ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

SCHOOL OF APPLIED NATURAL SCIENCE
APPLIED CHEMISTRY PROGERAM

# PHYTOCHEMICAL INVESTIGATION OF THE ROOTS OF *RUMEX NERVOSUS*

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August, 2015
ADAMA, ETHIOPIA

# PHYTOCHEMICAL INVESTIGATION OF THE ROOTS OF *RUMEX NERVOSUS*

THESIS SUBMITED TO THE SCHOOL OF GRADUATE STUDIES ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY(ORGANIC CHEMISTRY STREAM)

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August, 2015

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## ABBREVIATITIONS

| | |
|---|---|
| AROM | Fungal pentafunctional complex of enzymes 2-6 in the shikimate pathway. |
| CC | Column Chromatography |
| $^{13}$C-NMR | $^{13}$Carbon Nuclear Magnetic Resonance |
| CS | Chorismate synthase |
| E-4-P | Erythrose-4-phosphate |
| d | doublet |
| m | multiplet |
| DAHP | 3-deoxy-D-arabino-heptulosonate-7- Phosphate |
| DEPT | Distortionless Enhancement by Polarization Transfer |
| 3-DHS | 3-dehydroshikimate |
| 3-DHQ | 3-dehydroquinate |
| Fig | Figure |
| $^{1}$H-NMR | Proton Nuclear Magnetic Resonance |
| IR | Infrared spectroscopy |
| NAD | New and Approved Drugs |
| NCI | National Cancer Institute |
| NMR | Nuclear Magnetic Resonance |
| ppm | parts per million |
| PTLC | Preparative Thin Layer Chromatography |
| Rf | Retardation factor |
| RN | *Rumex Nervosus* |
| t | triplet |
| TLC | Thin Layer Chromatography |
| TM | Traditional Medicine |
| UV-Vis | Ultraviolet-Visible |
| WHO | World health organization |

# Acknowledgement

I express my sincere gratitude and appreciation for my research advisor **Dr. Teshome Abdo** for his ongoing involvement, contributions, and support throughout this research process. **Dr. Teshome Abdo** knew when to encourage my efforts and more importantly, when not to intervene. I thank him from my heart for endless hours of toil over draft after draft, editing, directing, encouraging, and with consistent reminders for patience. His experience in paying attention to minute details in writing was a great asset in keeping me focused on the final products. I am indeed lucky to have him as a research mentor and friend.

I wish to express my deep appreciation to **Dr. Aman Dekbo** for his time, support, encouraging, guidance and technical advice. I have received during the completion of this research project.

My thanks and appreciation also goes to **Adama Science and Technology University Applied chemistry Progeram** for assistance in materials for the extraction and isolation of sample.

My thanks is also expressed to **Addis Ababa University Chemistry Department** for running spectra of the sample.

I am grateful to my family, friends and others who deserve to be acknowledged for their support and continued encouragement in making this endeavor a reality. This is an opportunity to express my most humble appreciation and gratitude to each and every one of them.

# Abstract

*Rumex nervosus,* belongs to the family of polygonaceae, which is traditionally used for the treatment of infectious diseases. Ground root parts of *Rumex nervosus* were subjected to exhaustive extraction successively with petroleum ether and methanol. The solvent from each extract was evaporated under reduced pressure using rotavapour to obtain petroleum ether and a methanol extract. Chromatographic purification of the methanol extracts by Column Chromatography followed by Preparative Thin Layer Chromatography using Chloroform**:** methanol (9**.**5**:**0**.**5) gave a compound coded as **RN-6**. The structure of this compound (4-ethylheptyl benzoate) was characterized by means of $^{1}$H NMR, $^{13}$C NMR, UV and IR spectral data.

# 1. INTRODUCTION

## 1.1 Preamble

The history of plant based health care goes back to antiquity and as old as human civilization. Plants have been primary source of medicines in the traditional health care systems around the globe, till recently and even currently in most of the developing countries. The approach to characterization and isolation of active ingredients from plants started in the late $19^{th}$ century. Consequently chemical substances isolated are currently used as important drugs as such or as their derivative(s) today. From 1983 to 1994, 39% of the New Approved Drugs (NAD) was of natural origin i.e. original natural products, products derived semi synthetically from natural products and synthetic products based on natural molecules [1]. Parallel to synthetic drug demand, the global natural products market is growing exponentially. The global demand for botanical and plant derived drugs were estimated to increase from 19.5 billion in 2008 to 32.9 billion USD in 2013, with a compound annual growth rate of 11.0% [2]. The number of higher plant species on this planet is estimated at 250,000 [3] of these, only around 6% have been screened for biological activity, and of which only 15% are reported to be phytochemically characterized [4]. Asia, especially the southern region shares about 20% of the all known vascular plants in the Globe. This includes 7000-8000 species of medicinal plants. Out of the 17,500 flowering plant species found in India, over 1600 are used in traditional medicine [5]. According to world health organization (WHO), 65-80% of the global population use plants and plant products for their primary health care [6]. The Investigations on therapeutic applications of plants have led to the discovery of several clinically applicable drugs (e.g., digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine etc.).

Elucidation of the structure of active principles paved the way for synthesis and derivatization for compounds with higher efficacy and lower adverse effects (e.g., Metformin, nabilone, oxycodon, taxotere, teniposide, verapamil, amiodarone etc) [7]. Thus plants continue to engage the attention of scientists associated with drug discovery. Evidences accumulated thus clearly show that plants are rich source of bioactive chemical entities. Many phytochemicals are capable of modulating biochemical pathways of higher animals. However phytochemicals can be beneficial or harmful. There is sufficient traditional knowledge to substantiate this but further studies are required to index plants with beneficial and adverse effects. Based on the traditional knowledge, some plants and plant products are documented to be non-toxic and therapeutically potent. This knowledge can be exploited to develop cheaper plant based product/formulation for preventive health and disease management. However, scientific evidences need to be created for their efficacy through pharmacological and chemical studies.

## 1.2. Plant Based Traditional Health Care: an Overview

According to WHO, Traditional Medicine (TM) refers to health practices, approaches, knowledge and belief incorporating plant, animal and mineral based medicine, spiritual therapies, manual techniques and exercises applied singularly or in combination to treat, diagnose and prevent illness or wellbeing [8]. Naturopathic, Ayurveda, Reiki, Chinese, Native American medicine, Homeopathic medicine, etc. are some examples of traditional health care systems. Fossil records date, use of plants as medicines at least to the middle Paleolithic period, some 60,000 years ago [9].

One of the oldest records related to the plant based medicine is the Papyrus Eber written nearly 1500 BC and contains information of more than 500 natural ingredients [10]. Similarly, Asia's one of the traditional systems of health care is Chinese medicine that primarily use plants for disease management. Shennong Bencao Jing is the first Chinese herbal book which was compiled during the Han Dynasty but dating back to a much earlier date, possibly 2700 B.C., that lists 365 medicinal plants and their uses. During the middle age, many medical texts have been written. Causes and Cures were written by a $12^{th}$ century Benedictine nun. `Herbals and medical texts migrated from Asia to middle East and West. The knowledge and applications of herbals greatly increased during the period 15 to $17^{th}$ century. The books published during that period include Great herbals, 1526; General history off plants, 1597 etc. Indian Materia Medica includes about 2000 drugs of natural origin almost all of which are derived from different traditional systems and folklore practices [11]. "Indian traditional medicine "Ayurveda" is a store house of knowledge on traditional health care.

