Phytochemical investigation, and biological evaluation of roots and aerial parts extract of *Ranunculus multifidus* and *in silico* study of the interaction of some of its constituents with selected protein targets

Terfo Yilma Mikre

A Thesis Submitted to the Department of Applied Chemistry

School of Applied Natural Sciences

Presented in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Applied Chemistry (Medicinal Chemistry)

Office of Graduate Studies

Adama Science and Technology University

November, 2022

Adama, Ethiopia

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Major Advisor: Professor Aman Dekebo (PhD)

Co-Advisor: Dr. Endale Mulugeta (PhD)

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Declaration and Recommendation

Declaration

I hereby declare that this Master thesis entitled "Phytochemical investigation, and biological evaluation of roots and aerial parts extract of *Ranunculus multifidus* and *in silico* study of the interaction of some of its constituents with selected protein targets" is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

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Recommendation

I/we, the advisor(s) of this thesis, hereby certify that I/we have read the revised version of the thesis entitled "Phytochemical investigation, and biological evaluation of roots and aerial parts extract of *Ranunculus multifidus* and *in silico* study of the interaction of some of its constituents with selected protein targets" prepared under my/our guidance by Terfo Yilma submitted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Chemistry (Medicinal chemistry). Therefore, I/we recommend the submission of a revised version of the thesis to the department following the applicable procedures.

_____	_____	_____
Major Advisor	Signature	Date
_____	_____	_____
Co-advisor	Signature	Date

Approval Page of M.Sc. Thesis

I/we, the advisors of the thesis entitled "Phytochemical investigation, and biological evaluation of roots and aerial parts extract of *Ranunculus multifidus* and *in silico* study of the interaction of some of its constituents with selected protein targets" and developed by Terfo Yilma, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Terfo Yilma have read and evaluated the thesis entitled "Phytochemical Investigation, and Biological Evaluation of Roots and Aerial parts extract of *Ranunculus multifidus* and *in silico* study of the interaction of some of its constituents with selected protein targets" and examined the candidate during the open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Chemistry (Medicinal chemistry).

Chairperson	Signature	Date
Internal Examiner	Signature	Date
External Examiner	Signature	Date

Final approval and acceptance of the thesis are contingent upon the submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

Department Head	Signature	Date
School Dean	Signature	Date
Office of Postgraduate Studies, Dean	Signature	Date

ACKNOWLEDGEMENTS

I am grateful to the Almighty of God for the good health and well-being that were necessary to complete this thesis. Surely goodness and mercy shall follow me all the days of my life. Throughout the writing of this thesis, I have received a great deal of support and assistance. My genuine apologies, if I miss mentioning some who deserve to be acknowledged!

I would first like to express my deepest appreciation to thank my advisor, Professor Aman Dekebo (PhD), whose expertise was irreplaceable from the beginning of the research up until the thesis was made to this final form. He made every effort possible to help me by providing constructive criticism, financial support, frequently visits during laboratory work with fatherhood and friendly advice during the research and academic stay in general. I'm really indebted!

I would also like to extend my deepest gratitude to my co-advisor, Dr. Endale Mulugeta (PhD) for his very critical comments, and taking time for the proofreading of this thesis including the proposal. Especial thanks go to Dr. Venkatesh Perumal (PhD) for his support during molecular docking analysis study. I am thankful to Adama Science and Technology University for giving me the opportunity to pursue my M.Sc. study. I also thank the World Academy of Science (TWAS) for sponsoring my MSc research. I am also thankful to Addis Ababa University for the help in NMR sample analysis.

The last, but not the least my deepest gratitude might be goes to the haramaya university college of health and medical science for giving opportunity to join a pharmacy school which give me a great opportunity to view medicinal chemistry as a major and fascinating discipline in pharmaceutical industry. The pharmacy staff who shows a great interest to update myself through this discipline and the willingness of department head Mr. Jemal Abdalla to use equipment and material for this research. Mr. Fitsum W/Gabriel a head of medical microbiology to facilitate their laboratory for antibacterial investigation and Dr. Meseret a head of biology department for their cooperation to perform antifungal investigation.

In addition, I would like to thank my parents for their wise counsel and sympathy. Your love and guidance are with me in whatever I pursue. I wish to thank my friends and supportive colleagues; Wegene Diriba, Yalew Amare, Bethlehem Behailu, Abate Kassaw, Teshome Assefa, Dibaba Beyena, Teshome Fikre, Tewodros, Tadele, Yohannes Tadiwos, and Lami and Guta who shared materials and thank you indeed for being the source of unending inspiration.

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LIST OF ACRONYMS AND ABBERVIATIONS

ADT	Auto Dock Tools
A549	Human lung cancer cell
AgNPs	Silver nanoparticle
AQS	Anti-quorum sensing
ATP	Adenosine tri-phosphate
ASTU	Adama science and technology university
ATCC	American type culture collection
BHA	Butylated hydroxyl anisole
BEL-7470	Human hepatoma cancer cell
CC	Column chromatography
$CDCl_3$	Deuterated chloroform
CFU	Colony forming unit
CPFX	Ciprofloxacin
COX-1	Cyclooxygenase enzyme
^{13}C-NMR	Carbon-13 Nuclear magnetic resonance
d	Doublets
d6-Acetone	Deuterated acetone
DCM	Dichloromethane
DEPT-135	Distortion less enhancement by polarization transfer at angle of 135°
DFT	Density function theory
DMSO	Dimethyl sulphoxide
DNA	Deoxy ribonucleic acid
DPPH	1-1- Diphenyl-2-picrylhydrazyl
D.R. Kongo	Democratic republic of Kongo
DSM	German collection of microorganisms
EI-MS	Electron ionization mass spectroscopy
GSH	Glutathione
HL-60	Human leukemia cells
HR-EIMS	High resolution electron ionization mass spectroscopy
IC_{50}	Half maximal inhibitory concentration

IR	Infrared spectroscopy
j	Coupling constant
KB	Human epithelial carcinoma cell
5-LOX	Lipoxygenase enzyme
M	Multiplet
MCF7	Human breast cancer cell
MD	Molecular dynamics
MHA	Muller-Hinton agar
MS	Mass spectroscopy
MTCC	Microbial type culture collection and gene banks (India)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDA	Potato-dextrose agar
PDB	Protein data bank
PRDX5	Human peroxiredoxin 5
Ppm	Parts per million
Rf	Retention factor
ROS	Reactive oxygen species
RSA	Radical-scavenging assay
s	Singlet
SNNPRS	Southern Nation, Nationality, and Peoples' Regional State
STD	Sexually transmitted disease
t	Triplet
TB	Tuberculosis
THPs	Traditional health practitioners
TLC	Thin layer chromatography
TMS	Tetramethyl silane
TWAS	The world academy of science
UTI	Urinary tract infection
UV-Vis	Ultraviolet-visible spectroscopy
WHO	World health organization
δ	Chemical shift

ABSTRACT

Medicinal plants contain a wide range of secondary metabolites that can be used to treat chronic and infectious diseases. Infectious and inflammation diseases are still a growing public health concern, especially with the emerging challenge of drug resistance to the current drugs. Ranunculus multifidus, commonly known as Etse siol (in Amharic) was a perennial herb, traditionally used treating malaria, asthma and healing of wound etc. In view of its medicinal uses, the powdered root was successively extracted with n-hexane, dichloromethane and methanol yield 1.125, 1.58, and 6.096%, respectively. The powdered aerial part was extracted with methanol and successively fractionated with n-hexane, chloroform, ethyl acetate, n-butanol and afford 4.54,10.87,26.27,15.15,38.08%, respectively. Phytochemical test revealed the presence of flavonoids, phenols, tannins, saponins, and steroids in both extract of the plant. The silica gel column chromatography has led to the isolation of four compounds from methanol extract designated as RMR01, RMR02, RMR08 and F59 and three compounds from ethyl acetate fraction coded as RML01, RML02, and RML03. The structures were elucidated using spectroscopic methods such as ^{1}H-NMR,^{13}C-NMR and DEPT-135. The extracts and isolated compounds were evaluated in vitro antibacterial activities using disc diffusion method against B. subtilis, S. aureus, E. coli, and P. aeruginosa. Strong inhibition zone (13±0.82 mm, 12.5±1.5 mm,12±0.34 mm, and 11±0.45 mm) and (11.5± 0.5 mm ,11±0.45 mm, 10±0.98 mm, 10±0.26 mm) against B. subtilis, S. aureus, E. coli, and P. aerugin osa compared to standard ciprofloxacin (24±0.15 mm,23±0.34 mm, 22.5±0.52 mm,22±0.00m m) for compound RML02 and RML 01, respectively. The antifungal activities of extracts and isolated compounds were done using disc diffusion methods against two fungal strains. RMR02 and RMR08 shows inter mediate inhibitory with inhibition zone of (5.33±0.58 mm, and 7.33 ±0.42mm) to C. albicans and (9.12±0.76mm, and 5.77±0.68 mm) for A. niger at concentration of 3 mg/ml respectively. RML01, RML02 and RML03 shows good inhibitory zone with (14.43±0.40mm ,11.33 ±0. 58 mm, and 9.93±0.13 mm) against C. albicans and (15.17±0.76 mm, 14.67±0.59 mm, and 16.1± 0.27mm) to A. niger compared to standard fluconazole (21±1.5 mm) and ketoconazole (23.43 ±0.65 mm) at 1mg/ml respectively. The antiradical activities of extracts and pure compounds were done using DPPH assay and MeOH, and n-hexane root extracts shows 97.57%, 71. 95%, and n-butanol and ethyl acetate fraction shows (9 4.187%) and (90.78 %) respectively, which was comparable to ascorbic acid (98.59%) at 100 µg/mL. The pure com pounds also have promising anti-radical activities with maximum inhibition for RML03 (90.13%), RML02 (89.91%) and RMR08 (90.02 %). The Insilico molecular docking of some isolated compounds was done using AutoDock vina with some selected protein (tyrosyl-tRNA synthetase and human peroxiredoxin 5). The result shows compound RML03 and RML01 had the highest binding affinity (-9.8 kcal/mol) and (-8.7 kcal/mol) relative to CPFX (-8.8 kcal /mol). Insilico antioxidant molecular docking revealed RML01 and RML03 showed highest bin ding affinities -6.2 and -5.9 kcal/mol, respectively. The present finding of these pharmacologic ally important secondary metabolites from R. multifidus extracts brings the future attention of experts to look more on the medicinal importance of the plant.

Key words: Medicinal plants**,** Ranunculus multifidus, antimicrobial activities, antioxidant activities, phytochemical screening, *Insilico* molecular docking.

CHAPTER-ONE

1. INTRODUCTION

1.1.Background of the study

The need for novel chemical compounds to treat human diseases is ever-increasing (Akpotu *et al*., 2017; Zeleke *et al*., 2020). The rapid development of drug-resistant microbes, the discovery of new cases of life-threatening infections, and the constant recurrence of diseases have pushed for advances in the field of drug discovery (Muluye *et al.*, 2019). In principle, there are three pathways for discovering new pharmacologically significant compounds: rational drug design, where the drugs are purposefully tailored toward specific targets in the microbial cell (Kaaniche *et al*., 2018); combinatorial chemistry, which involves the synthesis of a combinatorial library of compounds, which are then tested against the cellular target to determine the most potent compounds; and natural product-based drug discovery, by isolating bioactive compounds from biological sources, especially from plants and microorganisms (Negussie *et al*., 2009).

In drug development, virtual screening like drug-likeness, ADMET, and DFT analysis is a computational method to find compounds that are likely to exhibit physiological activity in a short time and at a low cost using various *in silico* simulation methods (Anza *et al*., 2021).

Medicinal plants have been used to treat a wide range of diseases throughout the history of human beings (Anza *et al*., 2021; Galma *et al*., 2021). Today it plays a role in primary health care as a therapeutic remedy in many developing countries. This is mainly because most of these herbals are available and affordable, and have little or no side effects (Anza *et al.*, 2021; Taher *et al*., 2021). In the 21st century, studies have focused on the new group of bioactive components in medicinal plants, which have protective effects against cell oxidation to alleviate certain degenerative diseases (Raziq *et al*., 2016).

Up to 80-90% of the African population has been reported to rely on traditional medicine as a major provider of health care (Abebe, 2016). Traditional medicine is defined by the WHO as the total of all knowledge and practice, whether explicable or not, used in the diagnosis, prevention and elimination of physical, mental or social imbalances, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing (Issel & Cook, 1993).

The phytochemicals obtained from medicinal plants have also been used as precursors and can be modified synthetically to improve their therapeutic activities (Wink, 2015). Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans (Sharma *et al*., 2020). Natural products such as alkaloids, flavonoids, tannins, resins, glycosides, and oils among others reported to be bioactive against a vast range of pathogens and have therapeutic activities like antibacterial, antiviral, anti-oxidants, and anti-fungal (Asghar *et al.*, 2009). Hence, a great deal of interest has been generated recently in the isolation, characterization, and investigation of the biological activity of these phytochemicals (Wang & Chen, 2013).

Antibacterial therapy has been challenging because of the alarming rate in the rising of infections caused by bacteria coupled with their resistance to most first-line antibiotic agents (Zeleke *et al.,* 2020). This is a serious threat to human health in the world in the 21st century and urgently calls for continuing research to find out compounds that possess better antimicrobial activities with a broad spectrum. Therefore, searching for new drugs to treat disease and inflammations without causing significant side effects on patients is very important and it was the current task for which all researchers worked on.

Ethiopia has a long history of traditional health care based largely on rich, though unstandardized, pharmacopeia drawn mostly from plants used both by the peoples in the home in self-administration and by traditional health practitioners (THPs) (Bekele, G., & Hazare, S. T. 2017). *Ranunculus multifidus* is one of the traditional medicinal plants dominantly found in Africa including Ethiopia (Bhatti *et al*., 2015). This plant is called the common buttercup in South Africa and it is widespread species of flowering plant in the family of *Ranunculaceae.* It is native to Sub- Saharan Africa except West Africa, Madagascar, and the Arabian Peninsula (Bhatti *et al*., 2015). The most common use of *Ranunculus* species in traditional medicines is to treat rheumatism, intermittent fever, and rubefacient (Aslam & Uzair, 2015).

The selection of plants for natural product research can be based on certain criteria like ethno medicinal values, endemic nature of the plant to a particular country, the plant that has large biodiversity, and having unusual biology which means its ability to survive in harsh climatic condition and having a novel strategy of survival is seriously considered (Najmi *et al.*, 2022). *Ranunculus multifidus* can also have been selected based on one or more than one criterion

depicted above. Especially the diversified traditional use of the plant in Ethiopia can be a major criterion to select this plant to be researched more.

1.2.Statement of the Problem

Infectious diseases caused by bacteria, fungi, viruses, and parasites continue to pose a threatening challenge to public health (Cos *et al*., 2006). The Discovery and development of new antibiotics are very important because of the continuous appearance of drug-resistant pathogenic bacteria to many commercially produced synthetic antimicrobial agents (Zeleke *et al*., 2020). This suggests the need of searching for alternative antimicrobial agents. Medicinal plants play a role in the treatment of various human and livestock ailments in rural Ethiopia; it needs further scientific evidence of their traditional medication (Taher *et al*., 2021).

Over-production of reactive oxygen species (ROS) results in oxidative stress which is a harmful process that damages cell structures, including lipids, membranes, proteins, and DNA (Putri *et al*., 2018). Oxidative stress has been culpably involved in various diseases including Atherosclerosis, cancer, cardiovascular, and neurodegenerative disorders (Raziq *et al*., 2016). The *in vivo* carcinogenic effects of synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) have been posing another problem that many researchers also worked on. Therefore significant effort has been spent to find out natural antioxidants over synthetic compounds and elimination of synthetic antioxidants (Bhatti *et al.*, 2015).

Polyphenols are widely distributed in plants and play an important role in medicine (Niedzwiecki *et al*., 2016). Flavonoids and phenolic compounds are significant constituents of the human diet and many of them are natural antioxidants (Taher *et al.*, 2021). To the best of our knowledge a comprehensive biological study, isolation of compounds, and molecular docking study of the roots and aerial extracts of *Ranunculus multifidus* had not been reported so far, particularly in Ethiopia. However, the local people in the country use this plant for various ethnomedicinal purposes without having proper scientific evidence concerning the chemical composition and biological uses of the plant. Despite the above-mentioned reason, and the multivariate ethno medicinal application of *R. multifidus*, the study was aimed at phytochemical analysis, biological evaluation, and molecular docking study of isolated compounds from the roots and aerial extracts of the plants to find new lead compounds for the drug discovery program.

1.3.Significance of the Study

Plant-derived medicines have made large contributions to human health and well-being. This study was focused on the phytochemical investigation, Antimicrobial (antibacterial and anti-fungal) analysis, antioxidant activities test, and molecular docking of its constituents to some selected bacterial and human enzymes studies on the pure compounds isolated from the root and aerial extract of *R. multifidus*. Since the species have many traditional uses in Ethiopia at different locations and some laboratory investigation provides as it has an important secondary metabolite that acts as anti-inflammatory, anti-malaria, and anti-quorum sensing (AQS) effect the research might have a significant effect on scientific communities.

The scientific validation of traditional therapeutic values and indigenous knowledge of the Ethiopian medicinal plants claimed by traditional healers are so crucial that assist to provide a basic understanding of the plants' efficacy and lend further support to the widespread use of the traditional medicine in health care systems. Hence the importance of the present study was laid in providing a scientific rationale to support the traditional therapeutic claims of the selected traditional medicinal plants by investigating their chemical constituents and examining antimicrobial potency. The outcome of the study was also expected to pave a way for the scientific communities to carry on further studies on drug design and developments by providing novel bioactive compounds, which can be used as lead compounds. The present studies in general were having the following significance.

- ✓ Validating the medicinal potency of the selected plant species;
- ✓ Providing a scientific rationale to support the claimed ethnomedicinal knowledge of the selected plant species;
- ✓ Laying a scientific base for both modern and traditional medicines by providing a bridge between the modern and traditional claims of the medicinal application of the plant species.

1.4. Objectives of the Study

1.4.1. General Objective

- The major objective of this study was to investigate the phytochemical composition and examine biological activities of the roots and aerial parts of *Ranunculus multifidus*.

1.4.2. Specific Objectives

- To extract the bioactive compounds from root of *R. multifidus* successively with n-hexane, dichloromethane, methanol (MeOH) using cold maceration methods.
- To extract the bioactive compounds from aerial parts of *R. multifidus* using liquid-liquid partitioning protocol using different solvents of different polarity.
- To conduct preliminary phytochemical screening on crude extracts and fractions of the plant.
- To isolate the chemical constituents from the crude extracts of the root and aerial part of *R. multifidus* by using silica gel CC.
- To characterize the isolated compounds by employing spectroscopic methods using ^{1}H-NMR (^{1}D), ^{13}C-NMR, and DEPT-135.
- To evaluate *in vitro* antibacterial activities of the root crude extract and aerial fractions, and isolated compounds of *R. multifidus* against selected Gram-positive bacterial strains (*S. pyrogens/B.subtilis* and *S. aureus*), and Gram-negative bacteria (*E. coli*, and *P. aeruginosa*) as well as anti-fungal activities against fungal strains of *C. albicans* and *A. niger* using disc diffusion technique.
- To examine antioxidant activities of crude extract and isolated compounds from the roots and aerial extracts of *R. multifidus* using DPPH radical scavenging methods.
- To investigate ligand-protein docking of the isolated compounds from the root and aerial fraction using AutoDock Vina 4.2.6 version software.

CHAPTER -TWO

2. LITERATURE REVIEW

2.1 General Description of Medicinal Plants

Medicinal plants have been a major source of cures for human diseases since the time of immemorial (Guyasa *et al*., 2018). As modern techniques, for example, genomics, high-throughput screening, and target-oriented drug development strategies, have not yet fulfilled expectations that appeared promising upon their introduction, chemotherapeutics remain the cornerstone of patient management and will likely remain so for the foreseeable future (Guyasa *et al*., 2018).

Traditional healers use medicinal plants for treatments of a variety of illnesses caused by protozoan, bacteria, fungi, viruses, and helminths (Pandey *et al*., 2017). Traditional knowledge used to solve health problems of mankind and animals exists in all countries of the world. The use of medicinal plants as the source of remedies for the treatment of many diseases dates back to prehistory and people of all continents have this old tradition (Anza *et al*., 2021; Guyasa *et al*., 2018). The use of plants as medicines is not restricted to one particular region or continent. Plants as medicines are capitalized in African, Greek, Islamic, Chinese, Indian, and Western systems of medicine. This indicates the consensus among the nations on the use of plants as medicines (Solomou *et al.*, 2016).

The first official recognition of traditional medicine as an important participant in primary health care was expressed in the World Health Organization's Primary Health Care Declaration of Alma Ata in 1978 (Ameh *et al*., 2010). Traditional medicine according to the declaration is the total of all the knowledge, and practices used in diagnosis, prevention, and elimination of physical, mental, or social imbalance and relies exclusively on practical experience and observation handed down from generation to generation verbally or through writing (Shai, 2007).

The medicinal plant typically contains mixtures of different compounds that may individually or in combination improve health (Galma *et al*., 2021). In previous studies, many plant extracts have been shown to possess a combinatorial antimicrobial activity, including improving antibiotic efficacy (Muhammad & Salih, 2020). The importance of medicinal plants lies not only in their chemotherapeutic effect but also in their role as a source of model pharmacophore for drug

development. In addition to plant constituents being used directly as therapeutic agents, they can be utilized as starting material for drug synthesis (Kamboj & Saluja, 2011).

2.2. The Family of *Ranunculaceae*

Ranunculaceae (buttercup or crowfoot family) is a family of over 2,000 known species of flowering plants taxa under 60 genera, distributed worldwide (Ucuncu *et al*., 2020). The largest genera are *Ranunculus* (600 species), *Delphinium* (365), *Thalictrum* (330), *Clematis* (325), and *Aconitum* (300) (Azam *et al.*, 2019). People use this family ethnomedicinally for many years due to their phytochemical constituents.

For instance, *Clematis burgensis* (*Ranunculaceae)* (fitti, Afan Oromo, Ethiopia) is endemic and grows in open montane forest and forest borders, along roads and streams (Anza *et al*., 2021). Its leaves are reported to be used for the treatment of otorrhea and eczema. *Anemone is* distributed in the mountain of 1,000-2,300 m above sea level of Shanxi and Hebei province in China, and it is also distributed in Korea and reported for having antimicrobial, anti-inflammatory, and cytotoxic activities (Mei *et al.*, 2012).

Various Consolida species (*Ranunculaceae* family) have been extensively employed as herbal medicines for hundreds of years to treat multiple kinds of diseases, such as traumatic injury, rheumatism, sciatica, enteritis, stomach ache, ringworm, scabies, and other skin diseases (Yin, 2020). *Ranunculus multifidus is* also reported to have various ethnomedicinal uses for treating various human ailments, such as in the treatment of cancer (Ayele, 2018), malaria (Sirak *et al.*, 2021), eczema, toothache, anti-inflammatory (Dilebo *et al*., 2010), stomach ache, wounds, eye diseases, urinary tract infection (Hashemi *et al*., 2021), tuberculosis (TB) (Madikizela *et al.*, 2017), shingles, sores and sexually transmitted disease (STD) (Wet *et al*., 2012) and others.

2.3. Botanical Descriptions of *Ranunculus multifidus*

Ranunculus multifidus is a perennial herb with stems up to 1m long: belongs to the family of *Ranunculaceae*. The leaves are partly in a basal rosette, 2-pinnate with the ultimate leaflets, irregularly toothed, and hairy (Almerekova *et al.*, 2020). It has yellow flowers, 20 mm in diameter, on a long, branched, mostly leafless, hollow flowering stem. The herbs dominantly grow in wet grass land and on river banks and vlei margins at altitudinal range up to 2000 m above sea level. The flowering time of this herbs is august to February. The plant is native to many African country including Ethiopia (Kebebew & Mohamed, 2017).

Ranunculus multifidus has several vernacular or common names in different African countries where it is found or in its geographical areas of occurrence (Maroyi, 2017). Literature survey showed there are more than two or three vernacular names in a different country, but the plant mainly has many common names in South Africa followed by Ethiopia. Local people rarely name plant species that they do not use for food or medicinal values (Maroyi, 2017). The plant has many traditional medicinal uses in different parts of Ethiopia with different vernacular names. The common and dominant name of the plant known in Amharic is ‘’Etse siol’, which is directly taken from the Geez language and translated as ‘wuha akur’ which indicates the geographical habitat of the plant as it mainly around a watery environment and wetland. Other common name given for this plant in different parts of Ethiopia includes; Gubduu caaffee/Abba warqee (Jimma Zone) (Begashawu *et al*., 2016), Kartassa (Bale Zone) (Yineger *et al.*, 2008), Sherit (Meinit ethnic in SNNPRS (Megersa *et al.*, 2019), Chadera (Bench ethnic SNNPRS) (Giday *et al*., 2009), Qoricha dhibee Simbiraa (Arsi tiyo) (Aman *et al*., 2020), and others.

In Africa, the root, leave, and stem of *R. multifidus* are reported to possess diverse medicinal values to treat various human and animal ailments either alone or in the combination with other medicinal plants. Many studies have been done on the traditional treatments of various gynecological and obstetric problems around the world (Wet & Ngubane, 2014). In northern Thailand, the species is regularly used for the treatment of dysmenorrhea (menstrual cramps) and amenorrhea (absence of menstrual period) followed by the relieving of morning sickness in pregnant women and for promoting fetal stabilization (Srithi *et al*., 2012).

The Research by De Wet (2013) revealed that *E. elephantine* root decoction is taken orally in combination with *Ranunculus multifidus* (Whole plant) as a remedy for shingles as well as sores with another traditional medicinal plant in combination therapy (Wet & Ngubane, 2014). The boiled one handful of *Ranunculus multifidus* (whole plant) in 1L of water for 30 min, sieved and taken half a cup three or four times a day to treat genital warts and gonorrhea (Hutchings, 1989). According to Abebe (2016) the root of *R.multifidus* can be used for the treatment of external tumor/cancer in Ethiopian traditional practitioners (Abebe, 2016). According to Moges and Balakrishnan's report (2014), *R.multifidus* has nutritional values around Guassa Community in (Debre birhan) and they reported as the plant contains fat (4.9%) in its root and leaves (Moges & Balakrishnan, 2014).

In Sigmo district jimma, Oromia region *R. multifidus* is used by soaking its leaves in water and taken orally for GIT treatment and they put the sap from the leaf for the toothache treatment (Begashawu *et al.*, 2016). The dried root was concocted, mixed with butter, and taken orally around Bale national park for treatment of asthma by traditional health practitioners (Yineger *et al.*, 2008). The herbs are also used in the treatment of hepatitis B in Arsi zone, tiyo district Oromia (Aman *et al*., 2020). This and many other reports indicate that the plant was selected for research based on the ethnomedicinal report obtained from literature mining and unstructured interviews with the local traditional herbalist.

2.4. Phytochemistry of the Genus *Ranunculus*

Methanolic extract (10 g) of aerial parts of *R. muricatus* was fractionated on the column using silica gel 60 (40–63μm) as stationary phase, ethyl acetate and methanol were used as mobile phase and resulted in compounds such as caffeoyl-β-D-glucopyranoside **(1)**, 1,3 -dihydroxy-2-tetracosanoyl amino-4-(E)-nonadecene **(2)** (Azam *et al.*, 2019). The ethyl acetate fraction of the whole ethanolic extract results in the isolation of tricin7-O-β-D-glucopyranoside **(3)**, protocatechuyl aldehyde **(4)**, isoscopoletin **(5)**, anemonin **(6)**, β-sitosterol **(7)** (Nazir *et al.*, 2013). Protocatechuic acid **(8)**, luteolin factors **(9)** (Aslam & Uzair, 2015), and ranuncoside also isolated from this plants using different solvents **(10)** (Raziq *et al.*, 2016).

R. ternatus was reported to possess benzophenones compounds such as; methyl (R)-3-[2-(3,4-dihydroxybenzoyl)-4,5-dihydroxyphenyl]-2-hydroxypropanoate **(11)** and n-butyl (R)-3-[2-(3,4-dihydroxybenzoyl)-4,5-dihydroxyphenyl]-2-hydroxypropanoate **(12)**, were isolated from the root along with the two others compounds vanillic acid **(13)** and gallic acid **(14)** (Deng *et al*., 2013). They also reported that compound **(11)** shows good activity against tuberculosis, while the activity of a 1:1 mixture of compound **(11)** and **(14)** is better than that of **(11)** alone.

