Phytochemical, *In vitro* Biological, and Computational Analyses of Bioactive Compounds from Leaf Extracts of Some Selected Ethiopian Medicinal Plants

Mosisa Dejene Chala

A PhD Dissertation Submitted to the

Department of Applied Biology

College of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy in Applied Biology (Specialization in Health Biotechnology)

Office of Graduate Studies
Adama Science and Technology University

June, 2025

Adama, Ethiopia

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Approval of Board of Examiners

We, the undersigned, members of the board of examiners of the final open defense by consigned chairperson and we have read and evaluated his dissertation entitled" Phytochemical, *In Vitro* Biological, and Computational Analyses of Bioactive Compounds from Leaf Extracts of Some Selected Ethiopian Medicinal Plants "and examined the candidate.

This is, therefore, to certify that the dissertation has been accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy (Ph.D) in Health Biotechnology.

Main Supervisor /Advisor	Signature	Date
Co-Supervisor/Co-Advisor	Signature	Date
Chairperson	Signature	Date
Internal Examiner	Signature	Date
External Examiner	Signature	Date

Declaration

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

Name:_____

Signature:_____

This PhD dissertation has been submitted for examination with our approval as dissertation supervisors.

Name of Main Supervisor: _____

Signature:_____

Name of Co-Supervisor: _____

Signature: _____

Date of submission: …….

Supervisor's/Advisor's Approval Sheet

To: Applied Biology department

Subject: Dissertation submission

This is to certify that the dissertation entitled " Phytochemical, *In Vitro* Biological, and Computational Analyses of Bioactive Compounds from Leaf Extracts of Some Selected Ethiopian Medicinal Plants " submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Biology (Specialization in Health Biotechnology), the graduate program of the department of Applied Biology, and has been carried out by Mosisa Dejene Id.No.PGR/19454/12, under our supervision. Therefore, we recommend that the student has fulfilled the requirements and hence hereby he can submit the dissertation to the department.

Name of Major Advisor/Supervisor	Signature	Date
Name of Co-Advisor/Co-Supervisor	Signature	Date

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Acronyms and Abbriviations

AAU	Addis Ababa University
ADT	Auto Dock Tools
ADME	Absorption Distribution Metabolism and Excretion
ADMET	Absorption Distribution Metabolism, Excretion,Toxicity
AMP	Adenine Mono Phosphate
ASTU	Adama Science and Technology University
ATCC	American Type Culture Collection
CC	Column Chromatography
CC_{50}	Half Maximal Cytotoxicity Concentration
CD4	Helper T cells (T-Lymphocytes)
CDC	Centers for Disease Control
CLSI	Clinical and Laboratory Standard Institute
COPD	Chronic Obstructive Pulmonary Disease
2D	Two Dimensional Space
3D	Three Dimensional Space
d	Doublet
dd	Doublet of a Doublet
DALYS	Disability – Adjusted Life Years
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphox
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DZI	Diameter Zones of Inhibition
EDTA	Ethylene Diaminetetra Acetic Acid
EFSA	European Food Safety Authority
FT-IR	Fourier Transform-Infra Red
GC-MS	Gas Chromatography-Mass Spectrometry

HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HU	Haramaya University
IC_{50}	Half Maximal Inhibitory Concentration
J	Coupling Constant
LD_{50}	Half Maximal Lethal Dose
MAP	Medicinal and Aromatic Plant
MHA	Mueller-Hinton Agar
MTT	Methylthiazolyl diphenyl-tetrazolium bromide
MZI	Minimum Zones of Inhibition
NMR	Nuclear Magnetic Resonance
PDA	Potato Dextrose Agar
PDB	Protein Data Bank
RNA	Ribonucleic Acid
RI	Retention Index
RPM	Revolutions Per Minute
S	Singlet
spp	Species
t	Triplet
TLC	Thin Layer Chromatography
UV-VS	Ultraviolet-Visible Spectroscopy

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Publications

Published articles

1. **Mosisa Dejene**, Aman Dekebo, Kero Jemal, H.C. Ananda Murthy, and S. Giridhar Reddy. Phytochemical screening and evaluatition of anti-oxidant, anti-inflammatory and anticancer activities of leaves of *Vernonia amygdalina*, *Otostegia integrifolia*, and *Salvia rosmarinus* (**Green Chemistry letters and review**-TGCL-2024-0007.R2, **India**). (https://doi.org/10.1080/17518253.2024.2438069.)
2. **Mosisa Dejene**, Kero Jemal, Getachew Tegegn, Muhdin Aliye , Lemma Teshome and Aman Dekebo. Antimicrobial Activity, Docking and ADMET Profiling of *Salvia rosmarinus* Compounds on a Targeting DNMT1 and HPV type 16 E6 in Cervical Cancer. **(the ornamental plant research journal**-OPR-S2024-0015, **Korea)**. (https://www.maxapress.com/article/doi/10.48130/opr-0024-0026.)

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3. **Mosisa Dejene,** Aman Dekebo, Kero Jemal, Mo Hunsen , Getachew Tegegn, Muhdin Aliye, A. Ilyina , A. Khasanov, L. Esaulkova, and V. Zarubaev .Phytochemical composition, antimicrobial and antiviral activities of leaves of *Vernonia amygdalina* and *Otostegia integrifolia*.[**Journal of Ethno biology and Ethnomedicine**.https://www.google.com/search?q=journal+of+ethnobiology+and+ethno medicine zarubaev@gmail.com,**China**]

Manuscript to be submitted

4. **Mosisa Dejene**, Kero Jemal, Muhdin Aliye and Aman Dekebo. Chemical Composition, Antimicrobial, Antioxidant, Bio-pesticide Activities and Molecular Docking Studies of Major Compounds from Essential Oils of *Vernonia amygdalina, Otostegia integrifolia* and *Salvia rosmarinus*.[The American Chemical Society(ACS) is a scientific society based in the United States. https://en.wikipedia.org/wiki/American_Chemical_Society)].

Abstract

*The relationship between humans and herbal medicinal plants has a long history. Nowadays, interest in medicinal and aromatic plants is increasing due to rising consumer demand and their various culinary and medicinal applications. This trend is fueled by challenges such as antimicrobial resistance and the emergence of opportunistic pathogens, which complicate the treatment of infectious diseases. In addition, cancer remains a significant research focus because of its considerable impact on public health. In Ethiopia, there is a notable gap in addressing herbal medicines for drug-resistant diseases and cancers, making this a critical issue in health biotechnology research today. Successively, the primary aim of this research is to explore the phytochemicals and assess the in-vitro antimicrobial and anticancer activities of the leaves extracts obtained by maceration method of Vernonia amygdalina, Otostegia integrifolia, and Salvia rosmarinus, which are among the most commonly used medicinal plants by traditional healers. The essential oils (EOs) obtained from the leaves of V. amygdalina, O. integrifolia, and S. rosmarinus by hydro-distillation method were analyzed by GC-MS. In vitro techniques were employed to evaluate various biological activities, including antimicrobial, anticancer, antioxidant, anti-inflammatory, anti-protozoa, and antiviral effects. This was accomplished using different assays: agar disc diffusion for assessing antimicrobial activity, methylthiazolyl diphenyl-tetrazolium bromide (MTT) bioassay for anticancer effects, and diphenyl-1- picrylhydrazyl (DPPH) bioassay for measuring antioxidant activity. Additionally, the inhibition of albumin denaturation and proteinase was measured for anti-inflammatory activity, while broth inhibition on peptone-yeast extract-glucose (PYG) bioassay for assessing anti-protozoa effects and micro-inhibition assays on Madin-Darby canine kidney (MDCK) cells were performed to evaluate antiviral effects. The anti-proliferative activities of three plant extracts were examined against HeLa cells, which are continuously cultured. For the in silico analysis, drug-likeness and Absorption, Distribution, Metabolism, and Excretion – Toxicity (ADMET) profiling were assessed using Biovia-2020 software, which facilitated molecular docking tests on the compounds under investigation. Statistical analysis was conducted using GraphPad Prism version 8.0.1 (244). Phytochemical screening tests identified presence of alkaloids, steroids, terpenoids, flavonoids, tannins, and saponins in the leaves of the three studied plants. Six pure compounds were isolated using column chromatography: compound **1** from the petroleum ether and chloroform (9:1) fraction of V. amygdalina, and compounds **2** -**4** from O. integrifolia and **5** and **6** from chloroform: methanol (1:1) fractions of S. rosmarinus. The petroleum ether leaf extracts of V. amygdalina exhibited significant antimicrobial activity against P. aeruginosa, with an inhibition zone of 17.80 ± 0.2 mm. However, among all the tested extracts, the petroleum ether leaf extracts of S. rosmarinus showed strong antimicrobial activity, with inhibition zones of 21.37 mm against S. aureus and 20.83 mm against C. albicans. The study found that the petroleum ether extract of S. rosmarinus and the chloroform: methanol (1:1) extract of O. integrifolia exhibited strong anti-proliferative effects on HeLa cancer cells. Specifically, the anti-proliferation rates were 93.73% and 91.02%, respectively, when tested at a concentration of 1250 µg mL⁻¹. In the three plants, the petroleum ether extracts from leaves demonstrated higher IC_{50} (µg mL^{-1}) values for DPPH radical- scavenging activities, ranging from 10 to 30 µg mL⁻¹, compared to the positive control ascorbic acid. The chloroform: methanol (1:1) extract of S. rosmarinus leaves exhibited the highest anti-inflammatory activity against albumin denaturation, with an IC_{50} value of 0.58 µg mL^{-1}. This was followed by V. amygdalina, which had an IC_{50} value of 0.81 µg mL⁻¹ in comparison, the positive control, aspirin, exhibited an IC_{50} value of 4.59 µg mL⁻¹. Similarly, the extracts of V. amygdalina and O. integrifolia in chloroform/methanol (1:1) demonstrated significant inhibitory activity against proteinase, with IC_{50} values of 0.77 µg mL⁻¹ and 0.86 µg mL⁻¹, respectively, both showing higher activity than the positive control. The in-vitro study of Salvia rosmarinus extracts chloroform: methanol (1:1) showed strong amoebicidal activity against Acanthamoeba castellanii, resulting in less than 10% viability. Among all the tested antiviral activities, the in vitro studies of V. amygdalina extracts using aqueous, chloroform:*

*methanol (1:1), and petroleum ether have shown a selectivity index (SI) greater than 10, indicating their potential as antiviral candidates against influenza A (H1N1).The essential oils (EOs) from the leaves of V. amygdalina, O. integrifolia, and S. rosmarinus contained main components: dihydro-2H-pyran (93.6%), 3-hexanol (19.63%), and camphene (23.75%), respectively. The EOs derived from S. rosmarinus and V. amygdalina exhibited the most potent antifungal properties against C. albicans. Among all the tested EOs, the EO from Salvia rosmarinus leaves showed the strongest antioxidant activity, as indicated by its DPPH free radical scavenging capacity, with an IC_{50} value of 0.6927 μg mL^{-1}. The in silico study revealed that the extracted EOs dihydro-2H-Pyran was highly bound to two bacterial strains: S. pyogenes (PDB ID: 4D8E) with a minimum binding energy of –3.3 kcal mol^{-1} and E. coli (PDB ID: 6F86) with –2.7 kcal mol^{-1}. In comparison, Amoxicillin had binding energies of –6.1 kcal mol^{-1}, and –5.9 kcal mol^{-1} , respectively. Compounds micromeric (**5**) and benzocaine (**6**) isolated from a methanol:chloroform (1:1) extract of S. rosmarinus leaves, indicating high binding affinities for potential anticancer activities: DNMT1 (PDB ID: 4WXX) enzyme with a minimum binding energy of -8.4 kcal mol^{-1} for micromeric and -5.3 kcal mol^{-1} for benzocaine, and HPV type 16 E6 enzyme interacted with compound **5** at -10.1 kcal mol^{-1} and with compound **6** at -6.5 kcal mol^{-1}, similar to the reference compound Jaceosidin had binding energy of -7.8 kcal mol^{-1}, and -8.8 kcal mol^{-1}, respectively. Furthermore, a docking study of compounds **2** - **4** isolated from methanol: chloroform (1:1) extracts of O. integrifolia leaves showed good binding affinity for P. falciparum (PDB ID: 1LDG) and Influenza A virus (6ML8): Compound **4** had the highest binding energy at -6.7 kcal mol^{-1}, compared to the reference drug Artesunate at -6.3 kcal mol^{-1}. and -6.8 kcal mol^{-1}, compared to the reference drug Amantadine HCl at -4.6 kcal mol^{-1} for P. falciparum (PDB ID: 1LDG) and Influenza A virus (6ML8), respectively. The results not only contribute to our understanding of the three studied plants' medicinal potential but also provide promising directions for their future research and therapeutic development. Further biochemical studies on the three investigated plants may lead to the discovery of more potent secondary metabolites. Our findings support the traditional use of these plants for treating infectious diseases and cancer with other like oxidative agents and inflammation.*

Keywords: Antimicrobial, Anticancer, *Otostegia integrifolia*, phytochemicals, *Salvia rosmarinus, Vernonia amygdalina*

CHAPTER 1

1 INTRODUCTION

1.1 Background

Humans have used plants for thousands of years to treat various infections, and in-home customers in many developing and developed countries(Stashenko and Martinez, 2018; Mehani & Ladjel, 2012). Remarkably, the widespread use of medicinal plants for health purposes has increased dramatically due to their great importance to the public health (El-Beltagi and Badawi, 2013) .Up to 80% of the population in Africa uses traditional medicine for their primary health care, which is mainly due to high cost of modern drugs, inadequacy and inaccessibility of modern health services, and cultural acceptability of traditional medicine (Chota *et al*.,2020). The plant species found on our planet searched by authors; about 20,000 of them are used for medicinal purposes, according to a World Health Organization (WHO) report. Some plants like aromatic plants are a source of fragrances, flavors, cosmeceuticals, health beverages, and chemical terpenes (Hemeg *et al*., 2020; Iftikar, 2019). Currently, Ethipia and many other countries in Africa have a division, department, or task force on traditional medicine, usually attached to their Health Ministries (Demie *et al*., 2018).

Ethiopia is rich in biodiversity with high endemism(Kindie & Tamiru, 2021;Tugume *et al*., 2016). The richness of flora and fauna is favorable to the diverse ecological setting, climate, and topography found in the country. The Ethiopian flora is estimated to contain 6500 to 7000 species of higher plants, of which about 12 percent are endemic (Endale *et al*., 2013). Of these, 800-1,000 species are experienced in the traditional healthcare system (Endale *et al*., 2013). Ethiopia has the fifth owning flora in tropical Africa (Stashenko and Martinez, 2018; Karunamoorthi, 2014; Endale *et al*., 2013; Momoh and Muhamed, 2012). Moreover, national product research is often based on ethnobotanical information, and many of the drugs used today were developed from medicinal plants used by indigenous societies(Chota *et al*.,2020; Demie *et al*., 2018; Berhan *et al*., 2006) . Conversely, research on medicinal plants in Ethiopia is limited, with few studies on chemical composition and anti-microbial properties (Busse and Tefera, 2013; Momoh and Muhamed, 2012). Recently, researchers in the field of health biotechnology

have begun to explore a new more source of organic naturally generated medicinal materials. These natural organics have been found to have no side effects on human health or the environment(Shimira *et al*., 2022). Here, the existing study selected three herbal medicines called *Vernonia amygdalina*, *Otostegia integrifolia*, and *Salvia rosmarinus* in Ethiopia. Consequently, *V. amygdalina,* locally called *Grawa* in Amharic and it is the most commonly used in Ethiopia in the preparation of local beer called *tella* as fumigant and firewood (Habtamu & Melaku, 2018). In Ethiopia, wild plants are occasionally cultivated for their medicinal uses, which include treating diarrhea, skin wounds, fever, mastitis, and worm infections (Habtamu & Melaku, 2018; Ijeh & Ejike, 2011).

Otostegia integrifolia Benth more commonly known as Abyssinian rose and locally in Amharic known as *Tinjute* . It used for the treatment of several diseases as antimalarial, anthelmintic, insecticides, anti-cough, and thyroid inhibiting properties of the essential oil distilled from the leaves (Chekol & Desta, 2018). The authors have further indicated that stigmasterol of the chemical constituents of *O. integrifolia* can be useful in the prevention of certain cancers, including ovarian, and colon cancers. In addition, the plant is well-known for its pleasant odour, omnipotent medicinal values (Tadesse *et al*., 2012). *Salvia rosmarinus* Spenn is popularly identified as rosemary and it was used for the treatment as antitumor, antioxidant, and analgesic activities and effects on stress, and anxiety (Hameed & Mohammed, 2017; Hamidpour, 2017; Andrade *et al*., 2016). Rosemary extracts have been utilized for disease prevention due to their hepatoprotective and therapeutic potential for Alzheimer's disease, as well as their antiangiogenic effect. The European food safety authority (EFSA) has reviewed the safety of rosemary extracts (Veenstra & Johnson, 2021; Nieto *et al*., 2018).

The pharmaceutical potential in *V. amygdalina* , *O. integrifolia* and *S. rosmarinus* have consituted some of the phytochemicals including alkaloids, tannins, saponins, flavonoids, phenols, polyphenols, steroid, terpenoid , phlobatannin, and glycoside (Veenstra & Johnson, 2021; Chekol & Desta, 2018; Abate & Yayinie, 2018; Andrade *et al*., 2016). In some African countries including Ethiopia, *V. amygdalina, O. integrifolia,* and *S. rosmarinus* are among the medicinally significant plants used against anti-diabetic, anti-allergic effect, anti-gastrointestinal disorders, helminths infections, promote wound healing and fever, release constipation,

neuroprotective (stresses), and immunological activity (for HIV patients increased CD_4 count) (Veenstra & Johnson, 2021, Chekol & Desta, 2018). Natural products from *V. amygdalina, O. integrifolia,* and *S. rosmarinus* were used in the past to treat edema, wounds, tumors, and tumor necrosis with no side effects because they are edibles. Antioxidants found in aromatic plants and spices stabilize free radicals that cause diseases like cancer, diabetes, and heart attacks(Abate & Yayinie, 2018; Hamidpour, 2017). Cancer is the uncontrolled growth of cells that can be caused by various factors and can be fatal. There are over 100 types of cancer, and it affects various cells in the body. Prevention is key to reduce the risk of developing cancer (Malvezzi *et al* 2020),Abate & Yayinie, 2018). Treatments include chemotherapy, radiotherapy, and surgery(Malvezzi *et al* 2020). Traditional medicine is preferred by 65-80% of people in non-industrialized countries due to its affordability, accessibility, and cultural significance. Researchers have screened thousands of plant species for anti-cancer drugs with nearly 1,000 medicinal plants showing potential as therapeutic agents(Malvezzi *et al* 2020).

Hence,one potential approach to treat cervical cancer is to inhibit the activity of the DNA methyltransferases (DNMT1) and human papilloma virus (HPV type 16 E6) enzymes specifically (Kaur *et al.,* 2021, Brooijmans and Kuntz, 2003 , Rhee *et al*., 2000). Over 50% of clinical drug forms worldwide originate from plant compounds (Yoshino *et al.*, 2023). In the past, developing new drugs was a lengthy and costly process. However, with the emergence of bioinformatics, the use of computer-based tools and methods has become increasingly important in drug discovery. One such method is molecular docking and ADMET profiling which involves the structure of a drug to screen for potential candidates (compounds screen from herbal medicine plants). This approach is known as structure-based drug design and can save both time and resources during the research process (Brooijmans and Kuntz, 2003).

Despite the vast pharmacological activities of traditional knowledge of *V. amygdalina, O. integrifolia,* and *S. rosmarinus* in developing countries as primary health care. Still, there is a gap in overcoming the treatment of various shooting drug resistances of nosocomial diseases (hospital-acquired diseases), and cancer is a heavy economic, and social burden, and it is a hot point in the field of health biotechnology and medical research (CDC, 2020; Adams *et al*., 2007). Therefore, the current study was designed to analyze chemical composition and

evaluate antimicrobial activities of the extracts and essential oils of leaves of *V. amygdalina*, *O. integrifolia* , and *S. rosmarinus* using the common pathogenic bacterial species; three from Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) and three from Gram-positive (*Staphylococcus aureus*, *Streptococcus pyogenes* and *Staphylococcus epidermidis*) and two fungal species (*Aspergillus niger*, *Candida albicans*). Moreover, this study included the anticancer, antioxidant, anti-inflammation, anti-amoeba, anti-viral, and antimalaria properities of the extracts of plants using *in vitro* and *in silico* studies.

1.2 Statement of the problem

Ethiopia has a rich biodiversity of higher plants (Demie *et al*., 2018). Of these, 800-1000 plant species are being used in the traditional health care system making the country one of the most diverse floristic regions in the world (Asmamaw and Achamyeleh, 2018; Demie *et al*., 2018). In Africa, the situation is not different, over 80 % of the population depends directly on plants for their primary healthcare requirements (Tugume *et al*., 2016). Therefore, the acceptance of customers and use of herbal medicine has increased globally (Stashenko and Martinez, 2017). Two decades, researchers estimated that there were about 400,000 species of plants worldwide, including about a quarter or a third have been used by companies for medicinal purposes (Mehani & Ladjel, 2012) . As a consequence, the chemical compositions of these medicinal plants need to be deeply investigated. This composition may vary due to several factors, including climate, geography, environment, stage of plant growth, and extraction methods (Sobrinho *et al*., 2020 ; Awadh and Alhamzy, 2017). However, in Ethiopia the research conducted so far on most of these medicinal plants has been insufficient, primarily focusing on producing inventories and checklists,and these studies have remained largely survey-based, with their outputs consisting mainly of plant listings (Busse and Tefera, 2013;Asmamaw and Achamyeleh, 2018). Despite the wide spectrum of medicinal uses (Bouzid, 2013; Taylor *et al*., 2013; Tadesse *et al*., 2012), the bioactive constituents and biological activities have not yet been well described. Also, the reported research studies on the phytochemical constituents and antimicrobial activity of traditional medicinal plants found in Ethiopia are focused on a few plant species. Moreover, to our knowledge, no detailed research activities have been reported so far on the chemical constituents and biological activities on some medicinal plants in Ethiopia. Taking this into account, the need to conduct a comprehensive scientific investigation on the chemical

constituents anticancer and antimicrobial activities of the three selected medicinal plants namely *V. Amygdalina*, (bitter leaf), *O. integrifolia* (tinjute) and *S. rosmarinus* (rosemary) based traditional knowledge claimed by traditional healers is very crucial.

In the same way, the health conditions of people remain the primary health concern, representing 41% of the global disease burden (Nigatu *et al*., 2012). Then again, due to the increasing resistance of infectious diseases to antibiotics and cancer with a heavy social burden is a hot point in the field of medical research (CDC, 2020; Adams *et al*., 2007) there is a growing focus on healthy biologically active components. These components, which include both nutrient and non-nutrient molecules found in plant extract oils, can produce safe physiological effects. Research is emphasizing the use of these herbal medicines derived from various plant species, as they may present a promising new source of antimicrobial activity (Hemeg *et al*., 2020). It is therefore greatly needed to conduct a comprehensive scientific investigation on chemical composition and biological activities of the extracts such as solvent fractions, essential oils and pure compounds of the leaves of these plants.

1.3 Objectives

1.3.1 General objective

The general objective of this dissertation work was to investigate the phytochemical composition and examine *in vitro* antimicrobial and anticancer activities of leaf extracts of some medicinal plants and study molecular docking of their identified compounds against selected protein targets.

1.3.2 Specific objectives

The specific objectives of this dissertation study were to:

- Isolate compounds from the leaf extracts of *V.amygdalina* , *O.integrifola* and *S. Rosmarinus* using thin- layer chromatographic (TLC) techniques and structural elucidation of isolated compounds using NMR;
- Examine *in vitro* antibacterial and antifungal activities of leaf extracts of *V.amygdalina* ,*O.integrifola* and *S. Rosmarinus* against some selected human

pathogenic Gram negative and Gram positive bacteria; and selected human pathogenic fungi;

- Evaluate *in vitro* anticancer, antioxidant, anti-inflammatory, and anti-influenza virus subtypes A activities of leaf extracts of *V.amygdalina* , *O.integrifola* and *S. Rosmarinus*;
- Examine *in vitro* anti-*Acanthamoeba* species from chloroform:methanol(1:1) extracts of *S. Rosmarinus* leaves;
- Extract essential oils from the plant's leaves using the hydro-distillation technique to identify their chemical compositions using GC-MS and evaluate their antimicrobial, and antioxidant activities;
- Predict the binding capacity of dihydro-2H-Pyran, one of the compounds selected from the essential oil docking against *E. coli* DNA , gyrase B (PDB ID: 6F86), and *S. pyogenes*; SpeB (PDB ID: 4D8E) with the help of AutoDock 2021;
- Predict anticancer activities of compounds isolated from *S.rosmarinus* leaves targeting enzymes caused in cervical cancer called DNA methyltransferases (DNMT1) and human papillomavirus (HPV) type 16 E6 in human cervical cancer;
- Predict anti-malarial activities and anti-influenza-A virus activities of compounds isolated from *O.integrifolia* leaves targeting enzymes called PDB ID: 1LDG and PDB ID: 6ML8 by respectively using *in silico* method;
- Perceive the drug-likeness, ADME and toxicity characteristics of some of isolated compounds by SwissADME, PreADMET and OSIRIS property explorer software.

1.3 Significance of the study

Validating the ethnobotanical uses of Ethiopian medicinal plants scientifically evaluated is needed to provide a basic understanding of the plant's efficacy and further support the widespread use of traditional medicine in health care systems. Hence the importance of the present dissertation study may lay in providing a scientific rationale to support the purported folkloric usages of some of the selected central parts of Ethiopia, the Oromia regional state, the southwest Showa zone indigenous people use medicinal plants for various traditional medicinal purposes by investigating their chemical constituents and examining their *in vitro* antimicrobial and anticancer activity. This dissertation study may have the following

significance:

1. Providing information about extraction, phytochemical compositions of leaves of studied plant species; and anticancer, antimicrobial activities, antiviral of the three plants leaves extract; and *in-vitro* anti-*Acanthamoeba* species from Chloroform:methanol(1:1) extracts of *S. Rosmarinus*;
2. Providing information in health biotechnology on molecular docking analysis so as to have better understanding of the molecular mechanisms of interaction between the compounds with promising antibacterial, anticancer, antimlarial activity and selected protein domains;
3. Providing information on drug-likeness, ADME predictions and toxicity profile of the isolated compounds to understand the reason for their interaction and their drug properties
4. Providing drug "lead" candidates which may be used for designing of new analogues having either better therapeutic potency or reduced cytotoxicity effect;
5. Exploring eco-friendly treatment options for drug-resistant of the nosocomial diseases using antibiotics through biotechnological and nanotechnological applications.
6. Laying a scientific base for both the modern and traditional medicines by providing a bridge between the modern and traditional ethno-medicinal claims about the medicinal uses of the selected plant species; and
7. Growing biodiversity of the herbal medicinal plants and which will be spent for conservation of - these plant species be gradually destroyed at different region of Ethiopia.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Botanical and ethnobotanical aspect of the studied plants

2.1.1 *Vernonia amygdalina*

Vernonia amygdalina, locally called *Ebicha* in Afan Oromo, *Grawa* in Amharic, and *bitter leaf* in English (worldwide) and it is quite commonly used in Ethiopia in the fermentation of homegrown beer (*tella*). It is also utilized as fire wood (Ugbogu *et al.*, 2021; Habtamu & Melaku, 2018),and its leaf extracts commonly found in cosmotics, food and pharmaceuticals (Figure 1). It is a perennial shrub (Table 1) that belongs to the family Asteraceae and grows throughout tropical Africa. *V. amygdalina* is drought tolerant (though it grows better in a humid environment) (Ijeh & Ejike, 2011). *V. amygdalina* can grow to 6 - 8 m tall (Figure 2). Its leaves are 6 mm in diameter and 20 cm long, and they are dark green. They are consumed in a wide variety of delicious foods in African countries (like Nigeria, Congo, and South Africa), and *V. amygdalina* in Ethiopia occasionally wild plants are cultivated for their medicinal uses. It is probably the most used medicinal plant in the genus *Vernonia*. Traditional medicine(Table 1) practitioners use the plant as an anti-helminth, anti-malarial, laxative, digestive tonic, appetizer, and febrifuge, and it is also used for the topical treatment of wounds (Ugbogu *et al.*, 2021).

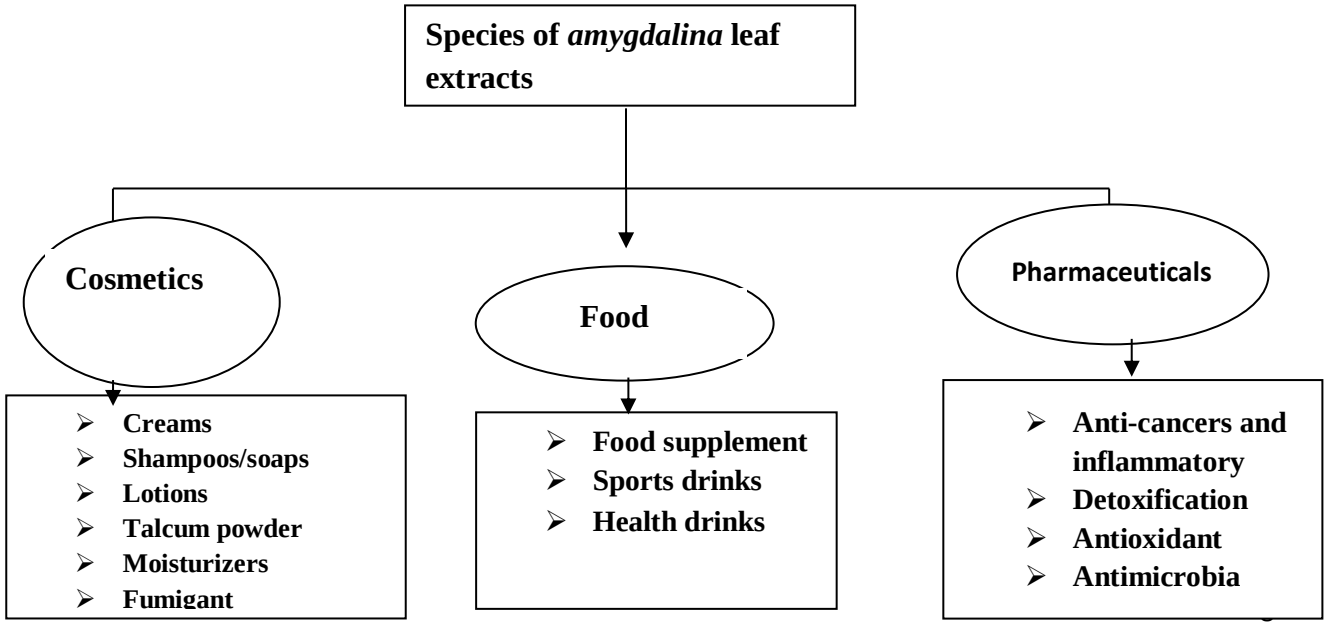


Figure 1: The utility of species of *V.amygdalina* leaf extracts(Ugbogu *et al.*, 2021)

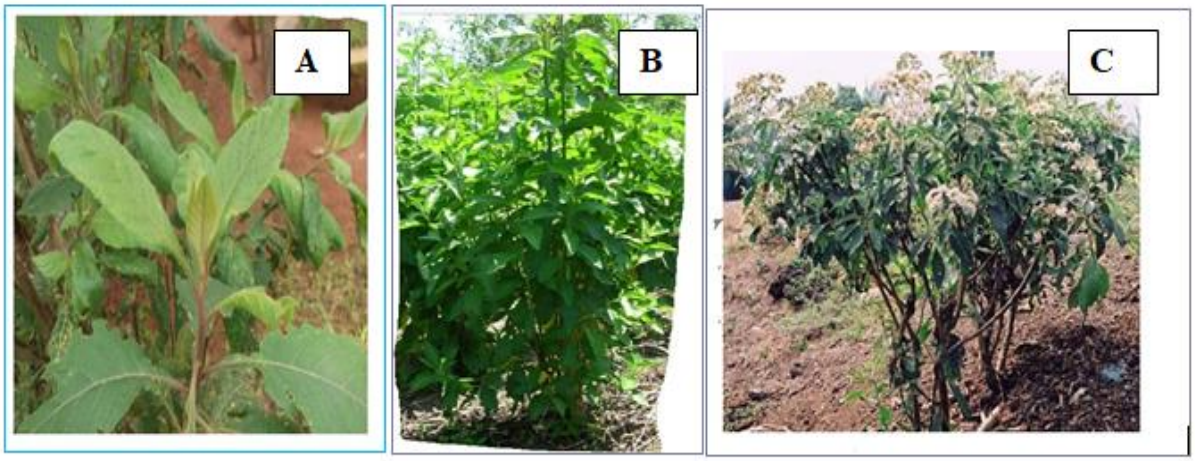


Figure 2: Picture of *Vernonia amygdalina L. (bitter leaf*) A: Seedlings; B: Young; C: Grownup (Ugbogu *et al.*, 2021)

2.1.2 *Otostegia integrifolia*

Otostegia integrifolia Benth (synonym of *Rydinglia integrifolia* Benth) (Figure 3) is found in Ethiopia called *tinjute* (vernacular name of the local native language, Amharic) and the *Lamiaceae* family which are endemic to the northern part of tropical Africa, South-western and Central Asia. Genus *Otostegia* belongs to the family *Lamiaceae*, which consists of about 180 genera and over 3500 species (Karunamoorthi, 2014). The *Otostegia* comprises about twenty species among them 15 are endemic to the northern part of tropical Africa and Southwestern and Central Asia, while the remaining five species have been reported in the flora of Ethiopia (Karunamoorthi, 2014; Endale *et al*., 2013). *O. integrifolia* Benth family of Lamiaceae *is* an erect perennial shrub (table 1), much branched and spiny, 1–3 m tall (Figure 3), the greyish color of the leaves, pink or yellow flowers, a shrub, often with paired spines at the nodes. Its leaves are sessile or shortly petiolate and reach 2–9 cm long, and it is found in the dry evergreen woodlands and moist agro-climatic zones known as Dega (Chekol & Desta, 2018). The plant grows in the wild, and it is cultivated in gardens. It grows on mountain bushlands and woodlands overgrazed slopes at altitudes ranging from 1300 to 2800 m (Chekol & Desta, 2018;Endale *et al.*, 2013). *O.*

integrifolia is used as a traditional medicine(Table 1) to treat inflammation, and rheumatic conditions, inhibit diarrhea, oral infections, antiulcer, antispasmodic, antidepressant, anxiolytic, insecticides and insect repellent, ophthalmia, sedative habit activities, tremor and ptosis, fumigant for pots and houses (Chekol & Desta, 2018 ; Shewamene *et al*., 2015; Khan & Syed, 2013 ; Berhan *et al*., 2006). In Saudi Arabia, the irritated eyes of cows, goats, and sheep can be rinsed with leaf infusion of *O. integrifolia,* and the roots of *O. integrifolia* are used for treating lung diseases, too (Karunamoorthi, 2014). *O. integrifolia* was reported to be a control means for livestock ticks, lice, and flea infestation in ethno-veterinary practice and acaricidal activity (Kemal *et al*., 2020).

In addition, *O. integrifolia* is common in Ethiopia, where it is used locally to smoke and sterilize utensils and used as ingredient to flavor different spices. According to the local understanding, the practice of smoking the vessel by burning wooden chips of specific trees and shrubs has the advantage of imparting a special taste and odor to the product, and disinfecting the equipment of vessels, thus reducing the numbers of micro-organisms and thereby extending the shelf life of the product(Karunamoorthi, 2014; Endale *et al*., 2013). Clothes smoked; perhaps the most common practice is the ritual custom(Table 1) that a mother smoked with *O. integrifolia* (*Tinjute*) on the tenth day after giving birth to a child. She is "cleansed" with the smoke postpartum so that process mother can leave her confinement and again resume a normal daily life (Berhan *et al*., 2006).

Figure 3: Picture of *Otostegia integrifolia* Benth Pleasant odour; *Tinjute* leaf (Mosisa took photo April 28, 2022)

2.1.3 *Salvia rosmarinus*

Salvia rosmarinus (Rosemary) previously called *Rosmarinus officinalis* is an aromatic evergreen shrub in the Lamiaceae family (Figure 4). Native to the Mediterranean region, rosemary is now a day cultivated around the world due to its use as a natural food preservative and flavoring agent (Hamidpour, 2017). It can flourish up to an altitude of 1500 m above sea level. The plant grows from 60 to 200cm (Shimira *et al*., 2022). The investigators further remarked that *Salvia rosmarinus* belongs to xeromorphic species that thrive naturally on cliffs, sand, and stony zones close to the sea across the globe, including Africa, Europe, and Asia. *S.rosmarinus* is a dense bush, branched, evergreen, and blue–white flower, reaching a height of about 1 m -2m(Figure 4). It is characterized by leaves 1–4 cm long and 2–4 mm wide, sessile, leathery, linear to linear-lanceolate, with curved edges, dark green upper side and granulosa and page bottom tomentous, with prominent midrib, and very characteristic smell (Andrade *et a*l., 2016). *S.rosmarinus* is used as a traditional medicine (Table 1) to perfume in ointments, shampoos, and soaps. The flowers are laid in clothes and cupboards to destroy moths. The leaves are crushed into meats, fish, potato salads, and other flavors (Veenstra & Johnson, 2021;Hamidpour, 2017).

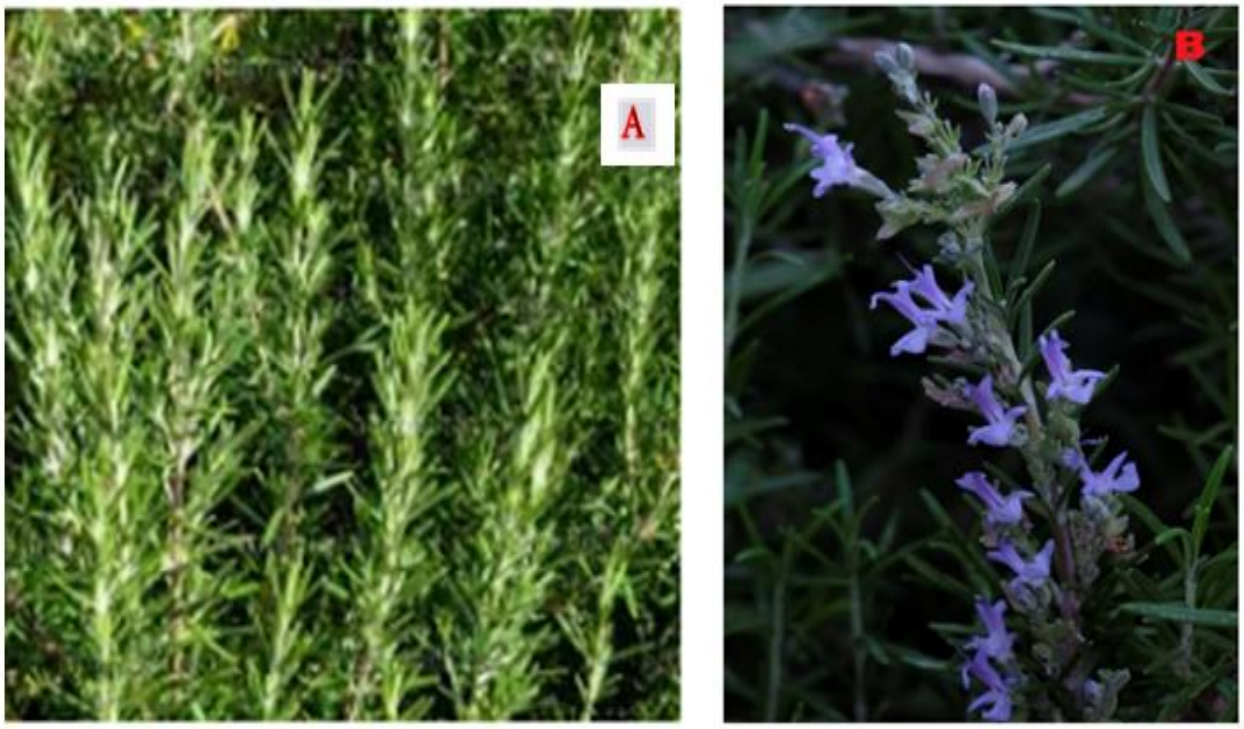


Figure 4: Picture of *Salvia rosmarinus* (*Rosemary*) plants A. Leaves B. Flowers *(Shimira et al*., 2022)

Table 1 : Some botanical and ethnobotanical aspects of the studied plants

Scientific name	Family	Venacular name (Amharic)	Habit	Traditional medicinal uses(treatements)	PU	Reference
				For topical treatment of wounds,Anti-inflammation	L	(Ugbogu *et al*., 2021).
V.amygdalina	Asteraceae	Grawa	HP	For animal skin-cyst, insecticides ,Intestinal worms, cough and hemorrhoids	L	(Nigatu *et al.*, 2012)
				For STD like wart of fungi, diarrhea, skin wounds, fever, mastitis, and worm infections	L, F	(Habtamu & Melaku, 2018)
O.integrifolia	Lamiaceae	Tinjute	HP	For gum diseases, ophthalmia,antimosquito repellency ,inhibit diarrhea, oral infections,antiulcer, insecticides	L	(Chekol & Desta, 2018; Shewamene,, 2015)
				For livestock ticks, lice, and flea infestation in ethno-veterinary practice and acaricidal activity	L	(Kemal *et al*., 2020)
S. rosmarinus	Lamiaceae	Rosemary	SH	For perfume in ointments, antidepressant,antispetics, food preservative and flavoring agent	L, F	(Hamidpour, 2017)
				For preventing obesity and skin diseases and non-pathological states, such as aging	L, S, F	(Malvezzi De Macedo *et al*., 2020)

Notes: PU-part used, SH-Shrub, HP-herb perennial, L-Leaves, S-Stems, F-Flowers

2.2 The pharmaceutical reported from the studied Plants

It is crucial to take our medications to protect against various infectious diseases that require different pharmacotherapeutics (drugs).Those drugs are sourced from natural; synthetic; and other sources. Primarily, natural sources of medicines are derived from plants. Medicines such as Morphine, digoxin, quinine, atropine, nicotine, reserpine, and caffeine, etc were originated from

plants. Accordingly Insulin, thyroid extract, heparin, anti-venom, gonadotropins and antitoxic, etc were obtained from animals. Liquid paraffin, magnesium sulfate, magnesium trisilicate, kaolin, Ca, I and Cl, etc were from mineral and penicillins are derived from microorganism (Bacteria and fungi, isolated from the soil) are sources of antibacterial substances. Second sources of druges are synthetic drugs which include Analgesics, hypnotics, anticancer drugs, e.g. Paracetamol, Aspirin, etc. In the third group, other sources in health biotechnology applications of drugs obtained using genetic engineering (DNA recombinant technology),for example., insulin and growth hormones, genes; and hybridism techniques (monoclonal antibodies) (Kothiyal *et al*., 2019).

Traditional medicine is an evolutionary process as communities (Andrade *et al*., 2016) and individuals continue to discover new techniques that transform practices. Drug discovery using natural products is an issue in the current situation for those diseases resistant to antibiotics. Therefore, for thousands of years, people have used naturally occurring herbal plants like bitter leaf (genus *Vernonia*), tinjute(genus *Otostegia*), and rosemary(genus *Salvia)*, to treat diseases and relieve pain (Shimira *et al*., 2022; Berhan *et al*., 2006; Patwardhan, 2005). A phytochemical screening of vernonia (Ugbogu *et al*., 2021), integrifolia (Tadesse *et al.*, 2012)and rosmarinus (Andrade *et al*., 2016) species characterized the presence of alkaloids, flavonoids, tannins, glycosides and terpene as possible bioactive compounds .

2.2.1 Pharmacological uses of *V. amygdalina*

V. amygdalina have several medical, industrial, and food uses for humans and animals. The plant has been used as a tonic in curing fever, constipation, and many illnesses in ancient times and in herbal medicine (Ugbogu *et al*., 2021). Tonics from this medicinal plant have been used for curing sexually transmitted diseases like the wart of fungi. The Congolese maximize *V. amygdalina's* medicinal potential by using it to treat cough and hemorrhoids (Nigatu et al., 2012). The leaves are frequently utilized in the treatment of malaria in Ethiopia. *V. amygdalina* is among the medicinally significant plants used against anti-microbial (Ugbogu *et al*., 2021; Habtamu & Melaku, 2018;Karunamoorthi & Tsehaye, 2012).

Vernonia species are resources of various classes of natural products with biological activities (Hemeg *et al*., 2020). The root of *V. amygdalina* is also used for treating gingivitis and

toothache due to its proven use in the cure of microbial activity (Adedapo *et al*., 2014) .Therefore, *V. amygdalina* is well known as a medicinal plant with several uses including for the treatment of diabetes, fever reduction, and recently for a non-pharmaceutical solution to persistent fever, headache, and joints pain associated with AIDS (an infusion of the plant is taken as needed) (Adedapo *et al.,* 2014). The investigators further remarked that *Vernonia* species are widely used in dietary supplements and have been investigated for the control of diseases caused by free oxidative like mention in *Moringa oleifera* (Gupta *et al.,* 2005) such as cancer, coronary heart disease, and even altitude sickness. Oxidative stress is an occurrence caused by a disproportion between the production of reactive species such as reactive oxygen species (ROS) or reactive nitrogen species (RNS), and the detoxifying proficiency of a biological system (Pizzino *et al.*, 2017).

Several scientific studies of *Vernonia s*pecies have found that as antioxidative, anti-inflammatory, and anticancer properties (Ugbogu *et a*l., 2021). Conventionally, technology Practices in herbal extracting and manufacturing processes for various pharmacotherapeutic (Table 2) uses depend upon the nature of the herbs of geographical origin, the organ to extract and distill botanical variety (Zenebe *et al*., 2015).

2.2.2 Pharmacological uses of *O. integrifolia*

Naturally, the species of *Otostegia* are medicinally decisive and used as an antimosquito repellent, antimicrobial, antihyperglycemic, anti-cancer, and antioxidant activity to prevent different kinds of sickness and disorders (Karunamoorthi, 2014). Besides, its chemical constituents have also been shown to possess antiulcer, antispasmodic, antidepressant, anxiolytic, and sedative activities (Chaudhary *et al*., 2009). Moreover, the genus *Otostegia* is used to treat children's gum diseases, ophthalmia in men, and curing wounds in men and breast (Shewamene,, 2015). *O. integrifolia* has insecticidal properties (Table 2) and is often widely used to as folk medicine and a fumigant for pots and houses (Karunamoorthi, 2014). In additional, the leaves of *O. integrifolia* are used for the treatment of several diseases like malaria. In an ongoing search for effective, safe, and cheap antimalarial agents from plants, the 80% methanol leaf extract of *O. integrifolia* was tested for its *in vivo* antimalarial activity (table 2) against *Plasmodium berghei* by the reputed antimalarial effect of *O. integrifolia* that may be attributed in

whole or in part to the presence of the diterpenoid and otostegindiol (Chekol & Desta, 2018; Karunamoorthi, 2014).

O. integrifolia species are resources of various classes of natural products with biological activities: antifungal, abortifacient, antidote (Hemeg *et al*., 2020; Tadesse *et al.*, 2012) and the roots of *O. integrifolia* are used for treating lung diseases (Karunamoorthi, 2014). Despite this fact, many medicinal plants are threatened with extinction and indigenous cultures are being disrupted and destroyed (Karunamoorthi, 2014) and today, *O. integrifolia* is one of them(Table 2). Moreover, there is an inextricable link between cultural and biological diversity.Therefore, *O. integrifolia* is well-known for its pleasant odor, omnipotent medicinal values, and insecticidal properties.

2.2.3 Pharmacological uses of *Salvia rosmarinus*

S. rosmarinus plants have always played an essential role in the history of medicine. There are more than 20 varieties (Hamidpour, 2017). *S. rosmarinus* is a Mediterranean woody evergreen shrub used as an ornamental plant across the planet. As a result, they have shown a significant contribution to the development of contemporary medicine (Veenstra & Johnson, 2021).

S. rosmarinus (*rosemary*) is commonly used for brain and nervous system conditions: it stimulates by having caffeine; cardiovascular conditions: it improves circulation, raises blood pressure, and stimulates the weak heart subject to palpitation when consumed in small doses; gastrointestinal circulatory systems: in conditions of bad breath, and stomach upset; promotes proper digestion, toning, and calming effect on the digestion; reproductive system conditions: stimulates the sexual organs; respiratory system: colds and colic; Other uses: the oils used as known that the secondary metabolites of plants have therapeutic effects; many have been used in the treatment of different diseases, such as obesity and skin diseases as well as in the treatment of non-pathological states such as aging (Malvezzi De Macedo *et al*., 2020)

S. rosmarinus (*rosemary*) has been extensively studied for its anti-microbial activity against Gram-negative and Gram-positive bacteria. According Oliveira, (de Oliveira *et al*., 2019) investigated the anti-microbial activity of rosemary extract(RE) (200 mg/mL in propylene glycol) against *C. albicans, S. aureus, E. faecalis, S. mutans,* and *P. aeruginosa* in planktonic

cultures and against biofilm formation (Albalawi *et al*., 2017). Rosemary extract showed the strongest activity against *C. albicans* with a minimum inhibition concentration (MIC), and a minimum microbicidal concentration (MMC) of 0.78 mg/mL and 3.13 mg/mL, respectively. Additionally, RE showed strong activity against *P. aeruginosa* with both an MIC and MMC of 6.25 mg/mL. *S. aureus, S. mutans,* and *E. faecalis* growths were inhibited at >25 mg/mL, but a microbicidal concentration was not reached. The study also revealed that RE was effective against biofilm formation of *C. albicans, P. aeruginosa, S. aureus,* and *S. mutans*. In poly-microbial films, *C. albicans* was cultured with the other four bacteria for 48 hours in study and treated with 200 mg/mL RE for 5 min.

The most significant reduction in microbial growth with RE occurred in *C. albicans* plus *E. faecalis* and *C. albicans* plus *P. aeruginosa* ($P \leq 0.0001$) (Veenstra & Johnson, 2021). Methanol and ethanol RE were also evaluated against eight different bacterial strains grown on agar, including *E. coli, S. aureus, P. aeruginosa, B. cereus, E. faecalis, C. albicans, V. fluvialis*, and *V. damsel* (Veenstra & Johnson, 2021). The methanol extract showed a higher degree of inhibition against bacterial growth (Table 2) compared to the ethanol extract. The authors noted that the presence of carnosic acid (CA) in the methanol extract was likely the reason for the increased anti-microbial activity. Several studies have associated the concentration of CA in RE with its effectiveness against microbial growth (Veenstra & Johnson, 2021) .

S. rosmarinus extract also has been intriguing anti-tumorigenic activity. In one study, the extract was found to strongly inhibit skin tumorigenesis in mice by preventing carcinogens from binding to epidermal DNA (Hamidpour, 2017). The author added to this anti carcinogenic effect is caused by the extract's antioxidant activity. Major compounds in the plant's extract such as carnosic acid, carnosol, and rosemarinic acid, have been shown to induce apoptosis within these cancer cells, possibly through the production of nitric oxide. These anti-proliferative and anti-tumorigenic activities of *R. officinalis* can be utilized in future cancer treatments(Table 2) and warrant further investigation (Hamidpour, 2017).

