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Debre Berhan University
College of Natural and Computational Sciences
Department of Chemistry

MSc Thesis

Phytochemical Analysis and Determination of Antioxidant and Antibacterial Activities of Leaf extracts of *Rumex nervosus* (Embacho)

A Thesis submitted to the School of graduate studies, Debre Berhan University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry

BY

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The thesis entitled “Phytochemical analysis and determination of antioxidant and antibacterial activities of leaf extracts of *Rumex nervosus* (Embacho)”, by Workineh Yimer Tesfu is approved for the degree of “Master of Science in Chemistry.

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List of Abbreviations

AA	Ascorbic acid
Abs	Absorbance
Ac	Absorbance of the control
As	Absorbance of the sample
DPPH	1, 1- diphenyl -2-picrylhyorazyl
CHL	Chloroform
ME	Methanol
Mg	Milligram
HEX	hexane
Ppm	part per million
R.	Rumex
ROS	Reactive Oxygen Spcies
UV	Ultra Violet
WHO	World health organization
µg	Micro gram
nm	Nano meter

Abstract

In Ethiopia there are many species of medicinal plants which may contain disease-curing bioactive compounds which are distributed all over the world especially, in the south and south-western part of the country. The traditional medicine still plays an important role in the primary health care in Ethiopia. *Rumex nervosus* is one of the most common weed plants in Ethiopia and used in traditional medicines. In this study the leaves of *Rumex nervosus* were extracted using hexane, chloroform and ethanol in successive extraction method and the presence of phytochemicals was checked. The antioxidant activity of the extracts of *Rumex nervosus* was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (2mM). The antibacterial activities of the extracts were also investigated against Gram positive, and Gram negative bacteria using Muller Hinton Agar medium by disc paper method. Ethanol extract of *Rumex nervosus* showed slightly better antibacterial activity against a Gram positive bacteria Staphyloccus aureus and Listeria monocytogens and a Gram negative bacteria Escherichia coli and Salmonolla typhymurium. Hexane extract had relatively low zone of antibacterial activity in all tested bacteria. Generally ethanol extract had good antioxidant and slightly better antibacterial activity than chloroform and hexane extracts.

Key words: *Rumex nervosus*, phytochemical, antioxidant activity, antibacterial activities, DPPH

CHAPTER ONE

1. INTRODUCTION

1.1 Background of the study

The plant kingdom has been the best source of remedies for curing a variety of disease and pain. This is why medicinal plants have played a key role in the worldwide maintenance of health. Traditional herbal medicine is intimately related to the Mexican popular culture; its use has origins based on ancestral knowledge. Natural products of higher plants are an important source of therapeutic agents; therefore, many research groups are currently screening the different biological activities of plants [1].

According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Use of herbal medicines represents a long history of human interactions with the environment. Plants used in traditional medicine contain a wide range of ingredients that can be used to treat chronic as well as infectious disease. Reactive oxygen species (ROS) may cause great damage to cell membranes and DNA, including oxidation that causes membrane lipid peroxidation, decreased membrane fluidity and DNA mutations leading to cancer, degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus and other. Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain [2].

Obesity is a disease with serious public health implications, associated with insulin resistance, type 2 diabetes, hypertension, dyslipemia and atherosclerosis. Due to the side effects associated with the currently available anti-obesity medications and limited efficacy, much attention has been focused on developing new anti-obesity agents through herbal medicines that would minimize the side effects. Several animal studies and clinical studies with many herbal medicines have been performed, and some studies reported significant improvements in controlling body weight without any noticeable adverse effects [3, 4]. *Rumex nervosus* is one of medicinal plants used to treat different disease.

1.2. Statement of the problem

In our country Ethiopia different plants have been used as traditional medicine to treat different disease. *Rumex nervosus* is one of medicinal plant in the North Africa and Asia .*Rumex nervosus* plant is the multi-*use plants in Ethiopia traditional medicine which encourage researches to carry out phyto chemical and bioassay studies. Rumex nervosus is traditionally used for the treatment of eye disease, Taeniacapitis, Hemorrhoids,* Infected wounds, Arthritis Diarrhea, Typhus and Eczema. There are some reports on antimicrobic activity on the family of Rumex nervosus, But its potential as anti-oxidant agent is not yet confirmed, Further more. It seems that phytochemical studies for characterization of chemical constituents of the plant are unexploited area of research in the case of Awi Zone in my work place can be observed not available as medicine, It would be interest investigate the chemical constituents of the leaves *of Rumex nervosus* plant.

1.3. Significance of the study

Recently, there has been much research emphasis on the antioxidant and antibacterial properties of plants. Plants with these attributes are good resources for general health maintenance and well-being. Anti-bacterial and antioxidant agents of plant origin have been studied from various plant extracts with the objective of developing antibacterial and antioxidant drugs. This is because plants are richest in bioactive compounds and these bioactive compounds are responsible for the antibacterial and antioxidant effect of plants. Evaluation of plant products for pharmacological and medicinal effects is of growing interest as they contain many bioactive substances which have therapeutics potential. The result of this study will indicate the antioxidant and antimicrobial ability of the leaf extracts of *R. nervosus* and it shows a kind of phytochemicals present in leaf parts of this plant. Generally, this study will strengthen the medicinal use of leaf *R. nervosus*.

1.4. Objectives of the study

1.4.1. General objectives

The main objective of the study was to investigate the phytochemical analysis, antioxidant and antibacterial activities of *Rumex nervosus* leaf extract.

1.4.2. Specific objectives

The specific objectives of this study were: -

- ✓ Extract the leaf of *Rumex nervosus* using hexane, chloroform and ethanol.
- ✓ Screen out the major phytochemicals of the leaf of *Rumex nervoss*
- ✓ Determine antioxidant and antibacterial activities of ethanol, chloform and hexane extracts leaf of *Rumex nervosus*

CHAPTER TWO

2. LITERATURE REVIEW

2.1. The family Polygonaceae

Polygonaceae comprises about 1200 species distributed into 50 genera; the largest genera are Polygonum, Rheum and Rumex. The family distributed worldwide but most diverse in the North Temperate Zone. Members of the Polygonaceae are herbs, shrubs, or small trees. Family Polygonaceae distributed in temperate region particularly in the northern parts of the world. Rumex nervosus is a perennial herb distributed in Yemen, Saudi Arabia, Ethiopia, Somalia, Kenya and Tanzania. It has been used traditionally for treatment of many inflammatory diseases, diarrhea, wounds, typhus, rabies, and skin disorders [5].

Table 1*:* classification of Rumex nervosus

Kingdom	**Plantae**
Division	Magnoliophyta
Classe	Magnoliopsida
Ordre	Polygonales
Family	Polygonaceae
Genus	Rumex
Species	Rumex nervosus

Figure 1**:** Rumex nervosus plant

2.2. The genus Rumex

Rumex genus includes more than 200 species, distributed in temperate regions particularly in the northern areas of both parts of the world. In different countries Rumex species have been used

traditionally as antibacterial, anti-inflammatory, antitumor, anti-dermatitis, diuretic, tonic, laxative, astringent in hemorrhoids bleeding, anti-rheumatic, hepatoprotective, analgesic, antipyretic, purgative and anthelmintic. Rumex species are used as food plants by the larvae of a number of Lepidoptera species. The leaves of most species contain oxalic acid and tannin, and many have as tringent and slightly purgative qualities. Some species with particularly high levels of oxalic acid are called sorrels (including sheep's sorrel, Rumex acetosella, common sorrel, Rumex acetosa and French sorrel, Rumex scutatus), and some of these are grown as pot herbs or garden herbs for their acidic taste. Rumex species contains anthracene derivatives like chrysophanol, physcion, emodin, aloe-emodin, rhein; which are the main biologically active compounds responsible for anti-cancer, cytotoxic, genotoxic and mutagenicity properties [6].

2.2.1. Traditional use of Rumex species

Plants belonging to the genus Rumex have been used traditionally either as edible plants or for the treatment of several diseases in many parts of the World. The above ground parts of numerous species (e.g. R. acetosa and R. patientia) are gathered mainly in the spring and used as vegetables [7, 8]. The rhizomes of R. abyssinicus are used to refine butter and give it a yellow color, and in Kenya it has been used as a source of a yellow dye which renders cellulose fibres red-brown when applied in the presence of sodium carbonate [9,10].

For medicinal applications, mainly decoctions or infusions are prepared from the plant parts, but there are other utilization processes too, e.g. the fresh young leaves of R. nepalensis are rubbed over the affected areas after injury from stinging nettles [11]. In Europe, mainly R. acetosa, R. acetosella, R. alpinus, R. confertus, R. crispusand R. obtusifolius are used for the treatment of different diseases. These plants are applied in Hungary and in Romania for constipation, diarrhoea, swellings, sores, rashes and wounds and as an astringent. In traditional Austrian medicine, R. alpinusleaves and roots have been used internally for the treatment of viral infections [8]. Several Rumex species (R. dentatus, R. hastatus, R. nepalensis, R. japonicusand R. aquaticus) have been used in the Traditional Chinese Medicine for the therapy of different conditions, including bacterial and fungal infections, coughing, headache, fever, eczema, dysentery, diarrhoea, constipation, jaundice, haematemesis and uterine haemorrhage [12, 13, 14]. In Africa, the H_2O extracts of R. abyssinicus, R. usambarensis and R. bequaertii roots have been utilized as remedies

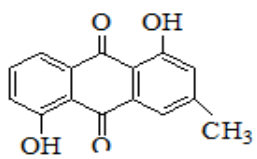
for various types of stomach disorders, while the extracts of R. abyssinicus are drunk to control mild diabetes, and as an antihypertensive, diuretic and analgesic agent [9, 10]. The extracts of R. hymenosepalus and R. maderensis are used as a “blood depurative” or “blood purifier” [15, 16]. R. hastatusis traditionally taken for the treatment of sexually transmitted diseases, including AIDS [17].