Other phyto compounds also reported from this species at a different time from different parts of the plants using different solvents and extraction techniques include; bilobetin **(15)**, sternbin **(16)**, methyl paraben **(17)**, 4-O-D-glucopyranosyl-p-coumaric acid **(18)**, linocaffein **(19)**, kaya flavone **(20)**, podocarpus flavone A **(21)**, isoginkgetin **(22)**, amentoflavone **(23)**, robusta flavone-4′-methyl-ether **(24)**, and others (Aslam & Uzair, 2015).

Using different chromatographic techniques R(+)-dalbergic phenol **(25)**, R(+)-4-methoxy dalbergione **(26)**, methyl-3,4,5-tri-hydrobenzoate **(27)**, 4-hydroxy-2-methoxy benzoic acid **(28)**

,p-hydroxycinnamic acid **(29)**, ranupenin-3-galactoside **(30)** were isolated from whole parts of the plants by different researchers (Aslam & Uzair, 2015) and others.

R. sceleratus is a wet land plant and a widely used traditional Chinese medicinal herb that has been researched intensively. It has many pharmacological effects, such as antibiosis, anti phlogistic, and the relief of articular effusion and its major phytoconstituents isolated from this species include anemonin **(6)** from whole plants, flavone derivatives, 2-phenyl-4H-chromenone **(31)** from the stem, tricin **(32)** from stem and leaves, 5-hydroxy tryptamine **(33)** from the whole plant, ranunculin **(34)**, isoscopoletin **(35)**, scoparone **(36)** (Mei *et al*., 2012), protocatechuic aldehyde **(4),** protocate chuic acid **(37)**, apigenin **(38)**, protoanemonin **(39)** (Asghar *et al.*, 2009) and stigmasterol **(40)**.

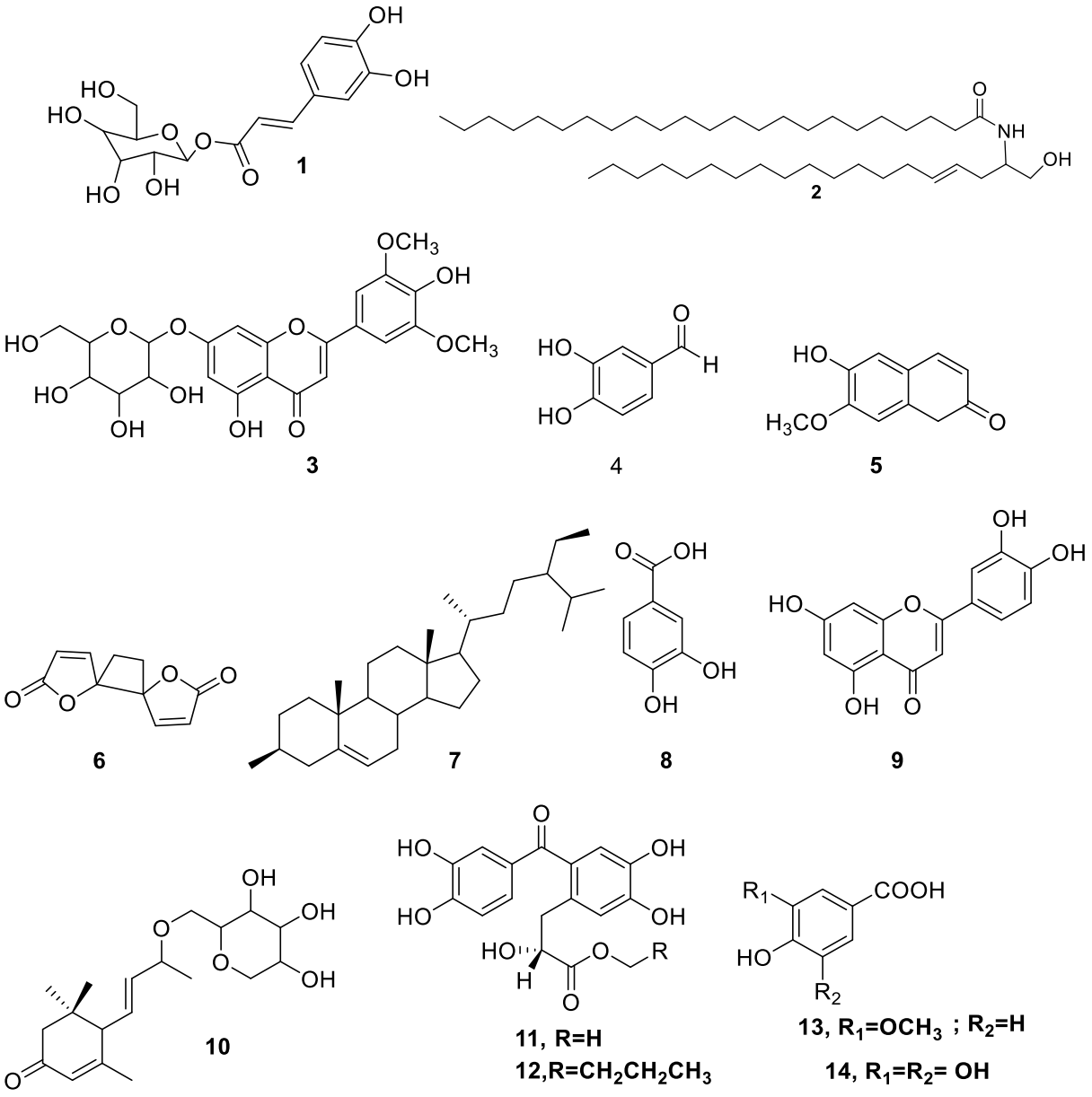
Protoanemonin isolated from the aerial parts of *R. bulbosus* was investigated for its antimicrobial properties and shows a promising effect. The combinations of protoanemonin and antibiotic was also investigated and the striking result was obtained with the protoanemonin-cefamandole combination against a pathogenic strain of *S.aureus* (Ordoñez *et al*., 2003).

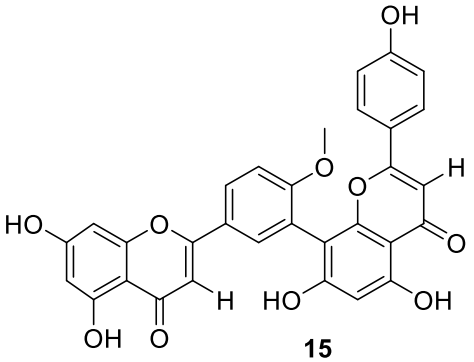
According to Mare's report, hexadecanoic acid **(45),** *β*-sitosterol **(7)**, and anemonin **(6)** were isolated from whole plants of *R.sceleratus* using different solvents (Aslam & Uzair, 2015).

Four new flavonoid glycosides, 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-galactopyranosyl]-7-*O*-*β*-D-glucopyranosyl kaempferol **(41)**, 3-*O*-(*α*-L-arabinopyranosyl-(1→2)-{4-*O*-[(*E*)-caffeoyl]-*β*-D-galactopyranosyl})-7-*O*-*β*-D-glucopyranosyl quercetin **(42)**, 3-*O*-{2-*O*-[(*E*)-caffeoyl]-*α*-L-arabinopyranosyl-(1→2)-*β*-D-galactopyranosyl}-7-*O*-*β*-D-glucopyranosyl-kaempferol **(43)**, and 3-*O*-{2-*O*-[(*E*)-caffeoyl]-*α*-L-arabinopyranosyl-(1→2)-*β*-D-galactopyranosyl} kaempferol **(44)**, together with two known compounds were isolated from the aerial parts of *R. Chinensis* (Goo, 2022).

R. hirtellus used in the treatment of febrifacient, anthelmintic can also be reported to have four known compounds from its butanol fraction of aerial extracts. Namely, *R (+)-dalbergic phenol **(46)**, 5-hydroxy tryptamine* **(33)**, *hexadecanoic acid* **(41)**, and *β-amytrin **(47)*** (Kamboj & Saluja, 2011). The species were also reported as it used for the treatment of antirheumatic, and rubefacient. The chloroform extract (86 g) of *R.laetus* was subjected to column chromatography using different solvent systems (Hussain *et al*.,2009) and afforded three known flavone jacein **(48)**, jacedin-5-*O*-β-D-glucoside **(49)**, and centaurein **(50)**. According to Ahmed and Arsalan (2020) one known coumarin, 6,7-dimethoxy coumarin **(51)**, β-amytrin **(47)**, and β-sitosterol-3-

O-β-D-glucoside **(52)**, have been isolated from different parts of this plant using different solvents for the first time (Ahmad & Arsalan, 2020). (Figure:1) shows structures of compounds reported from different species of the genus *Ranunculus.*





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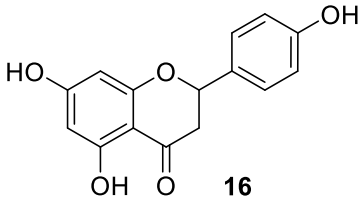

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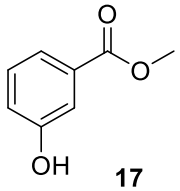

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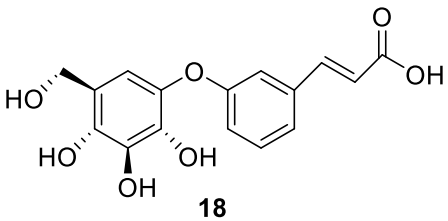

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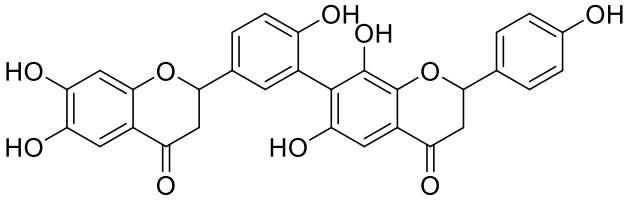

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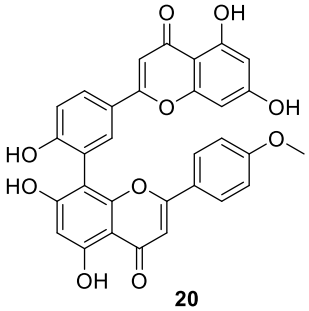

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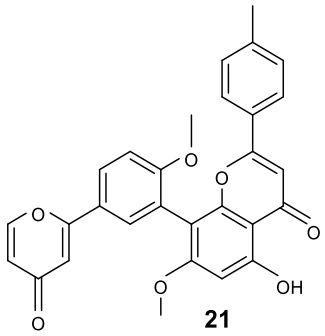

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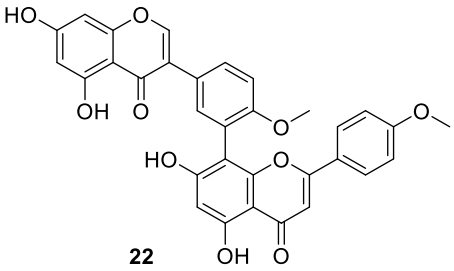

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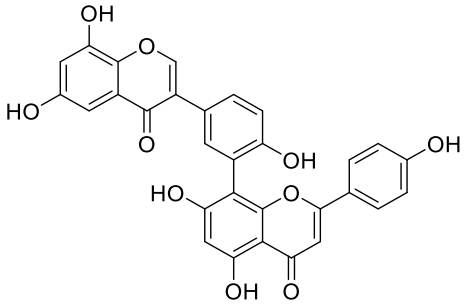

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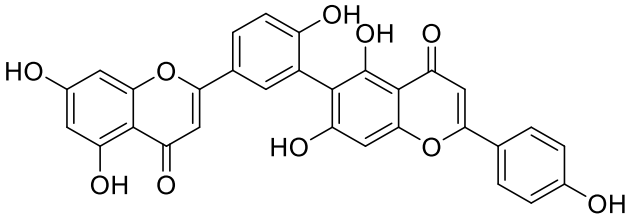

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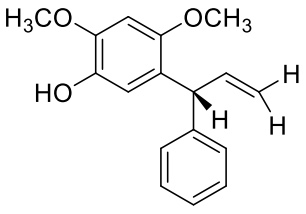

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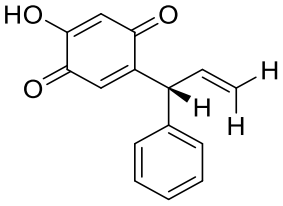

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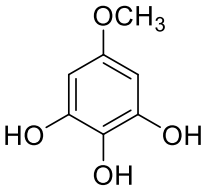

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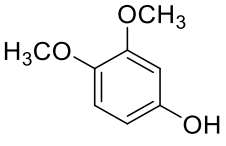

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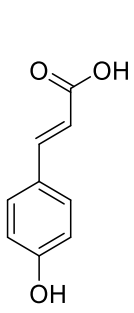

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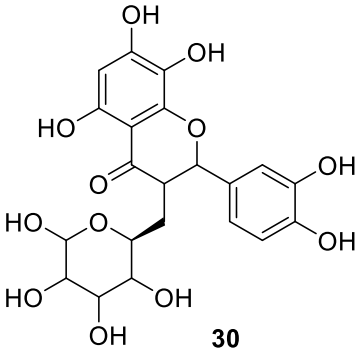

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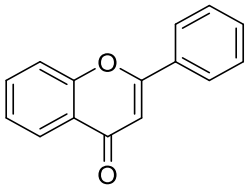

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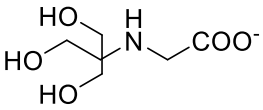

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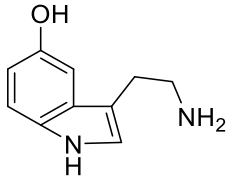

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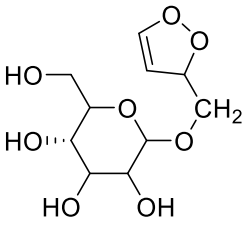

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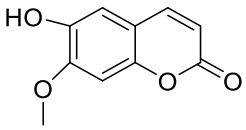

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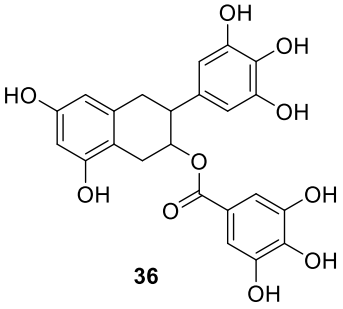

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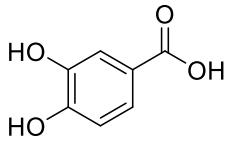

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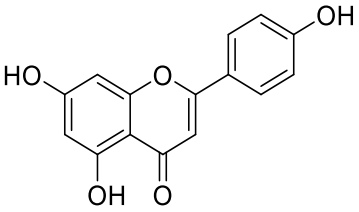

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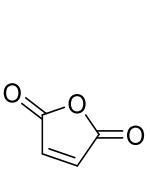

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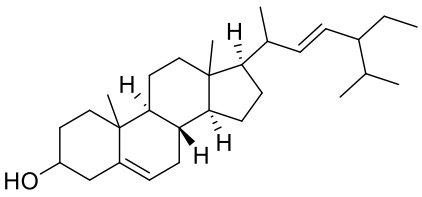

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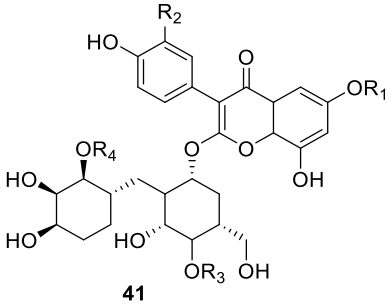
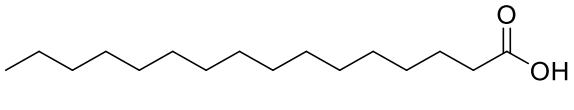


41

41, R_1=β-D-Glc, R_2=R_3=R_4=H, **42**, R_1= β-D-Glc, R_2=OH, R_3=Caff, R_4=H, **43**, R1= β-D-Glc, R_2=H, R_3=H, R_4= Caff, **44**, R_1= R_2=R_3=R_4=Caff

Where, Glc and Caff stands for glucose and caffeol moiety in structure



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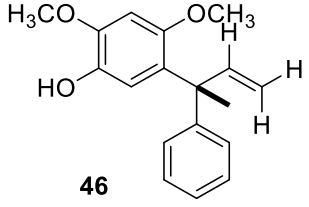

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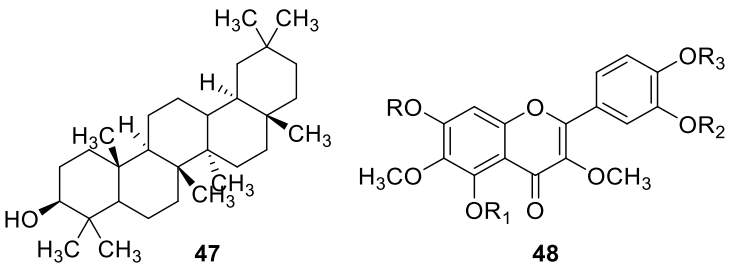


48; R= Glc, R_1=R_3=H, R_2=CH_3, **49**; R = R_3=H, R_1 = Glc, R_2= CH_3 , **50**, R = Glc, R_1 = R_2 = H, R_3 = CH_3 and where Glc stands for glucose moiety in structural analogs.

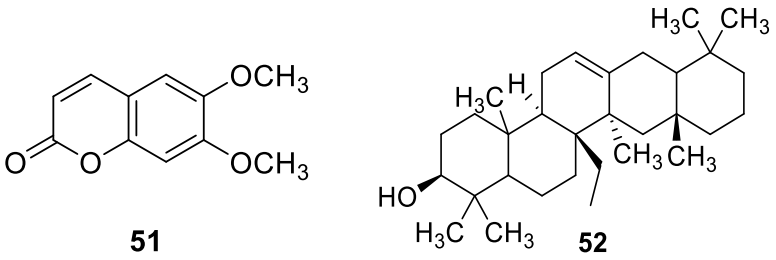


Figure 1: Some chemical compounds reported from the genus *Ranunculus*

To the best of our knowledge of literature mining only anemonin, a dimeric lactone was isolated from leaf parts of *R. multifidus*. This compound has strong anti-malarial activities by increasing oxidative stress in the parasite, since it interacts with the thiol group of the precursor amino acid L-cysteine, along with the cysteine residue of GSH itself by a Michael type addition (figure. 2)

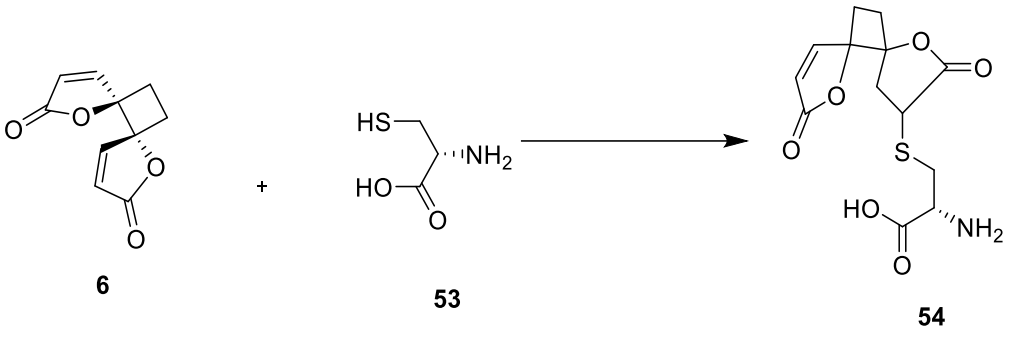


Figure 2: Possible Michael-type addition reaction between anemonin **(6)** and L-cysteine **(53)**

Meanwhile, L-cysteine is relevant as a substrate in the synthesis of GSH (Bouvier *et al*., 2009). The capability of anemonin to interact with this important amino acid may suggest its potential to increase oxidative stress and inhibit growth in the parasites.

2.5. Biological Activities of the *Ranunculus* Species

The various parts of the genus *Ranunculus* have been reported to have antibacterial, anti-inflammatory, anti-oxidant, cytotoxic, antiviral, insecticidal, anti-fungal, anti-tuberculosis, and other properties (Azam *et al*., 2019).

2.5.1. Antibacterial Activities

Different species of *Ranunculus* were reported to have antibacterial activities in different places by researchers. Methanolic extract of aerial parts of *R.muricatus was* reported to possess antibacterial activity (Azam *et al*., 2019). Acetone and methanol fractions of *R. sericeus*, had moderate antimicrobial activity against tested human and plant pathogenic bacteria such as *P. aeruginosa, B.subtilis, S. aureus, E. faecalis* (Atci, 2018).

Khalid *et al.* (2019) reported that silver nanoparticles (AgNPs) were synthesized from leaf extract of *R. laetus* which have enhanced antibacterial potential against *P.aeruginosa, E.coli, S.aureus and B.subtilis* compared to aqueous leaf extract (Khalid *et al.*, 2019). Extracts of *R. bulbosus* reported anti-*citrobacter* constituents in its ethyl acetate and methanol extracts. The zone of inhibition obtained from ethyl acetate extract (40.00±0.57mm) which was higher than *chloramphenicol (*20.67±6.80 mm) and t*etracycline* (13.33±0.57 mm) (Nazir *et al*., 2013).

2.5.2. Anti-fungal Activities

Acetone and methanol fractions of *R.sericeus*, had moderate anti-fungal activity against fungi *Rhizoctonia sp., Alternaria sp.,* and *Fusarium sp.* (Atci, 2018). Anemone (*Anemone cathayens is*), was reported to be a source of many bioactive compounds isolated from ethanolic extract, which act as antimicrobial, including pathogenic fungi. Chloroform extract of fresh leaves of *R.sceleratus* results in protoanemonin and shows strong fungicidal activities (Ali Esmail Al-Snafi, 2022). The antifungal activity of *Ranunculus sceleratus* root extracts were investigated against *Trichophyton mentagrophytes* (MTCC 8476), *T. rubrum* (MTCC 8477), *T. tonsurans* (MTCC 8475), *microsporum gypseum (M. gypseum)* (MTCC 8469) and *M. fulvum*. The growth inhibition zone of the chloroform extract ranged from 15 to 23 mm (MIC 1.25-205mg/ml), and the growth inhibition zone of the methanol extract ranged from 10 to 21 mm (MIC 20.5-5 mg/ml) against all the tested fungi, while the water extract showed antifungal activity against only *T. mentagrophytes* (8mm), *M. gypseum* (9mm) and *M. fulvum* (8mm) (MIC>10.0 mg/ml). *R. arvensis* L. aerial extract shows anti-fungal activity, and the results shows that extract from

this plant has significant antifungal activity against *C. albicans* with a good result as compared with *A. niger* with growth inhibition zone ranging from 14 to 21 mm for fungal strains (Khalid *et al*., 2019).

The leaves extract of *R. laetus* was tested for antifungal activity against two human pathogenic fungal strains, *C. albicans* and *F. solani*, depicting promising results as compared to standard *clotrimazole* with zones of inhibition found between 18 and 25 mm at 100 mg/ml concentration against both of the selected fungal strains, *clotrimazole* depicted nearly 46 mm inhibition zones against both of the strains at 1 mg/ml (Ahmad & Arsalan, 2020). n-hexane extract of flower of this plant was found most active, particularly against *C. Albicans*.

2.5.3. Anti-inflammatory Activities

Anemone (*Anemone cathayensis*), was reported to be a source of many bioactive compounds isolated from ethanolic extract, which acts as an anti-inflammatory (Wang *et al*.,2012). From the aerial part of *R.sceleratus,* phytol, β-sitosterol, stigmasterol, and others were isolated and reported to inhibit cyclooxygenase-1 (COX-1), 5-lipoxygenase (5-LOX) and human leukocyte elastase activities (Dilebo *et al*., 2010). There is also a report that suggests *Ranunculus japonicum* possesses (Analgesic and Anti-inflammatory) activities on mice tests.

R. constantinapolitanus and *Ranunculus pedatus* possess wound healing and anti-inflammatory properties using *in vivo* tests (Goo, 2022). According to Dilebo *et al., (*2010) 80% methanol extract from the aerial part of *R. multifidus* extracts showed edema inhibition at both 300 and 500 mg/kg doses. It exhibited better edema inhibition than the reference drug Indomethacin (10 mg/kg) three hours after carrageenan injection (Dilebo *et al.*, 2010). The anti-inflammatory activities of these plants may partially justify the rationale for their traditional use in the treatment of skin diseases.

2.5.4. Antioxidant Activities

Phytochemical analysis of *R. muricatus* and subsequent isolation and characterization technique revealed a new metabolite, named Ranuncoside **(10)** which showed potent free radical scavenging activity (Raziq *et al*., 2016). The compound exhibited lipoxygenase and xanthine oxidase inhibitory properties and therefore may provide a natural source of new drug lead for the therapeutic management of diseases associated with exaggerated activities of lipoxygenase and xanthine oxidase enzymes (Raziq *et al.*, 2016).

2.5.5. Anticancer and cytotoxic activities

Cytotoxicity of *R. sieboldii* aerial extracts using methanol and acetone solvents was evaluated using *in vitro* MTT assay on four different human tumor cell lines (KB, BEL-7407, A549, HL-60) (Aslam & Uzair, 2015). The methanol extracts shows promising for epithelial cancer cells (KB) and lung cancer cell (A549) (Aslam & Uzair, 2015). Anemone (*Anemone cathayensis*), was reported to be a source of many bioactive compounds isolated from ethanolic extract of its root, which act as anticancer (breast cancer and external tumor cells) and cytotoxic activity (Wang *et al*., 2014). *Ranunculi ternatus* (whole part) extracted using chloroform and ethyl acetate has anticancer activities on human breast cancer cells *in vitro* and it withdraws and inhibits MCF7 cells growth via apoptosis (Aslam & Uzair, 2015). Acetone and methanol fractions of *R. sericeus* were tested on its dermal irritant activities and show no acute or chronic irritant activity was observed at all applied dose levels (20, 40, 80, and 120 µl at a concentration of 10 mg/ml) (Atci, 2018).

R. ternatus has been reported to be effective against malignant lymphoma, leukemia, pulmonary tuberculosis, breast tumor, goiter, lung, gastric, esophageal tumors (Yun *et al*., 2021). The cytotoxic activities of ethyl acetate extracts of the root were evaluated using MTT assay and exhibited significantly reduced the viability of Jurkat cells in a dose-dependent manner with half-maximal inhibitory concentration (IC_{50} was 0.20 mg/mL). Treatment also resulted in improved hepatic, renal, and hematologic parameters. The results demonstrate the anticancer effects of *R. ternatus* both *in vitro* and *in vivo* with no apparent toxicity (Yun *et al*., 2021).

2.5.6. Anti-malarial Activities

The anti-malarial activity of the extract of the young stem of *R. japonicus* was evaluated *in vitro* using both chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) strains; *in vivo* activity was evaluated in *Plasmodium berghei*-infected mice via oral administration followed by a four-day suppressive test focused on biochemical and hematological parameters (Yun *et al*., 2021). Exposure to extracts of *R. japonicus* resulted in significant inhibition of both chloroquine-sensitive (3D7) and resistant (Dd2) strains of *P. falciparum*, with IC_{50} values of 6.29 -2.78 and 5.36-4.93 g/mL, respectively (Yun *et al.,* 2021). Administration of *R. japonicus* also resulted in potent anti-malarial activity against *P. berghei* infected mice with no associated toxicity.

R. multifidus has been reported to have anti-malarial activities against the parasite (Sirak *et al*., 2021; Vonthron-Sénécheau *et al*., 2003). A previous study demonstrated that the whole plant of

R. multifidus extracted with dichloromethane to methanol (1:1), possesses *in vitro* anti-malarial activity against a chloroquine-sensitive strain of *Plasmodium falciparum* with an IC_{50} value of 2.3 µg/mL (Vonthron-Sénécheau *et al*., 2003). The most recently reported article suggested that 80% methanol (RM-M) and hydro-distilled (RM-H) extracts of fresh leaves from *R. multifidus* and its major constituent anemonin were tested for their *in vivo* anti-malarial activity against *Plasmodium berghei* in mice which confirmed the potential of the plant extracts to prevent or mitigate a primary attack due to malaria (Sirak *et al*., 2021).

There is also a report on anemonin isolated from the hydro-distilled extract of the leaves of *R. multifidus* displayed significant anti-leishmanial activity with IC_{50} values of 1.33 nM and 1.58 nM against promastigotes at concentration of 1.24 nM and 1.91 nM against amastigotes of L. *aethiopica* and L. *donovani*, respectively (Sirak *et al.*, 2021).