Table 2: Some pharmacological activities of studied medicinal plant species

Scientific name	Pharmacological activities	Plant part/extract	Reference
	Anti-microbial	Ethanol and acetone extracts of roots, stems and flower	(Ugbogu *et al*., 2021; Habtamu & Melaku, 2018;Karunamoorthi & Tsehaye, 2012)
	Antioxidative	Acetone extracts of leaves	(Ugbogu *et al*., 2021;Adedapo, 2014).
V.amygdalina	Anti-inflammatory	Acetone extracts of leaves	(Ugbogu *et a*l., 2021;Adedapo, 2014).
	Antinociceptive properties	Acetone extracts of leaves	(Adedapo, 2014)
	Antidiabetic effect	Aqueous extracts of leaves	(Michael *et al*., 2010)
	Anti-microbial activity	Ethanol extracts of leaves	(Asghari *et a*l., 2006)
O.integrifolia	Antimalarial activity	Methanol extracts of leaves	(Endale e*t al*.,, 2013)
	Antioxidative properity	methanol, and petroleum ether extracts of leaves	(Abate & Yaynie, 2018)
	Anti-inflammatory		
	Antidepressant property	Aqueous and n-hexane extracts of leaves	(Chaudhary *et al.*, 2009)
	Antioxidant and Antimicrobial Properties	Methanolic extract of leaves	(Dagnaw *et al*., 2023;El-Beltagi and Badawi, 2013)
S. rosmarinus	Lung carcinoma (A549)	Methanolic extract of leaves	(De Oliveira, 2019)
	Anti-oxidant	Aqueous extracts of leaves	(De Oliveira, 2019)
	Antidepressant property	Aqueous extracts of leaves	(Malvezzi De Macedo *et al*., 2020)
	Effect of Digestibility	Aqueous and alcoholic extracts of leaves	(Tawfeeq *et a*l,, 2019)
	Gastric carcinoma**(**AGS, cancer cells)	Methanol extracts of leaves	(Karimi, 2017)

2.3 Phyotochemicals reported in the studied plant species

2.3.1 Phytochemicals of the *V.amygdalina*

Phytochemicals are naturally found by application of extractions to human beings and found in the body parts of herbal medicinal plants such as in leaves, stems, barks, and roots that have defense mechanisms and protect from various diseases. Phytochemicals are subdivided into primary and secondary metabolites. Chlorophyll, proteins, and common sugars are included in primary constituents, and secondary metabolites are tannins, flavonoids, quinones, alkaloids, glycosides, saponins, steroids, and terpenoids. According to Para Sujana, et al (2013), the secondary metabolites are classified as phenolic compounds (tannins, quinone, flavonoids, flavonols and flavones, xanthones, and stilbenes); the nitrogen compounds(alkaloids, chlorophylls, indoles, amino acids, amines, cyanogenic glycosides, purines, pyrimidines and cytokinins); and terpenoids (essential oils, carotenoids, diterpenoids and gibberellins, triterpenoids and steroids). Investigations of medicinal plants should be carried out because these secondary metabolites are responsible for the medicinal activity of the plants (Table 2). The object of phytochemicals and herb plant chemistry has developed in recent years as a distinct discipline, somewhere between natural product organic chemistry and plant biochemistry, and is closely related to both. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by herb plants. Phytochemicals deal with the chemical structures of these substances, their biosynthesis, metabolism, natural distribution, and biological function. Phytochemicals are found abundantly in the leaves of *V. amygdalina* (Figure 5)

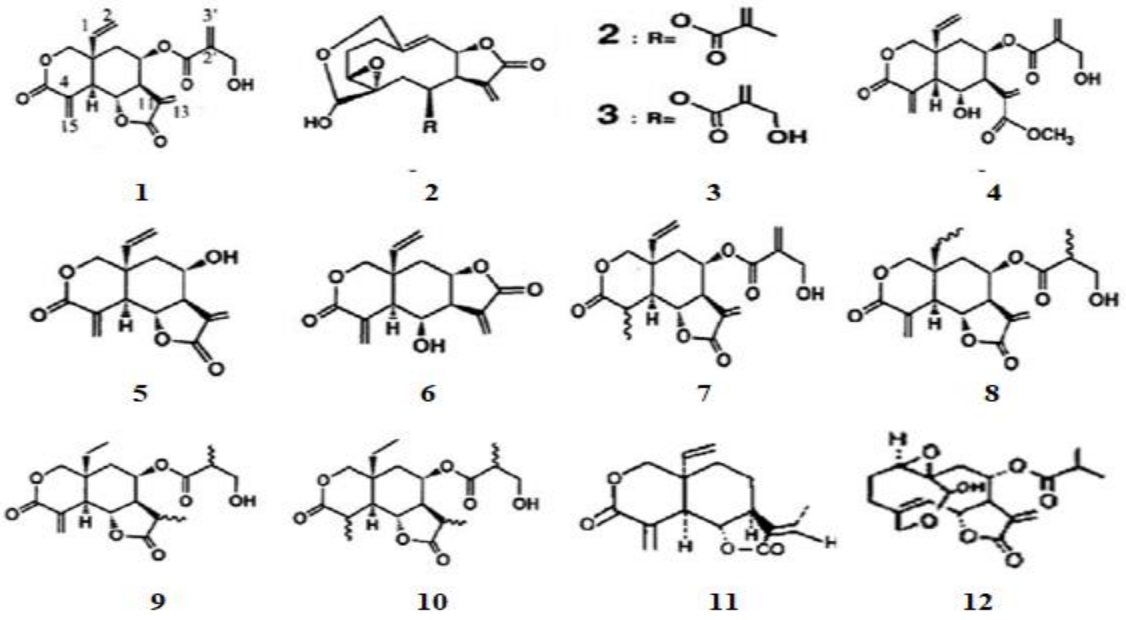


Figure 5: Structures of some phytochemicals found in the leaves of *V. amygdalina* (Yeap *et al*., 2010)

[Compounds **1**-vernodalin; **2**-Vernolide; **3**-hydroxyvernolide; **4**-vernodalol; **5**-vernolepin;**6**-Vernomenin; **7**-dihydrovernodalin; **8**-tetrahydroovernoodalin;.**9**-hexahydrovernodalin; **10**-octahydrovernodalin; **11**- dihydrovernodalin; and **12**- vernomygdin]

Leaves of *V. amygdalina* plant constitutes sesquiterpene lactones such as vernolide, vernodalol, vernolepin, vernodalin, hydroxyvernolide and flavonoids luteolin, luteolin 7-O-β-glucoroniside and luteolin 7-O-β-glucoside (Yeap *et al.*, 2010).

2.3.2 Phytochemicals of the *O. integrifolia*

Phytochemicals are found abundantly in the leaves of *O.integrifolia* (Figure 6). The presence of different bioactive compounds such as flavonoids, phenols, terpenoids, saponins, steroids, and glycosides of theirs high polarity. However, alkaloids and tannins were absent in all extracts of the leaf parts of the plant (Chekol & Desta, 2018) in different solvent extracts (Methanol extract, ethyl acetate and petroleum ether) of the leaves of *O. integrifolia* were reported using qualitative tests.

Similarly, other researchers reported 40 different constituents, including monoterpenes, sesquiterpenes, diterpenes, and their derivatives essential oil and chloroform extract from air-

dried leaves of *O. integrifolia* (Karunamoorthi, 2014). Among these compounds, the five of them such as axinyssene, otostegindiol, pretostegindiol, pentatriacontane, and stigmasterol are observed to be the potential sources of pharmaceutics and medicine and used to treat various illnesses (Figure 6).

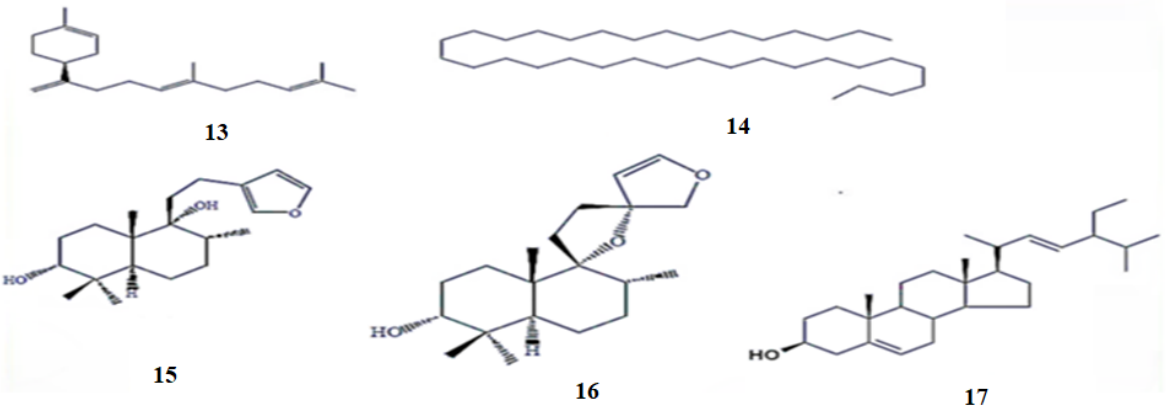


Figure 6: Structures of some phytochemicals found in the *O. integrifolia* plant (Karunamoorthi, 2014)

[Demonstrated that compounds **13**-Axinysene; **14**-Pentatriacontane; **15**-Otostegindiol; **16**-Preotostegindiol; and **17**-Stigmasterol]

2.3.3 Phytochemicals of the *Salvia rosmarinus*

Phytochemicals found abundantly in the leaves of *S. rosmarinus* contain secondary metabolites (Figure 7) with therapeutic potential, such as carnosol and carnosic, rosmarinic, ursolic, oleanolic, and micromeric acids (Figure 7). These compounds have been applied topically (used on the skin) and studied for their anti-inflammatory capacity, wound-healing potential, effects on tissue survival, anti-skin-cancer effects, antinociceptive effects, antifungal effects, and UV-protective activity. The triterpenes ursolic, oleanolic, and micromeric acids exhibit the strongest anti-inflammatory activity of all the secondary metabolites (Malvezzi De Macedo *et al.*, 2020; Carbone *et al.*, 2019). In addition to the gross extracts, it is possible to use rosemary essential oil for topical applications (Pintore *et al.*, 2002) . The authors further indicated that the structure of the major constituents of the oil are β-pinene, 1, 8-cineole, borneol, camphor, limonene, and verbenone.

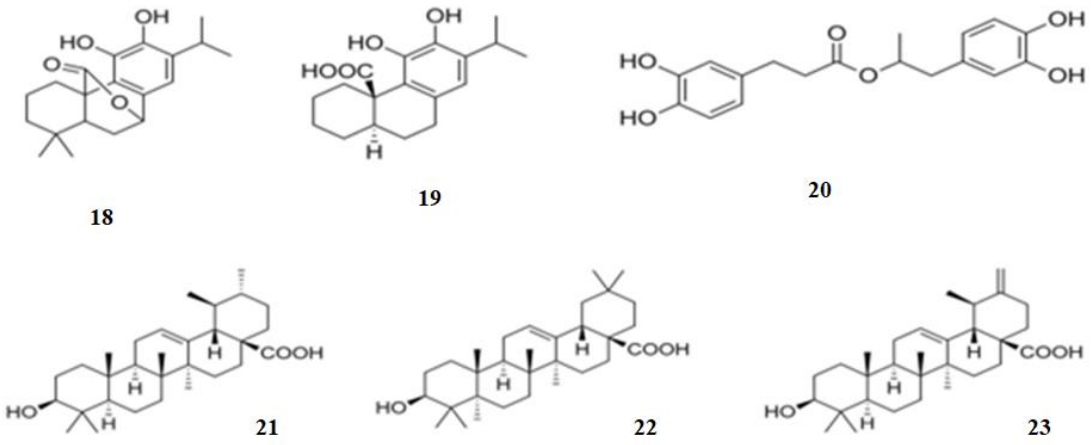


Figure 7: Structures of some phytochemicals found in the leaves of *S. rosmarinus* (previously called *R. officinalis*) secondary metabolites (Malvezzi De Macedo *et al.*, 2020):

[Figure 7 demonstrated that compounds **18**-carnosol, **19**-carnosic acid, **20**-rosmarinic acid, **21**-ursolic acid, **22**-oleanolic acid, and **23**-micromeric acid].

2.4 Extraction of phytochemical (bioactive components)

Phytochemical refer to the massive number of primary and secondary metabolic compounds present in plants (Figure 8). Plants are the reservoirs of naturally occurring chemical compounds and structurally diverse bioactive molecules. The biologically active compounds that are present in plants are known as phytochemicals. These phytochemicals are derived from different parts of plants like leaves, barks, seeds, seed coats, flowers, roots, and pulps, and are used as sources of direct medicinal agents. The reason is why; plants are recognized in the pharmaceutical industry is due to their broad spectrum of structural diversity, geographical origin, harvest time, age of the plant, soil type, and extreme conditions, and their wide range of pharmacological activities can affect the quantity and quality of the chemical composition of the plants. The extraction of bioactive compounds from the plants and their quantitative and qualitative estimation is utilized for the exploration of new biomolecules to be used by the pharmaceutical and agrochemical industry directly or can be used as a lead molecule to synthesize more potent molecules (Altemimi *et al*., 2017).

Extraction is the crucial first step in the analysis of medicinal plants because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization (Sasidharan *et al.*, 2011). The basic operation steps included pre-washing, air drying of plant materials, and grinding to obtain a homogenous sample often improving the kinetics of analytic extraction and increasing the contact of the sample surface with the solvent system. Proper actions must be taken to ensure that potential active constituents are not lost, distorted, or destroyed during the readiness of the extract from plant samples. The selection of a solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol, or ethyl-acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some instances, extraction with hexane is used to remove chlorophyll (Sasidharan *et al*., 2011).

General methods of extraction of medicinal plants are maceration, infusion, digestion, decoction, percolation, hot continuous extraction (Soxhlet), etc. On the other hand, extraction steps (Zenebe *et al.*, 2015;Hashimoto, 2011) stated that for medicinal ingredients from plant material, the following sequential steps are involved, size reduction, extraction, filtration, and concentration drying. The current study will also follow and use the standards of general methods for the specifics of separation phytochemicals for constituents of the leaves of *V.amygdalina, O. integrifolia*, and *S. rosmarinus*.

According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries and involving 12 mega-biodiversity countries (Sasidharan *et al*., 2011) like Brazil, China, Colombia, Costa Rica, India, Indonesia, Kenya, Mexico, Peru, the Philippines, South Africa and Venezuela. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of the bioactive compound, toxicological evaluation, and clinical evaluation.

Extraction is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites and leave the insoluble cellular marc. The purpose of standardized extraction

procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstruum. The extract thus obtained, after standardization, may be used as a medicinal agent in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form as tablets and capsules. These products all contain a complex mixture of many medicinal plant metabolites (phytochemical analysis): such as alkaloids, flavonoids, quinones, saponins, steroids, tannins, terpenoids, and glycosides-lignans (vallinols)(Figure 8). Phytochemical analysis involves both qualitative and quantitative analysis. While qualitative analysis shows the presence or absence of a phytochemical, quantitative analysis accounts for the quantity or the concentration of the phytochemical present in the plant sample (Ezeonu & Ejikeme, 2016). To be used as a modern drug, an extract may be further processed through various biotechnology methods to isolate individual biochemical entities (Kothiyal *et al*., 2019 ; Hashimoto,2011).

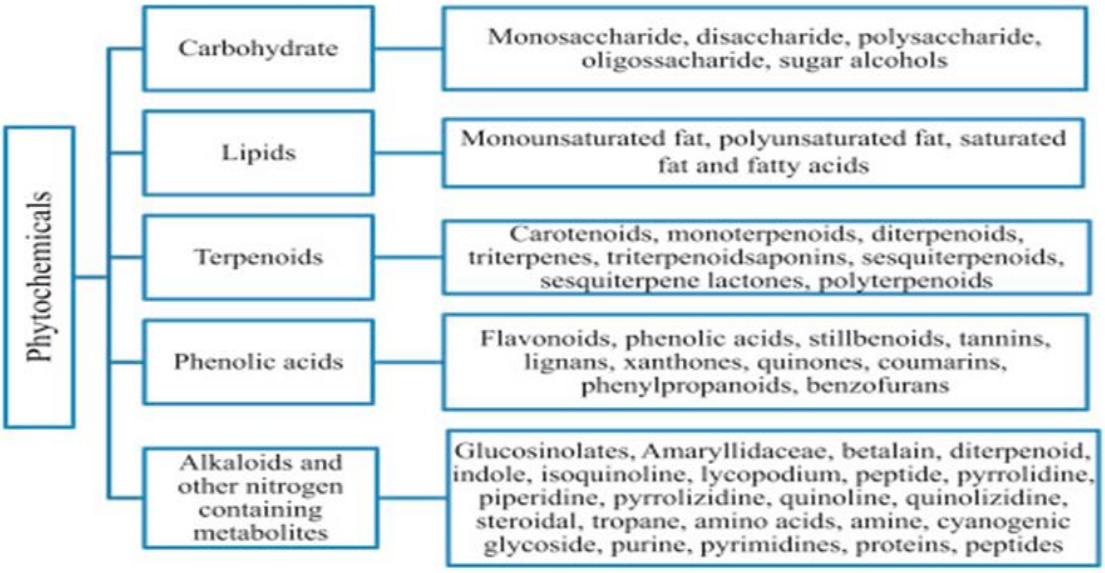


Figure 8: Classification of phytochemicals in food technology (Huang *et al.*, 2016)

2.5 Infectious diseases

Infectious diseases are illnesses caused by the invasion and multiplication of pathogens like microbes. The microbial world is vast, diverse, and dynamic (Alberts, *et al* 2002). The Earth hosts over 10^{30} microorganisms, representing the largest component of the planet's biomass. Microbes include bacteria, archaea, mollicutes, fungi, microalgae, viruses, and protozoa, and many more organisms with a wide range of morphologies and lifestyles. All other life-forms

depend on microbial metabolic activity. Microorganisms have colonized virtually every environment on earth ranging from deep sea thermal vents, polar sea ice, desert rocks, guts of termites, roots of plants, to the human body (Alberts *et al* 2002). The human body is a complexe perform most cell functions and thriving ecosystem. It contains about 10^{13} human cells and also about 10^{14} bacterial, fungal, and protozoan cells, which represent thousands of microbial species (Prakash *et al.*, 2012). These microbes, called the normal flora, are usually limited to certain areas of the body, including the skin, mouth, large intestine, and vagina.

However, pathogens are usually distinct from the normal flora.Commonly, infectious diseases are caused by pathogens, which include bacteria, fungi, protozoa, worms, viruses, and even infectious proteins called prions (Alberts *et al.*, 2002). They have developed highly specialized mechanisms for crossing cellular and biochemical barriers and for eliciting specific responses from the host organism that contribute to the survival and multiplication of the pathogen. Therefore, in order to survive and multiply in a host, a successful pathogen(Prakash *et al .*, 2012) must be able to: (1) colonize the host; (2) find a nutritionally compatible niche in the host body; (3) avoid, subvert, or circumvent the host innate and adaptive immune responses; (4) replicate, using host resources; and (5) exit and spread to a new host. Under severe selective pressure to induce only the correct host cell responses to accomplish this complex set of tasks. Then, pathogens have evolved mechanisms that maximally exploit the biology of their host organisms, and the host may need synthetic and natural drugs to motivate the immune system to overcome the pathogens.

2.6 Oxidative stress

Oxidative stress is an imbalance in the body where there are too many free radicals (unstable molecules) and not enough antioxidants to neutralize them. This imbalance can lead to cellular and tissue damage, potentially contributing to chronic diseases and aging. Unstable molecules are chemical substances whose atoms remove at least one electron from another atom in a chemical reaction. As per this definition, oxidizing agents are the reactants that undergo reduction in redox reactions. Oxidizing agents are also vital to many biological processes such as metabolism and photosynthesis. Oxidizing agents are O_2, O_3 , hyderoxyl,carbonyl and X_2

(halogens) .Oxidizing agents when increased in the body's blood and intracellular fluids cause precursors of diabete, cancer and inflammation (Hamidpour, 2017) .

Our lifestyle and environmental factors affect us, such as poor diet, excess weight, lack of exercise, stress, smoking, pollution and excessive alcohol consumption can also lead to chronic inflammation,cancer and diabete and so on. Our diet has a huge effect on how healthy we are. A diet that is overloaded with highly processed carbohydrates (sugar, flour and all the products made from them) and the excess consumption of trans fats that are found in many processed foods are contributing to chronic inflammation in the body. When we consume simple carbohydrates such as sugar, our blood sugar rises rapidly. In response, your pancreas secretes insulin; a hormone that prompts cells to absorb blood sugar for energy or storage. If the cell is full and does not need glucose, the full cells reject the extra glucose, blood sugar rises producing more insulin and the glucose converts to be stored fat and free radicals like –OH, O, and NO and -SH to converts to formed precursors of inflammation, cancer, diabete and stress in our body (Li *et al.*, 2024; Hamidpour, 2017). Therefore, the consumption of *V.amygdalina* (Ugbogu *et al*.,2021) , *O.integrifolia* (Tadesse *et al*., 2012) and *S.rosmarinus* (Hamidpour, 2017) plants materials in cancer prevention at early is believed to contribute immensely to the improvement of the health of human beings may be occuried.

2.7 Inflammatory diseases

Inflammatory diseases are conditions where inflammation, the body's natural response to injury or infection.Accordingly, inflammation is part of the body’s immune response. It’s the defense mechanism of body tissue reacting to kick start the healing process (Bunnett, 2006). It is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. There are 2 types of inflammation: acute inflammation and chronic inflammation. Under acute inflammation examples are sore throat (cold or flu), skin blister, appendicitis, gastric ulcer, injury to body or a scratch/cut to the skin and chronic inflammation examples are arthritis, heart disease, asthma, allergies, crohn’s disease, diabetes, cancer, neurological and autoimmune diseases **.** Inflammatory states invariably induce hypoalbuminemia as a consequence of increased capillary escape of serum albumin and other plasma solutes into the interstitium and into cells. The primary signs of inflammation include: pain, heat, swelling,

redness and loss of function (Bunnett, 2006). This is associated with an increased volume of fat-free mass due to increased total water content of serum, interstitium, and, possibly, cells. In longstanding conditions, blood volume may be normal, but plasma volume increases due to diminished red cell mass. Together, fat-free mass constitutes by far the largest part of the distribution volume of albumin leading to hypoalbuminemia(Thalacker-Mercer *et al.*,2007).

Whereas the fractional synthesis rate (FSR) of albumin in plasma is increased in hypoalbuminemia, absolute synthesis rates may not increase due to a shorter turnover time and/or fecal/urinary losses of albumin, which have a lowering influence on whole body albumin mass (Thalacker-Mercer *et al.*,2007). As a result, the drop in serum albumin concentration may not be compensated by the increase in the FSR of serum albumin. An increase in serum albumin FSR appears to be a beneficial response in inflammatory conditions in which an immune response, cell proliferation, tissue healing, and growth are required. Here, albumin plays a scavenging and antioxidative role in the interstitial space. In cells, albumin can also be degraded at an accelerated rate, providing amino acids as building blocks for cell proliferation and matrix deposition. Low serum albumin (secretion from liver cells) levels are, therefore, an indicator of the severity of inflammation (Soeters *et al* 2019). Preexisting inflammation is an important factor interfering with the success of medical and surgical treatment, diminishing the adequacy of the response to trauma and disease and reducing quality of life and longevity. In critical illness, spontaneous rises or decreases in serum albumin levels from the range 3.4-5.4 g/dL and the accompanying shrinking (weight loss) or increasing (weight gain) total (Soeters *et al* 2019).

On the other hand, proteolytic enzymes (proteases) are enzymes that break down protein and it has role in inflammation processes. Proteolytic enzymes break down proteins in the body or on the skin. This might help with digestion or with the breakdown of proteins involved in swelling and pain. Some proteolytic enzymes that may be found in supplements include bromelain, chymotrypsin, ficin, papain, serrapeptase, and trypsin. Proteolytic enzymes are used for a long list of conditions including cleaning wounds on the skin, help with digestion, pain and swelling, and many other conditions during inflammation (Bunnett, 2006; Thalacker-Mercer *et al.*,2007).

2.8 Cancer

The process by which normal, healthy cells transform into cancer cells is termed carcinogenesis or oncogenesis. Cancers start when cells in the body begin to grow out of control (Adams *et al*., 2007). Cancer with a heavy economic and social burden is a hot point in the field of medical research (CDC, 2020; Adams *et al*., 2007). Some remarkable achievements have been made; however, the exact mechanisms of tumor initiation and development remain unclear. Cancer is a complex, whole-body disease that involves multiple abnormalities in the levels of DNA, RNA, protein, metabolite, and medical imaging. In this study, we were tested and used *in vitro* the anti-cancer properties of leaf extract of *V. amygdalina*, *O. integrifolia*, and *S. rosmarinus* to validate its medicinal importance. According Kamiloglu, *et al* (2020), cell viability assays are very influential and used for screening the response of the cells against a drug or a chemical agent. In particular, the pharmaceutical industry widely uses viability assays to evaluate the influence of developed agents on the cells. Cancer is a whole-body disease and a global burden disease(IHME, 2019). It has needed special attention, for example, when we look cervical cancer, it is a common cancer in women and a prominent cause of death (Sul'ain, 2019). In Ethiopia, cervical cancer is a big deal for women aged 15 to 44, coming in as the second most common cancer (ICO, 2023).

2.8.1 Cervical cancer

Cervical cancer is the third most common cancer associated with breast cancer in women worldwide(IHME, 2019). The burden of this cancer with continuing improvement in screening methods and vaccination programs in developed countries, and the disparity of burden between women in developed countries and women in resource-poor settings become even more profound. Currently, >85% of cervical cancer deaths occur in low and middle-income countries. Tragically, cervical cancer is the leading cause of cancer deaths in women in the developing world (Rhee *et al*., 2000). The author has further remarked that aberrant methylation of tumor-suppressor genes' promoters can shut down their important functions and play a big role in causing cervical tumors.

There are various cervical cancer repressor genes (proteins turn off or reduce gene expression from the affected gene) such as CCNA1, CHF, HIT, PAX1, PTEN, SFRP4, and TSC1. The genes play a crucial role in causing cervical cancer by regulating transcription and expression through promoter hypermethylation, leading to precursor lesions during cervical development and malignant transformation (Siegel, 2022). The process of DNA methylation is primarily carried out by a group of enzymes known as DNA methyltransferases (DNMT1). It has been reported that DNMT1 (PDB ID: 4WXX), a protein responsible for DNA methylation, can contribute to the development of cervical cancer. DNMT1 inhibits the transcription of tumor suppressor genes, facilitating tumorigenesis, which finally develops into cervical cancer. Tumor suppressor gene transcription is inhibited by DNMT1, which helps cancer grow and eventually leads to cervical cancer. Repressive genes' hypermethylation may be decreased, their expression can be increased, and the phenotype of malignant tumors can be reversed by inhibiting the DNMT1 enzyme.

However, new technologies have been recently developed for more rapid, cost-effective, and sensitive cervical cancer screening. This study of leaf extracts of *V. amygdalina*, *O. integrifolia*, and *S. rosmarinus* may be cheap, found everywhere, and weigh up which has the potential to promote greatly lower cervical cancer incidence in the developing world. Nearly all cases of cervical cancer can be connected to infection with human papillomavirus (HPV). HPV types are divided into low-risk or high-risk strains depending on their oncogenic potential. Low-risk strains of HPV may be asymptomatic or may cause anogenital warts, whereas high-risk strains are oncogenic. Over 99% of precancerous lesions (cervical dysplasia) and cervical carcinomas occur by high-risk HPV infection (Kumar *et al*.,2015). More than 200 strains of HPV have been identified, of which approximately 40 infect the anogenital region(Rodríguez *et al*., 2008).
. The authors further remarked that a 15 to 18 HPV strains have been classified as high-risk genotypes. In addition, authors have remarked that virtually all cervical neoplasias and cancers are attributable to high-risk HPV genotypes, and approximately 70% of all cervical cancer cases are attributable to types 16 and 18.

Superfluous , infection by HPV type 16 E6(PDB ID: 4XR8) has been correlated with a greatly increased genital risk of precursor cervical cancer worldwide (Silva *et al*., 2020; Mok, 2009),and

responsible for 50% of squamous cell carcinomas and 55-60% of all cervical cancers, whereas type 18 causes about 20% of cervical adenocarcinomas. Other oncogenic strains of HPV include types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, which combined cause 25% of cervical carcinomas (Kumar *et al.*,2015). Infection with certain HPV types causes a proportion of cancers of the anus, vulva, vagina, penis, and oropharynx as well (CDC, 2020). Almost all cervical cancers through anatomical body structures are either squamous cell carcinoma or adenocarcinoma (Rodríguez *et al.*, 2008). The main steps known to be necessary for cervical carcinogenesis include HPV infection, HPV persistence, progression to dysplasia, and invasion (CDC, 2020; Kumar *et al.*,2015).

One potential approach to treat cervical cancer is to inhibit the activity of the DNMT1 and HPV type 16 E6 enzymes specifically (Brooijmans and Kuntz, 2003). Over 50% of clinical drug forms worldwide originate from plant compounds (Yoshino *et al.*, 2023). In the past, developing new drugs was a lengthy and costly process. However, with the emergence of bioinformatics, the use of computer-based tools and methods has become increasingly important in drug discovery. One such method is molecular docking and ADMET profiling which involves using the structure of a drug to screen for potential candidates. This approach is known as structure-based drug design and can save both time and resources during the research process(Silva *et al.*, 2020; Rhee *et al*, 2009; Brooijmans and Kuntz, 2003). Structural-based drug designing addresses ligand binding sites with a known protein structure (Brooijmans and Kuntz, 2003). Using free binding energies, a computational method known as docking examines a large number of molecules and suggests structural theories for impeding the target molecule (Yoshino *et al.*, 2023). Therefore, the purpose of this research is to assess the antimicrobial activity of extracts, molecular docking and ADMET Profiling in anticancer properties of compounds isolated from the medicinal plants, on a targeting DNMT1 and HPV type 16 E6 in human cervical cancer.

2.8.2 Types of cervical cancer

Cervical cancers and cervical pre-cancers are classified by how they look in the labs with a microscope. The main types of cervical cancers are squamous cell carcinoma and adenocarcinoma. Most (up to 9 out of 10) cervical cancers are squamous cell carcinomas. These cancers develop from cells in the exocervix. Squamous cell carcinomas most often begin in the

transformation zone (where the exocervix joins the endocervix). Most of the other cervical cancers are adenocarcinomas. Adenocarcinomas are cancers that develop from glandular cells. Cervical adenocarcinoma develops from the mucus-producing gland cells of the endocervix. Less commonly, cervical cancers have features of both squamous cell carcinomas and adenocarcinomas. These are called adenosquamous carcinomas or mixed carcinomas. Although almost all cervical cancers are either squamous cell carcinomas or adenocarcinomas, other types of cancer also can develop in the cervix. These other types, such as melanoma, sarcoma, and lymphoma cancer occur more commonly in other parts of the body (Kumar *et al*.,2015).

2.8.3 The prospect of cervical cancer

Cancer of the cervix is the most common cancer and the leading cause of death by cancer in women in developing countries (Islami *et al.*, 2024). Cervical cancer is caused by human papillomavirus (HPV) contracted through sexual activity, especially at a young age (ICO, 2023) . HPV can cause pre-cancerous cells in the cervix, which can turn into cervical cancer within 10-20 years if left untreated(Fahad and Shameem, 2018). According to the World Health Organization (WHO) cancer is one of the leading causes of death worldwide, which accounted for 7.6 million deaths (around 13%) of the world's population in 2008 (Islami *et al.*, 2024). They have furthermore estimated that the worldwide deaths are likely to rise to over 11 million in 2030 (Apollinaire *et al*., 2016; Berrington & Lall, 2012). Cervical cancer is caused by the Human Papilloma Virus (HPV) which forms warts in the throat and genital area. Although it is the most prevalent cancer in African black women it is also the most preventable cancer through screening and treatment of precancerous lesion and the use of vaccines. Natural products are being tested for the treatment of cancer as conventional cancer treatments such as chemotherapy that destroys cancerous as well as healthy cells. However, natural products are being tested for cancer treatment as conventional therapies damage healthy cells. *V. amygdalina*, *O. integrifolia*, and *S. rosmarinus* were utilized to treat edema, wounds, tumors, and tumor necrosis with no side effects because they are edible. Antioxidants in aromatic plants and spices stabilize free radicals that cause diseases like cancer, diabetes, and heart attacks. They can also alleviate issues like weight changes, dyslipidemia, cerebral ischemia, and hepato-nephrotoxicity (Abate & Yayinie, 2018). Essential oils (EOs), extracted from *V. amygdalina, O. integrifolia,* and *S. rosmarinus* leaves treat health problems such as malaria, coughs, worms, infections, colon wounds, and

rheumatism. Ethiopian women use the plant after childbirth to wash their bodies, treat them as antimicrobial, and minimize wounds (Busse and Tefera 2013). They have anti-inflammatory and antioxidant properties that can lower blood sugar levels and inhibit thyroid function (Chekol & Desta, 2018).

The plant (*Combretum caffrum*) more commonly known as the "bush willow" causes the shutdown of tumours and tumour necrosis (Cragg & Newman, 2005). Herbs and spices have been used for thousands of years as medicines. Through scientific research it is indicated that the bioactive components present in herbs and spices can reduce the risk of cancer through their antimicrobial, antioxidant, and antitumorogenic activity and their ability to directly suppress carcinogen bioactivation (Beecher, 1999). Herbs and spices were tested for their cytotoxic activity on cervical cancer (*HeLa*) cells (immortal cell line used in scientific research the first cells that could be easily shared and multiplied in a lab setting). Primary prevention of cervical cancer is essentially based on healthy lifestyles and HPV vaccination.

2.9 *Acanthamoeba* species

Acanthamoeba spp. are free-living amoebas present in the environment, particularly in soil and water (Khan *et al*., 2019). Several species of *Acanthamoeba* have been characterized (Chelkha et al., 2020), and most of the human pathogenic species are classified into T4 genotype, for example, *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, and *Acanthamoeba triangularis* (Chelkha *et al*., 2020). Free-living amebae belonging to the genus *Acanthamoeba* are protozoa ubiquitously in nature such as water and soil. The protozoa are causative agents of several diseases including granulomatous amebic encephalitis (Kalra *et al*., 2020; Mitsuwan *et al*.,2020) and amoebic keratitis (Jercic *et al*., 2019).

The occurrence of *Acanthamoeba keratitis* in contact lens users can cause severe vision loss and complete blindness (Jercic *et al*., 2019). In addition, the infection caused by the organism is severe in immunocompromised patients. *Acanthamoeba* species has only two stages in its life cycle, cysts and trophozoites. The trophozoite measures between 25-50 µm and can be recognized by the presence of slender, spine-like projections called acanthopodia. It has a single nucleus containing a dense nucleolus and reproduces by binary fission. It feeds on a variety of

organisms such as bacteria, fungi, and other protozoa (Chelkha *et al.*, 2020). Trophozoite is a vegetative amoeba form moving by amoeboid locomotion. Cyst form is dormant stage that survives in harsh environment conditions such as lack of nutrients.

Acanthamoeba cysts are classified into three groups including astronyxids, polyphagids, and culbertsonids (Anwar *et al*., 2018). The cysts contain two strong layers of cyst wall including ectocyst and endocyst walls. *Acanthamoeba* cysts have been reported to resist to antimicrobial substances (Strassmann and Shu, 2017;Coulon *et al*., 2010). It affects the eye, skin, and central nervous system. The resilient cyst form can survive for years under the most adverse conditions such as extreme temperatures, pH, drying, and chemical exposures. The trophozoites are the infective form, however, both cysts and trophozoites can gain entry into the body through a variety of means (Chelkha *et al*., 2020).Therefore, the treatment of *Acanthamoeba* infections is difficult due to its double walled cyst layers. Plant-derived compounds have been focused to treat *Acanthamoeba* infection.

So far, several drugs have been approved by the United States Food and Drug Administration, but standard therapeutic management of *Acanthamoeba*-infected patients is not yet available (Khan *et al*., 2019). The two most commonly used first-line drugs for *Acanthamoeba* treatment, especially in *Acanthamoeba* species (its life cycle, cysts and trophozoites), still use chlorhexidine and polyhexamethylene biguanide. However, identifying new compounds and screening natural extracts for amoebicidal activity are still attractive approaches for further studies. It could provide an alternative drug for *Acanthamoeba* treatment or be used as a complementary treatment for *Acanthamoeba* infection in the future.

CHAPTER- 3

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 General

All the NMR (^{1}H, ^{13}C and DEPT-135) experiments were performed on a BRUKER ACQ 400 AVANCE spectrometer operating at 400 MHz (for ^{1}H) and 100 MHz (for ^{13}C and DEPT-135) equipped with a 5 mm proton probe under a temperature of 298 K with topspin software (version 2.1, USA). All chemical shifts (δppm) of the spectra were recorded relative to an internal TMS reference. Obtained spectral data after acquisition were further processed using MestReNova software (Mestrelab Research S.L., version 12).In the GC-MS instrumentation, the GC analyses were done on 7890B (Agilent Technologies, USA) equipped with an HP 5MS non-polar column (internal diameter of 30 m × 250 μm and 0.25 μm of film thickness). Helium was used as carrier gas with a flow rate of 1 mL/min. The injector temperature was set at 230°C and the injection mode was a split mode with 10:1 of split ratio. The oven temperature was initially set at 40°C for 5 min and was elevated to 250 °C with a rate of 6 °C/ min held for 20 min. The total run-time was 60 min. The MS part was run on 5977A Network (Agilent Technologies) and operated in the EI mode at 70 eV. All spectra were scanned from 50-650 m/z range and compounds were identified by comparing their mass spectra with those possible compounds searched from the database NIST11 (National Institute of Standards and Technology, Gaithersburg, USA). Relative amounts of identified compounds were calculated based on the peak areas of the total ion chromatograms (TIC).

3.1.2 Chemicals, Apparatuses and Instruments

3.1.2.1 Chemicals

Thin Layer Chromatography (TLC) visualizing reagents (iodine vapor, Wagner's reagent, vanillin/MeOH/H_2SO_4), analytical grade organic solvents (n-hexane, petroleum ether , chloroform/methanol (1:1), and methanol), organic reagents bovine albumin fraction, 10% fetal bovine serum (FBS, trypsin-EDTA, inorganic reagents carrier gas (helium) , Tris HCl buffer (pH

7.4), 70% per chloric acid ($HClO_4$), MEM with trypsin, MTT, Dulbecco's modified Eagle's medium (DMEM), culture media (PYG medium, MHA , PDA etc.), and others (trichloroacetic acid, DMSO, 5% CO_2, Gel-type baits formulation standard, 0.5% Tween 20 standard, Ketoconazole standard, Cisplatin standard, Aspirin standard, Gentamycin synergized Azole standard, Ciprofloxacin standard, 230-400 mesh size silica gel, ascorbic acid, acetic acid, DPPH free radical, Phytochemical screening tests (used throwaway mercuric chloride and potassium iodide in water (Mayer's reagent) , chloroform, Conc.H_2SO_4 , glacial acetic acid, ferric chloride , methanol/ethanol,KOH,or NaOH, conc. HCl, acetic anhydride , chloroform, alcoholic α-naphthol, lead acetate, and H_2O), anhydrous sulphate, were used.

3.1.2.2 Apparatuses and instruments

The apparatus and instruments used in this study include: Using Column chromatographic (CC), Pre-coated aluminum, thin layer chromatography (TLC) sheet silica gel 60 F254 (Merck), TLC chamber, waring commercial laboratory blender (Torrington, CT., USA), suction filtration and Petri plates, digital melting point (SMP10, Bibby Scientific, UK), RPM-orbital shaker (Hy-5A, Movel Scientific Instrument CO. Ltd., China), rotary evaporator (Rotary vacuum, Jainsons, India), incubator (Binder B28, Germany), autoclave (tuttnauer 3150EL, Israel), UV- lamp cabinet (UVP Chromato-Vue C-70G, Analytik Jena, USA), UV-Vis spectrophotometer (517nm using a UNICO CE4001 UV/VIS Cecil, Cambridge, England), inverted microscope (Biolinkz, India), EI GC-MS spectrometry (7890B and 5977A, Agilent Technologies, USA), ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopy were employed.

3.2 Methods

3.2.1 Description of the study area

The medicinal plant materials and leaves samples were gathered from the Becho, Southwest Shewa Zone in the Oromia Regional State of Ethiopia. Becho is situated in the Southwest Zone of the Showa and is approximately 80 km away from the route to Jimma from Addis Ababa. The coordinates of the area are 8°40'N and 38°13'E, with an altitude of 2193 meters above sea level in the Oromia region, as shown in Figure 9 (Becho woreda,2015).

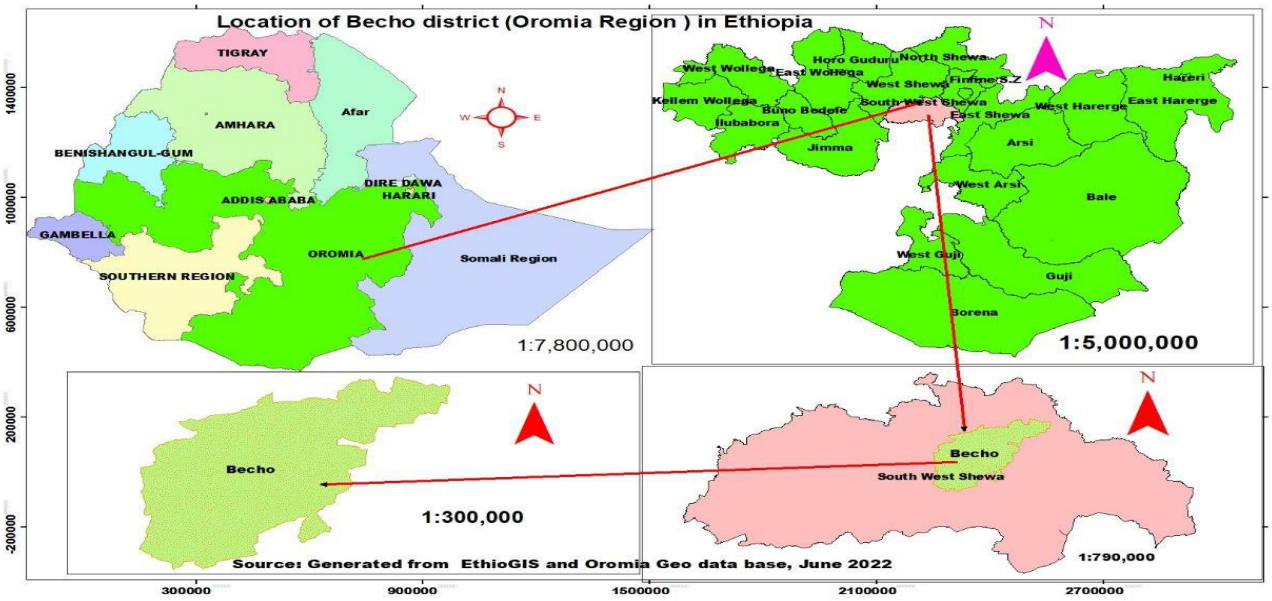


Figure 9: Map of study area (Becho) from where *V. amygdalina*, *O. integrifolia*, and *S. rosmarinus* leaves Samples were collected (Becho woreda,2015).

3.2.2 Collection and authentication of selected plant materials

In November 2022, during the dry season, healthy upper (airial part) plant materials were collected from three different plants-namely *Vernonia amygdalina*, *Otostegia integrifolia*, and *Salvia rosmarinus* in Becho distinct (8°40'N38°13'E), Southwest Shewa Zone, Oromia, Ethiopia. Leaves are the candidates of samples which have contained a high concentration of bioactive compounds and relatively easy to dry, which can be beneficial for preserving the extracted compounds. From the official data of Becho woreda (2023) found that vertisols (black soils) are the dominant up about 85% of the soils. Physically, black soil is characterized by a high content of clay and organic matter, precipitation leading to excellent water holding capacity and a deep cracking pattern during dry periods. Chemically, black soil boasts high levels of nitrogen, phosphorus, and other essential nutrients, making it a fertile soil type. The moisture content was recorded as 0.012±0.15 g water/g dry samples. The plant materials were authenticated by botanist at National Herbarium, Addis Ababa University and were given voucher numbers 3/2-2/MD001-80/8060/15, 3/2-2/MD002-80/8060/15, and 3/2-2/MD003-80/8060/15 for *Vernonia amygdalina*, *Otostegia integrifolia*, and *Salvia rosmarinus* respectively. The collected plant specimens were then deposited in Addis Ababa University's National Herbarium. The dried plant

samples were ground into powder with electrical blender. Powdered samples of the three plants were stored in an airtight container and kept at 4^{o}C refrigerator until extraction process was commenced.

3.2.3 Experimental Sites

The solvent extraction, phytochemicals screening tests, isolation of compounds, chromatographic techniques(CC), UV-V, and GC-MS were conducted in the Laboratory of the Department of Applied Chemistry and Biology at Adama Science and Technology University (ASTU), and antimicrobial activities were carry out at the Laboratory of the Department of Biology, Haramaya University (HU). NMR analyses were done at Institute of Material Chemistry Chungnam National University, Daejeon, South Korea, Biochemistry and Molecular Biology at Kenyon College, Columbus, Ohio, United States of America (USA) and Department of Chemistry, Addis Ababa University (AAU). The anti-cancer, anti-amoeba, and anti-viral activities were done at the Institute Amrifa-School of Engineering, India, Walailak University, Nakhon Si Thammarat-Thailand, and Virology Department, St. Petersburg Pasteur Institute, 14 Mira st., St. Petersburg, Russia, respectively.

3.2.4 Test microbial and cell line cultures

Six human bacterial pathogens, viz., three from Gram-positive (*Staphylococcus aureus* serotype ATCC 25923, *Staphylococcus epidermidis* ATCC14990, and *Streptococcus pyogenes* ATCC 19615) and three from Gram-negative (*Escherichia coli* serotype ATCC 25922, *Klebsiella pneumoniae* ATCC e13883, and *Pseudomonas aeruginosa* ATCC 5702) and two pathogenic fungal species (*Aspergillus niger* serotype ATCC 11414, and *Candida albicans* ATCC 16404) were obtained from Ethiopian Public Health Institute (EPHI). HCK1T cell lines and Cervical cancer cell lines (*HeLa* cells) were purchased from NCCLS, Pune, India. *Acanthamoeba* serotype WU19001,a strain from the recreational reservoir at Walailak University, Nakhon Si Thammarat-Thailand. Influenza virus A/Puerto Rico/8/34 (H1N1) and Madin-Darby Canine Kidney (MDCK) cells line were purchased from ICN Biochemicals Inc. Aurora, Ohio, china.

3.2.5 Extraction and isolation

Total extraction of each plant material was conducted by mixing with organic solvents in the ratio of 1:10 (plant material/solvent, g/mL). Briefly, dried and ground plant material was defatted

with n-hexane (3x), filtered using suction filtration followed by solvent evaporation using rotary evaporator. The remaining marcs, after n-hexane extractions, were successively extracted with petroleum ether, chloroform: methanol (1:1), and methanol solvents followed by filtration and concentration. The general procedure applied for the preparation of these crude extracts used to isolate general phytochemical constituents, is shown below (Figure 10). Obtained crude extracts were subjected to *in vitro* anti-microbial, anti-oxidant, anti-cancer, anti-inflammation, anti-viral and anti-amoeba activities, and chromatographic fractionations.

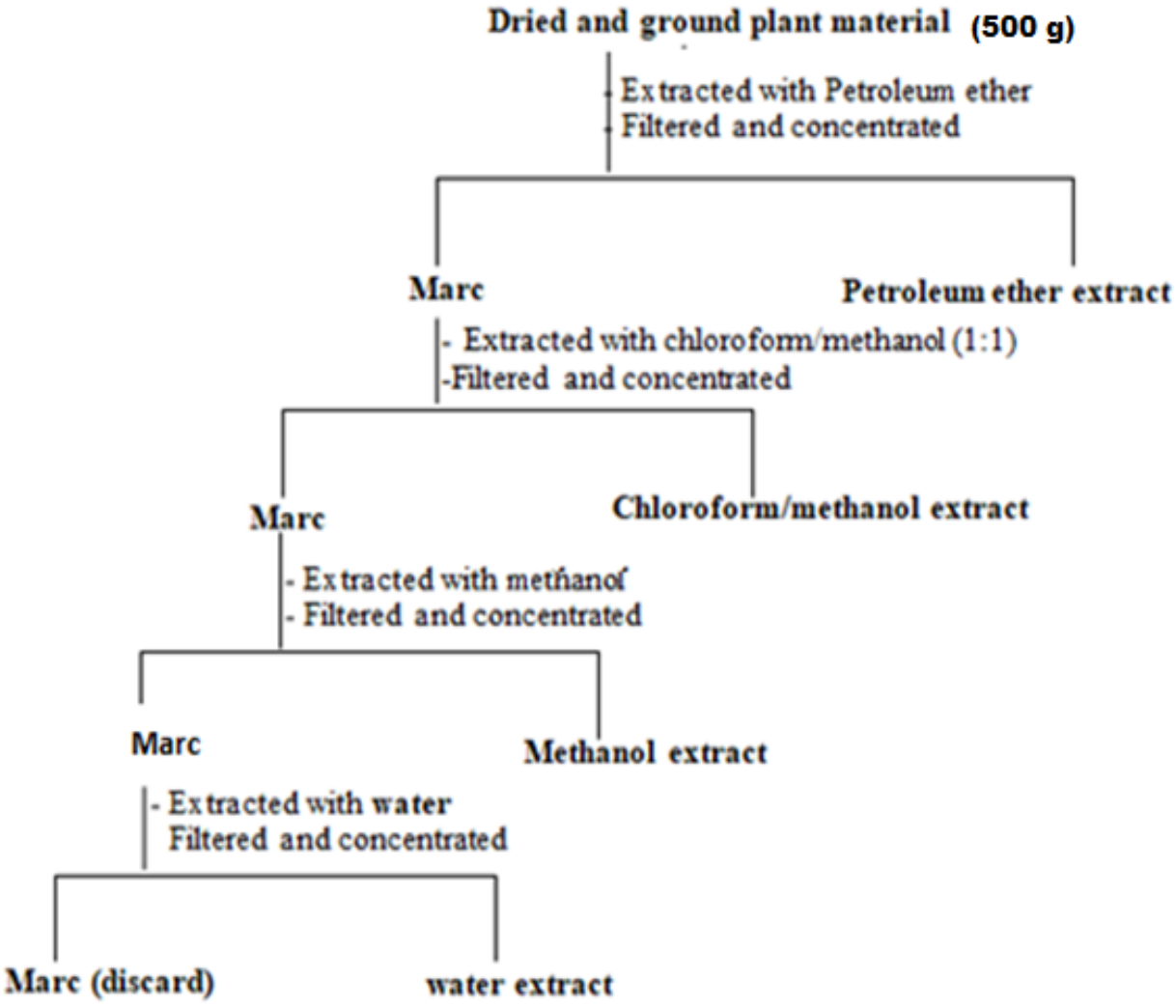


Figure 10: General procedure for preparation of crude plant extracts with increasing polarity

3.2.5.1 Extraction and isolation of *Vernonia amygdalina* leaves

Shed dried leaves powder (250g) was defatted with n-hexane (1.5 L, 3x) for 24 hr, filtered by suction filtration, concentrated by Rota-vapor at reduced pressure, and weighed (3 g).The marc after n-hexane extraction was extracted with petroleum ether, filtered, and concentrated affording 29 g. The remaining marc after petroleum ether extracts of the leaves (218 g) was further extracted with chloroform: methanol (1:1) and methanol found 30 g and 24 g respectively. Obtained crude extracts of the plant leaves were subjected to *in vitro* anti-microbial, anti-oxidant, anti-cancer, anti-inflammation, anti-viral, and anti-amoeba activities assay and TLC guided fractionation.