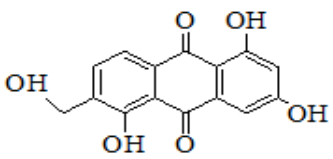
2.2.2. Previously isolated compounds from the genus *Rumex*

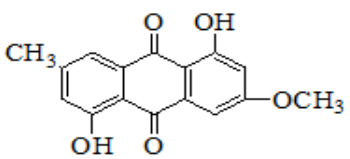
The genus Rumex is characterized by the accumulation of anthraquinones, flavonoids, steroids, Terpenes, phenoic compounds and Alkaloids/ there are many different kinds of the secondary metabolites that have been isolated and reported from genus Rumex. Among those compounds, some of them will be discussed as follows.

2.2.2.1. Anthraquinones

A large number of species belong to the genus Rumex are the rich sources of hydroxyl anthraquinones. The type and level of hydroxy-anthraquinones in plants widely varies depending upon the genetic factors and environmental conditions. The three important anthraquinones that isolated from roots of R. crispus which are 1, 5-dihydroxy-3-methylanthraquinone (**1**), 1, 3, 5-trihydroxy-6hydroxymethylanthraquinone(**2**) 1.5.-dihydroxy-3-methoxy-7-methylanthraquinone **(3)[**19]. The anthraquinone derivatives used as secondary metabolites important for plants have been detected and isolated chromatographically. For example, emodine (**4**) physcion(**5**), physcion-1-O-β-D-glycopyranoside (**6**), nepodine (**7**), rhein (**8**), physcion-8-O-β- D-glycopyranoside (**9**) were isolated from the R. acetosa, R. acetosella, R. confertus, R .crispus, R. hydrolapathum, R. obtusifolius and R. nepalensis[20]. Likewise, Rhein-dianthrone-D-glycoside **(10**) was identified in leaves, roots and fruits of R. crispusand R. obtusifolus. Similarly, 3-acetyl-5-hyroxy-7-methoxy-2-methyl-1,4-naphthaquinone (**11**) was reported in R. japonicusand Aloe-emodine acetate **(12**), which is an anthraquinone derivative was reported in R. acetosa[21]







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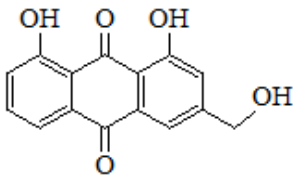

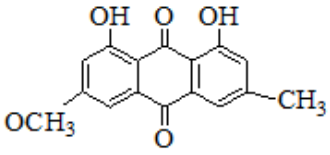

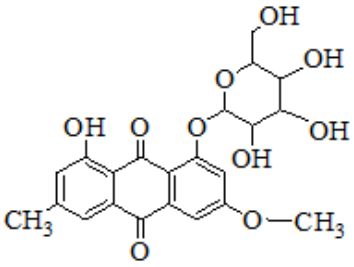

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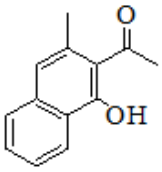

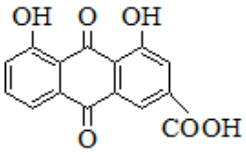

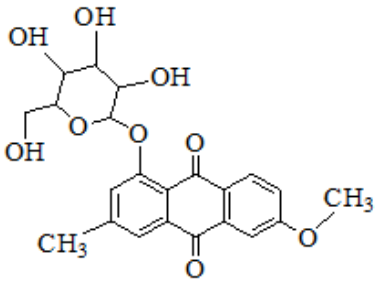

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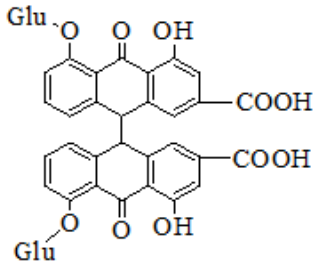

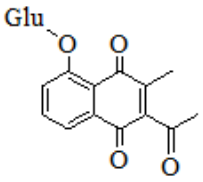

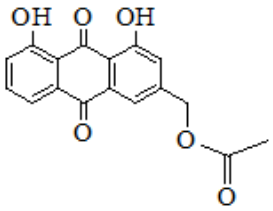

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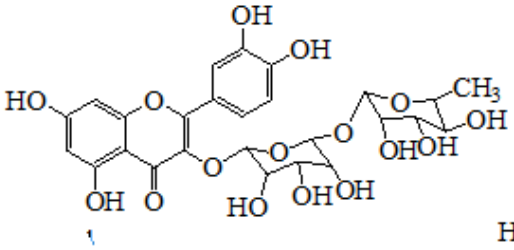
Figure 2**:** Previously isolated Anthraquinones from genus Rumex.

2.2.2.2. Flavonoids

An important class of secondary metabolites also called as flavonoids also occur in the genus Rumex. Flavonoids are polyphenolic compounds occurring in plants. These are derivatives of a large heterogenous group of benzo-γ-pyron and are present in fruits, vegetables and medicinal plants [22]. These have attracted the attention of the researchers over the last several decades and

their biological activities like anti-oxidant, apoptosis-induction and anti-inflammatory activity have been noticed. These activities show the beneficial effects of flavonoids in different human pathologies, including hypertension, inflammatory conditions even cancer [23]. The aerial parts of R. acetosahave been investigated to contain flavonoids like rutin (**13**), hyproside (**14**), quercetin (**15**), quercitrin (**16**), avicularin (**17**), vitexin (**18**), Orientin (**19**), and iso-orientin (**20**). Similarly, R. japonicushave been investigated to contain quercetin (**15**), quercitrin (**16**), iso-quercitrin (**21**) [24] along with kaempferol-3-O-β-D-glucoside **(22**) and catechin (**23**). These compounds are found to act as therapeutic agents for diabetic complication and related disease. Likewise, simple halogenated flavan-3-ol, called as 6-chloro catechin (**24**) isolated and reported from the plant *R. patientia* [23].



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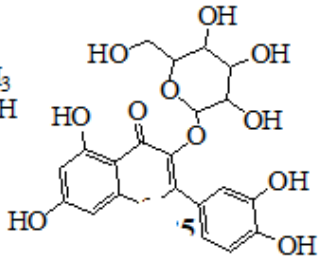

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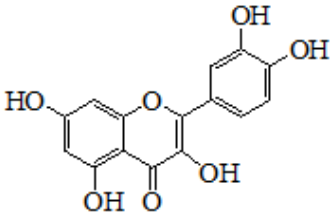

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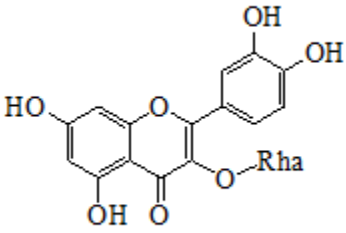

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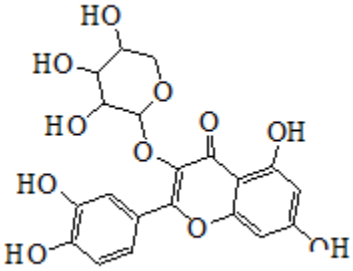

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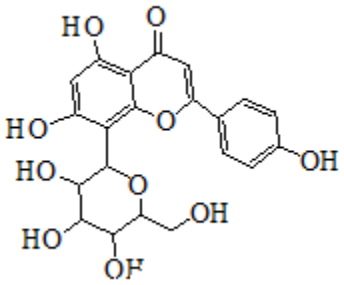

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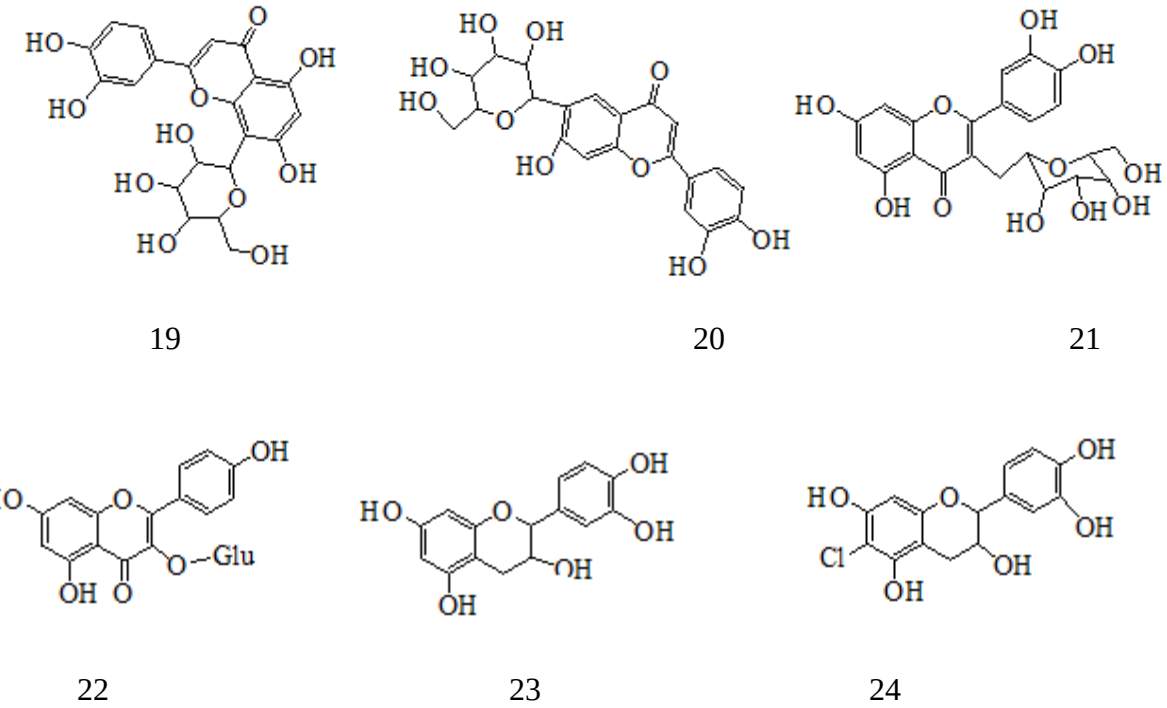
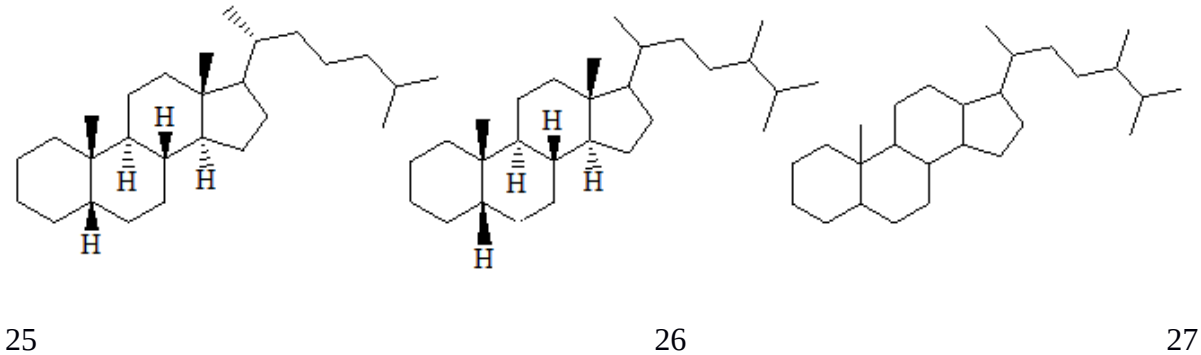