CHAPTER- THREE

3. MATERIALS AND METHODS

3.1. Chemicals and Apparatus

All solvents and chemicals used were analytical grades and extra pure which do not need extra purification techniques. Methanol (99.99% GC grade), n-hexane (99%), Dichloromethane (98%), Ethanol (100% GC grade Mumbai, India), Chloroform (98%), n-Butanol (99.8%), and DPPH (Sigma) were purchased from Addis Ababa (Fine Chemical. PLC), DMSO (Sigma, USA), Ethyl acetate (98% UNI-CHEM chemical reagents). The other chemicals which were used for Phytochemical screening were 1%NH_3, 5% $FeCl_3$, NaOH, KOH, HCl, H_2SO_4, Wagner's reagent (KI and I_2), and Fehling's solution (A and B), Vanillin in H_2SO_4 spraying, and Iodine solution, reagents used for visualizing spots on thin-layer chromatography plates were purchased from Alchem private trading limited company, Addis Ababa (sub-city Kirkos). Standard drug such as ciprofloxacin, ketoconazole, fluconazole and L-ascorbic acid for comparison of biological activities purchased from the commercial market as required with standard purity.

Items used for extraction and isolation of compounds include rotavapor (Germany), Orbital mini-shaker (Italy), Erlenmeyer flask, Vacuum pumps for filtration (Germany), Watchman filter papers-No1, Funnels, Beakers, Petri dish of medium size, Vials of (30ml), Column chromatography larger and smaller size, Test tube, Water bath, Refrigerators, Stand, Round bottom flask, Spatula, Stirrer, Sterile Cotton /Gauze, Analytical balance (Poland), Capillary tube, Oven, Ruler, Pencil, Pen, Reagent bottle, Pasture pipette, Spatula, Separatory funnel of large size, Electronic balance, Dropper, Lyophilizer (Alpha 1-2LD plus Martin Christ Co. Ltd., Germany) Electrical blender (Germany), and others.

Analytical TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck, Germany) on an aluminum plate. Detection of spots on TLC was done either by spraying with visualizing reagents or by observation under a UV lamp at 254 and 365 nm. Purification of compounds was done using column chromatography over silica gel (60-120 mesh). The NMR spectra was measured using Bruker Avance 400 spectrometer operating at 400 MHz, using $CDCl_3$ and Acetone-d6 as a solvent and TMS as an internal standard for recording chemical shift in ppm respectively.

3.2. Description of the sample collection site

The plant was collected from Bekoji which is a town in central Ethiopia located in the Arsi Zone of the Oromia Region, its coordinates are 7°31'60" N and 39°15'0" E in DMS (Degrees Minutes Seconds) or 7.53333 and 39.25 (in decimal degrees), with an elevation of 2810 m. Its UTM position is EJ23 and its joint operation graphics reference is NB37-03 (Source gate map .com).

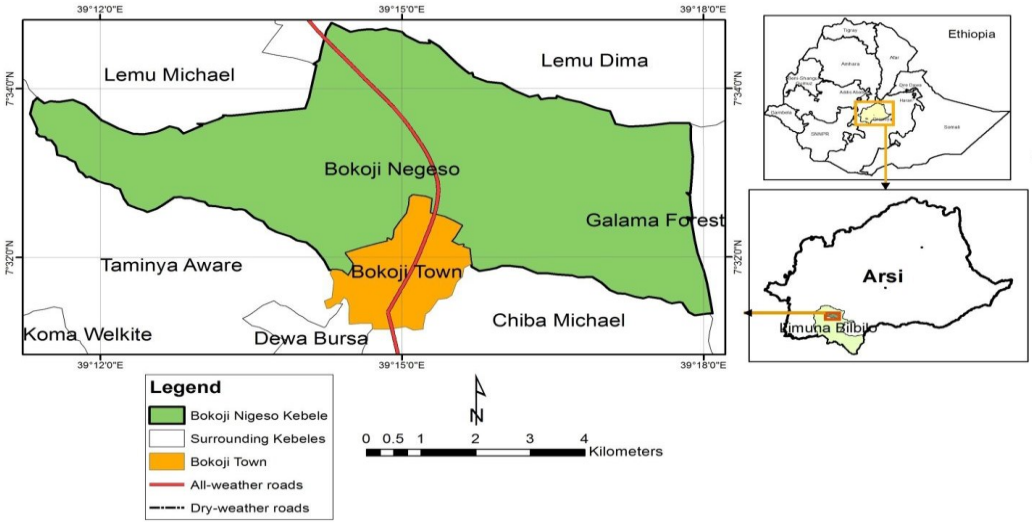


Figure 3: Map of plant collection site (Mesay *et al*., 2017).

3.3. Plant material collection and identification

The fresh root and aerial part as well as botanical specimens of *Ranunculus multifidus* were collected and packed in the plastic bag from sultana village, Bekoji Woreda, Oromia, Ethiopia, which is 224 km from Addis Ababa in November 2021. The plant material was authenticated by Melaku Wondafrash and a voucher specimen (Voucher No: TY001) was deposited in the National Herbarium, Department of Botany, Addis Ababa University, for future references.


(a)

(b)

Figure 4: Root (a) and Aerial part (b) of *R. multifidus* (Picture taken by Terfo Yilma at Sultana Village of Bekoji district on November 2021).

3.4. Extraction and isolation

3.4.1. Maceration of root

The shade-dried root and aerial part were ground to powder using an electric blender. The powdered plant material was stored in the plastic bag until used for extraction. The powdered root (400 g) was macerated for 72h through occasional shaking at 230 rpm using an orbital mini-shaker with n-hexane at room temperature. The extract was filtered by Whatman no.1 filter paper mediated by vacuum pumps and concentrated by a rotary evaporator at a reduced temperature (40 ℃). The marc left was further extracted by the same procedure using dichloro-methane, and then concentrated. Similarly, the marc from DCM extraction was further extracted by the same procedure using MeOH, filtered, concentrated to get methanol extract. The crude extracts (n-hexane, DCM, and methanol) were analyzed by TLC. The whole process of extraction was depicted in the following schematic representation as shown in (figure 5).

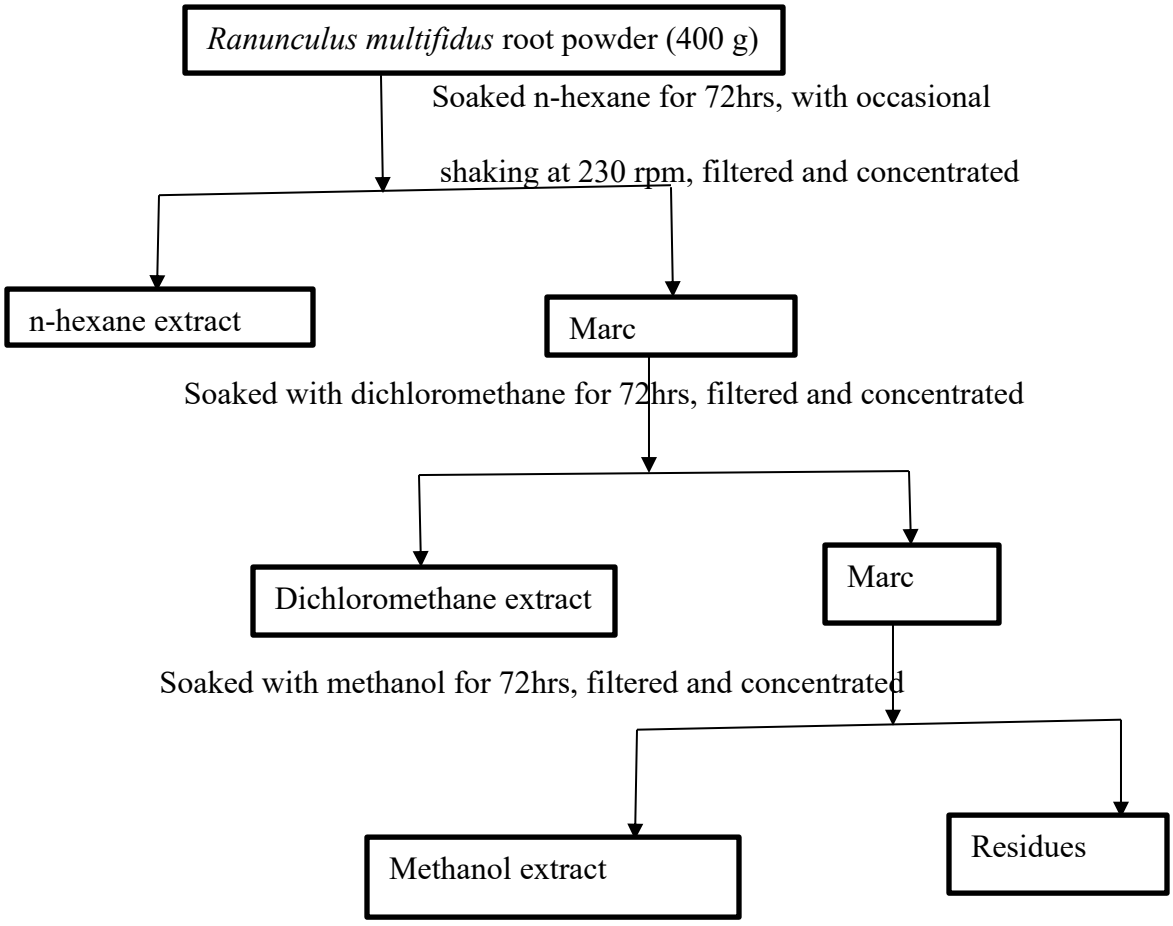


Figure 5: Schematic representation of maceration for extraction of root of *R. multifidus.*

3.4.2. Extraction of aerial part using liquid-liquid partitioning techniques

The extraction of compounds from aerial parts was performed according to Khan. (2010) with slight modification (Khan *et al*., 2010). The 1kg of a powdered aerial part (1kg) was taken and extracted with methanol at room temperature, the total filtrate was concentrated to dryness in a vacuum at 40 ℃ leaving behind the methanolic extract (120g). The methanolic extract was then suspended in water and partitioned successively in n-hexane, chloroform, ethyl acetate, and n-butanol. The whole procedure of liquid-liquid fractionation was shown in (Figure 6).

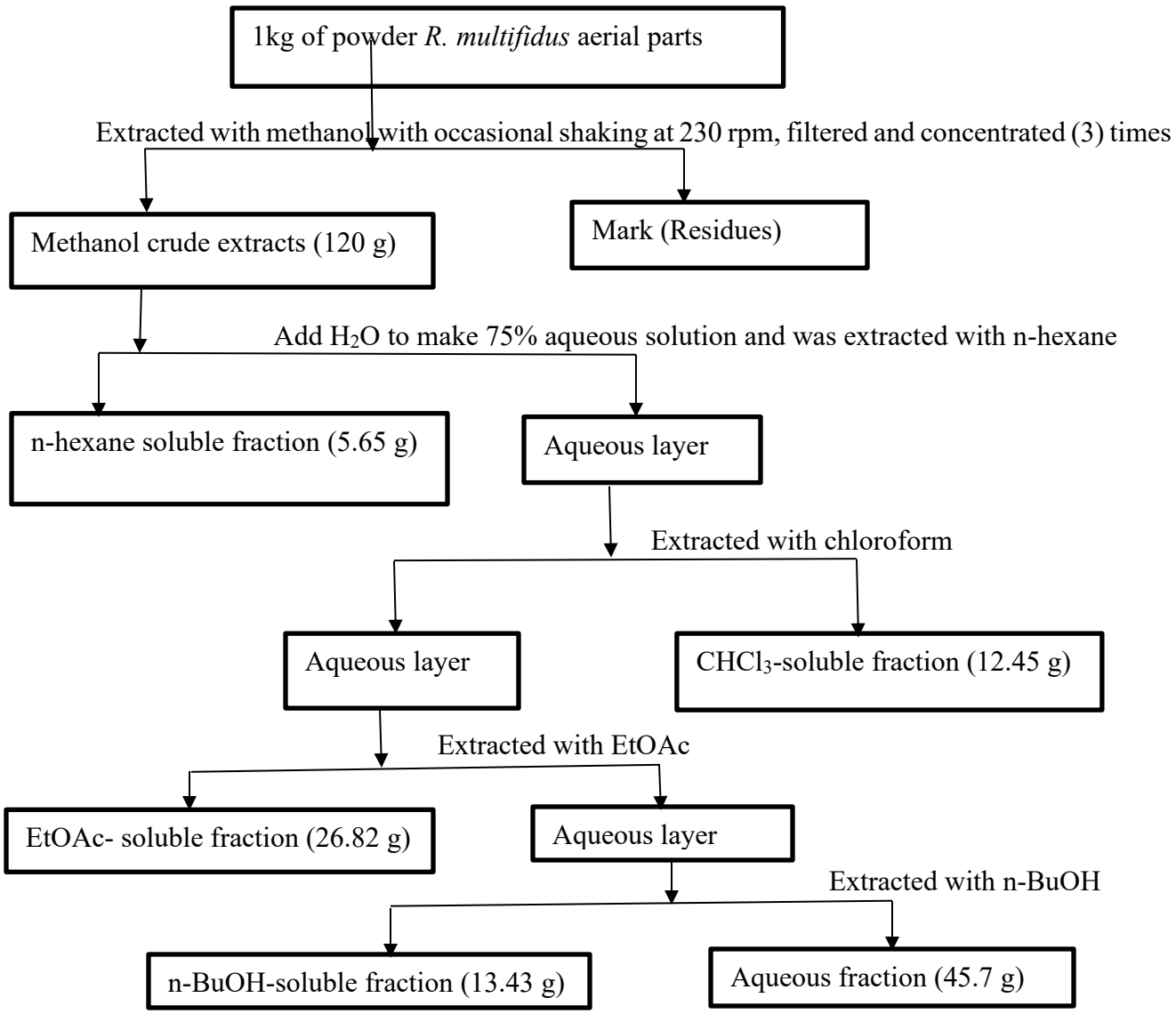


Figure 6: Schematic representation of liquid-liquid partitioning of aerial part of *R. multifidus*

3.5. Phytochemical screening

The word “Phyto” is the Greek word for plant. Phytochemicals are not only nutritive plant chemicals they have protective or disease preventive properties and also protect humans from host diseases (Kumar *et al*., 2014). Qualitative screening of various extracts of the plant was performed for the identification of various classes of chemical constituents in the plant based on the standard procedures with slight modifications as required.

3.5.1 Test for flavonoids: To 5 mg portion of crude extracts of n-hexane, DCM, and MeOH extract, and each fraction of aerial extracts were dissolved in 15 mL of 96% ethanol and filtered. Then 3 mL of the filtrate were in a test tube, and 1mL of potassium hydroxide solution was added. A dark yellow color indicated the presence of flavonoid (Chintalapani *et al*., 2018).

3.5.2 Test for saponins: To 5 mg of crude extracts and fractions of all solvent, 5 mL of distilled water was added and shaken while heating to boil. The formation of foam to a length of 1cm indicated the presence of saponins and steroids (Geetha & Geetha, 2014).

3.5.3 Test for phenols and tannins: The presence of phenolics and tannins in each extract of n-hexane, DCM, and MeOH and each fraction of aerial extract was screened according to the following methods with slight modification.

Sodium hydroxide test: Five mg of extract was dissolved in 0.5 mL of 20% sulfuric acid solution. Followed by the addition of a few drops of aqueous sodium hydroxide solution, it turns blue which indicates the presence of phenols (Geetha & Geetha, 2014).

Ferric chloride test: To each extract above and 0.5 mL of 5% ferric chloride was added. The development of dark bluish-black color indicates the presence of tannins (Geetha & Geetha, 2014).

3.5.4 Test for terpenoids (Salkowski test): To 5 mg of each extract was added 2 mL of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A Reddish-brown coloration of the interface indicates the presence of terpenoids (Ayoola *et al.*, 2008).

3.5.5 Test for steroids: The presence of steroids was conducted following the methods of Anandharaj *et al*. (2021), one mg of the extracts was dissolved in 10 mL of chloroform and an equal volume of concentrated H_2SO_4 was added to the sides of the test tube. The upper layer turns red and the H_2SO_4 layer showed yellow with green fluorescence. This indicates the presence of steroids (Anandharaj *et al*., 2021)

3.5.6 Detection of phytosterols (Salkowski test): 5 mg crude extract (n-hexane, DCM, MeOH) and each fraction was treated with a few drops of chloroform and filtered. To the filtrate, a few drops of concentrated H_2SO_4 was added, shaken, and allowed to stand for a few min. The appearance of the golden yellow color indicates the presence of triterpenes (Ayoola *et al.*, 2008).

3.5.7. Test for cardiac glycosides (Keller-Killiani test): To 5 mg crude extract (n-hexane, DCM, MeOH) and each solvent fraction were diluted to 5 ml of water then 2 ml of dilute glacial acetic acid containing one drop of ferric chloride solution was added. Then 1mL of concentrated sulfuric acid was added. A brown ring at the interface indicated the presence of a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Ayoola *et al*., 2008).

3.5.8 Test for Alkaloid: A 5 mg of crude extract (n-hexane, DCM, MeOH) and fractions were separately stirred with 1 % HCl (0.5 mL) in a water bath for 5 min and filtered. To these filtrates Wagner's reagent (Iodine in potassium iodide solution) (1 mL) was added. The formation of an orange-colored precipitate indicates the presence of alkaloids (Dey *et al*., 2010).

3.5.9. Test for anthocyanin

NaOH Test: 2 ml of crude extract and fractions were treated with 2 M NaOH and observed the formation of blue-green color indicating the presence of anthocyanin (Kumar *et al*., 2014).

3.5.10. Test for quinones: A small amount of the extract was treated with conc. HCl and checked for observing the formation of a yellow color precipitate which indicates the presence of quinones (Kumar *et al*., 2014).

3.5.11. Test for reducing sugars (Fehling's test): The aqueous ethanol extract (0.5 g in 5 mL of water) was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a color reaction (Chintalapani *et al.*, 2018)

3.5.12. Test for anthraquinones: To 5 mg of the extract was boiled with 10 mL of sulfuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 mL of chloroform. The chloroform layer was pipetted into another test tube and 1 mL of dilute ammonia was added. The resulting solution was observed for color changes (Ayoola *et al*., 2008).

3.6. Isolation of compounds

3.6.1 TLC Analysis.

Analytical TLC was applied in the detection and monitoring of compounds through a separation process and was used to optimize solvent systems for column chromatography. The band separation on the TLC describing the separation of compounds were detected under UV absorbance at 254 nm (absorption) and 365 nm (fluorescence), followed by spraying the TLC plates with vanillin-H_2SO_4 reagent and subsequent heating at 80°C. The selection of an appropriate solvent system for particular plant extracts was achieved by analyzing the Rf values of compounds in a different solvent system. The TLC analysis of the methanol extract indicated the best resolution using ethyl acetate and methanol (8:2) solvent system compared with another solvent system for methanol root extracts and (6:4) n-hexane to ethyl acetate fraction of aerial extracts.

3.6.2. Column chromatography

As described earlier, the crude extracts were analyzed by TLC to choose the best mobile phase for column chromatography. Then 18 g of methanol crude extract was adsorbed on an equal amount of silica gel (mesh size 60-120), subjected to silica gel chromatography (180 g silica gel) using n-hexane for column packing and elution was carried out with an increasing gradient of ethyl acetate in n-hexane and methanol in ethyl acetate as eluent (Table 1).

Different fractions were collected and concentrated over a rotary evaporator. The composition of the fractions collected was monitored by TLC and characteristics color. The spots were detected by their UV absorption and fluorescence under 254 and 365 nm respectively, as well as by spraying 1% vanillin in sulphuric acid and iodine chamber for UV active constituents. Fractions that showed the same Rf value and the same characteristic color on TLC were combined and further purified. The column was then eluted with the increasing polarity of the solvent system.

Table 1: Chromatographic fractionations of the methanol root crude extract.

Solvent system	Ratio of solvent	Fractions collected	Volume (ml)
n-hexane	100%	F_1-F_{14}	20
n-hexane/EtOAc	90:10	F_{15}-F_{28}	20
n-hexane/EtOAc	80:20	F_{29}-F_{37}	‘’
n-hexane/EtOAc	70:30	F_{38}-F_{46}	‘’
n-hexane/EtOAc	60:40	F_{47}-F_{60}	‘’
n-hexane/EtOAc	50:50	F_{61}-F_{74}	‘’
n-hexane/EtOAc	40:60	F_{75}-F_{93}	‘’
n-hexane/EtOAc	30:70	F_{94}-F_{106}	‘’
n-hexane/EtOAc	20:80	F_{107}-F_{119}	‘’
n-hexane/EtOAc	10:90	F_{120}-F_{135}	‘’
n-hexane/EtOAc	0:100	F_{136}-F_{159}	‘’
EtOAc/MeOH	90:10	F_{160}-F_{192}	‘’
EtOAc/MeOH	80:20	F_{193}-F_{214}	‘’
EtOAc/MeOH	70:30	F_{215}-F_{227}	‘’
EtOAc/MeOH	60:40	F_{228}-F_{242}	‘’
EtOAc/MeOH	50:50	F_{243}-F_{250}	‘’
EtOAc/MeOH	40:60	F_{251}-F_{259}	‘’
EtOAc/MeOH	30:70	F_{260}-F_{270}	‘’
EtOAc/MeOH	20:80	F_{271}-F_{280}	‘’
EtOAc/MeOH	10:90	F_{281}-F_{290}	‘’
MeOH	100%	F_{291}-F_{300}	‘’

3.6.3. Purification of compounds from fractions

TLC analysis of the fractions was done and some of the fractions that have the same TLC profile were combined. Further purification was done using repeated column chromatography and preparative thin-layer chromatography (PTLC) to get single pure compound from the collected fractions.

3.6.4. Isolation of compounds from ethyl acetate fraction of aerial extract.

Based on the preliminary results of the phytochemical profile and biological activity of the ethyl acetate fraction was selected for the isolation of active principles. Additionally, the TLC profile of ethyl acetate fractions obtained after liquid-liquid partitioning from methanol extract of aerial parts of *R. multifidus* also seems promising band separation.

Then 15 g of ethyl acetate fraction was adsorbed on an equal amount of silica gel (mesh size 60-120), subjected to silica gel chromatography (180 g silica gel) using n-hexane for column packing and elution was carried out with an increasing gradient of ethyl acetate in n-hexane and

methanol in dichloromethane as eluent (Table 2). Different fractions were collected and composition of the fractions collected was analyzed by TLC and UV lamp. The spots were detected by their UV absorption and fluorescence under 254 and 365 nm respectively, as well as by spraying 1% vanillin in sulphuric acid and iodine chamber for UV inactive constituents. Fractions that showed the same Rf value and the same characteristic color on TLC were combined and further purified. The column was then eluted with the increasing polarity of the solvent system. Most of the experimental techniques such as separation, isolation and purification were conducted at the laboratory of the Department of Applied Chemistry, Adama Science and Technology University. Whereas spectroscopic analysis such as ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 of the isolated compounds were done in Addis Ababa University.

Table 2: Chromatographic fractionations of the ethyl acetate fraction of aerial extracts

Solvent system	Ratio of solvent	Fraction collected	Volume (ml)
n-hexane	100%	F_1-F_{14}	20
n-hexane/EtOAc	90:10	F_{15}-F_{28}	20
n-hexane/EtOAc	80:20	F_{29}-F_{37}	‘’
n-hexane/EtOAc	70:30	F_{38}-F_{46}	‘’
n-hexane/EtOAc	60:40	F_{47}-F_{60}	‘’
n-hexane/EtOAc	50:50	F_{61}-F_{74}	‘’
n-hexane/EtOAc	40:60	F_{75}-F_{93}	‘’
n-hexane/EtOAc	30:70	F_{94}-F_{106}	‘’
n-hexane/EtOAc	20:80	F_{107}-F_{119}	‘’
n-hexane/EtOAc	10:90	F_{120}-F_{135}	‘’
n-hexane/EtOAc	0:100	F_{136}-F_{159}	‘’
CH_2Cl_2/MeOH	90:10	F_{160}-F_{192}	‘’
CH_2Cl_2/MeOH	80:20	F_{193}-F_{214}	‘’
CH_2Cl_2/MeOH	70:30	F_{215}-F_{227}	‘’
CH_2Cl_2/MeOH	60:40	F_{228}-F_{242}	‘’
CH_2Cl_2/MeOH	50:50	F_{243}-F_{250}	‘’
CH_2Cl_2/MeOH	40:60	F_{251}-F_{259}	‘’
CH_2Cl_2/MeOH	30:70	F_{260}-F_{270}	‘’
CH_2Cl_2/MeOH	20:80	F_{271}-F_{280}	‘’
CH_2Cl_2/MeOH	10:90	F_{281}-F_{290}	‘’
MeOH	100%	F_{291}-F_{300}	‘’

3.7. Antimicrobial investigation

3.7.1. Antibacterial activity test

Antimicrobial activities of the root extracts and aerial fractions as well as the pure compounds of *R. multifidus* were investigated in *vitro* antimicrobial activities using the Kirby Bauer Disc diffusion technique against test bacterial and fungal strains (Khalid *et al*., 2019). Agar well diffusion method is used to determine whether a bacterium is resistant or susceptible to the crude extracts and pure compounds. This method was selected due to its simplicity, capacity to analyze multiple samples simultaneously, and its ability to work well with defined inhibitors (Manoharan *et al*., 2003). The activity of the plant extracts was tested against four American Type Culture Collection (ATCC) bacterial strains. In the process, two Gram-positive bacteria *Streptococcus pyrogens* ATCC12696, or *B. subtilis* ATCC6633 and *Staphylococcus aureus* ATCC25923 and two Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used to evaluate the antibacterial activities. The medium was prepared using Muller–Hinton agar (MHA). The autoclaved medium was poured into sterile plates (35-40 mL/plate) and the plates were allowed to solidify under the sterile condition at room temperature. After solidification, the plates were seeded with overnight grown culture approximately 1.5 X 10^8 CFU/mL of bacterial strain and 10^4 CFU/ mL for fungal strain by swabbing evenly onto the surface of the medium with a sterile cotton swab. *Ciprofloxacin* was used as a positive control while DMSO was used as a negative control. The four bacterial strains were tested with 50, 100, and 200 mg/mL crude concentration and (250, 300, and 350 µg/ml) for pure compounds using the paper disc-diffusion method (Tadesse *et al.,* 2016) with slight modification. For each concentration, 6 mm diameter Whatman filter paper discs were soaked with 100 µL solution of the above concentration for each extract and then these saturated paper discs were inoculated at the center of each petri dish having a bacterial lawn with the help of sterile Cork borer (Nazir *et al*., 2013). The plates were incubated at 37℃ for 48hrs, and the inhibition zone that appeared around the paper disc in each plate was determined by measuring the diameter of the inhibition using a caliper.

3.7.2. Antifungal activity test

The anti-fungal activity was conducted against two common human pathogenic fungal strains *Candida albicans* (ATCC 26555) and *Aspergillus niger* (ATCC 10864) which were incubated on Potato dextrose agar (PDA) for three days at 30℃ (Gizaw *et al*., 2022; Tran *et al*., 2020).

The anti-fungal activity was carried out by the agar well diffusion method. Different dilutions of crude extracts of root and aerial fractions (10, 50, and 100 mg/mL) and (3mg/mL, 2mg/mL and 1mg/mL in DMSO) for pure compounds were tested while *fluconazole* and *ketoconazol*e (50 µL, 1 mg/mL in DMSO) was used as the standard drug. The zone of inhibition was recorded in the same fashion above for bacterial strain using a caliper. The results were expressed as mean value ± standard deviation. The bacterial and fungal strain was collected from Harar health science medical laboratory and Haramaya university plant pathology department respectively and the work was done in collaboration with the medical microbiology laboratory and biology department at Haramaya University.

3.8. Antioxidant activity test

3.8.1. Evaluation of the DPPH radical scavenging assay (RSA)

Antioxidants play an important role as health protection factors. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease (Kalbessa *et al*., 2019). The DPPH assay was used to assess the free radical scavenging activity of crude and isolated pure compounds. With this method the radical scavenging power of an antioxidant compound was measured with the principle of a decrease in absorbance due to the donation of hydrogen from the sample to DPPH radical to produce stable DPPH-H and the color change from purple to yellow was observed as shown in (figure 7) below.

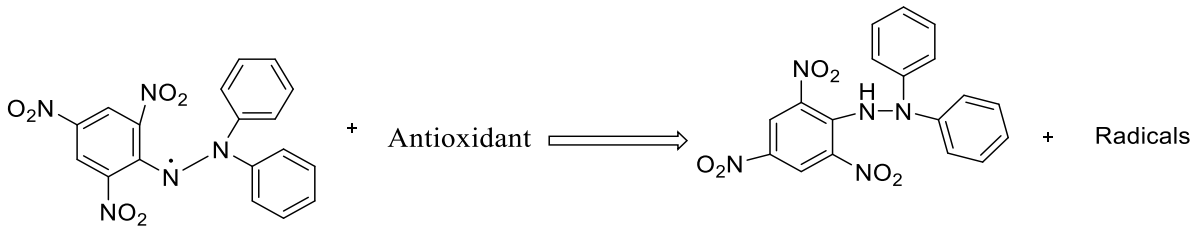


Figure 7: The structure of the DPPH radical and the scavenged radical.