Prior to fractionation, the TLC profiles of the five different *Vernonia amygdalina* leaves extract, petroleum ether, chloroform: methanol (1:1), and methanol were observed using the developing solvent systems: petroleum ether/ $CHCl_3$(1:1), $CHCl_3$/MeOH(3:1), $CHCl_3$/MeOH (3:2), $CHCl_3$/MeOH (1:1) and $CHCl_3$/MeOH (1:3). UV-lamp (254 and 365 nm) followed by iodine vapor was applied to visualize the developed TLC chromatograms (Figure 11)

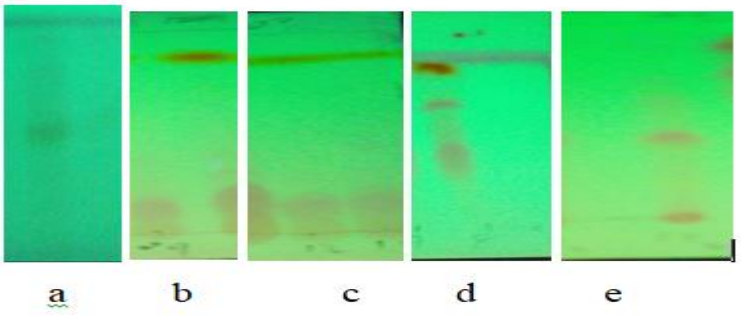


Figure 11: TLC profiles of *n*-hexane (a), chloroform (b), chloroform: methanol (1:1; c), petroleum ether /$CHCl_3$/MeOH (d) and methanol (e) crude extracts of the leaves of *Vernonia amygdalina.*

Taking into consideration the observed TLC profiles (Figure 11) and keen to target the phytochemicals with medium and higher polarity, the petroleum ether: chloroform /methanol (9:1) extracts were selected for silica gel CC fractionation as follows.

Fractionation of petroleum ether: $CHCl_3$ /MeOH (9:1) *V.amygdalina* **leaf extracts**

The petroleum ether: chloroform/ methanol (9:1) extract of *Vernonia amygdalina* leaves

extract (8 g) was dissolved in MeOH (25 mL) and adsorbed on 10 g silica gel. The adsorbed sample was chromatographed over column chromatography packed with silica gel (120 g). Elution was accompanied with petroleum ether: chloroform with increasing polarity and sevenity five fractions were collected (Table 3). Collected fractions were subjected to TLC analysis with different ratio of petroleum ether: chloroform as developing solvent and UV-lamp (254 and 365 nm) and iodine vapor as visualization methods. And fractions with similar spot (s) were combined and their TLC analysis was performed (Figure 12).

Table 3: Collected fractions of chloroform: methanol (1:1) leaves extract of *Vernonia amygdalina*

Fraction No.	Eluents	Volume (mL)
1-15	100% petroleum ether	**25 each**
16-28	100% petroleum ether	**25 each**
29-35	petroleum ether / $CHCl_3$ (9:1)	**25 each**
36-47	petroleum ether / $CHCl_3$(1:1)	**25 each**
48-55	petroleum ether / $CHCl_3$(1:9)	**40 each**
51-75	100% Chloroform	**40 each**

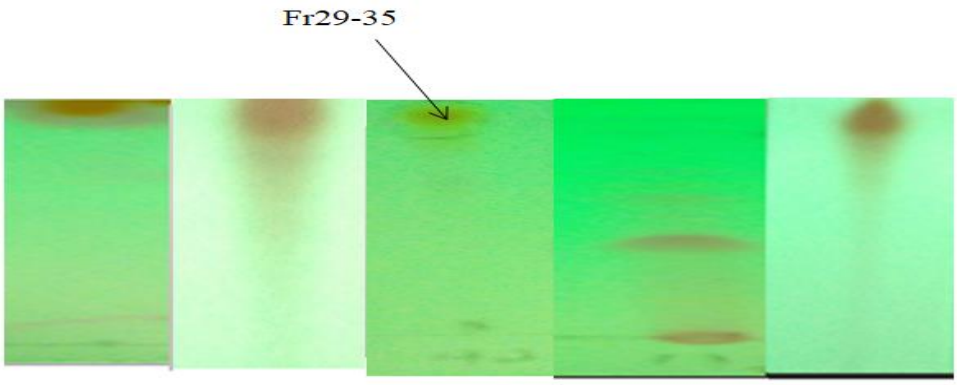


Figure 12: TLC profiles of combined fractions of petroleum ether: chloroform (9:1) leaves extract of *Vernonia amygdalina*

The TLC profile of fr29-35 (Figure 12) appeared as single spot on TLC with R_f value of 0.51; petroleum ether: chloroform (9:1) was used chloroform as eluent and afforded compound **1** (11 mg).

3.2.5.2 Extraction and isolation of *Otostegia integrifolia* leaves

The petroleum ether, chloroform: methanol (1:1) and methanol extracts of the leaves

were first subjected to TLC analysis using petroleum ether / $CHCl_3$ (3:1), $CHCl_3$/MeOH (1:1), petroleum ether /$CHCl_3$/MeOH (3:1:0.1) and 100% methanol, respectively, as developing solvents and the spots visualzed under UV-lamp (254 and 365 nm) followed by vanillin/H_2SO_4 as visualization methods. Then, to target compounds with medium and high polarity, those crude extracts expected to have such compounds and taking their TLC profiles (Figure 13) into consideration, were fractionated over silica gel column chromatography.

Figure 13: TLC profiles of *n*-hexane (a), chloroform (b), chloroform: methanol (1:1; c), petroleum ether /$CHCl_3$/MeOH (d) and methanol (e) crude extracts of the leaves of *Otostegia integrifolia*

Fractionation of chloroform: methanol (1:1) of *Otostegia integrifolia* leaves extract

The chloroform: methanol (1:1) leaves extract (5 g) was dissolved in MeOH (20 mL) and adsorbed on 10 g silica gel. The adsorbed sample was chromatographed over column chromatography packed with silica gel (130 g). Elution was accompanied with $CHCl_3$/MeOH with increasing polarity and one hundred five fractions were collected (Table 4). Collected fractions were subjected to TLC analysis with different ratio of $CHCl_3$/MeOH as developing solvent and UV-lamp (254 and 365 nm) and iodine vapor as visualization methods. And fractions with similar spot (s) were combined and their TLC analysis was performed (Figure 14).

Table 4: Collected fractions of chloroform: methanol (1:1) leaf extracts of *Otostegia integrifolia*

Fraction no.	Solvent system	**Volume (mL)**
1-6	100% petroleum ether	**15-25**
7-20	100% petroleum ether	**10-40**
21-25	100% petroleum ether	**25-40**
26-39	$CHCl_3$/MeOH (3:1)	**15-25**
40-44	$CHCl_3$/MeOH (3:1)	**10**
45-55	$CHCl_3$/MeOH (3:2)	**10**
56-75	$CHCl_3$/ MeOH (1:1)	**10-25**
76-85	$CHCl_3$/ MeOH (1:1)	**10-30**
86-90	$CHCl_3$/ MeOH (2:3)	**10-25**
91-94	$CHCl_3$/ MeOH (2:3)	**10**
95-101	$CHCl_3$/MeOH(1:3)	**10**
102-105	**100% Methanol**	**10-25**

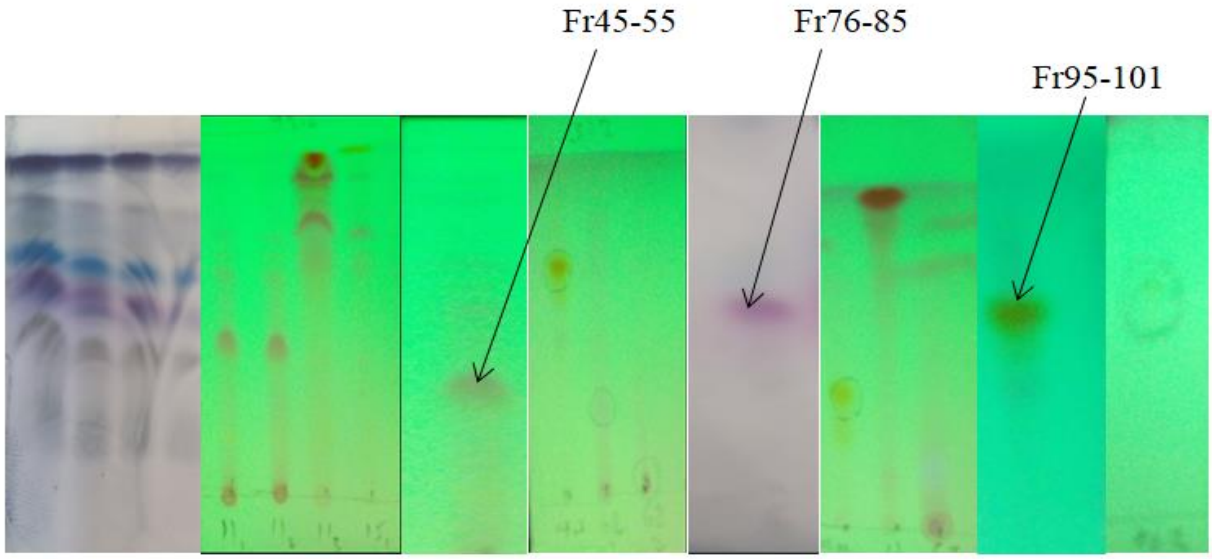


Figure 14: TLC profiles of combined fractions of chloroform: methanol (1:1) leaves extract of *Otostegia integrifolia*

The TLC profile of fr45-55 and fr76-85 (Figure 14) appeared as single spot on TLC with R_f value of both 0.4 [$CHCl_3$/MeOH (3:2)] and [$CHCl_3$/ MeOH (1:1)] using methanol as eluent and afforded compound **2** (16 mg) and compound **3** (10 mg), respectively. The TLC profile of fr95-101 (Figure 14) appeared as single spot on TLC with R_f value of 0.42 [$CHCl_3$/ MeOH (1:3)] using methanol as eluent and afforded

compound **4** (26 mg)

Compound 2: white gel (16 mg); R_f 0.4 ($CHCl_3$/MeOH; 3:2); ^1H, ^{13}C and DEPT- 135 NMR spectral data: See Table 8; EI MS *m/z* (relative abundance): 320.5 - matched with the molecular formula of $C_{20}H_{32}O_3$ (calcd. MW, 320),

Compound 3: Dark brown gel (10 mg); R_f 0.4 ($CHCl_3$/ MeOH (1:1); ^1H, ^{13}C and DEPT- 135 NMR spectral data: See Table 9; EI MS *m/z* (relative abundance): 313.5 [M-3]$^-$ matched with the molecular formula of $C_{20}H_{31}O_2$ (calcd. MW, 313)

Compound 4: white gel (26 mg); R_f 0.42 ($CHCl_3$/MeOH (1:3); ^1H, ^{13}C and DEPT- 135 NMR spectral data: See Table 10; EI MS *m/z* (relative abundance): 320.5 $^-$ matched with the molecular formula of $C_{20}H_{32}O_3$ (calcd. MW, 320). It was similar to that of otostegindiol, except that the former instead of the furan ring signals exhibited signals typical of a β, β -disubstituted dihydrofuran ring.

3.2.5.3 Extraction and isolation of *Salvia rosmarinus* leaves

Finely ground *Salvia rosmarinus* leaves (0.5 kg) was defatted with n-hexane (3x 2.5 L) via shaking on orbital shaker(RPM) for 24 hr followed by filtration with suction filtration and solvent evaporation by rotary evaporator leading to the green n-hexane extract residue (8 g). The marc, after petroleum ether extraction; the extract was filtered using suction filtration and the filtrate was concentrated using rotary evaporator affording a dark green petroleum ether extract (34 g). Marc of petroleum ether extract was subjected to chloroform: methanol (1:1, mL) extraction using same method mentioned above and a dark green extract residue (30 g) was obtained after filtration and solvent evaporation. The remaining marc was further extracted with methanol for 24 hr on orbital shaker (RPM) followed by filtration and concentration resulting a green residue (22 g). Finally, the remaining marc of methanol extract was mixed with distil water and shaken over night; extract was then filtered and concentrated resulting in green residue (8 g). Prior fractionation, the TLC profiles of the five different *Salvia rosmarinus* leaves extract, n-hexane, petroleum ether, chloroform, chloroform: methanol (1:1), and methanol were observed using the developing solvent systems: petroleum ether/ $CHCl_3$(1:1), $CHCl_3$/MeOH(3:1), $CHCl_3$/MeOH (3:2), $CHCl_3$/MeOH (1:1) and

$CHCl_3$/MeOH (1:3). UV-lamp (254 and 365 nm) followed by iodine vapor was applied to visualize the developed TLC chromatograms (Figure 15)

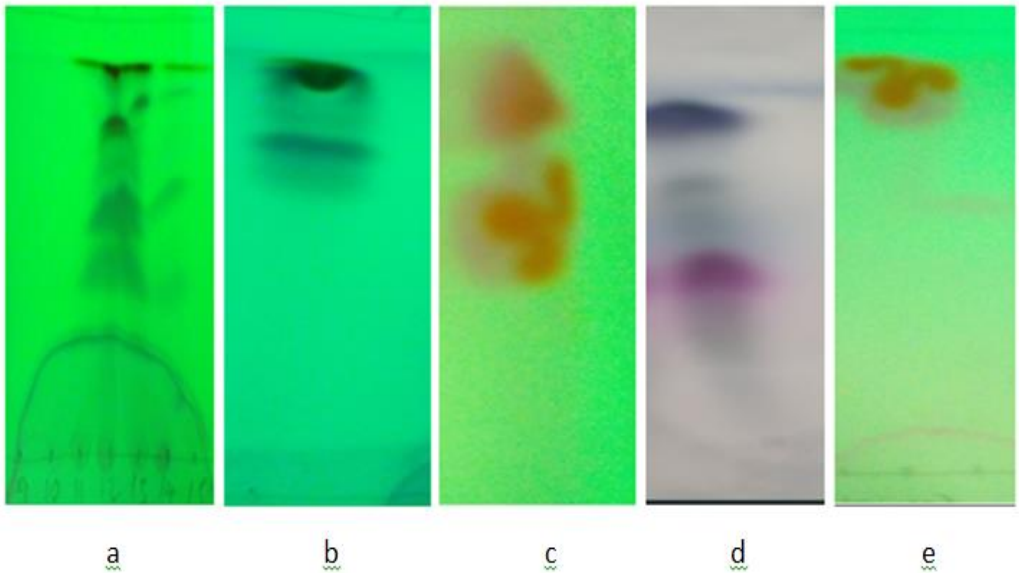


Figure 15: TLC profiles of n-hexane (a), petroleum ether (b) chloroform (c), chloroform: methanol (1:1; d), and methanol (e) crude extracts of the leaves of *Salvia rosmarinus.*

Taking into consideration the observed TLC profiles (Figure 15) and keen to target the phytochemicals with medium and higher polarity, due to capturing a wider range of compounds the chloroform: methanol (1:1) extracts were selected for silica gel CC fractionation as follows.

Fractionation of chloroform: methanol (1:1) leaf extracts of *S. rosmarinus*

Dark green chloroform extract (16 g) of *S. rosmarinus* leaves was disolved in chloroform (100 mL), adsorbed on silica gel (20 g) and dried using rotary evaporator. Adsorbed sample was then applied onto column packed with silica gel slurry (150 g). One hundred ten fractions were collected using the eluents petroleum ether / $CHCl_3$/MeOH with different ratio (Table 5). And their TLC profile was analyzed using petroleum ether/$CHCl_3$ (3:1, 1:1 and 1:3), $CHCl_3$/MeOH (3:1, 1:1 and 1:3) and visualized under UV-lamp assisted iodine vapor. Similar fractions were merged and re-analyzed their TLC analysis (Figure 16).

Table 5: Collected fractions from chloroform: methanol (1:1) extract of *S. rosmarinus* leaves

Fraction No.	Eluents	Volume (mL)
1-15	100% petroleum ether	**25 each**
16-28	100% petroleum ether	**25 each**
29-35	petroleum ether / $CHCl_3$ (3:1)	**25 each**
36-47	petroleum ether / $CHCl_3$(1:1)	**25 each**
48-55	petroleum ether / $CHCl_3$(1:3)	**40 each**
51-75	100% Chloroform	**40 each**
76-80	$CHCl_3$ /MeOH (3:1)	**40 each**
81-85	$CHCl_3$ /MeOH (1:1)	**40 each**
86- 93	$CHCl_3$ /MeOH (1:1)	**40 each**
94-106	$CHCl_3$ /MeOH (1:3)	**40 each**
107-102	100 % Methanol	**25 each**
103-110	**100 % Methanol**	**25 each**

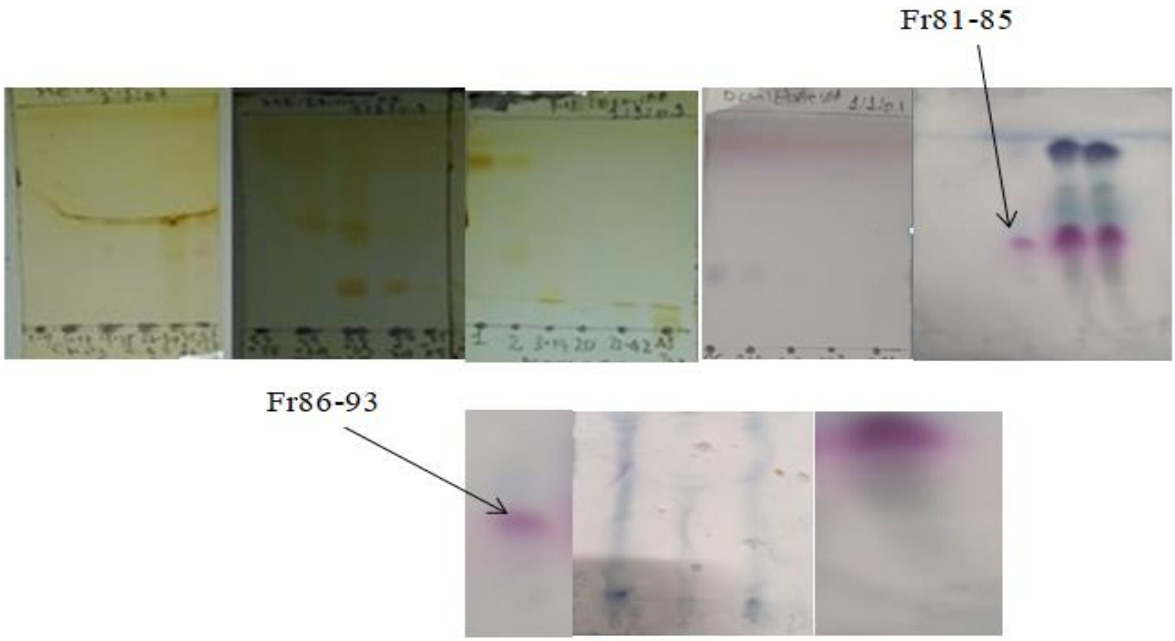


Figure 16: TLC profiles of combined fractions of chloroform: methanol (1:1) leaf extracts of *S. rosmarinus*

The TLC profile of fr81-85 and fr86-93 (Figure 16) appeared as single spot on TLC with R_f value of 0.42 and 0.49 with [$CHCl_3$/MeOH (1:1)] and [$CHCl_3$/ MeOH (1:1)] using methanol as eluent and afforded compound **5** (10 mg) and compound **6** (18 mg), respectively.

Compound 5: Dark brown gel (10 mg); R_f 0.42 Methanol/Chloroform (1:1); ^{1}H, ^{13}C and DEPT- 135 NMR spectral data: See Table 11; EI MS *m/z* (relative abundance): 454.7 - matched with the molecular formula of $C_{30}H_{46}O_3$ (calcd. MW, 455),

Compound 6: Dark brown gel (18 mg); R_f 0.49 ($CHCl_3$/ MeOH (1:1); ^{1}H, ^{13}C and DEPT- 135 NMR spectral data: See Table 12; EI MS *m/z* (relative abundance): 147.2 matched with the molecular formula of $C_9H_7O_2$ (calcd. MW, 147)

3.2.6 Qualitative determination of the phytochemical constituents

Phytochemicals are naturally occurring compounds found in plants. The term typically refers to the wide variety of secondary metabolites present in nature. Screening the phytochemical profile of crude extract metabolites for further analysis was done by referencing the guide scores (Amabie *et al*., 2024; Andrade *et al*., 2016).

Tests for alkaloids:-*Mayer's Test:* A small amount of the samples (2 ml) were treated with Mayer's Reagent using one or two drops, resulting in the appearance of a white precipitate indicates the presence of alkaloids.

Test for steroids:- *Libermann Burchard* Reaction: A 2 ml extract were mixed with chloroform. To this 1-2 ml acetic anhydride and 2 drops of concentrated sulphuric acid were added from the side of the test tube. First red, then blue and finally green color appearance indicates the presence of steroids.

Test for Glycoside:-*Keller-Killiani Test:* A fraction of the extracts were treated with 2 ml of glacial acetic acid and a drop of ferric chloride solution in a test tube. It was carefully underplayed with 1ml concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violet ring might appear below the ring which the acetic acid layer and a greenish ring might form.

Test for Coumarins:- A 2 mg of the plant material was dissolved in methanol/ethanol, and alcoholic KOH or NaOH, which has a yellow color that disappears when adding conc. HCl indicates the presence of coumarins.

Test for Terpenoids:- *Libermann Burchard Test:* A 2 ml of the extracts were treated with a few drops of acetic anhydride, boiled, and cooled. Concentrated sulphuric acid was added along the sides of the test tube. A brown ring at the junction of two layers with a green upper layer shows

the presence of steroids, and the formation of a red color indicates the presence of triterpenoids; and *Salkowski's Test*:

A fraction of the extracts were treated with 1ml chloroform followed by a few drops of concentrated sulphuric acid. Shaken well and allow stand for 5 min. The red color in the lower layer indicates the presence of sterols, and the formation of the yellow-colored lower layer indicates the presence of triterpenoids.

Test for Flavonoids:- *Shinoda Test*: (Magnesium hydrochloride reduction test): A 2ml of the extract was treated with Magnesium turnings and Conc. HCl added drop wise, pink, scarlet, crimson red, or occasionally green to blue after a few minutes the presence of Flavonoids; and Ferric Chloride Test: A fraction of the extracts were dissolved with a few drops of ferric chloride solution. The formation of an intense green color indicates the presence of flavonoids.

Test for Carbohydrate:- *Molisch Test:* A 3 ml of the test solution with a few drops of alcoholic α-naphthol solution in a test tube and the 1 ml of concentrated sulphuric acid was added carefully along with a side of the test tube. The formations of a violet ring at the junction indicate the presence of carbohydrates; and *Fehling's Test:* Fehling solution A and Fehling solution B were combined in equal amounts. A few drops of the sample were added and boiled. The presence of reducing sugar indicated at brick-red precipitate.

Test for Tannins:- Ferric Chloride Test: Some extracts were dissolved in distilled water to this solution 2ml of 5% ferric chloride solution was beaded. The formation of blue-green color indicates the presence of tannins; and *Lead Acetate Test*: Some extract with a few drops of lead acetate solution was added. The formation of a precipitate indicates the presence of tannins.

Test for Saponins:-*Foam test:* A 2ml extracts were diluted with 6ml water in a test tube. The mixtures were shaken vigorously and it was observed for the formation of persistent foam that confirms the presence of saponins.

3.2.7 *In vitro* antimicrobial activity

The antibacterial and antifungal activities of different crude extracts obtained from *Vernonia amygdalina, Otostegia integrifolia,* and *Salvia rosmarinus* plant leaves were evaluated by the disk diffusion method (Agency, 2022). Two Gram-positive bacteria namely *Staphylococcus*

aureus serotype (ATCC 25923) and *Staphylococcus epidermidis* (ATCC14990); and three Gram-negative bacteria, namely *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 5702) and *Klebsiella pneumonia*(ATCC e13883) were inoculated overnight at 37ºC in Muller–Hinton Agar (MHA) medium and two fungi strains of *Candida albicans* (ATCC 16404) and *Aspergillus niger* (ATCC 11414) were inoculated overnight at 27-30 ºC in the Sabouraud Dextrose Agar (SDA) culture medium (CLSI, 2015).

Briefly, the test was performed in sterile Petri dishes (100 mm diameter) containing solid and sterile Muller–Hinton Agar medium (25 ml, pH 7) and Sabouraud Dextrose Agar (SDA) for bacteria and fungi respectively. The pure extracts were placed on the surface of the media that had previously been a sterile microbial (one microbe per Petri dish) after being adsorbed on sterile paper discs (5 µL per Whatman disc of 6 mm diameter). To prevent test samples from eventually evaporating, all the Petri dishes were sealed with sterile laboratory para films. They were then incubated at 37ºC for 24 h, and the zone diameter of the inhibition was measured by microplate readers and represented in millimeters. Ciprofloxacin antibiotic reference (manufactured by Wellona Pharma Ciprofloxacin tablet, India) was used as a positive control and DMSO was used as negative control for antibacterial activity test, while Ketoconazole 2% (made in Bangladesh Incepta Pharmaceuticals Ltd) was used as positive control and 10 µL of 0.2% agar as negative control for antifungal activity test (Hemeg *et al*., 2020). Three repetitions of the crude extract sample were used to precisely measure the inhibitory halo diameter (in mm), which was then expressed as mean ± standard deviation to assess the anti-microbial activity.

Total flavonoid content determined from the leaf extracts.

The absorbance of the reaction mixtures was measured against blank at 570 nm wavelength with a Varian UV-Vis spectrophotometer. In the linear equation obtained from the standard curve, Y is the absorbance of the sample obtained from the UV-visible spectrophotometer. X is the concentration of the Standard from the calibration curve. From the value of X, the total Flavonoid content (mg/g) C is calculated by the following equation 1 (Regmi and Sharma, 2023).

$$C = X * V/N \quad (1)$$

Where: V=volume of extract taken in mL: N=weight of plant extract in g. In our case, V = 1mL and N = 0.002 g (2mg), and the value of X calculated from the standard curve

3.2.8 *In vitro* anticancer activity

To study the anticancer activities of the phytochemicals, human cervical cancer using continuous cells culture of *HeLa* cell lines were purchased from NCCLS, Pune, India and used. The extract solvents were used petroleum ether and CH_3Cl_3: MeOH (1:1). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Himedia, India) supplemented with 10% fetal bovine serum [(FBS), PanBiotech, Germany]. Medium was contained to cell culture medium: DMEM high glucose medium- (AL057S, Himedia), standard: DMSO [(PHR1309, Sigma)] and Cisplatin (232120, Sigma). An inverted microscope (Biolinkz, India) was used for the study. The samples were incubated for 24 h at 37 °C in a humidified atmosphere consisting of 5% CO_2 in the air for 2–3 days before use. To subculture the cells they were washed with phosphate-buffered saline (PBS), and the cells were detached by treating them with trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) for 10 min and then by adding a complete medium to inhibit the reaction. MTT assay (4060, Himedia) was used to measure cellular metabolic activity: indicating viability, proliferation, and cytotoxicity. For this purpose, 96-well plates were used and 20,000 cells were added to each well with a volume of 200 µl cell suspension. To determine the cytotoxicity effects on the non-cancerous cell lines like one immortalized human cervical keratinocyte line (HCK1T) as normal cervical epithelium cell lines. Since *HeLa* cells were chosen as cancer model cell lines, then HCK1T cell lines could be used as normal correspondence to co-relate the result. To avoid exposure to light, the plates were covered with aluminum foil (Chirumamilla & Taduri ,2023 ; Sul'ain *et al*.,2019; Mosman, 1983). Positive control: medium with cervical cancer cells and Cisplatin; Negative control: medium with cervical cancer cells but without the experimental drug/compound; the culture medium may reduce the MTT to formazan. Occasionally, it was necessary to pipette up and down to completely dissolve the MTT formazan crystals, especially in dense cultures. The absorbance was measured at 570 nm using a spectrophotometer or ELISA reader, and the IC_{50} value was determined using logarithmic regression equation 2.

$$Y = M\ln(x) + C \qquad 2$$

Where, Y = 50, M and C values were derived from the viability graph.

In order to determine the number of cells viable that means cell viability refers to the proportion of healthy, living cells within a population; we measured the absorbance at 570 nm. We took measurements for samples at concentrations of 250, 500, 750, 1000, and 1250 µg/mL. We calculated the concentration required for a 50% reduction in viability (IC_{50}) both graphically and by measuring the absorbance at 570 nm using a spectrophotometer. To eliminate any errors, we used wells containing cell-free samples as blanks. To express the effect of the samples on cervical cancer cell proliferation, we calculated the percentage cell viability using equation 3.

$$\% \text{ Cell Viability} = \frac{\text{OD of Sample}}{\text{OD of Control}} \times 100 \qquad 3$$

Anticancer Assay for cancerous cervical cell line (*HeLa* cell):- *HeLa* cell is the name of the human cell line. In our study, *Hella cell* was selected as preferable as continuous cell lines (immortalized cell lines) are generally more robust and easier to work with than primary cells. They have unlimited growth potential and are prolific, allowing for extensive applications in scientific study, and are a quick, easy way to get basic information for cancerous of cervical cells (Beecher, 1999).

Validation of cell viability assays:- The experiment was practiced in Stellixier Biotechnology Pvt Ltd in Bangalore, India a niche preclinical research and development service provider, Vested in the hands of remarkably skilled professionals and Project reported by an inventor named: Giridhar Reddy in the institute/organization Amirta School of Engineering; with the code SBPL-CCS-2022- 23-125 date 29-06-2023.

3.2.9 *In vitro* Free-radical scavenging activity

To test the antioxidant activity of the phytochemicals, diphenyl-1-picrylhydrazyl (DPPH) bioassay was used, and the DPPH powder was procured from Mumbai Company, India. The methods used for the study were based on previous research with minor modifications (Awadh and Alhamzy, 2017 ; Momoh and Muhamed, 2012) for the experiment on radical scavenging activity, 10mg of each crude extract (petroleum ether (100%), chloroform/methanol (1:1), and methanol) was diluted in 75 mL of methanol. Then, 10 µl, 15 µl, 20 µl, 25 µl, and 30 µl of the diluted test solutions were added to 2 ml of 0.004% DPPH solution each. These mixtures were kept in the dark for 30 min at 37ºC. After incubation, the absorbance was measured at 517 nm

using a UNICO 2100 UV/VIS spectrophotometer. The tests were repeated thrice to ensure accuracy. A 2 mg vitamin C solution in 1 mL methanol was used to serve as a positive control. Finally, the percentage of DPPH scavenged was calculated using Equation 4.

$$\text{Percent of DPPH scavenged (\%)} = \frac{(\text{Ao} - \text{A})}{\text{Ao}} \text{x } 100 \quad 4$$

A_o represents the control's absorbance (which includes all reagents except for the test compound), and A represents the tested sample's absorbance at 517 nm.

The IC_{50} value represents the concentration that reduces the initial DPPH absorbance by 50%. By plotting inhibition percentages against sample concentrations, the IC_{50} can be calculated using GraphPad Prism version 8.0.1 (USA).

3.2.10 *In-vitro* Anti-inflammatory Activity

The study was conducted *in vitro* by measuring the inhibition of albumin denaturation and anti-proteinase activity. The extract solvents were used CH_3Cl_3: MeOH (1:1). The methods used were based on previous studies, with some minor modifications (De Oliveira, 2019, Rastogi *et al.*,2018 ; Hosseini and Ghorbani,2015; Nigatu *et al.*, 2012). A negative control was prepared using DMSO, while aspirin (Sigma-Aldrich, Singapore) was used as a positive control drug, at a concentration of 100 μg mL^{-1}.

3.2.10.1 Inhibition of Albumin Denaturation

One milliliter of a 1% aqueous solution of bovine albumin fraction was combined with 1 mL of the test extract from CH_3Cl_3: MeOH (1:1) to create the reaction mixture. After adjusting the pH of the reaction mixture to 6.3, it was heated to 51°C for 30 min and then incubated for 20 min at 37°C. A Jasco V-730 UV-Vis spectrophotometer from the USA was used to measure the absorbance of the sample at 660 nm after it was cooled to room temperature. The percentage inhibition of protein denaturation was calculated using Equation 5, and the results were reported as IC_{50} values. (IC_{50} values refer to the concentration required to achieve a 50% inhibition.):

$$\text{Percentage inhibition (\%)} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \text{x } 100 \quad 5$$

Where, $A_{control}$ is the absorbance of negative control (DMSO) and A_{sample} is the absorbance of the extract.

3.2.10.2 Anti-proteinase Activity

In the reaction mixture, one mL of 20 mM of Tris HCl buffer (pH 7.4) and 0.06 mg of trypsin were added. After five min of incubation at 37°C, 1 mL of 0.7% (w/v) casein was added to the mixture. After another 20 min of incubation, 2 mL of 70% per chloric acid ($HClO_4$) was added to the reaction mixture to bring it to a stop. To collect the supernatant, the reaction mixture was centrifuged for 10 min at 6,000 rpm and 4°C. The absorbance of the supernatant was measured at 210 nm using a spectrophotometer (V-730 UV-Vis Spectrophotometer, JASCO, USA). The percentage inhibition of proteinase was calculated using the following equation 6, and the results were reported as IC_{50} values:

$$\text{Percentage inhibition (\%)} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \text{x } 100 \qquad 6$$

3.2.11 *In-vitro* Anti protozoal activity

3.2.11.1 *S. rosmarinus* extracts against *Acanthamoeba spp*

Anti-*Acanthamoeba* activity of *S. rosmarinus* of leaves extract against the trophozoites and cysts of *Acanthamoeba* spp. was determined by Bunsuwansakul *et al*., (2019) with minor modification. Each of trophozoites and cysts was adjusted to a final concentration of 2×10^5 cells mL^{-1}. One hundred microliters of the parasite suspension were added into 96 well microtiter plates, containing 100 µL of serially diluted plant extract. The plates were incubated at room temperature for 24 h. One percent DMSO was included as a negative control. Inhibitory activity was carried out using a trypan blue exclusion assay. The relative percentage of parasite viability was defined as (mean of the treated parasite/mean of the control) × 100. Meanwhile, the morphology of the trophozoite and cyst forms of *A. triangularis* and *A. castellanii* after treatment with *S .rosmarinus* extract was investigated preliminarily by inverted microscopy.

3.2.11.2 Determination of MIC of *S rosmarinus* extract against *Acanthamoeba spp*

Minimal inhibitory concentration (MIC) of *S. rosmarinus* extract against *A. triangularis* and *A. castellanii* was investigated by broth microdilution method (Boonhok *et al*.,2022). Each of trophozoites and cysts was adjusted to a final concentration of 2×10^5 cells mL^{-1}. A total of 100 µL of the parasite suspension were added into 96 well microtiter plates, containing 100 µL of serially diluted *S. rosmarinus* extract. Then, 1% DMSO and Chlorhexidine were included as a negative and positive control, respectively. The plates were incubated at room temperature for 24 h. The MIC values were defined as the lowest concentration that >90% growth inhibition of the zoites and cysts, compared with the negative control.

Cultivation of *Acanthamoeba organism:-* a severe infection of human *Acanthamoeba triangularis* WU19001, a strain from the recreational reservoir at Walailak University, Nakhon Si Thammarat-Thailand and *Acanthamoeba castellanii* ATCC50739 (reference pathogen strain) were used in this study. The parasite was grown in Peptone Yeast Glucose (PYG) medium [20 g proteose peptone, 2 g yeast extract, 0.98 g $MgSO_4 \cdot 7H_2O$, 0.35 g $Na_2HPO_4 \cdot 7H_2O$, 0.34 g KH_2PO_4, 0.02 g $(NH4)_2Fe\ (SO_4)_2 \cdot 6H_2O$, 18 g glucose]. The trophozoites were observed after 72 h of incubation at room temperature. Trophozoites were centrifuged at 4000 rpm for 5 min and re-suspended in fresh PYG thereafter. For counting, 50 µL of cell suspension was mixed with 50 µL of trypan blue. Viability was investigated under the inverted microscope based on the principle of the dye can cross the membrane of dead cells with blue color, but not intact membrane of viable cells with colorless appearances.

Screening:-The *S.rosmarinus* extracts (final concentration of 2 mg mL^{-1}) were screened for their anti-*Acanthamoeba* activity by the broth method in a 96-well polystyrene microtiter plate. The stock solutions (100 mg mL^{-1}) of extracts were diluted to 4 mg mL^{-1} with PYG medium. Then, 100 µL of extract was transferred to each well. The aliquot of 100 µL (2×10^5 trophozoites mL^{-1}) trophozoites were added to give a final concentration (2 mg mL^{-1}) and incubated at 25°C for 24 h. The relative percentage of parasite viability was defined as (mean of the treated parasite divided by mean of the control) × 100.

3.2.12 *In-vitro* Anti-Viral activity

Cytotoxicity assay:-The microtetrazolium test (Mitra *et al.*, 2016) was used to study the cytotoxicity of the extracts. Briefly, series of three-fold dilutions of each extract in MEM (3.7 – 300 µg/mL) were prepared. The Madin-Darby Canine Kidney (MDCK) cells line (widely was used as epithelial cell model) were incubated for 48 h at 36°C in 5% CO_2 in the presence of the dissolved specimens. The cells were washed twice with phosphate-buffered saline (PBS), and a solution of 3-(4, 5-dimethylthiazolyl-2) 2, 5-diphenyltetrazolium bromide (ICN Biochemicals Inc. Aurora, Ohio) (0.5 µg/ml) in PBS was added to the wells. After 1h incubation, the wells were washed and the formazan residue was dissolved in DMSO (0.1 ml per well). The optical density in the wells was then measured on a Thermo Multiskan FC plate reader at the wavelength of 540 nm and plotted against the concentration of extracts. Each concentration was tested in three parallels. The 50% cytotoxic concentration (CC_{50}) of each extract was calculated from the data obtained.

Cytoprotection assay:-The extracts were dissolved in 0.1 ml DMSO to prepare stock solutions, and final serial solutions (300 – 3.7 µg/ml) were prepared by adding MEM with 1 µg/ml trypsin. Extracts were incubated with MDCK cells for 1 h at 36°C. Each concentration of the extracts was tested in triplicate. The cell culture was then infected with influenza virus A/Puerto Rico/8/34 (H1N1) (moi 0.01) for 48 h at 36°C in the presence of 5% CO_2. After this, cell viability was evaluated by MTT assay as described above. Based on the results of photometry, 50% inhibiting concentration (IC_{50}), the concentration that provided protection of 50% cells comparing to the wells without extracts, was calculated for each extract as well as the selectivity index (SI, the ratio of CC_{50} to IC_{50}). For calculations, GraphPad Prism software was used. The specimens having demonstrated SI of 10 or higher were considered active and prospective for further development (Indrayanto, 2022).

3.2.13 Extraction of essential oils (EOs)

The collected fresh leaves of *V. amygdalina, O. integrifolia*, and *S. rosmarinus* were air-dried in a shaded place at room temperature (25^0C). Briefly,500 g of each powder of the sample was hydro-distilled using a Clevenger-type apparatus for 3 hr (Ruiz-Ciau *et al.*, 2017). The essential oil from each plant was collected, dried under anhydrous sulphate and stored at 4^oC until used

further to bioactivities(Shimira *et al.*, 2022). Each extraction was performed at least three times. The application performed and expressed following simplified schemed activities (Figure 17).

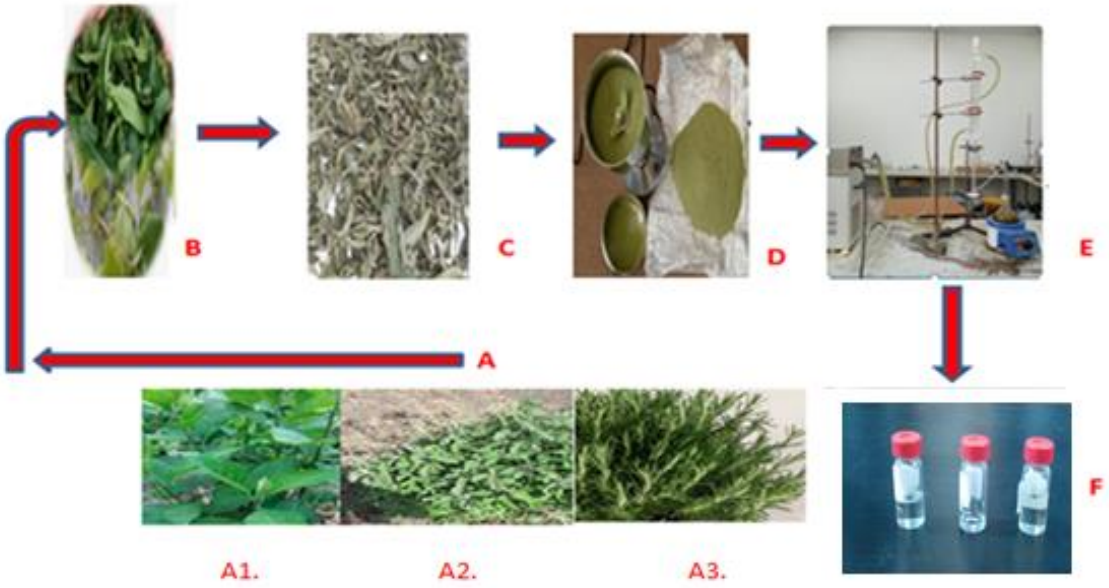


Figure 17: Essential oils extraction

[They were taken photos directly and separately through the three plants. (**A**) Fresh leaves of the three plants (A1: *V. amygdalina*, A2: *O. integrifolia*, and A3: *S rosmarinus*); (**B**)Collected the samples each (**C**) Air dried in a shaded place ; (**D***)* Prepared powdering. Samples (used micro grinding machine); and (**E**) Hydro distillation using a Clevenger-type apparatus and (**F**) Essential oils]

3.2.13.1 Chemical analysis of the essential oils.

The chemical composition of essential oils(EOs) was analyzed using GC-MS, as described by (Van Vuuren *et al.*, 2010). GC/MS (7890B) analysis was done by using a GC (7890B, Agilent Technologies, USA) coupled with an MS (5977A Network, Agilent Technologies). The GC had an HP 5MS column (non-polar column, Agilent Technologies), 30 m × 250 µm internal diameter (i.d.) and 0.25 µm film thickness. The carrier gas was helium flowing at a rate of 1 mL min^{-1}. The sample was dissolved in hexane to give a 1% v/v solution. The injector temperature was set to 230ºC, and the injection mode was splitter mode with a split injection technique (split ratio of 10:1). We held the initial oven temperature at 40˚C for 5 min and then raised it to 250˚C at a rate of 6˚C min-1, maintaining this temperature for 20 min. The total runtime was 60 min, and we recorded the mass spectrum in EI mode at 70 eV, scanning the 50-500 m z^{-1} range. Inlet temperature and MSD detector temperatures were set at 270˚C and 290˚C, respectively.

Identification of the components was achieved based on their linear retention indices(Adams *et al*., 2007) and comparing mass spectra of the compounds with those in the HP Chemstation library, WILEY275, NBS75K, Library (the NIST-database, USA).The relative amounts (RA) of individual components of the oil were expressed as percent peak area relative to the total peak area.

3.2.13.2 *In vitro* anti-bacterial and anti-fungal assays

Four strains of bacteria and one fungus strain were cultured overnight in specific culture media. The two Gram-positive bacteria were *Staphylococcus aureus* serotype (ATCC 25923) and *Streptococcus pyogenes* (ATCC 19615). The two Gram-negative bacteria were *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 5702). The single fungus strain was *Candida albicans* (ATCC 16404). The bacteria were grown in a nutrient Muller-Hinton agar (MHA) (25 ml, pH 7) culture medium at 37ºC, while the fungus strain was grown in a Sabouraud Dextrose Agar (SDA) culture medium at 27-30ºC. As a consequence, using agar may affect their biological activities and efforts of the zone of inhibition (Lafraxo *et al*.,2022).

We conducted a study for the antibacterial and antifungal properties of various essential oils (EOs) through the disc diffusion method(EUCAST, 2021). The media was prepared and used for culturing using standard methods Muller Hinton Agar (CLSI, 2015) and Sabouraud Dextrose Agar (SDA) modified (Balouiri *et al*.,2016) for bacteria and fungi respectively. The antimicrobial activity of EO was evaluated by the agar diffusion method. It consists of placing 6 mm paper disks saturated with 5 µL EOs (1 mg L^{-1}) antimicrobial agents on a lawn of bacteria and fungi seeded on the surface of agar medium and positive controls and then placed on Petri dishes culture before being incubated for 24 h at 37°C and measuring the presence or absence of a zone of inhibition around the disks. Ciprofloxacin antibiotic reference (manufactured by Wellona Pharma Ciprofloxacin tablet, India) and Gentamycin (manufactured by Lexicare Pharma Pvt. Ltd in India) synergize with Azoles (Lexicare Pharma in India) against drug-resistant *C. albicans* were dissolved in distilled water by small concentrations(µg mL^{-1})as described by Clinical & Laboratory Standards Institute , document M27-A3 (Lu *et al*., 2018) used as positive controls, and DMSO used as a negative control for microbes. Fungus, received before inoculating a 5 % v/v inoculum size of a 100 ml process would mean we were used and added 5 ml of the inoculum to 95ml of the prepared media (10 µL of 0.2% agar) (Lu *et al*.,

2018). The culture was then evenly seeded on the agar surface using a sterile glass spreader. The EO samples in antibacteria and antifungus activities were measured by determining the mean inhibition halo diameter (in mm) from three repeats and reported as mean ± standard deviation.

3.2.13.3 Free-radical scavenging activity

The diphenyl-1-picrylhydrazil (DPPH) assay was used to determine the antioxidant (free-radical scavenging) activity of the essential oils (Kamath and KizhedathS,2018). DPPH radical scavenging activity is one of the most widely used methods for screening the antioxidant activity of plant leaves (EUCAST, 2021). To carry out the experiment, 12.5 ml of essential oils were diluted in 125 ml of methanol. Subsequently, 62.5 µl, 125 µl, 250 µl, 500 µl, and 1000 µl of the diluted test solutions were added to 2 ml of 0.004% DPPH solution each. The mixtures were then incubated for 30 min at 37ºC in the dark. After incubation, the absorbance was measured at 517 nm using a UNICO 2100 UV/VIS spectrophotometer. The tests were repeated three times, and a solution of 2 mg vitamin C in 1 ml methanol was utilized as a positive control. Finally, the percentage of DPPH scavenged was calculated using the following equation 6:

$$\text{Percent of DPPH scavenged } (\%) = \frac{(\text{Ao} - \text{A})}{\text{Ao}} \text{x } 100 \qquad 6$$

In this equation, the control's absorbance (which included all reagents except for the test compound) is denoted as Ao and the tested sample's absorbance at 517 nm is denoted as A. Additionally, the analysis allows for the calculation of the sample concentration that results in 50% inhibition (IC_{50}), which is determined by plotting the inhibition percentages against the sample concentrations (Graph Pad Prism version 8.0.1 USA, graphpad.com). The samples were tested in triplicates with a UV/VIS spectrophotometer, and the antiradical activity of essential oils from *V. amygdalina*, *O. integrifolia*, and *S. rosmarinus* was valued using the DPPH bioassay and the powder DPPH made in Mumbai Company, India. The three essential oils were tested using a similar biological system, and their code numbers were MD001, MD002, and MD003, respectively.

3.2.14 *In-silico* molecular docking study

The molecular docking studies were carried out using *AutoDock Tools* (ADT) (Narramore *et al.*, 2019; Trott and Olson, 2010) which is a free graphic user interface (GUI) for the *AutoDock Vina*

program. The compounds selected for the molecular docking study were Compounds **5** and **6** isolated from *Salvia rosmarinus*, compounds **2**, **3** and **4** isolated from *Otostegia integrifolia* and 3, 4-dihydro-2H-Pyran identified as a common and principal compound from the essential oils of *V. amygdalina*, *O. integrifolia*, and *S. rosmarinus*. The structures of the compounds were drawn using ChemOffice tool (Chem Draw 16.0) assigned with proper 2D and 3D orientation and energy of each molecule was minimized using ChemBio3D. The energy-minimized ligand molecules were used as input for AutoDock Vina-20 to carry out the docking simulation.

Identification and Selection of Target Proteins

Cervical cancer-causing protein was identified through protein data bank formats. The protein molecule structure of DNA (cytosine-5)-methyltransferase 1 (DNMT1), protein data bank identity (PDB ID: 4WXX) and HPV type 16 E6 (PDB ID: 4XR8) - a protein known to cause cervical cancer. The protein molecule structure of bacteria that cause human diseases via the crystal structure of receptor molecule *E. coli* gyrase B (PDB ID: 6F86) and *S. pyogenes* SpeB (PDB ID: 4D8E), malaria causes the protein molecule structure of *Plasmodium falciparum* (*PfLDH*) - The protein molecule structure of *Pf*LDH (residues ALA18-ALA329) *plasmodium falciparum* l-lactate dehydrogenase complexed with NAD+/NADH and oxamate were downloaded from the Protein Data Bank identity (PDB ID: 1LDG) formats (Silva *et al.*, 2020) and the protein crystal was identified structure of the hem agglutinin from H1N1 influenza A virus with cofactor retrieved from the protein data bank(PDB ID: 6ML8, resolution: 2.92 Å) and influenza A virus (Denver/57 bound to the CO5 antibody, classification viral protein(PDB ID: 6ML8) (Silva *et al.*, 2020).The stability of the proteins molecule was assessed using Rampage (Wang *et al*.,2016).

Selection of ligand molecules

Phytochemical constituents of *Vernonia amygdalina*, *Otostegia integrifolia*, and *Salvia rosmarinus* plant leaves were used to select as a source of secondary metabolites (ligands) and the ligands were used, selected and based on the trial of bioactivities. Ligand molecules were obtained through plant extraction, and isolation, and realized with PubChem (https://pubchem.ncbi.nlm.nih.gov/). The ligands were downloaded in Silver diamine fluoride format (SDF) and then converted to PDB format using an online SMILES translator

(https://cactus.nci.nih.gov/translate/). The downloaded files were in PDB format, which was utilized for running various tools and software (Wang *et al.*,2016).

Preparation of protein molecule

The Biovia Discovery Studio Visualizer software was used to analyze the protein molecule. The protein molecule was converted into the PDB format and its hierarchy was analyzed by selecting ligands and water molecules. Both the protein molecule and the water molecules lost their attached ligands during the analysis. Then, the protein preparation was done using the reported standard protocol (Narramore *et al*., 2019) by removing the co-crystallized ligand, selected water molecules and cofactors. Finally, the protein's crystal structure was saved in a PDB file (Wang *et al.*,2016). The ligand energy was minimized and changed to PDBQT format. The protein was docked with the ligand and screened based on minimum binding energy. The optimal ligand was selected for final docking using AutoDock Vina and Biovia Discovery Studio Client 2020. Once the docking process was complete, the results were analyzed based on several parameters, including absolute energy, clean energy, conf number, mol number, relative energy, and pose number. Then, the output PDBQT file was added. The docked structure was visualized and the "molecule" option was changed to "molecular surface" under the "shown as" menu.

