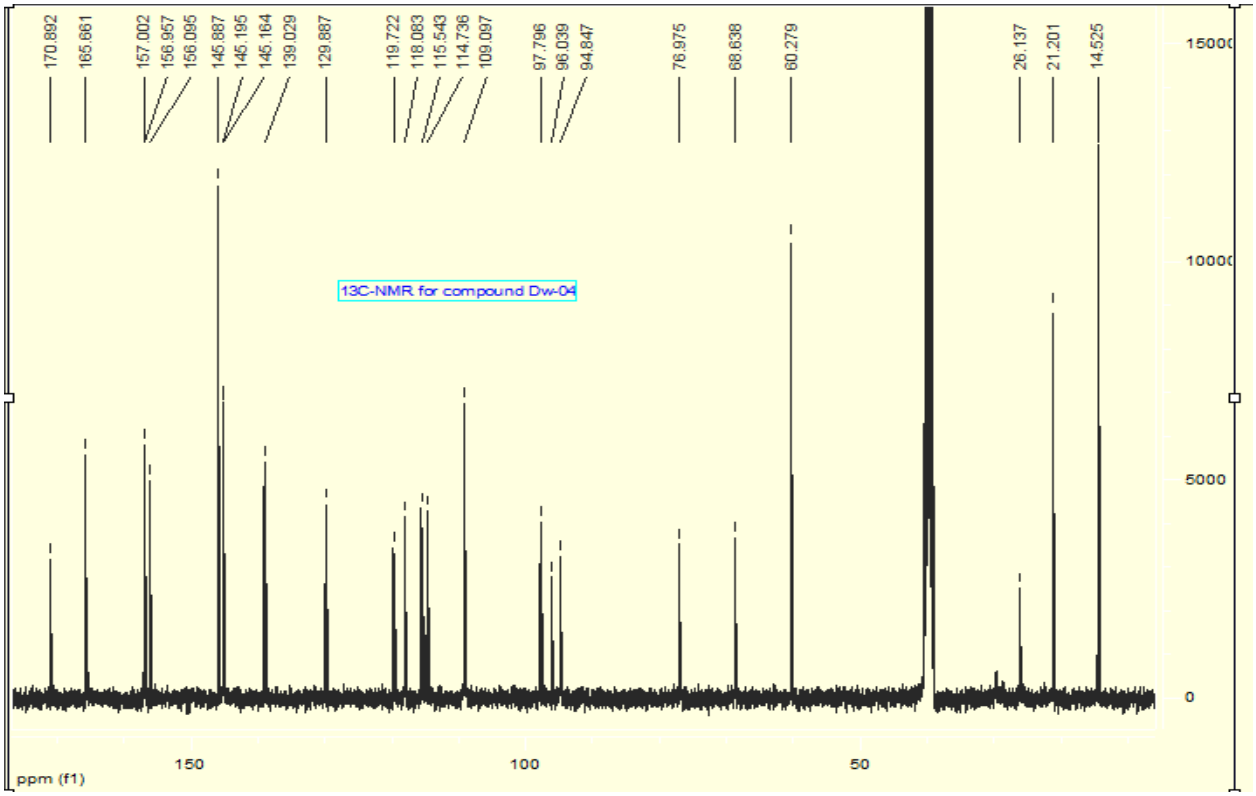
Appendix 2a FT-IR for compound 17(Dw-04)