Figure 3**:** Previously isolated Flavonoids from genus Rumex.

2.2.2.3. Steroids

Steroids are also other classes of secondary metabolites that have been isolated and reported from some species of Rumex [23]. For instance, β-cholestan (**25**), α-cholestan (**26**) and stigmastane (**27**) were isolated from R. induratus[15]. Similarly, β-sitosterol (**28**) and β-sitosterol-3-O-β-D-glycoside

(29) were isolated from R.patientia [25].



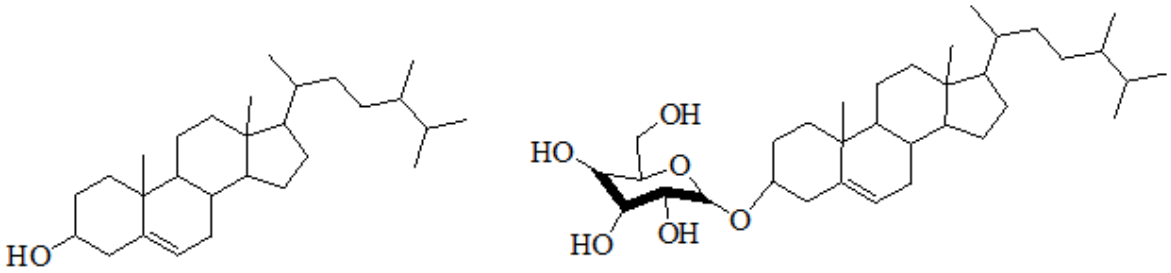


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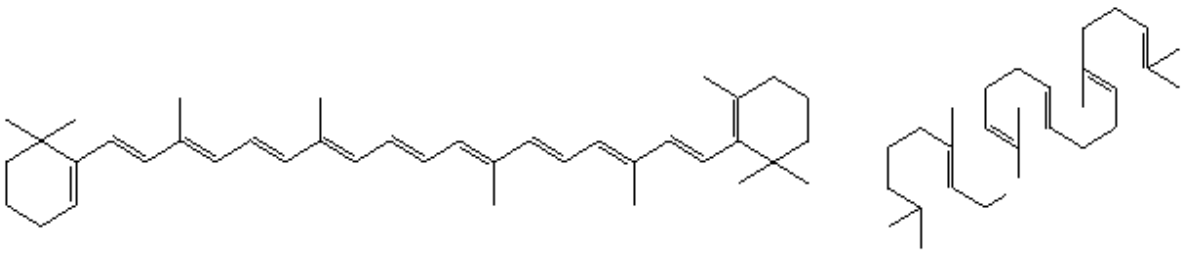
Figure 4: Previously isolated Steroids from genus Rumex.

2.2.2.4. Terpenes

Another class of naturally occurring medicinally and economically important compounds are terpenes. These class of compounds also have been isolated and reported from the leaves of R. induratus and some other species of the genus Rumex. For instance, β-carotene (**30**) occurs in leaves of R. crispus and is used as anti-tumour agent. Likewise, Squalene (**31**) a tritepene has been isolated and reported from the R. induratus. It has pleasant and bland taste. Squalene is used as tumor inhibitor [26], anti-oxidant, anti-aging in sun blocks etc. [27]. Similarly, some other terpenoids like Limonene **(32**), menthol **(33**) and (E)-piperitol (**34**) were obtained from R. induratus. Furthermore, leaves of R. induratus have been investigated to contain α– pinene (**35**), camphene (**36**), myrtenol (**37**), camphor (**38)** [18].



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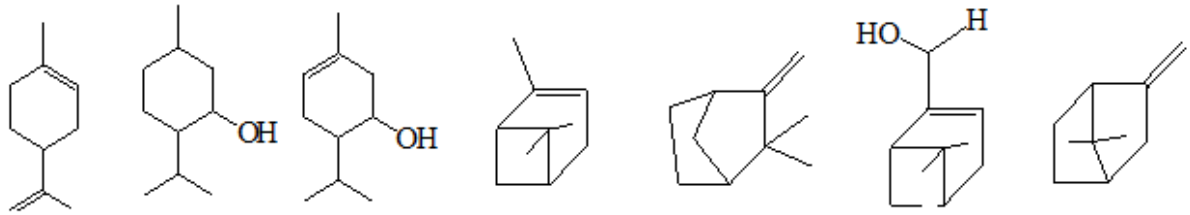

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Figure 5: Previously isolated Terpenes from genus Rumex.

2.2.2.5 Phenolic compounds

Phenolic compounds are the largest category of phytochemicals and the most widely distributed in the plant kingdom. Phenols, sometimes referred to as phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) attached to an aromatic hydrocarbon group. The three most important groups of dietary phenolics are flavonoids, phenolic acids, and polyphenols. They have relatively higher acidities than aliphatic alcohols and some are germicidal while others like estradiol are estrogenic. The simplest of the class is phenol (C_6H_5OH). The subdivision of polyphenols into tannins, lignins and flavonoids is also based on the variety of simple polyphenolic units derived from plant metabolism of the shikimate pathway;[28] Flavonoids are the largest group of plant phenols and the most studied. Phenolic acids form a diverse group that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids. Phenolic polymers, commonly known as tannins, are compounds of high molecular weight that are divided into two classes: hydrolyzable and condensed tannins.[29]

2.2.2.6 Alkaloids

Alkaloids are a structurally diverse group of over 12,000 cyclic nitrogen-containing compounds that are found in over 20% of plant species [30] Alkaloids are basic in nature and mostly colorless solid crystalline substances with a bitter taste. They are soluble in organic solvents but insoluble in water whereas their salts are soluble in water and insoluble in organic solvents. The name of alkaloids derived from the “alkaline” and it was used to describe any nitrogen-containing base,[31]

2.3. Rumex nervosus

R. nervosus is a woody bush up to 2 m long with group of stems arising from the ground, and its

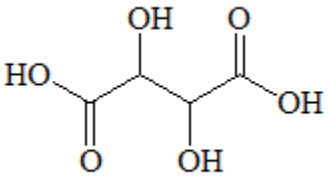
bark has a gray color while its leaves little succulent. It is a perennial plant with green and lanceolata leaves, little and terminal raceme blooms, and delivering huge, prominent, rose-pink fruits, and it recreates either by seed or self-spreading. It is spread on a wide range of altitudes and is common on road sides and in overgrazed regions [33]. Rumex nervosus is commonly found near and around the terraces of high altitude areas (above 1000 m.). It is a perennial herb distributed in Yemen, Saudi Arabia, Ethiopia, Somalia, Kenya and Tanzania. Rumex nervous leaves are an edible, consumed by some people in Saudi Arabia. In Eritrea the leaves and stem of this herb is used for traditional medicine by the practitioner mostly on highland and on the villages. It is used for purifying the body by women (traditionally known as 'tish') as substituent of olive tree. To do this, the leaves are put on fire then they cover the patient body with that hot leaves and blanket so that the vapors and smoke surround all the body [29]. The charcoal of the stem if blended with egg yolk then could be utilized as solution for skin smolders; furthermore, the margarine if added could be used to treat skin irritation. R. nervosus roots used as powder on cut edge to cure a small, hard, benign growth on the skin, which is called Wart. Likewise, its root is used as nectar glue dressing to cure the stomach throb and as powder blended with softened butter against diarrhea.

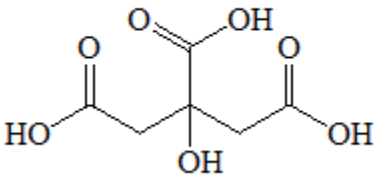
Methanolic extracts got from the root and leaves of R. nervosus plants often used to treat helminthiasis, antidiarrheal, while its bark and leaf if smashed could be used to lessen the swelling impact [28]. The leaves, stems and sometimes roots of Rumex nervosus are used as traditional medicines, for the eye disease, taeniacapitis, haemorrhoids, infected wounds, arthritis, diarrhea, typhus, eczema, rabies, inflammatory diseases, abscess, skin disorders and gynecological disorders [29]. R. nervosusis applied to cure acne, and as a hypoglycaemic and ophthalmic antiseptic agent. It is also used for the treatment of wounds, eczema, typhus and rabies. The methanol, water and chloroform extracts of the leaf, bark, stem and root parts of R. nervosus were reported to possess anti-inflammatory activity and antibacterial activity against several bacteria including S. aureus and P. aeruginosa. [10, 30].