A most authentic compilation of the teaching of Sushrutha, called "Sushrutha Samhitha" contains description about 1120 illness and 700 medicinal plants [12]. Charak Samhita' and 'Sushruta Samhita' described not only the medicinal properties of individual plants but also the poly herbal and herb mineral preparations. These resources, that emphasize the doctrines for life with healthy mind and body, were evolved through day-to-day life experiences as a part of cultural heritage of India. Besides Ayurveda, there are several other complementary and alternative systems of medicine like Homeopathy, Siddha and Unani, which are also practiced and developed with the course of time in India, where plants and plant-based formulations are employed for health care and disease treatments.

In "Ayurveda" and almost all other traditional systems of medicine, the predominant raw materials used are plants that they play a major role and constitute the backbone. These systems are based on experience and interaction with nature and natural resources. TM is rapidly growing health care system with great economic importance. In developing countries majority of the population uses TM to meet their health care needs. In several regions viz. Asia and Latin America, people use TM as a result of their historical background and cultural beliefs. Meanwhile, in many developed countries, TM is becoming more and more popular.

In developing countries, broad use of TM is often attributable to its accessibility and affordability and by concern about the adverse effects of chemical drugs used in allopathic. Therefore many people trust the gentleness of TM in managing diseases than allopathic medicine. Moreover together with demand for TM, concerns over the safety, efficacy and quality of TM, products and practices related to it have also been raised. Since TM has developed with in different cultures in different regions, there has been no parallel development of standards and methods. Therefore scientific evidence to prove the rationale of using these formulations in health care is essential to develop and to preserve the cultural heritage. Approaches like high-throughput screening, phytochemical profiling, quality control and standardization of raw materials and formulations, pharmacokinetics, pharmacovigilance and clinical trials of herbal therapeutics will not only help to prove the rationale of using these systems but also to get maximum benefits of the natural resources [13].

## 1.3. Importance of Natural Product

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as secondary metabolites, often are differentially distributed among limited taxonomic groups within the plant kingdom. Their functions, many of which remain unknown, are being elucidated with increasing frequency. The primary metabolites, in contrast, such as phytosterols, acyl lipids, nucleotides, amino acids, and organic acids, are found in all plants and perform metabolic roles that are essential and usually evident [14].

The term ‘natural product’ is commonly used for those organic compounds of natural origin that are unique to one organism, or common to small number of closely related organism. Natural products are secondary metabolites of an organism [15]. Compounds and extracts derived from the natural product have found uses in medicine, agriculture, cosmetic, and food in ancient and modern societies around the world. Therefore, the ability to access natural products, understand their usefulness, and drive applications has been a major driving force in the field of natural product research [16]. Individual secondary metabolites may be common to a number of species or may be produced by only one organism.

Relative species often have related patterns of secondary metabolite production; according to the secondary metabolite they produce species can be subdivided in different parts. Such classification is known as chemotaxonomy. Two plants may be found to have identical physical make up which Botanists use for classification, but differ in the secondary metabolites they produced. Such different situation is known as chemo types [17]. Natural product chemistry covers the chemistry of naturally occurring organic compounds, their biosynthesis, function in their environment, metabolism and structure elucidation and synthesis [18].

Natural products are generally classified according to structure, physiological activity, taxonomy, and biochemical origin [19]. The World Health Organization (WHO) estimated about 80% of the people in the developing countries relies on traditional medicine for primary health care needs, of which a major proportion corresponds to plant extracts [20]. Synthetic organic chemists have then been able to produce the molecules in vitro and so produce them on large scales [21].

Natural products in fact do have a function in the organism from which they originate. Many of them are now considered to have vital roles as mediators of ecological interactions, thereby ensuring the continual survival of a particular organism. The ability to synthesize an array of secondary metabolites, which may repel or attract other organisms, is once facet of the survival strategy [22]. Flower color, pollination and deterrent properties in plants and chemical defense in termite may explain some of the importance of secondary metabolites for the survival of an organism [23].

Therefore, there is no similar chemical investigation on *R.nervosus* was result in the identification of bioactive and a fragrance constituent as far as the knowledge of the researcher is concerned.

## 1.4. Objective of the study:

### 1.4.1. General Objective: -

The general objective of this study is to isolate and elucidates structures of some major constituents from the root of *Rumex nervosus*.

### 1.4.2. Specific Objectives: -

The Specific objective of this study is:

- To extract the plant roots using different solvents
- To isolate secondary metabolites from the root of *Rumex nervosus* using chromatographic techniques
- To elucidate the structure of the isolated compounds using a combination of spectroscopic methods

# 2. LITRATURE REVIEW

## 2.1. Phytochemical Studies

Phytochemical studies of plants, animals and microorganisms helps in order to have deeper understanding of the factor underlying for the growth, development and differentiation of their chemistry out comes [24]. Plant and animals produce a very large numbers of chemicals. These chemical products can be classified in to primary and secondary metabolites. Primary metabolites are those, which are common to all species and can be subdivided in to proteins, carbohydrates, lipids and nucleic acids. Primary metabolites are essentially ubiquitous and certainly essential for life and they function in cycle [25].

The secondary metabolite are often referred to as ‘’natural products’’ they can be subdivided in to trepenoids, alkaloids, steroids, shikimates and polyketides. The classification is based on the means by which the materials were made. In principle, secondary metabolites are non- essential to life but they definitely contribute to the species fitness of survival. Different plant and animal species can employ different biosynthetic route to produce the same metabolites [26, 27]. The contribution of natural products to the development of medicine could be demonstrated by the amount of plant-derived drugs being used. In general 40% of modern drugs are said to be of natural origin. Many antecedents, plant growth regulating hormones, fungicides and agricultural chemicals are developed from natural products from time to time [28].

## 2.2. Medicinal Use of the Genus *Rumex* Species

Natural product is a major source of drugs, with more than 25% of the pharmaceuticals in use today derived from natural product, therefore interest in natural product research remain strong [29]. The Family of Polygonaceae has about 40 genera and 800 species. These include *Rheum* (50 species), *Rumex* (about 180 species). *Fagopyrum* (15 species), *Coccoloba* (150 species.) and *Polygonum* [30]. They are herbs, shrubs, climbers or rarely trees.

Several species of *Rumex* have important medicinal properties and they have been the subject of several pharmalogical investigation. The *Rumex genus* comprises several species of which leaves and roots have been used in traditional medicine for inflammation, blood purification, constipation, purgative and tonic in Turkish traditional medicine [31]. *Rumex nervous* leaves are an edible, consumed by some people in Saudi Arabia. Phytochemical screenings of *Rumex* species have revealed the presence of antraquinone derivatives, flavonoids, terpenes, organic acids and naphtalane derivatives [32]. The roots of *Rumex nervosus* used as anti-microbial and anti-inflammatory activity [33]. *Rumex nervosus* is commonly known as athrob or uthrub in the Arabian Peninsula. It has been used for medication in many diseases such as ophthalmic, pharyngitis, stomach-ache, dysentery, wounds and diarrhea and also has been considered as medicine for livestock [34, 35].

## 2.3. Botanical Background of *Rumex nervosus*

*Rumex nervosus* is commonly found near and around the terraces of high altitude areas (above1000m.). Genus *Rumex* is a genus of about 180 species of annual, biennial and perennial herbs in the buck wheat family Polygonaceae. Members of this family are very common perennial herbs growing mainly in the northern hemisphere, but various species have been introduced almost everywhere.