3.2.15 *In silico* drug-likeness and toxicity predictions

Drug likeliness properties of the screened ligands were evaluated using the SwissADME online server. SMILE notations were obtained from PubChem and submitted to the SwissADME web server for analysis. The drugs were subjected to Lipinski's rule of five) (Banerjee *et al*., 2018) for analysis. Lipinski's rules of five were selected for final docking through AutoDock Vina and Biovia Discovery Studio Client 2020. The BIOVIA Discovery Studio Client 2020 is the leading visualization tool for analyzing protein and modeling data. The drugs were analyzed using Lipinski's rule of five for docking with AutoDock Vina and Biovia Discovery Studio Client 2020. To predict the ligands' organ toxicities, toxicological endpoints, and LD_{50} values, we utilized ProTox II and OSIRIS Property Explorer (Balouiri *et al*.,2015; Zarubova *et al*.,2015). The compounds were compared with antimicrobial agents amoxicillin, anti-cancer agents Jaceosidin, anti-malaria agents Artesunate and anti-influenza A virus agents Amantadine HCl standard drugs owned by Hikma Pharmaceuticals Plc, US (1978).

3.2.16 Data analysis

The experiments were conducted in triplicates. The mean ± SEM was used to express the results. For all comparisons, the level of $p \leq 0.05$ was considered significant. All statistical analyses were performed using Graph Pad PRISM Version 8.0.1(244) from Graph Pad Software Inc. USA and means were presented in bar and line charts.

3.2.17 Biosafety

Biosafety was secured on key containment measures, including microbiological practices, safety equipment, and facility safeguards (CDC, 2020). These were protected lab personnel, the sorro und, and the public from infectious microorganisms.Accordingly, effective risk assessments were designed and based on the right practices and equipment to prevent laboratory-associated i nfections (LAIs) following the standard guidelines (CDC, 2020).

3.2.18 Ethical Considerations

The study focused on the safety and well-being of *in vitro* models, rather than *in vivo* models involving animals. However, we obtained the necessary approval and clearance letters from ASTU reference number 7/2-2-2/7m74/3615/14 and for study collected data following the standard protocol in compliance with ASTU guidelines (ethical review board).

CHAPTER 4

4 RESULTS

In this study, three plant species, namely, *Vernonia amygdalina, Otostegia integrifolia,* and *Salvia rosmarinus* leaves were extracted through successive solvent extraction and investigated for their general phytochemical screening, constituents, and *in vitro* antimicrobial and anticancer activities (Table 12 and 13). Besides, *in vitro* for anti-oxidant, anti-inflammatory, anti-amoeba, and anti-viral activity of the leaf extracts of the three plants. During this study, again the essential oils of the plants leaves were extracted to evaluate different bioactivities against bacteria, fungi, and free radical scavenging activities. The overall results obtained from the present finding are conversed below.

4.1 Extraction yields

The petroleum ether, chloroform: methanol (1:1) and methanol extracts of *Vernonia amygdalina, Otostegia integrifolia,* and *Salvia rosmarinus* leaves each finely ground (0.5 kg) and yielded the following results: Petroleum ether 29 g, 18 g and 34 g by respectively, chloroform: methanol (1:1) extract 30 g, 30 g and 30 g, respectively and methanol extract 24 g,22 g and 22g by respectively. The powdered (0.5 kg) leaves of *Vernonia amygdalina, Otostegia integrifolia,* and *Salvia rosmarinus* were hydrodistilled and yielded concentrated, colorless essential oils with pleasant odors: A 25.1 g (0.05% w/w), 40.04 g (0.08% w/w), and 50.89 g (0.102% w/w), respectively.

4.2 Preliminary phytochemical analysis

Phytochemical screening of the extracts was conducted to investigate the presence (+) and absence (-) of alkaloids, steroids, glycoside, coumarins, terpenoids, flavonoids, carbohydrates, tannins, and saponins (Table 6). The extracts of *V. amygdalina, O. integrifolia,* and *S. rosmarinus* leaves were found to contain commonly known bioactive constituents such as alkaloids, steroids, terpenoids, flavonoids, tannins, and saponins (Table 6). The study also found that these flavonoids are related to natural phenolic compounds that have antioxidant and anti-inflammatory propertiest (Table 7). Our study clearly identified the presence of tannins and

saponins in all the three extracts, specifically in *V. amygdalina*. In our study, presence of coumarins was detected only in *S. rosmarinus*. Glycosides are present in *V. amygdalina* and *S. rosmarinus* and absent in O. *integrifolia.* Terpenoids were found commonly in all the three plants leaves.

Table 6: Phytochemical screening tests result of different grade solvents extract of *the target plant leaves*.

			Extracts		
Botanical name	**Phytochemicals**	**Phytochemical screening tests**	**Petroleum Ether**	**Chloroform/ Methanol(1:1)**	**Methanol**
Vernonia amygdalina	Alkaloids	Wagner's test	+	+	+
	Steroids	Libermann Burchard	+	+	+
	Glycoside	Keller-Killiani Test	+	+	+
	Coumarins	Appirade test	-	-	-
	Terpenoids	Libermann Burchard Test	+	+	+
	Flavonoids	Shinoda Test	+	+	+
	Carbohydrate	Fehling's Test	+	+	+
	Tannins	Lead Acetate Test	+	+	+
	Saponins	Foam test	+	+	+
Otostegia integrifolia	Alkaloids	Wagner's test	+	+	+
	Steroids	Libermann Burchard	+	+	+
	Glycoside	Keller-Killiani Test	-	-	-
	Coumarins	Appirade test	-	-	-
	Terpenoids	Libermann Burchard Test	+	+	+
	Flavonoids	Shinoda Test	+	+	+
	Carbohydrate	Fehling's Test	+	+	+
	Tannins	Lead Acetate Test	+	+	+
	Saponins	Foam test	-	+	+
Salvia rosmarinus	Alkaloids	Wagner's test	+	+	+
	Steroids	Libermann Burchard	+	+	+
	Glycoside	Keller-Killiani Test	-	+	-
	Coumarins	Appirade test	-	+	+
	Terpenoids	Libermann Burchard Test	+	+	+
	Flavonoids	Shinoda Test	+	+	+
	Carbohydrate	Fehling's Test	-	+	+

	Tannins	Lead Acetate Test	+	+	+
	Saponins	Foam test	+	+	+

Key: + indicates highly present, - indicates absence

4.3 Structural elucidation of isolated compounds

4.3.1 Compound 1 isolated from leaves of *V. amygdalina*

The phytochemical investigation of different extracts of *V. amygdalina* leaves led to the isolation and identification of compound **1** (Figure 18) from the leaves *V. amygdalina*

Compound 1:

Compound **1** was obtained (11 mg) as yellow oil with R_f value of 0.51 with petroleum ether: $CHCl_3$ (9:1) as a mobile phase. The ^{1}H-NMR spectroscopic data (400 MHz, $CDCl_3$, and Appendix 1.1) varied between δ 0.9 to 5.4 .The triplet signal observed at δ 2.3 is attributed to methylene protons attached to a carboxyl group. The multiplet signal at δ 2.0 (m, 2H) are integrated for the allylic methylene protons. The signal showed at δ 2.8 (J = 6 Hz) attributed to the methylene protons flanked between the two double bonds. The intense broad singlet signal due to many overlapping methylene protons was observed at δ 1.3 which was supported by the appearance of an intense carbon signal at δ 29.7 in the ^{13}C-NMR spectrum. The spectrum showed signals for olefinic proton at δ 5.4 (*brs*, 2H, H-9 and H-12). The proton signal showed at δ 0.9 suggesting the presence of terminal methyl proton. The ^{13}C-NMR (101 MHz, $CDCl_3$, Table 7 and Appendix 1.2) spectral data with the aid of DEPT-135 (Appendix 1.3) revealed the presence of four olefinic signals at δ 130.2, 130.0, 128.0, and 127.9. The ^{13}C NMR also showed the presence of 18 signals, including 12 methylene carbons at δ 34.1, 31.9, 31.5, 29.7, 29.4, 29.3, 27.2, 27.2, 25.6, 24.9, 22.7, and 22.6. The most downfield and up field signals at δ 178.8 and 14.1 belong to the carboxyl and terminal methyl groups, respectively. Additionally, two olefinic carbons are present at δ 130.2, and 128.0. Thus, based on the above spectral data, the structure of compound **1** (Figure 18) was identified as linoleic acid. Linoleic acid is an omega-6 fatty acid (Table 7).

4.3.2 Compounds 2, 3 and 4 isolated from leaves of *O. integrifolia*

The phytochemical investigation of different extracts of *O. integrifolia* leaves led to the isolation and identification of three compounds **2**, **3** and **4** (Figure 18) from the leaves *O. integrifolia*

Compound 2:

Compound **2** was white gel (amorphous) obtained (16 mg) from chloroform/Methanol leaves extract and its *Rf* 0.40 (*n*-hexane: EtOAc, 6:4) as eluent. The ^{1}H NMR (400 MHz, $CHCl_3$, Table 7, Appendix-2.1) spectrum of compound **2** showed the presence of three methyl singlets at 0.94 (3H), 0.91 (3H), and 0.82 (3H), a methyl doublet at 0.88 (J = 9.6 Hz), and the presence of a methine carbinol signal at δ (brs, 3.35). The ^{1}H NMR also showed typical signals of furan moiety at ծ 6.25 (1H, brs, H-14), 7.31 (1H, brs, H-15) and 7.19 (1H, brs, H-16), this suggesting the presence of two of the double bonds due to the furan ring. The remaining proton signals were overlapping in the range of ծ 1.24– 1.92. The ^{13}C-NMR (400 MHz, $CHCl_3$, Table 8, Appendix -2.2) spectrum along with the DEPT-135 (Appendix-2.3) of compound **2** showed the presence of 20 carbons, including four methyls, six methylenes, two aliphatic methines, and four quaternary carbons. The spectrum also revealed an oxygenated methine carbon signal at δ 75.9 (C-3). The methine carbons of the furan moiety were revealed at ծ 110.9 (C-14), 142.7 (C-15) and 138.4 (C-16). The spectral results provided above were in good agreement with those for Otostegindiol (Table 7). Thus, compound **2** (Table 7, Figure 18) was elucidated to be Otostegindiol; this compound has been reported the same species.

Compound 3:

It was isolated as an amorphous (10 mg); from chloroform/Methanol leaves extract; R_f 0.4 ($CHCl_3$/MeOH; 1:1). The ^{1}H NMR (400 MHz, DMSO, Table 7, Appendix -2.4) spectrum of compound **3** showed typical signals of furan moiety at ծ 6.31 (1H, brs, H-14),7.48 (1H, brs, H-15) and 7.35 (1H, brs, H-16), this suggesting that three of the double bonds were because of the furan ring. The proton signal at δ 3.57 (brs, 1H, H-3) corresponds to the oxygenate1d sp^3 methine proton. The ^{1}H and ^{13}C NMR (400 MHz, $CHCl_3$, Table 7) data of compound **3** were identical to those of **2** with the exception of the appearance of an additional oxygenated methine carbon signal to compound **3**. The ^{13}C NMR (DMSO, 101 MHz, Table 7, Appendix -2.5) spectrum in combination with DEPT-135 (Appendix -2.6) displayed 20 well-resolved carbon signals

including three methyl, six methylene, three methine (Of these two methine groups, which are oxygenated), and four quaternary carbons of which one sp^2, one sp^3 oxygenated methine and two sp^3 aliphatic quaternary carbons appeared at ð 126.3, 76.3, 42.9 and 37.7 attributed to C-13, C-9, C-4, and C-10, respectively, whereas the olefinic methine carbons of the furan moiety were revealed a signal at δ 111.5, 143.3, and 138.7 corresponding to C-14, C-15 and C-16, respectively. The spectral data for compound **3** is similar to the data reported for Otostegindiol (Table 7), with the only difference being the presence of an additional hydroxyl at C-6 in compound **3** (Table 7).

Compound 4:

Compound **4** was isolated as an amorphous (26 mg); from chloroform/Methanol leaves extract and its *Rf* value of 0.42 (*n*-hexane: EtOAc, 6:4) as eluent. The ^{1}H NMR (400 MHz, $CHCl_3$, Table 7, Appendix -2.7) data of compound **4** were identical to those of 2 except that the furan ring signals were replaced by ð 6.12 (brs, ^{1}H, H-15), and 5.72 (brs, ^{1}H, H-14), suggesting of a β,β-disubstituted dihydrofuran ring (Tesso, and Konig, 2004). The two doublet proton signals at 4.38 (d, J = 8.6 Hz, 1H), 4.11 (d, J = 8.4 Hz, 1H) which are AB system consistent to the methylene group of the dihydrofuran ring (H-16). The analysis of the ^{13}C NMR spectrum (Appendix -2.8) spectrum in combination with DEPT-135 (Appendix -2.9) displayed signals corresponding to four quaternary carbons, including two olefinic carbon signals, and two oxygenated aliphatic quaternary carbons, two aliphatic methine carbon signals, one oxygenated methylene carbon signals, six methylene carbon signal and four methyls groups. The signal at δ 75.9 is attributed to sp^3 oxygenated methine at C-3. The NMR spectral data of compound **4** is in good arrangement with data reported for pretotostegindiol, the same species (Table 7).

Table 7: ^{1}H NMR and ^{13}C NMR data of compound **1**, **2, 3, 4** and ^{13}C-NMR data DMSO,in ppm) ($CDCl_3$. δ *in ppm)* (Tesso and Konig, 2004 ; Simopoulos, 2004)

Position	1		2		2*	3		4		4*
	δH^a	δC	δH	δC	δC	δH	δC	δH	δC	δC
1	-	178.8		24.7	25.1		25.0		24.9	25.1
2	2(t,J=7.8Hz)	34.1		25.1	25.6		25.9		25.1	25.2
3	2(m,2H)[b]	24.9	3.35 (brs, 1H)	75.9	76.3	3.57 (brs, 1H)	79.6		75.9	76.1
4		29.2		42.8	43.2	-	42.9		42.1	42.4
5		29.3		39.6	39.8		49.0		39.7	39.8
6		29.4		21.2	21.6		74.3		21.2	21.2
7		29.7		31.1	31.4		31.2		31.5	31.6
8		27.2		36.5	37.1		36.5		36.7	37.3
9	5.4(brs,1H)	130.2	-	76.9	77.5	-	76.3		94.1	93.3
10		128.0	-	37.6	37.8	-	37.7		37.7	37.7
11	2.8(t,J=6.0Hz)	25.8		35.1	35.5		35.7		31.9	32.0
12	5.4(brs,1H)	128.0		21.6	21.9		21.8		36.0	35.6
13		130.2	-	125.7	126.1	-	126.3		88.5	93.0
14		27.2	6.25 (1H, *brs*)	110.9	111.3	6.31 (brs, 1H)	111.5	5.72 (brs, 1H)	102.6	107.6
15	1.3(m,2H)[b]	29.7	7.31 (1H, *brs*)	142.7	143.2	7.48 (brs, 1H)	143.3	6.12 (brs, 1H)	141.7	147.8
16		22.7	7.19 (1H, *brs*)	138.4	138.9	7.35 (brs, 1H)	138.7	4.38 (d, *J* = 8.6 Hz, 1H), 4.11 (d, *J* = 8.4 Hz, 1H)	78.7	81.6
17		31.7		16.2	16.5	0.74 (d, *J* = 7.9 Hz)	16.5		17.5	17.6
18	0.9(t,J=7.3Hz)	14.1		22.5	22.6	0.82 (s, 3H)	22.9		22.4	22.4
19				28.7	29.0	0.82 (s, 3H)	29.4		28.2	29.5
20				16.3	16.8	0.85 (s, 3H)	16.8		22.3	21.1

[a] coupling constant(J) in Hz are indicated in parentheses; [b] overlapping signals;Literature report 2*= Otostegindiol; and 4* = Pretotostegindiol

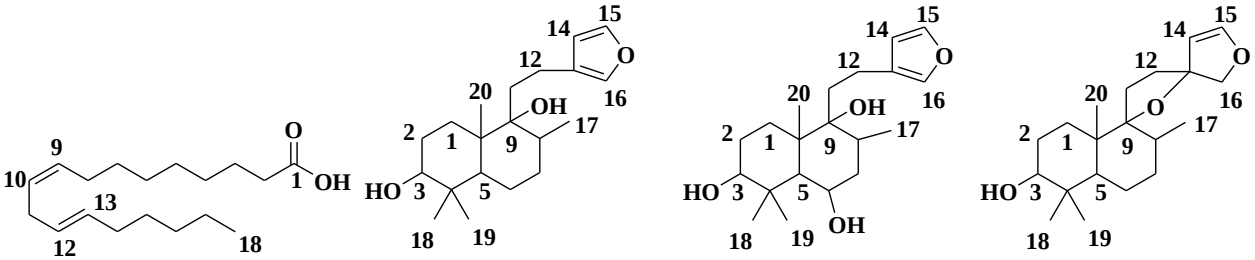


Figure 18: Structure of isolated compounds from the leaves of *V. amygdalina* compound **1** and *O. integrifolia* compound **2-4**

4.3.3 Compounds 5 and 6 isolated from leaves of *S. rosmarinus*

The phytochemical investigation of different extracts of *S. rosmarinus* leaves led to the isolation and identification of two compounds **5** and **6** (Figure 19).

Compound 5:

Compound **5** (10 mg) was isolated as an amorphous from the Methanol/Chloroform (1:1) leaves extract of *Salvia rosmarinus*. TLC profile showed a spot at Rf 0.42 with Methanol/Chloroform (3:2) as a mobile phase. Two compounds were characterized using NMR spectroscopic methods (Figure 19). The ^{1}H-NMR spectrum (600 MHz, MeOD, Table 8, Appendix – 3.1) of compound **5** showed the presence of one olefinic proton signal at δ 5.3 (t, J = 3.7 Hz, 1H), two deshielded protons at δ 4.7 (m, 1H), and 4.1 (m, 1H) associated with the C-30 *exocyclic* methylene group, and one O-bearing methine proton at δH 3.2 (m, 1H), and six methyl protons at δ 1.14 (s, 3H), 1.03 (d, J = 6.3 Hz, 3H), 1.00 (s, 3H), 0.98 (s, 3H), 0.87 (s, 3H), and 0.80 (s, 3H). A proton signal at δ 2.22 (d, *J* = 13.5 Hz, 1H) was attributed to methine proton for H-18. Other proton signals integrating for 20 protons were observed in the range δ 2.2 to 1.2. The proton decoupled ^{13}C-NMR and DEPT-135 spectra (151 MHz, CDOD, (Appendix -3.2 and Appendix -3.3) of compound **5** revealed the presence of 30 well-resolved carbon signals, suggesting a triterpene skeleton. The analysis of the ^{13}C NMR spectrum displayed signals corresponding to six methyl, nine methylene, seven methine, and eight quaternary carbons. Among them, the signal observed at δ 125.5 (C-12) belongs to olefinic carbons. The methylene carbon showed signals at δ_C 39.9, 28.5, 18.1, 36.7, 23.9, 30.4, 26.5, 32.9, and 38.6. The quaternary carbons showed a signal at δ_C 39.4, 41.9, 38.4, 138.2, 41.8, and 47.8. The signals of exocyclic methylene carbon signals appeared at δ 153.1 and 103.9. The spectrum also showed sp^3 oxygenated methine carbon at δ 78.3 and carboxyl carbon at δ 180.2. The spectrum revealed signals due to methyl groups at δ_C 27.4, 16.3, 15.0, 20.2, 22.7, and 16.4. The remaining carbon signals for aliphatic methines were showed at δ_C 55.3, 55.2, 53.0, and 37.1. The NMR spectral data of compound **5** (Figure 19, Table 8) was matched arrangement with data micromeric acid, the same species.

Table 8: Comparison of the ^{13}C-NMR spectral data of compound **5** and micromeric acid (MeOD, δ in ppm) .

Position	**NMR data of compound 5**		**(Abdel-Monem *et al.*, 2017)**
	^{1}H-NMR	**^{13}C-NMR**	**^{13}C-NMR**
1		38.60	39.9
2		27.8	28.5
3	3.2 (m, 1H)	78.3	80.3
4	-	39.4	39.9
5		55.3	56.7
6		18.1	18.3
7		36.7	34.2
8	-	41.9	40.7
9		53	48.8
10	-	38.4	38.2
11		23.9	24.6
12	5.3 (t, *J* = 3.7 Hz, 1H)	125.5	127.7
13	-	138.2	138
14	-	41.8	43.3
15		30.4	29.1
16		26.5	25.6
17	-	47.8	48
18	δ 2.22 (d, *J* = 13.5 Hz, 1H)	55.2	56.1
19		37.1	38.7
20	-	153.1	152.8
21		32.9	33.5
22		39.0	40.1
23		27.4	29.4
24		16.3	16.9
25		15.0	16.6
26		20.2	18.3
27		22.7	24.6
28	-	180.2	177.8
29		16.4	17.3
30	4.7 (m, 1H), and 4.1 (m, 1H)	103.9	106.5

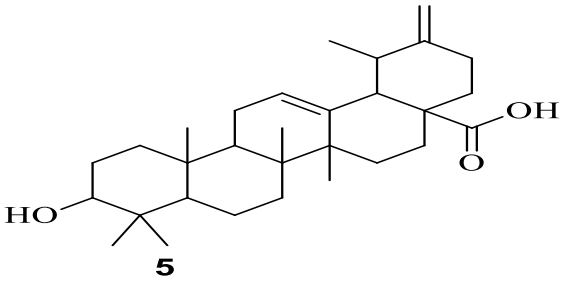
Compound 6:

Compound **6** was found as an amorphous add(18 mg), TLC profile showed a spot at Rf 0.4 with chloroform/Methanol leaves extract; Compound **6** (18 mg) was obtained as a white amorphous isolated from Methanol/Chloroform (1:1) by column chromatography after purification from Petroleum ether fraction. The ^{1}H NMR (600 MHz, DMSO, (Appendix -3.4) spectral-data showed two doublets at 7.79 (d, *J* = 8.7 Hz, 2H), and 6.90 (d, *J* = 8.7 Hz, 2H) which are evident for the

presence of 1,at 4-disubstituted aromatic group. The oxygenated methylene and terminal methyl protons were shown at ð 4.25 (q, *J* = 7.1 Hz, 2H) and 1.29 (t, J = 7.1 Hz, 3H), respectively. The ^{13}C-NMR spectrum, with the aid of DEPT-135 (151 MHz, DMSO, Table 9, Appendix -3.5 and Appendix -3.6) spectra of compound **6** showed the presence of well-resolved 7 carbon peaks corresponding to 9 carbons including 3 quaternary carbons, 1 oxygenated methylene carbon, 1 terminal methyl carbon, and two symmetrical aromatic methine carbons. The presence of quaternary carbon signals was shown at *δ* 120.9 (C-1), 148.2 (C-4), and ester carbonyl at *δ* 166.0 (C-7). The symmetry aromatic carbons signal was observed at *δ* 131.4 (C-2, 6), and 116.8 (C-3, 5). The oxygenated methylene and terminal methyl carbons appeared at ð $_C$ 60.4 (C-8) and 14.7 (C-9), respectively. The spectral results provided above were in good agreement with those for benzocaine reported by Alotaibi *et al*., (2014) . Accordingly, compound **6** was characterized to be benzocaine (4-Aminobenzoic acid-ethyl ester) (Table 9, Figure 19 and Appendix -3.1, Appendix -3.2, Appendix -3.3), this compound has never been reported before from the leaves of *Salvia rosmarinus.*

Table 9: Comparison of the ^{1}H-NMR, and ^{13}C-NMR spectral data of compound **6** and benzocaine (DMSO, δ in ppm)

Position	**NMR data of compound 6**		**(Alotaibi *et al*., 2014 ; Nathan *et al*., 1990)**	
	^{1}H-NMR	**^{13}C-NMR**	**^{1}H-NMR**	**^{13}C-NMR**
1	-	120.9	-	119
2	7.79 (d, *J* = 8.7 Hz, 2H)	131.4	7.86 (d, *J* = 7.6 Hz)	132
3	6.90 (d, *J* = 8.7 Hz, 2H)	116.8	6.83 (d, *J* = 7.6 Hz)	114
4	-	148.2	-	151
5	6.90 (d, *J* = 8.7 Hz, 2H)	116.8	6.83 (d, *J* = 7.6 Hz)	114
6	7.79 (d, *J* = 8.7 Hz, 2H)	131.4	7.86 (d, *J* = 7.6 Hz)	132
7	-	166.0	-	169
8	4.3 (q, *J* = 7.1 Hz, 2H)	60.4	4.3 (q, *J* = 7.0 Hz)	61
9	1.3 (t, J = 7.1 Hz, 3H)	14.7	1.36 (t, *J* = 7.0 Hz)	15



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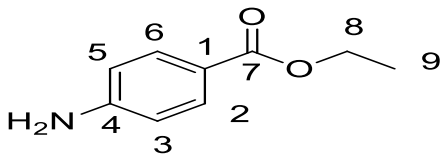

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Figure 19: Structure of isolated compounds from the *leaves of Salvia rosmarinus.*

4.3 Antimicrobial activity assay

The prepared of antimicrobial activity of the different extracts (Table 10-12) was examined against the same human pathogens stated at three different concentrations (100, 75, and 50 µg mL^{-1}). Resulted zone of inhibition, expressed as mean ± standard deviation (in mm) of each tested extract including the antibiotic agent against each pathogen, is presented in Tables 10-12 for the studied plant leaf extracts.

4.4.1 Antimicrobial activity of extracts derived from *V. amygdalina*

As shown in Table 10, all the tested concentrations (75-100 µg mL^{-1}) of the petroleum ether leaves extracts of *V. amygdalina* showed an activity (inhibition zone of ≥ 7 mm) against the bacterium *P.aeruginosa* with the higher inhibitory value (17.80 ± 0.2 mm) followed by methanol extracts against the bacterium *K.pneumoniae* with the high inhibitory value (16.87±0.23mm). The chloroform: methanol (1:1) extracts against the bacterium *P.aeruginosa* was (14.57±0.37 mm) at the maximum dose of 100 µg mL^{-1} (Table 10). The chloroform: methanol (1:1) leaf extracts of *V. amygdalina* exhibited the highest inhibition value (15.90±0.14 mm) against *C. albicans* among all the extracts tested at the maximum concentration of 100 µg mL^{-1}. Similarly, the petroleum ether leaf extract showed significant inhibition (12.83±0.15 mm) against *A.niger* at the same concentration (Table 10).

Petroleum ether extract of *V. amygdalina* exhibited significant activity against *P. aeruginosa*, with a high inhibition value of 17.80 ± 0.2 mm, surpassing the inhibition value of Ciprofloxacin used as a positive control (16.00 ± 0.00 mm) (Table 10, Appendix 4.1). The petroleum ether, chloroform : methanol (1:1), and methanol extracts of *V. amygdalina* demonstrated significant activity against all the microbes tested in this study at 100 µg mL^{-1}, with inhibition zones ranging from 7 mm to 18 mm (Table 10). The petroleum ether extract of *V. amygdalina* was significantly active against *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, and *K. pneumoniae*, as well as the fungi *C. albicans* and *A.niger*, at a concentration of 100 µg mL^{-1}. Both the chloroform: methanol (1:1) and petroleum ether extracts showed significant activity against *C. albicans* and *A.niger*, respectively, at concentrations of 50-100 µg mL^{-1}, exhibiting inhibition zones of 11-15 mm and 8-13 mm, respectively (Table 10).

Table 10: Comparison of Mean zone of inhibition (MZI) leaf extracts of *Vernonia amygdalina*

Petroleum ether extracts

Samples and standard antibiotics	Concentration(µg mL^{-1}) of extract in 99.8% DMSO	Average values of the zone of inhibition (mm)						
		Gram-positive(+) bacteria		Gram-negative (-) bacteria			Fungus	
		S. aureus	*S. epidermidis*	*E. coli*	*P.aeruginosa*	*K.pneumoniae*	*C. albicans*	*A.niger*
V. amygdalina	**50**	5.93±0.06	9.70±0.75	9.40±0.10	8.83±0.76	2.83±0.49	5.07±0.25	7.63±0.15
	75	7.77±0.15	12.27±0.46	11.97±0.55	15.17±0.76	3.97±3.00	7.77±0.25	9.87±0.15
	100	12.60±0.10	13.13±0.61	13.60±0.10	17.80±0.20	9.50±0.50	9.73±0.23	12.83±0.15
Standard antibiotics	**Cipro.**	14.83±0.76	18.33±0.29	18.17±0.76	16.00±0.00	14.33±0.29		
	Ketocon.						15.90±0.17	16.23±0.40
Chloroform/Methanol(1:1) Extracts								
V. amygdalina	**50**	8.38±0.02	8.33±0.01	5.87±0.66	6.73±0.12	5.50±0.41	10.50±0.41	3.60±0.08
	75	10.68±0.02	9.36±0.00	6.80±0.08	9.53±0.12	8.37±0.26	13.03±1.81	6.63±0.39
	100	13.50±0.08	12.64±0.03	9.67±0.12	14.57±0.37	12.63±0.12	15.90±0.14	9.70±0.22
Standard antibiotics	**Cipro.**	15.50±0.71	16.00±0.00	17.1±0.24	18.00±0.00	17.13±0.19		
	Ketocon.						17.00±0.82	16.37±0.26
Methanol Extracts								
V. amygdalina	**50**	6.33±0.29	10.50±0.50	5.20±0.20	7.20±0.35	12.73±0.38	0.00±0.00	0.00±0.00
	75	8.70±0.30	13.13±0.23	6.83±0.29	9.43±0.40	15.60±0.53	6.83±0.21	0.00±0.00
	100	11.90±0.17	15.73±0.25	10.73±0.23	13.33±0.29	16.87±0.23	8.80±0.20	6.67±0.15
Standard antibiotics	**Cipro.**	16.00±0.00	18.10±0.36	14.73±0.25	19.00±1.00	19.00±1.00		
	Ketocon.						14.37±0.32	9.33±0.31

Mean values of Flavonoids (mg/g) by 570nm

		Petroleum ether Extracts	Chloroform/Methanol	Methanol Extracts
V. amygdalina	**50**	0.690	0.697	0.802
	75	0.772	0.871	0.882
	100	0.947	0.944	0.925

Key: Bacteria: *S. aureus*, *Staphylococcus aureus; S.epidermidis*, *Staphylococcus epidermidis E. coli*, *Escherichia coli; P. aeruginosa, Pseudomonas aeruginosa;* and *K.pneumoniae*, *Klebsiella pneumonia*. Fungus*: C. albicans*, *Candida albicans;* and *A.niger*, *Aspergillus niger* .Antibiotics: Cipro, Ciprofloxacin; Ketocon, ketoconazole (Nizoral); DMSO 99.8%, Dimethyl sulfoxide.

V. amygdalina exhibited a notably high zone of inhibition, with diameters of 17.80 ± 0.20 mm for the petroleum ether extract and 16.87 ± 0.23 mm for the methanol extract, demonstrating antimicrobia properties against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, both Gram-negative bacteria (Table 10, Appendix 4.1). The chloroform: methanol(1:1) extract of *V. amygdalina* exhibited high activity against the Gram-positive bacteria *S. aureus* and *S.*

epidermidis, with inhibition zones of 14 mm and 16 mm, respectively, compared to the positive control, which produced inhibition zones of 16 mm and 18 mm. In this study, the petroleum ether, chloroform:methanol(1:1), and methanol extracts of *V. amygdalina* demonstrated significant activity against all the microbes tested at 100 µg mL^{-1}, with inhibition zones ranging from 7 to 18 mm (Table 10).

The different extracts, using various solvents of various polarity indexes, have been attributed to specific biological activities. For example, the antimicrobial activities of *V. amygdalina* extracts may be due to alkaloids, terpenoids, flavonoids tannins, and saponins in natural products (Table 6). From literature, flavonoids are a class of phenolic compounds used as anti-infections and were reported to possess antioxidant, antifungal, antiviral, and antibacterial activity. Here, the results of this study revealed that *V. amygdalina* exhibited the highest inhibition value (15.90±0.14 and 12.83±0.15 mm) against *C. albicans* and *A.niger*, respectively. Thus, the results of this study revealed that *V. amygdalina* contain flavonoids (69 -93%) (Table 10) .

4.4.2 Antimicrobial activity of extracts derived from *O. integrifolia*

The extracts from *O. integrifolia* were evaluated *in Vitro* activities against microbes from the Gram-positive bacteria (*S. aureus*, and *S.epidermidis*), Gram-negative bacteria (*E. coli*, *P. aubergines*, and *K. pneumoniae*), and fungi (*C. albicans* and *A.niger*) (Table 11, Appendix 4.2). The petroleum ether extracts exhibited significant activity against all microbes tested in our study at 100 µg mL^{-1}, with inhibition zones ranging by negotiating from 7 to 16 mm (Table 11), except for *E. coli* and *A.niger*, which showed lower inhibition zones among the bacteria and fungi, respectively. The chloroform/methanol (1:1) extracts exhibited significant activity against all the microbes tested in our study at 100 µg mL^{-1}, with inhibition zones ranging by negotiating from 10 to 18 mm (Table 11), except for *P. aeruginosa*, which showed no inhibition. The methanol extracts of *O. integrifolia* leaves demonstrated significant activity, particularly against *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, and *K. pneumoniae*, as well as against the fungi *C. albicans* and *A.niger*, at 100 µg mL^{-1}, exhibiting inhibition zones ranging from 8 to 16 mm (Table 11).

Determining the given solvent extracts in *O. integrifolia* plants resulted relatively high comparable with positive (+) control. Specially, the *O. integrifolia* petroleum ether leaves extracts against drug resistance human pathogenic bacteria S. *aureus, S. epidermidis, P.aeruginosa*, and *K.pneumoniae* were minimum zone of inhibition (MZI) recorded that 12.13±0.42, 14.87±0.15, 10.93±0.12, and 15.97±0.06 mm study-tested at the concentration 100 µg mL^{-1}, respectively and against human pathogenic fungi *C. albicans* with minimum zone of inhibition (18.17±0.29 mm) which was used from fungal against *C. albicans* MZI recorded that 18.17±0.29 mm higher than the positive control (12.83±0.76mm) at 100 µg mL^{-1}. The *O. integrifolia* of chloroform/methanol (1:1) extracts were found to have strong activity against *S. aureus* (12.30±0.26 mm) higher than the positive control (10.67±0.58) at 100 µg mL^{-1}.

The methanol extracts of leaves in our study *O. integrifolia* was found overall MZI recorded by 15.17±0.29 mm and 16.97±0.06 mm in under study bacterium of *S. aureus* and *S. epidermidis,* respectively the stronger activity than the positive control. The *O.integrifolia* extracts showed better antifungal activities than the Gram-negative bacteria (Table 11). Therefore, the three extracts, using various solvents of different polarity indexes, have been attributed to specific biological activities. For example, the antimicrobial activities of *O. integrifolia* extracts may be due to the presence of alkaloids, terpenoids, flavonoids, tannins, and saponins in natural products (Table 6).

The highest antibacterial results recorded a mean inhibition with diameters of 17 mm and 16 mm at a concentration of 100 mg mL^{-1} against *S. epidermidis* and *K. pneumoniae*, respectively, and in methanol and petroleum ether extracts, respectively. The petroleum ether extract of *O. integrifolia* was able to inhibit the growth of *S. epidermidis, P.aeruginosa, K.pneumoniae* and *C. albicans,* with inhibition zones of 15 mm, 11mm, 16mm, and 18 mm, respectively. The petroleum ether extracts showed good efficacy against all the tested microbes, particularly Gram-negative bacteria and fungi (Table 11).

Table 11: Comparison of Mean zone of inhibition (MZI) leaf extracts of *Otostegia integrifolia*

Petroleum ether extracts

Samples and standard antibiotics	Concentration(µg mL^{-1}) of extract in 99.8% DMSO	Average values of the zone of inhibition (mm)						
		Gram-positive(+) bacteria		Gram-negative (-) bacteria			Fungus	
		S. aureus	*S. epidermidis*	*E. coli*	*P.aeruginosa*	*K.pneumoniae*	*C. albicans*	*A.niger*
O.integrifolia	**50**	0.00±0.00	10.67±0.76	0.00±0.00	7.83±0.42	12.50±0.10	0.00±0.00	0.00±0.00
	75	0.00±0.00	12.13±0.42	0.00±0.00	8.87±0.64	14.10±0.46	15.67±1.15	0.00±0.00
	100	12.13±0.42	14.87±0.15	0.00±0.00	10.93±0.12	15.97±0.06	18.17±0.29	5.83±0.29
Standard antibiotics	**Cipro.**	10.00±0.00	14.77±0.25	14.00±0.00	10.67±0.58	14.00±0.00		
	Ketocon.						12.83±0.76	9.50±0.50
Chloroform/Methanol(1:1) Extracts								
	50	6.83±0.76	0.00±0.00	6.07±0.40	0.00±0.00	0.00±0.00	10.50±0.71	13.33±0.29
O.integrifolia	**75**	8.33±0.58	0.00±0.00	8.13±0.23	0.00±0.00	11.17±0.76	13.17±0.57	15.50±0.50
	100	12.30±0.26	10.33±0.58	9.80±0.20	0.00±0.00	12.40±0.35	14.50±0.87	17.60±0.53
Standard antibiotics	**Cipro.**	10.67±0.58	14.00±0.00	15.00±0.00	10.33±1.15	13.00±0.00		
	Ketocon.						15.33±0.58	18.00±0.00
Methanol Extracts								
	50	11.13±0.23	12.77±0.21	0.00±0.00	7.07±0.31	0.00±0.00	15.17±0.29	6.63±0.71
O.integrifolia	**75**	12.77±0.25	14.93±0.12	0.00±0.00	7.87±0.81	0.00±0.00	16.17±0.29	7.40±0.36
	100	15.17±0.29	16.97±0.06	10.67±1.15	8.67±0.58	12.77±0.68	16.43±1.25	7.80±0.17
Standard antibiotics	**Cipro.**	15.00±0.00	14.33±0.58	12.33±0.58	11.67±0.58	14.00±0.00		
	Ketocon.						19.00±0.00	11.00±0.00

Mean values of Flavonoids (mg/g) by 570 nm

		Petroleum ether Extracts	Chloroform/Methanol	Methanol Extracts
O.integrifolia	**50**	0.606	0.697	0.802
	75	0.702	0.821	0.870
	100	0.917	0.904	0.920

Key: Bacteria: *S. aureus*, *Staphylococcus aureus; S.epidermidis*, *Staphylococcus epidermidis E. coli*, *Escherichia coli; P. aeruginosa, Pseudomonas aeruginosa;* and *K.pneumoniae*, *Klebsiella pneumonia*. Fungus*: C. albicans*, *Candida albicans;* and *A.niger*, *Aspergillus niger* .Antibiotics: Cipro, Ciprofloxacin; Ketocon, ketoconazole (Nizoral); DMSO 99.8%, Dimethyl sulfoxide

The petroleum ether, chloroform: methanol (1:1), and methanol extracts of *O. integrifolia* exhibited significant activity against all the tested fungal pathogens at 100 µg mL^{-1} that showed inhibition zone of ≥ 7 mm resulted in an inhibition zone ranging from 8 to 18 mm (Table 11, Appendix 4.2),except *A.niger* under study-tested of petroleum ether leaves extracts. Petroleum

ether extracts of the leaves were used at a concentration of 100 mg mL^{-1}, resulting in impressive inhibition zone diameters of 11 mm for *P. aubergines*, and 16 mm for *K. pneumoniae*, respectively.

The Chloroform/Methanol (1:1) extracts of *O. integrifolia* leaves were the high significantly active against *C. albicans* and *A.niger* with 11-15 mm and 13-18 mm, respectively at 50-100 µg mL^{-1} and also methanol extracts of *O. integrifolia* leaves were the high significantly active against *C. albicans* and *A.niger* with 15-18 mm and 7- 8 mm, respectively at 50-100 µg mL^{-1}. Our study found that at a concentration of 50 µg mL^{-1}, Petroleum ether, extracts did not display any significant inhibition effects against the tested microbes Viz. *S. aureus, E. coli, C. albicans* and *A.niger*. Chloroform/Methanol (1:1) extracts did not display any again through bacterium Viz. *S. epidermidis; P.aeruginosa, and K.pneumoniae* and methanol, extracts did not also display any through bacterium Viz. *E. coli,* and *K.pneumoniae* .This implies that the samples have a dose-dependent inhibitory effect on the pathogens. The leaves of *O. integrifolia* have been found to possess remarkable antimicrobial properties against Gram-negative bacteria in different extracts such as *P. aubergines*, and *K. pneumoniae* with 10.93±0.12 mm and 15.97±0.06 mm in Petroleum ether: *E.coli* and *K. pneumoniae* with 9.80±0.20 mm and 12.40±0.35 mm in Chloroform/Methanol (1:1); and *E.coli*, *P.aeruginosa* and *K. pneumonia* with 10.67±1.15 mm, 8.67±0.58mm, and 12.77±0.68 mm in methanol, respectively.

However, in our study *O. integrifolia* has been found to possess a remarkably high zone of inhibition with diameters of 15.17±0.29 mm and 16.97±0.06 mm antimicrobial properties against *S. aureus,* and *S.epidermidis* of Gram-positive bacteria, respectively.The results are summarized in Appendix -4.2. Methanol extract was found to have a wide range of bioactive compounds including favonoids because of its high polarity, the mean values of flavonoids content of methanol extract of *O. integrifolia* was 92 mg g^{-1} while that the petroleum ether, and chloroform: methanol (1:1) was 91.7 mg g^{-1}, and 90 mg g^{-1}, respectively contains more flavonoids at a concentration of 100 mg mL^{-1}(Table 11).

The petroleum ether, and chloroform: methanol (1:1) and methanol extracts from the leaves of *O. integrifolia* show differences in the inhibition of growth of bacterial and fungial strains. The

absolute values of the diameter zones of inhibition (DZI) varied from 10 to 15.97±0.06 mm, 6 to 18.17±0.29, and 9 to 16.97±0.06, respectively at concentration of 100 µg mL^{-1}. On the other hand, in this study found that the highest inhibition in petroleum ether extract against *Candida albicans* (18.17±0.29 mm) and also the highest inhibition in petroleum ether extract against Gram negative bacteria (*P.aeruginosa* and *K.pneumoniae*) compare to the positive controls at concentration of 100 µg mL^{-1} (Table 11).

4.4.3 Antimicrobial activity of extracts derived from *S.rosmarinus*

The extracts and isolated compounds from *Salvia rosmarinus* were evaluated *in-vitro* against microbes from Gram-positive bacterial (*S. aureus*, and *S.epidermidis*), Gram-negative bacteria (*E. coli*, *P. aubergines*, and *K. pneumoniae*) and fungi (*C. albicans* and *A.niger*) (Table 12, Appendix 4.1). The petroleum ether extracts exhibited significant activity against all the tested microbes at 100 µg mL^{-1}, resulting in an inhibition zone ranging from 7 to 21 mm (Table 12). The Chloroform/Methanol (1:1) extracts were significantly active against *E. coli* and *K. pneumonia* and *A.niger* at 100 µg mL^{-1} with inhibition zone ranged from 12 to 14 mm (Table 12). Methanol extracts exhibited significant activity against *S. aureus, E. coli* bacteria, and *A.niger* fungi at 100 µg mL^{-1}. The inhibition zone was recorded to be 11 to 13 mm (Table 12). Additionally, our findings indicated that the mean values of flavonoids (mg/g) tested were 92.2%, 90.4%, and 94.0% for petroleum ether, chloroform/methanol (1:1), and methanol extracts, respectively. Flavonoids acted as inhibited bacterial growth, disrupting cell membranes, and interfering with biofilm formation. This suggests that the groups of phenolic compounds evaluated that play a significant role in antimicrobial activities, particularly against antibiotic-resistant strains.

Table 12: Comparison of Mean zone of inhibition (MZI) leaf extracts of *Salvia rosmarinus.*

Petroleum ether Extracts

Samples and standard antibiotics	Concentration(µgmL^{-1}) of extract in 99.8% DMSO	Average values of the zone of inhibition (mm) Gram-positive(+) bacteria		Gram-negative (-) bacteria			Fungus	
		S. aureus	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*K.pneumoniae*	*C. albicans*	*A.niger*
S.rosmarinus	**50**	18.50±0.50	15.33±0.58	0.00±0.00	0.00±0.00	10.00±0.00	15.93±0.12	4.47±0.50
	75	19.87±0.06	17.00±0.00	9.33±0.29	10.53±0.50	10.93±0.12	18.87±0.23	5.47±0.50
	100	21.37±0.78	17.50±0.50	11.47±0.5	13.17±0.29	12.43±0.51	20.83±0.76	6.70±0.10
Standard antibiotics	**Cipro.**	21.33±1.15	18.33±0.58	9.33±0.58	12.30±0.52	15.00±0.00		
	Ketocon.						22.00±1.00	10.67±0.58
Chloroform/Methanol(1:1) Extracts								
S. rosmarinus	**50**	6.00±0.40	0.00±0.00	10.33±0.00	0.00±0.00	9.70±0.00	0.00±0.12	8.47±0.50
	75	6.00±0.50	0.00±0.00	11.33±0.29	0.00±0.50	12.50±0.12	0.00±0.23	10.67±0.50
	100	6.47±0.06	0.00±0.00	14.17±0.50	7.33±0.29	14.17±0.51	0.00±0.76	12.67±0.10
Standard antibiotics	**Cipro.**	15.00±0.00	11.00±1.00	11.33±0.58	10.00±0.52	12.67±0.00		
	Ketocon.						7.00±1.00	13.67±0.58
Methanol Extracts								
S. rosmarinus	**50**	9.17±0.29	5.50±0.50	0.00±0.00	7.50±0.00	0.00±0.00	6.57±0.12	0.00±0.50
	75	9.90±0.10	6.93±0.12	9.33±0.29	8.50±0.50	0.00±0.00	8.70±0.23	0.00±0.50
	100	11.63±0.55	7.97±0.06	11.47±0.50	9.90±0.10	0.00±0.00	10.83±0.76	13.13±0.10
Standard antibiotics	**Cipro.**	13.00±0.00	11.50±0.50	14.20±0.58	13.33±0.29	10.00±0.00		
	Ketocon.						12.00±1.00	15.00±0.58

Mean values of Flavonoids (mg/g) by 570 nm		**Petroleum ether Extracts**	**Chloroform/Methanol (1:1) Extracts**	**Methanol Extracts**
S. rosmarinus	**50**	0.736	0.797	0.862
	75	0.902	0.881	0.890
	100	0.922	0.904	0.940

Key: Bacteria: *S. aureus, Staphylococcus aureus; S.epidermidis, Staphylococcus epidermidis E.coli, Escherichia coli; P aeruginosa, Pseudomonas aeruginosa;* and *K. pneumoniae, Klebsiella . pneumoniae*. Fungus*: C. albicans, Candida albicans;* and *A.niger, Aspergillus niger* .Antibiotics: Cipro, Ciprofloxacin; Ketocon, ketoconazole (Nizoral); DMSO 99.8%, Dimethyl sulfoxide

Determining the three solvent extracts in *S.rosmarinus* plants resulted relatively high comparable with positive (+) control. Specially, the *S.rosmarinus* petroleum ether leaves extracts against drug resistance human pathogenic bacteria S. *aureus, S. epidermidis, E. coli, P.aeruginosa*, and *K.pneumoniae* were minimum zone of inhibition (MZI) recorded that 21.37±0.78, 17.50±0.50, 11.47±0.50, 13.17±0.29, and 12.43±0.51 mm, respectively and against human pathogenic Fungi *C. albicans* and *A.niger* were minimum zone of inhibition (MZI) recorded that 20.83±0.76 and 6.70±0.10 mm, respectively which was used from bacteria against *S. aureus* MZI recorded that 21.37±0.78mm higher than the positive control (21.33±1.15 mm) (Table 12).

The *S.rosmarinus* of chloroform/methanol (1:1) extracts were found to be effective against *E. coli* (14.17±0.50 mm) and *K.pneumoniae* (14.17±0.51 mm) higher than the positive control 11.33±0.58 and 12.67±0.00 mm, respectively. The methanol extracts of leaves in our study plant was found to be recorded that was less than the positive control (Table 12). The *Salvia rosmarinus* extracts showed better antifungal activities than the Gram-negative (-) bacteria (Table 12).

Salvia rosmarinus subjected to fractionations in the three solvents (Table 12). The highest antibacterial results were recorded a mean inhibition with diameters of 21 mm and 14 mm at a concentration of 100 mg mL^{-1} against *S. aureus* and *E. coli/K. pneumoniae*, respectively. After testing, overall it was found that the high active petroleum ether extract of *Salvia rosmarinus* was able to inhibit the growth of *S. aureus* and *C. albicans*, with inhibition zones of 21 mm and 20 mm, respectively. The Petroleum ether extracts showed good efficacy against all tested microbes, particularly Gram-positive bacteria and fungi (Table 12).This is noteworthy because Gram-negative bacteria generally exhibit greater resistance to antimicrobial agents. petroleum ether and chloroform/methanol (1:1) extracts of the leaves were used at a concentration of 100 mg mL^{-1}, resulted in impressive inhibition zone diameters of 11 mm and 14 mm against *E. coli*, 13 mm and 7 mm against P. *aubergines*, and 12 mm and 14 mm against K. *pneumoniae*, respectively.

Our study found that at a concentration of 50 µg mL^{-1}, petroleum ether, chloroform/methanol (1:1), and MeOH extracts did not display any significant inhibition zone effects against the tested microbes. This implies that the samples had a dose-dependent inhibitory effect on the pathogens.

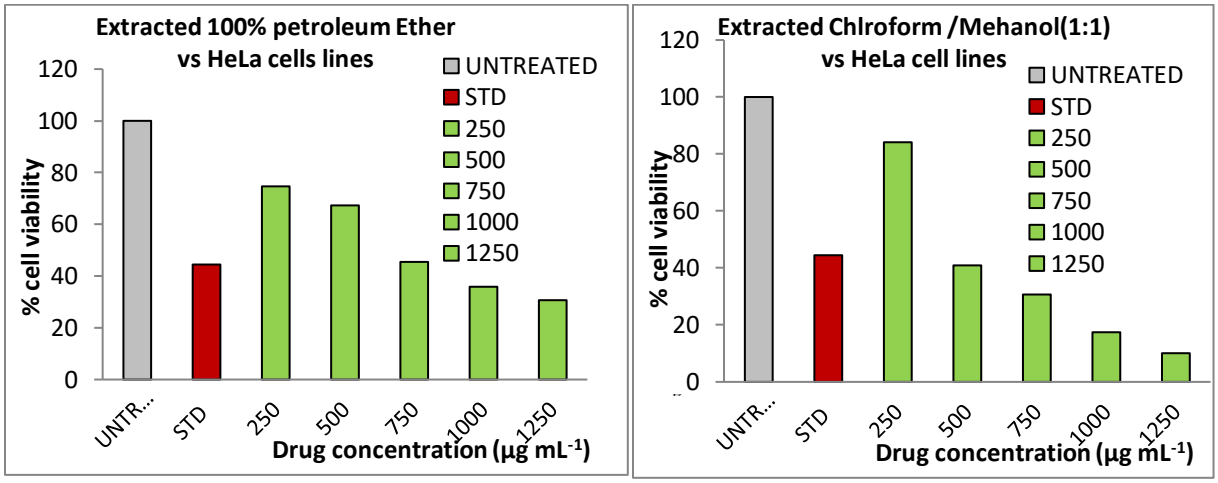
The leaves of *Salvia rosmarinus* have been found to possess remarkable antimicrobial properties against Gram-negative bacteria in different extracts such as *E. coli*, *P. aubergines*, and *K. pneumoniae* with 14.17±0.50 in chloroform/methanol (1:1), 13.17±0.29 in Petroleum ether and 14.17±0.51 in chloroform/methanol (1:1), respectively. The results are summarized in appendix 4.3. *Salvia rosmarinus* in our study at a concentration of 100µg mL^{-1}, the methanol extract demonstrated both maximum and minimum antibacterial zones against *E. coli* 11.47±0.50. Conversely, the test conducted with petroleum ether exhibited good zone of inhibition by increasing concentration. In study, *Salvia rosmarinus* have been found to possess remarkable high zone of inhibition with diameters of 21.37±0.78 and 17.50±0.50 mm antimicrobial properties against *S. aureus*, and *S.epidermidis* of Gram-positive bacteria, respectively (Table 12, Appendix -4.3); and evaluated the antifungal activity of petroleum ether extracts from *Salvia rosmarinus* against two human pathogenic fungi, namely *C. albicans* and *A.niger*. The findings showed that at a concentration of 100 µg mL^{-1}, the extracts were able to inhibit the growth of *C. albicans* 20.83±0.76 resulting in minimum zone of inhibition. The findings showed in chloroform: methanol (1:1) and methanol extracts of leaves of *Salvia rosmarinus* that résistance way acted with Gram-negative bacteria like *Pseudomonas aeruginosa;* and *Klebsiella pneumoniae.* However, *Staphylococcus epidermidis* of Gram-positive bacteria under chloroform/methanol (1:1) extracts had the same antimicrobial résistance.