2.3.1. Previously isolated compounds from Rumex nervosus

Previously, revealed that the R. nervosus flowers had many chemical ingredients like polyphenolic components which showed many biological activities. Carbohydrates and/or

glycosides, sterols and/ or triterpenes, catechol tannins, saponins, steroids flavonoid and glycosides were found in all investigated parts, except saponins in roots [28]. Tartaric acid **(39)** and citric acid **(40)** were isolated from R. nervosus [18]





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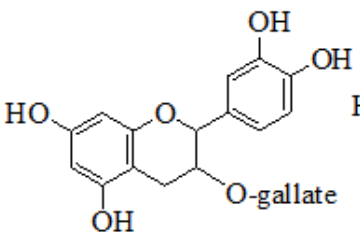
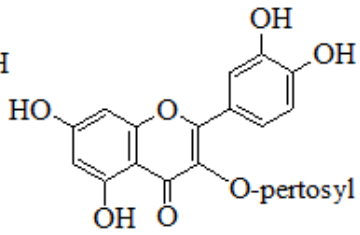
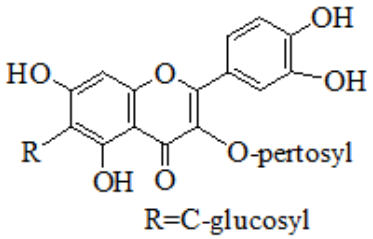
39 40

Figure 6 previously isolated compounds from Rumex nervousus

They are different flavonoids: flavonols, flavones, flavanones, and flavanol, were isolated from flowers of R. nervosus. Some of these are (Epi)catechinO-gallate (**41**), QuercetinO-pentoside (**42**), Luteolin-6-C-glucoside (**43**), Apigenin-6-C-glucoside (**44**), Apigenin-8-C-glucoside (**45**), Quercetin-3-O-glucoside (**46)** ,Quercetinacetylglycoside (**47**), Quercetin-3-O-rhamnoside (**48**), Quercetin-3-O-rutinoside (**49**), Quercetin-3-acetyl rhamnoside (**50**), Hesperetin (**51**), Naringenin (**52**), Apigenin-6-C-glucoside-7-O-glucoside (**53**) and Liquiritin (**54**)[31, 32].







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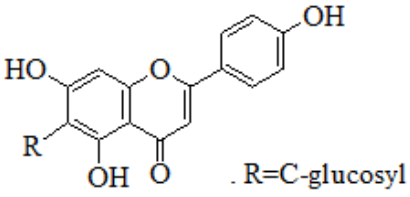

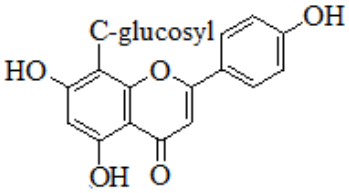

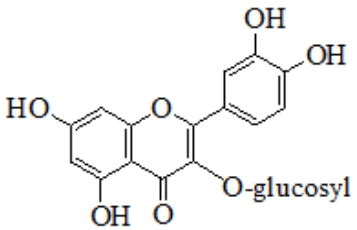

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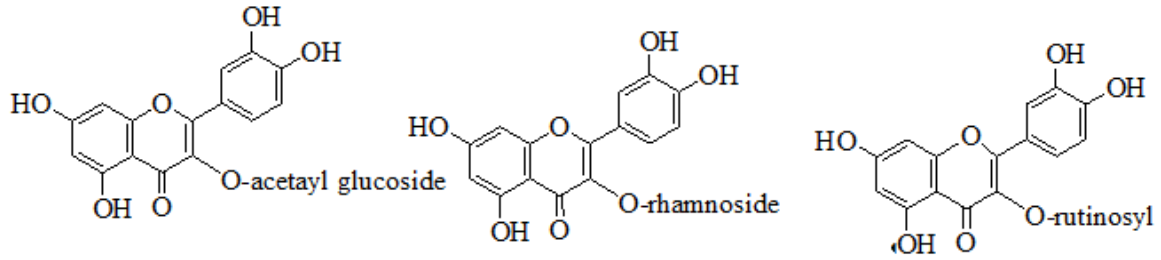

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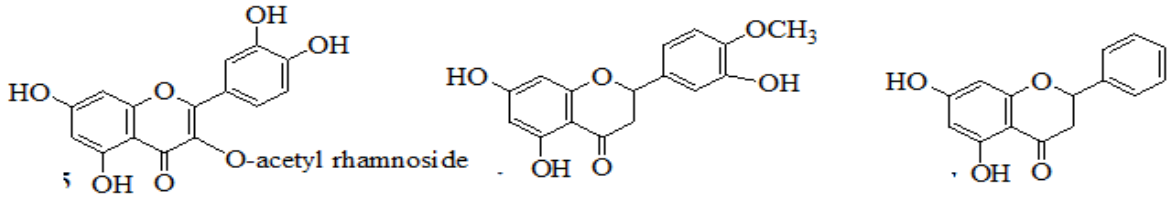
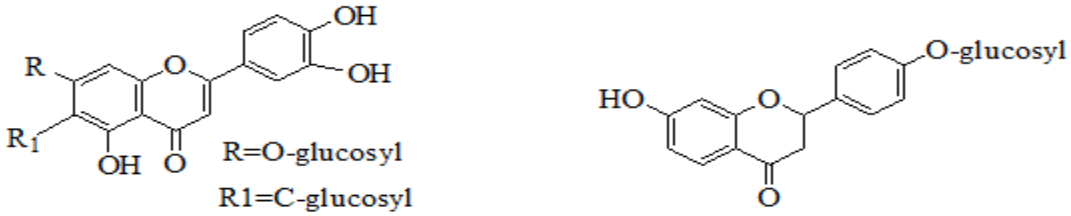


50 51 52



53 54

Figure 7 : previously isolated flavonoids from genus Rumex nervousus

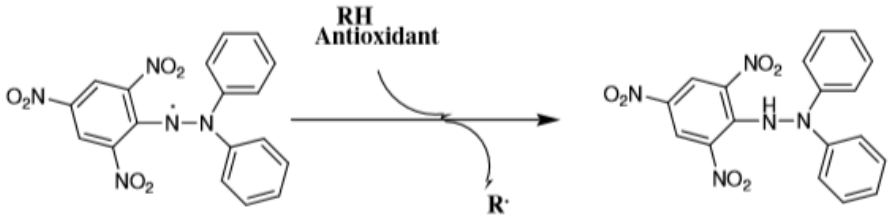
2.4 Antioxidants

Antioxidant is defined as “any substance, when present in low concentration compared to that of an oxidizable substrate, significantly delays or inhibit the oxidation of that substrate.[”37] Antioxidants are molecules that contain one or more free electrons that can be donated to stabilize ROS and they can reduce the oxidative stress in cells. A more biologically relevant definition of antioxidants is “synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. Antioxidants play an important role as health protecting

factor. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties[.38] Medicinal plants contain various types of antioxidants, mostly polyphenols and flavonoids which exhibit high antioxidant activity. The “antioxidant activity” measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay; it is inappropriate and misleading to generalize the data as indicators of “total antioxidant activity”. Antioxidant agents are closely associated to the prevention of degenerative diseases, such as cardiovascular and neurological illnesses, oxidative stress malfunctions and cancer [39].

2.5 2-Diphenyl-1-picryhydrazyl radical assay (DPPH)

The DPPH assay is popular in natural product antioxidant studies. One of the reasons is that this method is simple and sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. The molecule of 1, 1-diphenyl2-picryl-hydrazyl (DPPH) radical has a spare electron over the molecule which makes the molecule stable as it does not dime rise like most of other free radicals. Shows bellow the mechanism by which DPPH free radical accepts hydrogen from antioxidant. The antioxidant effect is proportional to the disappearance of DPPH free radical in test samples. DPPH free radical is one of the few stable and commercially available organic nitrogen radicals [.40] Monitoring DPPH free radical with UV spectrometer has become the most commonly used method because of its simplicity and accuracy. DPPH free radical shows strong absorption maximum at 517 nm (purple). The color turns from purple to yellow followed by the formation of DPPH up on absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Therefore the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm [.40]



2, 2-Dipheryl-1-picrylhydrazyl Free radical (DPPH)

2, 2-Dipheryl-1-picrylhydrazyl (DPPH-H)

Figure 8. Free radical conversion of DPPH to DPPH-H by antioxidant compound.

2.6 Antibacterial activity

Antibacterial of plant origin have enormous therapeutic potential. And they are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antibacterial. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products were present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, Trepan, phenol compounds, flavonoids, steroids, resins, fatty acids and gums which are capable of producing definite physiological action on body. Compounds extracted from different parts of the plants can be used to cure atherosclerosis, ischemic heart disease, ageing, diabetes mellitus coughing, headache, fever, eczema, dysentery, diarrhea, constipation, jaundice, hematemesis and uterine hemorrhage and other. [14, 41].

2.6.1 Paper Disc method

. The paper disks (6 mm in diameter; BD Diagnostic Systems) impregnated with diluted antibiotic solution was placed on the surface of each Mueller-Hinton agar (MHA) plate using a sterile pair of forceps. Then the plates were incubated aerobically and the diameter of zone inhibition was measured by a ruler. Based on the diameter of the inhibition zone. The bigger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial [42].

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant materials

Fresh leaves of *Rumex nervosus* were collected from Adis kidame, which is located in Awi zone, Amhara regional state, Ethiopia in March 2020. The plant material was identified and authenticated by botanist in Department of biology, Debre Berhan University.