Some of the species which are found in Ethiopian are *Rumex nervosus, R. epalensis, R. crispus, R. vesicarius, R.abyssinicus, 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.nepalensis, R.maritimus, R.alpinus, R.palustris and R.obtusifolius* [36]. Traditionally in Ethiopia, the leaves, stems and sometimes roots of *Rumex nervosus* are used as traditional medicines, for the eye disease, taeniacapitis, haemorrhoids, infected wounds, arthritis, eczema, abscess and gynecological disorders.

## 2.4. Shikimic Acid

Shikimic acid is the key intermediate of large group aromatic natural products. For the first time, shikimic acid was isolated in 1885 from fruits of aniseed – a plant originating from Japan – *Illicium anisatum* and the fruit of *I. religiosum*, whose Japanese name was "*shikimi-no-ki*" (*shi* º four; *kimi* º seasons; *no* º of; *ki* º tree, literally "tree of four seasons") [37] . Shikimic acid has since been found in many plants, bacteria, yeasts and moulds. It is estimated that this group accounts for 35% of plant dry mass, and that one-fifth of the carbon fixed by plants is channeled to shikimic acid metabolites, such as the lignins. Shikimic acid is the precursor of three important aromatic amino acids: phenylalanine, tyrosine, and tryptophan (Figure 1). The acid, in turn, being simultaneously the key metabolite of a pathway leading to the formation of chorismate, has given rise to the name of the pathway. The shikimic acid pathway, also referred to as shikimate pathway, occurs in cells of various groups of microorganisms, plants [38] and parasites [39]. It has not been detected in animal organisms and for this reason; the shikimate pathway constitutes a good source for designing antimicrobial agents.

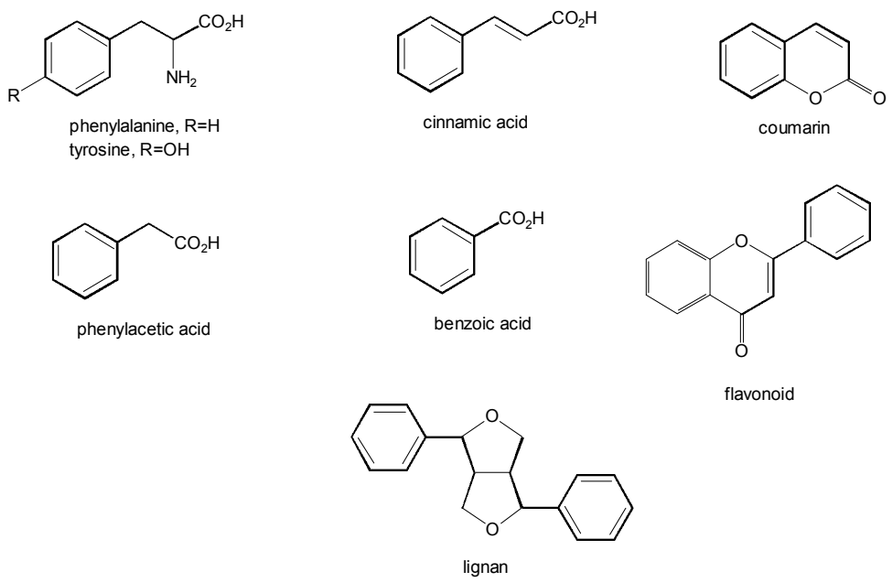



Figure 1 Shikimates comprise a large group of aromatic natural products

### 2.4.1. The Shikimate Pathway

The shikimate pathway is an essential metabolic pathway involved in the synthesis of aromatic compounds in plants, apicomplexan parasites, fungi and microbes [40, 41]. Animals obtain their aromatic compounds from their diet. Challenge the view that the shikimate pathway is not found in animals by showing its presence in metazoans using phylogenetic analysis [42]. However, no functional data has been provided for the identified genes. The interest in the shikimate pathway enzymes as potential targets for non-toxic herbicides and anti-microbial compounds dates back more than 25 years ago. In 1972, the company Monsanto reported the discovery of glyphosate (*N*-phosphonomethylglycine), which became a billion dollar herbicide, and in the 1980s following Monsanto's model, more commercially important herbicides that targeted other amino acid biosynthetic pathways became available [42]. Even though glyphosate was discovered as an herbicide, later on it was also tested as an antiparasitic agent. In vitro growth of *Toxoplasma gondii*, *Plasmodium falciparum* (malaria) and *Cryptosporidium parvum* was inhibited by glyphosate [39, 43]. This effect on *T. gondii* and *P. falciparum* was reversed by treatment with *p*-aminobenzoate, which suggests that the shikimate pathway supplies folate precursors for their growth. However, since its introduction, resistance to glyphosate has been reported, including several species of cyanobacteria that are equipped with a resistant form of 5-enolpyruvylshikimate-3- phosphate (EPSP) synthase up to the millimolar range [44]. Therefore, new efforts have concentrated on more successful drug and herbicide design strategies. Apart from inhibitor discovery, the shikimate pathway is also of high interest for metabolic engineering since the phenolic products of the pathway are extensively used in the industry as antioxidants for processed foods [45]. Shikimic acid, one of the intermediates in the shikimate pathway, is used as a precursor for the synthesis of Tamiflu (Oseltamivir phosphate), the most prescribed flu medicine [46].

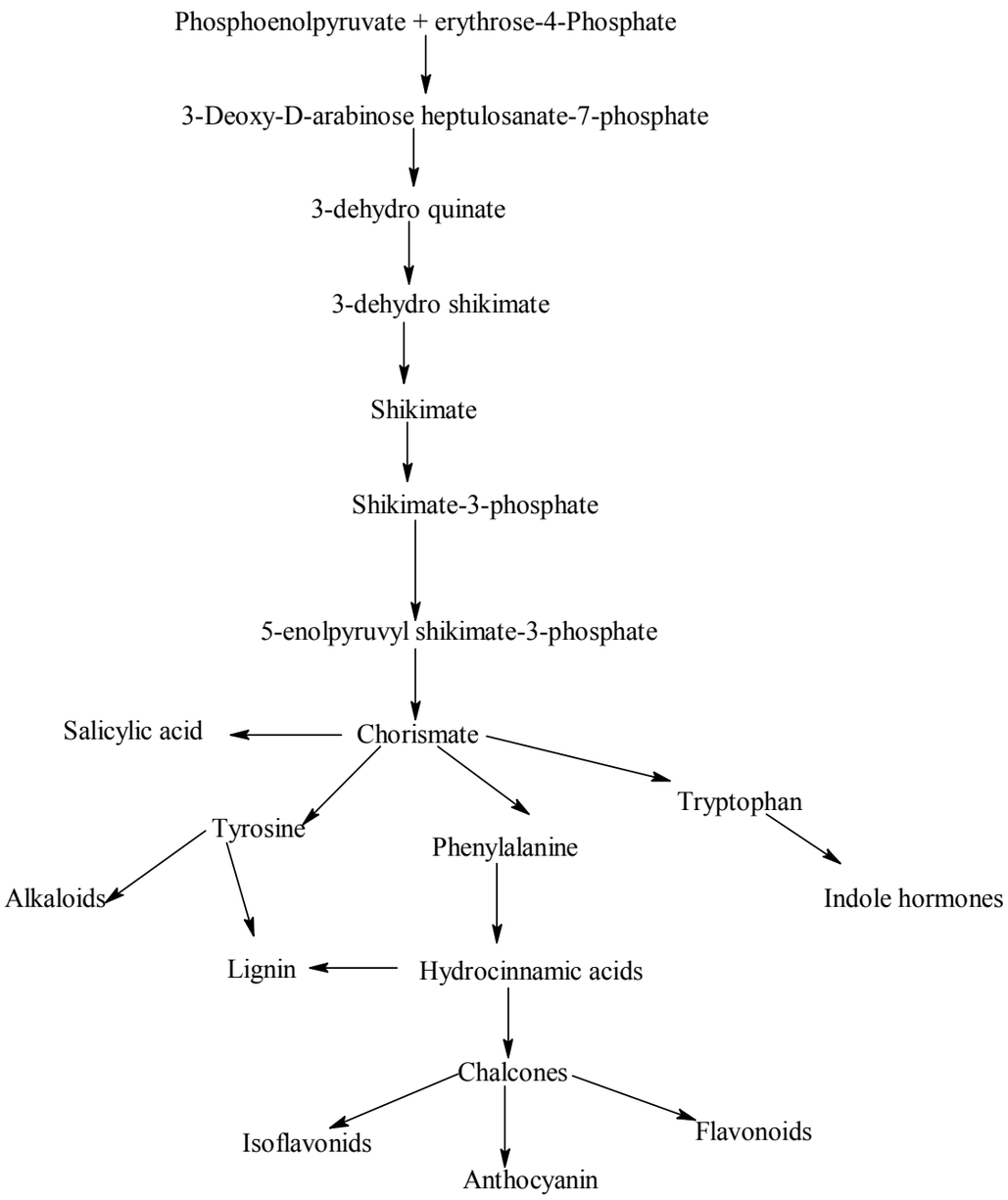
In plants, the shikimate pathway provides the precursors for the synthesis of the indole hormones and phenylpropanoids, which are currently of high interest due to their potential as nutraceuticals such as ferulic acid and genistein. However, despite its potential the metabolic engineering of the shikimate pathway has encountered a lot of challenges because regulation of the pathway in eukaryotes is much more complex than in microbes due to this secondary metabolism branching [40, 47, 48].