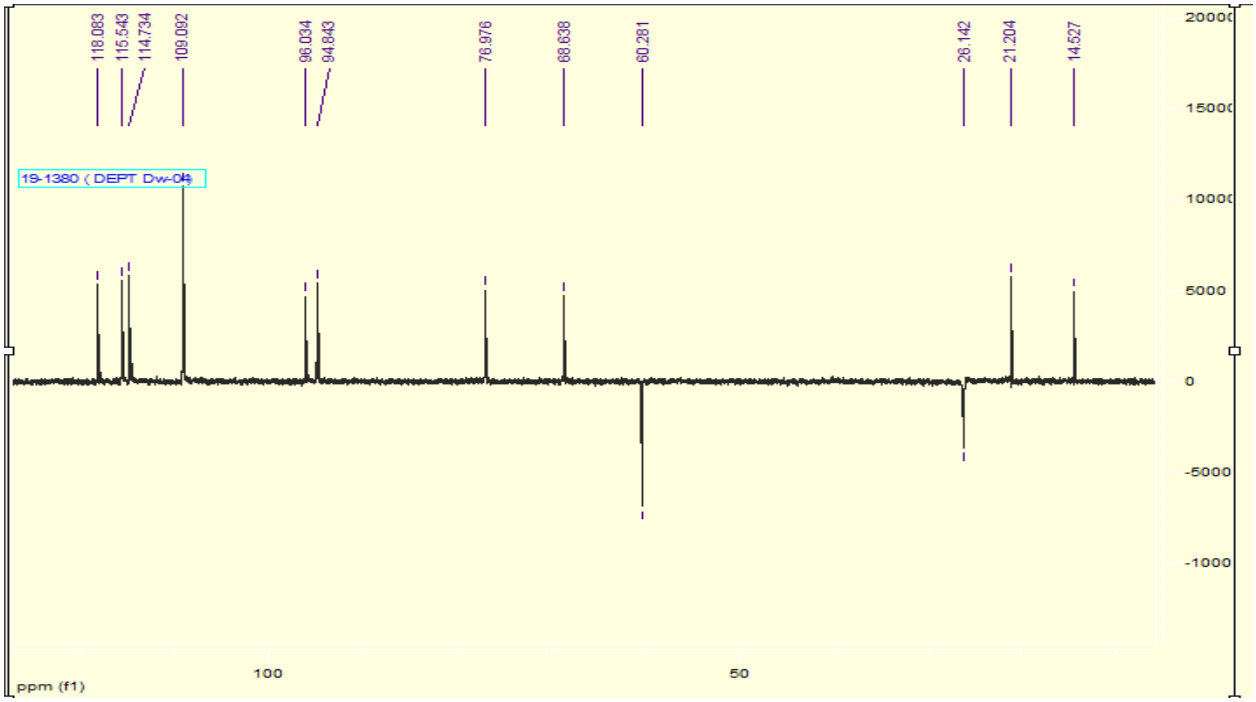
Appendix 2b 1H-NMR for compound 17(Dw-04)



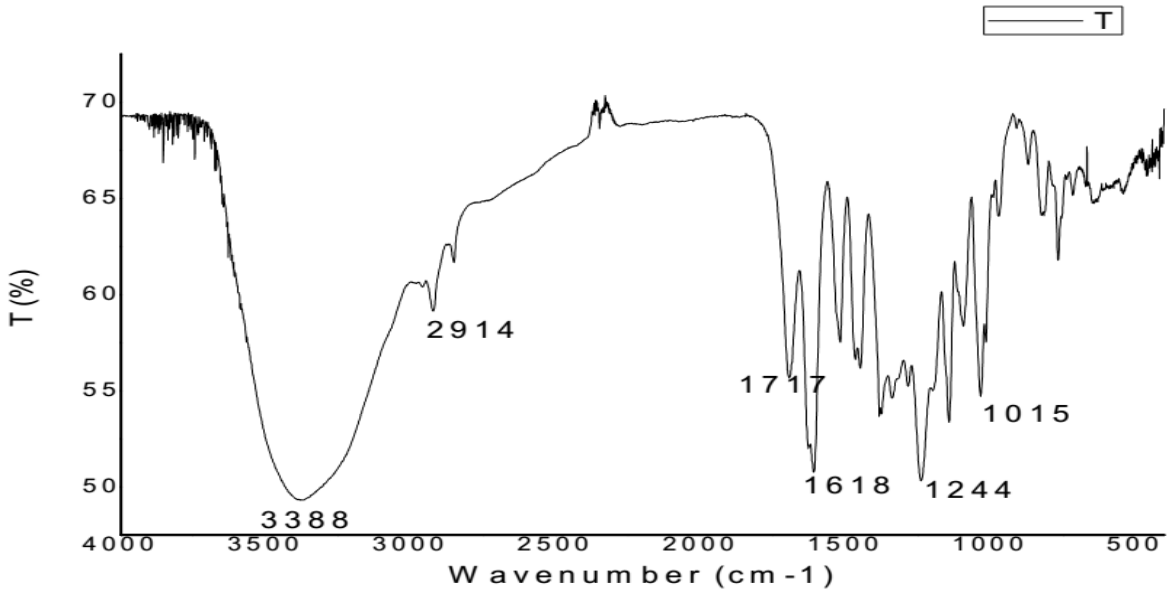
Appendix: 2c ^{13}C-NMR for compound 17 (Dw-04)