4.5 *In vitro* anticancer activity

In our study, continuous cell culture of *HeLa* cells was identified and used for anti-cancer activities by *in vitro* method. The three plants' crude extracts inhibited the growth of *HeLa* cell lines, as shown by the reduction of the percentage of cell viability after the treatment of *HeLa* cells similarly suited to cervical cancer cells for 24 h. In this MTT assay, our results showed that, the percentage viability of *HeLa* cells significantly decreased after being treated with crudes of each extracted plant at increasing concentrations (250–1250 µg mL^{-1}) in a concentration-dependent manner (Figure 20-23, Appendix 5.1, 5.2 and 5.3).

In addition, the extracts of the three plants also showed anti-cancer activity in time-dependent manners with distinct IC_{50} values of the studied cell lines in the incubation time (24 h) (Figure 20-22). This anticancer effect of the extracts could be due to the molecular action of each plant

extracted having similar act on the cancer cell line but differed in the inhibited growth of cancer cells. For example, in our study an extract containing the values of IC_{50} =89.93 ±0.00 and IC_{50} = 91.02 ±0.02 from extracts of chloroform/methanol (1:1) of *V. amygdalina* and *O. integrifolia*, respectively. Hence,in our study the chloroform/methanol (1:1) extracts of *V. amygdalina* and *O. integrifolia* exhibited strong active anticancer activity (potent anti-proliferation) and *S. rosmarinus* the values of IC_{50} = 93.73 ±0.00, was more potent anti-proliferation or anticancer activities compared to the petroleum ether extract of the three plants (Figure 22).



A B

Figure 20: Effects of *Vernonia amygdalina* on *HeLa* cell viability.

[*HeLa* cancer cells were treated with 100% petroleum ether (Figure 23A) and chloroform/methanol (1:1) (Figure 23B) concentrations of the extract for 24 h to measure viability.UNTR= untreated and STD=Standard drug which is positive control]

The study revealed that petroleum ether extracts of *S. rosmarinus* and chloroform: methanol (1:1) extracts of *O. integrifolia* showed strong anti-proliferative effects on *HeLa* cancer cells, with anti-proliferation rates of 93.73% and 91.02% respectively, when tested at a concentration of 1250 mg mL^{-1}(Figure 20-21). Similarly, the chloroform/methanol (1:1) extracts of *V. amygdalina*, and *S. rosmarinus* also exhibited significantly high anti-proliferative effects, with anti-proliferation rates of 89.93%, and 86.91%, respectively, at the same concentration of the extract for 24 h. However, when we compare the positive (+) control with the samples, we found that the positive (+) control had a lower score than the samples at the same concentration of the

extract, as shown on Figure 20-22. This may be shown no specific antidote to use in cisplatin toxicity (Either no preventing the absorption of the toxin or no inhibition of conversion of the toxin to more toxic metabolites). Additionally, high scores were shown for overall dose concentrations of chloroform/methanol (1:1) on cervical cancer cell lines (*HeLa* cell lines), particularly in the case of *Otostegia integrifolia* at a concentration of 250 to 1250µg mL^{-1} viability after 24 h (Figure 24). Here, in our study Figure 23 - 26 showed the abundant anti-cancer properties against a variety of cervical cancer cells(*HeLa* cell lines) using varied concentrations in MTT assay of chloroform/methanol (1:1) of *O. integrifolia* extract cytotoxic effects devoid of demonstrating substantial damage to normal cells.

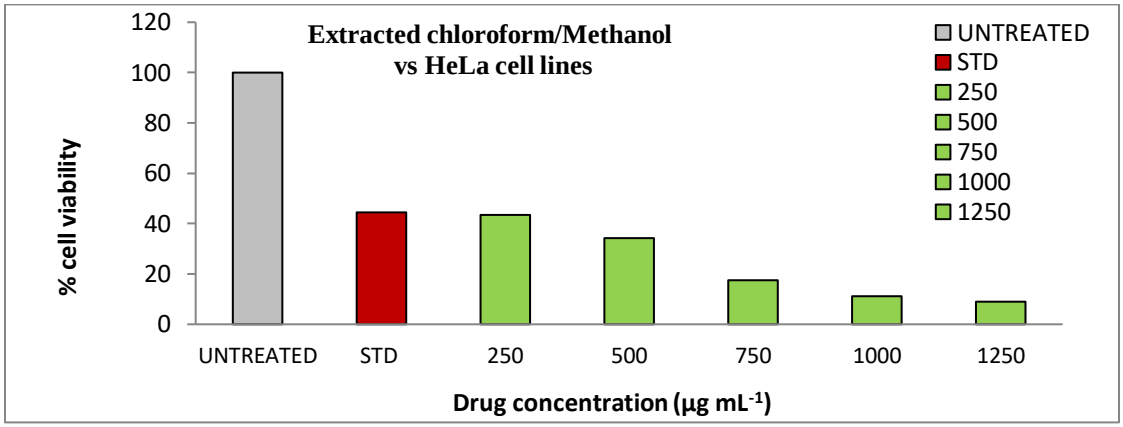
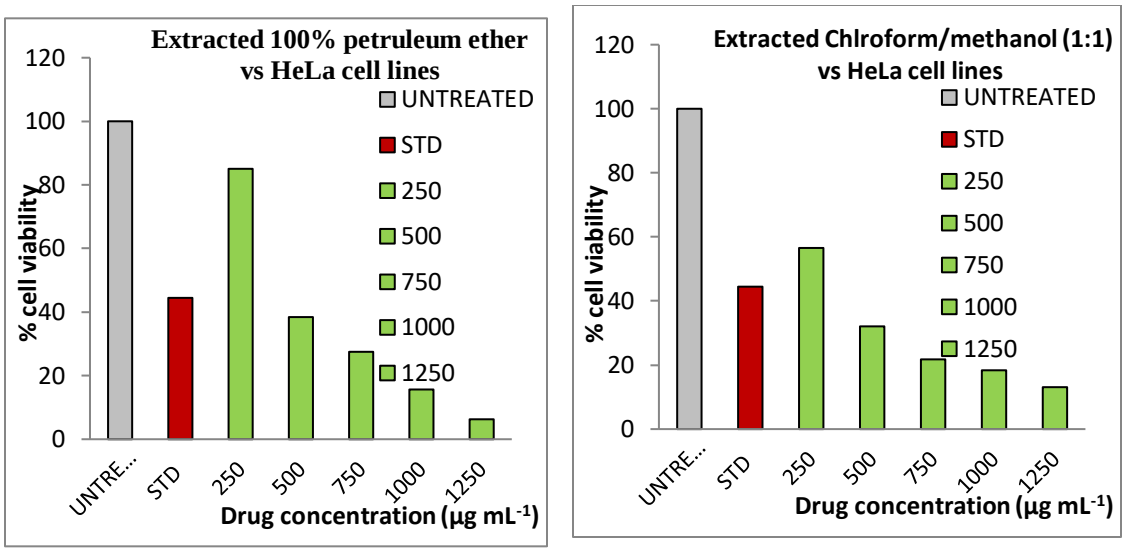


Figure 21: Effects of *Otostegia integrifolia* on *HeLa* cell viability.

[Cells were treated with chloroform/methanol (1:1) extract concentrations to test *HeLa* cell line viability after 24 hrs. UNTR= untreated and STD=Standard drug which is positive control]

Appendix 5.3 demonstrated the plentiful anti-cancer properties of *S. rosmarinus* extract against various cervical cancer cells (*HeLa* cell lines). The study utilized different concentrations in an MTT assay of petroleum ether and a chloroform/methanol (1:1) mixture. The cytotoxic effects of the extract were observed to cause minimal damage to the cancerous cells (Figure 22).



A B

Figure 22: Effects of *Salvia rosmarinus* on *HeLa* cell viability.

[*HeLa* cancer cells were treated with 100% petroleum ether and chloroform/methanol (1:1) the extracts for 24 h to measure viability. UNTR= untreated and STD=Standard drug which is positive control]

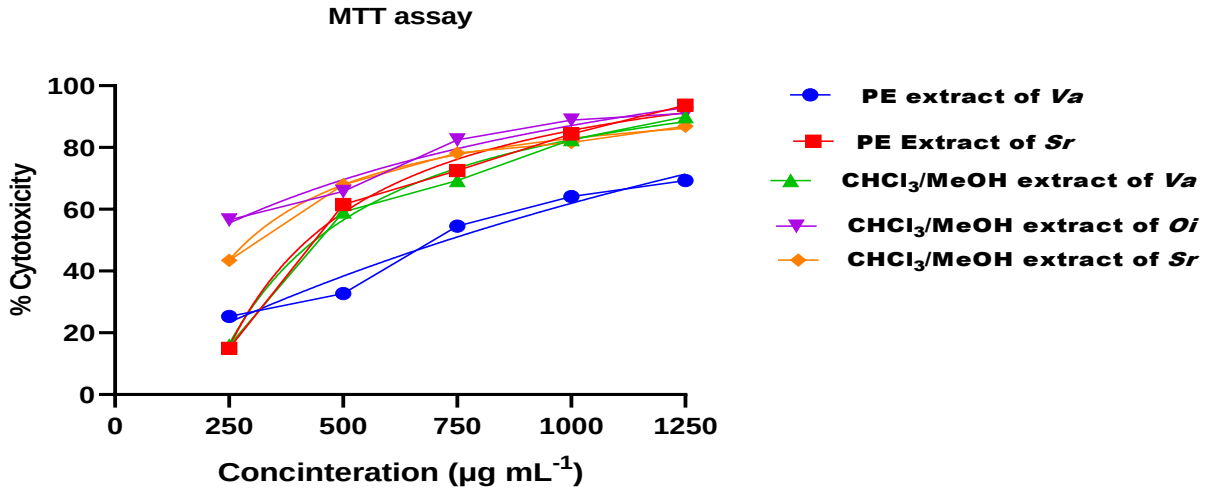


Figure 23: The percentage of cytotoxicity of *HeLa cell* lines at different concentrations.

[The line graphs represent the three extracts derived from three plants, cisplatin (the positive control) with 55.54 %Cytotoxicity (Atangwho *et al.*, 2013). The extracts are as follows: PE extract of *Va*= petroleum ether extract of *Vernonia amygdalina*; PE extract of *Sr*= petroleum ether extract of *Salvia rosmarinus*; $CHCl_3$/MeOH extract of *Va* =chloroform/methanol extract of *Vernonia amygdalina*;

$CHCl_3$/MeOH extract of *Oi* =chloroform/methanol extract of *Otostegia integrifolia* and $CHCl_3$/MeOH extract of *Sr* =chloroform/methanol extract of *Salvia rosmarinus*.]

In this study, most the samples (Figures 20, 21, 22) can be explained based on ISO 10993-5. Percentages of cell viability greater than 80% are considered non-cytotoxicity, weak cytotoxicity: 80% – 60%; moderate cytotoxicity 60% – 40%; and strong cytotoxicity below 40%. Then, the three plants naturally constituted high concentration of terpenoids (Table 6). We found that chloroform/methanol (1:1) extracts of *O. integrifolia* resulted in significant increases in cytotoxicity levels on *HeLa* cell viability. The results were particularly good at concentrations of 250, 500, 750, 1000, and 1250 µg mL^{-1}, with recorded percentages of 56.55%, 65.76%, 82.49%, 88.85%, and 91.02%, respectively. These results were even better than those of the positive control of Cisplatin (55.54%). Therefore, the current study high scores were shown for overall dose concentrations particularly in chloroform/methanol (1:1) extract of *Otostegia integrifolia* at a concentration of 250 to 1250µg mL^{-1} viability after 24 h (Figure 21 and 23).

Table 13: Summary of cell growth inhibition of crude extracts of *Vernonia amygdalina, Otostegia integrifolia,* and *Salvia rosmarinus* on *HeLa* Cell Lines.

		Cell growth inhibition, IC_{50} (µg mL^{-1}) ±SEM
Botanical Name	**Extraction solvents**	*HeLa* cell
V. amygdalina	Petroleum ether	>100
	Chloroform/Methanol(1:1)	70.28±0.01
O. integrifolia	Chloroform/Methanol(1:1)	66.14 ±0.01
S. rosmarinus	Petroleum ether	69.16 ± 0.01
	Chloroform/Methanol(1:1)	56.74 ± 0.02
Cisplatin		14.7 ±1.2

IC_{50} values are presented as means±SEM; SEM, Standard Error Mean, Cisplatin is the positive control

Based on Table 13, the IC_{50} value of petroleum ether and chloroform/methanol (1:1) extracts of *Vernonia amygdalina* on *HeLa* cell was 104.0 µg mL^{-1} and 70.28±0.01µg mL^{-1}, respectively. The IC_{50} value of Chloroform/Methanol (1:1) extracts of *Otostegia integrifolia* on *HeLa* cell was 66.14 ±0.01µg mL^{-1}. The IC_{50} value of petroleum ether and chloroform/methanol (1:1) extracts

of *Salvia rosmarinus* on *HeLa* cell was 69.16 ± 0.01µg mL^{-1} and 56.74±0.016µg mL^{-1}, respectively while the extract seemed to have high growth inhibition on *HeLa* cell when the graph exhibits linear trend in all concentrations (Figure 23). Therefore, they can be described as overall extracts that had a strong anti-proliferative effect when their concentrations have been increased (Figure 20-22).

On the other hand, as the positive control drug, Cisplatin appeared to have less value of IC_{50} (55.54 ± 0.00 µg mL^{-1}) to the *HeLa* cancer cell line. So, our present study, on the three plants under study corroborates the traditional medicinal values claimed for each plant leaf.

4.6 *In vitro* Antioxidant activities

Leaf extracts of *V. amygdalina, O. integrifolia,* and *S.rosmarinus* at concentrations ranging from 10 to 30 µg mL^{-1} exhibited concentration-dependent increases in DPPH radical scavenging activities. Petroleum ether of the plants showed the highest radical scavenger activities of 96.03%, 96.90%, and 93.49% for *V. amygdalina, O. integrifolia,* and *S. rosmarinus* respectively at 30 µg mL^{-1}(Table 14). However, the standard ascorbic acid concentrations at 10 and 30 µg mL^{-1} showed free radical scavenging activities of 46.31% and 89.52%, respectively, as shown in Table 14.

Table 14: DPPH radical scavenging activities (%) of the leaves extracts of *V. amygdalina, O. integrifolia* and *S. rosmarinus*

Extraction solvents	Concentration (µg mL^{-1})	First Absorbance (A_c) Uv 517nm	DPPH assay results of the extracted samples: Leaves were dried at 37^{0}C with samples were practiced by different solvents extracted									Positive(+) control: Ascorbic Acid		
			V. amygdalina			*O. integrifolia*			*S. rosmarinus*					
			A	%RSA	SD	A	%RSA	SD	A	%RSA	SD	A	%RSA	SD
	30	1.259	0.050	96.03	0.005	0.290	96.90	0.007	0.082	93.49	0.290	0.132	89.52	0.055
	25	1.259	0.142	88.72	0.003	0.248	80.30	0.023	0.095	92.45	0.016	0.237	81.18	0.015
petroleum	20	1.259	0.169	86.58	0.003	0.340	72.99	0.009	0.131	89.59	0.003	0.303	75.93	0.020
ether	15	1.259	0.228	79.51	0.002	0.386	69.34	0.046	0.242	80.78	0.001	0.615	51.12	0.022
	10	1.259	0.258	79.51	0.002	0.407	67.67	0.002	0.440	65.05	0.003	0.676	46.31	0.018
	IC_{50} (µg mL^{-1})		3.440			8.199			8.084			36.62		
	30	1.259	0.220	82.53	0.005	0.399	68.31	0.004	0.505	59.89	0.011	0.132	89.52	0.055
chloroform/	25	1.259	0.246	80.46	0.002	0.462	63.30	0.001	0.607	51.79	0.002	0.237	81.18	0.015
methanol	20	1.259	0.280	77.76	0.004	0.511	59.41	0.011	0.699	44.48	0.001	0.303	75.93	0.020
(1:1)	15	1.259	0.316	74.90	0.002	0.555	55.92	0.006	0.721	42.73	0.006	0.615	51.12	0.022
	10	1.259	0.340	72.99	0.001	0.568	54.88	0.013	0.731	41.94	0.006	0.676	46.31	0.018
	IC_{50} (µg mL^{-1})		2.108			4.177			8.893			36.62		
	30	1.259	0.580	53.93	0.001	0.444	64.73	0.003	0.136	89.20	0.210	0.132	89.52	0.055
	25	1.259	0.644	47.26	0.005	0.480	61.87	0.010	0.330	73.79	0.002	0.237	81.18	0.015
Methanol	20	1.259	0.693	44.96	0.003	0.517	58.94	0.016	0.370	70.61	0.010	0.303	75.93	0.020
	15	1.259	0.719	42.89	0.002	0.560	55.52	0.005	0.510	59.49	0.065	0.615	51.12	0.022
	10	1.259	0.736	41.54	0.021	0.627	50.20	0.004	0.400	36.46	0.001	0.676	46.31	0.018
	IC_{50} (µg mL^{-1})		4.758			5.022			25.87			36.62		

Each value is a mean± standard deviation (%) of three biological replicates. **A_c** -Absorbance control, **A**-Absorbance of the samples**, RSA**-Radical Scavenging Activity and **SD**-Standard deviation

Determining the IC_{50} (µg mL^{-1}) value of the three solvent extracts of *V. amygdalina*, *O. integrifolia,* and *S. rosmarinus* plants resulted in values lower than the positive (+) control. The IC_{50} (µg mL^{-1}) values of *V. amygdalina*, *S.rosmarinus,* and *O.integrifolia* petroleum ether extracts were 3.440, 8.084, and 8.199, respectively, which was lower than the positive control and a more potent antioxidant. The IC_{50} (µg mL^{-1}) values of *V. amygdalina, S. rosmarinus*, and *O. integrifolia* of chloroform/methanol (1:1) extracts were found to be 2.108, 4.177, and 8.893, respectively. The methanol extracts of the plants were found to be 4.758, 5.022, and 25.87, respectively (Table 14).

The extracts of *Vernonia amygdalina*, *Otostegia integrifolia*, and *Salvia rosmarinus* demonstrated stronger antioxidant activities compared to the positive control. These crude extracts or their constituents may have the potential to be used as antioxidant agent, especially in food industries (Table 14). The free radical scavenging activity of methanol extracts of *S. rosmarinus* in our present study was stronger (89.20%) at 30 µg ml^{-1}. On the otherhand, in our study, the petroleum ether extracts exhibited the highest antioxidant properties compared to the methanol extracts (Table 14).

4.7 *In vitro* Anti-inflammatory activities

4.7.1 Inhibition of albumin denaturation

In the present study, the *in-vitro* anti-inflammatory activity of *V. amygdalina, O. integrifolia,* and *S. rosmarinus* extracts were measured for inhibitory activity against protein denaturation. In biochemistry, denaturation is the process where proteins lose their quaternary, tertiary and secondary structures that are present in their native state. As shown in Table 15, the highest inhibitory activity against albumin denaturation was observed in S. *rosmarinus* of the chloroform/methanol (1:1) extracts (IC_{50} 0.58 µg mL^{-1}), followed by *V. amygdalina* extracts and *O. integrifolia* chloroform/methanol (1:1) extracts with IC_{50} values of 0.81µg mL^{-1} and 5.01µg mL^{-1}, respectively. *Vernonia amygdalina* and *Salvia rosmarinus* leaves extracts displayed significantly higher protein protection 74.90% and 75.34%, and 70.50% and 73.84%, respectively than aspirin, a standard anti-inflammatory drug, since aspirin inhibited 69.97% and 73.39% of albumin denaturation at a concentration of 10µg mL^{-1} and 15µg mL^{-1}, respectively (Table 15). This may be shown no specific antidote to use in aspirin toxicity. However, *Otostegia integrifolia* leaf extracts exhibited relatively lower activity IC_{50} values of 5.01 µg mL^{-1} compared to aspirin (IC_{50} values of 4.59 µg mL^{-1}) to inhibit albumin denaturation (Table 15).

Table 15: *In vitro* anti-inflammatory activity of *V. amygdalina, O. integrifolia*, and *S. rosmarinus* in Chloroform/Methanol (1:1) extracts.

Botanical Name	Extraction solvents	Concentration (µg mL^{-1})	First Absorbance (A_c) Uv517 nm	***In vitro* anti-inflammatory activity**						Positive(+) control		
				IC_{50} values (µg mL^{-1})								
				Albumin Denaturation			Proteinase Inhibition			Aspirin		
				A	%	SD	A	%	SD	A	%	SD
V. amygdalina	Chloroform/Methanol(1:1)	30	2.98	0.625	79.19	0.265	0.625	79.03	0.324	0.396	86.71	0.019
		25	2.98	0.663	77.75	0.001	0.652	78.12	0.001	0.440	85.23	0.014
		20	2.98	0.711	76.14	0.001	0.700	76.51	0.010	0.578	80.60	0.007
		15	2.98	0.735	75.34	0.001	0.729	75.56	0.001	0.793	73.39	0.002
		10	2.98	0.748	74.90	0.001	0. 753	74.73	0.001	0.895	69.97	0.001
		IC_{50} (µg mL^{-1})		0.81			0.86			4.59		
O. integrifolia	Chloroform/Methanol(1:1)	30	2.98	0.215	92.79	0.001	0.410	86.24	0.010	0.396	86.71	0.019
		25	2.98	0.260	91.28	0.010	0.433	85.47	0.001	0.440	85.23	0.014
		20	2.98	0.290	90.27	0.010	0.452	84.83	0.001	0.578	80.60	0.007
		15	2.98	0.725	75.67	0.001	0.496	83.36	0.001	0.793	73.39	0.002
		10	2.98	0.747	74.93	0.001	0.533	82.11	0.001	0.895	69.97	0.001
		IC_{50} (µg mL^{-1})		5.01			0.77			4.59		
S. rosmarinus	Chloroform/Methanol(1:1)	30	2.98	0.842	71.74	0.001	0.455	85.07	0.001	0.396	86.71	0.019
		25	2.98	0.825	72.32	0.001	0.471	84.19	0.144	0.440	85.23	0.014
		20	2.98	0.858	71.21	0.001	0.500	83.22	0.010	0.578	80.60	0.007
		15	2.98	0.869	73.84	0.001	0.533	82.11	0.001	0.793	73.39	0.002
		10	2.98	0.912	70.50	0.001	0.645	78.36	0.001	0.895	69.97	0.001
		IC_{50} (µg mL^{-1})		0.58			1.30			4.59		

All **IC_{50}** values are a mean± standard deviation (%) of three biological replicates, **A_c** -Absorbance control, **A**- Absorbance of the samples and **SD**-Standard deviation

4.7.2 Anti-proteinase activity

As presented in Table 15, the highest anti-proteinase activity was obtained in leaf [chloroform/methanol (1:1)] extracts of *O. integrifolia* with IC_{50} value of 0.77 µg mL^{-1}, followed by chloroform: methanol (1:1) leaf extracts of *V. amygdalina* (IC_{50} value of 0.86 µg mL^{-1}), and chloroform/methanol leaf extract of *S. rosmarinus* (IC_{50} value of 1.30 µg mL^{-1}). In the proteinase inhibition assay, it is observed that the bioactive compounds in chloroform/methanol (1:1) extracts bound to the trypsin molecule. This indicates that the anti-inflammatory activity of these extracts is noteworthy. Interestingly, all the plant extracts investigated exhibited a greater

inhibitory activity against proteinase compared to aspirin (IC_{50} value of 4.59 µg mL^{-1}) (Table 15).

4.8 *In-vitro* Anti *Acanthamoeba* activity

The chloroform/methanol (1:1) leaf extracts showed the strongest anti-Acanthamoeba activity against both the trophozoites and cysts (Table 16 and 17). After 24 h, 78% and 92 % trophozoite viability was significantly decreased following the treatment with extract at increasing concentration(Appendix 6.1 and 6.2) against *A. triangularis* and *A. castellanii*, respectively compare to the control. The MIC values of *S. rosmarinus* leaf extracts against *A. triangularis* and *A. castellanii* trophozoites were 22.22 and 7.89 µg mL^{-1}, respectively . While MICs of the extract against *A. triangularis* and *A. castellanii* cysts were 125 and 62 µg mL^{-1}, respectively. The MIC values of *S.rosmarinus* against *A. triangularis* and *A.castellanii* trophozoites and cysts are presented in Table 16 and 17.

Table 16: Screening of anti-*Acanthamoeba* activity of *S. rosmarinus* extracts against *A. triaugularis* WU19001 and *A. castellanii* ATCC50739 trophozoites.

Extract	Part	Extraction Solvent	Solubility in %	After 24h, % of Viablity (Trophozoites)			
				A. triangularis		*A. castellanii*	
Salvia rosmarinus	**Leaves**	**$CHCl_3$/MeOH (1:1)**	**DMSO**	**avg.**	**sd**	**avg.**	**Sd**
				22.22	**0.00**	**7.89**	**7.02**
		1%DMSO		**99.77**	**8.66**	**100.00**	**3,60**
		Chlorhexidine (Growth control)		**6.25**	**0.00**	**6.25**	**0.00**

All MIC (µg mL^{-1}) values are a mean± standard deviation (%) of the biological treatments

Table 17: Screening of anti-*Acanthamoeba* activity of *S. rosmarinus* extracts against *A. triaugularis* WU19001 and *A. castellanii* ATCC50739 cysts.

Extract	Part	Extraction Solvent	Solubility in %	After 24h, % of Viablity (cysts)			
				A. triangularis		*A. castellanii*	
Salvia rosmarinus	**Leaves**	**$CHCl_3$/MeOH (1:1)**	**DMSO**	**avg.**	**sd**	**avg.**	**Sd**
				125	**0.00**	**62**	**5.00**
		1%DMSO		**100.00**	**8.66**	**100.00**	**3,60**
		Chlorhexidine (Growth control)		**25.00**	**0.00**	**25.00**	**0.00**

All **MIC** (µg mL^{-1}) values are a mean± standard deviation (%) of the biological treatments

The amoebicidal activity of *S.rosmarinus* against *A.castellanii* trophozoites was shown to be highly prominent for its benefit to health (% viability < 10) (Table 16). However, the cysts were not completely inhibited by the extract due to the resistance form of the cyst walls.

4.9 *In-vitro* Antiviral activity

The specimens that demonstrated a selectivity index (SI) of 10 or higher were considered active and prospective results.

Table 18: Antiviral activity results of *V. amygdalina* and *O. integrifolia* leaves extracts through P.Ether, Chloroform/Methanol (1:1) and aqueous solvents and *S. rosmarinus* leaves extracts through Chloroform/Methanol (1:1) and Methanol against influenza(A).

Specimen	**Extracts' Solvents**	**Biological properties against influenza(A) virus in MDCK cells**		
		CC_{50}, µg/mL	**IC_{50}, µg/mL**	**SI**
V. amygdalina	**Pet. Ether**	**251±16**	**12.5±2.2**	**20**
	Chlr/MeOH(1:1)	**251±14**	**25.8±3.0**	**10**
	Aqueous(H2O)	**>300**	**7.9±1.0**	**38**
O. integrifolia	**Pet. Ether**	**10.6±0.5**	**>3.7**	**3**
	Chlr/MeOH(1:1)	**38.1±1.8**	**32.3±3.7**	**1**
	Aqueous(H2O)	**10.2±0.6**	**2.5±0.4**	**4**
S. rosmarinus	**Chlr/MeOH(1:1)**	**10.6±0.2**	**>3.7**	**3**
	MeOH	**10.2±0.6**	**2.5±0.4**	**4**
Rimantadine		**358±27**	**63.0±4.9**	**6**

CC_{50} , -half-maximal cytotoxic concentration .IC_{50},- Half-maximal inhibitory concentration. SI,- Selectivity index

At this time, empirical knowledge based on the ethnomedical benefits of *V. amygdalina*, *O. integrifolia*, and *S. rosmarinus*, coupled with bioassay-guided fractionation has the potential to identify novel antivirals that could be used against influenza virus. As a result, the extracts of aqueous, chloroform: methanol (1:1), and petroleum ether of *V. amygdalina* leaves of the selectivity index showed high activity against influenza virus (Table 18). Importantly, the virus used in the study, influenza A/Puerto Rico/8/34 (H1N1), was resistant to rimantadine (Table 18). All three extracts of *V. amygdalina* appeared much more active against the virus. Indeed, they possessed SI's two- to six-fold higher than rimantadine. The plant leaves extract which have

shown a good activity have a potetial as candidate drugs (Table 18) which might need further study.

4.10 Extraction of essential oils (EOs)

4.10.1 Chemical composition of EOs

The chemical composition of essential oils (EOs) of *V. amygdalina, Otostegia integrifolia*, and *S. rosmarinus* leaves were identified by GC-MS in Appedix-7.1, Appedix-7.2, and Appedix-7.3, respectively. The EOs of the three plants had distinct aromas. The results given for the three studied plants' EOs are shown in Tables 20, 21, and 22, respectively. For each constituent, their linear retention index (LRI) values were calculated. In this study, the overall results of the three plants' EOs class of compounds; Namely: hydrocarbon terpenes/ terpenoids (monoterpenes $C_{10}H_{16}$, and sesquiterpenes $C_{15}H_{24}$); oxygenated molecules their derivatives (alcohols, ethers, aldehydes, esters, ketones, and acids); and heterocyclic compounds. In the essential oil from *V. amygdalina* leaves 15 compounds were identified, corresponding to over all 99.5%, containing 93.3% of terpene derivates and other non-terpenes (6.7%) (Table 19) .

Table 19: GC-MS analysis of the essential oil of *V. amygdalina* leaves

Compounds	Molecular Formula	LRI*	LRI**	Peak Area (%)[a]	References
Dihydro-2H-Pyran	**C_5H_8O**	**-**	**500**	**93.60**	**-**
α- cadinol	**$C_{15}H_{26}O$**	**1640**	**1655**	**4.20**	**(Babushok *et al*.,2011)**
Benzaldehyde	**C_6H_5CHO**	**926**	**928**	**1.22**	**(Babushok *et al*.,2011)**
2-Hexenal	**$C_6H_{10}O$**	**853**	**841**	**0.15**	**(Tadesse *et al*.,2012)**
N-Methylpyrrolidone	**C5H9NO**	**-**	**634**	**0.09**	**-**
α- pinene	**$C_{10}H_{16}$**	**937**	**940**	**0.06**	**(Anwar *et al*.,2009)**
o-Cymene	**$C_{10}H_{14}O$**	**1020**	**1014**	**0.03**	**(Taylor, *et al*., 2013)**
Isophytol	**$C_{20}H_{40}O$**	**1940**	**1936**	**0.03**	**(Taylor, *et al*., 2013)**
Limonene	**$C_{10}H_{16}$**	**1024**	**1018**	**0.02**	**(Souza *et al*., 2003)**
Linalool	**$C_{10}H_{18}O$**	**1085**	**1059**	**0.02**	**(Anwar *et al*.,2009)**
Propionic acid	**$C_3H_9O_2$**	**-**	**300**	**0.02**	**-**
1, 8-cineole	**$C_{10}H_{18}O$**	**1022**	**1032**	**0.02**	**(Babushok *et al*.,2011)**
3-octanol	**$C_8H_{18}O$**	**983**	**996**	**0.02**	**(Babushok *et al*.,2011)**
3-hexen-1-ol	**$C_6H_{12}O$**	**856**	**842**	**0.01**	**(Tadesse *et al*.,2012)**
Methyl salicylate	**$C_8H_8O_3$**	**1172**	**1178**	**0.01**	**(Babushok *et al*.,2011)**
Total identified				**99.5**	

[a] *Components are arranged in order of their elution from a HP 5MS capillary column; LRI* = Literature linear retention indices'; LRI**=Experimental linear retention indices'*

Otostegia integrifolia leaves constituted 13 compounds, containing 91.7% of terpene derivates and others (8.3%) (Table 20) .

Table 20: GC-MS analysis of the essential oil of *O. integrifolia* leaves

Compounds	Molecular Formula	LRI*	LRI**	Peak Area (%)[a]	References
3-Hexenol	$C_6H_{12}O$	836	830	19.63	(Babushok *et al.*,2011)
Linalool	$C_{10}H_{18}O$	1096	1090	11.18	(El Hamdaoui *et al.*,2018)
Benzaldehyde	C_6H_5CHO	926	928	8.41	(Babushok *et al.*,2011)
Acetyl-1-methylcyclohexene	$C_9H_{14}O$	-	900	8.24	-
1-Octen-3-ol	$C_8H_{16}O$	974	980	7.95	(Waterman,1996)
Caryophyllene oxide	$C_{15}H_{24}O$	1576	1583	7.05	(Souza *et al.*, 2003)
Methyl heptenone	$C_8H_{14}O$	-	799	6.45	-
Dihydro-2H-Pyran	C_5H_8O	-	500	6.36	-
Cembrene A	$C_{20}H_{32}$	1947	1938	6.25	(Waterman,1996)
1, 8-cineole	$C_{10}H_{18}O$	1022	1032	5.95	(Babushok *et al.*,2011)
α- Terpineol	$C_{10}H_{18}O$	1192	1193	5.77	(Babushok *et al.*,2011)
α- cadinol	$C_{15}H_{26}O$	1640	1655	3.30	(Babushok *et al.*,2011)
Total identified				96.5	

[a] *Components are arranged in order of their elution from a HP 5MS capillary column; LRI* = Theoretical linear retention indices'; LRI**=Experimental linear retention indices'*

In our study, the essential oils of *Vernonia amygdalina* showed that the main component was dihydro-2H-pyran (93.6%). *Salvia rosmarinus* leaves were composed of 28 compounds, with 67.9% of terpene derivates and hydrocarbons (32.1%) (Table 21) .

Table 21: GC-MS analysis of the essential oil of *S. rosmarinus* leaves

Compounds	Molecular Formula	LRI*	LRI**	Peak Area(%)[a]	References
Camphene	$C_{10}H_{16}$	**953**	**947**	**23.75**	**(Awadh and Alhamzy,2017)**
Dihydro-2H-Pyran	C_5H_8O	**-**	**500**	**20.36**	**-**
Limonene	$C_{10}H_{16}$	**1024**	**1028**	**9.14**	**(Souza et al., 2003)**
α-Copaene	$C_{15}H_{24}$	**1377**	**1380**	**8.89**	**(Awadh and Alhamzy,2017)**
α-Terpineol	$C_{10}H_{18}O$	**1192**	**1193**	**8.23**	**(Babushok et al.,2011)**
Carveol	$C_{10}H_{16}O$	**-**	**1022**	**4.09**	**-**
Myrtenol	$C_{10}H_{16}O$	**1182**	**1178**	**3.96**	**(Babushok *et al*.,2011)**
α –Cadinene	$C_{15}H_{24}$	**1526**	**1513**	**3.26**	**(Babushok et al.,2011)**
α –Thujone	$C_{10}H_{16}O$	**1089**	**1171**	**2.97**	**(Zámboriné et al.,2020)**
1-Hexanol	$C_6H_{14}O$	**854**	**802**	**2.96**	**(Babushok *et a*l.,2011)**
Citronellol	$C_{10}H_{20}O$	**1212**	**1201**	**2.54**	**(Babushok *et a*l.,2011)**
α-Terpinene	$C_{10}H_{16}$	**1010**	**999**	**2.25**	**(Babushok *et al*.,2011)**
Bornyl acetate	$C_{12}H_{20}O_2$	**1270**	**1244**	**0.88**	**(Babushok*et al*.,2011)**
α-Humulene	$C_{15}H_{24}$	**1456**	**1453**	**0.54**	**(Tung *et al*.,,2010)**
Cymophenol	$C_{10}H_{14}O$	**-**	**999**	**0.50**	**-**
D-Borneol	$C_{10}H_{18}O$	**1153**	**1124**	**0.45**	**(Babushok et al.,2011)**
Thymol	$C_{10}H_{14}O$	**1583**	**1582**	**0.44**	**(Alavez et al.,2022)**
α--Terpinyl acetate	$C_{12}H_{20}O_2$	**1119**	**1128**	**0.37**	**(Ghavam, 2022)**
δ-Carene	$C_{10}H_{16}$	**956**	**949**	**0.34**	**(Ghavam, 2022)**
Benzaldehyde	C_6H_5CHO	**926**	**928**	**0.32**	**(Babushoket al.,2011)**
Pinocarvone	$C_{10}H_{14}O$	**1140**	**1108**	**0.32**	**(Babushok *et al*.,2011)**
δ-Cadinene	$C_{15}H_{24}$	**1513**	**1522**	**0.31**	**(Babushok *et al*.,2011)**
α- Cadinol	$C_{15}H_{26}O$	**1640**	**1655**	**0.31**	**(Babushok *et al*.,2011)**
Levomenol	$C_{15}H_{26}O$	**1365**	**1368**	**0.30**	**(Babushok *et al*.,2011)**
□-Calacorene	$C_{15}H_2O$	**1530**	**1526**	**0.28**	**(Babushok *et al*.,2011)**
Eugenol methyl ether	$C_{15}H_{24}O$	**1326**	**1327**	**0.27**	**(Babushok *et al*.,2011)**
p-Cymene	$C_{10}H_{14}$	**1020**	**1024**	**0.26**	**(Waterman,1996)**
5-Undecanol	$C_{11}H_{24}O$	**-**	**1099**	**0.23**	**-**
Total identified				**98.52**	

[a] *Components are arranged in order of their elution from a HP 5MS capillary column; LRI* = Theoretical linear retention indices'; LRI**=Experimental linear retention indices'*

In the present study (Table 19- 21), the principal constituent of *Vernonia amygdalina*, *Otostegia integrifolia,* and *Salvia rosmarinus* essential oils were found to be dihydro-2H-pyran(93.6%), 3-hexenol (19.63%), camphene (23.75%), respectively.

4.10.2 Antimicrobial activities of the EOs evaluation

The results of antimicrobial activity for *Vernonia amygdalina, Otostegia integrifolia,* and *Salvia rosmarinus* essential oils are presented in Figure 24. This study demonstrated that the essential oils of the three plants possess antibacterial properties, effectively inhibiting *S. pyogenes* by *V. amygdalina* (11.0±2.2) and *O. integrifolia* (9.7±0.6) samples, as well as *E. coli* by *S. rosmarinus* samples (12.3±0.6). Additionally, the findings indicate that the essential oils of samples from *V. amygdalina, O. integrifolia,* and *S. rosmarinus,* respectivily were also effective in inhibiting the growth of *C. albicans* (11, 9 and 11.7 mm). These results are presented in Figure 24 and Appedix-7.4B.

The antibiotic reference products (Ciprofloxacin - positive test) indicated a high zone of inhibition (Appedix-7.4A). The negative control was 10 µL of 0.2% agar, while Gentamycin synergized Azole was used as a positive control with 5µg mL^{-1} in the presence of fungal strains. The zone of inhibition ranged from 9 to 27 mm found in the three EOs of the three plants (Figure 24).

Our recent study *Otostegia integrifolia* showed no activity (MZI value of 125 to 1000 µg ml^{-1}) against *E. coli* strains, and the use of rosemary essential oils exhibited good inhibitor effect against *E. coli* bacteria(EO at concentration 1000 µg mL^{-1}) but did not have any impact on the growth of *P. aeruginosa*. Overall, *Pseudomonas aeruginosa* was the most resistant to the essential oils from the three plants. In the present study, the antifungal activities of extracts from *Vernonia amygdalina, Otostegia integrifolia,* and *Salvia rosmarinus* were comparable to Gentamycin in synergy with Azole.

Candida albicans strains showed enhanced inhibitory effects when higher oil concentrations of these three plants, used ranging from 125 to 1000 µg mL^{-1}. In general, the three examined essential oils exhibited notable fungicidal activity. The essential oil of *Salvia rosmarinus* exhibited significant antifungal activity. In several cases, the opportunistic infections by *Candida* albicans in recent times are immune-compromised and longsuffering, especially those pretentious by cancer, diabetes, and AIDS.

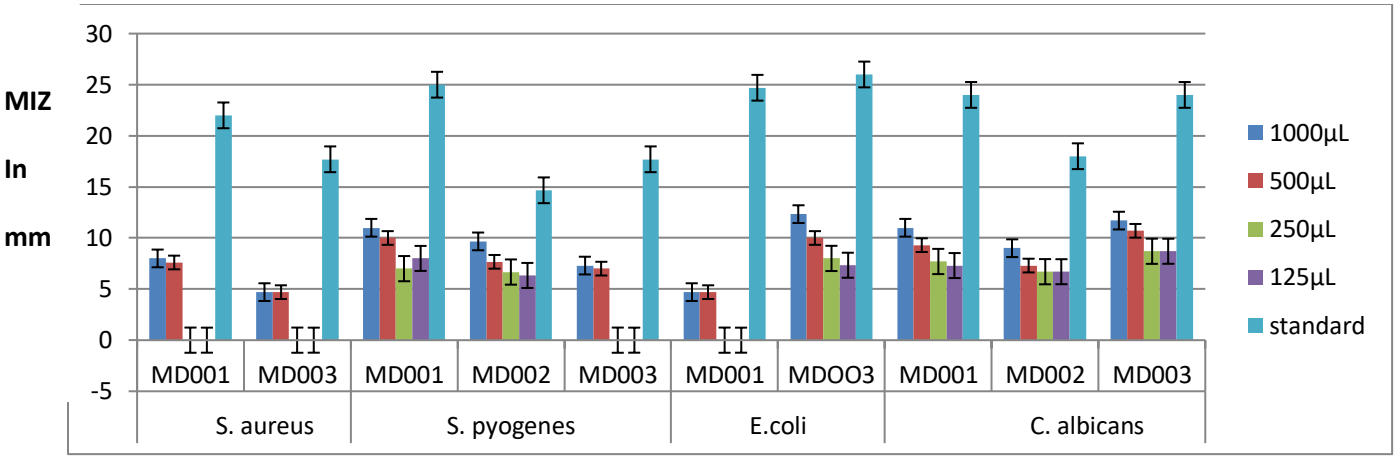


Figure 24: Microbes resistance to essential oil relative to standard antibiotics

(MIZ=minimmum inhibition zones, mm=millimeter)

4.10.3 Free radical scavenging activities of the EOs

In the DPPH scavenging assay, the reaction of the three essential oils (EOs) was accompanied by a color change in the DPPH solution, measured at 517 nm after 30 min in the dark (Table 22). The degree of discoloration indicated their antioxidant efficacy. Free radical scavenging capacity of the tested essential oils increased in a concentration dependent manner (62.5 to 1000 µgmL^{-1}) (Table 22 and Figure 24).

Table 22 shows the antioxidant activities of essential oils of leaves determined by DPPH scavenging values for 50% inhibition (IC_{50}) resulting in the essential oils ranging 0.82-0.95(82-95%). The essential oils from *Vernonia amygdalina* and *Salvia rosmarinus* showed good radical scavenging activity with IC_{50} of 94.14% and 95.23% respectively (Table 22). In the present study, the antioxidant activity of *Otostegia integrifolia* essential oil was significantly lower compared to the other essential oils and ascorbic acid . EOs were predicted as better exemplary fits for radical scavenging activity.Hence, in our study, the DPPH scavenging activity of *Salvia rosmarinus* showed relatively good inhibition, with an IC_{50} value of 0.6927 µg mL^{-1}.

Table 22: DPPH radical scavenging activities (%) of the leaves extracts of EOs of *V. amygdalina, O. integrifolia* and *S. rosmarinus*

Concentration (µg mL^{-1})	First Absorbance (A_0) of the Control in Uv -517	DPPH assay results of the extracted samples									Positive(+) control		
		Leaves were dried at 37^{0}C and EOs practiced by hydro-distillation											
		V. amygdalina			*O. integrifolia*			*S. rosmarinus*			Ascorbic Acid		
		A	% RSA	SD	A	% RSA	SD	A	% RSA	SD	A	% RSA	SD
1000	1.468	0.086	94.14	0.00082	0.293	80.02	0.0008	0.070	95.23	0.0082	0.041	97.21	0.17933
500	1.468	0.08	95.07	0.0082	0.114	92.23	0.0008	0.094	93.80	0.00082	0.033	97.75	0.00082
250	1.468	0.263	82.08	0.00082	0.123	91.62	0.0008	0.074	94.96	0.00082	0.034	97.68	0.00082
125	1.468	0.254	82.70	0.00081	0.206	85.97	0.0008	0.099	93.26	0.00082	0.047	96.80	0.21150
62.5	1.468	0.067	95.44	0.0008	0.293	80.04	0.0008	0.089	93.94	0.00082	0.035	97.62	0.00082
IC_{50} (µg mL^{-1})		1.089			5.205			0.6927			0.002		

Each value is a mean± standard deviation (%) of three biological replicates. A_0 -Absorbance control, A-Absorbance of the samples, RSA-Radical Scavenging Activity, and SD- standard deviation.

This was compared to the IC_{50} of 0.002 µg mL^{-1} ($P < 0.05$) observed for the positive control. The variations in the radical scavenging activities of the essential oils explored in our investigation were found relatively higher ranging from 0.82-0.97 (82-97%)(Table 22 and Figure 31). Thus, in the present study, *S. rosmarinus* shows strong radical scavenging activities in all concentrations tested in the sample followed by *V. amygdalina* (medium) and *O. integrifolia* (weak). Figure 31 depicts the antioxidant activity of the three essential oils and showed excellent antioxidant activity. On the other hand, our investigation revealed significant variations in the chemical composition of essential oils within the same species due to intrinsic features and extrinsic factors.

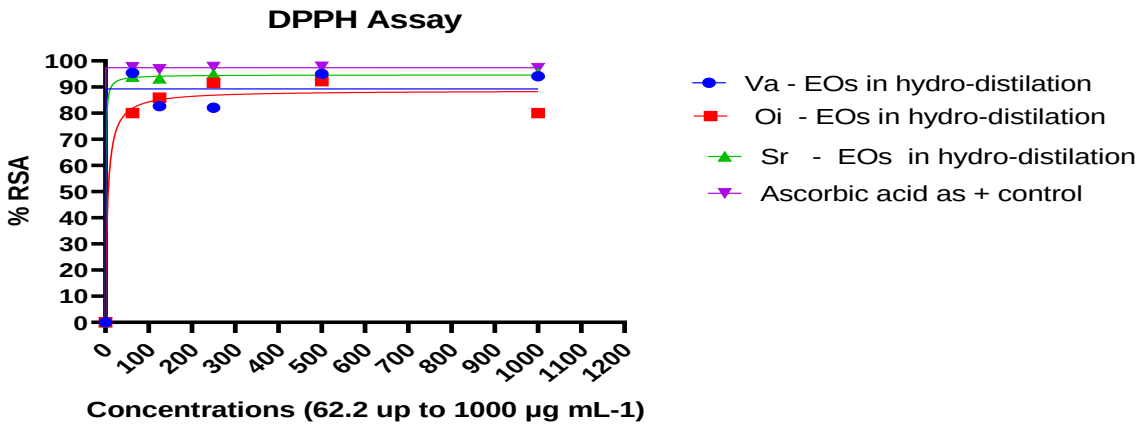


Figure 25: Radical Scavenging Activity (%) of the of the essential oil from the leaves of three of plants *V. amygdalina (Va), O. integrifolia (Oi),* and *S. rosmarinus (sr)*

4.10.4 Molecular docking studies compound obtained from EOs

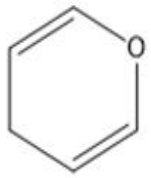
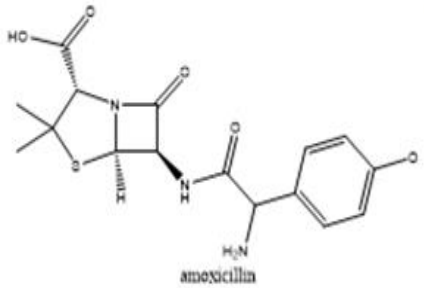
The candidates' bacteria favorable for predicting the potential binding mode were selected *E. coli* DNA gyrase B (PDB ID: 6F86), and *S. pyogenes*; SpeB (PDB ID: 4D8E), using molecular docking analysis software (Narramore *et al*.,2019).The study also compared the results with those of standard anti-bacterial agents with amoxicillin (Table 23 and Table 24). A compound identified had a minimum fixing energy – 3.3kcal mol^{-1}, displayed in Table 23. It was compared to amoxicillin (– 6.1 kcal mol^{-1}). The results of the molecular docking analysis showed good reliable matching amino acid residue interactions compared to amoxicillin (Table 23). On the other hand, the same compound identified had a minimum fixing energy – 2.7kcal mol^{-1}, shown in Table 24 compared to amoxicillin (– 5.9 kcal mol^{-1}). The results of the molecular docking analysis offered reliable matching amino acid residue interactions compared to amoxicillin (Figure 26 and Figure 27).

As a result, our run-through was selected to identify dihydro-2H-pyran (IUPAC name 3, 4-dihydro-2H-pyran) molecule from three plants were found for the study, and their derivative amoxicillin was used as a positive regulator medicine. Lab-practiced biological activities were a good treat for *S. pyrogens* in the *V. amygdalina, O. integrifolia,* and *E. coli* in *Salvia rosmarinus* (Table 24; Figure 27); and the molecular docking analysis was performed using the method of Auto Dock tools (version 4.2) to predict the potential binding mode of the identified compound

into *S. pyogenes*; SpeB (PDB ID: 4D8E), and *E. coli* DNA gyrase B (PDB ID: 6F86) in microbe species and the active sites were obtained from the protein data bank identity (PDB ID) (Burley *et al*.,2017). *In silico,* dihydro-2H-pyran was seen to inhibit disease-causing bacteria and life-threatening form antibacterial activity and the prediction was found in 2D/3D structuraes or molecular modeling technique interactions with microbes (Figure 26 and Figure 27). The characterization in which Pyran molecules were done dockings with *S. pyogenes* by protein name found at SBD-Site, sphere, ID 0, visible-Yes, visibility locked-No, color-255 0 0, parent 4D8E, XYZ-11.446440 12.555160 -5.864320, radius 12.434794, transparent-Yes. In addition to these, the molecular docking results were analyzed based on the binding energy (kcal mol^{-1}) and the number of binding interactions between catalytic amino residues of the compound (Table 23).