3.1.2. Chemicals and reagents

The chemicals and reagents used for this study were distilled water, chloroform (99.9 %, Fisher Scientific UK Limited, UK), methanol, petroleum ether, ferric chloride (10 %), Wagner's reagent (Iodine in potassium iodide), aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_{2)}$, hydrochloric acid (HCl), sulfuric acid (H_2SO_4) (products of Loba Chemie Pvt. Ltd, Germany), sodium hydroxide (NaOH), nitric acid (HNO_3), sodium carbonate, iodine, NaH_2PO_4, Na_2HPO_4, phosphoric acid, bromine, Ascorbic acid, DPPH, ammonia solution. There may be Gram positive, and Gram negative bacteria.other chemicals and reagents which will be used during the experiment.

3.1.3. Instruments and apparatus

The necessary apparatus and instruments used for this study are electronic beam balance for mass measurement, vacuum rotary evaporator for concentrating the filtrate to dryness UV – visible spectrophotometer (Agilent technologies, Cary 60 UV-Vis), volumetric flask, beaker, round bottom flask, Whiteman No.1 filtrate paper, aluminum foil, micropipette, incubated agar, cuvettes , Petridish, autoclaves. forceps swaps and others were used for different purposes.

3.1.4. Preparation and Extraction of the plant materials

The collected fresh leaves of *R. nervosus* were washed and air dried at room temperature for a week under shade until it became well dried for grinding. The dry plant materials was taken separately and ground to a powdered. 200 g of leaf powder was mixed with 750 mL hexane, chloroform and ethanol solvent using successive extraction and soak for 24 h on rotary electrical

shaker. After that the extract was filtered by Whatman (no.1) filter paper from residue and the extract was concentrated using rotary vacuum evaporator to obtain the crude extract. Extracts was stored in closed and dark place for the next experiment.

Figure 9**:** Preparation and extraction of *Rumex nervosus.*

3.2.2. Preparations of stock solutions and reagents

3.2.2.1. Reagent preparation

DPPH reagent: - DPPH (0.004 g) powder was taken into volumetric flask (100 mL) and then methanol was added up to the mark in the flask to prepare DPPH (0.002 %) solution.

Ferric chloride solution (5 %): ferric chloride (5 g) was dissolved in 100 mL distilled water.

Lead acetate (10 %, w/v):- lead acetate (10 g) was dissolved in distilled water (100 mL).

Muller Hinton Agar:- The media used for antibacterial test was prepared by dissolving 3.8 g of Muller Hinton agar in 100 mL of volumetric flask and distilled water was added up to the mark.

Wanger's reagent: - 2 g of iodine powder was measured and mixed with 6 g of potassium iodide (KI) salt. Then the mixture was transferred into 100 mL volumetric flask and filled distilled water up to the mark.

Phosphate buffer (0.2M) with pH 6.6:- 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of sodium dihydrogen phosphate, 0.24 g of potassium dihydrogen phosphate was taken in a 1,000 mL standard flask and add 800 mL of distilled water and adjust the pH 6.6 using hydrochloric acid and adjust the volume with distilled water [39]

3.2.2.2. Preparations of stock solutions

800 ppm stock solutions of ascorbic acid was prepared by dissolving 0.01 g of ascorbic acid with 12.5ml methanol in a separate 100 mL of volumetric flask and distilled water was added up to the mark. 800 ppm stock solution of extracts of hexane, chloroform and ethanol were prepared by dissolving 0.01 g extract by 12.5 mL methanol in a separate 100 mL of volumetric flask and distilled water was added up to the mark.

. 200mg of each extract were dissolved in 1ml DMSO to prepared 200 ppm sample solution for anti-bacterial activity.

3.2.3. Qualitative phytochemical analysis

For qualitative analysis of leaf extracts of Rumex nervosus different phytochemical tests were performed [33, 34, 35, 36, and 37].

3.2.3. 1. Detection of alkaloids (Wagner's Test)

Filtrates are treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/ reddish precipitate indicates the presence of alkaloids.

3.2.3.2. Detection of phenols (Ferric Chloride Test)

Extracts was treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

3.2.3. 3 Detection of flavonoids (Lead acetate test)

A small amount of extract was treated with lead acetate and observed for the formation of white/ yellow precipitate indicates the presence of flavonoids.

3.2.3.4 Detection of terpenoid (Liebermann-Burchard test)

One ml extracts was treated with chloroform, acetic anhydride and added drops of H_2SO_4 and observed for the formation of dark green color indicates the presence of terpenoids.

3.2.3.5. Test for steroids (Salkowski's test)

2 mL of chloroform was added, and then 1 mL of concentrated H_2SO_4 acid was added carefully along the sides of the test tubes. A red color produced in the chloroform layer and confirms the presence of steroids.

3.2.4. Antioxidant capacity assay

3.2.4.1. DPPH radical scavenging assay

Free radical scavenging activity was evaluated on the basis of the scavenging activity of DPPH after measuring the reduction of absorbance around 517 nm by the method described before by using different concentrations (50, 100, 200, 400, and 800 µg/mL) of the plant extracts and ascorbic acid which were prepared separately. The assay mixture contains a total volume of 6 mL and consists of 4 mL of the extracts, 2 mL of freshly prepared DPPH solution (2 mM in methanol).The contents were mixed vigorously shake for 10s and incubated at room temperature in the dark (wrapped with aluminum foil) for 30 min. The absorbance was measured at 517 nm using a spectrophotometer. Ascorbic acid was used as control. The radical scavenging activity of samples corresponded to the intensity of quenching DPPH. The results were expressed as percentage inhibition [49].

$$\% \textbf{ Inhibition} = \frac{(\textbf{Ac} - \textbf{As})}{\textbf{Ac}} \textbf{X100}$$

Where Ac = absorbance of the control As = absorbance of the sample

3.2.5. Antimicrobial activity test

Antimicrobial effect of R. nervosus extracts were tested against the four bacteria (two Gram negative bacteria, Escherichia coli (ATCC 25922) and Salmonella typhymurium (ATCC 13311) and two Gram positive bacteria, Staphyloccus aureus (ATCC 25923) and Listeria monosaytozens (ATCC 19115) by paper disk method using Muller Hinton agar media. All materials used for antimicrobial activities (Petridish, flask, forceps, swaps, beakers, loop) were first sterilized by autoclaves at 121 ^{0}C for 15 minutes. Nutrient agar media was prepared by mixing agar powder with distilled water in 100 mL flask. This solidified media was transferred to the sterilized Petri dish. Then, the microbial were introduced in to the Petri dish containing solidified agar by inoculating loop and microbial were refreshed (incubated) at 37^{0}C for 24 h [51]. Finally the refreshed microbial were transferred and distributed evenly in to the sterilized Petri dish containing Muller Hinton agar for bacteria by swapping method . In this Petri dish, solutions of

plant extracts with concentration (100 and 200 µg/mL) were introduced by paper disk method [44]. The standard antibiotic, gentamycin was also applied to the center of the agar plate. At the end of the day, the Petri dishes were observed for zone of inhibition after 24 h incubation at 37 ^{0}C and the diameter of the inhibited zone was measured. The diameter of inhibited zone formed was compared with the standard drug gentamycin. The antibacterial test was carried out at Debre Berehan University, microbiology laboratory center, Debre Berehan, Ethiopia.

3.3. Analytical methods

Calibration curves of standards were constructed to determine each extract of sample from the Linear equations derived from each calibration curves in the form of

$Y = ax + c$ ……1

Where, a = slope, c = y-intercept

Inhibition (% I) in DPPH assay of the extracts were calculated in equation "2".

$$\% \text{ Inhibition} = \frac{(Ac - As)}{Ac} X100 \quad \text{……..2}$$

Where Ac is the absorbance of the control and As is the absorbance of the sample

3.4 Over all methods of the experiment

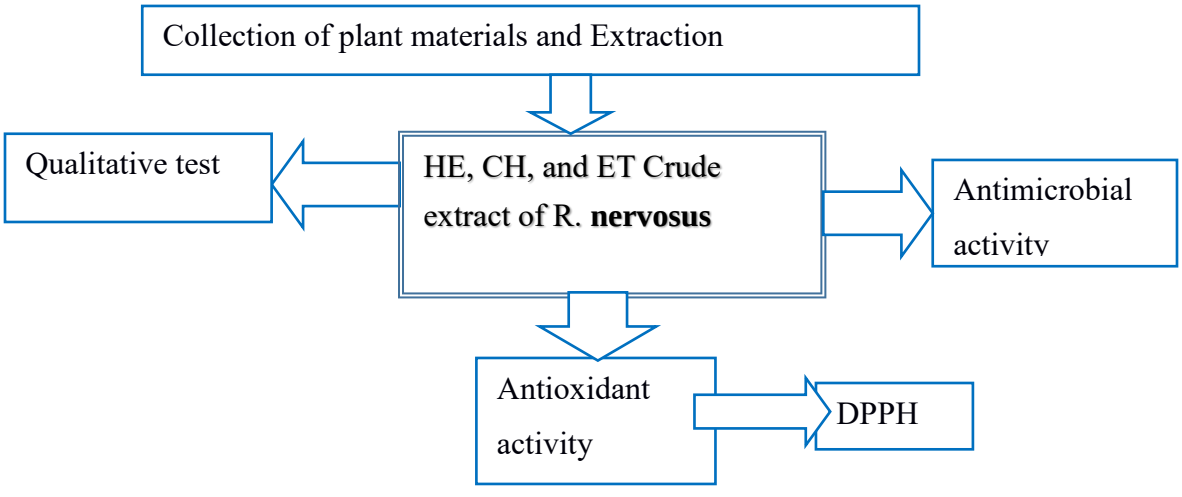


Figure 10**:** Over all methods of the experiment.