The shikimate pathway consists of seven enzymatic steps that terminate with the production of chorismate (scheme 1). The committed step of the shikimate pathway is the aldol condensation of phosphoenol pyruvate (PEP) and erythrose-4-phosphate (E4P) to form 3-deoxy-D-Arabino-Heptulosonate 7-phosphate (DAHP) catalyzed by the enzyme DAHP synthase. The pathway terminates with the synthesis of chorismate, which branches into the production of tyrosine, phenylalanine and tryptophan as well as folates, ubiquinones, salicylic acid, terpenoids, flavonoids, benzylisoquinoline alkaloids [49], indole hormones and lignin in plants [39]. These compounds are involved in signaling, growth, UV protection, pathogen defense and structural support in plants [40, 41]. Furthermore, benzylisoquinoline alkaloids serve as precursors for semi-synthetic drugs [49]. DAHP synthase, the first enzyme of the pathway, interlinks glycolysis and the pentose-phosphate pathway with the shikimate pathway. This enzyme exists in three isoforms in most organisms and as such, it is a key regulatory point for the shikimate pathway. It condenses phosphoenol pyruvate (PEP) from glycolysis, and erythrose 4- phosphate (E4P) from the pentose phosphate pathway to form DAHP. 3-dehydroquinate (3-DHQ) synthase catalyzes the cyclization of 3-DHQ from DAHP and 3-DHQ dehydratase forms 3-dehydroshikimate (3-DHS), which is converted to shikimate by shikimate dehydrogenase. Shikimate is phosphorylated by shikimate kinase to form shikimate 3 phosphate (S3P). S3P is converted to 5-*enol*pyruvylshikimate 3-phosphate (EPSP) by EPSP synthase. The seventh and ultimate step of the pathway is catalyzed by chorismate synthase (CS) to form chorismate [40]. Chorismate is used to synthesize the aromatic amino acids as well as a variety of aromatic compounds such as: folates, ubiquinones, salicylic acid etc [39, 40, 41].

In bacteria, a separate enzyme catalyzes each step of the shikimate pathway, but this is not the case in fungi, apicomplexan parasites and plants. Fungi and *Taxoplasma gondii* have a pentafunctional AROM complex that includes the second to the sixth steps of the pathway [50, 51]. It has been suggested that *arom* is the ancient supergene form that was either lost or replaced in several eukaryotic lineages raising interesting questions about the differential regulation of this pathway in the different taxa [39]. This phylogenetic path of the shikimate pathway places plants in an interesting taxonomic context with all the reactions being catalyzed by separate enzymes with the exception of dehydroquinase and shikimate dehydrogenase that form a bifunctional enzyme with two independent active sites [48].

The aromatic amino acids are a direct product of the shikimate pathway, and apart from their role in protein synthesis, they also serve as precursors for a wide range of metabolites such as flavonoids, anthocyanins, lignin and indole hormones that are involved in signaling, UV protection and structural support in plants [40, 41]. Shuttling of aromatic amino acids into secondary pathways is generally not observed in microbes, even though there is some evidence for their participation in antibiotic synthesis and other secondary metabolites [52, 53]. Because the intermediates of the shikimate pathway serve as precursors for many branch pathways, the study of the regulation mechanism of this pathway in plants is both interesting and necessary for the engineering of the pathway as well as for drug, vaccine or herbicide development.

All the shikimate pathway enzymes are predicted to be localized in the plant chloroplast using ChloroP [54], which predicts a characteristic N-terminal transit peptide and from data present in the plastid proteome databank which also indicates the localization of the shikimate pathway enzymes in the chloroplast [55]. In addition, chorismate synthase has been shown to be inactive in the cytosol in the presence of the cleavable transit peptide and chorismate is not synthesized in the cytosol [56].




Scheme 1 An Over View of the Shikimate Pathway

## 2.5. An Overview of the Chromatographic Methods

Chromatography is the method of choice in handling the problem of isolation of a compound of interest from a complex natural mixture. Therefore, the chromatographic methods used during the present work were briefly described.

### 2.5.1. Thin Layer Chromatography (TLC*)*

TLC involves the use of a particulate sorbant spread on an inert sheet of glass, plastic, or metal as a stationary phase. The mobile phase is allowed to travel up the plate carrying the sample that was initially spotted on the sorbant just above the solvent. Depending on the nature of the stationary phase, the separation can be either partition or adsorption chromatography. Different spots equivalent to different analytes are characterized by their retardation factor (Rf) values. This is defined as a measure of the fraction of the distance moved by an analyte in reference to the distance moved by the solvent and is calculated as follows.

Rf= distance moved by the analyte⁄distance moved by solvent front.

The advantage of TLC is that the samples do not have to undergo the extensive cleanup steps, and the ability to detect a wide range of compounds, using reactive spray reagents. Non destructive detection (fluorescent indicators in the plates, examination under a UV lamp) also makes it possible for purified samples to be scraped off the plate and be analyzed by other techniques [57, 58, 59].

### 2.5.2. Preparative Thin Layer Chromatography (PTLC)

This is a modified version of analytical TLC used in the separation and isolation of analytes from small quantities of sample. Often it is used in conjunction with column chromatography as a final purification step of relatively less complex mixtures [60]. The major difference from analytical TLC is in the thickness allowing the application of larger volumes of sample. After development the plates are visualized under UV and the band with the analytes of interest is scraped off together with the adsorbent using a spatula. The analyte is separated from the adsorbent by filtration and concentrating [61].