This idea was proved similarly by (Burley *et al*.,2017) affinity of a drug was its ability to bind to its biological target (receptor, enzyme, transport system, etc.) of resistances' bacteria and DNAgyrase is dominant for bacterial survival and it is essential to consider bacterial DNAgyrase as a key target of antibacterial agent (Schoeffler & Berger,2008). So, the molecular docking study was carried out to examine the binding interactions of the isolated compound from EO with the pocket of DNA gyrase and compared it with amoxicillin. The compound forms GLY-77 and THR-165 (Hydrogen bonds) in additional residual interaction with GLU 50 (Electrostatic) and ILE-78 (Hydrophobic) in the active site of the target protein with the docking score (-3.3 kcal mol-1)(Table 24) and, again, it shown to have comparable binding score amino acid interactions compared to amoxicillin (–6.1 kcal mol^{-1}).

Table 23: Molecular docking results of 3,4-dihydro-2H-Pyran with *S. pyogenes;* SpeB (PDB ID: 4D8E)

Compound	Ligands	Binding affinity (kcal/ mol)	Residual interactions		
			H-bond	Hydrophobic/ Electrostatic	Van der Waals
 3, 4-dihydro-2H-Pyran	C_5H_6O	-3.3	GLY-77 (2.74368), THR-165 (3.3525)	Electrostatic Pi-Anion- GLU-50 (3.81671), Hydrophobic Pi-Alkyl- ILE-78 (5.13563)	ARG-76, GLY-75,ASP-73,ALA-47,ASN-46
 amoxicillin	$C_{16}H_{19}N_3O_5S$	-6.1	Val-120:HN (1.86984), Ser-121:HN (2.48365), Ser-121: HG (2.2538), Ile-94:O (2.78615), Val-97:O (2.33003), Glu-50: OE1(1.93625) Gly-119:CA (3.46709)	-	ARG-76, ASP-49, ASN-46, LUE-98, VAL-118

The compound (3, 4-dihydro-2H-Pyran) have shown slighter binding affinity but similar residual interaction profile with amino acid residues ARG-76 and ASN-46. Based on the molecular docking analysis results, the compound has shown comparable residual interactions with amoxicillin and therefore this compound might have potential to be promising antibacterial agent.

As a result, the molecular docking of dihydro-2H-Pyran within the binding sites of *S. pyogenes* was analyzed, and the results were compared with standard antibacterial agent amoxicillin (Figure 35). The compound has shown similar residual amino acids binding interactions with SER-219, ASP-275 and THR-217 (Hydrogen bonds) and no additional residual interactions within the binding pocket. The compound was found to have minimum binding energy –2.7 kcal mol^{-1} (Table 24) comparable result with those of amoxicillin (– 5.9 kcal mol^{-1}). Hence, the

compound might have a good potential as antibacterial agent and the *in silico* result was in agreement with *in vitro* result.

Therefore, the free energy was produced at the binding site in dihydro-2H pyran molecules with *S. pyogenes* less than the derivatives (amoxicillin) (Table 23), and again this was true with *E.coli* (Table 24). Prominent the result, free energy is measured in kilojoules per mole called Gibbs free energy. If its value is negative, the system will tend to do work spontaneously, as in an exothermic chemical reaction.

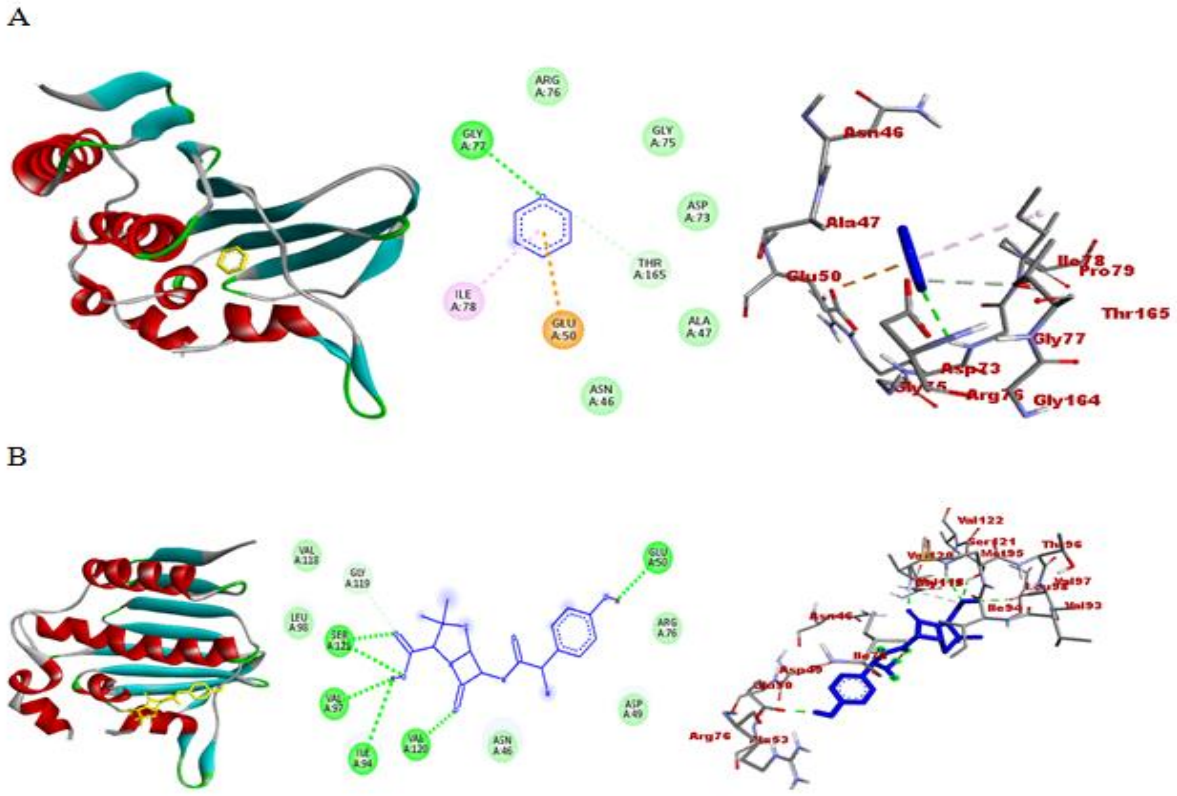


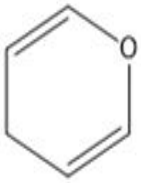
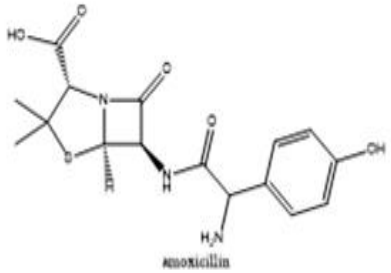
Figure 26: The 2D and 3D binding interactions between 3, 4-dihydro-2H pyran against *S. pyogenes;* SpeB (PDB ID: 4D8E) and amoxicillin (standard) against *S. pyogenes;* SpeB (PDB ID: 4D8E)

[The structure figure represents an understudy of the 2D and 3D binding interactions between the compound and bacteria chosen from in-vitro activities. The structures are as follows: A and B - the compound 3, 4-dihydro-2H-Pyran against S. pyogenes (PDB ID: 4D8E) and amoxicillin (standard)

against S. pyogenes (PDB ID: 4D8E), respectively. Chemical structures were drawn by ChemDraw Pro 16.0 Suite (PerkinElmer, USA) and analyzed by the BIOVIA Discovery studio 2020 Client].

Exothermic reaction is the energy transferred to the surroundings and the temperature of the surroundings increases and the system will tend to do work (binding) with dihydro-2H-Pyran molecules at the oxidative part bind to its biological target protein SER A: 219 on *S. pyogenes* and *E.coli* and inhibit the bacterial activities and killed bacteria (Figure 26-27). This showed us that the lower the system's potential energy, the more stable it is. "Thermodynamically favorable" means from high energy to low energy, or, put another way, from less stable to more stable, and again if free energy decreases, the reaction can proceed. If the free energy increases, the reaction can't proceed. A reaction is favored if the free energy of the system decreases. A reaction is not favored if the free energy of the system increases (Bozin *et al*.,2007).

Table 24: Molecular docking results of compounds of EO (3, 4-dihydro-2H-Pyran) from the three plants with *E.coli* at the site of DNA gyrase B (PDB ID: 6F86)

Compounds	Ligands	Binding affinity (kcal/ mol)	H-bonds	Residual interactions	
				Hydrophobic/ Electrostatic	Van der Waals
 3, 4-dihydro-2H-Pyran	C_5H_6O	-2.7	SER-219(2.1612), ASP-275(3.59044), THR-217(3.43484)	-	LEU-218, ARG-287, ASP-273, TYR-216
 amoxicillin	$C_{16}H_{19}N_3O_5S$	-5.9	SER285(2.93281), GLN304(2.45343), GLN332(1.98062), GLN332(2.44212), GLY339(2.97775), SER285(3.37916)	Hydrophobic Pi-Sigma-VAL-334:CG2(3.66063), Hydrophobic Pi-Sigma-VAL-334:CG1:B(3.57604)	TYR-389, GLY-284 ,SER-391,ALA-283

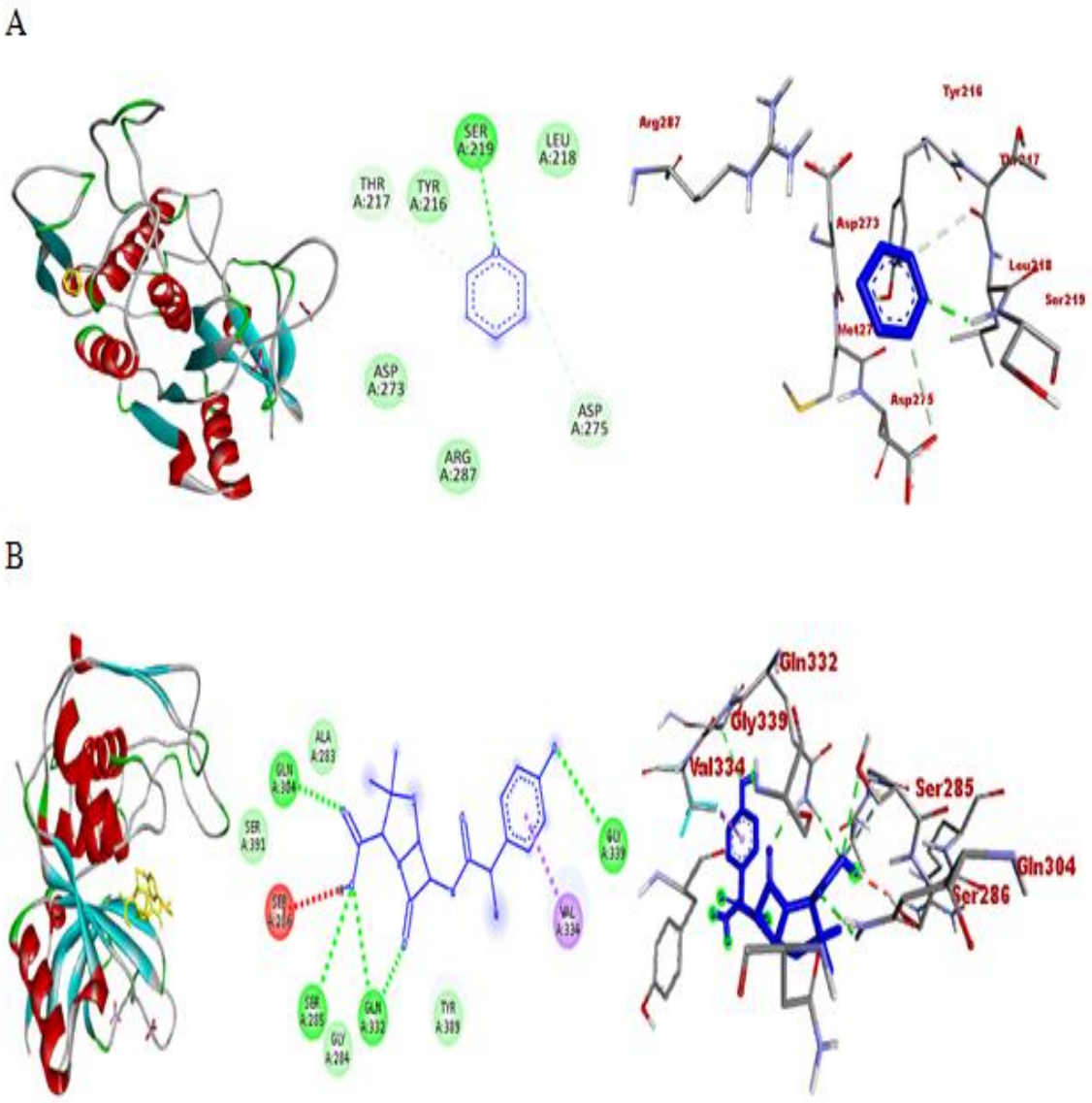


Figure 27: The 2D and 3D binding interactions between 3, 4-dihydro-2H pyran against *E.coli* at the site of DNA gyrase B (PDB ID: 6F86) and amoxicillin (standard) against *E.coli* at the site of DNA gyrase B (PDB ID: 6F86)

[The structure figure represents an understudy of the 2D and 3D binding interactions between the compound and bacteria chosen from in-vitro activities. The structures are as follows: A and B - the compound 3,4-dihydro-2H-Pyran against E.coli (PDB ID: 6F86) and amoxicillin (standard) against E.coli (PDB ID: 6F86)., respectively. Chemical structures were drawn by ChemDraw Pro 16.0 Suite (PerkinElmer, USA) and analyzed by the BIOVIA Discovery studio 2020 Client].

4.10.5 *In silico* drug likeness and toxicity predictions result

Drug likeness a prediction of Pyran molecule (C_5H_6O) was fulfilled Lipinski's rule of five with zero violations, molecular weight: belong to the group small molecule (Table 25), because it was directly related with very soluble, high diffusion with transported in aqueous media like blood, intracellular fluid, skin and gastrointestinal and it was high ligand (binding per atom) and lipophilic (linking potency of lipophilicity) efficiency (Table 26).

Table 25: Drug likeness predictions of compound 3,4-dihydro-2H-Pyran from the three plants computed by SwissADME.

Molecule's Name	Ligands	Mol. Wt. (g mol^{-1})	NHD	NHA	NRB	TPSA (Ă 2)	log P	Lipinski's rule of five with zero violations
Pyran	**C_5H_6O**	**82.1**	**0**	**7**	**0**	**9.23**	**1.43**	**0**
Amoxicillin	**$C_{16}H_{19}N_3O_5S$**	**365.4**	**4**	**28**	**5**	**158.26**	**1.05**	**0**

NHD -number of hydrogen donors, NHA- number of hydrogen acceptors, NRB - number of rotatable bonds, TPSA- total polar surface area, and log P- octanol-water partition coefficients

Table 26: ADME predictions of dihydro-2H-pyran, computed by SwissADME and PreADMET

Molecule's Name	Ligands	Skin Permeation value (log Kp) cm s^{-1})	GI Absorption	BBB Permeability	Inhibitor Interaction					
					Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
Pyran	**C_5H_6O**	**-5.95**	**High**	**Yes**	**No**	**No**	**No**	**No**	**No**	**No**
Amoxicillin	**$C_{16}H_{19}N_3O_5S$**	**0.87**	**-**	**-**	**Yes**	**No**	**No**	**No**	**No**	**No**

GI - gastrointestinal, BBB - blood brain barrier, P-gp - P-glycoprotein, and CYP - cytochrome-P.

Table 27: Toxicity prediction of dihydro-2H-pyran computed by ProTox-II and OSIRIS property explore.

Molecule's Name	Ligands	LD_{50} (mg kg^{-1})	Toxicity class	Organ toxicity					
				Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity	Irritant
Pyran	C_5H_6O	1070	4	Inactive	Active	Inactive	Inactive	Inactive	Inactive
Amoxicillin	$C_{16}H_{19}N_3O_5S$	15000	6	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive

Toxicity prediction of compound pyran was computed by ProTox-II and OSIRIS property and it was explored as toxicity predicted level 4 (Table 28); LD_{50}: 1070 mg kg^{-1} (Table 28) and it was computed by SwissADME and PreADMET organ toxicity relatively inactive by all except active in carcinogenicity (Table 27).

4.11 Prediction of anticancer compounds 5 and 6 isolated from *S. rosmarinus* leaves

4.11.1 Binding mode of compounds 5 and 6 docked against DNMT1 enzymes

The crystal structure of human DNMT1 (351-1600), classification transferase, resolution: 2.62 Å, visible yes, transparent yes, PDB ID 4WXX, XYZ -12.800500 34.654981 -24.870231 and radius 59.081291. A study was conducted to investigate the binding interaction of the isolated compounds **5** and **6** of the leaves of *Salvia rosmarinus* with the binding sites of the DNMT1 enzyme in human cervical cancer (PDB ID 4WXX), using molecular docking analysis. The study also compared the results with those of standard anti-cancer agents Jaceosidin (Table 28 and Figure 28).The reason why is standard Jaceosidin (contains three methoxy groups and one hydroxyl) used in mechanism of action as multifaceted and involved anticancer induces apoptosis (eliminate unwanted cells by inhibition DNMT1 enzymes) in cancer cells, inhibits cell proliferation, and may block the Akt pathway, which is involved in cell survival, migration, and invasion.

The compounds isolated had a final fixing energy extending from -5.3 to -8.4 kcal mol^{-1}, as shown in Table 28. It was compared to Jaceosidin (– 7.8 kcal mol^{-1}). The results of the molecular docking analysis showed that compound **5** (-8.4 kcal mol^{-1}) showed the highest binding energy values compared to the standard drugs jaceosidin (– 7.8 kcal mol^{-1}). Compound **6** has shown lower docking affinity (–5.3 kcal mol^{-1}) but good matching amino acid residue interactions compared to Jaceosidin. After analyzing the results, it was found that the isolated compounds had similar residual interactions and docking scores with Jaceosidin (Table 28).

Table 28: Molecular docking results of ligand compounds **5** and **6** against DNMT1 enzyme (PDB ID: 4WXX).

Ligands	Binding affinity (kcal mol^{-1})	Residual interactions		
		H-bond	Hydrophobic/ Electrostatic	Van der Waals
5	-8.4	ARG778(2.85249), ARG778(2.97417),		Lys-889,Pro-879,Tyr-865,His-795, Cys-893,Gly-760,Val-759,Phe-892, Phe-890,Pro-884,Lys-749
6	-5.3	VAL894 (2.42832) ARG596(2.73996), ALA597(1.84126), ILE422(2.99493), THR424 (2.1965), ILE422 (2.93653)	ElectrostaticPi-Cation-ARG595 (3.56619), Hydrophobic Alkyl-ARG595 (4.15839), HydrophobicPi-Alkyl-ARG595(5.14967)	Asp-423,Glu-428,Gly-425,Ile-427, Trp-464,Phe-556,Gln-560,Gln-594, Glu-559,Gln-598,Ser-563
Jaceosidin	-7.8	ASP571(2.93566), GLN573(2.02126), GLU562(2.42376), GLN573(3.49555), GLU562(3.46629)	Hydrophobic Alkyl PRO574 (4.59409), Hydrophobic Alkyl-ARG 690(5.09748), Hydrophobic Pi-Alkyl- PHE576 (5.1314), Hydrophobic Pi-Alkyl-PRO574 (4.97072), Hydrophobic Pi-Alkyl- ARG690 (5.07356)	Glu-698,Cys-691, Ala-695,Pro-692, Val-658,Glu-566, Asp-565

Hence, compound **5** might have potential anti-cancer agents. However, anti-cancer *in-vitro* analysis has not been performed yet. Promising *in silico* results indicate that further research could be beneficial. The 2D and 3D binding interactions of compounds **5** and **6** against human cervical cancer of DNMT1 enzyme (PDB ID 4WXX) are presented in Figures 37. The binding interactions between the DNMT1 enzyme (PDB ID 4WXX) and compound **5** (Figure 28) and

compound **6** (Figure 28) were displayed in 3D. Compounds and amino acids are connected by hydrogen bonds (green dash lines) and hydrophobic interactions (non-green lines).

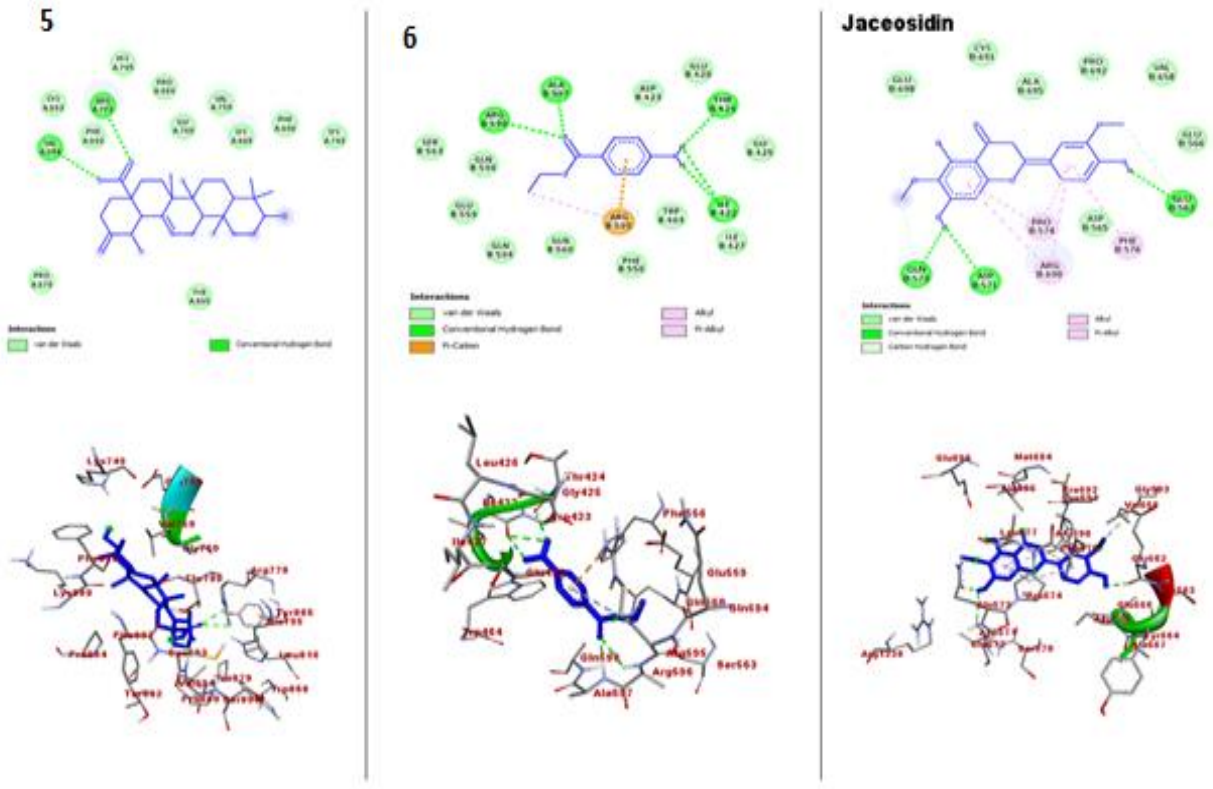


Figure 28: The 2D and 3D binding interactions of compounds against DNMT1 enzyme (PDB ID: 4WXX).
*[The 2D and 3D binding interactions of compound **5** and **6** represent against DNMT1 enzyme ,and Jaceosidin (standard)]*

4.11.2 Binding mode of compounds 5 and 6 docked against HPV type 16 E6 enzymes

Crystal structure of the HPV16 E6/E6AP/p53 ternary complex at 2.25Ă resolution, classification viral protein, PDB ID: 4XR8, visible yes, transparent yes, XYZ -43.202782 -39.085513 -29.194115, R-value observed 0.196, and Radius 65.584122. A study was conducted to investigate the binding interaction of the isolated compounds **5(**Sr-**101)** and **6(**sr-**102)** of the leaves of *Salvia rosmarinus* with the binding sites of the enzyme of human papilloma virus

(HPV) type 16 E6 (PDB ID 4XR8), using molecular docking analysis software. The study also compared the results with those of standard anti-cancer agents Jaceosidin (Table 29 and Figure 29). The compounds isolated had a bottommost fixing energy extending from -6.3 to -10.1 kcal mol^{-1}, as shown in Table 29. It was compared to Jaceosidin (– 8.8 kcal mol^{-1}). The results of the molecular docking analysis showed that, compound **5** (-10.1 kcal mol^{-1}) showed the highest binding energy values compared to the standard drugs jaceosidin (– 8.8 kcal mol^{-1}). Compound **6** has shown lower docking affinity (–6.3 kcal mol^{-1}) but good matching amino acid residue interactions compared to Jaceosidin. After analyzing the results, it was found that the isolated compounds had similar residual interactions and docking scores with Jaceosidin (Figure 29).

Hence, compound **5** and **6** might have potential anti-cancer agents of HPV as good inhibitors .However, anti-cancer *in-vitro* analysis has not been performed yet in HPV that causes cervical cancer agents. Promising *in silico* results indicate that further research could be beneficial. The 2D and 3D binding interactions of compounds **5** and **6** against human papilloma virus (HPV) type 16 E6 enzyme (PDB ID 4XR8) are presented in Figures 29. The binding interactions between the HPV type 16 E6 enzyme (PDB ID 4XR8) and compound **5** (Figure 29) and compound **6** (Figure 35) were displayed in 3D. Compounds and amino acids are connected by hydrogen bonds (green dash lines) and hydrophobic interactions (non-green lines).

Table 29: Molecular docking results of ligand compounds **5** and **6** against HPV type 16 E6 (PDB ID: 4XR8).

Ligands	Binding affinity (kcal mol^{-1})	H-bond	Residual interactions	
			Hydrophobic/ Electrostatic	Van der Waals
5	-10.1	ASN101(2.25622), ASP228(2.88341)	-	Asp-148,Lys-176,Lys-180,Asp-178,Ile-179,Tyr-177,Ile-334,Glu-382,Gln-336,Pro-335,Gln-73,Arg-383,Tyr-100
6	-6.5	TRP63(1.90011), ARG67 (2.16075), ARG67 (2.8181)	Hydrophobic Pi-Sigma-TRP341(3.76182), Hydrophobic Pi-Pi Stacked-TYR156(4.36581), Hydrophobic Pi-Pi T-shaped-TRP63(5.16561), Hydrophobic Pi-Pi T-shaped-TRP63(5.44632), Hydrophobic Alkyl PRO155 (4.34691), Hydrophobic Pi-Alkyl-TRP341(4.11391), Hydrophobic Pi-Alkyl-ALA64 (4.61525)	Glu-154,Arg-345,Asp-66,Met-331,Glu-112,Lys-16,Trp-231
Jaceosidin	-8.8	ARG146(2.06941), GLY70(3.49991), GLN73 (3.38801)	Electrostatic Pi-Cation-ARG67 (3.93442), Hydrophobic Pi-Alkyl-PRO49(5.40012)	Tyr-342,Tyr-79,Ser-338,Arg-129,Pro-335,Leu-76,Tyr-81,Ser-74,Tyr-71,Ser-80,Glu-46

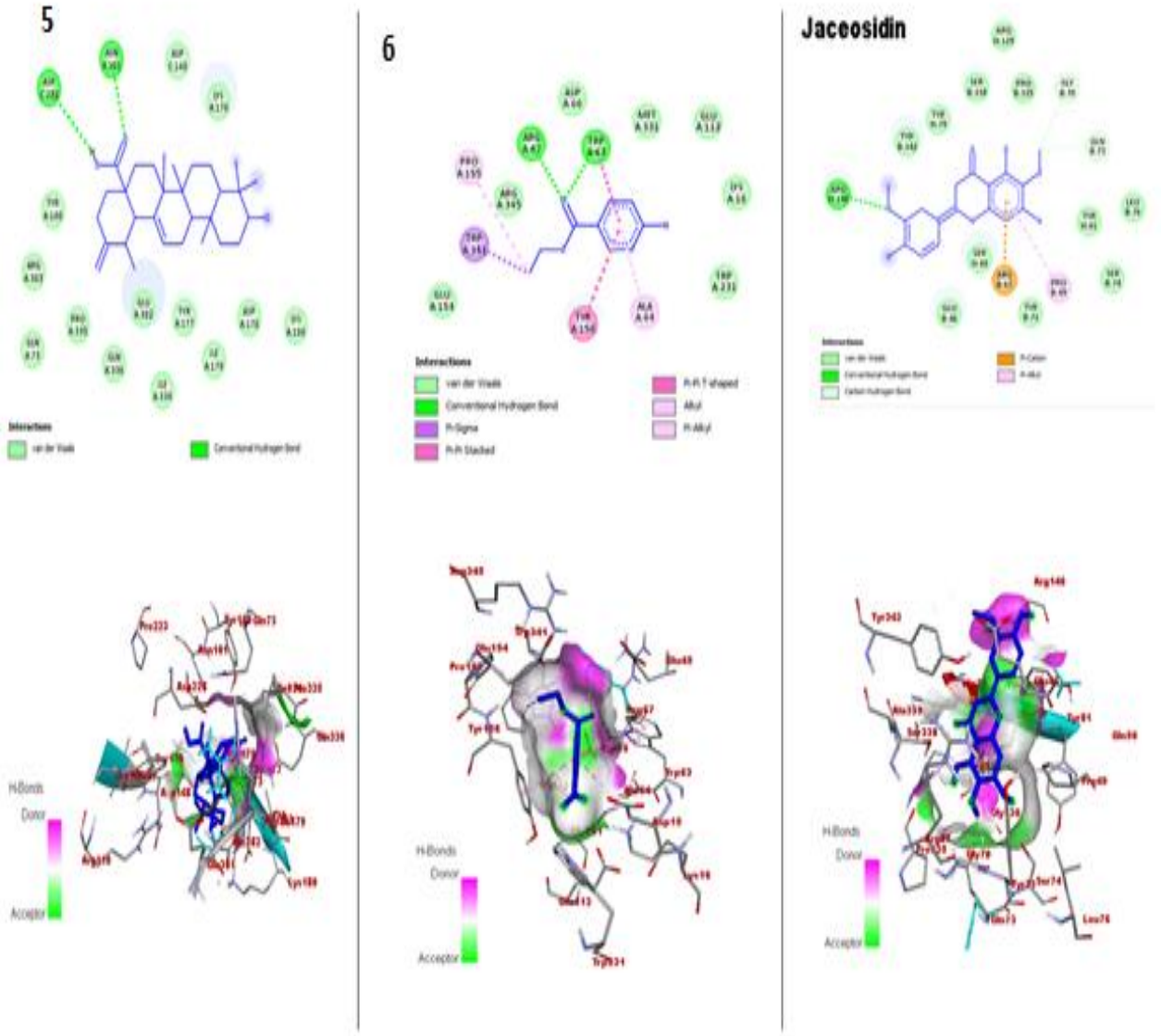


Figure 29: The 2D and 3D binding interactions of compounds against HPV type 16 E6 (PDB ID 4XR8).

*[The 2D and 3D binding interactions of compound **5** and **6** represent against HPV type 16 E6 enzyme, and Jaceosidin (standard)]*

4.11.3 Prediction of the ADMET profiling in compounds 5-6 drug-likeness and toxicity analysis

In-silico bioactivities of a drug, including drug-likeness and toxicity, predict its oral activity based on the document of Lipinski's Rule (Lipinski *et al*.,2012) was stated and the results of the current study ligands **5** and **6** showed that the compounds displayed conform to Lipinski's rule of five (Table 30).

Therefore, both compounds **5** and **6** should undergo further investigation as potential anti-cancer agents. Table 32 shows the acute toxicity predictions, such as LD_{50} values and toxicity class classification (ranging from 1 for toxic to 6 for non-toxic), for each ligand, revealing that none of them were acutely toxic. Furthermore, they were found to be similar to standard drugs. The isolated compounds **5** showed toxicity classification 4 (harmful if swallowed), while **6** showed even better toxicity prediction giving results of endpoints such as hepatotoxicity, mutagenicity, cytotoxicity, and irritant (Table 32). Both the isolated compounds were predicted to be in non-hepatotoxic, non-irritant, and non-cytotoxic. However, compound **5** has shown carcinogenicity and immunotoxicity (Table 32). Hence, based on ADMET prediction analysis, none of the compounds have shown acute toxicity, so they might be proven as good drug candidates.

Table 30: Drug-likeness predictions of compounds **5** and **6** computed by SwissADME .

Ligands	Formula	Mol.Wt. (g mol $^{-1}$)	NRB	NHA	NHD	TPSA (A°2)	Log P(iLOGP)	Log S (ESOL)	Lipinski's rule of five
5	$C_{30}H_{46}O_3$	454.68	1	3	2	57.53	3.56	-6.21	1
6	$C_9H_{11}NO_2$	165.19	3	2	1	52.32	1.89	-2.21	0
Jaceosidin	$C_{17}H_{14}O_7$	330.3	3	7	3	105	1.7	1	0

NHD -number of hydrogen donors, NHA- number of hydrogen acceptors, NRB - number of rotatable bonds, TPSA- total polar surface area, and log P- octanol-water partition coefficients, Log S- turbid metric of *solubility.*

Table 31: PreADMET predictions of compounds **5** and **6**, computed by SwissADME

Ligands	Formula	Skin Permeation value (logKp) cm s^{-1})	GI absorption	Inhibitor Interaction					
				BBB Permeability	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor
5	$C_{30}H46O_3$	-4.44	Low	No	No	No	No	No	No

6	**$C_9H_{11}NO_2$**	**-5.99**	**High**	**Yes**	**No**	**No**	**No**	**No**	**No**
Jaceosidin	**$C_{17}H_{14}O_7$**	**-6.13**	**High**	**No**	**No**	**Yes**	**No**	**Yes**	**Yes**

GI - gastrointestinal, BBB - blood brain barrier, P-gp - P-glycoprotein, and CYP - cytochrome-P.

Table 32: Toxicity prediction of compounds **5** and **6**, computed by ProTox-II and OSIRIS property explore

Ligands	Formula	LD_{50} (mg Kg^{-1})	Toxicity class	Organ toxicity					
				Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity	Irritant
5	**$C_{30}H_{46}O_3$**	**2000**	**4**	**Inactive**	**Active**	**Active**	**Inactive**	**Inactive**	**Inactive**
6	**$C_9H_{11}NO_2$**	**NA**	**NA**	**Inactive**	**Inactive**	**Inactive**	**Inactive**	**Inactive**	**Inactive**
Jaceosidin	**$C_{17}H_{14}O_7$**	**69**	**3**	**Inactive**	**Inactive**	**Inactive**	**Inactive**	**Inactive**	**Inactive**

NA- no available

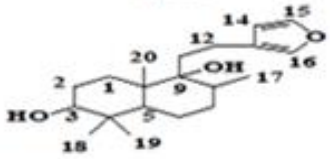
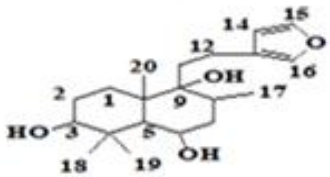
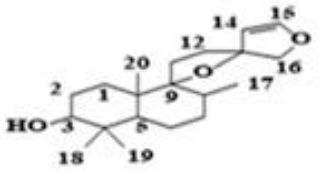
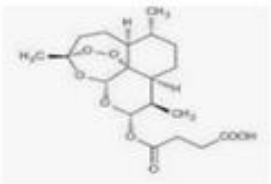
4.12 *In-silico* Antimalarial activities of compounds 2, 3, and 4 isolated from *O. integrifolia* leaves

4.12.1 Prediction of antimalarial activity

The crystal structure of *Plasmodium falciparum* (residues ALA18-ALA329), *PfLDH* that put for L-lactate dehydrogenase complexed with NADH and oxamate (1LDG), SBD-Site-Sphere, mutation no, classification oxidoreductase, resolution: 2.62 Å, visible yes, visibility locked no, transparent yes, PDB ID 1LDG, XYZ -31.660820 25.658680-35.929080 and Radius 12.205537. The study investigated the binding interaction of the isolated compounds **2, 3**, and **4** of the leaves of *Otostegia integrifolia* with the binding sites of the 1LDG enzyme in *Plasmodium falciparum* (PDB ID 1LDG), using molecular docking analysis using*AutoDock Tools* (ADT) Discovery Studio Client 2020 (Narramore *et al.*, 2019; Trott and Olson,2010). Molecular docking is a virtual tool that finds the best binding orientation of newly designed molecules (ligands) to their target protein molecules of interest. It has been useful for predicting the binding affinity with the biological efficacy of the targeted small molecules(Kitchen *et al.*,2004).What is more, *P. falciparum* lactate dehydrogenase (PfLDH) enzyme is a crucial malaria parasite enzyme involved in the glycolytic pathway, thus, has been considered as a potential molecular target (Kalita *et al.*,2021). On the other hand, malaria is a potentially deadly disease with many anti-malarial drugs that have been extracted

ineffective due to *Plasmodium falciparum* resistance concerns (Nurhanis *et al*.,2020). However, this study comparatively compared the results with those of standard anti-malaria agents Artesunate (Table 33 and Figure 30). The compounds isolated had a final fixing energy extending from -5.5 to -6.7 kcal mol^{-1}, as shown in Table 34. It was computed to Artesunate (– 6.3 kcal mol^{-1}). The results of the molecular docking analysis showed that compound **4** (-6.7 kcal mol^{-1}) showed the highest binding energy values compared with the standard drugs Artesunate (– 6.3 kcal mol^{-1}) and compound **2** (-5.9 kcal mol^{-1}) relatively come next binding energy. Compound **3** has shown lower docking affinity (–5.5 kcal mol-1) but good matching amino acid residue interactions compared to Artesunate. After analyzing the results showed that these compounds exhibited ethier comparable or higher binding affinity than the reference drug Artesunate. This study is similar to the literature in the results of 4-aminoquinoline Hybrids binding affinity with the reference drug chloroquine, amodiaquine, and hydroxychloroquine (Nurhanis *et al*.,2020).

Table 33: Molecular docking results of ligand compounds **2, 3** and **4** isolated from *O. integrifolia leaves* against *plasmodium falciparum* (PDB ID 1LDG)

S. No.	Ligands	Binding affinity (kcal/ mol)	Residual interactions		
			H-bond	Hydrophobic/ Electrostatic	Van der Waals
2	 1 (Oi-10)	-5.9	ILE31(2.17349), ASP53(2.27302)	Hydrophobic Pi-Alkyl- ILE31 (5.24416)	Ser-28,Met-58,Gly-29, Thr-97,Gly-32,Met-30, Thr-101,Lys-102,Phe-100, Gly-99,Ile-54,Val-55
3	 2 (Oi-34)	-5.5	GLY99(2.14924), THR97(3.67658), ILE31(2.87084)	Hydrophobic Pi-Alkyl- ILE31(4.72387)	Ala-244,Met-30,Thr-101,Lys-102,Phe-100,Gly-29,Gly-32
4	 3 (Oi-100)	-6.7	-	Hydrophobic Pi-Sigma- PHE100 (3.96033)	Thr-101,Gly-99, Val-55,Ile-54, Ile-119,Ala-98, Asp-53,Phe-52, Gly-27,Val-26
SD		-6.3	ASN140 (1.58395), ASN140 (2.41064), ASN140 (2.25997), PHE100 (2.74859)	Hydrophobic Alkyl- ILE31(4.39917)	Gly-99,Thr-139,Thr-101,Thr-97,Met-30,Ser-245,Tyr-247,Pro-246,Pro-250,Val-138,His-195,Arg-109,Leu-112,Asn-116,

Henceforward, compounds **2, 3**, and **4** might have potential anti-malaria agents of the target protein molecules (1LDG) in *plasmodium falciparum* as inhibitors. It may be that anti-malaria; *in-vitro* has not been performed yet in the plasmodium falciparum (*PfLDH*) enzyme that causes malaria. Promising *in silico* r*esults* indicate that further research could be beneficial. The 2D and 3D binding interactions of compounds **2, 3,** and **4** against *the plasmodium falciparum* (PDB ID 1LDG) enzyme are presented in Figure 36. Briefly, the binding interactions between the *plasmodium falciparum* (PDB ID 1LDG) enzyme and compound **2**, compound **3**, and compound **4** presented in Figure 36 were displayed in 2D and 3D, respectively. The ligands and targeted amino acids are connected by hydrogen bonds (green dash lines) and hydrophobic interactions (non-green lines) (Figure 36).

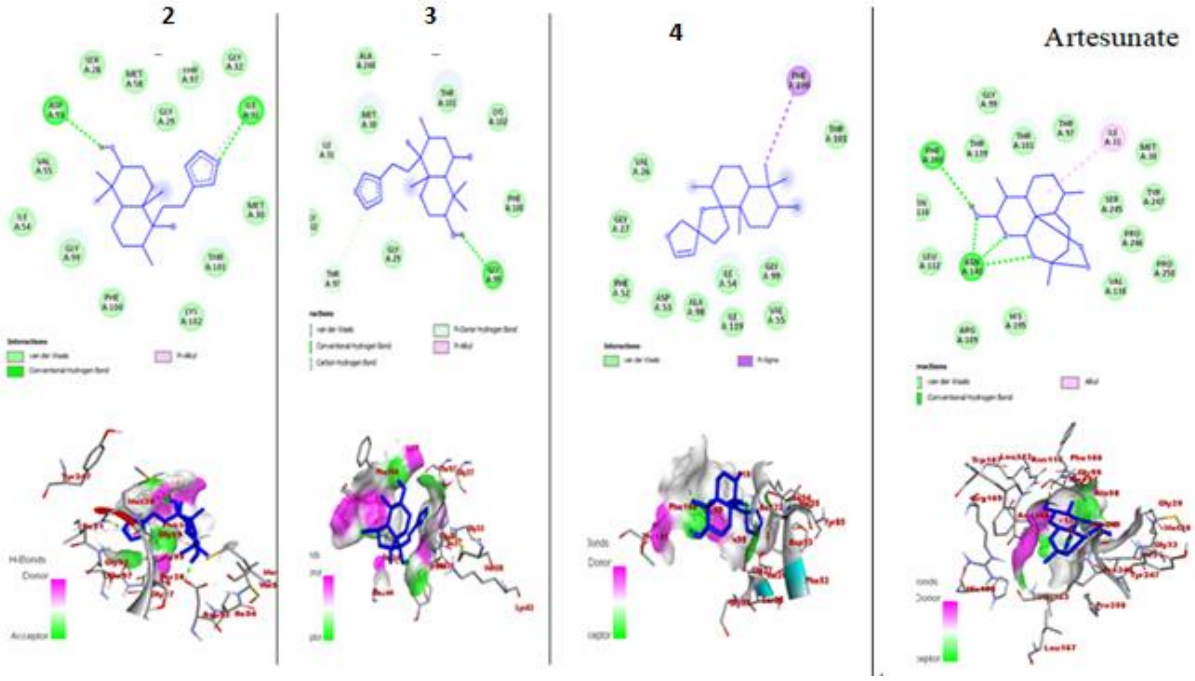


Figure 30: The 2D and 3D binding interactions of compounds **2, 3** and **4**; with the reference of Artesunate (SD) against *plasmodium falciparum* (PDB ID 1LDG).

*[The 2D and 3D binding interactions of compound **2, 3** and **4** isolated from O. integrifolia leaves against plasmodium falciparum (PDB ID 1LDG), and Artesunate (standard) against plasmodium falciparum (PDB ID 1LDG) enzyme]*

4.12.2 Prediction of the ADMET in compounds 2-4 drug-likeness and toxicity analysis

In-silico bioactivities of a drug predict its oral activity based on the document of Lipinski's Rule (Lipinski et al.,2012) was stated and the results of the current study showed that the compounds displayed conform to Lipinski's rule of five (Table 35). Therefore, all compounds **2**, **3,** and **4** should undergo further investigation as potential anti-malaria agents. Table 37 shows the acute toxicity predictions, such as LD_{50} values and toxicity class classification (ranging from 3 for harmful to 4 for corrosive), for each ligand, revealing that none of them were acutely toxic. PreADMET predictions of gastiroinstestanal absorption are high (Table 35, Figure 34). Furthermore, they were found to be similar to standard drugs. The isolated compounds **2** and **3** showed corrosive classification 3 (not harmful if swallowed) while **2** and **4** showed even better toxicity prediction giving results of endpoints active to such as

immunotoxicity (Table 36). All the isolated compounds were predicted to be non-hepatotoxic, carcinogenicity, non-cytotoxic and non-irritant. Hence, based on ADMET prediction analysis, none of the compounds showed acute toxicity, so they might be proven as good drug candidates. In literature, the bioavailability score (Martin,2005) calculates their drug-likeness properties, more than half of the compounds considered were expected to have a good bioavailability score (0.55) and obeyed Lipinski's rule >10% .Similarily, in this study was shown a good bioavailability score results were shown for predicting the drugs(0.55).However, the reference was scored 0.56. Then again, antimalarial were come to be from *O. integrifolia leaves* of the three compounds **2**, **3**, and **4** that were defined in terms of boiled-egg-model for gastrointestinal absorption and brain penetration (www.researchgate.net/figure/boiled-egg-mod): the three experimental molecules located in boiled-egg's yolk are predicted to permeate through the blood-brain barrier (BBB) passively (Table 35). However, the references molecules (Artesunate) located in the boiled-egg's white are expected to be passively absorbed by the gastrointestinal tract (Table 35). Compound **3** blue-dotted molecules are predicted to be effluated from the central nervous system (CNS) by the p-glycoproteins (**PGP+)**, and, when the compound **2** and **4** with the red-dotted molecules are not effluated (**PGP-)** (Figure 30).

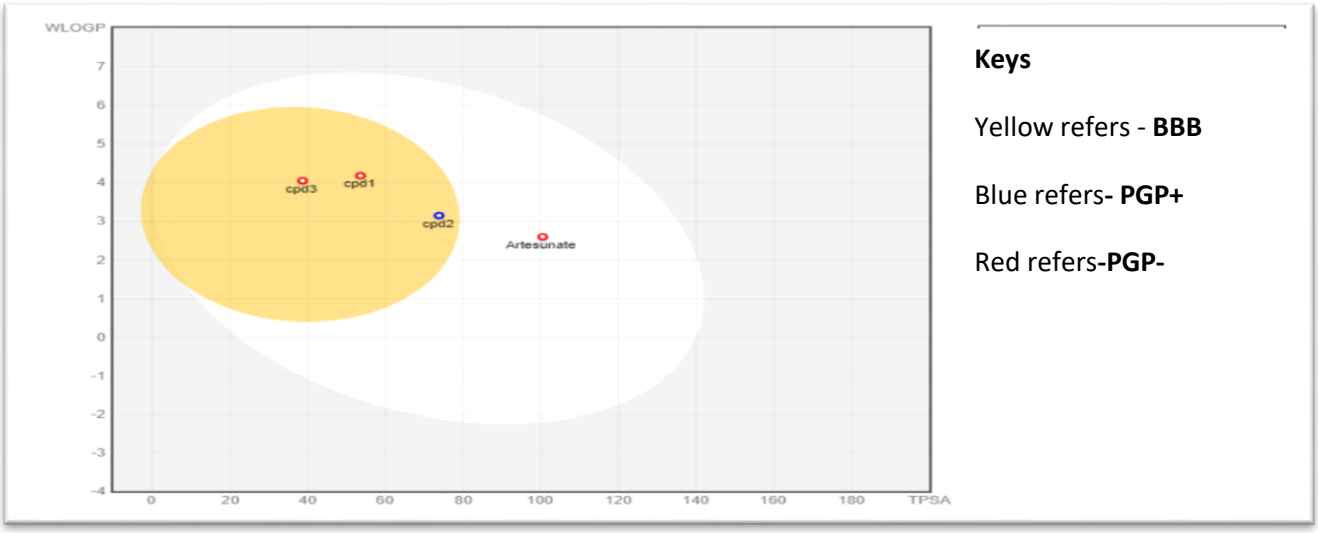


Figure 31: Boiled-egg model was demonstrated against *Plasmodium falciparum* via compounds **2, 3**, and **4** revive from *O. integrifolia* leaves in yolk egg passively permeate BBB and the standard (Amantadine HCl) passively permeate GIA in the white egg boiled portions.

[Drugs against Plasmodium falciparum via *the compounds **2, 3**, and **4** revive from O. integrifolia leaves represented the boiled-egg's model experimental was shown that passively permeate blood-brain barrier (BBB) in yolk egg boiled portions and the standard (Artesunate) passively permeate gastrointestinal absorption (GIA) in the white egg boiled portions.]*

Table 34: Drug-likeness predictions of compounds **2**, **3**, and **4** computed by SwissADME

Ligands	Formula	Mol.Wt. (g mol $^{-1}$)	Bio Availability score	Ghose	Veber	Egan	Muegge	NRB	NHA	NHD	TPSA (A°^{2})	Log P(iLOGP)	Log S (ESOL)	Lipinski's rule of five
2	$C_{20}H_{32}O_3$	320.47	0.55	yes	yes	Yes	yes	3	3	2	53.60	3.21	-4.46	0
3	$C_{20}H_{32}O_4$	336.47	0.55	yes	yes	Yes	yes	3	4	3	73.83	2.95	-3.82	0
4	$C_{20}H_{32}O_3$	320.47	0.55	yes	yes	Yes	yes	0	3	1	38.69	3.41	-4.25	0
Artesunate	$C_{19}H_{28}O_8$	384.42	0.56	yes	yes	Yes	yes	5	8	1	100.52	2.92	-3.08	0

NHD -number of hydrogen donors, NHA- number of hydrogen acceptors, NRB - number of rotatable bonds, TPSA- total polar surface area, and log P- octanol-water partition coefficients, Log S- turbid metric of *solubility.*

Table 35: PreADMET predictions of compounds **2**, **3**, and **4** computed by SwissADME

Ligands	Formula	Skin Permeation value (logKp) cm s^{-1})	GI Absorption	Inhibitor Interaction						
				BBB Permeability	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP2D4 Inhibitor
2	$C_{20}H_{32}O_3$	-5.24	High	Yes	No	No	No	No	Yes	No
3	$C_{20}H_{32}O_4$	-6.17	High	Yes	Yes	No	No	No	Yes	No
4	$C_{20}H_{32}O_3$	-5.52	High	Yes	No	No	No	No	No	No
Artesunate	$C_{19}H_{28}O_8$	-7.31	High	No	No	No	No	No	No	No

GI - gastrointestinal, BBB - blood brain barrier, P-gp - P-glycoprotein, and CYP - cytochrome-P.

Table 36: Toxicity prediction of compounds **2**, **3**, and **4** computed by ProTox-II and OSIRIS property explorer.