The radical-scavenging activities of the extracts and isolated compounds were determined using the DPPH method (Rajesh & Natvar, 2011). In the process, the n-hexane, dichloromethane, and methanol crude extracts of the root as well as the fractions of aerial extracts, and isolated pure compounds were dissolved in methanol to afford 1mg/mL. It was serially diluted in methanol to give a concentration of 500, 250, 125, and 62.5 µg/mL. To 1 mL of each concentration, 4 mL

DPPH (0.04% DPPH in MeOH) was added to make 100, 50, 25, and 12.5 µg/mL solutions. Then all the samples prepared were incubated in an oven at 37℃ for 30 min and then absorbance was recorded at 517 nm using a UV-Vis spectrophotometer (Jenwey 6858, England). A reaction solution without DPPH was used as blank, DPPH solution as a control, and L-ascorbic acid was used as standard. The percentage inhibition was calculated using the following formula:

% Inhibition (RSA)= [A control –A test] / A control × 100……. (1)

The DPPH radical scavenging activity of the samples is also expressed as IC_{50}, the concentration of the test compound to give a 50% decrease of the absorbance from that of the control solution (Kalbessa *et al.*, 2019). The IC_{50} values were calculated from the graph plotted from concentration versus %RSA determined using excel and interpreted as the smallest IC_{50} values mean the strongest radical scavenging concentration of extracts (Castro *et al*., 2014).

3.9. *In silico* molecular docking study of the isolated compounds

In the field of molecular modeling, docking is a method that predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex (Mishra *et al*., 2019). Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules. Molecular docking is a computational procedure that attempts to predict the non-covalent binding of macromolecules (receptor) and a small molecule (ligand) efficiently, starting with their unbound structures, structures obtained from MD simulations, or homology modeling, to predict the bound conformations and binding affinity (Adejoro *et al*., 2020).

To have a better understanding of the inhibitory mechanism as well as the mode of interactions of the isolated compounds, *in silico* antibacterial and antioxidant molecular docking analysis was accomplished using the ADT version 1.5.7 and Auto Dock Vina version 4.2.6 docking program (Trott, O., & Olson, A. J. (2009). In general, antimicrobial agents target the key components of bacterial metabolism to disable the bacteria such as cell walls, DNA gyrase, DNA-directed RNA polymerase, protein synthesis, and enzymes (Levine *et al*., 1998). DNA gyrase, an enzyme belonging to a member of bacterial topoisomerase, controls the topology of DNA during transcription, replication, and recombination by introducing transient breaks to both DNA strands (Zeleke *et al.*, 2020). The ATP-dependent bacterial type-II topoisomerase,

DNA gyrase, and DNA topoisomerase-IV contribute to the maintenance of the correct level of supercoiling in bacterial DNA (Smith & Mondragón, 2021).

The chemical structures of isolated compounds were drawn using the Chem Office tool (Chem Draw 16.0) assigned with proper 2D orientation, and the energy of each molecule was minimized using ChemBio3D. The energy-minimized ligand molecules are then used as input for Auto Dock Vina, to carry out the docking simulation (Adejoro *et al*., 2020).

The crystal structure of receptor molecule *S. aureus* tyrosyl-tRNA synthetase (PDB ID: 1JIJ) and human peroxiredoxin 5 enzymes (PDB: 1HD2) were downloaded from protein data bank. The protein preparation was done using the reported standard protocol *(*Mitra *et al.*, 2021), by removing the co-crystallized ligand, selected water molecules and cofactors, the target protein file was prepared by leaving the associated residue with protein by using auto preparation of target protein file Auto Dock Vina 4.2.6 (MGL tools 1.5.7). The graphical user interface program Auto Dock Vina 4.2.6 was used to set the grid box for docking simulations. The grid was set so that it surrounds the region of interest in the macromolecule. The grid box was constructed using 21x24x22, pointing in x, y, and z directions with center grid box of -11.422655x14.972690 x85.947621 Å, and grid point spacing of 0.375 Å for *s. aureus* tyrosyl-tRNA synthetase (PDB ID 1JIJ). In the same manner the grid box was constructed using 20x23x24, pointing in x, y, and z directions, respectively, with a grid point spacing of 0.375 Å. The center grid box of 7.023778x41.721111x34.408556 Å for human peroxiredoxin 5 (PDB ID :1HD2). Nine different conformations were generated for each ligand scored using Auto Dock Vina functions and were ranked according to their binding energies for both targeted proteins by Discovery studio visualizer and PyMOL (Garg *et al*., 2021) The molecular docking study was done in Adama Science and Technology University by Venkatesh Perumal (PhD).

CHAPTER-FOUR

4. RESULTS AND DISCUSSION

4.1. Determination of Extraction Yield

The extraction yield is a measure of the solvent efficiency to extract specific components from the original material. The percentage yield of crude extract of roots and aerial parts in the respective solvent were shown in Table 3 and Table 4, respectively. It could be calculated according to the following method.

%Yield = [Weight of crude/Weight of sample] x100……. (1)

Table 3: Percentage yield of the root crude extracts of *R. multifidus*:

Crude extract	Weight of extract in gram	Nature of crude	Yield in (%)
n-hexane	4.5g	Oily green	1.125
Dichloromethane	6.2g	Black jelly	1.58
Methanol	23.73g	Greasy Yellow	6.096

The methanol yields obtained from the root of *Ranunculus multifidus* were significantly higher than the n-hexane and dichloromethane extract. This clearly showed as the plant is rich in polar organic compounds.

Table 4: Percentage yield of fractions from aerial crude extract of *R. multifidus:*

Crude extract	Weight of extract in gram	Nature of fractions	Yield in (%)
n-hexane fraction	5.65	Green jelly	4.54%
Chloroform fraction	12.45	Black oily	10.87%
Ethyl acetate fraction	26.82	Yellowish jelly	26.27%
n-Butanol fraction	13.43	Dark black	15.15%
Aqueous fraction	45.7	Light honey	38.08%

From the percentage yield mentioned (Table 4) one can easily suggest that the aerial part of the plant mainly contain polar phytochemicals which are also manifested in root extracts of the plant as well.

4.2. Phytochemical screening

Phytochemical screening tests were done to determine the classes of compounds present in the crude extracts following standard experimental protocols. The preliminary phytochemical screenings on the root extract of *R. multifidus* and a different solvent fraction of aerial extract were tested in this study. The results suggested that flavonoids, saponins, phenols, tannins, terpenoids, steroids, phytosterols, and glycosides are present within the crude extracts with different ranges Table 5 and Table 6. The results obtained in this study suggest the identified phytochemical may be the bioactive constituents of this plant suggesting the plant is a valuable reservoir of bioactive compounds of substantial medicinal importance.

Table 5: Phytochemical screening of different crude extracts of the root of *R. multifidus:*

Phytochemical screening	Test type	n-hexane extract	DCM extract	MeOH extract
Flavonoids	Alkaline test	-	+	+
Saponins	Foam test	+	+	+
Phenols	Alkaline test	-	+	+
Tannins	Ferric chloride test	-	+	+
Terpenoids	Salkowski test	-	-	+
Steroids	-	+	-	+
Phytosterols	Salkowski test	+	+	-
Cardiac glycosides	Keller-Killiani test	+	+	+
Alkaloids	Wagner's test	-	+	-
Anthocyanin	-	-	-	+
Quinones	-	+	-	+
Reducing sugar	Fehling's test	-	+	+
Anthraquinones	-	+	-	-

+ = the presence of phytochemical constituents
- = the absence of phytochemical constituents

Table 6: Phytochemical screening of different fractions of aerial extracts of *R. multifidus:*

Phytochemical screening	Test type	n-hexane fraction	EtOAc fraction	$CHCl_3$ fraction	n-BuOH fraction	Aqueous Fraction
Flavonoids	Alkaline test	-	+	+	+	+
Saponins	Foam test	+	-	+	-	+
Phenols	Alkaline test	+	+	+	+	+
Tannins	Ferric chloride test	-	+	+	+	-
Terpenoids	Salkowski test	-	+	-	-	+
Steroids	-	-	+	-	+	+
Phytosterols	Salkowski test	-	+		-	+
Cardiac glycosides	Keller-Killiani test	-	+	+	+	+
Alkaloids	Wagner's test	-	-	+	+	-
Anthocyanin	-	-	+	-	-	+
Quinones	-	+	-	+	-	+
Reducing sugar	Fehling's test	-	+	+	+	+
Anthraquinone	-	-	+	+	+	+

+ = the presence of phytochemical constituents, - = the absence of phytochemical constituents

These secondary metabolites are known to be biologically active and play significant roles in the pharmacological effects of medicinal plants because the medicinal values of plants lied in these phytochemicals, which produce a definite pharmacological action on the human body. The presence of phenols could confer antioxidant and antimicrobial properties on the plant as reported by various researchers from the genus.

4.3. Structural elucidation of isolated compounds

The ethyl acetate fraction of the aerial part of *Ranunculus multifidus* was subjected to repeated silica gel column chromatography which has led to the isolation of three compounds coded as; RML01 (30mg), RML02 (25 mg) and RML03 (27mg). From this RML01 and RML03 obtained from fraction (F_{107}-F_{119}) and RML02 (F_{75}-F_{93}) from fraction after their TLC analysis. Herein is the description of characterization of each compound.

4.3.1. Characterization of RML01

RML01 was obtained as reddish amorphous from the CC of ethyl acetate fraction of the aerial extract of *R. multifidus*. The compound exhibited *Rf* value of 0.56 in its TLC with n-hexane: methanol (8:2) as a mobile phase (Figure 20).

The ^{1}H-NMR spectrum of RML01 (Appendix 1) showed the proton of H-3 appeared as a multiplet at δ 3.56 (m) revealed the existence of oxygenated methine and signals for olefinic proton at δ 5.31(m), 5.33 (m), and 5.36 (m). The H-4 appeared as doublet δ 2.10 (d) revealed aliphatic proton. Angular methyl proton at 0.83 (d,3H), 0.87 (t,3H), 0.93 (d,3H), and 0.89 (d,3H) corresponds to C-19 and C-26, C-27, and C-29 proton respectively.

The proton decoupled ^{13}C-NMR spectrum (Appendix 2) with the aid of DEPT-135 (Appendix 3) revealed the presence of forty-four carbon resonances of which seven are quaternary, fourteen methines, thirteen methylene and ten methyl groups. The spectrum showed signal at δ 173.24 due to acetyl carbonyl carbon. The ^{13}C-NMR has shown recognizable signals at δ 129 and 127.9 ppm which are assigned to C-5 and C-6 double bonds respectively. The presences of oxygenated methine carbons were evident at δ 69.03. The value at 19.21 corresponds to angular carbon atom of C-19, and the alkene carbon at 131.5 stands for C-20 and 129.9 for C-21. The NMR spectral data of RML01 compared to stigmasterol reported in the literature (Table 7).

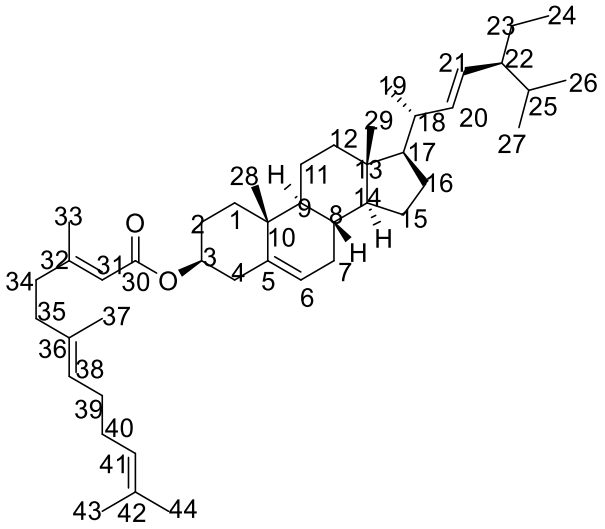
The alkene carbons appeared at δ 127.7, 128.3, 128.6, 129.6, 127.9 and 129.7 here was resulted from esterification of stigmasterol and 3,7,11-Trimethyldodeca-2,6,10-trienoic acid (Farnesoic acid) polyunsaturated fatty acid. This acid was also reported as it was found in *Solanum agrimoniifolium* and *solanum habrochaites* which know might fused to this compound at hydroxy functional group of stigmasterol at C-3 position due to esterification. As per literature survey the stigmasterol and β-sitosterol which are very common active secondary metabolite in many medicinal plants including the *Ranunculaceae* family of the plant obtained in mixture form (Pierre & Moses, 2015).

Stigmasterol reported previously from *Ranunculus sceleratus* which is a wet land plant and a widely used traditional Chinese medicinal herb that has been researched intensively (Mei *et al*., 2012). From the above information and literature support data the proposed structure of compound RML01 is stigmasterol-3-farnesoic acid (Figure 8).

Table 7: ^{1}H and ^{13}C NMR Chemical shifts values (δ) in ppm for compound RML01 recorded in d_6-Acetone (400MHz) with TMS as internal standard.

Carbon atom	^{13}C-NMR Experimental	^{13}C-NMR Literature	^{1}H-NMR Experimental	^{1}H-NMR Literature	Nature of Carbon
C-1	37.20	36.72	1.32(t,2H)		CH_2
C-2	31.75	32.0	1.60(q,2H)		CH_2
C-3	69.03	71.97	3.56(m,1H)	3.53(m,1H)	CH
C-4	33.75	42.35	2.10(d,2H)		CH_2
C-5	129.78	140.94			C
C-6	127.09	122.32	5.36(t,1H	5.31(t,1H)	CH
C-7	31.55	31.71			CH_2
C-8	32.46	29.24			CH
C-9	50.7	50.03			CH
C-10	39.21	36.16			C
C-11	22.34	21.12			CH_2
C-12	39.48	39.57			CH_2
C-13	32.62	40.45			C
C-14	62.85	57.76			CH
C-15	25.19	24.27			CH_2
C-16	28.88	28.83			CH_2
C-17	63.03	56.84			CH
C-18	37.11	36.5	1.32(t,1H)	1.29(d,1H)	CH
C-19	19.21	18.6	0.86(d,3H)	0.74(d,3H)	CH_3
C-20	131.53	138.6	5.30(t,1H)	5.20(m,1H)	CH
C-21	129.93	129.34	Olefinic proton		CH
C-22	27.79	28.5			CH
C-23	22.44	23.4			CH_2
C-24	13.48	12.2			CH_3
C-25	29.50	29.6			CH
C-26	19.21	19.4	0.87 (d,3H)	0.84(d,3H)	CH_3
C-27	19.21	19.3	0.93(d,3H)	0.97(d,3H)	CH_3
C-28	24.24	20.03			CH_3
C-29	13.69	12.4	0.89(d,3H)	0.83 (t, 3H)	CH_3
C-30	173.24				C
C-31	127.67				CH
C-32	128.03				C
C-33	24.75				CH_3
C-34	33.44				CH_2
C-35	25.32				CH_2
C-36	128.06				CH
C-37	24.87				CH_3
C-38	129.57				C
C-39	36.29				CH_2

C-40	31.35				CH_2
C-41	127.88				CH
C-42	129.74				C
C-43	29.42				CH_3
C-44	29.47				CH_3



(3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-((2*R*,5*S*,*E*)-5-ethyl-6-methylhept-3-en-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl (2*E*,6*E*)-3,6,11-trimethyldodeca-2,6,10-trienoate

Figure 8: Chemical structure of compound RML01

4.3.2. Characterization of RML02

RML02 was obtained as greenish oily from the CC of ethyl acetate fraction of the aerial extract of *R. multifidus*. The compound exhibited *Rf* value of 0.46 in its TLC with n-hexane: ethyl acetate:(1:1) as a mobile phase (Figure 20).

The ^{1}H NMR spectrum of RML02 (Appendix 4) exhibited the presence of three aromatic proton at δ 7.34 (d, J=8.5, 1H), 6.90 (d, J=10, 2Hz, 1H), and 6.80 (s, J=9, 2 Hz,1H) region, which also indication of ortho coupled and oxygenated methylene proton around 4.16 (dt, J = 26.9, 6.7 Hz, 1H) respectively. The proton at 2.41 (dd, J = 7.7 Hz, 2H) also shows the presence of methylene proton. Oxygenated singlet also observed for quaternary carbon due to hydroxyl group at C-10′. The presences of three methyl proton also observed at 1.31 (s, 3H), 1.01 (s, 3H) and 0.80 (t,3H). The proton NMR also revealed the presence of many overlapping methylene at 1.27(m, H).

The proton decoupled ^{13}C-NMR spectrum (Appendix 5) with the aid of DEPT-135 (Appendix 6) revealed the presence of twenty-four carbon resonances of which four are quaternary, two

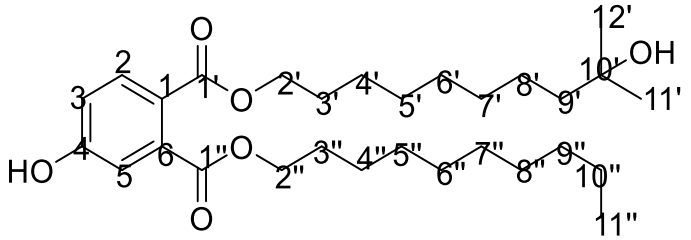
methines, fifteen methylene of which one is oxygenated and three methyl groups. There is a quaternary carbon signal around 168 ppm having small pick value due to weaker intensity. The peak at 144 ppm is due to quaternary olefinic oxygenated carbon with OH (C-4). The presences of oxygenated methylene carbons were evident at δ 64.60 correspond to C-2′ and C-2″. The chemical shift values at 77.21 ppm correspond to the quaternary carbons (C-10′). The NMR spectral data of RML02 was compared and the whole result was presented in (Table 8). This compound correlate with the Di-iso octyl phthalate which was isolated tannins from green rind of *Aloe vera* (Benzidia *et al*., 2019) and Dibutyl Phthalate isolated from *Ipomoea cornea* stem (Kumar & Islam, 2012), with exception of additional methylene and oxygenated quaternary carbon on this compound.

The pharmacology of this compound highly correlated with the pharmacological activities of related article above. Especially the antioxidant effects of Di-iso octyl phthalate using DPPH assay with percentage inhibition of free radical (74.17%) (Benzidia *et al*., 2019) and antibacterial activities of Dibutyl Phthalate reported against different strain especially on *P. aeruginosa* with zone of inhibition 7 mm at concentration of 400 (µg/ml) (Kumar & Islam, 2012), exactly related with compounds reported. The ethnomedicinal use of the *Ranunculus multifidus* also revealed the conciseness of information given for the above phthalate which was previously reported from *Holoptelea integrifolia* or an Indian elm used for wound treatment, eczema, malaria, intestinal cancer, urinary disorders in same manner reported for the plant of interest (Ganie & Yadav, 2014).

Table 8: ^{1}H and ^{13}C NMR Chemical shifts values (δ) in ppm for compound RML02 recorded in ($CDCl_3$ 400MHz) with TMS as internal standard.

Carbon atom	^{13}C-NMR Experimental	^{13}C-NMR Literature	^{1}H-NMR Experimental	^{1}H-NMR Literature	Nature of Carbon
C-1	129.89	132.35	s	s	C
C-2	129.73	128.84	7.34 (d, J=8.5, 1H)	7.74 (dd, J=10, 2 Hz, 2 H)	CH
C-3	115.89	120	6.90 (d, J=10, 2Hz, 1H)	7.56 (dd, J=10 ,2Hz, 2H)	CH
C-4	144.3	120	-	-	C
C-5	129.73	128.84	6.80 (s,1H)	7.74 (dd, J=10, 2 Hz, 2H)	CH

C-6	129.89	132.35	s	s	C
C1′=1″	168	167.7	s	s	C
C2′=2″	64.60	65.55	4.16 (dt, *J* = 26.9, 6.7 Hz,1 H)	t	OCH_2
C3′=3″	31.91	30.59	2.41 (dd, *J* = 7.7 Hz, 2H)	t	CH_2
C-4′	25.99		1.59 (dd, *J* =15,7.8 Hz ,2H)		CH_2
C-5′	29.35				CH_2
C-6′	29.59				CH_2
C-7′	29.69		1.27 (m, 8H)		CH_2
C-8′	28.77				CH_2
C-9′	44.73		2.41 (t, *J* = 7.7 Hz, 2H),		CH_2
C-10′	77.21		s		C
C-11′	14.09	13.70	1.31 (s, 3H),	1.29(q,3H)	CH_3
C-12′	13.75	13.70	1.01 (s, 3H).	0.98(s,3H)	CH_3
C-4″	25.73				CH_2
C-5″	29.17				CH_2
C-6″	29.65				CH_2
C-7″	29.29		1.27 (m, 14H)		CH_2
C-8″	29.53				CH_2
C-9″	22.67				CH_2
C-10″	22.36				CH_2
C-11″	17.31	19.18	0.80(t,3H)	0.87(t,3H)	CH_3



2-decyl 1-(9-hydroxy-9-methyldecyl) 4-hydroxyphthalate

Figure 9: Chemical structure of compound RML02

4.3.3. Characterization of RML03

RML03 was obtained as yellowish amorphous compound from the CC of ethyl acetate fraction of the aerial extract of *R. multifidus*. The compound exhibited *Rf* value of 0.52 in its TLC with n-hexane: ethyl acetate (8: 2) as a mobile phase (Figure 20).

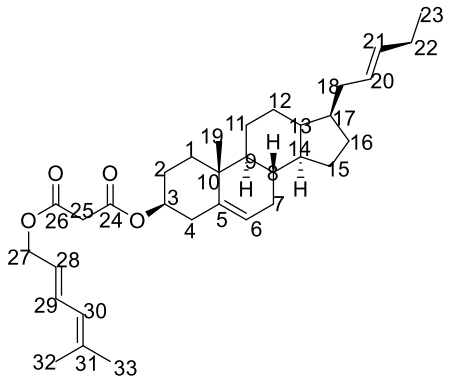
The ^{1}H-NMR spectrum of RML03 (Appendix 7) showed the proton of H-3 appeared as a multiplet at δ 3.53 revealed the existence of oxygenated methine and signals for olefinic proton at δ5.37(s), and methylene proton of H-4 at δ 2.85(s). Angular methyl proton at 0.85 (s, 3H) corresponds to C-19 and 0.84 (t,3H), 0.93 (d,3H), and 1.06 (s,3H) C-23, C-32, and C-33 proton respectively. Over all the spectral information of this compound was almost similar as depicted above for RML01 with slight modification of stigmasterol alcohol function with alcohol esterified malonyl moiety. The full information of the compounds was illustrated in (Table 9).

The proton decoupled ^{13} C-NMR spectrum (Appendix 8) with the aid of DEPT-135 (Appendix 9) revealed the presence of thirty-three carbon resonances of which five are quaternary, twelve methines, twelve methylene and four methyl groups. The spectrum showed signal at δ 172.45, 25.33 and 172.15ppm shows the malonyl moiety attached to hydroxy functional group on stigmasterol skeleton which also reported previously from *momordica charantia* leaf extracts with chemical shift values 167.15, 42.73, and 169.45 for C-24, C-25 and C-26 respectively (Mekuria *et al*., 2014). The ^{13}C-NMR has recognizable signals at 129.80 and 127.10 ppm which was assigned to C-5 and C-6 double bonds respectively. The presences of oxygenated methine carbons were evident at δ 69.04. The value at 13.69 ppm corresponds to angular carbon atom C-19, and the alkene carbon at 131.54 ppm stand for C-20 and 129.94 ppm for C-21. The NMR spectral data of RML03 was compared and the whole result was presented in (Table 9).

The alkene carbons appeared at 129.74, 128.06, 127.93, 127.69 ppm here was resulted from the esterification of malonic acid of stigmasterol skeleton to a medium chain primary fatty alcohol with two trans double bond at position 2, and 4 called (2E)-5-methylhexa-2,4-dienol. It is very difficult to obtain stigmasterol in pure state (Kamboj, A., & Saluja, A. K. 2011; Pierre & Moses, 2015), as mentioned in characterization of compound RML01 above. The extra peak of this compound emanated from solvent peak especially n-hexane peaks around 28s ppm co-eluted as impurity. From the above information and literature support data the structure of compound RML03 was proposed as stigmasterol with di-enol esterified malonic acid group (Figure 10).

Table 9: ^{1}H and ^{13}C NMR Chemical shifts values (δ) in ppm for compound RML03 recorded in d_6-Acetone (400MHz) with TMS as internal standard.

Carbon atom	^{13}C-NMR Experimental	^{13}C-NMR Literature	^{1}H-NMR Experimental	^{1}H-NMR Literature	Nature of Carbon
C-1	31.36	36.72			CH_2
C-2	31.76	32.0			CH_2
C-3	69.04	71.97	3.53 (m, 1H)	3.53 (m, 1H)	CH
C-4	33.75	42.35			CH_2
C-5	129.80	140.94			C
C-6	127.10	122.32	5.37 (s, 1H)	5.38 (s, 1H)	CH
C-7	29.03	31.71			CH_2
C-8	29.48	29.24			CH
C-9	29.16	50.03			CH
C-10	33.55	36.16			C
C-11	22.34	21.12			CH_2
C-12	29.35	29.57			CH_2
C-13	29.19	40.45			CH
C-14	62.85	57.76			CH
C-15	25.21	24.27			CH_2
C-16	22.44	28.83			CH_2
C-17	62.85	56.84			CH
C-18	26.94	36.5	1.20(m,1H)	1.29(m,1H)	CH_2
C-19	13.69	18.6	0.85(d,3H)	0.74(d, 3H)	CH_3
C-20	131.54	138.6	Olefinic proton		CH
C-21	129.94	129.34	Olefinic proton		CH
C-22	29.54	29.6	1.62 (m, 1H)		CH_2
C-23	13.48	12.2	0.84 (3H, t)		CH_3
C-24	172.45				C
C-25	25.33	-			CH_2
C-26	172.15	-			C
C-27	61.88	-			CH_2
C-28	127.69				CH
C-29	128.06				CH
C-30	127.93				CH
C-31	129.74				C
C-32	24.72	19.4	0.93(s, 3H)	0.84(d, 3H)	CH_3
C-33	24.87		1.06(s, 3H)	0.84(d, 3H)	CH_3



(3*S*,8*S*,9*S*,10*R*,14*R*,17*R*)-10-methyl-17-((*E*)-pent-2-en-1-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl ((*E*)-5-methylhexa-2,4-dien-1-yl) malonate

Figure 10: Chemical structure of compound RML03

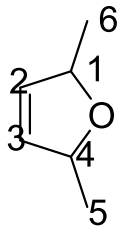
4.3.4. Characterization of RMR01

RMR01 (20mg) was obtained as bright green solid from the CC of methanol crude extract from the root of *R. multifidus.* The compound exhibited *Rf* value of 0.42 in its TLC with ethyl acetate: methanol (8: 2) as a mobile phase (Figure 20).

The ^{1}H NMR spectrum of RMR01 (Appendix 10) showed the proton of intense peak at 3.91 (p, J = 6.6 Hz, 1H) due to oxygenated methine and olefinic carbon at 4.18 ppm (d,1H) in the compound. The proton around 1.10 ppm (d, 3H) was due the methyl group. The proton decoupled ^{13}C-NMR spectrum (Appendix 11) with the aid of DEPT-135 (Appendix 12) revealed the presence of three carbon resonances of which two are methine, and two equivalent methyl groups. The ^{13}C-NMR has shown recognizable signals at 129.57 ppm and which are assigned to the equivalent C-2 and C-3 double bonds. The presences of oxygenated methine carbons were evident at δ 62.85 ppm corresponds to C-1 and C-4. The value at 24.85 ppm corresponds to two equivalent carbon atom C-5 and C-6 (Table 10). The carbon NMR and DEPT-135 indicates the compound has no methylene and quaternary carbon atoms. Based on the above spectral information structure of RMR01 was proposed as shown in (Figure 11).

Table 10: ^{1}H and ^{13}C NMR Chemical shifts values (δ) in ppm for compound RMR01 recorded in d_6-Acetone (400MHz) with TMS as internal standard.