CHAPTER FOUR

4. RESULT AND DESCUSSION

4.1. Percentage yields of extracts

The percentage yields of ethanol, chloroform and hexane extracts of ***Rumex nervosus*** leaves were 12.62 g (6.31%), 6.2 g (3.1%) and 3.4 g (1.7%), respectively(Table:2). Ethanol extract gives more yield than chloroform and hexane. Hexane extract gives lower yields than Chloroform and ethanol extracts. These results showed that more polar solvents extract more polar compounds and gives high yields but non polar solvents cannot extract polar compounds and as the result gives low yields.

Determination of the extraction yield is a measure of the solvent efficiency to extract specific components from the original material. The percentage yield of extract for each solvent was calculated using the formula:

$$percentage\ yield = \frac{weight\ of\ final\ extract}{initial\ weight\ of\ powder\ ed\ sample} \times 100$$

Table 2**:** Percentage yields of extracts.

Solvents	**Initial mass of powdered sample (g)**	**Mass of final extracts (g)**	**Percentage yield**	**Color**
Hexane	200	3.4	1.7	Green yellow
Chloroform	200	6.2	3.1	Dark green
Ethanol	200	12.62	6.31	Dark

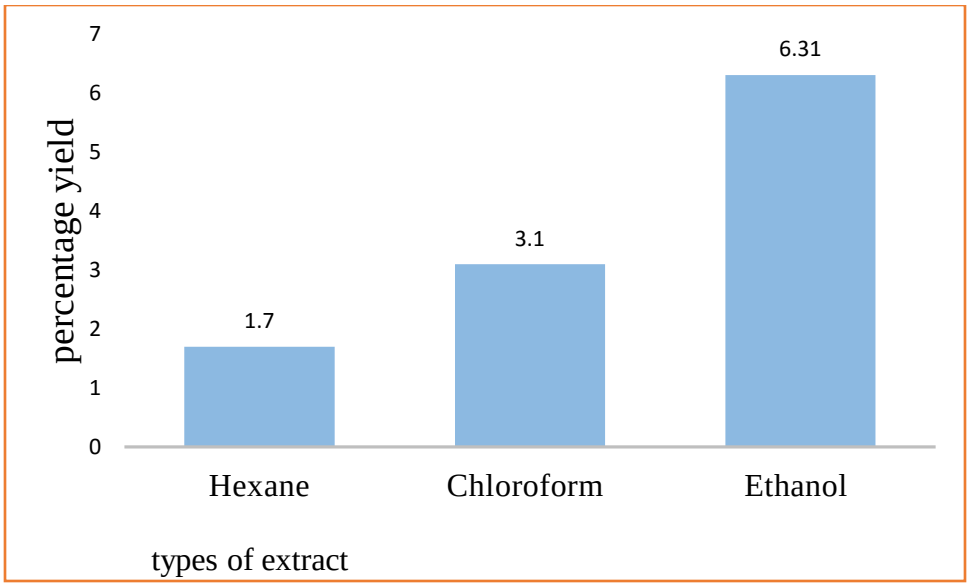


Figure 11 percentage yield of rumex nurvosus extract.

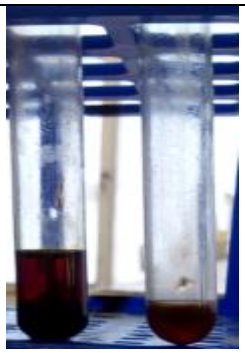
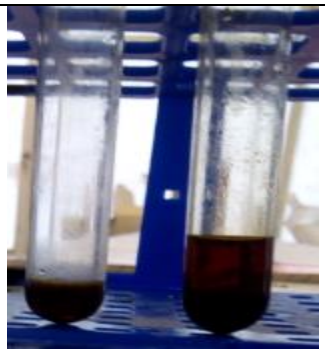
4.2. Qualitative phytochemical analysis

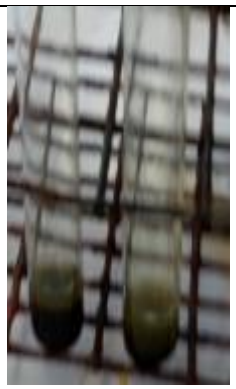
The qualitative analyses of bioactive compounds present in the three solvent extracts have been analyzed as follows. The presence and absence of useful bioactive substances such as, flavonoid, phenols, terpenoids, steroid, alkaloids, and other pharmacological active compounds in leaf extracts of ***Rumex nervosus*** were revealed by the confirmatory test, involving color changes.

Table 3**:** The qualitative phytochemical analysis of leaf extracts of *Rumex nervosus.*

Phytochemicals	**Types of test or Reagent**	**Ethanol**	**Chloro form**	**Hexane**
Alkaloids	Wagner's test	++	+	+
Phenols	Ferric chloride test	++	+	+
Flavonoids	Lead acetate test	++	+	+
Terpenoids	Lieberman-Burchard test	++	+	+
Steroids	Salkowski test	++	-	-

Key; ++ highly present += present, **-** = absent

Test	ET	CH	HE
Alkaloids			
Flavonoids			
Terpenoids			
Phenolic			

Steroids			

Key ET = Ethanol, CH = Chloroform, HEX = Hexane

Figure 12**:** Color observed in some phytochemical test

4.3 DPPH radical scavenging activity determination

Calibration curve was prepared to determine the antioxidant activity of *Rumex nervosus* leave extracts in terms of ascorbic acid equivalent using DPPH radical scavenging methods. Ascorbic acid equivalent antioxidant capacity is expressed as µg of AA per 12.5 mL of *Rumex nervosus* leave extracts samples. The calibration curve was plotted as absorbance verses different concentration of ascorbic acid (50 µg/ mL, 100 µg/ mL, 200 µg/ mL, 400 µg/ mL and 800 µg/ mL) and the value of the absorbance obtained corresponding to concentrations are given in Table 4.

Table 4 Standard of the absorbance of ascorbic acid in different concentration at 517 nm.

Ascorbic acid	**Absorbance**	**Inhibition%**
50	0.0291±0.000173	94.93913±0.030123
100	0.0276±0.0001	95.2±0.017391
200	0.0253±0.0001	95.6±0.017391
400	0.023±0.002	96±0.347826
800	0.017±0.002	97.04348±0.347826

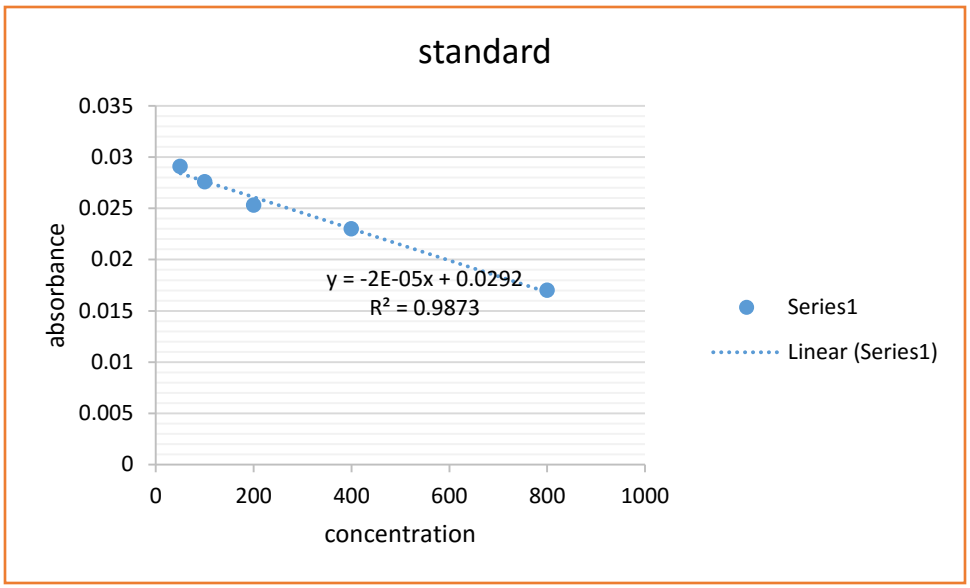


Figure 13 Calibration curve that shows the standard of the absorbance of ascorbic acid at different concentration at 517 nm.

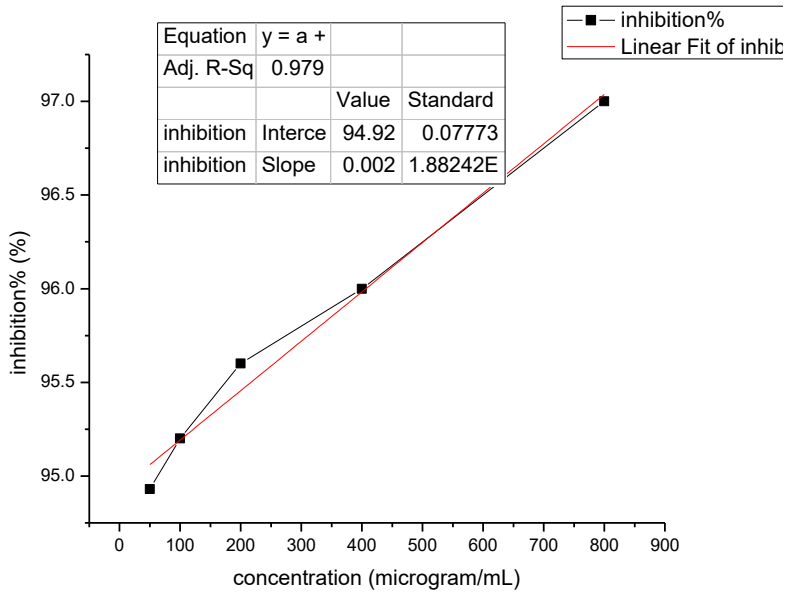


Figure 14 Calibration curve that shows the standard of the inhibition % of ascorbic acid at different concentration at 517 nm.

Table 5 absorbance of ethanol extracted at different concentration of 517nm.