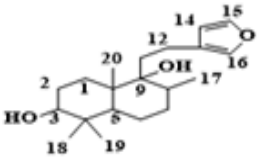
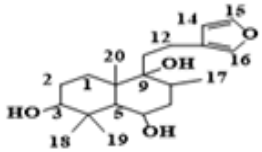
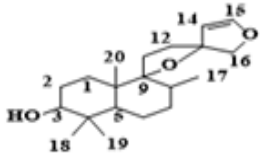
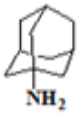
Ligands	Formula	LD_{50} (mg Kg^{-1})	Toxicity class	Organ toxicity					
				Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxi City	Irritant
2	$C_{20}H_{32}O_3$	104	3	Inactive	Inactive	Active	Inactive	Inactive	Inactive
3	$C_{20}H_{32}O_4$	104	3	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
4	$C_{20}H_{32}O_3$	2000	4	Inactive	Inactive	Active	Inactive	Inactive	Inactive
Artesunate	$C_{19}H_{28}O_8$	1000	4	Inactive	Inactive	Active	Inactive	Inactive	Inactive

4.13 Antiviral in-silico study compounds 2, 3, and 4 from O. integrifolia leaves

4.13.1 Prediction of antiviral activity

The crystal structure of hem agglutinin from H1N1 Influenza A virus A/Denver/57 bound to the C05 antibody recognition site. Method: x-ray diffraction, resolution: 2.92 Å, R-value observed: 0.204, Visibility Locked no, mutation no, Transparent Yes, classification viral protein PDB ID 6ML8, XYZ 5.251880 -45.787504 21.709299, and Radius 36.383682. Query: accession code is Q2IBI1 and database name 'UniProt' and scientific name of source organism *Homo sapiens*. The study investigated the binding interaction of the isolated compounds **2**, **3**, and **4** from the leaves of *Otostegia integrifolia* with the binding sites of the 6ML8 in Influenza A virus flu (PDB ID 6ML8), using molecular docking analysis using AutoDock Tools (ADT) (Narramore *et al.*, 2019, Trott and Olson, 2010). Hence, influenza A is a type of virus that causes influenza (the flu), a highly contagious respiratory illness as a potential molecular target (Britannica, 2024). On the other hand, Influenza spreads around the world in seasonal epidemics, resulting in the deaths of between 250,000 and 500,000 people every year, up to millions in some pandemic years and influenza is a potentially deadly disease due to vast genetic variability within influenza viruses and their highly error-prone RNA dependent RNA polymerase does raise concerns regarding the possible emergence of treatment resistant strains and generates further questions regarding their viral fitness and transmissibility as well as which strategies to employ in rapidly identifying and effectively treating these resistance variants (Lampejo, 2020). Similarly, the most recent influenza-A, H1N1 pandemic was thought to arise from a series of re-assortment events .However, this study comparatively compared the results with those of standard anti-influenza A virus agents Amantadine HCl (Table 37 and Figure 31). The compounds isolated had a final fixing energy extending from -6.0 to -6.8 kcal mol^{-1}, as shown in Table 38. It was computed to Amantadine HCl (– 4.6 kcal mol^{-1}). The results of the molecular docking analysis showed that compound **4** (-6.8 kcal mol^{-1}) showed the highest binding energy values compared with the standard drugs Amantadine HCl (– 4.6 kcal mol^{-1}) and compound **2** (-6.7 kcal mol^{-1}) relatively come next binding energy. Compound **3** has shown lower docking affinity (–6.0 kcal mol^{-1}) but good matching amino acid residue interactions compared to Amantadine HCl. After analyzing the results showed that **2 - 4** compounds exhibited ethier comparable or higher binding affinity than the reference drug Amantadine HCl (Table 37).

Table 37: Molecular docking results of ligand compounds **2**, **3** and **4** isolated from *O. integrifolia* leaves against Influenza A virus (PDB ID 6ML8)

	Ligands	Binding affinity (kcal/ mol)	Residual interactions		
			H-bond	Hydrophobic/ Electrostatic	Van der Waals
2		-6.7	ASN73 (2.28484), PHE112 (2.3863), GLU116(2.10092)	Hydrophobic Pi-Alkyl-ALA75 (4.23901)	Tyr-115,Gly-105,Asp-111,Thr-106,Gly-110,Lys-415,Asn-414
3		-6.0	THR106(3.56813), GLY110(3.45243)	Hydrophobic Pi-Alkyl-VAL236 (5.14514)	Pro-109,Phe-112,Asp-111,Glu-116,Tyr-115,Asn-73,Gly-105,Ala-75
4		-6.8	-	-	-
SD	Amantadine HCl 	-4.6	TRP247 (2.76723), ASP114(2.02462)	Hydrophobic Pi-Sigma-TRP247(3.64074), Hydrophobic Pi-Alkyl- TRP247(5.34797)	Tyr-222,Thr-248,Leu-249

In future, compounds **2** and **3** might have potential anti-viral agents of the target protein molecules (6ML8) in influenza A virus as inhibitors. Briefly, the binding interactions between the influenza A virus (PDB ID 6ML8) protein and compound **2** and compound **3** presented in Figure 45 were displayed in 2D and 3D, respectively. The ligands and targeted amino acids are connected by hydrogen bonds (green dash lines) and hydrophobic interactions (non-green lines)(Figure 38).However, in our results, the Compound **4** was shown by ligand no bonded between the acceptor of Oxygen atom and donor of Hydrogen atom that it unsatisfied type in donor and acceptor atoms process. Therefore, the compound **4** was not shown clear residual interaction in 2D and 3D structures even supposing the highest binding energy values (– 6.8 kcal mol^{-1}) compared to the standard drugs Amantadine HCl (– 4.6 kcal mol^{-1})(Table 37).

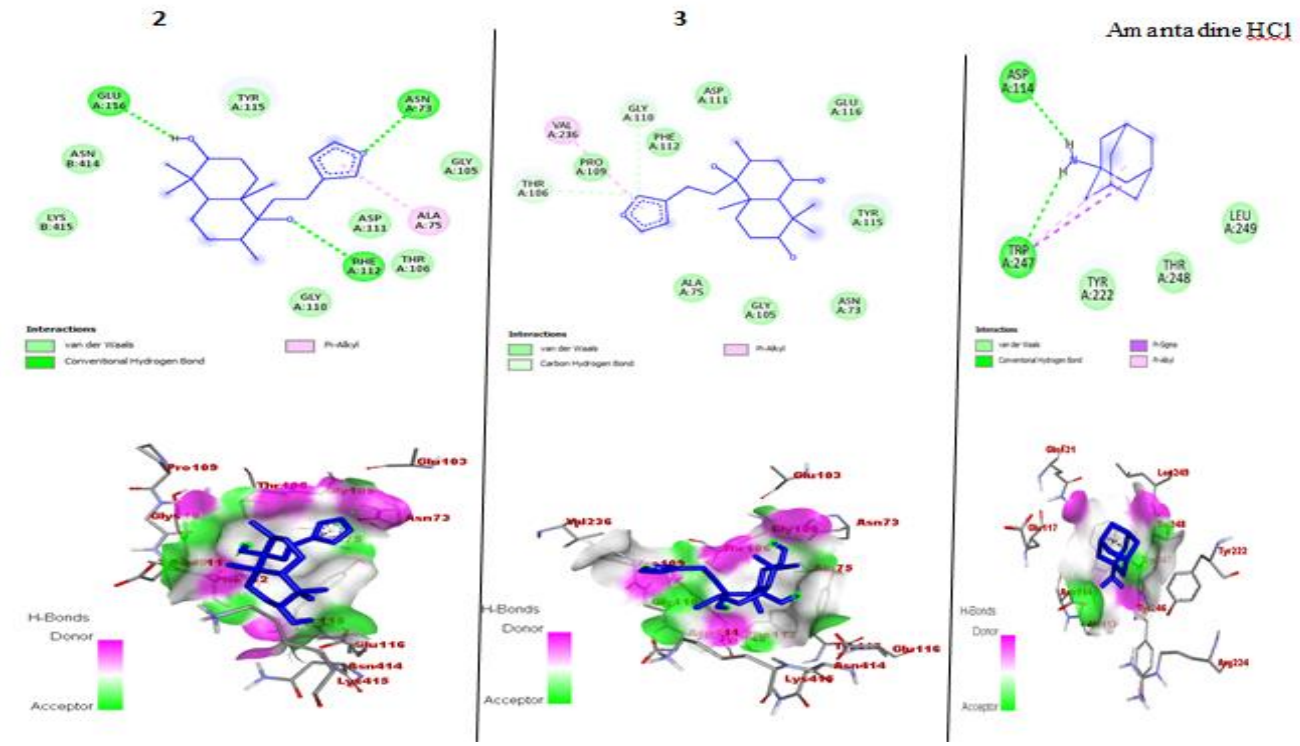


Figure 32: The 2D and 3D binding interactions of compounds **2** and **3**; with the reference of Amantadine HCl (SD) against influenza A virus (PDB ID 6ML8)

*[The 2D and 3D binding interactions of compound **2** and **3** isolated from O. integrifolia leaves against* influenza A virus *(PDB ID 6ML8), and Amantadine HCl (standard) against* influenza A virus *(PDB ID 6ML8) protein]*

4.13.2 Prediction of the ADMET profiling of compounds 2, 3, and 4 drug-likeness and toxicity analysis

In-silico bioactivities of a drug predict its oral activity based on the document of Lipinski's Rule (Lipinski *et al*.,2012) that stated the results of the current study where the compounds displayed conform to Lipinski's rule of five (Table 37). Therefore, all the compounds **2**, **3**, and **4** should undergo further investigation as potential anti-viral agents. Table 39 shows the acute toxicity predictions, such as LD_{50} values and toxicity class classification for each ligand, revealing that none of them were acutely toxic. PreADMET predictions of gastiroinstestan absorption are high (Table 39). Furthermore, they were found to be similar to standard drugs. The isolated compounds **3** showed corrosive classification 3 (not harmful if swallowed) while **2** and **4**

showed even better toxicity prediction giving results of endpoints active to such as immunotoxicity (Table 40). All the isolated compounds were predicted to be non-mutagenicity non-carcinogenicity, non-cytotoxic and non-nephron toxicity. However, all the isolated compounds were predicted to be high respiratory toxicity.Hence, based on ADMET prediction analysis, none of the compounds have shown acute toxicity, so they might be proven as good drug candidates. In literature, the bioavailability score (Martin,2005) calculates their drug-likeness properties, more than half of the compounds considered were expected to have a good bioavailability score (0.55) and obeyed Lipinski's rule >10% .Similarly, in this study was shown a good bioavailability score results for predicting the drugs(0.55) and all good matched with the reference scored (0.55) of it. Then again, anti-influenza A viruses were come to be from *O. integrifolia* leaves of the three compounds **2**, **3**, and **4** were defined in terms of boiled-egg-model for gastrointestinal absorption and brain penetration (Daina and Zoete, 2016): the three experimental molecules located in boiled-egg's yolk are predicted to permeate through the blood-brain barrier (BBB) passively(Figure 32). Similarly, the references molecules (Amantadine HCl) located in boiled-egg's yolk are predicted to permeate through the blood-brain barrier (BBB) passively (Table 40, Figure 32).

Table 38: Drug-likeness predictions of compounds **2**, **3**,and **4** computed by SwissADME.

Ligands	Formula	Mol.Wt. (g mol $^{-1}$)	Bio Availability score	Ghose	Veber	Egan	Muegge	NRB	NHA	NHD	TPSA (A°^{2})	Log P(iLOGP)	Log S (ESOL)	Lipinski's rule of five
2	$C_{20}H_{32}O_3$	**320.47**	**0.55**	**Yes**	**Yes**	**Yes**	**Yes**	**3**	**3**	**2**	**53.6**	**3.21**	**-4.46**	**0**
3	$C_{20}H_{32}O_4$	**336.47**	**0.55**	**Yes**	**Yes**	**Yes**	**Yes**	**3**	**4**	**3**	**73.83**	**2.95**	**-3.82**	**0**
4	$C_{20}H_{32}O_3$	**320.47**	**0.55**	**Yes**	**Yes**	**Yes**	**Yes**	**0**	**3**	**1**	**38.69**	**3.41**	**-4.25**	**0**
Amantadine	$C_{10}H_{18}ClN$	**187.71**	**0.55**	**Yes**	**Yes**	**Yes**	**No**	**0**	**1**	**1**	**26.02**	**0**	**-3.04**	**0**

NHD -number of hydrogen donors, NHA- number of hydrogen acceptors, NRB - number of rotatable bonds, TPSA- total polar surface area, and log P- octanol-water partition coefficients, Log S- turbid metric of *solubility.*

Table 39: PreADMET predictions of compounds **2**, **3**, and **4** computed by SwissADME

Ligands	Formula	Skin Permeation value (logKp) cm s^{-1})	GI Absorption	Inhibitor Interaction						
				BBB Permeability	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP2A4 Inhibitor
2	$C_{20}H_{32}O_3$	-5.24	High	Yes	No	No	No	No	Yes	No
3	$C_{20}H_{32}O_4$	-6.17	High	Yes	Yes	No	No	No	Yes	No
4	$C_{20}H_{32}O_3$	-5.52	High	Yes	No	No	No	No	No	No
Amantadine	$C_{10}H_{18}ClN$	-5.14	High	Yes	No	No	No	No	No	No

GI - gastrointestinal, BBB - blood brain barrier, P-gp - P-glycoprotein, and CYP - cytochrome-P.

Table 40: Toxicity prediction of compounds **2**, **3**, and **4** computed by ProTox-II and OSIRIS property explorer.

Ligands	Formula	LD_{50} (mg Kg^{-1})	Toxicity class	Organ toxicity							Eco toxicity
				Hepato toxicity	Carcinogenicity	Immuno toxicity	Mutagenicity	Cyto toxicity	Nephron toxicity	Respiratory toxicity	
2	$C_{20}H_{32}O_3$	1190	4	Active	Inactive	Active	Inactive	Inactive	Inactive	active	active
3	$C_{20}H_{32}O_4$	104	3	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	active	inactive
4	$C_{20}H_{32}O_3$	2000	4	Inactive	Inactive	Active	Inactive	Inactive	Inactive	active	inactive
Amantadine	$C_{10}H_{18}ClN$	530	4	Inactive	Inactive	inactive	Inactive	Inactive	Inactive	active	active

Compound **3** blue-dotted molecules are predicted to be effluated from the central nervous system (CNS) by the p-glycoproteins (PGP+), and, when the compound **2** and **4** with the red-dotted molecules are not effluated (PGP-) (Figure 32).

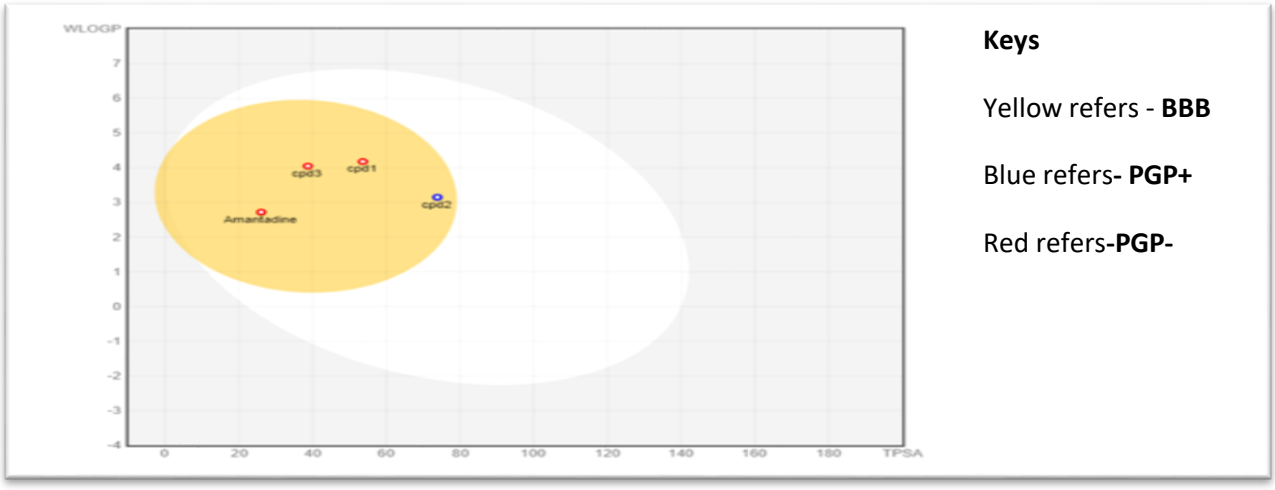


Figure 33: Boiled-egg model was demonstrated against influenza A viruses via compounds **2, 3**, and **4** revive from *O. integrifolia* leaves and the standard (Amantadine HCl) represented all are passively permeate blood-brain barrier.

[Drugs against influenza A viruses via *the compounds **2, 3**, and **4** revive from O. integrifolia leaves represented the boiled-egg's model experimental was shown that passively permeate blood-brain barrier (BBB) in yolk egg boiled portions.*

CHAPTER 5

5 DISCUSSION

The study titled "Phytochemical, *In Vitro* Biological, and Computational Analyses of Bioactive Compounds from Leaf Extracts of Some Selected Ethiopian Medicinal Plants" focuses on three medicinal plants: *Vernonia amygdalina*, *Otostegia integrifolia* Benth (also known as *Rydinglia integrifolia* Benth), and *Salvia rosmarinus* (formerly classified as *Rosmarinus officinalis*). The findings of the study were discussed by various subgroups.

Phytochemical Screening: Phytochemical refer to the massive number of secondary metabolic compounds present in plants. According to the study, certain phytochemicals found in the extracts of *V. amygdalina, O. integrifolia,* and *S. rosmarinus* leaves have the potential to halt the growth of cancer cells, and pathogens or even kill them (Stashenko and Martinez, 2018). In our study also we screened presence of flavonoids and alkaloids in the plants extracts.Flavonoids are related to natural phenolic compounds that have antioxidant and anti-inflammatory properties that can be found in the human diet (Table 6). In literature, alkaloids are found mostly in fungi and are known for their strong antimicrobial properties, which make them valuable in traditional medicine (Altundag, 2020; Apollinaire,2016; Kuber *et al*., 2013). Most alkaloids have a bitter taste and are used to protect against antimalarial, antiasthma, anticancer, antiarrhythmic, analgesic, antibacterial (Chirumamilla and Taduri , 2023; Altundag,2020 ; Truong *et al*.,2019).

In our study, steroids were found in *Vernonia amygdalina* leaves (Table 6).The natural steroid compound occurring both in plants and animals (Busse and Tefera, 2013). Steroids are found as a natural part of our body. They are hormones that help regulate our body's reaction to infection or injury, the speed of our metabolism, and more. In medicine, we can use artificial steroids called corticosteroids to help break fevers, bring down inflammation, and reduce pain. On the other hand, steroids were reported to have various biological activities like chronic obstructive pulmonary disease (COPD), and multiple sclerosis and imitate male sex hormones (Busse and Tefera, 2013). Likewise, in our study, Presence of coumarins was detected only in *S. rosmarinus*.

Conversely, in recent years, researchers have focused on the anticancer activity of coumarins, due to their high biological activity (Akkol e*t al*.,2020). Glycoside is also used for cardiac medicine and cancer treatment (Busse and Tefera, 2013). The same,in our study, glycosides are present in *V. amygdalina* and *S.rosmarinus* but absent in O. *integrifolia*. Terpenoids were found commonly in all the three plants leaves (Table 6). Terpenoids like mono-terpenes and sesquiterpenes are widely found in nature (more than 50,000 terpenoids) have been found in nature from plants that reduce tumors and cancers. Many volatile terpenoids such as menthol and perillyl alcohol are used as raw materials for spices, flavorings, and cosmetics (Liu, 2022).

Similarly, the results of this study revealed that the three plants *V. amygdalina, O. integrifolia,* and *S. rosmarinus* also contain flavonoids (Table 10-12), carbohydrate, tannins and saponins (Table 6). Flavonoids are commonly found in fruits and vegetables and are considered excellent antioxidants (Usunobun and Ngozi, 2016; Atangwho *et al*.,2013). According to literature, these flavonoids, terpenoids, and steroids activities include anti-diabetes, anti-inflammatory, anticancer, antibacterial, hepatic-protective, and antioxidant effects (Demie *et al*.,2018). Tannins are commonly found in most terrestrial plants (Tong, 2022) and have the potential to treat cancer, and HIV/AIDS as well as to treat inflamed or ulcerated tissues. Similarly, in our study, tannins were found in the three plants in higher concentration.

In-vitro **Antimicrobial activities:** Using a general rule, chemical agents showing an inhibitory zone of ≥ 7 mm are considered significantly active against the tested bacterial culture (Nascimento *et al*., 2000). In the present study also, the extracts were shown a mean of inhibition zones with a diameter less than 7 mm and were considered as not active against the tested bacterial pathogens at the tested concentration(s). The plant from the Asteraceae family was classified under the genus *Vernonia*, with the species *amygdalina* noted for its medicinal properties. Previous studies, by Dumas *et al*., (2020) have reported the antimicrobial activities of *V. amygdalina*, demonstrating its inhibitory effect on various bacteria, including *Staphylococcus aureus* and *Klebsiella pneumoniae*. In our study, *V. amygdalina* exhibited a notably good zone of inhibition, with diameters of 17.80 ± 0.20 mm for the petroleum ether extract and 16.87 ± 0.23 mm for the methanol extract, demonstrating antimicrobial properties against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, both Gram-negative bacteria (Table 10). The chloroform

extract of *V. amygdalina* showed strong activity against the Gram-positive bacterium *S. aureus*, with an inhibition zone of 21 mm. In our study, however, the chloroform: methanol(1:1) extract of *V. amygdalina* exhibited high activity against the Gram-positive bacteria *S. aureus* and *S. epidermidis*, with inhibition zones of 14 mm and 16 mm, respectively, compared to the positive control, which produced inhibition zones of 16 mm and 18 mm. Previous studies (Habtamu and Melaku,2018) reported that isorhamnetin and acetone extracts were active against all bacterial pathogens tested. On the contrary,of the extracts in the present study, the petroleum ether, chloroform:methanol(1:1), and methanol extracts of *V. amygdalina* demonstrated significant activity against all microbes tested at 100 µg mL^{-1}, with inhibition zones ranging from 7 to 18 mm (Table 10).

Other intellectual (Yusoff *et al*, 2020) evaluated acetone extracts in the antifungal activity of the leaf extracts of *V. amygdalina* against *Botrytis cinereal*. Therefore, the different extracts, using various solvents of various polarity indexes, have been attributed to specific biological activities like flavonoids (Demie *et al*.,2018). Similarly, the results of this study revealed that *V. amygdalina* exhibited the highest inhibition value (15.90±0.14 and 12.83±0.15 mm) against *C. albicans* and *A.niger*, respectively. Thus, *V. amygdalina* leaf extracts may show us the mean values of flavonoids (mg g^{-1}) by 570 nm of 69 -93% (Table 10).

Studies have reported the antimicrobial activities of *Otostegia integrifolia* (Chekol & Desta, 2018) showed that extracts of methanol exhibited inhibitory activity against Gram-positive bacteria including *S. aureus* and Gram-negative of *E. coli* and *S. typhi* with minimum zone of inhibition 13.5+0.40 , 13.9+0.16 and 10.1+0.04, respectively . However, in our study *Otostegia integrifolia* has been found to possess a remarkably large of inhibition with diameters of 15.17±0.29 and 16.97±0.06 in methanol extracts that exhibited antimicrobial properties *against S. aureus*, and *S. epidermidis* of Gram-positive , 10.67±1.15, 8.67±0.58, and 12.77±0.68 *E. coli, P. aeruginosa* and *K.pneumoniae* of Gram-nigetive bacteria, respectively (Table 11). The mean values of flavonoids content of methanol extract of *O. integrifolia* was 42 mg g^{-1}(Chekol & Desta,2018) while that in our study petroleum ether, chloroform: methanol (1:1) and methanol extracts was 92 mg g^{-1}, 90 mg g^{-1}, and 92 mg g^{-1} , respectively with more flavonoids at a concentration of 100 mg mL^{-1}.

The petroleum ether, chloroform: methanol (1:1) and methanol extracts from the leaves of *O. integrifolia* show differences in the inhibition of growth of bacterial and fungi strains. The absolute values of the diameter zones of inhibition (DZI) varied from 10 to 15.97±0.06 mm, 6 to 18.17±0.29, and 9 to 16.97±0.06, respectively at concentration of 100 µg mL^{-1}. However, some references evaluated the antimicrobial activities of three extracts of *O. persica* (hexane, followed by chloroform and methanol) against Gram positive and Gram negative strains using well plate(Anwar *et al*.,2009) .On the other hand, it was found that the highest inhibition was obtained with the ethyl acetate extract against *Candida albicans* (Asghari *et al*.,2006). Conversely, in this study we found that the highest inhibition in petroleum ether extract against *Candida albicans* (18.17±0.29 mm) and also (Amir *et al*.,2014) showed that methanolic extract of *O. persica* is effective against specially Gram positive bacteria (*S. aureus* and *B. subtilis*). However, in this study found that the highest inhibition in Petroleum ether extract against Gram negative bacteria (*P.aeruginosa* and *K.pneumoniae*) when compare with positive controls at concentration of 100 µg mL^{-1} (Table 11).

Studies have reported the antimicrobial activities of *Salvia rosmarinus* methanol extract showed (Dagnaw *et al*.,2023) that with good antibacterial activity and recorded at median concentration (65 µg mL^{-1}) a maximum and minimum zone with antibacterial activity against Gram negative bacteria *E. coli* 14 $\pm$ 0.71 mm and most of the petroleum ether test showed null zone of inhibition. However, in our study at a concentration of 100 µg mL^{-1}, the methanol extract demonstrated both maximum and minimum antibacterial zones against *E. coli* 11.47±0.50. According to Sepehri *et al.*,(2016), the ethanolic leaf extract of *Salvia rosmarinus* did exhibit activity against *C. albicans* strains. Conversely, in our study, we evaluated the antifungal activity of petroleum ether extracts from *Salvia rosmarinus* against two human pathogenic fungi, namely *C. albicans* and *A.niger*. Our findings showed that at a concentration of 100 µg mL^{-1}, the extracts were able to inhibit the growth of *C. albicans* 20.83±0.76 mm resulting in minimum zone of inhibition (Table 12).

Antimicrobial agents can be divided into groups based on the metabolism of the microbes themselves and drug efflux of the mechanism of antimicrobial activity (Reygaert, 2018). Thus, it

defined that the structure of the cell walls of microbes when exposed to an antimicrobial agent, there happen two main scenarios may occur regarding resistance and persistence. In the first scenario, resistant cells survive after non-resistant ones are killed. When these resistant cells regrow, the culture consists entirely of resistant bacteria. In the second scenario, dormant persistent cells survive. While the non-persistent cells are killed, the persistent cells remain.When regrown, any active cells from this group will still be susceptible to the antimicrobial agent. Our findings showed similarly acted in chloroform: methanol (1:1) and methanol extracts of leaves of *Salvia rosmarinus* that Gram-negative bacteria like *Pseudomonas aeruginosa;* and *Klebsiella pneumoniae.* However, *Staphylococcus epidermidis* of gram-positive bacteria under chloroform/methanol (1:1) extracts have similarly shown antimicrobial résistance (Table 12). This occurred due to intrinsic resistance that may make use of limiting uptake, drug inactivation, and drug efflux that need further study.

Alkaloids are found mostly in fungi and are known for their strong antimicrobial properties, which make them valuable in traditional medicine (Stashenko and Martinez, 2018 ;Apollinaire *et al*.,2016; Kuber *et al*.,2013). However, in our study, *S. rosmarinus* species have been possessed alkaloids (Table 6). Flavonoids are class of phenolic compounds commonly found in fruits and vegetables (Demie *et al*.,2018). Similarly, the results of this study revealed that *S. rosmarinus* contain flavonoids (74%-94%)(Table 12). Therefore, the extracts, using various solvents of different polarity indexes, have been attributed to specific biological activities. For example, the antimicrobial activities of *Salvia rosmarinus* extracts may be due to the presence of alkaloids, terpenoids, flavonoids tannins, and saponins in natural products (Table 6)

In-vitro **Anticancer activities:** According to previous literature reports, phenolic compounds such as flavonoids have long been reported in plants (Altundag,2020; Malvezzi *et al*.,2020) as chemo-preventive agents in cancer treatment (Sul'ain *et al*.,2019; Lifiani *et al*.,2018) .Stigmasterol, a component of *O. integrifolia*, may have cancer-preventing properties (Mosman,1983), especially for ovarian, prostate, breast, and colon cancers. Similarly, in our study, the three plants naturally constituted high concentration of terpenoids (Table 6). *In vivo* studies conducted on laboratory animals that were fed with stigmasterol showed that the absorption of both cholesterol and sitosterol led to a decrease in cancer incidence by 23% and

30%, respectively (Mosman,1983).The study, *O. integrifolia* was used for a dual purpose as synergy promotes better associating and enhancing cohesion with leukocyte proteinase, and solves the problems raised by proteinases involved in inflammatory mediator reactions. Congruently, we found that chloroform/methanol (1:1) extracts of *O. integrifolia* resulted in significant increases in cytotoxicity levels on *HeLa* cell viability. On the other hand, antiproliferation presented by IC_{50} values is a measure of the concentration of a drug or compound required to inhibit a particular biological or biochemical process by 50%. An extract is defined to be highly active if it has $IC_{50} < 10$ µg ml^{-1} (Chengo and Kiboi ,2016). Similarly, the current study high scores were shown for overall dose concentrations particularly in the case of *Otostegia integrifolia* at a concentration of 250 to 1250µg mL^{-1} viability after 24 h (Figure 24). As scientists gain more knowledge about the genetic changes that lead to cancer (Zarrinmehr *et al*.,2021), they are developing new drugs. So, our present study could be supported by using three plants under study .However, in the literature, the IC_{50} value of tamoxifen in Mouse fibroblast cell lines or the L929 cell lines (mouse control *in vitro*) was more well-known than Cisplatin in our study the IC_{50} value on women's cervical cell lines or the *HeLa* cell lines (Human control *in vitro*) with 55.54 ± 0.00µg mL^{-1} (Atangwho *et al*.,2013).

In order to determination of the cell death caused by these damages, there is a need to use for reliable, and reproducible short-term proliferation assays (that is cytotoxicity cell death or cell viability assays) that can easily understood in anticancer activities (López-García,2014). So as to anti-cancer, drugs have different cytotoxicity mechanisms such as the destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors. Proliferation assays are based on various cell functions (Karunamoorthi & Tsehay,2012 ; Vichai & Kirtikara, 2006 ; Rubinstein *et al*.,1990) . In our study used colorimetric assay and evaluated the inhibition of cancer through an increasing concentration-dependent manner. We investigated the cytotoxicity of petroleum ether and chloroform (1:1) crude extracts of *Vernonia amygdalina* leaves against human cervical cancer cell lines (*HeLa*) at various concentrations (250, 500, 750, 1000, and 1250 µg mL^{-1}), as shown in Appendix 5.1, and Figure 20, respectively. Similarly, we evaluated the anti-proliferative or anti-cancer activity of the other two plants (on the studied *HeLa* cell line. Before conducting the cytotoxic activity of the three plants on cancer cells, the effect of vehicle control (0.2%DMSO) in the completed medium on cell viability of *HeLa c*ell

lines was performed to ensure that the results of cytotoxicity of the three plants were not interfered with by the effect of vehicle control. The results illustrated that the vehicle control was not detrimental to the growth of cancer cells and could be utilized safely for dissolving crude extract of the three plants. Similarily, scholars remarcked that cell proliferation is generally measured by estimating the increase in the number of cells (cell count) over time starting with a specific number of parental cells (Chengo JK1 and Kiboi DM,2016).

The effect of the plants extracts on proliferation cells (viability cells or cytotoxicity cells) and greater than normal cell divisions are utilized to screen the response of cells to a chemical agent or drug. A substance that causes cell damage or death is considered cytotoxic, and the threshold for cytotoxicity/cell death varies from 20% to 50% across different literature *(Sul'ain et al*.,2019). Sometimes estimating the number of dead cells may be more sensitive than measuring viable cell (health cells) numbers using a marker. In this study, most the samples (Figures 20, 21, 22) can be explained based on ISO 10993-5. Percentages of cell viability greater than 80% are considered non-cytotoxicity, weak cytotoxicity: 80% – 60%; moderate cytotoxicity 60% – 40%; and strong cytotoxicity below 40% (Zarrinmehr *et al*.,2021). In our study, the three plants naturally constituted high concentration of terpenoids (Table 6). We found that chloroform/methanol (1:1) extracts of *O. integrifolia* resulted in significant increases in cytotoxicity levels on *HeLa* cell viability. The results were particularly good at concentrations of 250, 500, 750, 1000, and 1250 µg mL^{-1}, with recorded percentages of 56.55%, 65.76%, 82.49%, 88.85%, and 91.02%, respectively. These results were even better than those of the positive control of Cisplatin (55.54%). The reason behind of this may be shown no specific antidote to use in cisplatin toxicity. It means that either no preventing the absorption of the toxin or no inhibition of conversion of the toxin to more toxic metabolites.

On the other side, antiproliferation presented by IC_{50} (half-maximal inhibitory concentration) values is a measure of the concentration of a drug or compound required to inhibit a particular biological or biochemical process by 50%. Scholars reported an extract is defined to be highly active if it has IC_{50} < 10 µg ml^{-1}, active when the IC_{50} is between 10 µg ml^{-1} and 100 µg ml^{-1}, moderately active if the IC_{50} is between 100 µg $ml^{-1}$and 500 µg $ml^{-1}$and low activity if the IC_{50} is > 500 µg ml^{-1} (Chengo and Kiboi,2016). However, the current study high scores were shown

for overall dose concentrations particularly in chloroform/methanol (1:1) extracted the case of *Otostegia integrifolia* at a concentration of 250 to 1250µg mL^{-1} viability after 24 h (Figure 21 and 23).

In-vitro **radical scavenging activities:** In particular, *V. amygdalina*, *O. integrifolia*, and *S. rosmarinus* possessed flavonoids in our study. Flavonoids have been reported to interfere with the activities of the enzymes involved in radical scavenger (ROS) generation, quenching of free radicals, chelating transition metals, and rendering them redox inactive in the Fenton reaction (Rastogi *et al*.,2018; Usunobun and Ngozi,2016; Andrade *et al*.,2016). For example, *S. rosmarinus* with related to other biological activities by dual work such as cytoprotective and anticancer, primarily due to its ability to neutralize reactive oxygen species (Dagnaw *et al*.,2023; Shimira *et al*.,2022; Awadh *et al*.,2017;Andrade *et al*.,2016).Likewise, in our study, results for *S. rosmarinus* in the concentration of 10 and 30µg mL^{-1} showed the concentration dependent. The free radical scavenging activity of petroleum ether extracts of *V. amygdalina* in our present study, scavenging 79.51%, 79.51%, 86.58%, 88.72%, and 96.03% of DPPH radicals at concentrations of 10, 15, 20, 25 and 30 µg ml^{-1}, respectively (Table 14,Figure 29). According to the previous report, petroleum ether extracts indicated that *V. amygdalina* exhibit antioxidant activity, scavenging 75.9%, 93.9%, 97.1%, and 99.3% of DPPH radicals at concentrations of 0.01, 0.02, 0.05, and 0.1 µg mL^{-1}, respectively (Ugbogu *et al*.,2021). A similar study on *Otostegia integrifolia* reported that extracts using 90% aqueous methanol had higher antioxidant properties compared to those extracted with petroleum ether (Abate & Yayinie, 2018). However, the free radical scavenging activity of methanol extracts of *S. rosmarinus* in our present study was stronger (89.20%) at 30 µg ml^{-1}. In our study, the petroleum ether extracts exhibited the highest antioxidant properties compared to the methanol extracts (Table 14). The scavenging effect of methanolic extracts from *S. rosmarinus* (syn. *R. officinalis*) leaves on DPPH radicals was 80.2% at a concentration of 150 µg mL^{-1}, compared to the standard (Malvezzi *et al*.,2020).The authors further indicated that methanol extracts of *S. rosmarinus* have a noticeable effect on scavenging free radicals. In short, the biological activities of the three plant extracts are attributed to their constituents, extracted using solvents with different polarity indices. For example, the free radical scavenging activities of *V. amygdalina* extracts may be due to the presence of alkaloids, tannins, and saponins in natural products. *O. integrifolia* extracts may be

related to the presence of flavonoids and tannins in natural products. *S. rosmarinus* extracts may be related to alkaloids, terpenoids, flavonoids, and tannins (Table 6). In particular, *V. amygdalina, O. integrifolia*, and *S. rosmarinus* have possessed flavonoids in our study (Table 6). Flavonoids have been reported to interfere with the activities of the enzymes involved in radical scavenger (ROS) generation.So, in our study, results for *S. rosmarinus* in the concentration of 10 and 30μg mL^{-1} showed the highest free radical scavenger activities of 65.05% and 93.49%, respectively. *S. rosmarinus* is very rich in phytochemicals and overcome free radical (Table 6).

In-vitro **Anti-inflammation activities:** Studies have shown that inflammation and oxidative stress are closely associated with cancer and diabetes. Additionally, scientific research supports the role of polyphenols and flavonoids in the prevention of diabetes mellitus and inflammatory processes, as they can influence protein structures and have dual roles in these conditions (Paun ,2020). Similarly, in the present study, the secondary metabolites (like alkaloids, terpenoids, tannins, and saponins) are found rich in the three plants (Table 6). These metabolities can fight that causes fatigue, damage of DNA, headache, memory loss or brain fog, and decreased eyesight (Teixeira and Fari,2021) .The inflammatory agent overproduction mediates an increase in vascular permeability and leukocyte recruitment, causing edema formation and hyperalgesia (Teixeira and Fari, 2021). Some of inflammatory agent molecules include interleukins (IL-1β, IL-6, and IL-18), tumor necrosis factor-alpha (TNF-α), vasodilators, arachidonic acid metabolites, nitric oxide (NO), among others. However, the investigation and the herbal remedy under our study are important in relieving inflammation that may be happening in the body.

During the proteinase inhibition assay, it is believed that the bioactive compounds in chloroform/methanol (1:1) extracts binds to the trypsin molecule, indicating that the inflammatory activity is to be decreased (Assiry *et al*.,2022). It is interesting to note in our study all the plant extracts investigated demonstrated a higher inhibitory activity against proteinase higher than the control aspirin (IC_{50} value of 4.59 μg mL^{-1}) (Table 15). Most mastitis is an inflammatory condition of the breast, which may or may not be accompanied by infection (Teixeira and Fari, 2021; Bitew *et al*.,2010; Ricciutelli, 2006).The mastitis results when white blood cells (leucocytes), are released into the mammary gland, usually in response to an invasion of the teat canal by bacteria(like *Streptococcus agalactiae* is the most common cause of mastitis) (Ahmed,2020; Shoukat *et al*.,2018). Inflammation of the affected mammary tissue is

characterized, for example, there are changes in the composition and appearance of milk.(Ahmed,2020).In our study, the fresh leaf extracts of the three plants show promise as effective preventative measures and treatment options for inflammatory bovine mastitis pain. These extracts may provide relief due to their composition (Table 15), which includes terpenoids, flavonoids, and tannins (Table 6) and again their extracts may enhance synergy by promoting better cohesion with leukocyte proteinases and addressing issues related to proteinases involved in inflammatory mediator reactions. Inflammation and oxidative stress (including reactive free radicals and antioxidant defenses) are closely associated with cancer and diabetes. However, the involved mechanisms are not fully established due to the dual role of oxidative stress as both a signaling molecule and a damaging agent, affecting transcriptional control and cell cycle regulation. For example, aspirin and caffeine act synergistically; when combined, they provide greater efficacy and pain relief in patients compared to when used individually.Here, the study demonstrated significant effectiveness in reducing free radicals in patients due to the rich secondary metabolites found in the three reported plants (Table 15). Similarly, in the present study, the secondary metabolites are found rich in the three plants (Table 6 and 15). They may be used to overwhelm inflammatory responses like allergies (like itchy, sneezing, and stomach cramps), microbial infections (like mastitis), and autoimmune diseases (like rheumatoid arthritis, multiple sclerosis, psoriasis, and systemic lupus erythematous). Nonetheless, the investigation and the herbal remedy under our study are important in relieving inflammation that may be happening in the body. When we take the bovine mastitis inflammation of the affected mammary gland tissue is characterized by gross abnormalities in the udder (swelling, heat, redness, pain). For example, there are changes in the composition and appearance of milk. Abnormalities in milk may include flakes, clots, or a watery appearance and casein protein decreased in that gives milk color. In our study, the three plants contained terpenoids, flavonoids, and tannins (Table 6) may enhance synergy by promoting better cohesion with leukocyte proteinases and addressing issues related to proteinases involved in inflammatory mediator reactions (Table 15) goes to relief inflammation(pain) of the patients.

In-vitro **Anti-protozoa:** Previous studies have shown that Acanthamoeba cysts survive for several years (Sanguan *et al*.,2018; Sriram *et al*.,2008), and attacks eye lens and poses keratitis (Jercic *et al*.,2019; Bunsuwansakul *et al*.,2019). The amoebicidal activity of curcumin

against *Acanthamoeba triaugularis* was recently discovered (Boonhok *et al*.,2022). Similarly, in our study, the amoebicidal activity of *S.rosmarinus* against *Acanthamoeba castellanii* trophozoites was shown to be highly prominent for its benefit to health (% viability < 10) (Table 16). Diamidines and biguanides are often the first-line therapy for *Acanthamoeba keratitis* (List *et al*.,2021). However, in our present study, the cysts were not completely inhibited by the extract (Table 17); it may be happened due to the resistance form of the cyst walls. In addition; the treatment of this disease requires several months due to the stage of the cyst. Therefore, medical therapeutic intervention tends to produce more side effects in the long-term (Heredero *et al*.,2016) . It was suggested that combination of currently available drugs and/or natural products could be used as an alternative. The anti-acanthamoeba synergistic effect of chlorhexidine and cationic carbosilane dendrimers against the trophozoite and cyst forms of *A. polyphaga* has been reported (Heredero *et al*.,2016) . Currently, the synthesis of nanoparticles using plant extracts has been reported as one of the ways to improve the efficacy of these compounds in destroying the cysts (Bunsuwansakul *et al*.,2019) . Inhibition of *A. castellanii* cysts by *Curcuma longa* (turmeric) ethanol extract has been reported (Elkadery *et al*.,2018)strategy to kill Acanthamoeba. In addition, the aqueous extract of *Nigella sativa* in combination with chitosan nanoparticles has shown interestingly synergistic effects against *Acanthamoeba keratitis* (Anwar *et al*.,2020) . To support this, additional studies will be needed on *S. rosmarinus* in the efficacy in the treatment of Acanthamoeba infection.

On the other hand, studies have shown that the anti-amoebic activities of chloroform, methanol and water extracts from 12 Thai medicinal plants (39 extracts) commonly used by AIDS patients in southern Thailand were screened, at a concentration of 1,000 µg mL^{-1}, against Entamoeba histolytica strain HTH-56:MUTM and strain HM1:IMSS growing *in vitro* (Sawangjaroen *et al*.,2006). Conversely, in our present study, we found that chloroform/methanol (1:1) extracts of MIC values of *S. rosmarinus* against *A. triangularis* and *A. castellanii* trophozoites were 22.22 and 7.89 µg mL^{-1}, respectively. While MICs of the extract against *A. triangularis* and *A. castellanii* cysts were 125 and 62 µg mL^{-1}, respectively. Researchers suggested that combination of currently available drugs and/or natural products could be used as an alternative. To support this, additional studies may need on *S. rosmarinus* in the efficacy in the treatment of *Acanthamoeba* infection.

In-vitro **Antivirus activities:** In literature, some studies have addressed the antiviral activities of crude plant extracts (Grienke *et al*.,2014). Similarly, in our study, the extracts of aqueous, chloroform: methanol (1:1), and petroleum ether of *V. amygdalina* leaves of the selectivity index showed high activity against influenza virus (Table 18).The consistent of acting through time it will need further study. Influenza viruses are classified under the family *Orthomyxoviridae* and divided into three types: A, B, and C. Amongst the types, A and B are the predominant causes of human infections (Thi Nguyena *et al*.,2018). However, of these, ordered by the number H1N1, H2N2, and H3N2 of known deaths in years 1918, 1957 and 1968 , respectively that associated with pandemics and epidemics in human populations. The subtypes that have been confirmed in seasonal influenza viruses around the globe. Nonetheless, influenza viruses are becoming resistant to drugs due to the modification of the cell structure of the virus. Moreover, it has been confirmed that medicinal plants can be used against influenza. Similarly, this study focused on testing different extracts against influenza A viruse *in vitro*. The specimens that demonstrated a selectivity index (SI) of 10 or higher were considered active and prospective for further development (Indrayanto, 2022). However, it was to emphasize that for a drug to be ideally selective, the selectivity index, that is, the ratio between its cytotoxicity and its antiviral activity must be ≥ 10. If the SI is low (<1), the drug is toxic, and it cannot be utilized as a safe drug. Similarily, in the study the extracts of *V. amygdalina* leaves were shown to have good active SI as candidate drugs ≥ 10(Table 18).Importantly, the virus used in the study, influenza A/Puerto Rico/8/34 (H1N1), was resistant to rimantadine (Table 18), well-known inhibitor of viral proton pump M2. This was demonstrated in high value of IC_{50} and low SI (IC_{50}=63µM, SI=6). All three extracts of *V. amygdalina* appeared much more active against the virus. Indeed, they possessed SI's two- to six-fold higher than rimantadine. This suggests that the constituent(s) of these extracts is (are) directed against alternative target and has another mechanism of activity than reference compound.

For the reason that of viral infections affect three to five million patients annually*(Zakay-Rones et al.*,1995). While commonly used antivirals often show limited efficacy and serious adverse effects, herbal extracts have been in use for medicinal purposes since ancient times and are known for their antiviral properties and more tolerable side effects. Thus, naturally based pharmacotherapy may be a proper alternative for treating viral diseases. With that in mind,

considering the efficacy of the extract *in vitro* on all strains of influenza virus tested, the clinical results, its low cost, and absence of side-effects, this preparation could offer a possibility for safe treatment for influenza A and B (Zakay-Rones *et al.*,1995). Similarly, in our present study, we found *in vitro* antivirus activities of the extracts of aqueous, chloroform: methanol (1:1), and petroleum ether of *V. amygdalina* leaves extract against influenza A of the preparation that could offer a possibility for safe, absence of side effect, and low cost and edible ones.

Essential oils (EOs) component: In present study, *Vernonia amygdalina* contained dihydro-2H-pyran as the main component. However, this finding differs from privious reports on *Vernonia amygdalina* species essential oils (EOs), indicated sesquiterpenes (Sobrinho *et al.*,2020) and monoterpenoids (CLSI, 2015) as major compounds. Oppositely, it widely occurs in the plant kingdom and differently used as intermediate compounds (Kumar *et al.*,2017;Eskandari and Rafieian,2016;Rocha *et al.*,2011; Goel and Ram, 2009) and possesses diverse pharmacological activities like antioxidant, antitumor, antibiotic, antibacterial, antifungal, anti-allergic, hypo-lipidemic, immunomodulatory activities, and anti-cancer (Cimanga *et al.*,2002).In the present study (Table 20), the principal constituent of *Otostegia integrifolia*'s essential oil was found to be 3-hexenol (19.63%), consistent with other reports that also indicate the presence of another alcohol, 3-octenol (Tadesse *et al.*,2012) as a major constituent from the same species. In the current study (Table 21), the essential oil of *Salvia rosmarinus* had camphene (23.75%) as the main component. Similarly, a previous study by (Bozin *et al.*,2007) also reported monoterpenes as the primary constituents of this plant's essential oil. Some studies have addressed variations in the chemical composition of essential oils within the same species due to intrinsic features and extrinsic factors (Coimbra *et al.*,2023; Rahman ,2007). Our investigation also revealed significant differences in the chemical composition of essential oils within the same species compared to previous studies. The previous reports revealed that using odorous substances for medicinal reasons is as old as mankind (Reshna *et al.*,2023). Our present study demonstrated that the antimicrobial properties of essential oils got from hydero-distillation increased in a concentration-dependent manner. In this study, the overall results of the three plants' EOs class of compounds are comparable to those previously reported (Stashenko and Martinez,2018); namely: hydrocarbon terpenes/ terpenoids (monoterpenes $C_{10}H_{16}$, and sesquiterpenes $C_{15}H_{24}$); oxygenated molecules their derivatives (alcohols, ethers, aldehydes, esters, ketones, and acids); and heterocyclic compounds. On the other hand,

this finding differs from previous reports on *Vernonia amygdalina* species essential oils (EOs), which indicated sesquiterpenes. Climatic, and geographic conditions, environmental factors, vegetative cycle stage, and type of extraction are among the reasons which might be responsible for such differences (Stashenko and Martinez,2018).

***In-vitro* Antimicrobial activities of essential oils (EOs):** People have always been interested in EOs derived from herbs and spices because these oils have shown varying degrees of efficacy against different microbial strains with varying concentrations (Tadesse *et al*.,2012). Similarly, our present study showed that the anti-microbial properties of the essential oil increased in a concentration-dependent manner (Table 22). Previous studies indicated that EOs exhibit higher activity against Gram-positive bacteria than Gram-negative bacteria (Dhifi *et al*.,2016). This study does support the aforementioned findings that the essential oils of *Vernonia amygdalina* and *Otostegia integrifolia* were effective against *E.coli* and *P.aeruginosa* of bacteria. The previous study showed that the minimum zone of inhibition (MZI) of *Otostegia integrifolia* oil ranged from 5 to 100 µg ml^{-1} and 50 to 100 µg ml^{-1} against tested bacterial and fungal strains, respectively (Tadesse *et a*l.,2012). The oil (MZI value of 5 µg ml^{-1}) was found to be more potent than ciprofloxacin (MZI value of 10 µg ml^{-1}) against some *E. coli* strains. However, our recent study on Otostegia *integrifolia* showed no activity (MZI value of 125 to 1000 µg ml^{-1}) against *E. coli* strains (Table 22). On other hand, the antibiotic reference products (Ciprofloxacin - positive test) indicated a high zone of inhibition (Appedix-7.4A). Similarily,this study contrary previously reported (Lu et al.,2018).

Then again, the rosemary essential oils showed no significant effect on *P. aeruginosa* (Coimbra *et al*.,2023; Cimanga *et al*.,2002) and gave reasons that bacteria depending on the structure of the bacteria's outer membrane that allows hydrophobic compounds to diffuse through (Wheeler and Ratledge,1988) and the chemical composition, volatile molecules, and interactions also play a role in their antibacterial activity (Mmbengwa *et al*.,2009). Similarly, in our study, *Pseudomonas aeruginosa* was the most resistant to the essential oils from the three plants.

According to some scholars (Stashenko and Martinez,2018), the genus *Salvia* essential oil on the Gram-negative bacteria *E. coli* (MZI value of 62.50µg mL^{-1}) was not significant and four times weaker activity compared to the positive controls (MZI value of 3.90 µg mL^{-1}), but in our

research *Salvia rosmarinus* EOs at 1000 μg mL^{-1} concentration exhibited good inhibitory effect against *E. coli* strains (Table 22). *Escherichia coli* is a saprotrophic bacterial in the anthropoid intestine (Babushok *et al*.,2011) and in cases of *Hemorrhagic Cystitis*, around 80-90% of infections are resistant to *E. coli* (Dhifi *et al*.,2016) .The inhibition radiance diameter the essential oil of the *Salvia* genus has been identified to be between 13-16 mm, whereas our research has shown it to be between 8-13 mm for its inhibiting effect on *E. coli* (Table 22).