### 2.5.3. Column Chromatography (CC)

CC consists of a column of particulate material such as silica or alumina that has a solvent passed through it at atmospheric, medium or low pressure. The separation can be liquid/solid (adsorption) or liquid/liquid (partition). The columns are usually glass or plastic with sinter frits to hold the packing. Most systems rely on gravity to push the solvent through, but medium pressure pumps are commonly used in flash CC. The sample is dissolved in solvent and applied to the front of the column (wet packing), or alternatively adsorbed on a coarse silica gel (dry packing). The solvent elutes the sample through the column, allowing the components to separate.

Normally, the solvent is non polar and the surface polar, although there are a wide range of packing including chemically bound phase systems. Bonded phase systems usually utilize partition mechanisms. The solvent is usually changed stepwise, and fractions are collected according to the separation required, with the eluting products usually monitored by TLC. The technique is not efficient, with relatively large volumes of solvent being used, and particle size is constrained by the need to have a flow of several mls/min. The advantage is that no expensive equipment is required, and the technique can be scaled up to handle sample sizes approaching gram amounts [57, 58, 59].

## 2.6. Structural Elucidation Techniques

Structural elucidation involves the determination of factors such as atom number, number of bonds, configuration and conformation of pure compounds [62]. Most often the starting point is information from literature, the chemistry, and physical properties of compounds to be identified chemical spectroscopic techniques such as UV-Visible, Infrared (IR) and Nuclear magnetic resonance (NMR) spectroscopy have always been and continue to be used for structure elucidation. Advances in technology with time have allowed the use of milligram quantities of sample, high resolution and minimized analysis times [63]. The subsequent section looks at some of the structure elucidation techniques.

### 2.6.1. Infrared Spectroscopy

Infrared spectroscopy is useful in the identification of functional groups present in a compound [63]. This technique is based on the absorption of electromagnetic radiation at wave lengths ranging between 4000 and 400cm$^{-1}$. At this range of wave lengths functional groups give characteristic vibration, bending and stretching vibrations at characteristic wave lengths. This is recorded in a spectrum by the IR instrument and gives basic information on the structure [64].

### 2.6.2. Nuclear Magnetic Resonance Spectroscopy

This is the most comprehensive spectroscopic technique in organic chemistry giving detailed structural information useful for the complete identification of both simple and complex compounds [63] under radio frequency energy, nuclei with non-zero spin values ($^{1}$H,$^{13}$C,$^{31}$P,$^{15}$N) in static magnetic field absorb energy with frequencies of absorbed energy against the peak intensities. The following are some of the most useful experiments in NMR [64]. $^{1}$H-NMR (proton NMR) spectra shows the chemical shift values of protons against their intensities. The chemical shift values reveal the nature of different protons in a molecule and the splitting patterns give information on the number of neighboring protons [64]. A$^{13}$C-NMR spectra are a plot of the chemical shifts of different carbon environments against their intensities. The signals normally appear as singlets due to decoupling of attached protons. It is possible to classify the carbons in to primary, secondary and tertiary through an experiment called distortion less enhancement by polarization transfer [65].

# 3. MATERIALS AND METHODS

## 3.1. Instruments and Chemicals

IR spectrum was obtained as pellets on Perkin-Elmer Bx infrared spectrometer in the range 4000-400cm$^{-1}$. $^{1}$H NMR, $^{13}$C NMR spectra was recorded on a Bruker advance 400 MHz spectrometer with TMS as internal standard. The Ultra-Violet and Visible (UV-Vis) spectra was taken on GENESY'S 2PC UV-Vis scanning spectrometer (200-800nm). Melting points was recorded digital melting point apparatus. Silica gel with fluorescent indicator at 254 nm and aluminum cards with layer thickness 0.2 mm was used for TLC. Silica gel 60 (Merck), particle size 0.063-0.200 (70-230 mesh ASTM) was used for column chromatography. Compound on TLC was detected using eye protects by UV-Vis. PTLC(Preparative Thin Layer Chromatography) was used in the separation of analytes from small quantities of sample often it is used in conjunction with column chromatography as a final purification step of relatively less complex mixtures. Rotary evaporator was used to concentrate the samples. Petroleum ether, Methanol, Chloroform, Ethyl acetate, n-hexane were used as solvent.

## 3.2. Sample Collection

The plant *Rumex Nervosus* was collected from Deber Berhan, Amhara Region in the local distinct of Debersina and identified by Prof. Sebsibie Demissew of the National herbarium, Department of Biology, Addis Ababa University.