Ethanol	Absorbance	Inhabitation %
50	0.232±0.001155	59.65217±0.200817
100	0.228±0.002082	60.34783±0.362029
200	0.188±0.001528	67.30435±0.265657
400	0.159±0.003215	72.34783±0.559052
800	0.079±0.001528	86.26087±0.265657

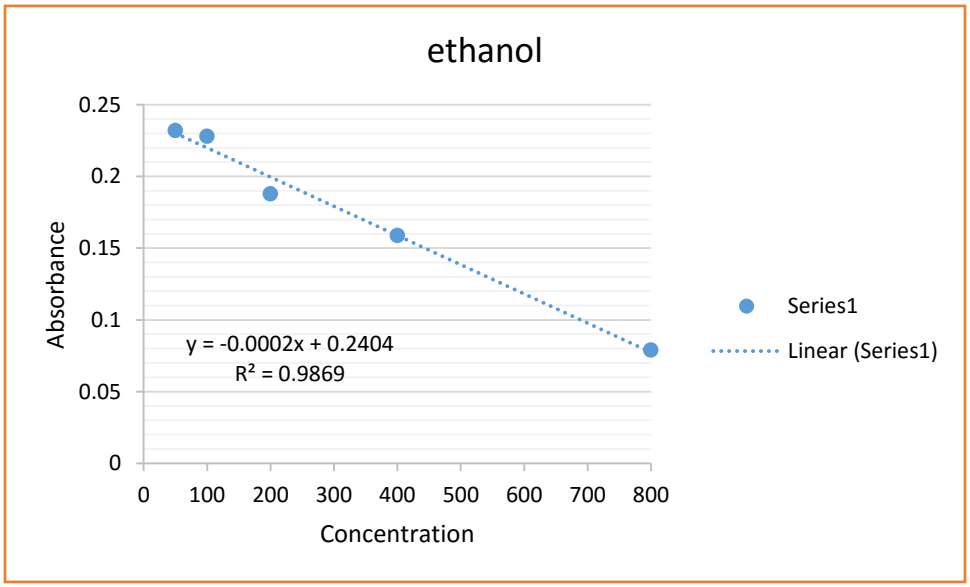


Figure 15 Calibration curve that shows the absorbance of ethanol extracted in different concentration at 517nm.

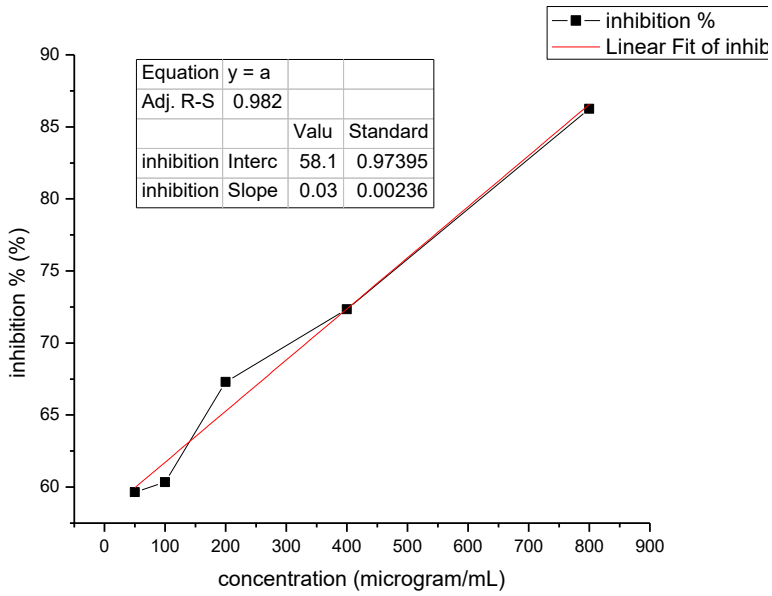


Figure 16 Calibration curve that shows the inhibition% of ethanol extracted in different concentration at 517nm.

Table 6 absorbance of chloroform extracted at different concentration of 517nm.

Chloroform	**Absorbance**	**Inhabitation %**
50	0.327±0.002	43.13043±0.347826
100	0.316±0.000099	45.04348±0.173913
200	0.271±0.0001	52.86957±0.347826
400	0.203±0.002646	64.69565±0.46131
800	0.127±0.002	77.91304±0.347826

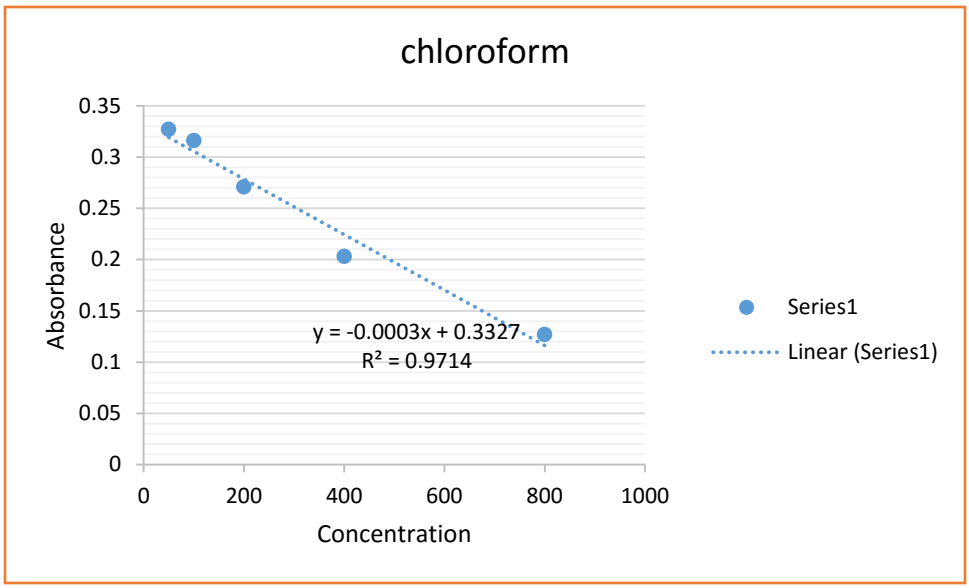


Figure 17 Calibration curve that shows the absorbance of chloroform extracted in different concentration at 517nm.

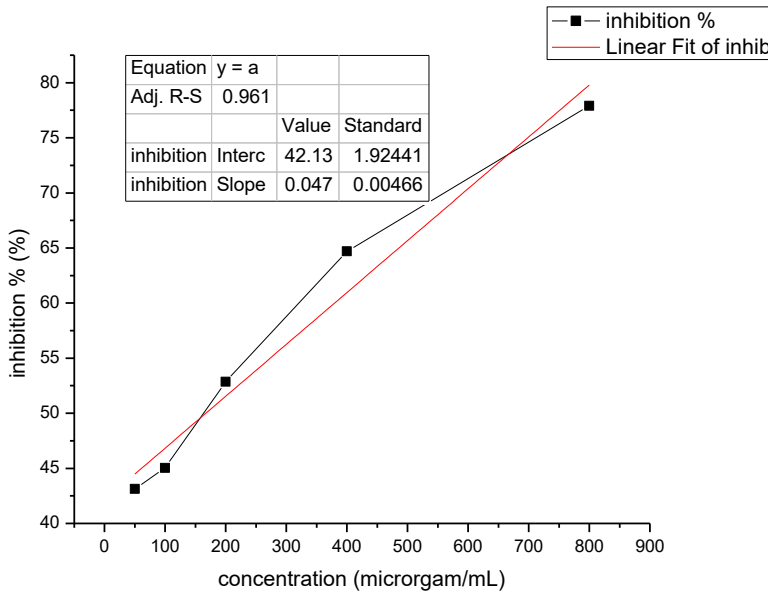


Figure 18 Calibration curve that shows the inhibition % of chloroform extracted in different concentration at 517nm

Table 7 absorbance of hexane extracted at different concentration of 517nm

Hexane	Absorbance	Inhibition %
50	0.358±0.003606	37.73913±0.627052
100	0.335±0.000099	41.73913±0.521739
200	0.311±0.0001	45.91304±0.347826
400	0.272±0,004	52.69565±0.265657
800	0.227±0.002646	60.52174± 0.173913

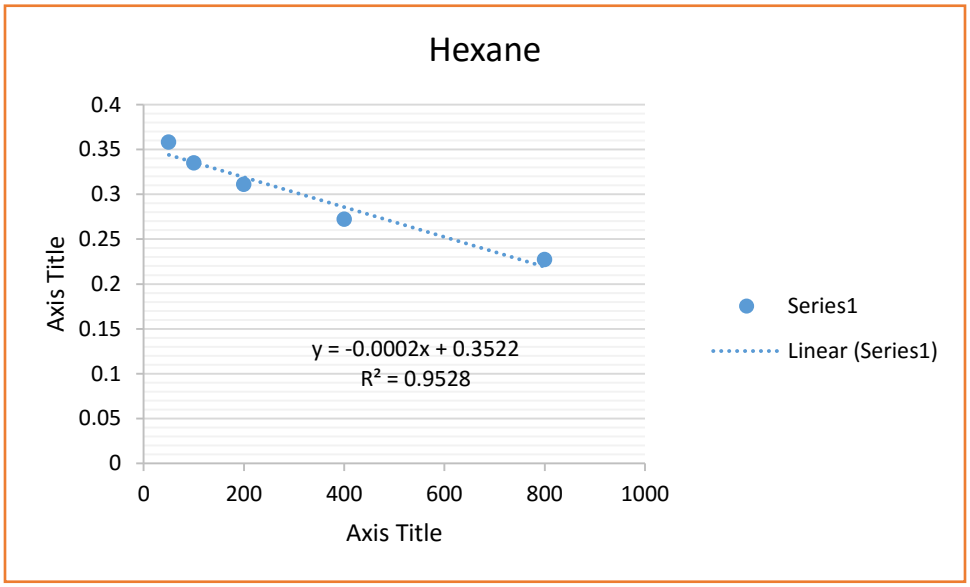


Figure 19 Calibration curve that shows the absorbance of hexane extracted in different concentration at 517nm.