Carbon atom	^{13}C-NMR Experimental	^{1}H-NMR Experimental	Nature of Carbon
C-1	62.85	3.91 (p, *J* = 6.6 Hz, 1H)	CH
C-2	129.57		CH
C-3	129.57		CH
C-4	62.85	3.91 (p, *J* = 6.6 Hz, 1H)	CH
C-5	24.85	1.10 (d, *J* = 2.6 Hz, 3H)	CH_3
C-6	24.85	1.10 (d, *J* = 2.6 Hz, 3H)	CH_3



2,5-dimethyl-2,5-dihydrofuran

Figure 11: Chemical structure of compound RMR01

4.3.5. Characterization of RMR02

RMR02 (20mg) was obtained as bright green solid from the CC of methanol crude from the root extract of *R. multifidus.* The compound exhibited *Rf* value of 0.43 in its TLC with ethyl acetate: methanol (7: 3) as a mobile phase (Figure 20).

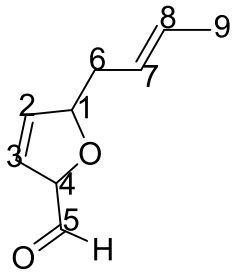
The ^{1}H NMR spectrum of RMR02 (Appendix 13) showed the proton of intense peak at 3.97 – 3.84 (m, 1H) due to oxygenated methine (H-1) and the olefinic proton also signified in the presence of alkene carbon at 4.74 ppm (m,1H) in the compound. The proton at 6.20 ppm shows the existence of aromatic proton correspondence to H-2 and the proton at 8.13 ppm might be proton of aldehyde functional group H-5 (CHO). The proton around 1.13 ppm (d, J = 2.8 Hz, 3H) was due the methyl group.

The proton decoupled ^{13}C-NMR spectrum (Appendix 14) with the aid of DEPT-135 (Appendix 15) revealed the presence of seven carbon resonances of which five are methine, one methylene carbon and one methyl groups. The ^{13}C-NMR has shown recognizable signals at 154.90 ppm and which are assigned to the equivalent C-2 and C-3 double bonds. The peak at 129.56 ppm and 119.6 ppm corresponds to C-7 and C-8 respectively. The presences of oxygenated methine

carbons were evident at 62.85 ppm corresponds to C-1 and C-4. The chemical shift value at 23.15 ppm corresponds to methylene carbon of C-6 and the chemical shift values at 24.85 and 205.42 ppm reveals the methyl carbon at C-9 and aldehyde carbon of C-5 respectively as shown in Table 11.

Table 11: ^{1}H and ^{13}C NMR Chemical shifts values (δ) in ppm for compound RMR02 recorded in d_6-Acetone (400MHz) with TMS as internal standard.

Carbon atom	^{13}C-NMR Experimental	^{1}H-NMR Experimental	Nature of Carbon
C-1	62.85	3.97 – 3.84 (m, 1H)	CH
C-2	154.90	Aromatic proton 6.20 (1H, d)	CH
C-3	154.90	Aromatic proton 6.20 (1H, d)	CH
C-4	62.85	3.97 – 3.84 (m, 1H)	CH
C-5	205.42	8.13(s)	CHO
C-6	23.15	2.07(m,2H)	CH_2
C-7	129.56	4.74(m)	CH
C-8	119.64	4.74(m)	CH
C-9	24.85	1.13 (d, J = 2.8 Hz, 3H)	CH_3



(*E*)-5-(but-2-en-1-yl)-2,5-dihydrofuran-2-carbaldehyde

Figure 12: Chemical structure of compound RMR02

4.3.6. Characterization of RMR08

RMR08 (22mg) was obtained as dark brown oily from the CC of methanol crude from the root extract of *R. multifidus.* The compound exhibited *Rf* value of 0.25 in its TLC with ethyl acetate: methanol (8: 2) as a mobile phase (Figure 20).

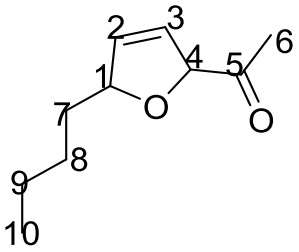
The ^{1}H NMR spectrum of RMR08 (Appendix 16) showed the proton of intense peak at 3.91 (dd, *J* = 11.8, 6.4 Hz, 2H) due to oxygenated methine (H-1, H-4) and the olefinic proton also signified in the presence of alkene carbon at 4.74 ppm (d,1H) in the compound. The proton at 2.06 (q, 2H), 1.30 (d, *J* = 2.3 Hz, 2H) and 1.24 – 1.13 (m, 3H) shows the existence of methylene

proton correspondence to H-7, H-8 and H-9 and the proton at 3.60 (s, 3H), and 0.89 (t, 3H) was the proton of H-6 and H-10 respectively.

The proton decoupled ^{13}C-NMR spectrum (Appendix 17) with the aid of DEPT-135 (Appendix 18) revealed the presence of eight carbon resonances of which two are methine, three methylene carbon, one is quaternary and two methyl groups. The ^{13}C-NMR has shown recognizable signals at 129.56 ppm and which are assigned to the equivalent C-2 and C-3 double bonds. The peak at 205.46 ppm corresponds to C-5 of ketone functional group. The presences of oxygenated methine carbons were evident at 62.86 ppm corresponds to C-1 and C-4. The value at 22.44, 29.48 and 31.74 ppm correspond to methylene carbon of C-9, C-8 and C-7 and the chemical shift values at 24.85 and 13.48 reveals the methyl carbon at C-6 and C-10 respectively with the whole information mentioned in Table 12.

Table 12: ^{1}H and ^{13}C NMR Chemical shifts values (δ) in ppm for compound RMR08 recorded in d_6-Acetone (400MHz) with TMS as internal standard.

Carbon atom	^{13}C-NMR Experimental	^{1}H-NMR Experimental	Nature of Carbon
C-1	62.86	3.91 (dd, *J* = 11.8, 6.4 Hz, 2H)	CH
C-2	129.56	Olefinic proton	CH
C-3	129.56	Olefinic proton	CH
C-4	62.86	3.91 (dd, *J* = 11.8, 6.4 Hz, 2H)	CH
C-5	205.46	-	C
C-6	24.86	3.60 (s, 3H),	CH_3
C-7	31.74	2.06 (q, 2H)	CH_2
C-8	29.48	1.30 (d, *J* = 2.3 Hz, 2H)	CH_2
C-9	22.44	1.24 – 1.13 (m, 2H)	CH_2
C-10	13.48	0.89 (t, 3H)	CH_3



1-(5-butyl-2,5-dihydrofuran-2-yl)ethan-1-one

Figure 13: Chemical structure of compound RMR08

4.3.7. Characterization of F59

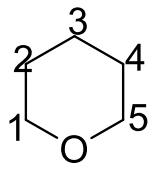
F59 (18mg) was obtained as colorless amorphous compound from the CC of methanol crude extract from root of *R. multifidus.* The compound exhibited *Rf* value of 0.46 in its TLC with n-hexane: ethyl acetate (6: 4) as a mobile phase (Figure 20).

The ^{1}H NMR spectrum of F59 (Appendix 19) showed the proton of intense peak at 3.90 ppm (t, J = 6.1 Hz, 2H) due to oxygenated methylene (H-1, H-5) and the proton at 1.60 ppm (m,2H) correspond to the two-equivalent methylene of C-2 and C-4. The proton at 1.17 – 1.08 (m, 2H) stands for another methylene proton of C-3 which is shielded due to less electronegative effect on its chemical shift values.

The proton decoupled ^{13}C-NMR spectrum (Appendix 20) with the aid of DEPT-135 (Appendix 21) revealed the presence of three carbon resonances of which of all are methylene carbon, and no other carbon type. The ^{13}C-NMR has shown recognizable signals at 62.85 ppm and which are assigned to the equivalent oxygenated carbon of C-1 and C-5. The peak at 29.21 ppm corresponds to C-2 and C-4 carbon atoms. The value at 24.85 ppm correspond to methylene carbon of C-3 with the whole information depicted in (Table 13). Based on the above spectroscopic data the structure of the compound was proposed as tetrahydro pyran (Figure 14). The information extracted from spectra and the pharmacology of the plant indicated in literature review particularly anti-quorum sensing and antibiofilm formation of which also reported for other pyran and furan from other plants (Srujana *et al*., 2012).

Table 13: ^{1}H and ^{13}C NMR Chemical shifts values (δ) in ppm for compound F59 recorded in d_6-Acetone (400MHz) with TMS as internal standard.

Carbon atom	^{13}C-NMR Experimental	^{13}C-NMR Literature	^{1}H-NMR Experimental	Nature of Carbon
C-1	62.85	69.5	3.90 (t, J = 6.1 Hz, 2H)	CH_2
C-2	29.21	27.7	1.60(m,2H)	CH_2
C-3	24.85	24.9	1.17 – 1.08 (m, 2H)	CH_2
C-4	29.21	27.7	1.60(m,2H)	CH_2
C-5	62.85	69.5	3.90 (t, J = 6.1 Hz, 2H)	CH_2



tetrahydro-2*H*-pyran

Figure 14: Chemical structure of compound F59

4.4. Antibacterial investigation of crude and pure isolate from root and aerial extracts of *Ranunculus multifidus*

The antibacterial activity of the crude extracts of the plant was determined by screening against two Gram-negative bacterial strains (*E. coli,* and *P. aeruginosa*) and two Gram-positive strains (*S. aureus* and *S. pyogenes)* using the disc diffusion method. *Ciprofloxacin* was used as a positive control for selected strains. The zone of inhibition values indicated that all the extracts exhibited a varied range of antibacterial activities against all the tested bacterial strains. In comparison, the antibacterial activity of crude extracts at 50 mg/mL was weaker than at 100 mg/mL which was again less than 200 mg/mL suggesting that the activity displayed has a dose-dependent manner in both root extracts and aerial fractions (Table 14) and (Table 15) respectively. The antibacterial activities of pure compounds isolated from methanol root extracts of R. *multifidus* designated as RMR01, RMR02, RMR08, and F59 and the compounds isolated from ethyl acetate fraction of aerial extracts coded as RML01, RML02, and RML03 were investigated using the same procedure as of a crude extracts and fractions, except substituting *Bacillus subtills* in place of *S. pyrogens.* The result shows moderate relative to its concentration of pure compounds and standards used since the *ciprofloxacin* has broad spectrum of activities against pathogenic strain. The results of antibacterial activities against all strain for each compound was promising at all concentration in concentration dependent manner as depicted in (Table 16).

Furthermore, the result showed better antibacterial activity against Gram-positive than the Gram -negative bacterial strains. The reason for the difference in sensitivity between Gram-positive and Gram-negative bacteria might be attributed to the differences in morphological constitution of these microorganisms. Gram-negative bacteria have an outer lipopolysaccharide membrane, and this makes the cell wall impermeable to antibacterial chemical substances. Gram-positive

bacteria on the other hand are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. Therefore, the cell walls of Gram-negative bacteria are more complex in layout than the Gram-positive ones acting as a diffusion barrier and making them less susceptible to the antibacterial agents (Carvalho *et al*., 2018).

The antibacterial activity of compounds might be caused by the formation of reactive oxygen species (ROS) which leads to oxidative stress and subsequent cell death of pathogenic strain. The formation of ROS is a common antibacterial activity adopted by the plant extracts (Desalegn *et al*., 2021). Another possible mechanism for the antimicrobial activity of extracts and compounds is through the attachment of extracts or compounds to the bacteria cell membrane via electrostatic forces. This interaction may distort the plasma membrane structure and damage the bacterial cell integrity, resulting in the leakage of intracellular contents and ends with cell death via apoptosis (Demissie *et al*., 2020; Desalegn *et al*., 2021).

Over all the antibacterial activities of the pure compounds isolated from *R. multifidus* interpreted as compound RML02 showed strong activity with a maximum zone of inhibition of 13±0.82 mm, 12.5±1.5 mm, 12±0.34 mm, and 11±0.45 mm against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*, respectively at concentration of 350 µg/mL. Compound RML01 showed the second most strong activity against *B. subtilis* (11.5±0.5 mm), *S. aureus* (11±0.45 mm), *E. coli* (10±0.98 mm), and *P. aeruginosa (*10±0.26 mm). Compound RMR08 showed the third most strong activity against *B. subtilis* (10.5±0.12 mm), *S. aureus* (10±0.0 mm), *E. coli* (9.5±1.1 mm), and *P. aeruginosa* (9±0.39 mm). Compound RML03 showed the fourth most strong activity against *B. subtilis* (10±0.37 mm), *S. aureus* (9.5±0.54 mm), *E. coli* (8.5±0.62 mm), and *P. aeruginosa* (8.5±0.8 mm). Compound RMR02 showed the fifth strong activity against bacterial strains, with zone of inhibition against *B. subtilis* (9±0.23 mm), *S. aureus* (8.5±0.68 mm), *E. coli* (8±0.0 mm), and *P. aeruginosa* (8±0.05 mm) at maximum concentration for all compounds.

The remaining two compounds isolated from root designated as RMR01 and F59 showed relatively weak activity comparative to others as shown in (Table 16). Further comparison was also made with literature reported for the antibacterial activities of this plant. The literature report showed that the methanol leaves extracts of *R. multifidus* and its chloroform and petroleum ether fraction shows strong inhibition against different pathogens such as *S. aureus* DSM7346 (22.00±0.50 mm), *P. aeruginosa* DSM1117 (23.33±0.67 mm), *E. coli* ATCC25722

(21.67± 0.83 mm), and *S. thyphimurium* ATCC 13311 (22.67±0.83 mm), *S. aureus* (23.33±0.33 mm), *P. aeruginosa* (23.00±0.50 mm), *E. coli* (23.00±0.50 mm) and *S. thyphimurium (*23.67± 0.33 mm) and *S. aureus* (26.67±0.83 mm), *P. aeruginosa* (22.67±0.33 mm), *E. coli* (23.00±0.50 mm), *S. thyphimurium (*23.33 ±0.33 mm) at 200 mg/mL concentration with the solvent listed respectively (Begashawu *et al*., 2016). This result was comparable to the current result even though the previous result somehow greater than the current results due to the morphological difference of strain used.

Table 14: Antibacterial activity of *R. multifidus* root crude extracts on selected bacterial strains:

Extracts Conc.(mg/ml)		Zone of inhibition in millimeter (mm)			
		S. aureus	*E. coli*	*P. aeruginosa*	*S. pyrogens*
		Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
n-hexane	200	8.99±1.13	7.11±0.13	-	1.11±0.23
	100	5.86±0.78	5.01±0.03	-	-
	50	4.17±0.26	3.29±0.19	-	-
Dichloromethane	200	7.65±0.42	6.78±0.32	3.83±0.32	2.78±0.21
	100	5.13±0.83	5.64±0.86	-	1.64±0.80
	50	3.49±0.44	3.52±0.21	-	-
Methanol	200	13.40±0.74	10.99±0.6	6.19±0.32	3.9±0.42
	100	8.93±1.12	6.37±0.39	4.15±0.06	1.37±0.52
	50	6.64±0.86	4.65±0.42	-	-
Standard drug	*CIP*	22.28±0.83	24.32±0.2	23.52±0.48	18.81±0.34

Where, CIP stands for *ciprofloxacin* as positive control and the test was done in three replicates.

Table 15: Antibacterial activity of *R. multifidus* aerial fraction on selected bacterial strains:

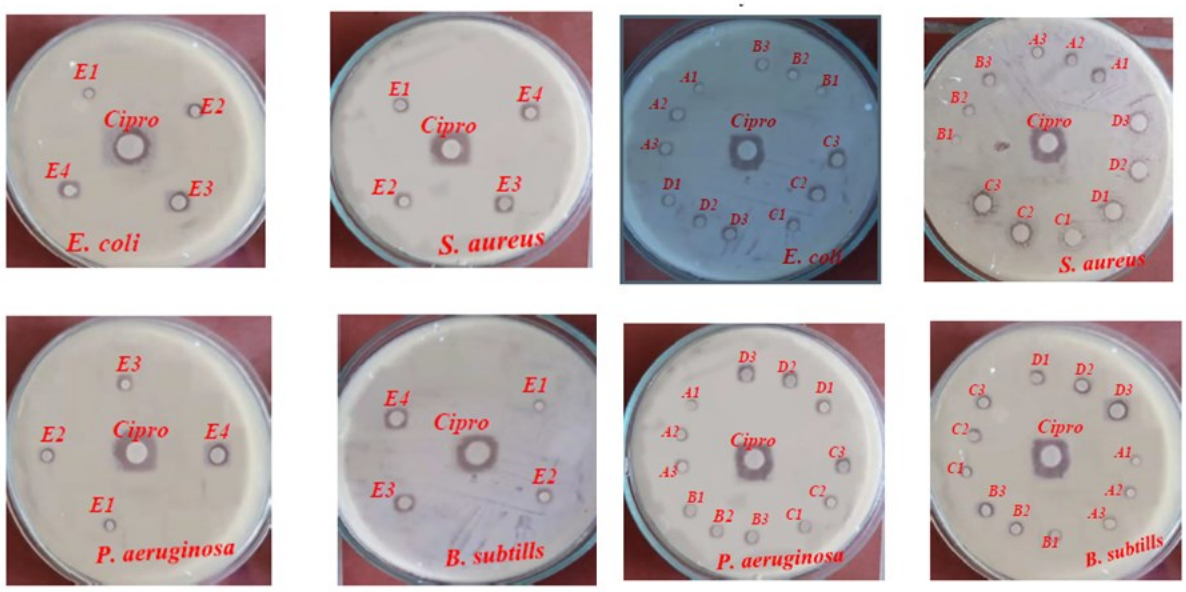
Extracts Conc.(mg/ml)		Zone of inhibition in millimeter (mm)			
		S. aureus	*E. coli*	*P. aeruginosa*	*S. pyrogens*
		Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
n-hexane fraction	200	-	-	-	-
	100	-	-	-	-
	50	-	-	-	-
EtOAc fraction	200	13.34±0.89	11.55±1.1	7.65±0.43	3.85±1.15
	100	8.76±0.5	8.53±0.48	5.72±0.11	2.73±0.81
	50	6.36±1.2	5.34±0.51	2.1±0.5	1.44±0.54
$CHCl_3$ fraction	200	7.13±0.56	6.86±0.28	-	-
	100	5.89±0.17	4.09±0.13	-	-
	50	-	2.76±0.25	-	-
n-BuOH fraction	200	7.13±0.56	6.43±0.61	-	4.3±0.16
	100	6.64±0.11	4.87±0.41	-	2.87±0.81
	50	4.39±0.30	3.91±0.11	-	1.91±0.18
Aqueous fraction	200	9.69±1.23	7.09±0.37	-	-
	100	6.62±0.57	3.75±0.25	-	-
	50	3.73±0.32	-	-	-
Standard drug	CIP	22.28±0.83	24.32±0.61	23.52±0.48	18.81±0.34

Where, CIP stands for *ciprofloxacin* as positive control and the test was done in three replication.

Table 16: Antibacterial activity of *R. multifidus* pure compounds on selected bacterial strains:

Compounds concentration in (µg/mL)			Bacteria Species and Zone of Inhibitions in (mm)			
			E. coli ATCC25922	*S.aureus* ATCC25923	*P. aeruginosa* ATCC27853	*B. subtilis* ATCC6633
RMR01	A1	250	1.5±0.12	2.5±0.17	-	3.5±0.22
	A2	300	2.7±0.23	4.2±0.32	1.75±0.73	5±0.33
	A3	350	3.5±0.52	5.5±0.56	2.5±0.34	6.5±0.41
RMR02	A1	250	7±0.15	7±0.55	6.5±0.33	7.5±0.66
	A2	300	7.5±0.23	8±0.53	7.5±0.18	8.5±0.54
	A3	350	8±0.0	8.5±0.68	8±0.05	9±0.23
RMR08	B1	250	7.5±0.54	8±0.76	7±0.74	8±0.27
	B2	300	8±0.73	9±0.46	8±0.65	9.5±0.90
	B3	350	9.5±1.1	10±0.0	9±0.39	10.5±0.12
F59	A1	250	2.57±0.40	3.5±0.12	1.5±0.32	4.5±0.14
	A2	300	3.75±0.11	5±0.23	2.53±0.13	5.25±0.43
	A3	350	5.15±0.61	6.5±0.52	4.05±0.24	6.82±0.56
RML01	C1	250	8±0.45	8±0.14	7.5±0.41	9±0.22
	C2	300	9±0.25	10±0.85	8.5±1.2	10±0.00
	C3	350	10±0.98	11±0.45	10±0.26	11.5±0.50
RML02	D1	250	8.5±0.13	9±0.44	8.5±0.46	10±0.49
	D2	300	10±0.54	11±0.36	10±0.75	11±0.06
	D3	350	12±0.34	12.5±1.5	11±0.45	13±0.82
RML03	E1	250	6.5±0.67	7±1.09	6.5±0.88	7±0.11
	E2	300	7.5±1.02	8.5±0.77	7±0.56	8.5±0.45
	E3	350	8.5±0.62	9.5±0.54	8.5±0.8	10±0.37
CIP			22.5±0.52	23±0.34	22±0.00	24±0.15

CIP was *ciprofloxacin* used as a positive control and the test was triplicates



Where, A_n, B_n, C_n, D_n and E_n stands for RMR02, RMR08, RML01, RML02, and RMRL03 respectively when ("n" stands for 1,2, and 3).

Figure 15: Disc diffusion antibacterial activity evaluation of the pure compounds against selected strains.

4.5. Antifungal activities of root extract and aerial fractions of *Ranunculus multifidus*

The antifungal activities of extracts and isolated compounds were evaluated for their antifungal effects against two human pathogenic fungal strains *C. albicans* and *A. niger*. The antifungal test was performed using the agar disc diffusion method in the same manner with standard protocol used in similar way as previously described by researchers (Nazir *et al*., 2013). The results obtained in this study revealed that all the studied crude extracts, fractions and isolated compounds presented antifungal activity. The antifungal activities in terms of zone of inhibition in (mm) were measured for the extracts and isolated compounds against selected strains and compared to the standard as shown in (Table 17 and Table 18). Methanol extract from root and ethyl acetate fraction from aerial extract had good inhibitory activity against both fungal strains. n-hexane extract did not have any activity since all the inhibition zones had 6 mm which was the same to the diameter of disc.

The pure compounds designated as RMR02 and RMR08 were isolated from methanol extract of the root shows intermediate inhibitory effects against selected fungal strain with inhibition zone of (5.33±0.58 and 7.33±0.42 mm) against *C. albicans* and (9.12±0.76 and 5.77± 0.68 mm)

for *A. niger* at concentration of 3 mg/ml respectively. In the same manner (RML01, RML02 and RML03) isolated from ethyl acetate fraction showed good inhibitory zone with (14.43±0.40, 11.33±0.58, and 9.93±0.13) mm against *C. albicans* at 3mg/ml and (15.17± 0.76 ,14.67± 0.59, and 16.1±0.27) mm against *A. niger* with the same concentration which was promising result compared to standard *fluconazole* (21±1.5 mm) and *Ketoconazole* (23.43±0.65 mm) at 1mg/ml for *C. albicans* and *A. niger* respectively.

The antifungal activities of the crude and pure isolates mostly target specific components of fungal plasma membrane or targeting sterols the synthesis machinery of cell wall components of fungal cells to combating fungal infections. The synergy among different phytochemicals especially in ethyl acetate fraction was being also projected on its effect against selected fungal strains.

Table 17: Antifungal activity of *R. multifidus* root crude extracts on the selected fungal strains:

Extracts and compounds Conc. (mg/ml)		Zone of inhibition in millimeter(mm)	
		C. Albicans	*A. Niger*
		Mean ±SD	Mean ±SD
n-hexane	100	-	-
	50	-	-
	10	-	-
Dichloromethane	100	6.92±0.45	-
	50	4.21±0.34	-
	10	2.13±0.23	-
Methanol	100	10.42±0.50	5.14±0.67
	50	7.44±0.48	3.10±0.12
	10	4.81±0.21	2.49±0.42
RMR02	3	5.33±0.58	9.12±0.76
	2	-	7±0.0
	1	-	4.53±0.50
RMR08	3	7.33±0.42	5.77±0.68
	2	5±0.0	-
	1	-	-
Standard drug (50µl, 1mg/ml)	*Fluconazole*	21±1.5	
	Ketoconazole		23.43±0.65

Table 18: Antifungal activity of *R. multifidus* aerial fraction on selected fungal strains:

Extracts and compounds Conc. (mg/ml)		Zone of inhibition in millimeter(mm)	
		C. Albicans	*A. Niger*
		Mean ±SD	Mean ±SD
n-hexane fraction	100	-	-
	50	-	-
	10	-	-
EtOAc fraction	100	14.5 ± 1.3	16.93±1.11
	50	7.38 ± 0.54	12.29±0.82
	10	4.46±0.51	6.32±0.71
$CHCl_3$ fraction	100	5.74±0.63	-
	50	2.42±0.40	-
	10	-	-
n-BuOH fraction	100	8.55 ±0.51	-
	50	4.89±0.10	-
	10	-	-
Aqueous fraction	100	10.47±0.50	13.19±1.06
	50	6.02±0.26	8.59±0.55
	10	-	4.09±0.11
RML01	3	14.43±0.40	15.17±0.76
	2	9.77±0.25	8.03±0.15
	1	5±0.20	4.43±0.60
RML02	3	11.33±0.58	14.67±0.59
	2	9.43±0.40	12.43±0.40
	1	7.73±0.25	10.13±0.42
RML03	3	9.93±0.13	16.1±0.27
	2	7.93±0.60	14.17±0.76
	1	5.53±0.42	10.1±0.85
Standard drug	Fluconazole	21 ±1.5	
	Ketoconazole		23.43±0.65

In all case the experiment done in triplicates.

Even though there was lack of literature previously reported from the plant on antifungal activities, further comparison was also made with literature reported for the compounds isolated from the genus. The anemonin isolated from the ethyl acetate fraction from *R. muricatus* was reported to have the inhibition effects against *A. niger* (Nazir *et al*., 2013). This compounds also isolated from the leaves of *R.multifidus* and reported to have antimalaria and anti-leishmaniasis activities (Sirak *et al.*, 2021).

In the same scenario the aqueous extracts of aerial parts from *R. arvensis* from the genus showed very optimal inhibition zone against *C.albicans* (21 mm) and *A.niger* (16 mm) reported before which support the current result of this paper (Hachelaf *et al*., 2013).

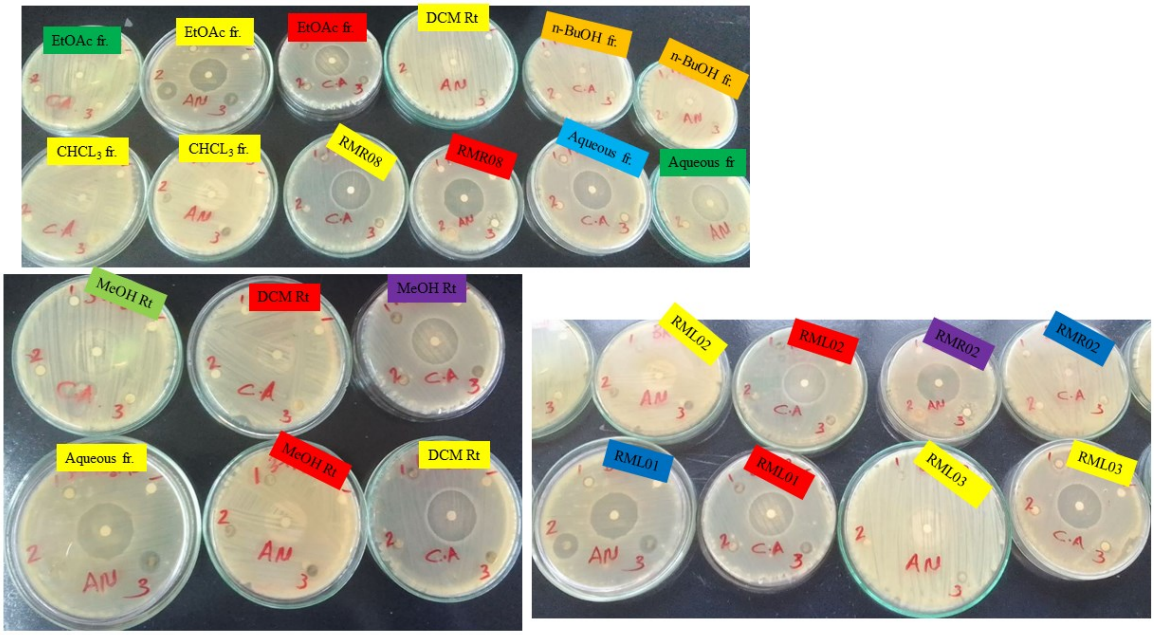


Figure 16: Disc diffusion antifungal activity evaluation of the crude (root and aerial fraction) extracts and isolated compounds against *Aspergillus niger* (AN), and *Candida albicans* (CA).