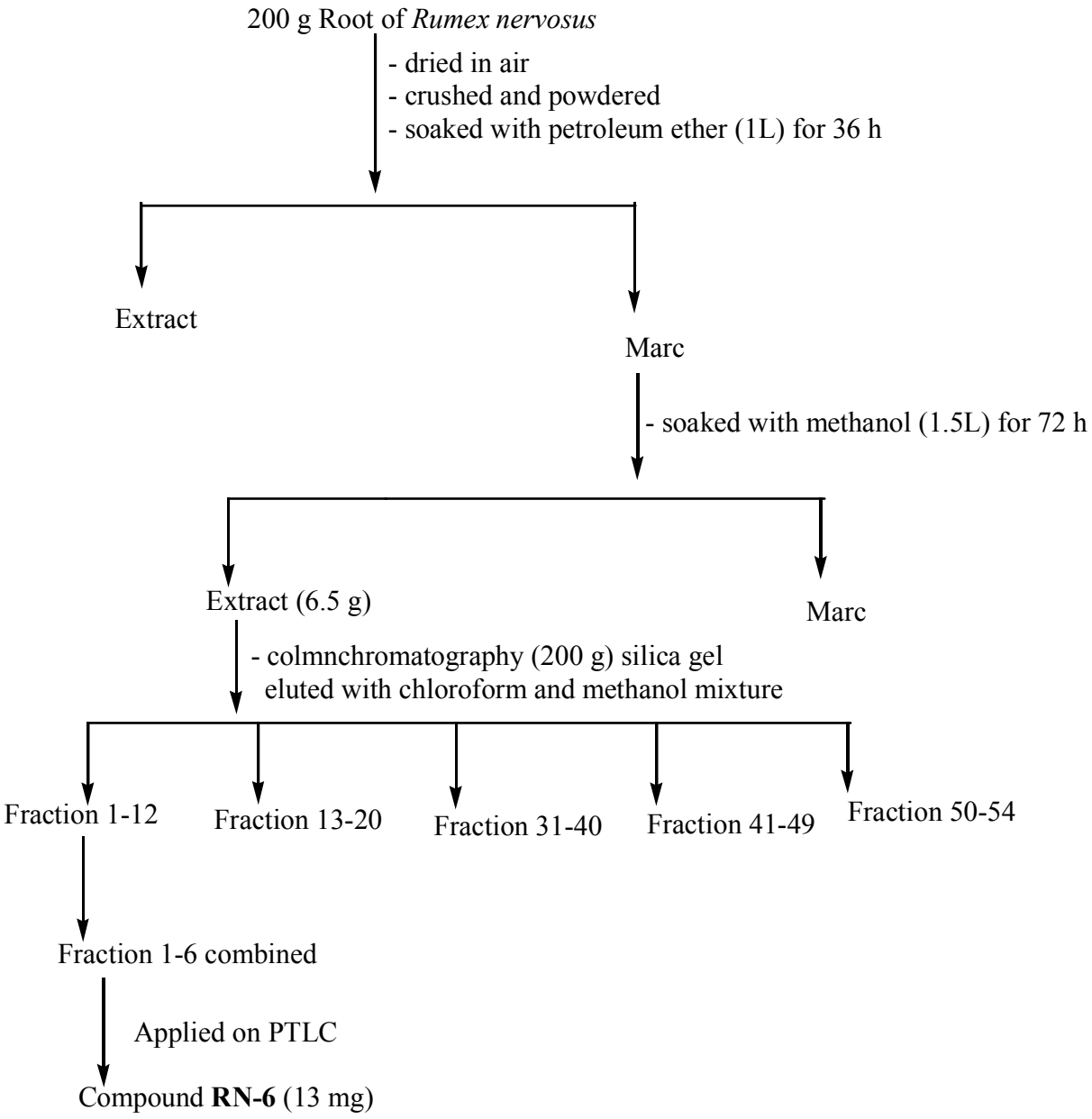
## 3.3. Extraction and Isolation

The air-dried and powdered plant material (200 g) was first soaked with petroleum ether for 36 h filtered and concentrated. The solvent free marc was then soaked with 1.5 L methanol for 72 h and the extract was filtered. This filtrate was evaporated under the reduced pressure using the Rota vapor and afforded 17g brown gummy extract. The methanol extract (6.5 g) was applied on a column chromatography packed with 200g silica gel. Isolation was carried out using the solvents chloroform and methanol with increasing polarity. The Colum was eluted using the following solvent system and 54 fractions were collected.

Table 1 Methanol extract fractions

| Solvent system | Ratio | Volume(ml) | Fractions |
|---|---|---|---|
| Chloroform | 100% | 100 | 1-12 |
| Chloroform-methanol | 9:1 | 100 | 13-20 |
| „ | 8:2 | 100 | 21-30 |
| „ | 7:3 | 100 | 31-40 |
| „ | 6:4 | 100 | 41-49 |
| „ | 1:1 | 100 | 50-54 |

Based on TLC analysis, fraction that showed the same characteristics of spots were combined. Fractions 1-6 (75.3mg) were combined and have four spots on TLC. The series combined fraction was subjected to Preparative Thin Layer Chromatography (PTLC) developed in chloroform-methanol (9.5: 0.5) mixture which yielded one spot with a white fluorescence under UV. The extraction of the plant and isolation of the compound is described in detail in the scheme below.




Scheme 2: Method of extraction of the plant material

# 4. Results and Discussion

## 4.1. Yield of Solvent Extract and Isolation of Roots of *Rumex nervosus*

The dried and powdered Roots (200 g) of *Rumex nervosus* subjected to exhaustive extraction successively with petroleum ether and methanol. The solvent from each extract was recovered under reduced pressure using rotavapor to obtain a methanol extract (17 g). Chromatographic purification of the methanol extract (6.5 g) yielded a compound coded; **RN-6**. The structure of this compound has been elucidated on the basis of spectroscopic evidences as described in the following section.

## 4.2. Characterization of Fraction RN-6

The UV spectrum of **RN-6** (appendix-1) shows absorbance peak at 276 nm which indicate the presence of a carbonyl substituted aromatic ring.

In the IR (KBr) spectrum (appendix-2) of the compound displayed the absorption band at δ 3439 $cm^{-1}$ may be due to moisture. The absorption band at δ 2925 $cm^{-1}$ indicates –C-H stretching alkayl groups. The absorption band at δ 1728 $cm^{-1}$ indicates the presence of ester carbonyl group attached to aromatic ring. The absorption band at δ 1276 $cm^{-1}$ indicates C-O stretching. The absorption band at δ 1125 $cm^{-1}$ indicates -C-C stretching of the compound.

The $^{1}$H-NMR Spectrum (appendix 3, Table 2), of the proton signals at δ 0.96 indicate the presence of two methyl group. The proton signals at δ 1.25, 1.29, 1.33, and 1.75 show the presence of five methylene groups. The proton signal at δ 1.47 multiplate indicate methine group. The proton signal at δ 4.25 triplets is due to oxygenated methylene carbon protons. The proton signals at δ 7.73-7.79 indicate protons of aromatic carbon.

Table 2. $^{1}H$ spectra data of compound **RN-6**

| Hydrogen No | δ(ppm) |
|---|---|
| 1 | 4.25 (2H, t, J= 12) |
| 2 | 1.75 (2H,m) |
| 3 | 1.25 (2H,m) |
| 4 | 1.47 (1H,m) |
| 5 | 1.25 (2H,m) |
| 6 | 1.33 (2H,m) |
| 7 | 0.96 (3H,d) |
| 8 | 1.29 (2H,m) |
| 9 | 0.96 (3H,d) |
| 3',7' | 7.97 (2H,dd,$J_1$= 3.2, $J_2$= 5.6) |
| 5' | 7.47 (1H,t) |
| 4',6' | 7.37 (2H,dd, $J_1$= 3.2, $J_2$= 5.6) |

The $^{13}C$ NMR and DEPT-135(appendix4, Table 3) indicate that compound **RN-6** has 14 carbons. The spectra show two methyl carbons at δ 10.97 and 14.7. Five methylene carbon at δ 23, 23.74, 28.93, 29.75 and 30.4.One methylene carbon that is attached with oxygen at δ 68.16. Two quaternary carbons at δ 167.79 and 132.45. Four methine carbons at δ 38.72, 130.9, 130.87, 128.81. Additionally the $^{13}C$ NMR spectrum of compound **RN-6**, indicate the presence of aromatic ring.

Table 3. $^{13}C$ and DEPT-135 spectra data of compound **RN-6**

| Carbon No | $^{13}C$ | DEPT-135 | Remark |
|---|---|---|---|
| 1 | 68.16 | down | $CH_2$ |
| 2 | 23.75 | down | $CH_2$ |
| 3 | 29.71 | down | CH |
| 4 | 38.74 | up | $CH_2$ |
| 5 | 30.37 | down | $CH_2$ |
| 6 | 23.00 | down | $CH_2$ |
| 7 | 14.07 | up | $CH_3$ |
| 8 | 28.93 | down | $CH_2$ |
| 9 | 10.97 | up | $CH_3$ |
| 1’ | 167.79 | - | Quaternary |
| 2’ | 132.45 | - | Quaternary |
| 5’ | 130.9 | up | CH |
| 3’,7' | 130.87 | up | CH |
| 4’6’ | 128.81 | up | CH |

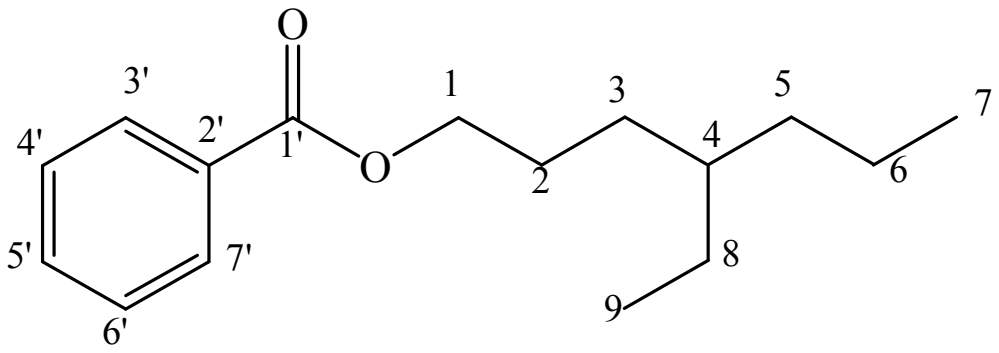



Figure 2 The proposed structure of Compound **RN-6** (4-ethylheptyl benzoate)

# 5. CONCLUSION AND RECOMMENDATION

The chloroform-methanol (9.5:0.5) extract of *Rumex nervosus* affords 13 mg of compound **RN-6**. Compound **RN-6** (4-ethylheptyl benzoate) was and further purified by chromatographic methods such as Column Chromatography, Thin Layer Chromatography, Preparative Thin Layer Chromatography and the structural elucidation of this compound was accomplished by means of a combination of spectroscopic methods. To the best of our knowledge there was no report on isolation and characterization of compounds from any part of *Rumex nervosus,* Compound **RN-6** is reported here for the first time from this species.