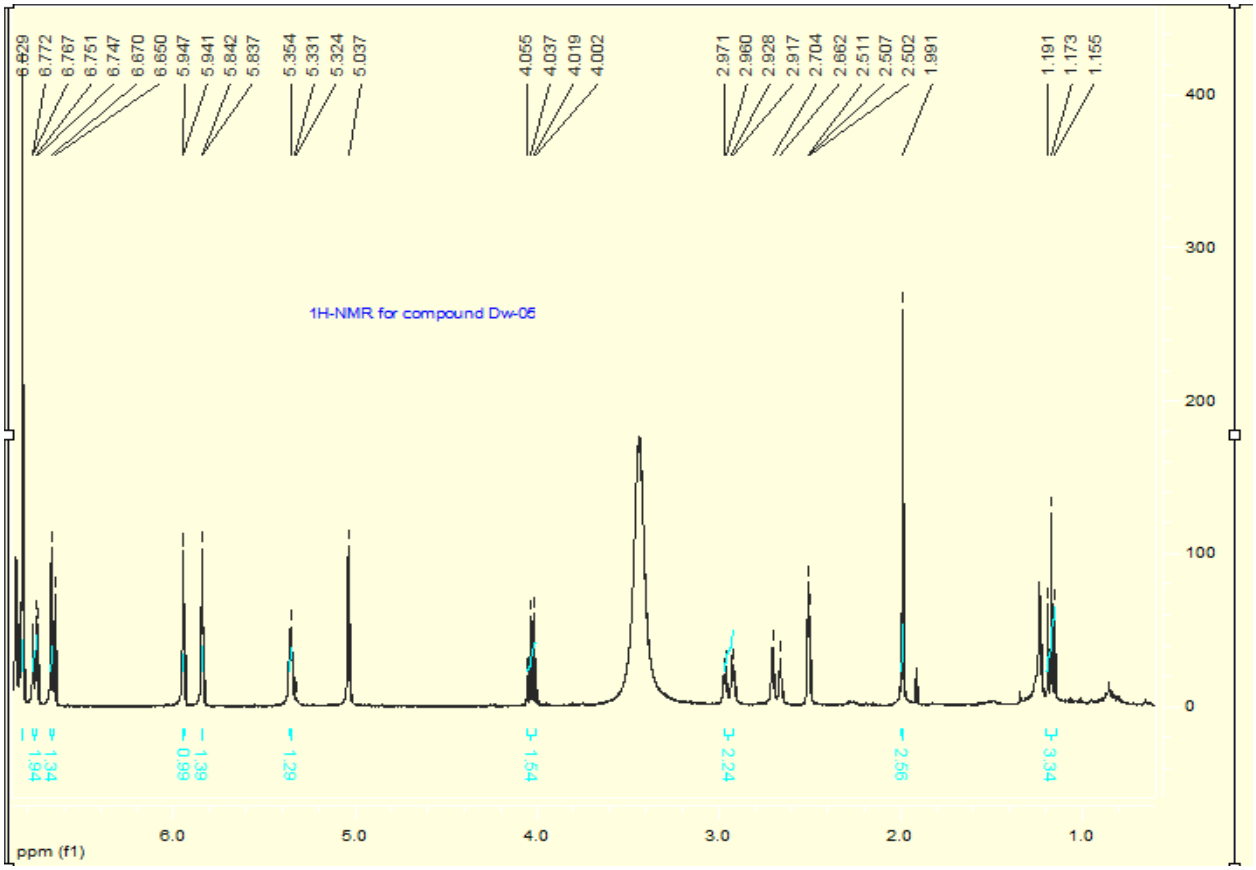
Appendix: 2d DEPT-135 for compound 17(Dw-04)