4.6. DPPH radical scavenging assay:

The DPPH assay was used to assess the free radical scavenging activity of the crude extract and isolated pure compounds. DPPH antioxidant assay is based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and for a visible deep purple color. A decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the extracts or compounds under investigation. *In vitro,* antioxidant assay of crude extracts of *R. multifidus* revealed the presence of antioxidant potential. The percentage of inhibition was observed in all the antioxidant models that free radicals were scavenged by the crude and pure isolated compounds in a concentration-dependent manner up to the given concentration. The data were compared to those obtained with the reference compound L-ascorbic acid.

The relationship between different crude extracts and isolated pure compounds from the root and aerial extracts and their scavenging activity for the DPPH radical was shown in (Table 19) and (Table 20) respectively. All of the investigated crude showed moderate to high radical scavenging activities compared with absorbance of the control (0.9118).

The DPPH radical-scavenging activities at 100 µg/mL were 97.57%, 71.95%, 38.45% and 98. 59% for MeOH, n-hexane, DCM and ascorbic acid respectively. From the result we conclude that methanol extracts have comparable antioxidant effect which could be attributed to its hydrogen-donating ability. In the reverse the DCM crude extracts shows insignificant radical scavenging ability compared to standard and n-hexane shows moderate antioxidant tendency.

Table 19: % Radical scavenging activity of the root crude extracts of *Ranunculus multifidus*

Conc. (µg/m)	Absorbance				DPPH % inhibition			
	n-hexane	DCM	MeOH	AA	n-hexane	DCM	MeOH	AA
12.5	0.29±0.012	0.71±0.08	0.22±0.04	0.02±0.002	68.24	22.25	75.99	98.44
25	0.28±0.015	0.68±0.060	0.14±0.002	0.01±0.003	69.53	22.45	84.39	98.49
50	0.26±0.002	0.64±0.05	0.06±0.002	0.013±0.010	71.69	30.02	93.57	98.54
100	0.25±0.039	0.56±0.096	0.02±0.006	0.012±0.001	71.95	38.45	97.57	98.59
Control						0.9118		

Where, DCM=Dichloromethane, MeOH= Methanol, A= Absorbance, AA=Ascorbic acid and %RSA= Radical scavenging activity, and the results were expressed as mean ±standard deviation.

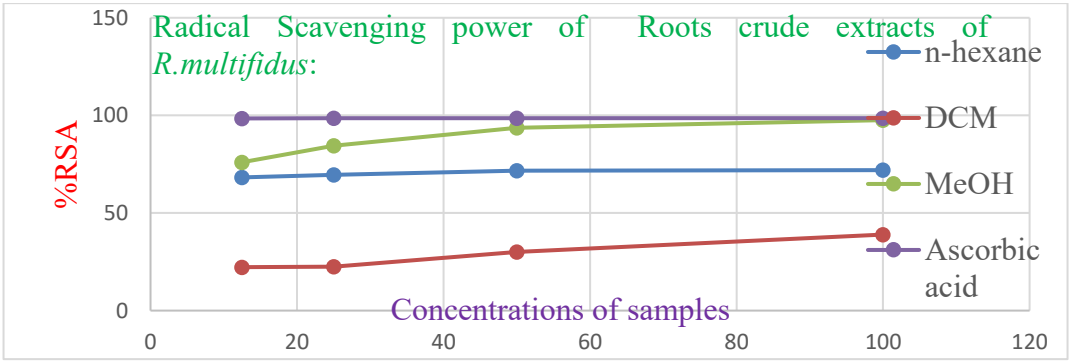


Figure 17: DPPH scavenging activity (%) of the root crudes extracts and the positive reference (L-Ascorbic acid).

Table 20: % Radical scavenging activity of the aerial fraction of methanolic extract of *Ranunculus multifidus*:

Conc.(µg/ml)	Absorbance					DPPH % inhibition				
	n-hexane fraction	EtOAc fraction	$CHCl_3$ Fraction	n-BuOH fraction	Aqueous fraction	n-hexane fraction	EtOAc Fraction	$CHCl_3$ Fraction	n-BuOH fraction	Aqueous fraction
12.5	0.95±0.001	0.17±0.031	0.77±0.026	0.16±0.003	0.70±0.002	-3.75	81.03	15.44	82.56	23.67
25	0.91±0.012	0.17±0.044	0.76±0.029	0.12±0.004	0.60±0.003	0.86	81.47	16.21	86.95	34.42
50	0.90±0.001	0.13±0.003	0.74±0.007	0.11±0.009	0.51±0.065	1.40	85.53	18.62	88.37	43.96
100	0.83±0.012	0.08±0.034	0.48±0.009	0.05±0.002	0.23±0.01	8.64	90.79	47.45	94.19	74.99
Control	0.9118									

Where, EtOAc = Ethyl acetate, n-BuOH = n-Butanol, $CHCl_3$ = Chloroform, and the results were expressed in mean ±standard deviation and the test was done in triplicates.

All of the investigated sample showed low to high radical scavenging activities compared with the absorbance of the control (0.9118). The scavenging activity of extracts could be attributed to their hydrogen-donating ability. To compare the result of inhibition of fractions the n-butanol (94.187%) and ethyl acetate (90.787%) fraction shows a promising and comparable radical scavenging power than others fraction relative to ascorbic acid (98.59%) at maximum concentration (100 µg/mL). The aqueous fraction also exhibited moderate hydrogen donating ability at 100 µg/mL with percentage inhibition of 74.99%. One the other way the n-hexane fraction shows almost very insignificant inhibition ability which might infer the fraction lacks polyphenolic groups in its constituents to donate a single hydrogen to snatch the radical and break chain reaction.

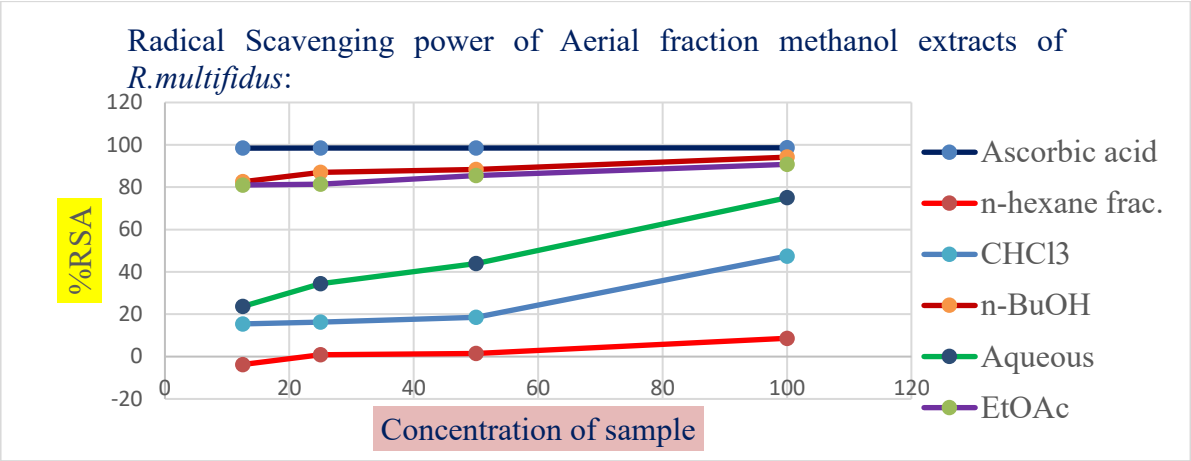


Figure 18: DPPH scavenging activity (%) of the aerial crudes extracts and the positive reference (L-Ascorbic acid)

The radical scavenging power of pure compounds was also investigated and the result revealed that the pure isolate has moderate antioxidant potential, but the pure compounds were showed less anti-radical activities compared to the roots crude and aerial fraction. This might be attributed due to the synergistic effects of phytochemical in crude extracts and might be the effects of storage condition like temperature, moisture contents and duration of storage since phenolic compounds responsible for scavenging radical are not totally stable and easily degraded during storage (Laher *et al.*, 2013; Zhang *et al*., 2021). Over all the antioxidant effects of all pure compounds were comparable to each other as depicted in (Table 21). To compare the antioxidant potential of compounds isolated from root and from ethyl acetate fraction of aerial extract, the ethyl acetate isolate was somewhat greater than from compounds isolated from methanol extract, but in all case the result was concentration dependent.

Table 21: % Radical scavenging activity of the pure compounds isolated methanol crude and ethyl acetate fraction of *R. multifidus:*

Conc. (µg/ml)	Absorbance						DPPH % inhibition					
	RML01	RML02	RML03	RMR01	RMR02	RMR08	RML01	RML02	RML03	RMR01	RMR02	RMR08
12.5	0.179	0.172	0.165	0.27	0.25	0.16	80.37	81.14	81.9	70.39	72.58	82.45
25	0.154	0.148	0.13	0.24	0.23	0.12	83.11	83.78	85.74	73.68	74.78	86.84
50	0.128	0.113	0.104	0.21	0.19	0.10	85.96	87.61	88.59	76.97	79.16	89.03
100	0.110	0.092	0.09	0.19	0.15	0.091	87.94	89.91	90.13	79.16	83.55	90.02
Control					0.9118							

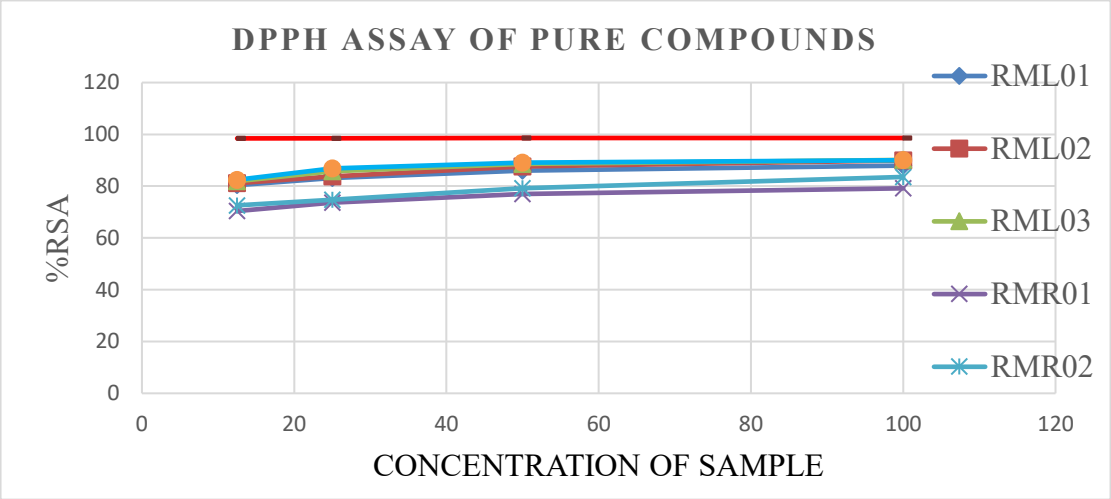


Figure 19: DPPH scavenging activity (%) of the compounds relative to the positive reference (L-Ascorbic acid).

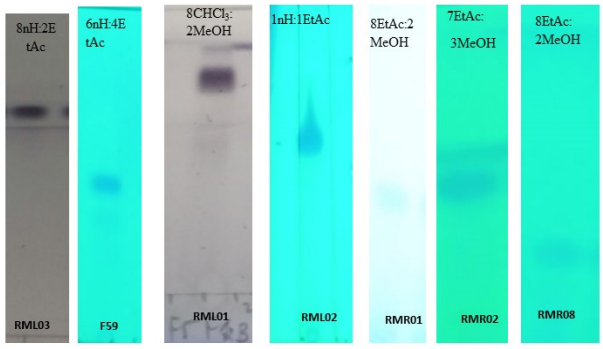


Figure 20: The TLC profiles of isolated compounds from root extract and aerial fractions of *R.multifidus.*

4.7. *In silico* molecular docking study

Molecular docking is generally employed to investigate the binding energy and to further validate the molecular mechanisms of ligands at the active site of a protein. To understand the binding mode of the ligands, the selected compounds were subjected to molecular docking against selected proteins using *S. aureus* tyrosyl-tRNA synthetase (PDB ID: 1JIJ) and human peroxiredoxin 5 (PDB ID:1HD2) using Auto-dock vina 4.2.6. A novel family of anti-oxidative proteins, designated as peroxiredoxin (PRDX), has been identified in the past two decades and currently comprises six members in mammals. Organisms living under aerobic conditions have developed to protect them from damage by reactive oxygen species (ROS) (Fujii *et al*., 2013). Among the peroxiredoxin family peroxiredoxin 5 is the most recent antioxidant drug targets in pharmaceuticals drug industry.

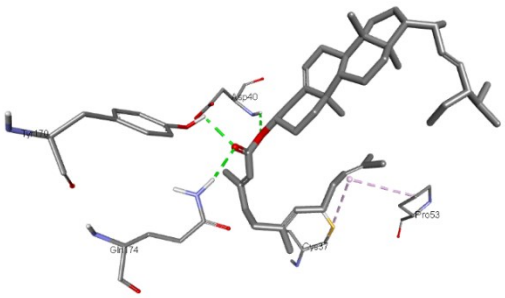
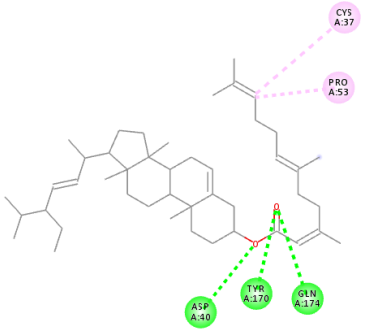
In order to support the experimental data and to understand the possible mechanism behind the antioxidant activity, molecular docking was done using human peroxiredoxin 5 enzymes (PDB: 1HD2), which has a broader activity against ROS and was mostly involved in stress protection mechanisms and cell differentiation. In this study, the molecular docking analysis of the isolated compounds RMR02, RMR08, RML01, RML02, and RML03 was carried out to investigate their binding interaction within the binding sites of human peroxiredoxin 5 and the results were compared with standard antioxidant ascorbic acid.

Staphylococcus aureus (S. aureus) can cause severe blood stream infection, endocarditis and skin infection with an annual incidence of twenty-six cases per one hundred thousand people and approximately 30% of them are lethal (Farshadfar *et al*., 2020). Among the different and diverse resistance mechanisms, *S. aureus* enzyme tyrosyl-tRNA synthetase (YRS) was selected as emerging target to be employed in this study (Farshadfar *et al*., 2020). *Ciprofloxacin* (CPFX) was utilized as a reference drug for docking studies. Binding affinity and interactions with different amino acids were presented in the (Table 22), and 3D and 2D binding interactions was depicted in (Figure 21). The result revealed compound RML03 had the highest binding affinity with the target (-9.8 kcal/mol), and RML01 showed comparable binding affinity (-8.7 kcal/mol) with that of standard drug, CPFX (-8.8 kcal/mol). CPFX exhibited hydrogen bonding interaction with Gln-174, Tyr-170, Tyr-36, Gln-190, Lys-84. Similarly, all the test compounds showed hydrogen bonding interaction with Gln-174, Tyr-170. Whereas, only RML02 showed similar

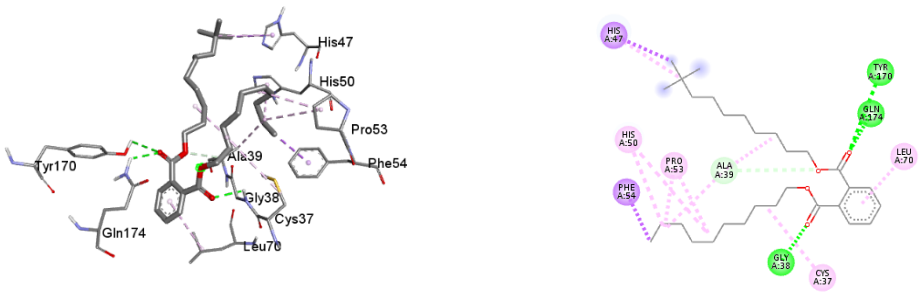
hydrophobic interaction like CPFX with Ala-39 and His-50. This molecular docking result was in correlation with the *in vitro* antibacterial activities.

Table 22: Binding affinity and amino acid interactions of test compounds and standard compounds with *S. aureus* tyrosyl-tRNA synthetase (PDB ID: 1JIJ).

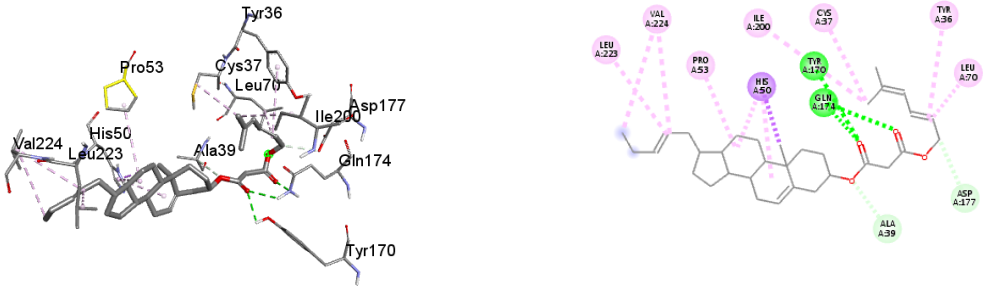
Compounds	Affinity (kcal/mol)	H-bond interactions	Residual Amino acid Interactions	
			Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions	Van-der Walls interactions
RML01	-8.7	Gln-174, Tyr-170, Asp-40	Cys-37, Pro-53	-
RML02	-6.8	Gln-174, Tyr-170, Gly-38	Ala-39, Leu-70, Cys-37, Pro-53, His-50, Phe-54, His-47	Ala-39
RML03	-9.8	Tyr-170, Gln-174	Leu-70, Tyr-36, Cys-37, Ile-200, Pro-53, Val-224, Leu-223, His-50	Ala-39, Asp-177
RMR02	-5.8	Gln-174, Tyr-170	Leu-70, Tyr-36, Cys-37	-
RMR08	-5.8	Gln-174, Tyr-170	TYR-36, Cys-37	-
Ciprofloxacin	-8.8	Tyr-36, Gln-190, Gln-174, Tyr-170, Lys-84	His-50, Ala-39, Asp-40	-

Compounds	**3D**	**2D**
RML01		

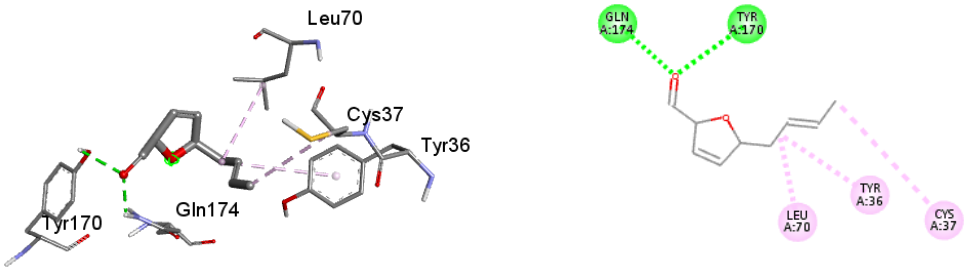
RML02



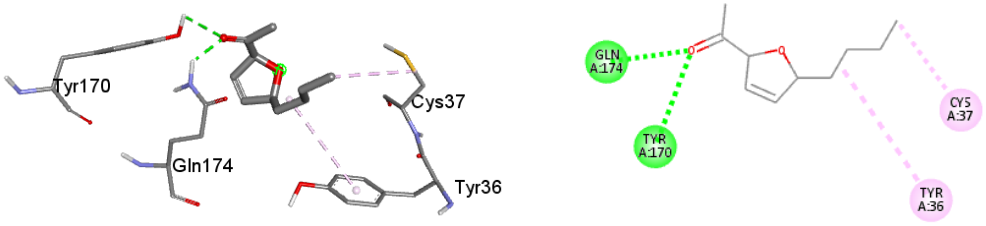
RML03



RMR02



RMR08



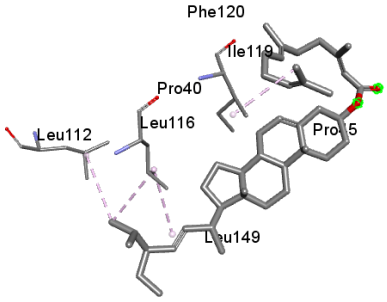
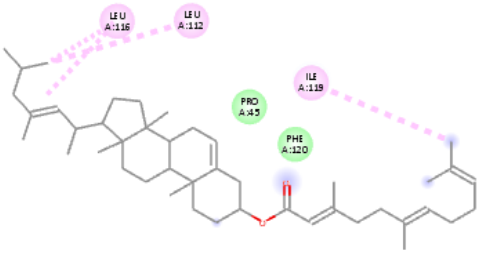
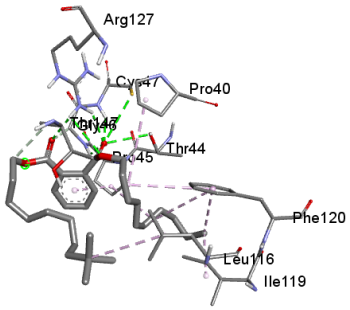
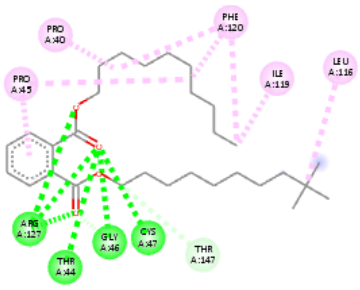
CPFX

Figure 21: Binding interactions (3D & 2D) of test compounds and standard compounds with *S. aureus* tyrosyl-tRNA synthetase (PDB ID: 1JIJ). Deep pink color shows pi-sigma interaction and faint color represent Vander Waals interaction

Human peroxiredoxin 5 (PRDX5) a member of the peroxiredoxin family of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydroperoxides. The encoded protein interacts with peroxisome receptor 1 and plays an antioxidant protective role in different tissues under normal conditions and during inflammatory processes (Declercq *et al*., 2001). Ascorbic acid (AA) was utilized as a reference drug for docking with PRDX5. Binding affinity and interactions with different amino acids are presented in the (Table 23), and 3D and 2D binding interactions was depicted in (Figure 22). Among the test compounds, RML01 and RML03 showed highest binding affinities -6.2 and -5.9 kcal/mol, respectively. Whereas, other test compounds showed comparable binding affinities with that of ascorbic acid (-4.5 kcal/mol). Compounds, RML02, RMR02 and RMR08 exhibited exactly similar hydrogen bonding interaction (Arg-127, Cys-47, Gly-46, Thr-44) with that of standard. Whereas, RML03 with Gly-46 and RML01 showed no hydrogen bonding interactions. The test compounds were showed hydrophobic interactions with Ile-119 and Leu-116, except RMR08 and standard compound didn't show any hydrophobic interactions. This result was in correlation with *in vitro* antioxidant of the compounds as depicted in (Table 21) and the result gives a clue for further works to find a lead compound for antioxidant activities. To compare the *Insilco* molecular docking with the previous report, even though it was the first work on the isolates from this plant the previously isolated compound anemonin showed the significant interaction with the thiol group of the precursor amino acid L-cysteine, along with the cysteine residue of glutathione (GSH). Most of the compounds isolated were showed the interaction to this residual amino acid in both selected human and bacterial protein which indicates the rationale behind using the plant for treatment of parasite infection and inflammation in traditional ways.

Table 23: Binding affinity and amino acid interactions of test compounds and standard compounds with human peroxiredoxin 5 (PDB ID: 1HD2).

Compounds	Affinity (kcal/mol)	H-bond interactions	Residual Amino acid Interactions	
			Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions	Van-der Walls interactions
RML01	-6.2	-	Ile-119, Leu-116, Leu-112	Pro-45, Phe-120
RML02	-4.4	Arg-127, Thr-44, Gly-46, Cys-47	Ile-119, Leu-116, Phe-120, Pro-120, Pro-40, Pro-45,	Thr-147
RML03	-5.9	Gly-46	Ile-119, Phe-120	-
RMR02	-4.2	Gly-46, Arg-127, Thr-44, Cys-47	Ile-119, Leu-116	-
RMR08	-4.4	Cys-47, Thr-44, Gly-46, Arg-127	-	-
Ascorbic acid	-4.5	Arg-127, Cys-47, Gly-46, Thr-44	-	-

Compds	**3D**	**2D**
RML01		
RML02		

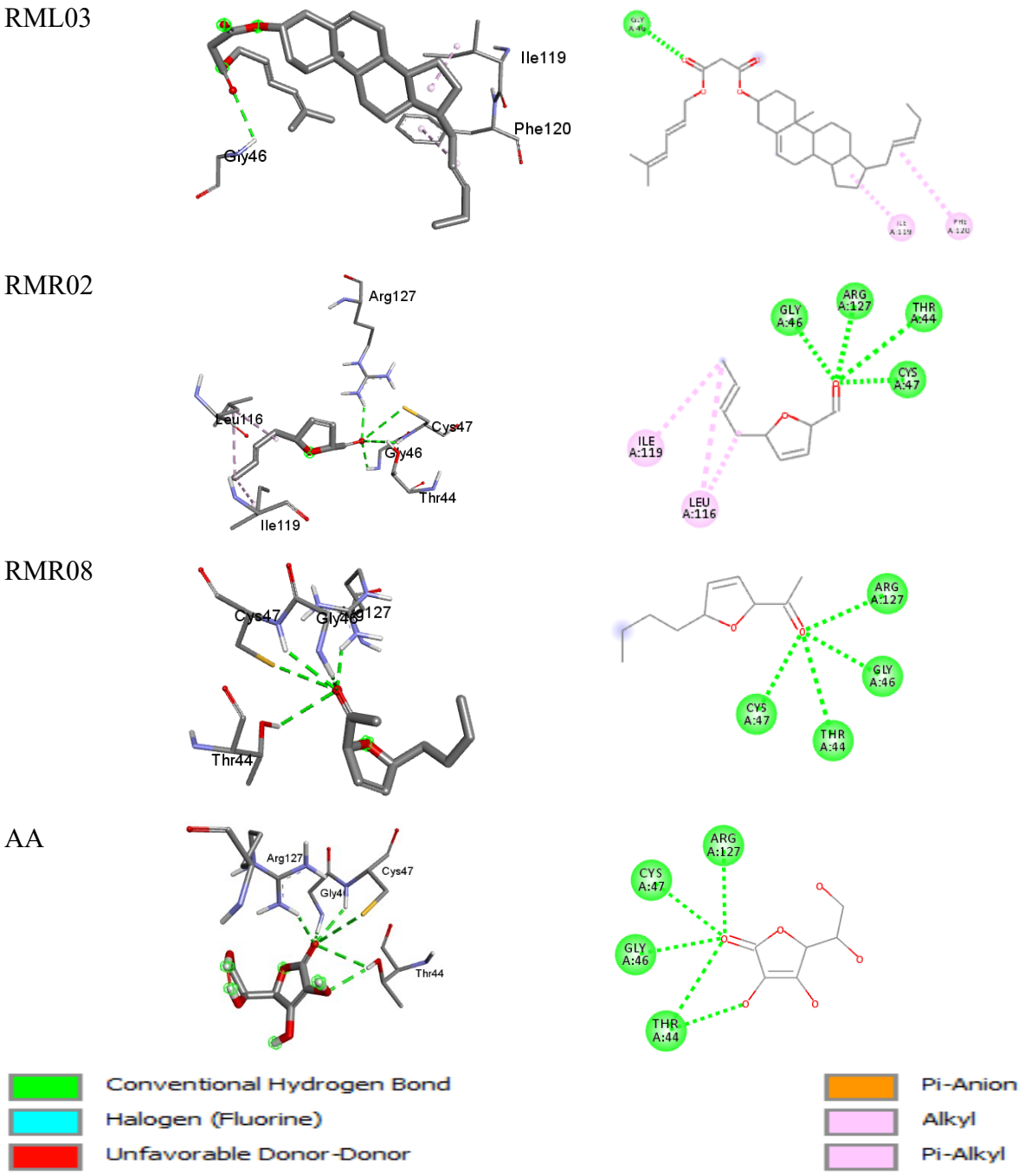


Figure 22: Binding interactions (3D & 2D) of test compounds and standard compounds with human peroxiredoxin 5 (PDB ID: 1HD2). Deep pink color shows pi-sigma interaction and faint color represent Vander Walls interaction.