In this study the extraction, isolation and structure elucidation of compound **RN-6** (4-ethylheptyl benzoate) was accomplished using chromatographic and spectroscopic method. The researcher recommended that advanced chromatographic techniques such as HPLC should be used to isolate more compounds from different extracts of the plants. 2D-NMR techniques are also required to elucidate structures of novel compounds isolated from the plant. Additionally antimicrobial tests should be conducted on crude extract fractions and isolated compounds from the plant.

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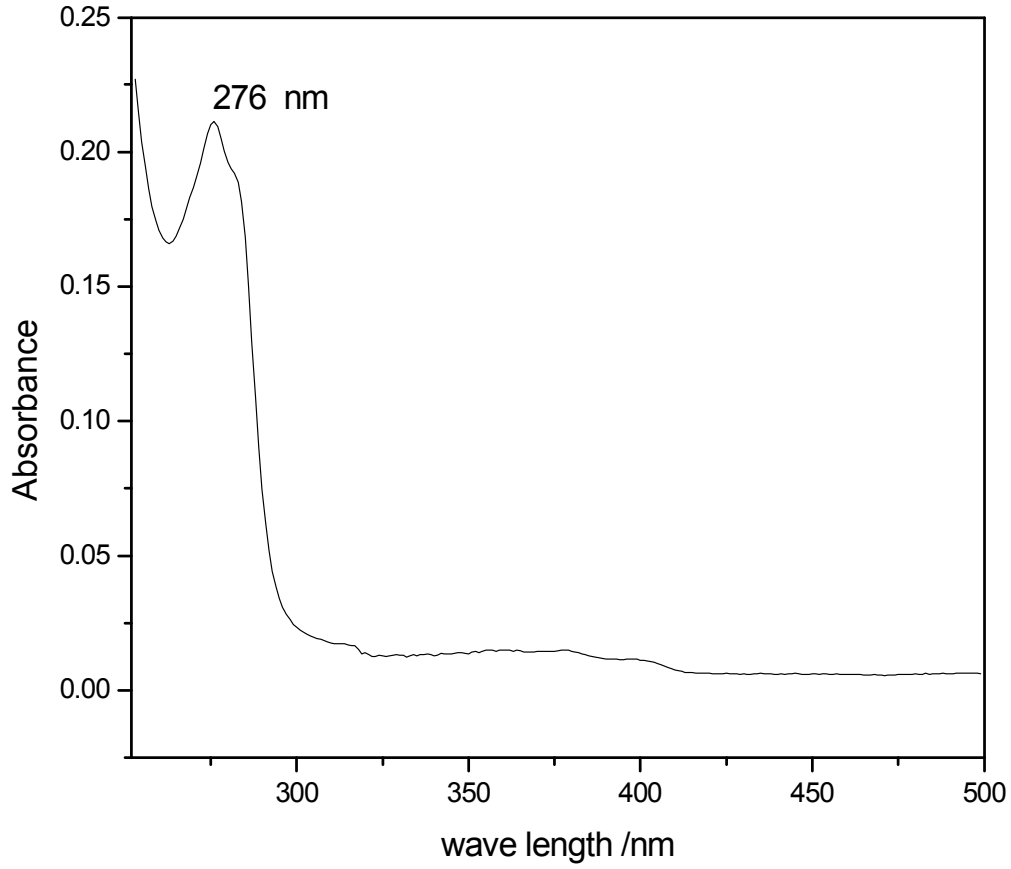
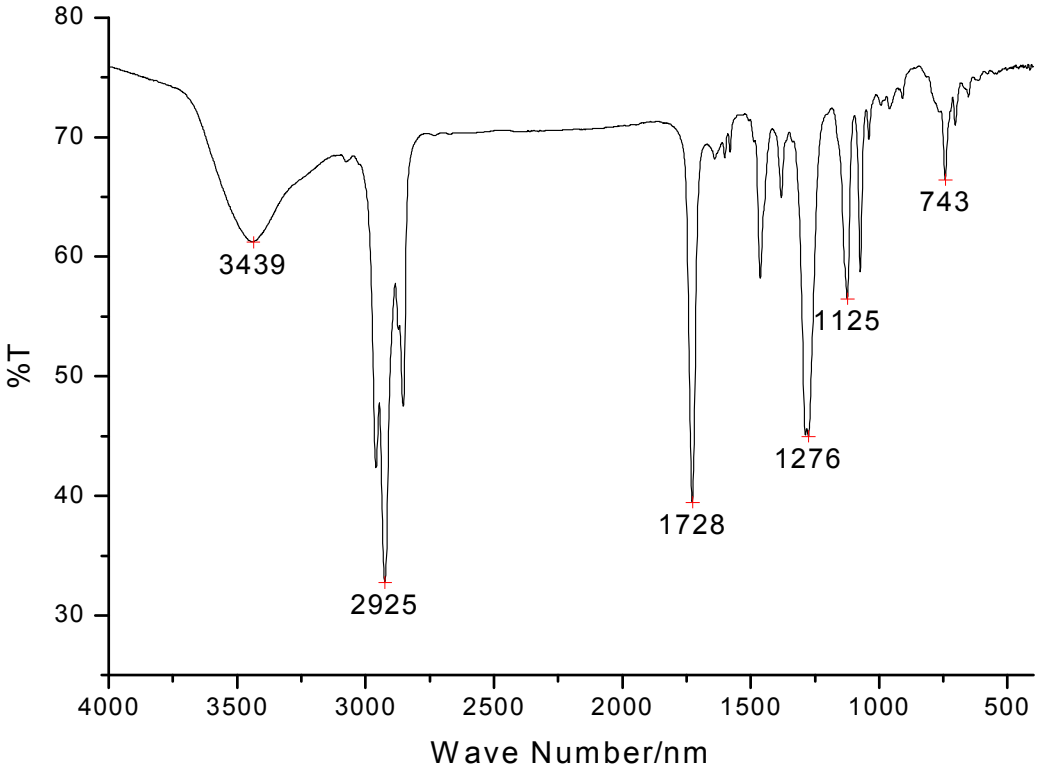
# 7. APPENDIXES