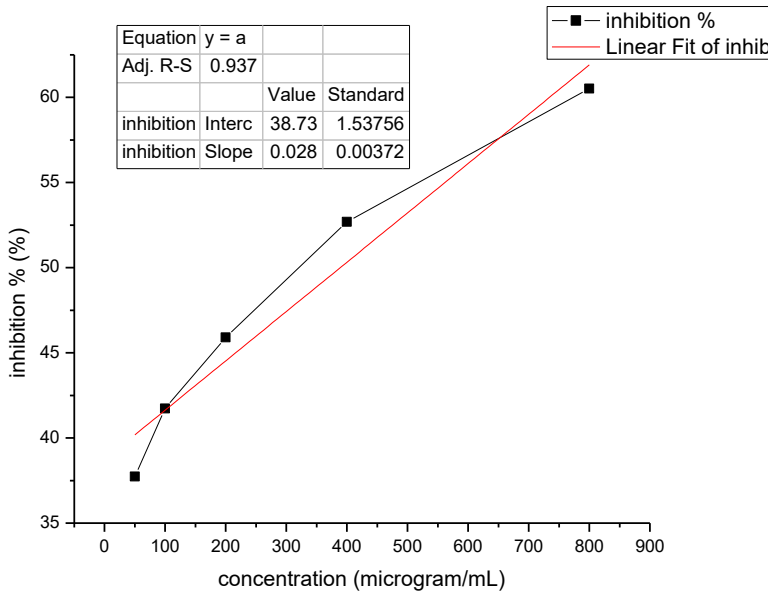


Figure 20 Calibration curve that shows the absorbance of hexane extracted in different concentration at 517nm.

Table 8 DPPH scavenging activity plant extract and standard

Concentration	**AA**	**ETH**	**CHL**	**HEX**
50	0.0291	0.232	0.327	0.358
100	0.0276	0.228	0.316	0.335
200	0.0253	0.188	0.271	0.311
400	0.023	0.159	0.203	0.272
800	0.017	0.079	0.127	0.227

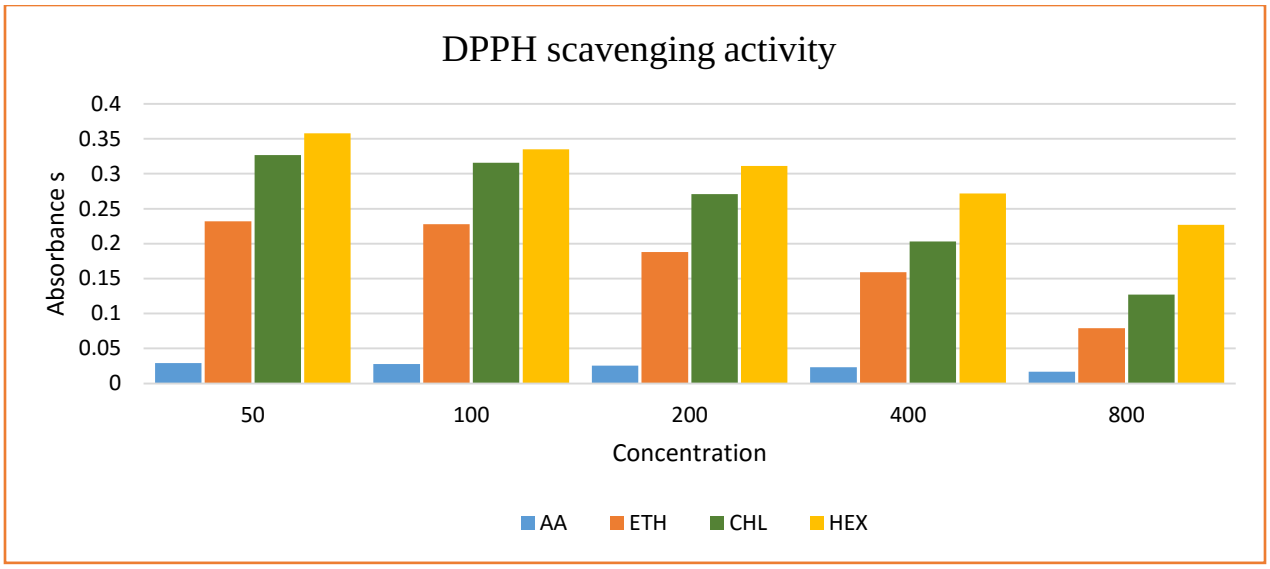
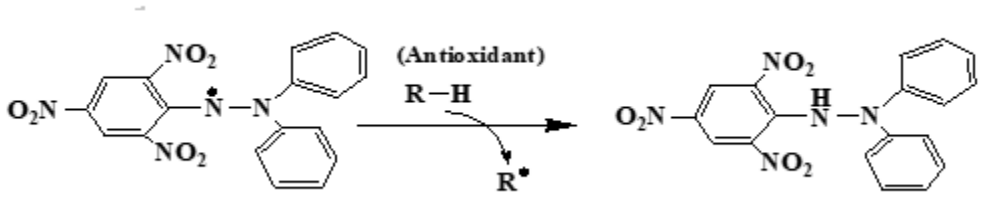


Figure 21 DPPH scavenging activity plant extract and standard

The antioxidant scavenging capacity of the plant extract can be measured by DPPH radical scavenging assay. The working principle of DPPH is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction. Freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm using methanol solvent. (Figure: 17 DPPH free radical stabilized due to the formation of strong hydrogen bonding with water. This purple color generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colorless /yellow product Figure**; 17** resulting in a decrease in absorbance at 517 nm.



DPPH

1,1-diphenyl-2-picrythydrazyl

Free radical form (purple color)

DPPH-H

1. 1---dipheryl –2- picrthydrazine

non-radical form (pale yellow)

For the DPPH radical scavenging assay different concentrations of ascorbic acid (50, 100, 200, 400, and 800 ppm) were used to construct the calibration curve. The calibration curve was constructed as a function of inhibition versus concentration of ascorbic acid. The absorbance at 517 nm and the percentage inhibition of ascorbic acid was given above in Table 4.

For the antioxidant activity study, different concentration of ethanol, chloroform, and hexane leaf extract of *Rumex nervosus* (50, 100, 200, 400 and 800 ppm) were screened with DPPH, a stable free radical, and shows strong radical scavenging activities. The activities of the test sample in DPPH scavenging assay can be expressed as a decrease in absorbance. This activity was increased by increasing the concentration of the sample extract. Figure17 showed that decreased in absorbance of the extract sample as the concentration of the each extract increased from 50 ppm to 800 ppm respectively. The change in absorbance of DPPH radicals caused by antioxidants is due to the reaction between the antioxidant molecules of the plant extract and the radicals. As the absorbance decreases, the free radical scavenging activity becomes high. The scavenging activities of the extracts of *Rumex nervosus* can be expressed using percentage inhibition of DPPH free radical. The percentage inhibition increases with increasing plant concentration.

4.4 Antibacterial activity test of leaf extract of *Rumex nervosus*

Antibacterial activity of hexane, chloroform and ethanol extract of *R. nervosus* was evaluated by using paper disk method in microbiology laboratory, Biology department at Debre Birhan University. Four bacteria were selected for the determination in which two of them were Gram negative bacteria (*Escherchia colia* and *Salmonella typhymurium*) and the remaining two of them were Gram positive bacteria *Listeria monocytogens* and *Staphylococcus aures*), and *Candida albicans* fungus was used. A series of two concentrations (100 and 200 ppm) for each extracts were prepared by using serial dilution method and paper disc method into incubated plates in which bacteria was cultured and inhibition zone (Table 9) values were recorded as shown below.

Table 9 Comparison of zone of inhibition among leaf extracts of *Rumex nervosus* and standards against drug resistant gram positive and gram negative bacteria.

Sample extract	Concentration of extracts (mg)	Zone of inhibition (mm) for bacteria			
		Gram positive		Gram negative	
		Listeria monocytogens	Staphylococcus aures	Escherchia coli	Salmonella typhymurium
Ethanol	200	10	9	10	8
	100	___	__	___	___
Chloroform	200	9	8	8	___
	100	___	7	___	___
Hexane	200	___	8	7	___
	100	___	___	7	___

As shown in the above Table 9,slightly better inhibition zone was recorded in ethanol extract than inhibition zone recorded in chloroform and hexane extracts of *Rumex nervosus* in all four bacteria whereas hexane has lower inhibition zone than ethanol and chloroform extract. All extracts had lowest antibacterial potential as compared with the antibiotics used. In this study slightly better antibacterial results were recorded at concentration of ethanol extract of *Rumex nervosus* showed slightly better antibacterial result against a Gram positive bacteria Staphyloccus aureus and Listeria monocytogens and Gram negative bacteria Escherichia coli and, Salmonolla typhymurium with zone of inhibition. Chloroform extract of *Rumex nervosus* showed a medium antibacterial activity against a Gram positive bacteria Staphyloccus aureus and Listeria monocytogens and Gram negative bacteria Escherichia coli and Salmonolla typhymurium with zone of inhibition. Hexane showed low zone of inhibition.

CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATION

5.1. CONCLUSION

Based on this study, antioxidant and antibacterial activities of *R*umex nervosus extracts were concentration dependent. As the solvent polarity increase antioxidant and antibacterial activity of the extract increased**.** Ethanol extracts were higher than chloroform and hexane extracts of *R. nervosus*. Ethanol extract showed powerful DPPH free radical scavenging activity compared to chloroform and hexane extracts. The antibacterial activity for ethanol extract is better compared to the other two extracts due to the presence of phenolics and flavonoids.

5.2. RECOMMENDATION

The result of this study suggested that the leaves of *Rumex nervosus* can be used as source of antioxidant and antibacterial, thus peoples can use the leaves of this plant for their primary health care needs to protect their body from resistance bacteria.

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