CHAPTER 5

5. CONCLUSIONS AND RECOMMENDATION

5.1. Conclusions

Traditional medicines have been used and continue to be used as an alternative approach on treatment for various diseases caused by bacteria, fungi, viruses, protozoa, and helminths for a long decade. The growing interest of peoples in substances of natural origin especially from medicinal plant is association with the increasing concern surrounding potentially harmful infectious disease. *R. multifidus* is one of these medicinal plants used traditionally to heal various infectious diseases in Ethiopia. In this study, the phytochemical screening tests showed that both root extracts and aerial fractions of the plants were rich in flavonoids, saponins, phenols, tannins, terpenoids, steroids etc. Chromatographic isolation of the root crude extracts furnished compounds coded as RMR01, RMR02, RMR08 and F59 whereas, the ethyl acetate fractions resulted RML01, RML02 and RML03. The extracts and isolated compounds were evaluated *in vitro* antibacterial, antifungal and antioxidant activities. The results revealed that RML02 has maximum zone of inhibition with (13±0.82 mm) against *B. subtilis*, and RML01 showed strong activity against *B. subtilis* (11.5±0.5 mm), and *S. aureus* (11±0.45 mm), compared to standard ciprofloxacin (24±0.15 mm and 23±0.34 mm). The antifungal result of (RML01, RML02 and RML03 with inhibitory zone of (14.43±0.40, 11.33±0.58 and 9.93±0.13 mm) against *C. albicans* and (15.17±0.76, 14.67±0.59, and 16.1±0.27 mm) against *A. niger* comparable to standard fluconazole (21±1.5 mm) and ketoconazole (23.43±0.65 mm) at 1mg/ml for *C. albicans* and *A. niger* respectively. The radical scavenging activities of pure compounds were promising with percentage activities of RMR08 (90.02 %), and RML03 (90.13 %), which was the strongest inhibition values compared to standard. The *in silico* molecular docking study result revealed that compound RML03 had the highest binding affinity with the target (-9.8 kcal /mol), and RML01 showed comparable binding affinity (-8.7 kcal/mol) with standard drug, CPFX (-8.8 kcal/mol). The *Insilico* antioxidant result of RML01 and RML03 showed highest binding affinities -6.2 and -5.9 kcal/mol, respectively relative, to ascorbic acid (-4.5 kcal/ mol). This result was in correlation with *in vitro* antioxidant activities of the compounds and the result gives a clues for further works to find a lead compound for antioxidant activities. The present finding in general brings the attention of experts to look more on the medicinal importance of this plant.

5.2. Recommendation

Based on the present study the following further works were recommended:

- Isolation of more bioactive compounds should be carried out especially from n-butanol fraction strongly recommended due to good %RSA result using advanced chromatographic techniques such as LC-MS and HPLC.
- Additional research efforts are also recommended to conduct "acid-base shake out" extraction techniques to obtain spectacular bioactive alkaloids from this plant.
- Determining minimum inhibitory concentration (MIC) of the crude extracts and isolated compounds should be conducted in addition to antimicrobial test using disc diffusion method.
- The antibacterial activity was carried out on four strains (*E. coli, P. aeruginosa, S. pyogenes/B.subtilis* and *S. aureus*), and antifungal activity was carried out on two strains (*C. albicans and A. niger*) therefore the isolated compounds should be done using more strains.
- Additional research efforts are also recommended to conduct structure-activity relationships, to have a better understanding of structural and chemical parameters required to develop related drug lead compounds.
- More spectroscopic techniques, such as 2D NMR and HR-MS should be used to confirm the structures of isolated compounds.
- It is necessary to evaluate anticancer, antinociceptive and anti-inflammatory activities of these isolated compounds.
- The *Insilco* pharmacokinetic parameters such as drug-likeness and ADMET profile of isolated compounds using (Swiss ADME and Po-Tox-II) was recommended.
- DFT analysis of isolated compound should be done using computational methods to know reactivity, stability and the energy of compounds to obtain a stable structure with minimum energy.

Research fund acknowledgement

This research thesis was funded by Adama science and technology university under the grant number of ASTU/SM-R/457/22, Adama, Ethiopia.

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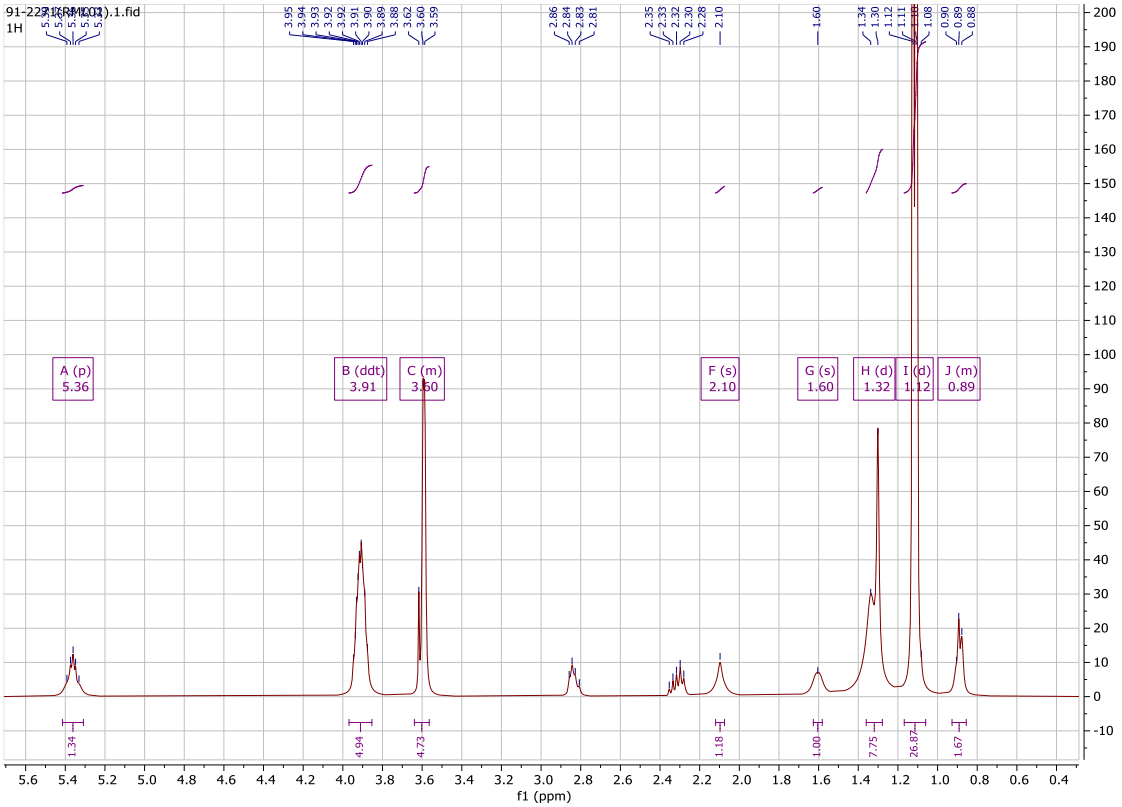
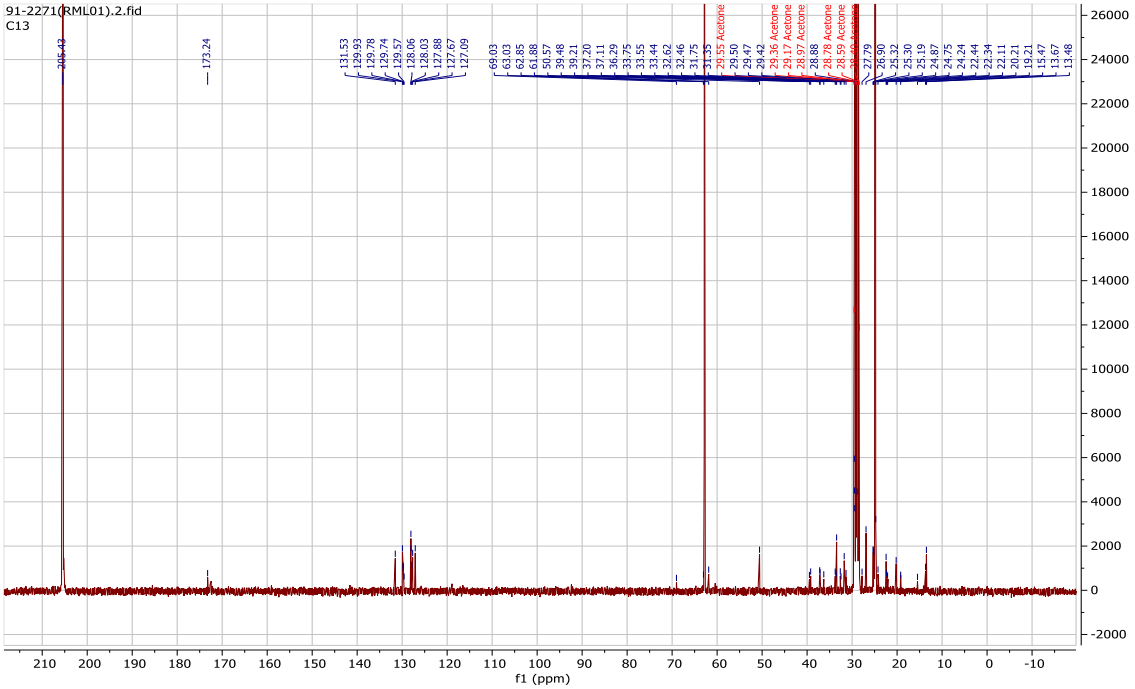
APPENDICES

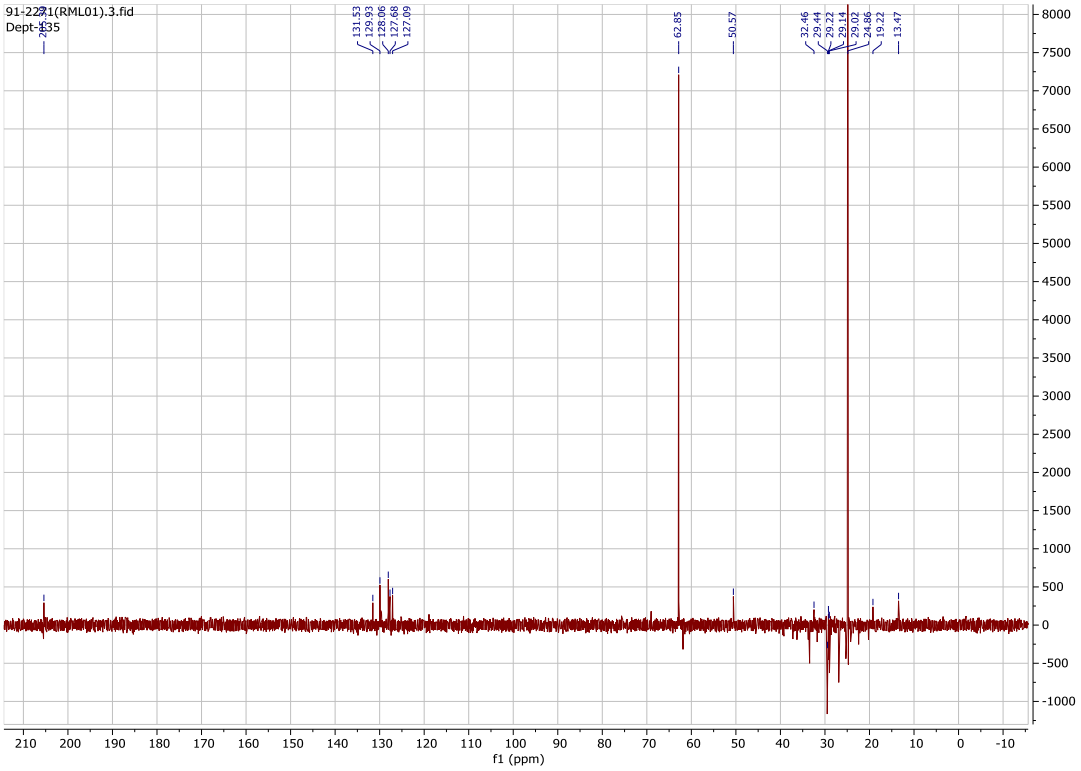
Appendix 1: ^{1}H-NMR (400 MHz, Acetone) spectrum of compound RML01



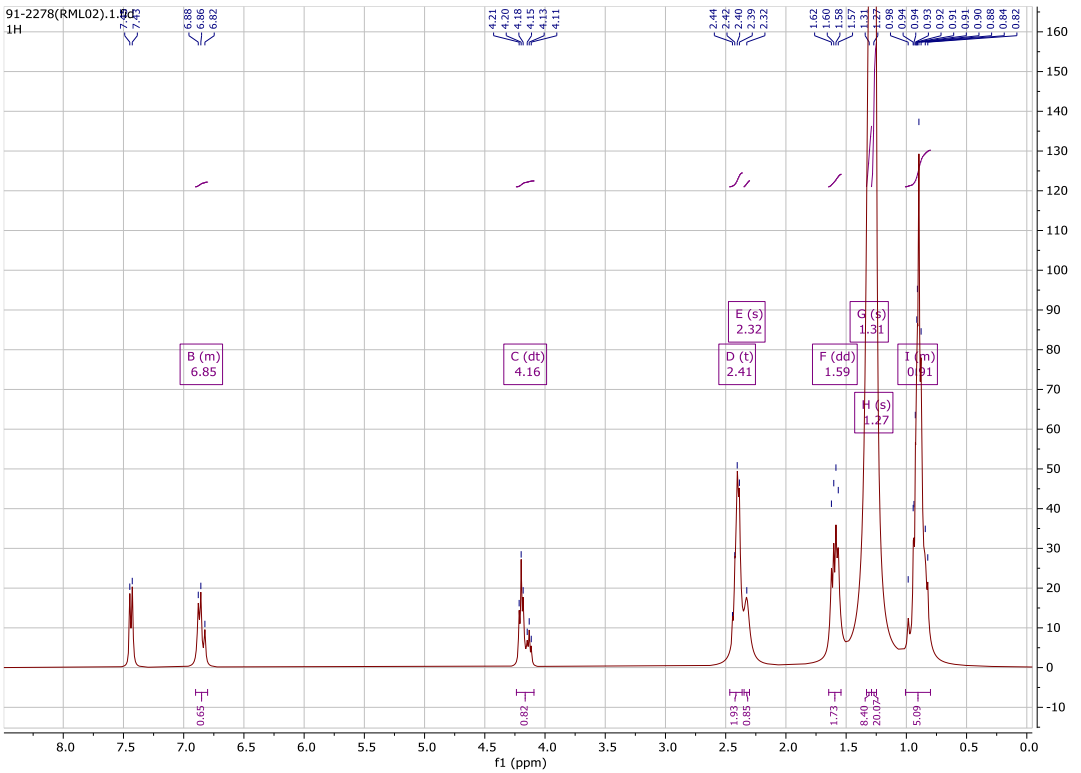
Appendix 2: ^{13}C-NMR (101 MHz, Acetone) spectrum of compound RML01



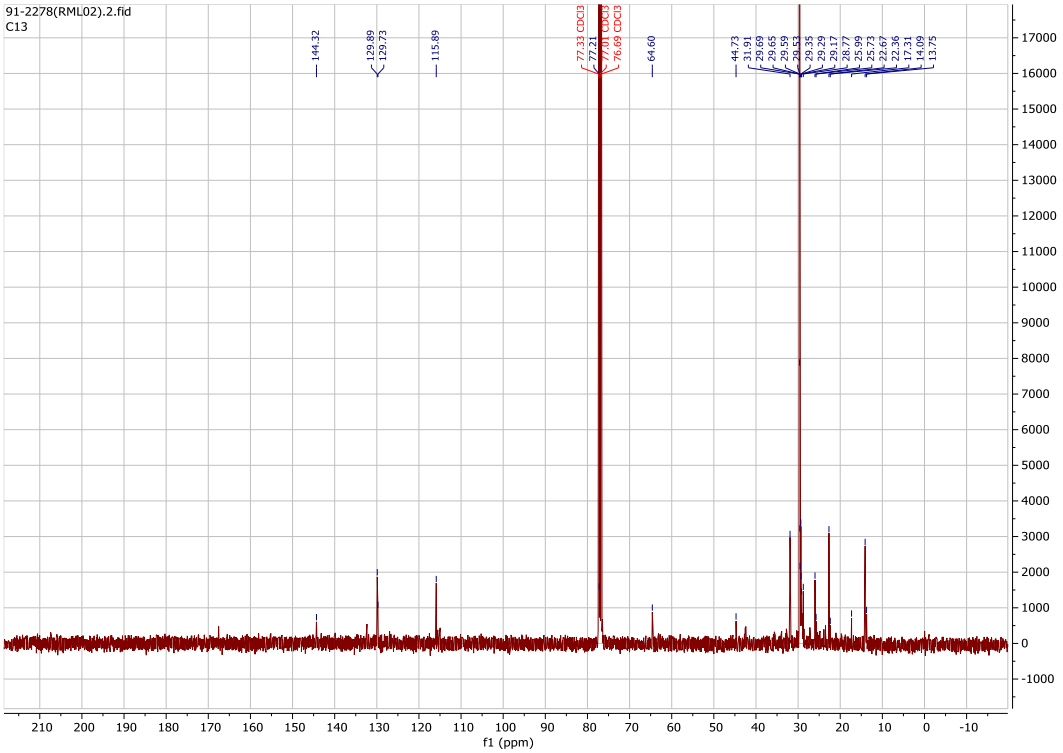
Appendix 3: DEPT-135 (101 MHz, Acetone) spectrum of compound RML01



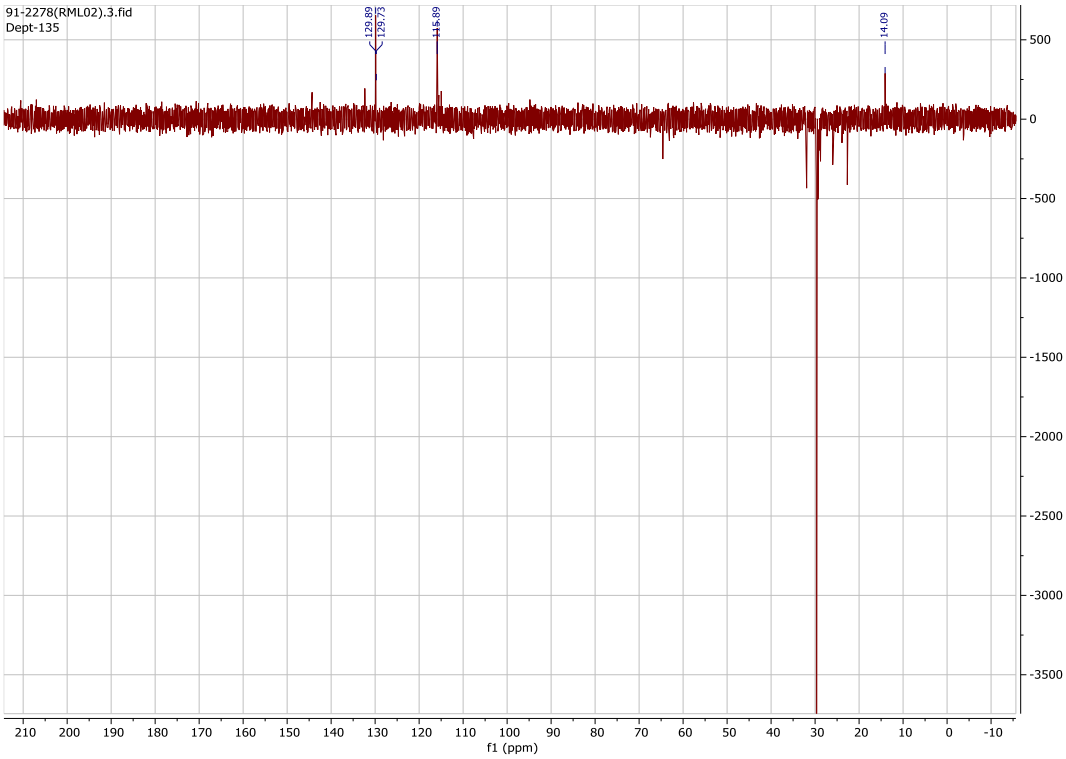
Appendix 4: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of compound RML02



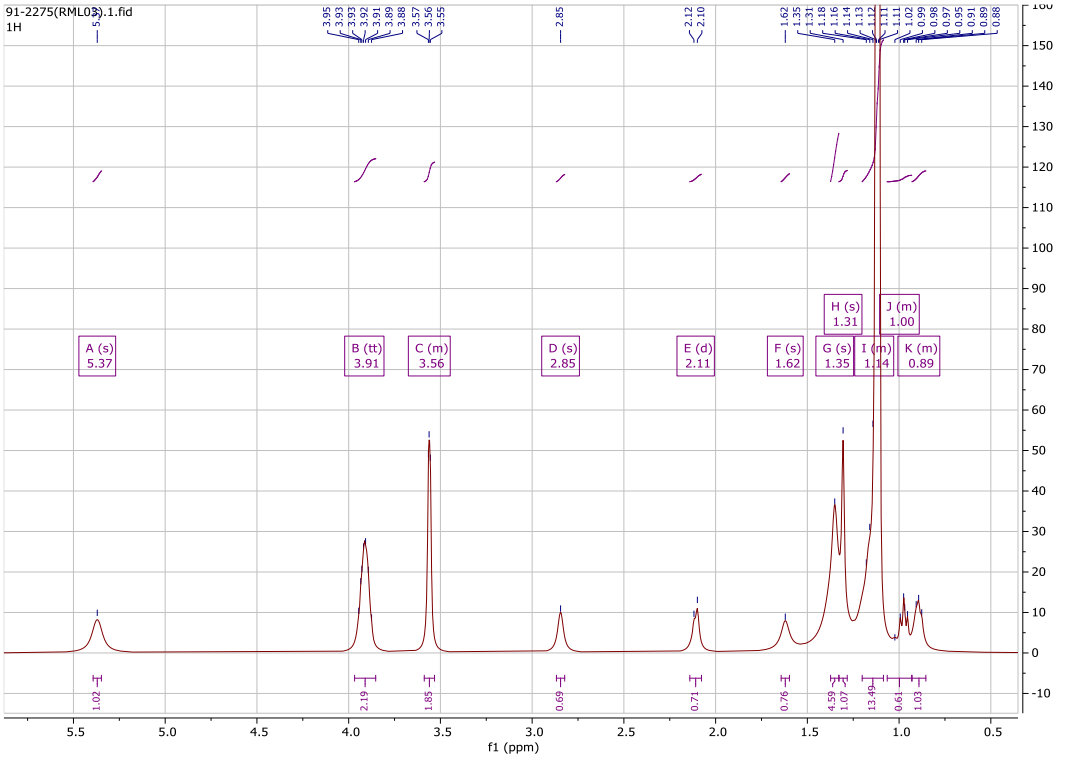
Appendix 5: ^{13}C-NMR (101 MHz, $CDCl_3$) spectrum of compound RML02



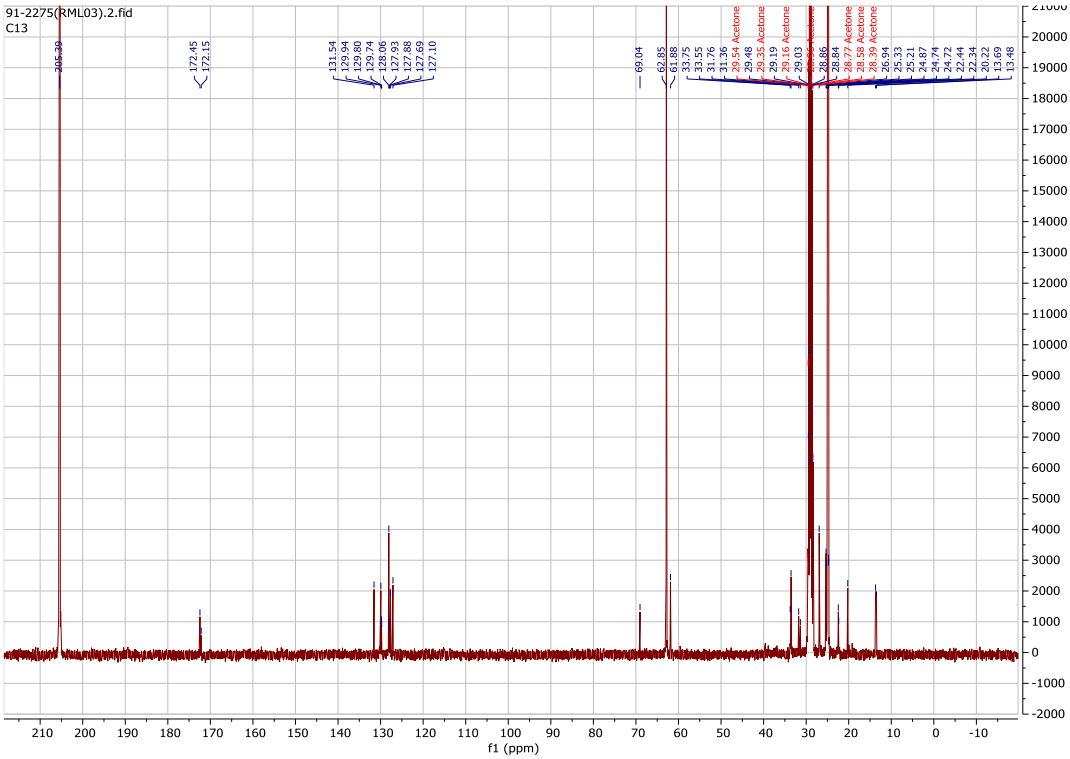
Appendix 6: DEPT-135 (101 MHz, $CDCl_3$) spectrum of compound RML02



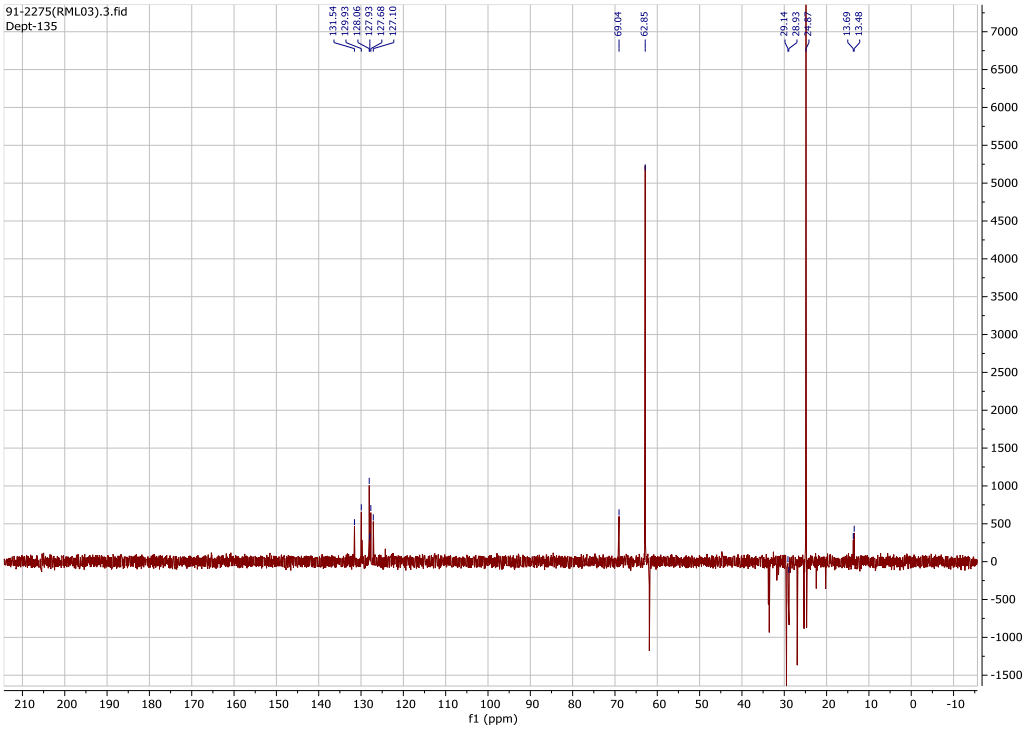
Appendix 7: ^{1}H-NMR (400 MHz, Acetone) spectrum of compound RML03