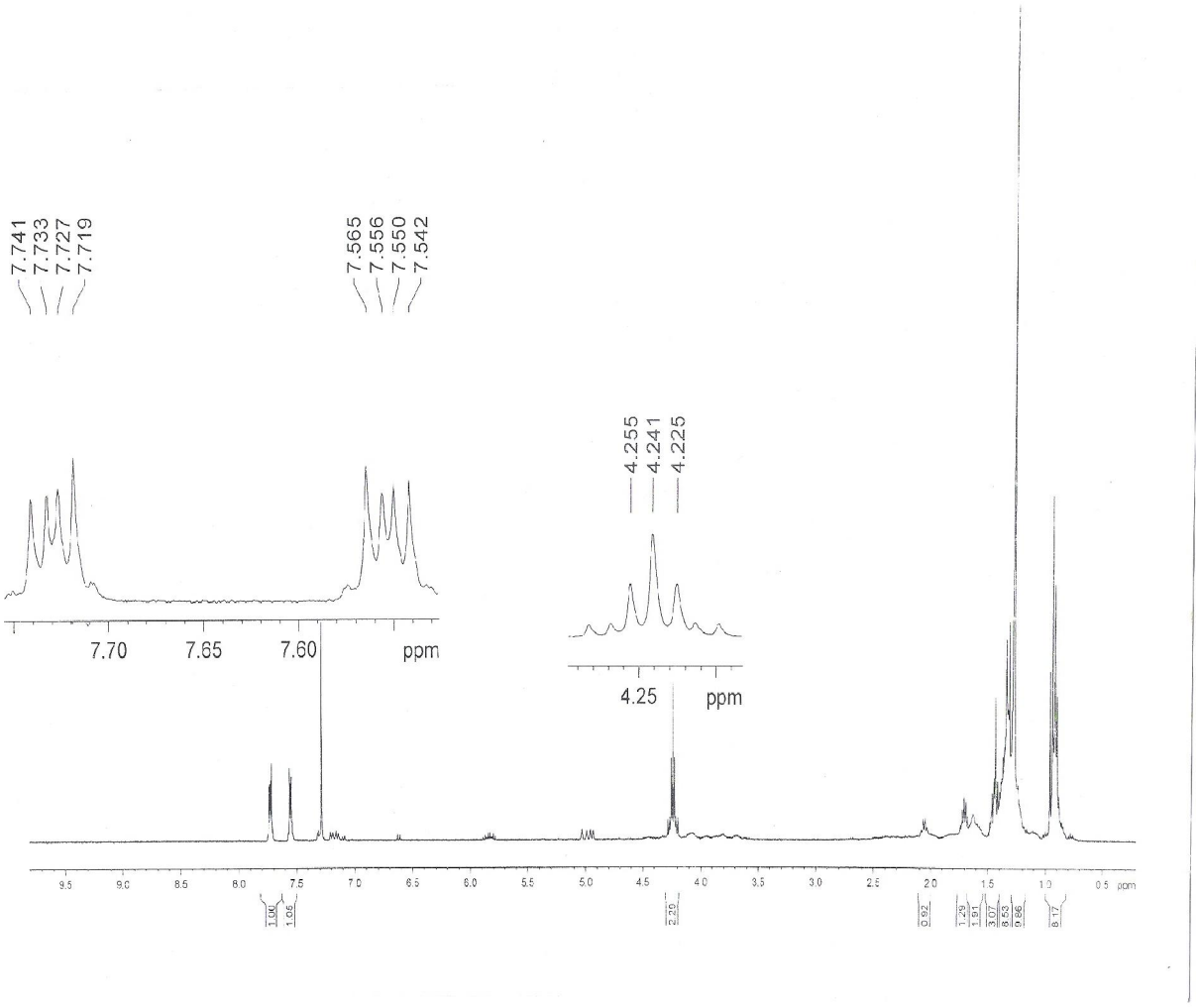
Appendix 1 UV-Visible Spectrum of Compound **RN-6**



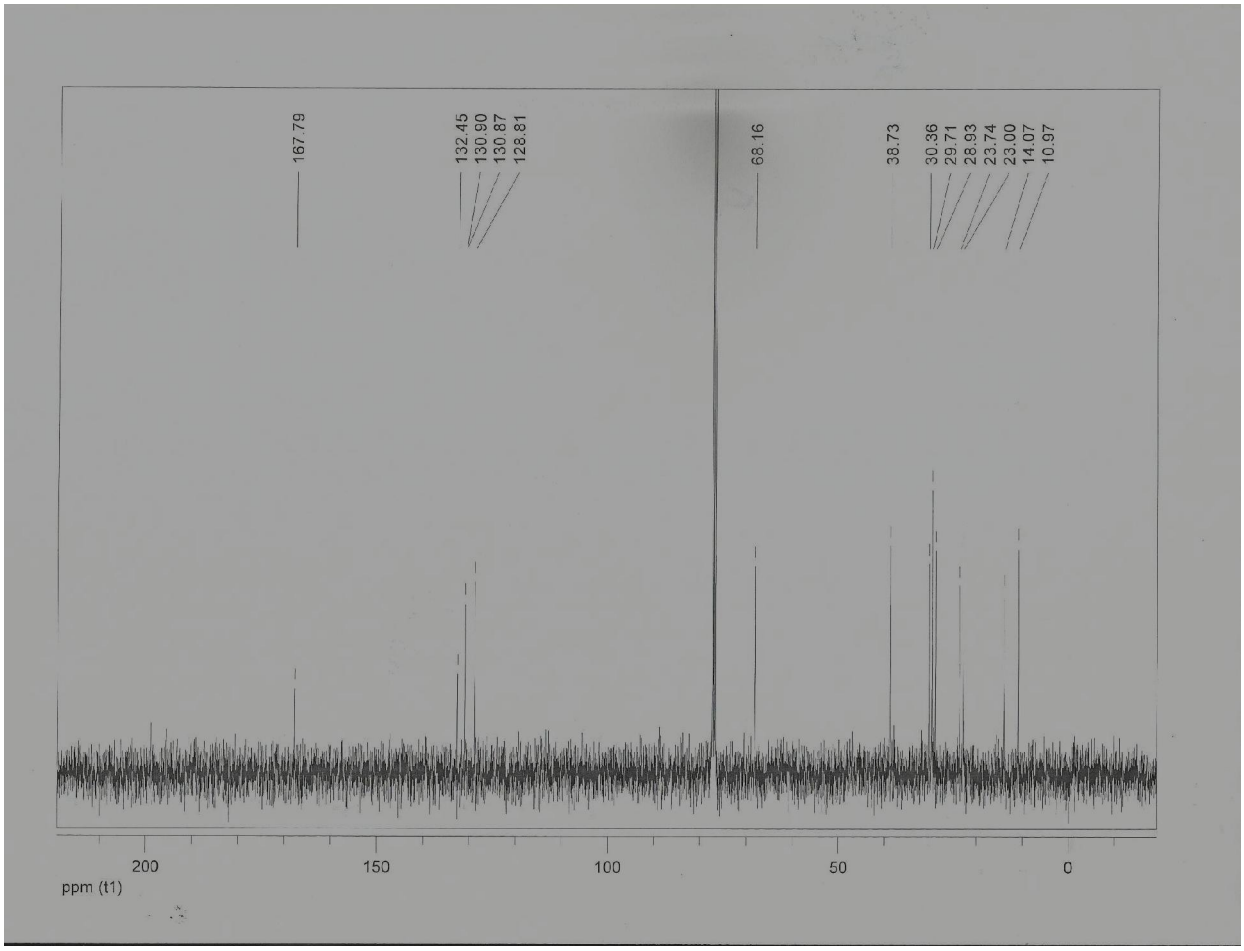
Appendix 2 IR Spectrum of Compound **RN-6**



Appendix 3 $^{1}$H NMR spectrum of compound **RN-6** in $CDCl_3$



Appendix 4 $^{13}C$ NMR spectrum of compound **RN-6** in $CDCl_3$



Appendix 5 DEPT-135 spectrum of compound **RN-6** in $CDCl_3$