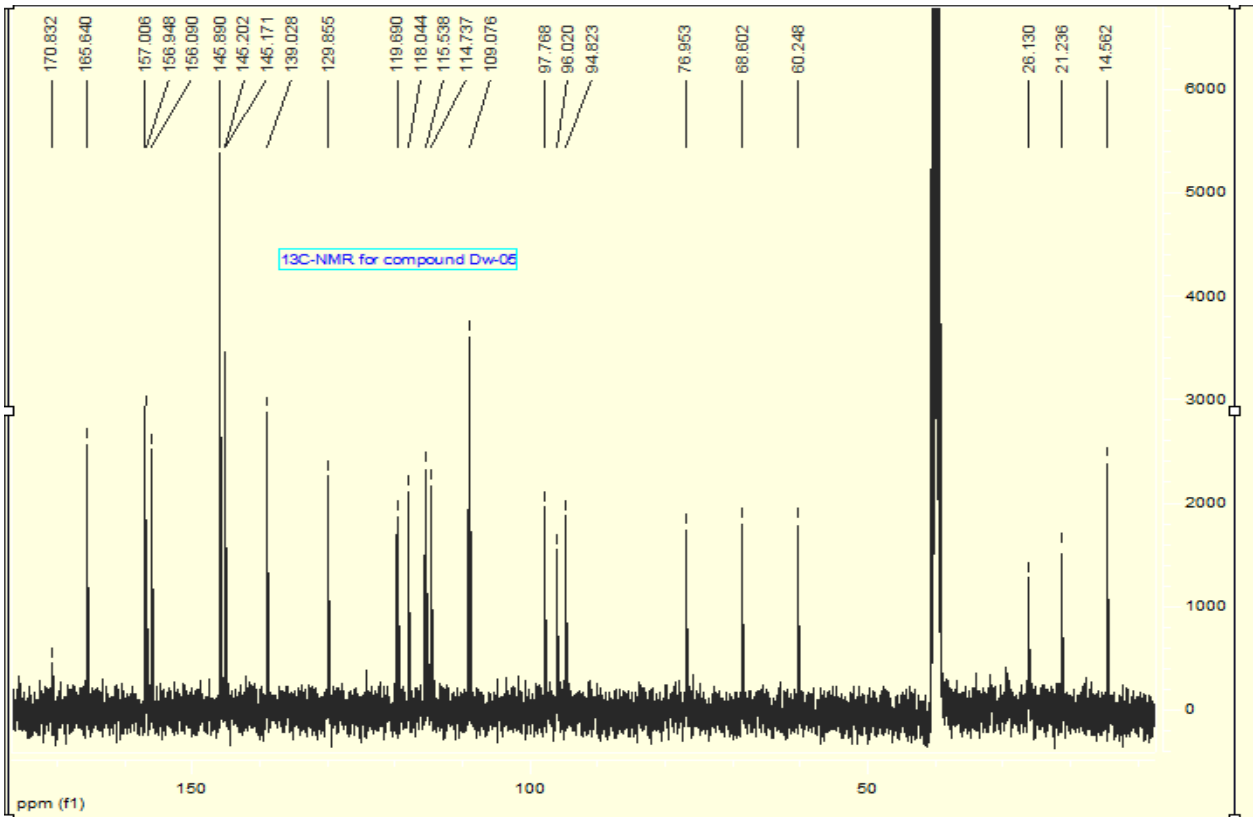
Appendix 3a FT-IR for compound 18(Dw-05)



Appendix 3b 1H-NMR for compound 18(Dw-05)



Appendix 13C-NMR for compound 18(Dw-05)



Appendix 3d DEPT-135 for compound18 (Dw-05)