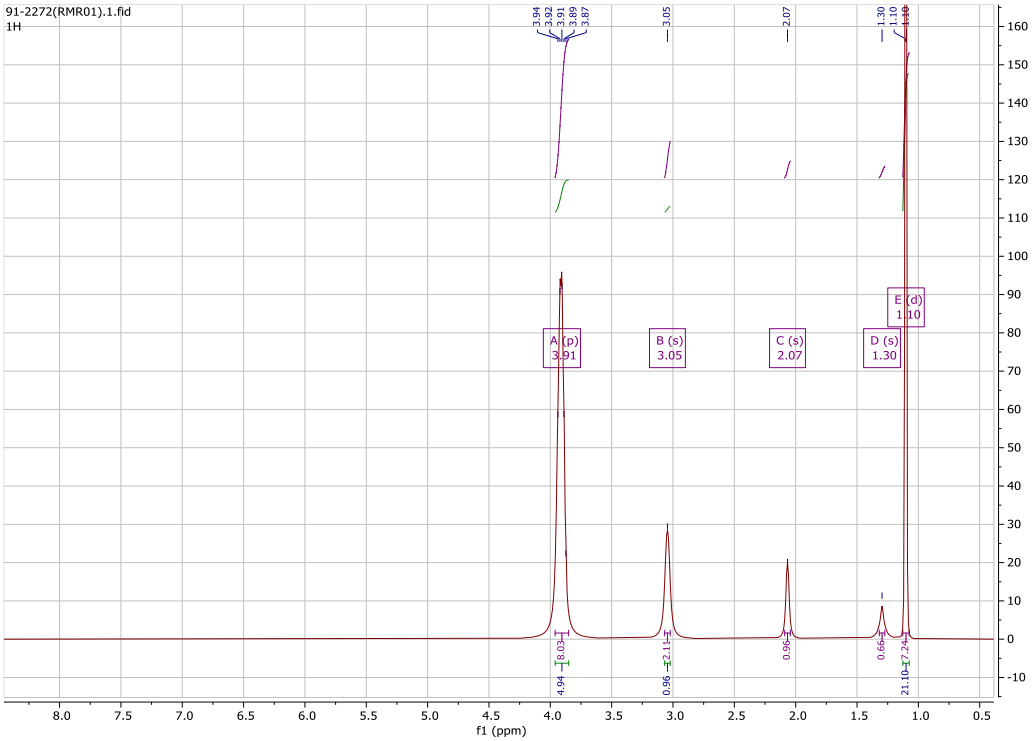
Appendix 8: ^{13}C-NMR (101 MHz, Acetone) spectrum of compound RML03



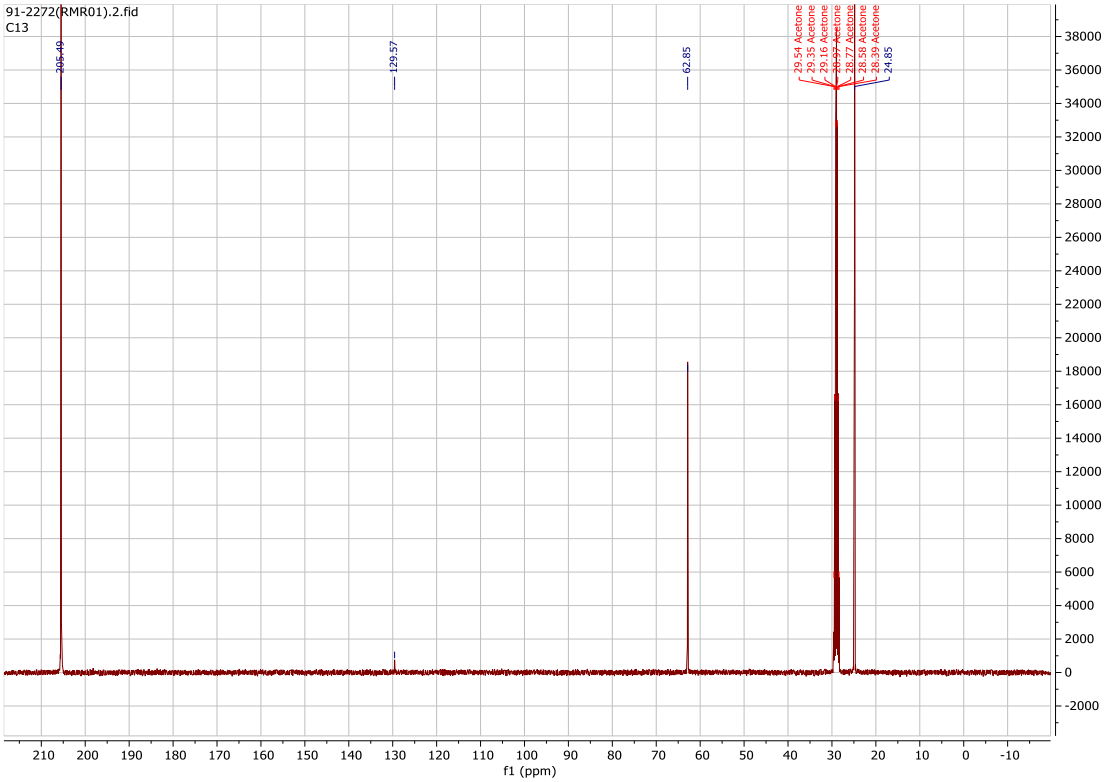
Appendix 9: DEPT-135 (101 MHz, Acetone) spectrum of compound RML03



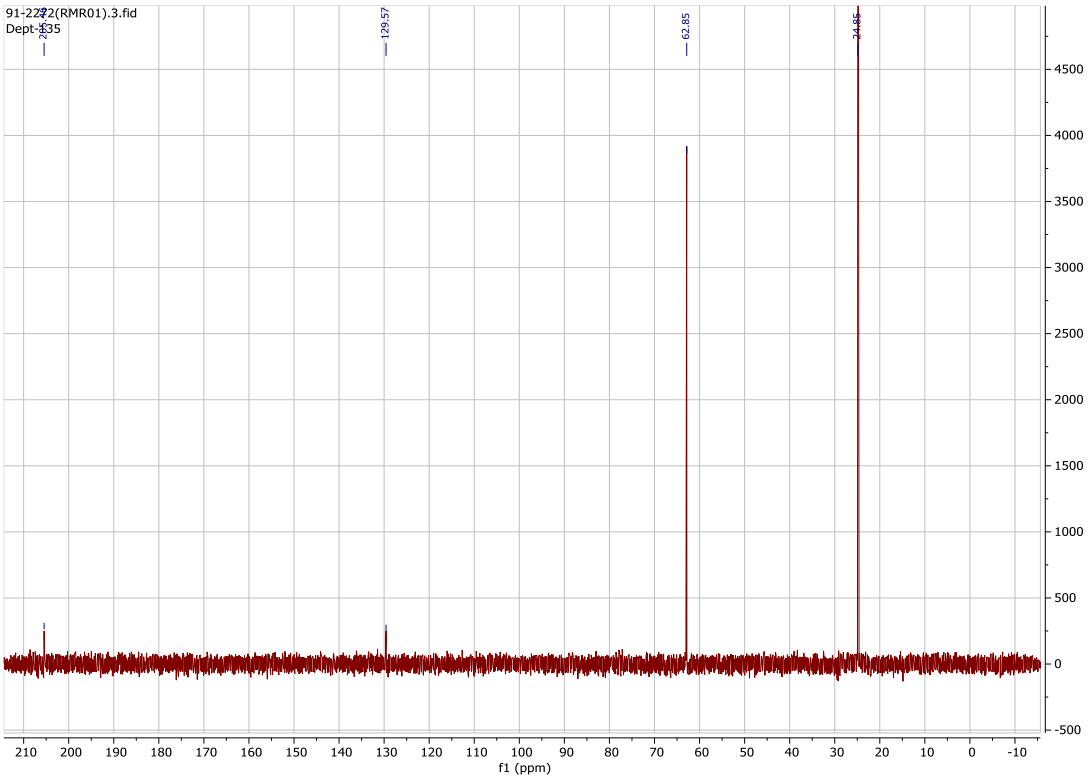
Appendix 10: ^{1}H-NMR (400 MHz, Acetone) spectrum of compound RMR01



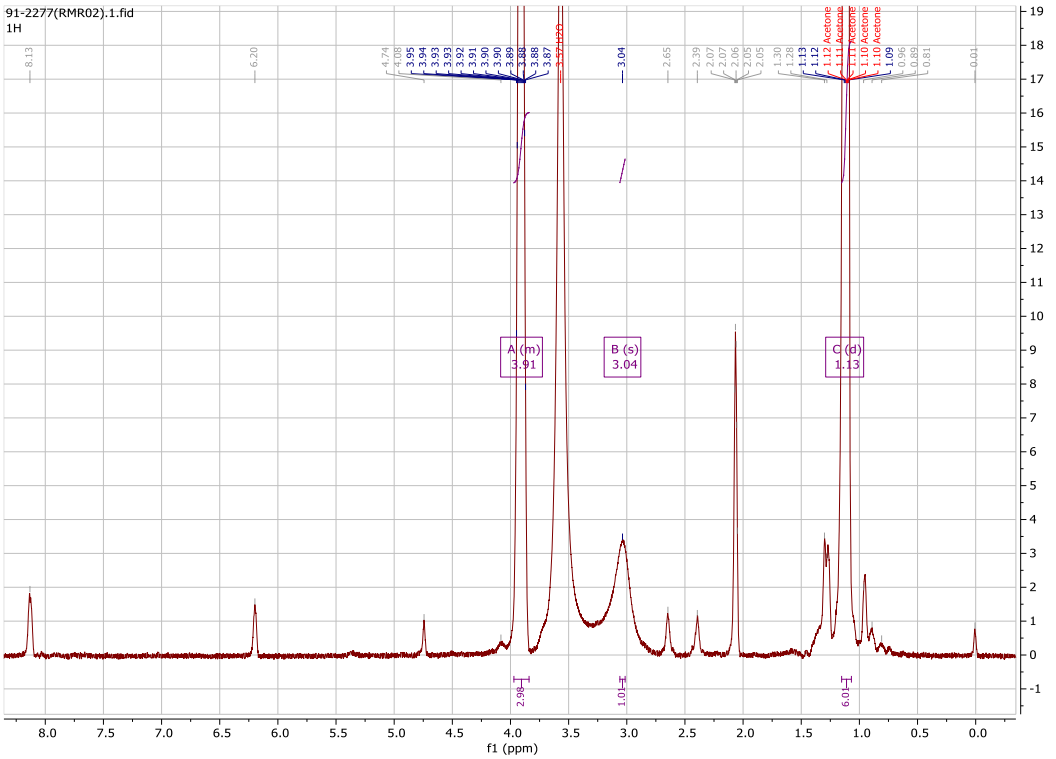
Appendix 11: ^{13}C-NMR (101 MHz, Acetone) spectrum of compound RMR01



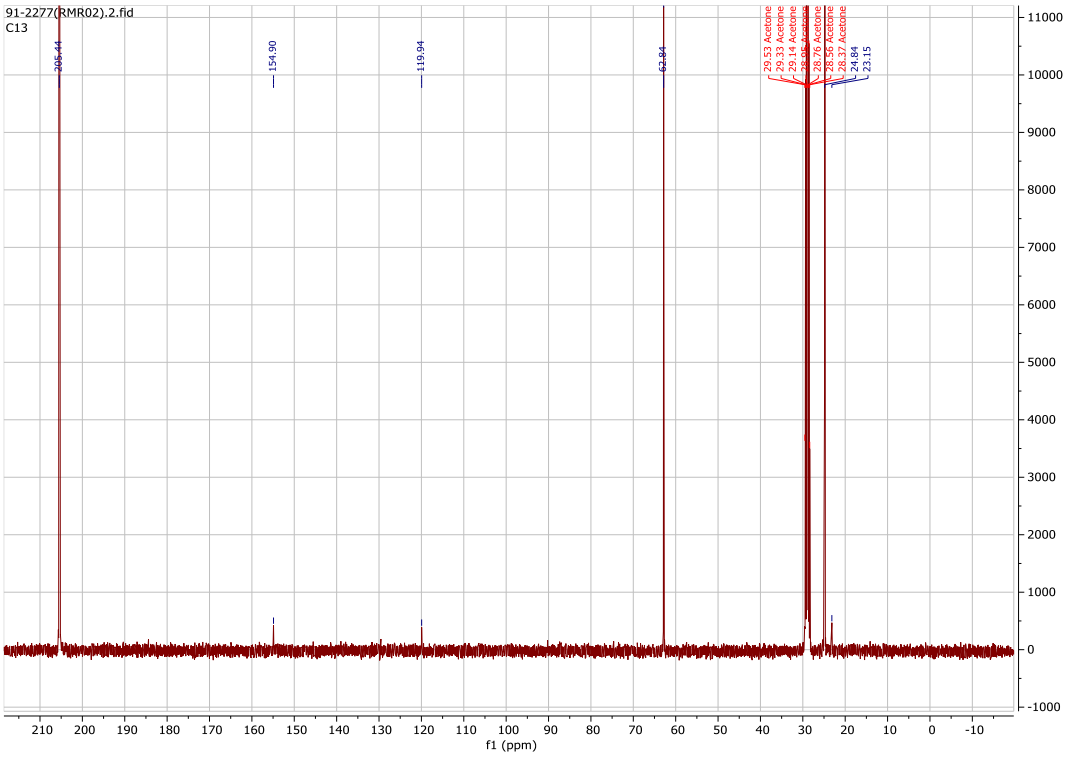
Appendix 12: DEPT-135 (101 MHz, Acetone) spectrum of compound RMR01



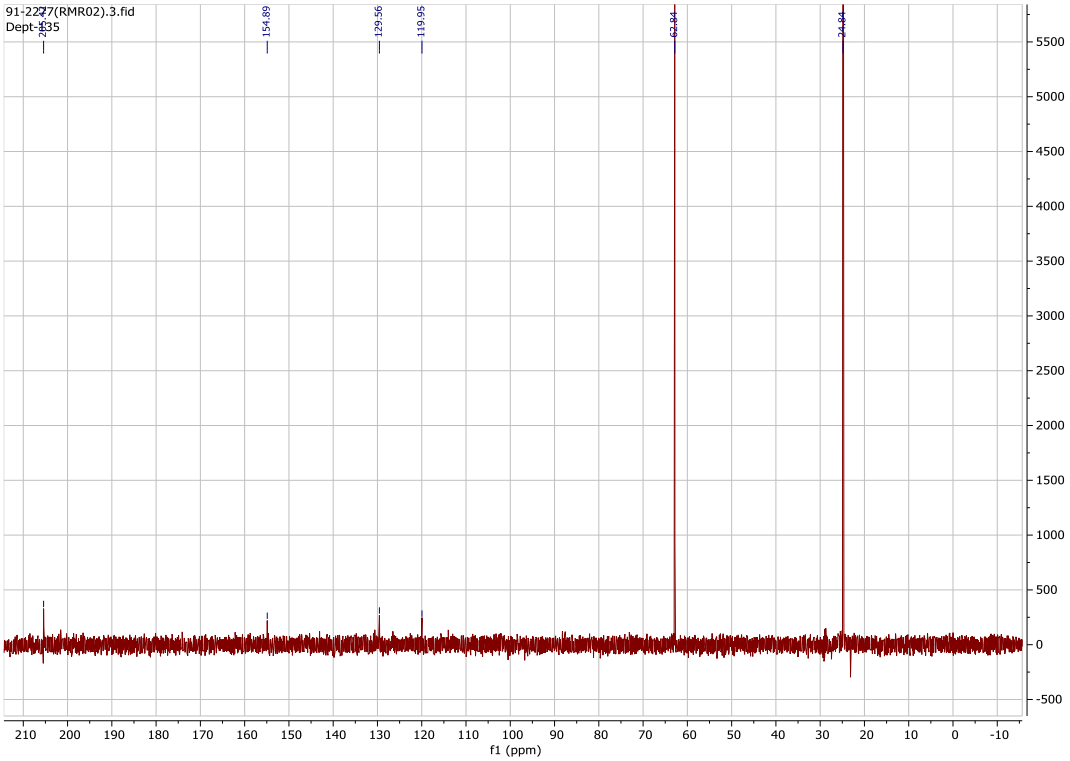
Appendix 13: ^{1}H-NMR (400 MHz, Acetone) spectrum of compound RMR02



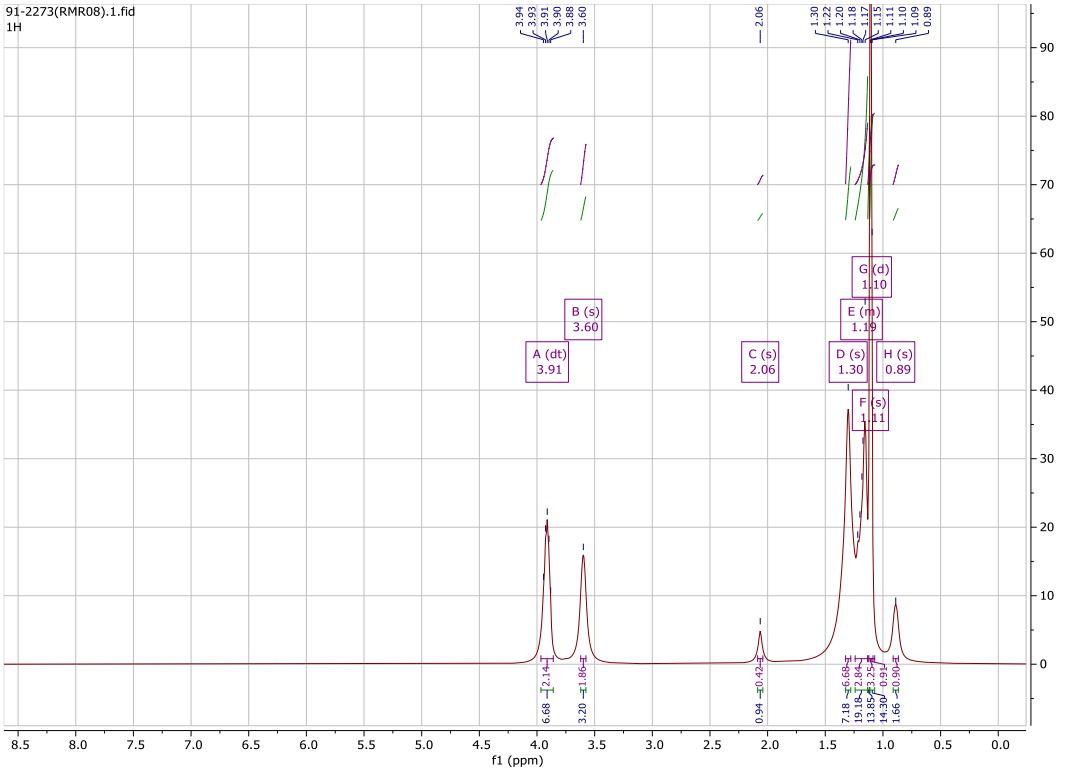
Appendix 14: ^{13}C-NMR (101 MHz, Acetone) spectrum of compound RMR02



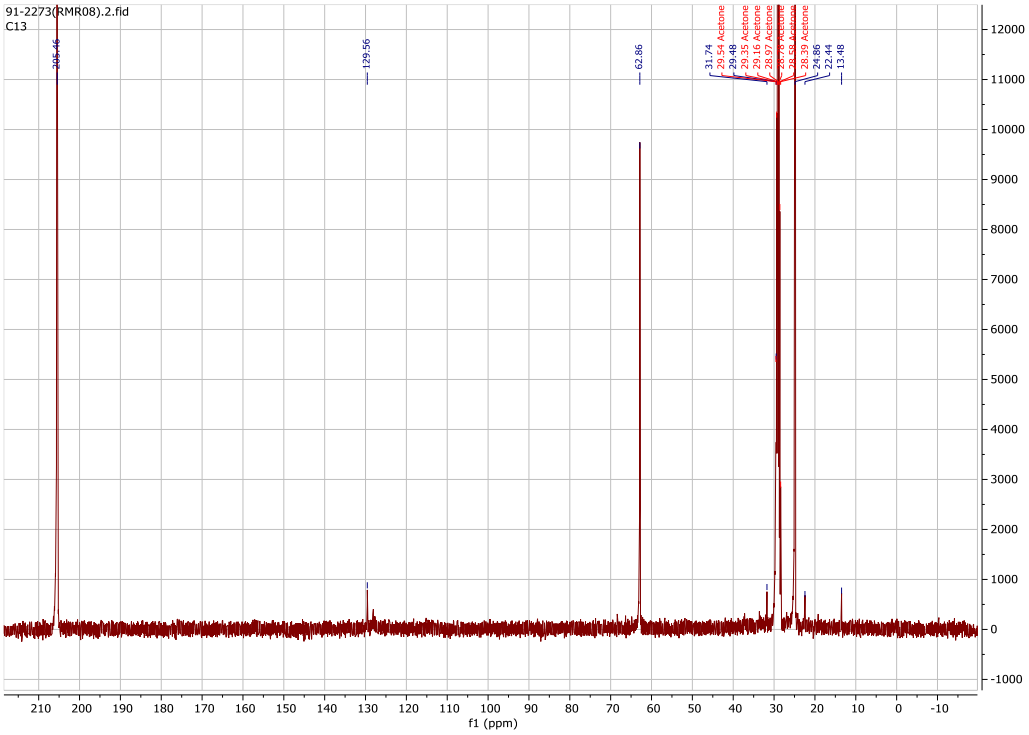
Appendix 15: DEPT-135 (101 MHz, Acetone) spectrum of compound RMR02



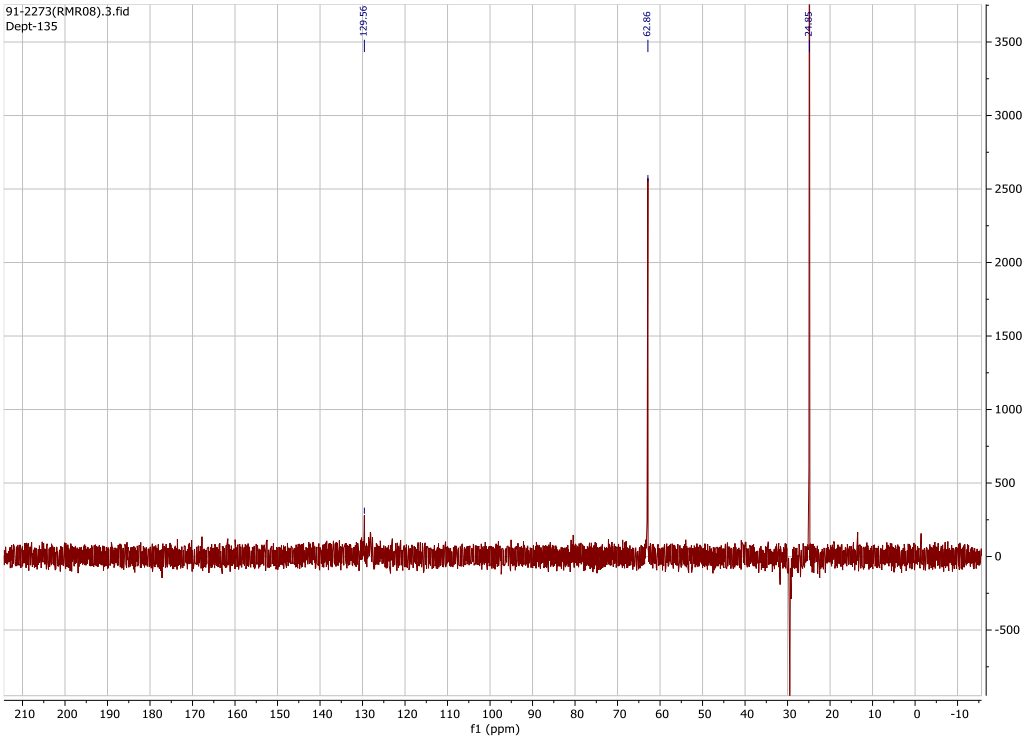
Appendix 16: ^{1}H-NMR (400 MHz, Acetone) spectrum of compound RMR08



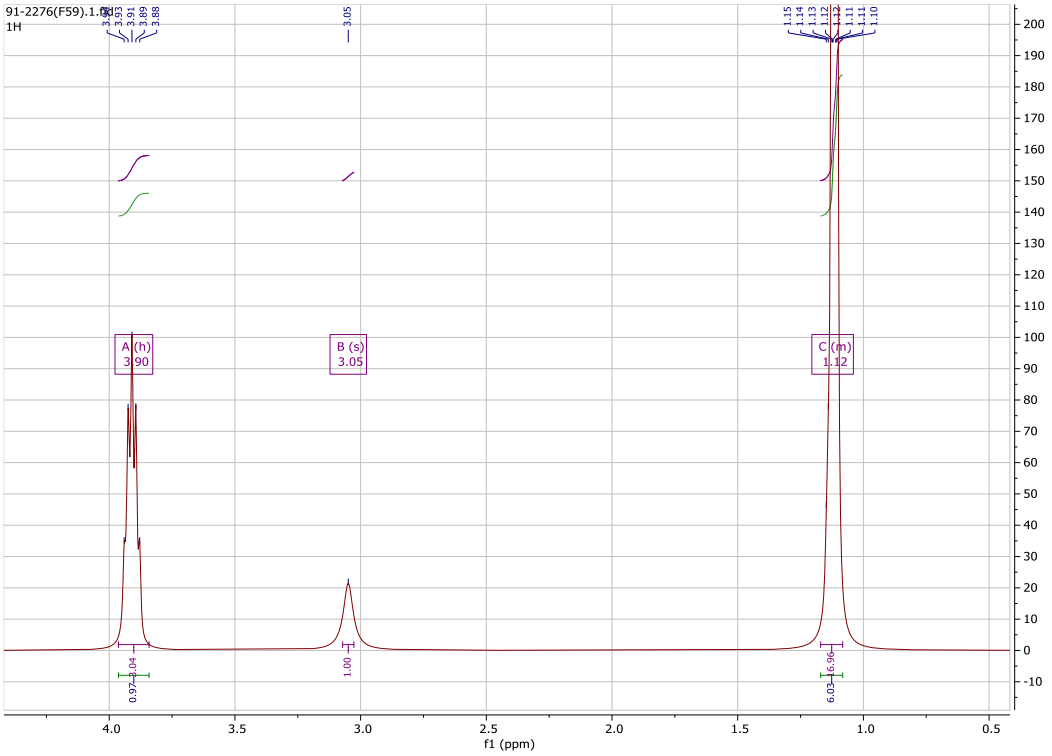
Appendix 17: ^{13}C-NMR (101 MHz, Acetone) spectrum of compound RMR08



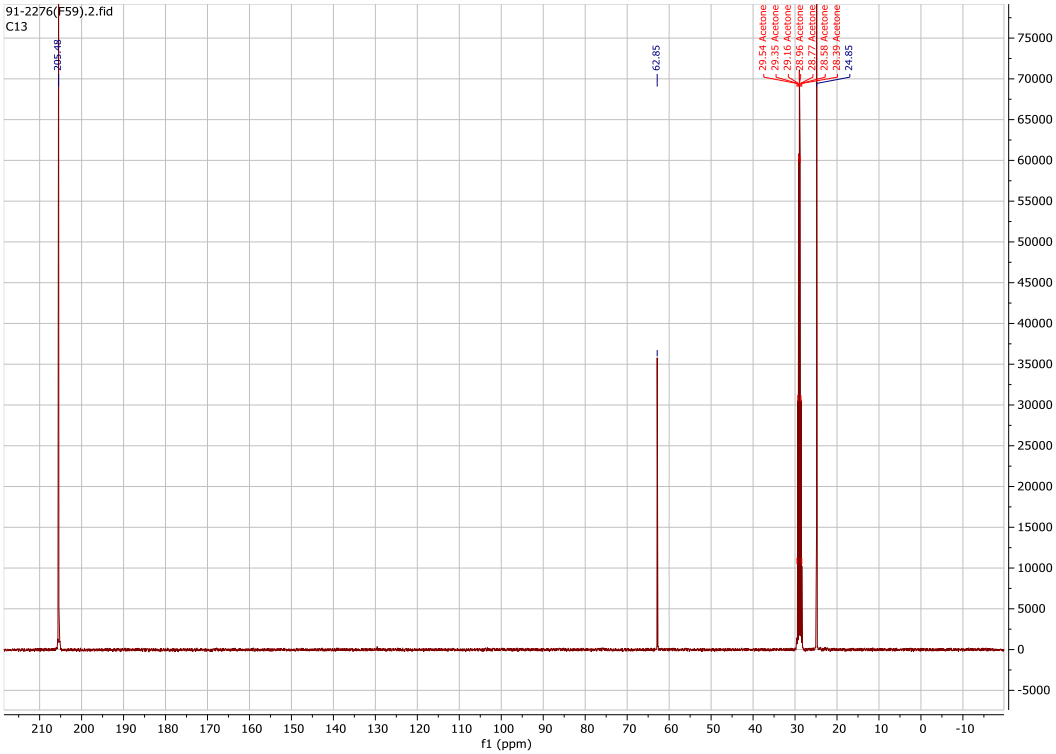
Appendix 18: DEPT-135 (101 MHz, Acetone) spectrum of compound RMR08



Appendix 19: ^{1}H-NMR (400 MHz, Acetone) spectrum of compound F59



Appendix 20: ^{13}C-NMR (101 MHz, Acetone) spectrum of compound F59



Appendix 21: DEPT-135 (101 MHz, Acetone) spectrum of compound F59