Otostegia integrifolia EO exhibited antifungal activity at concentrations of 50 to 100 μg mL^{-1}, which was comparable or superior to Griseofulvin against most of the fungal pathogens tested (Tadesse *et al*.,2012). In the present study, the antifungal activities of extracts from *Vernonia amygdalina*, *Otostegia integrifolia*, and *Salvia rosmarinus* were comparable to Gentamycin in synergy with Azole. *Candida albicans* strains showed enhanced inhibitory effects when exposed to higher oil concentrations of these three plants, ranging from 125 to 1000 μg mL^{-1} (Table 22). In general, three examined essential oils exhibited notable fungicidal activity. The essential oil of *Salvia rosmarinus* exhibited significant antifungal activity; however, in several cases, it showed a higher mean zone of inhibition (MZI), particularly against *Candida albicans* (Bozin *et al*.,2007) . In our study, we observed relatively similar results.The previous study showed that the increasing development of resistance of *Candida* species is a great challenge to the therapeutic and handling of skin contagions (De Oliveira,2019; Mendoza *et al*.,2017) and again it realized that causing severe opportunistic infections by *Candida albicans* in recent times is immune-compromised and long suffering, especially those pretentious by cancer, diabetes, and AIDS. The outcomes of obtainable EOs may have boosted progression in overcoming severe opportunistic infections by *Candida* species.

***In-vitro* radical scavenging activities of essential oils (EOs):** Some previous studies have addressed the variations in the radical scavenging activities of the essential oils explored between 0.50(50%) and 0.99(99%) are satisfactory (HDT, 2023).Similarly, in our investigation, it was found relatively higher ranging from 0.82-0.97 (82-97%). The exploring EOs of plants could result in bioactive activities of anti-oxidant activity was assigned as “strong” (testers that formed an intense with strong antioxidant properties) that produced an intense bright yellow zone, 'medium' for those forming a clear yellow spot, 'weak' for samples showing a faint yellow, and 'not active' for those with no visible yellow zone (Anwar *et al.*,2009). Thus, in the present

study, *S. rosmarinus* shows strong radical scavenging activities in all concentrations tested in the sample followed by *V. amygdalina* (medium) and *O. integrifolia* (weak). Previous reports indicated the EOs of various aromatic medicinal plants demonstrated interesting radical scavenging activities (Reshna *et al*.,2023;Hamidpour, 2017) and antimicrobial properties (Semeniuc *et al*.,2017;Tadesse *et al*.,2012). Similarly, in the present study, *S. rosmarinus* shows strong radical scavenging activities in all concentrations tested in the sample followed by *V. amygdalina* (medium) and *O. integrifolia* (weak). According to Erasto *et al*., (2008) reported that *Vernonia amygdalina* leaves exhibit appreciable radical scavenging antioxidant activities, unlike our findings for *Vernonia amygdalina* essential oils (Figure 24).

CHAPTER 6

6 CONCLUSIONS AND RECOMMENDATIONS

This study provides valuable insights into *Vernonia amygdalina* L, *Otostegia integrifolia* Benth , and *Salvia rosmarinus* Spenn biological activities, demonstrating significant anticancer and antimicrobial properties *in vitro* supported by EOs extract and compounds isolated in their leaves. The petroleum ether, chloroform/methanol (1:1), and methanol extracts from the leaves of the plants were studied *in vitro* for their antioxidant and anti-inflammatory properties. The petroleum ether leaf extract of *V. amygdalina* resulted in the isolation and identification of linoleic acid (**1**). Similarly, the methanol: chloroform (1:1) leaf extract of *O. integrifolia* led to the isolation of Otostegindiol (**2**), Otostegindiol (**3**), and pretotostegindiol (**4).** The chloroform: methanol (1:1) *S.rosmarinus* leaves extracts lead to isolation micromeric acid (**5**) and benzocaine (4-Aminobenzoic acid-ethyl ester) (**6**). Case in point,the petroleum ether extracts of leaves of *O.integrifola* and *S. Rosmarinus* showed good against *C. albicans* at a concentration of 100 µg mL^{-1} and the same solvent extracts of the three plants plants under study demonstrated significant activity against the tested *P.aeruginosa, K.pneumoniae* and *S. aureus* by respectively at a concentration of 100 µg mL^{-1}. Additionally, the essential oils extracted via hydro distillation exhibited strong antimicrobial activity at a concentration of 1000 µg mL^{-1}. The chloroform: methanol (1:1) extracts of leaves of the three plants were showed good against *HeLa* cervical cancer cell lines at a concentration of 1250 µg mL^{-1}. Likewise, the petroleum ether and chloroform: methanol (1:1) extracts of the three plants showed good antioxidant and anti-inflammation activities compared to the positive controls Ascorbic Acid and Aspirin respectively at a concentration of 30 µg mL^{-1}. The extracts of Aqueous, chloroform: methanol (1:1), and petroleum ether of *V. amygdalina* leaves showed high activity against influenza virus A and found a selectivity index (>10) and notably, the amoebicidal activity of *S. rosmarinus* in solvent of chloroform: methanol (1:1) extracts against trophozoites was found to be highly effective, with a significant reduction in viability (7.89%). Furthermore, high binding affinity was scored by compound **5** and **6** (-8.4 and -5.3 kcal/mol respectively) towards DNMT1 enzyme (PDB ID: 4WXX), **5** and **6** (-10.1 & -6.5 kcal/mol respectively), towards HPV type 16 E6 (PDB ID:

4XR8) at once against cervical cancer. These findings highlight its potential as a source of novel therapeutic agents. However, the researcher recommended that further pharmacological testing; likewise, *in vivo* microbial studies need for extracts that exhibited good *in vitro* microbial activity and compounds **2** to **4** and **5** to **6** isolation from *O. integrifolia* and *S. rosmarinus* respectively studies are necessary to fully evaluate its clinical applicability in cancer and infectious disease treatments. Based on our study results, we recommend that the results not only enhance our understanding of the medicinal potential of the three studied plants. However, it offers promising directions for future research and therapeutic development. The comprehensive analysis of both antimicrobial and anticancer activities, coupled with detailed phytochemical profiling, positions this study as a significant contribution to the field, while also highlighting the need for continued investigation through *in vivo* activities with the mechanisms of action and potential therapeutic applications of *V. amygdalina*, *O. integrifolia*, and *S.rosmarinus* and related species. Furthermore, scientific research also supports traditional healers' claims about their antimicrobial, anticancer, and therapeutic properties.

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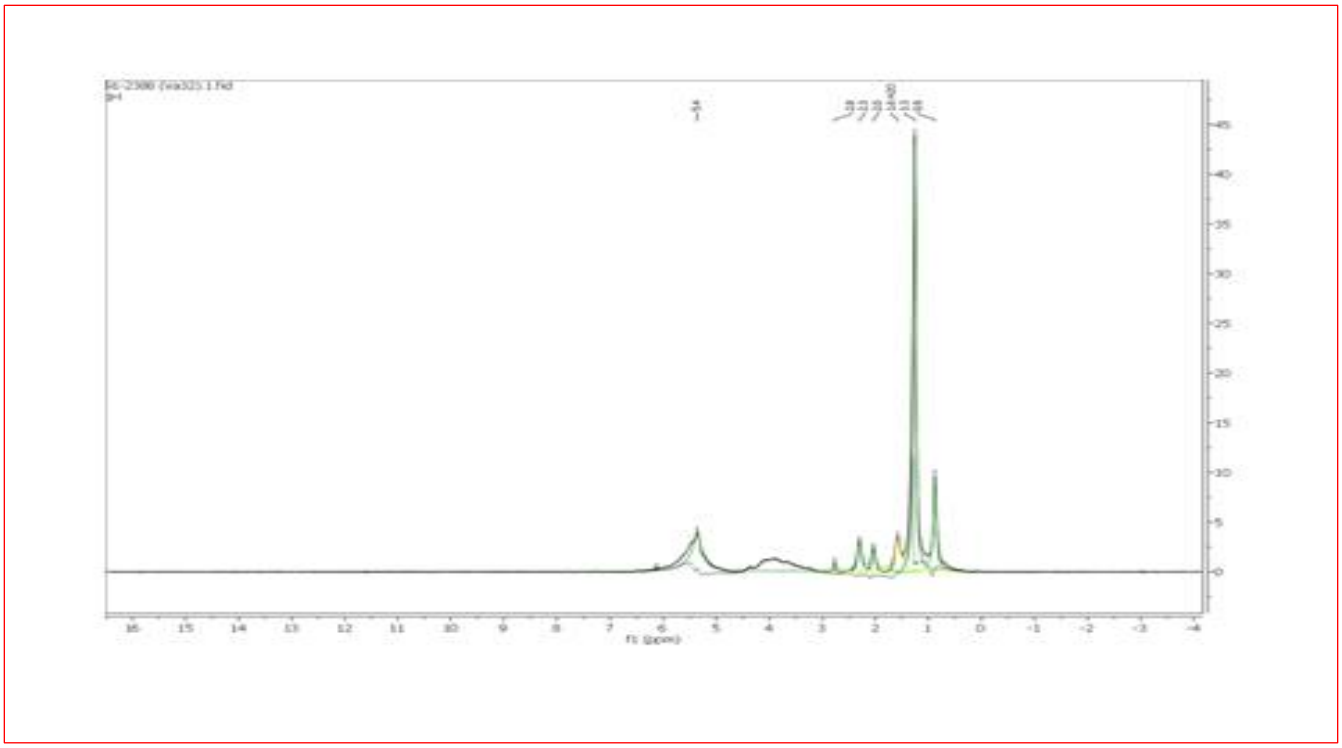
APPENDICES

APPENDIX 1: NMR spectra of compound 1

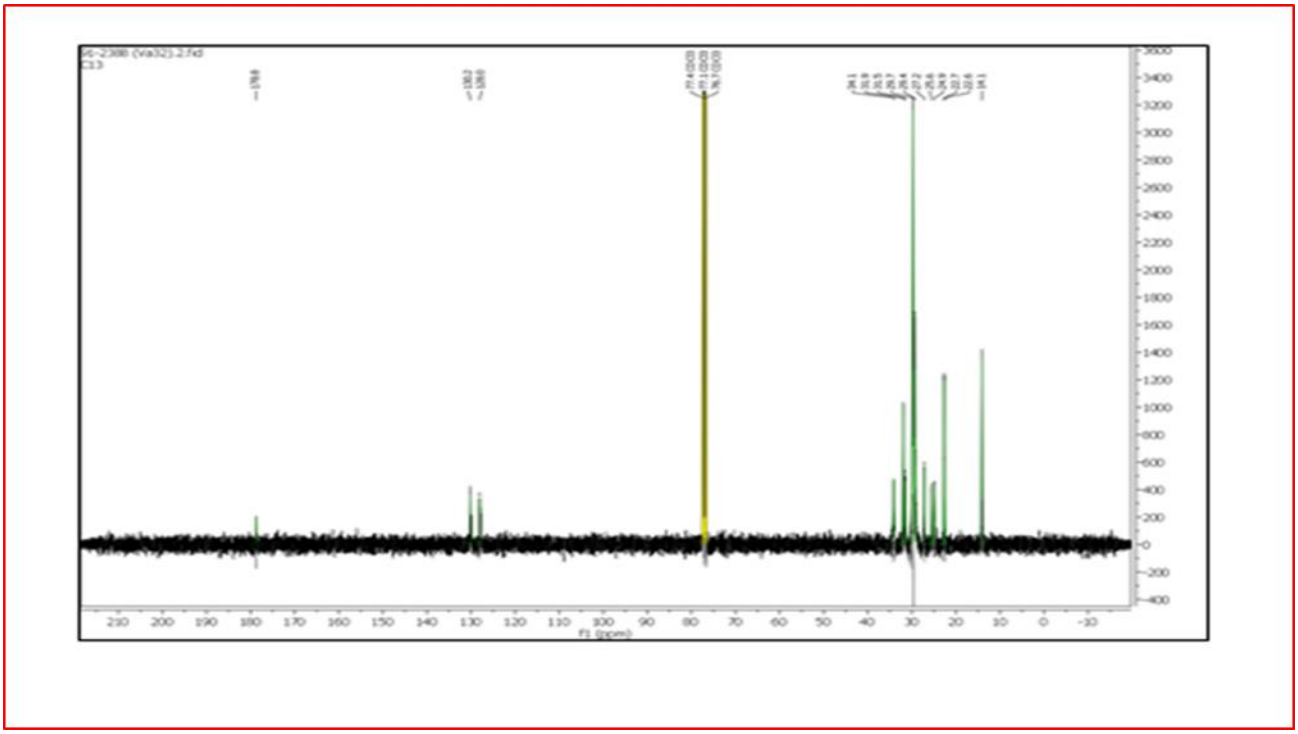
Supplementary Materials: Phytochemical screening by NMR spectroscopic analysis

The ^{1}H-NMR, ^{13}C-NMR and DEPT-135 NMR spectral data of compounds **1**

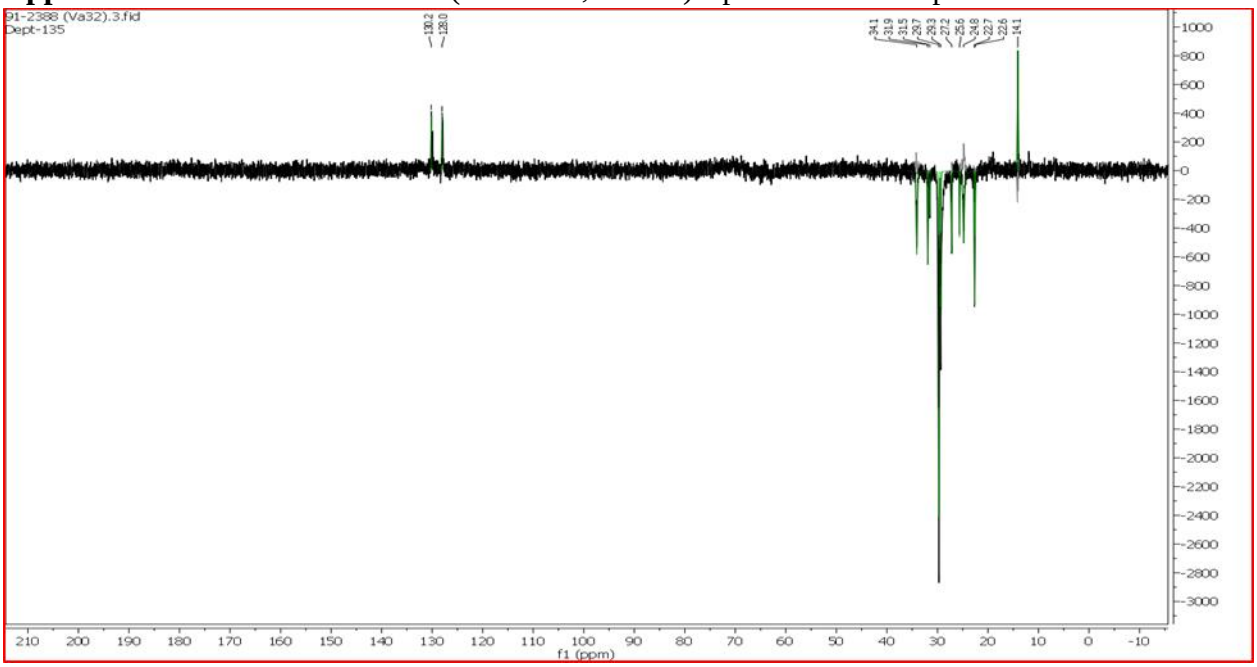
Appendix –1.1: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound **1**



Appendix – 1.2: ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of compound **1**



Appendix- 1.3: DEPT-135 NMR (101 MHz, $CDCl_3$) spectrum of compound **1**

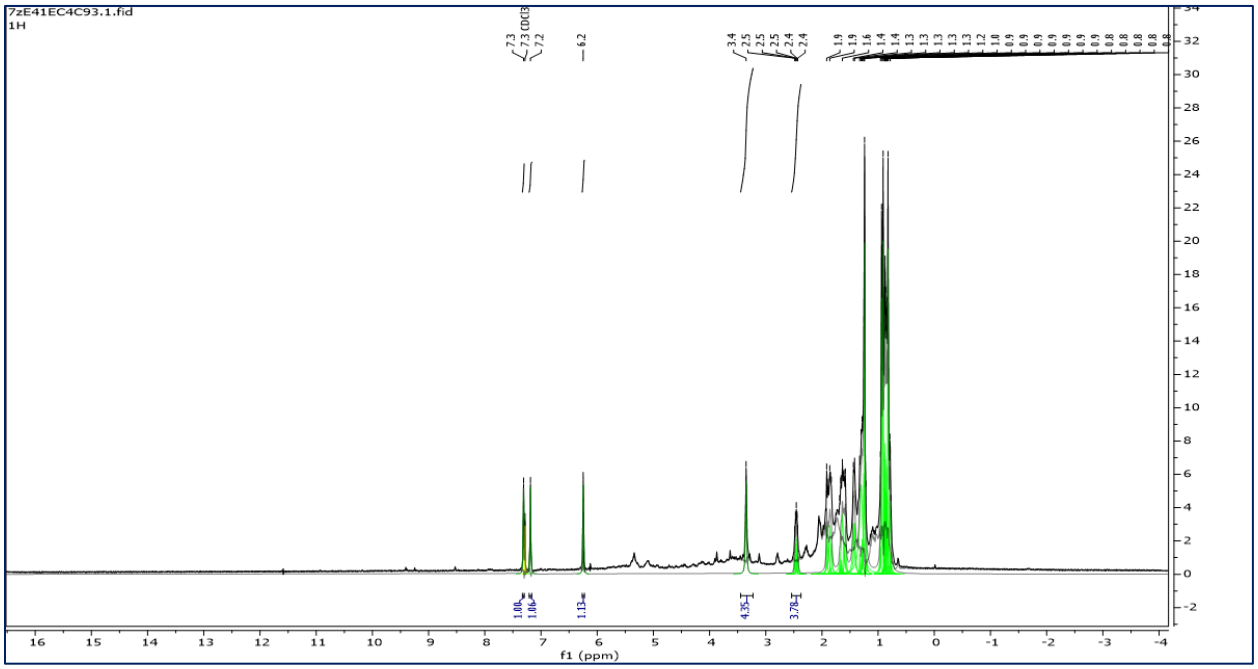


APPENDIX 2: NMR spectra of compounds 2-4

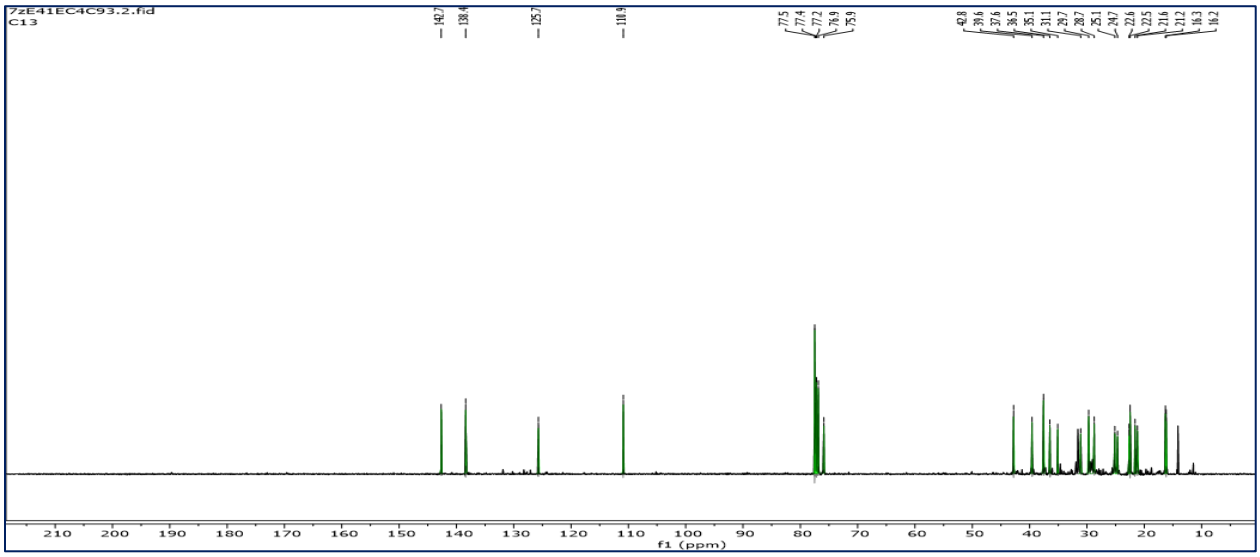
Supplementary Materials: Phytochemical screening by NMR spectroscopic analysis

The ^{1}H-NMR, ^{13}C-NMR and DEPT-135 NMR spectral data of compounds **2(**Oi-**10)**, **3(**Oi-**34)** and **4(**Oi-**100)**,

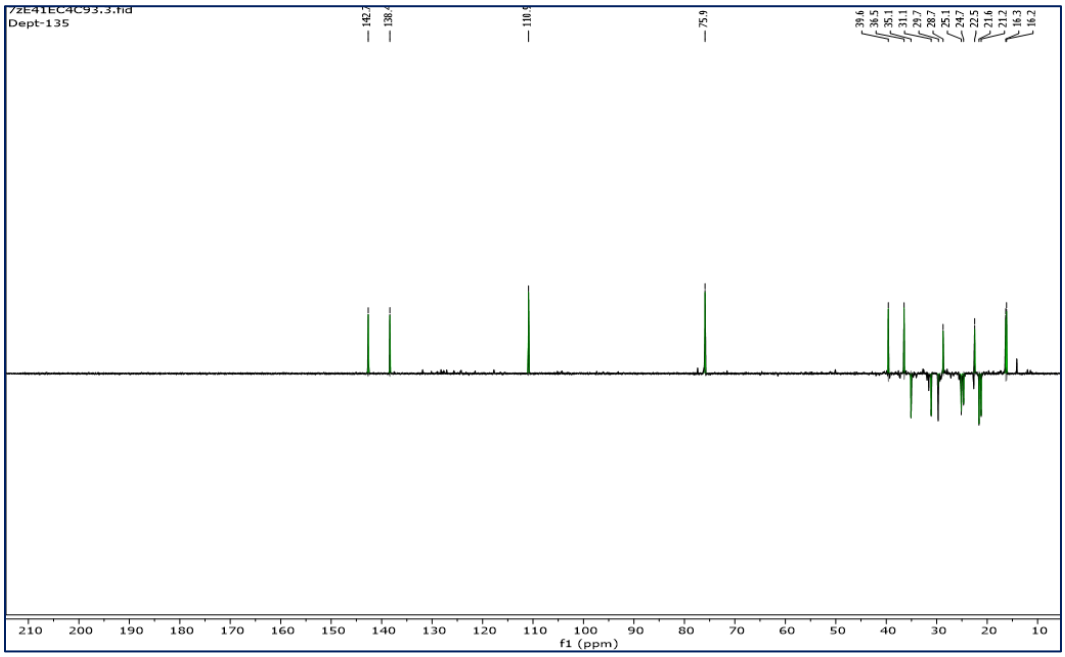
Appendix –2.1: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of **2**



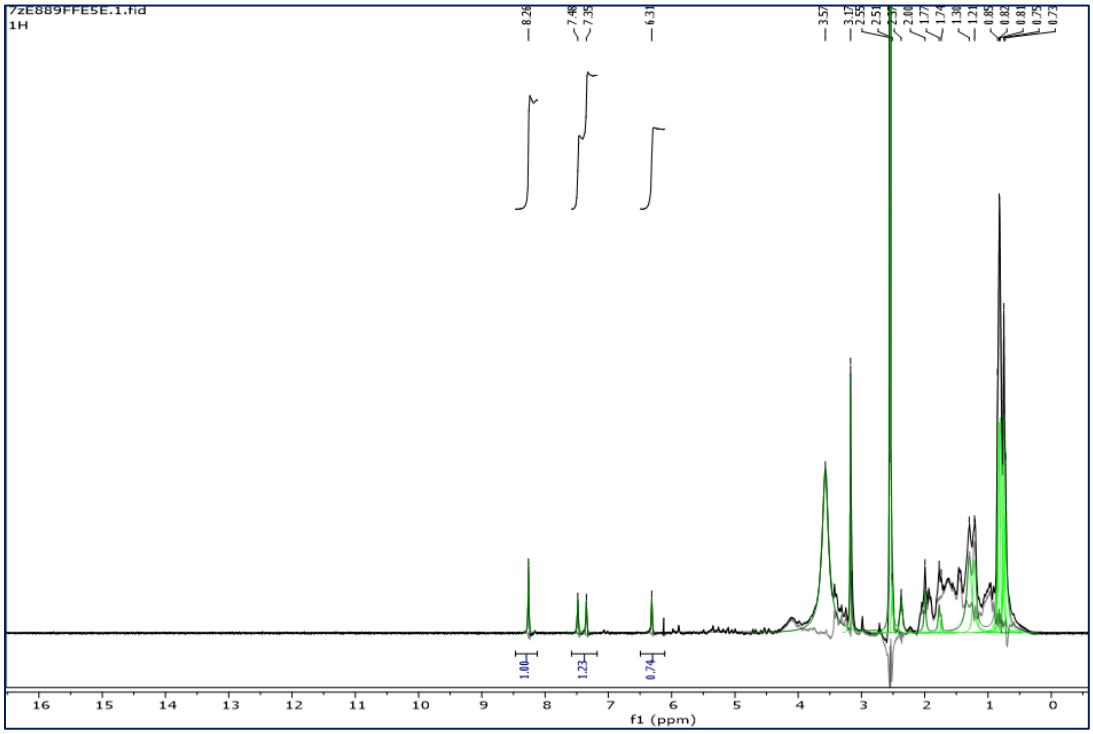
Appendix – 2.2: ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of **2**



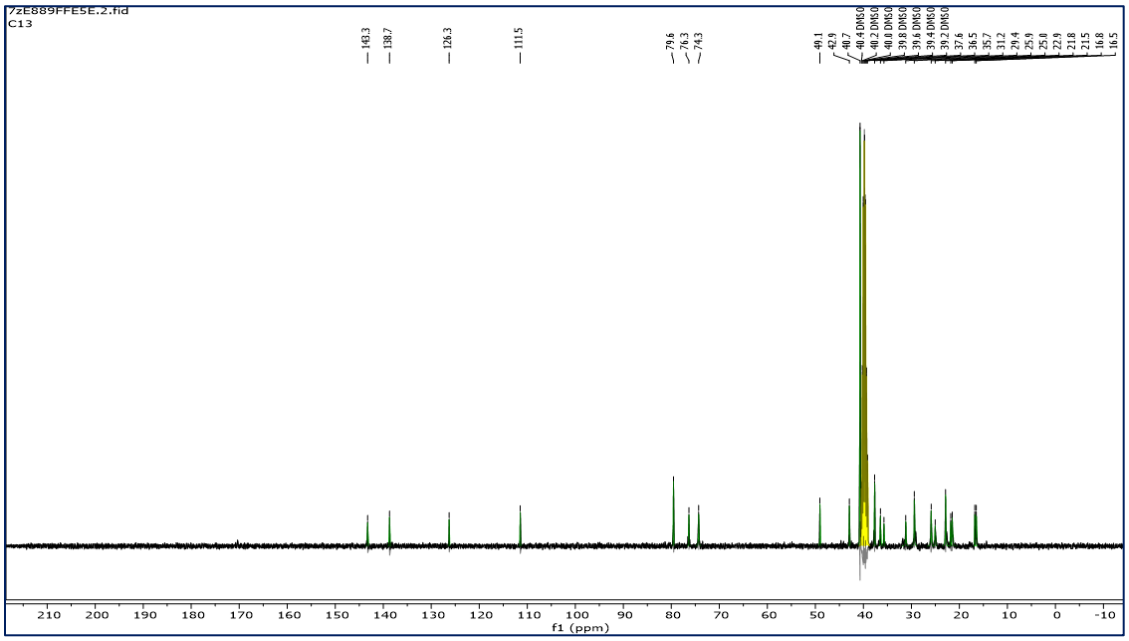
Appendix-2.3: DEPT-135 NMR (101 MHz, $CDCl_3$) spectrum of **2.**



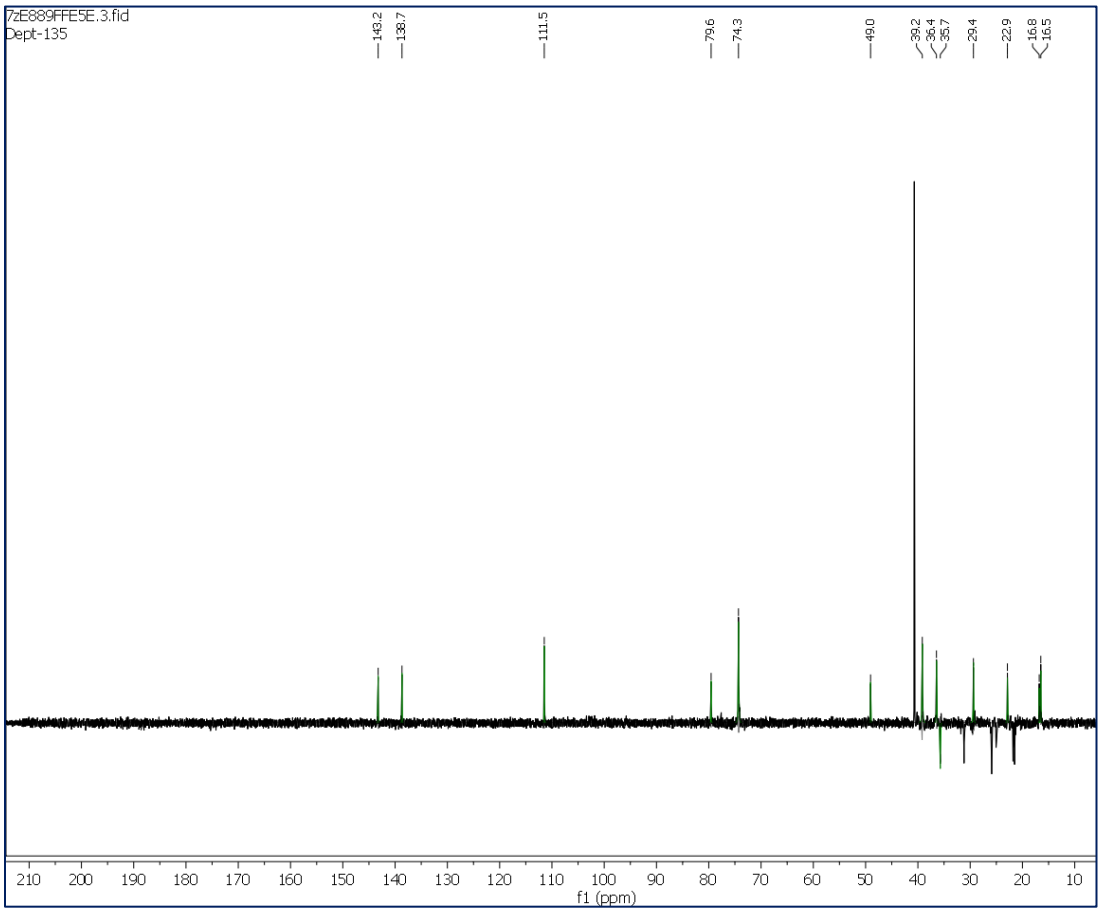
Appendix-2.4: ^1H NMR (400 MHz, DMSO) spectrum of **3**.



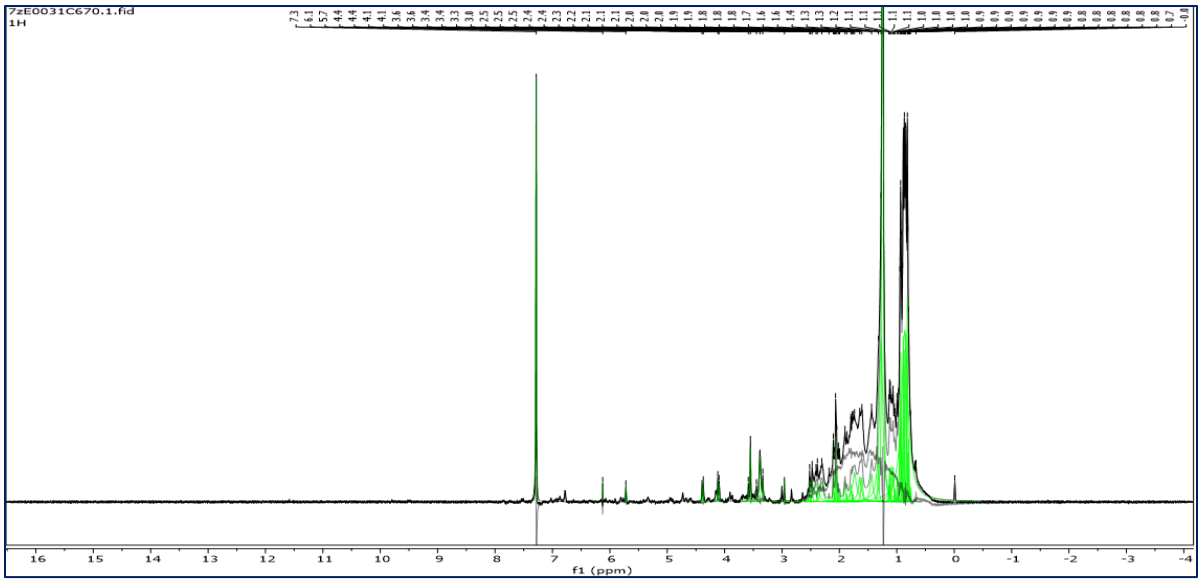
Appendix-2.5: ^{13}C NMR (101 MHz, DMSO) spectrum of **3.**



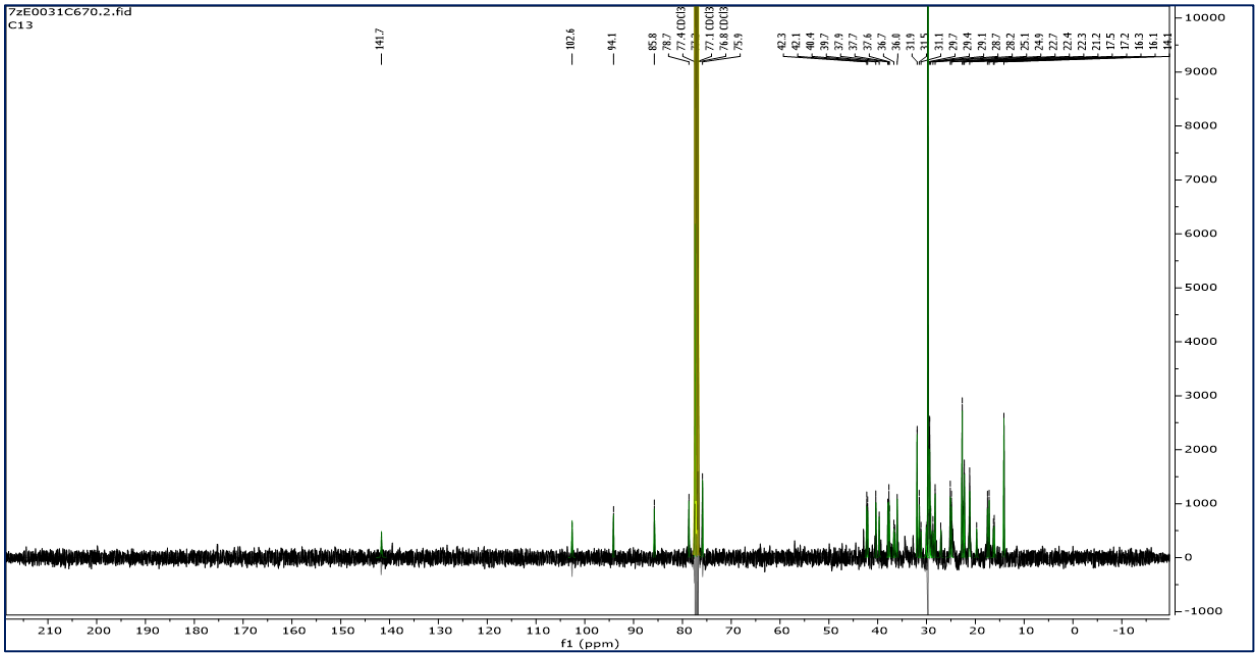
Appendix-2.6: DEPT-135 NMR (101 MHz, DMSO) spectrum of **3.**



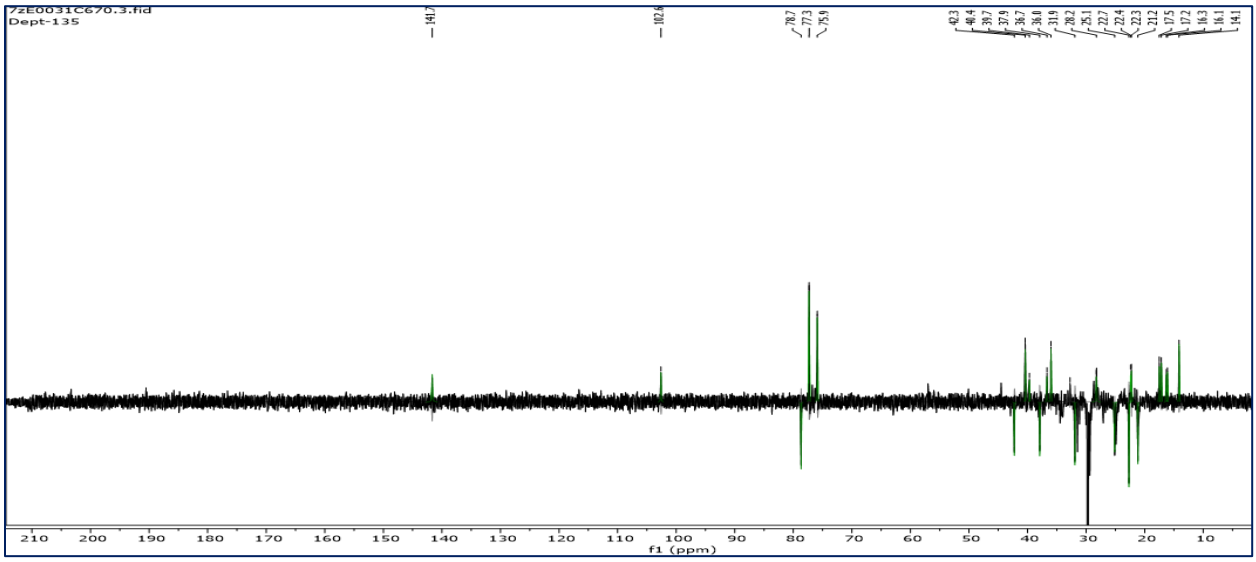
Appendix -2.7: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of **4**



Appendix -2.8: ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of **4.**



Appendix -2.9: DEPT-135 NMR (101 MHz, $CDCl_3$) spectrum of **4.**

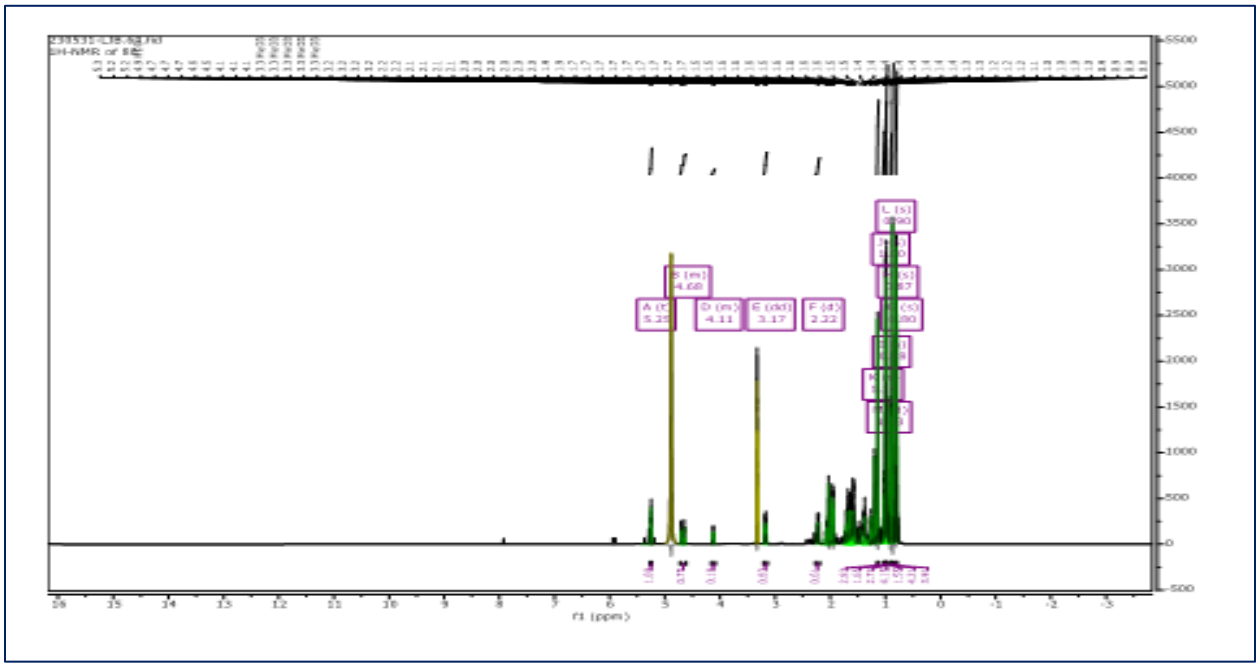


APPENDIX 3: NMR spectra of compounds 5 and 6

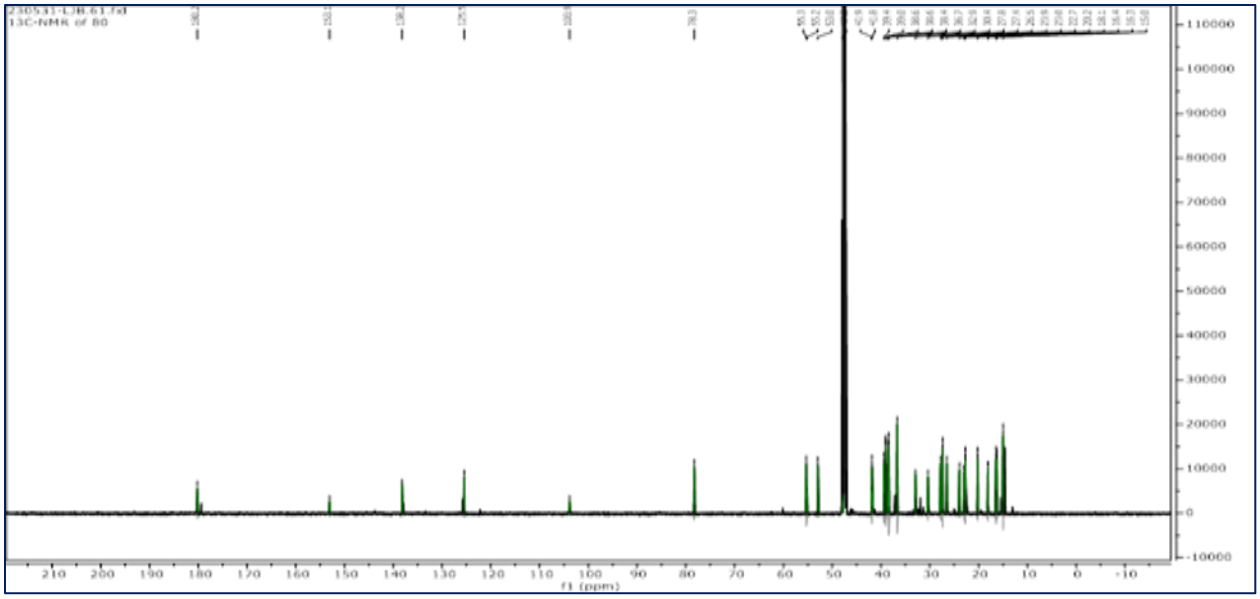
Supplementary Materials:

The ^{1}H-NMR, ^{13}C-NMR and DEPT-135 NMR spectral data of compounds **5(**Sr-**101)** and **6(**Sr-**102)**,

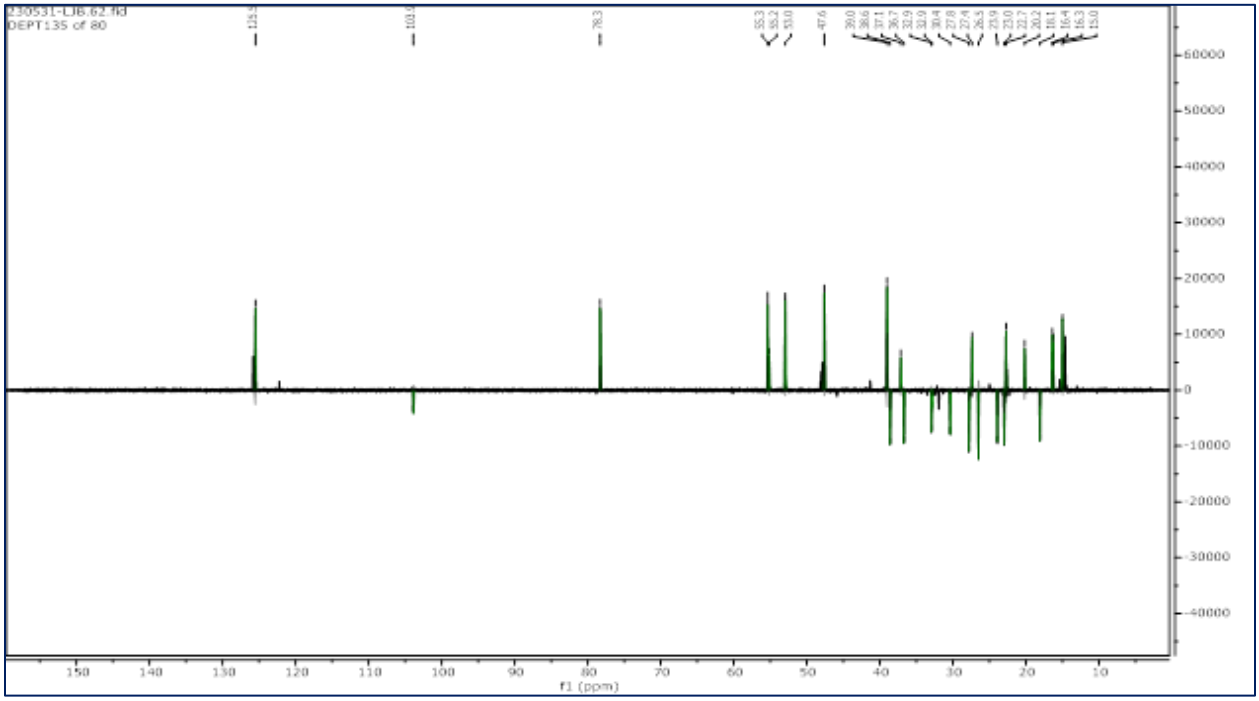
Appendix -3.1: ^{1}H NMR spectrum of compound 5.



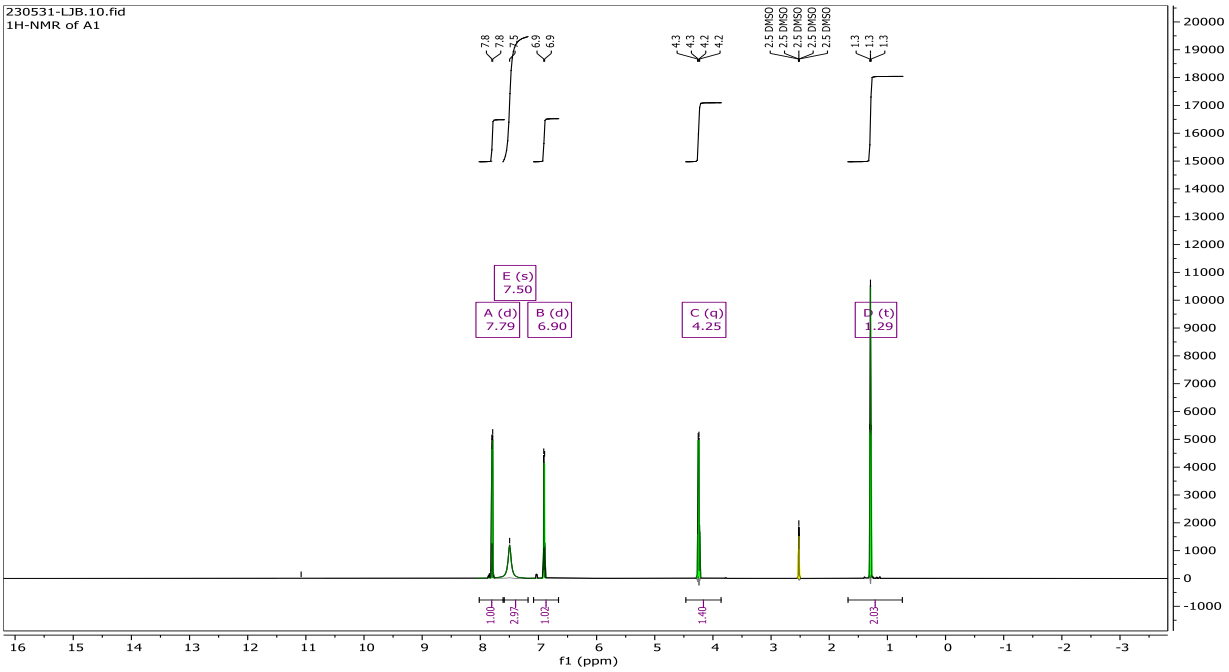
Appendix -3.2: ^{13}C NMR spectrum of compound 5.



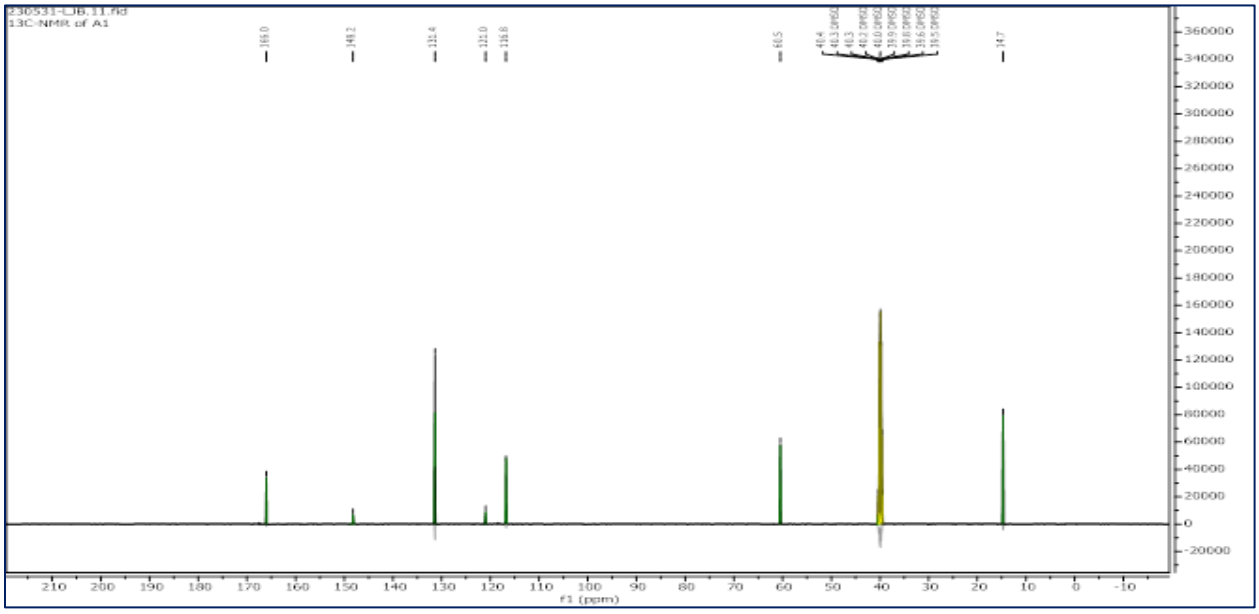
Appendix -3.3: DEPT-135 NMR spectrum of compound 5.



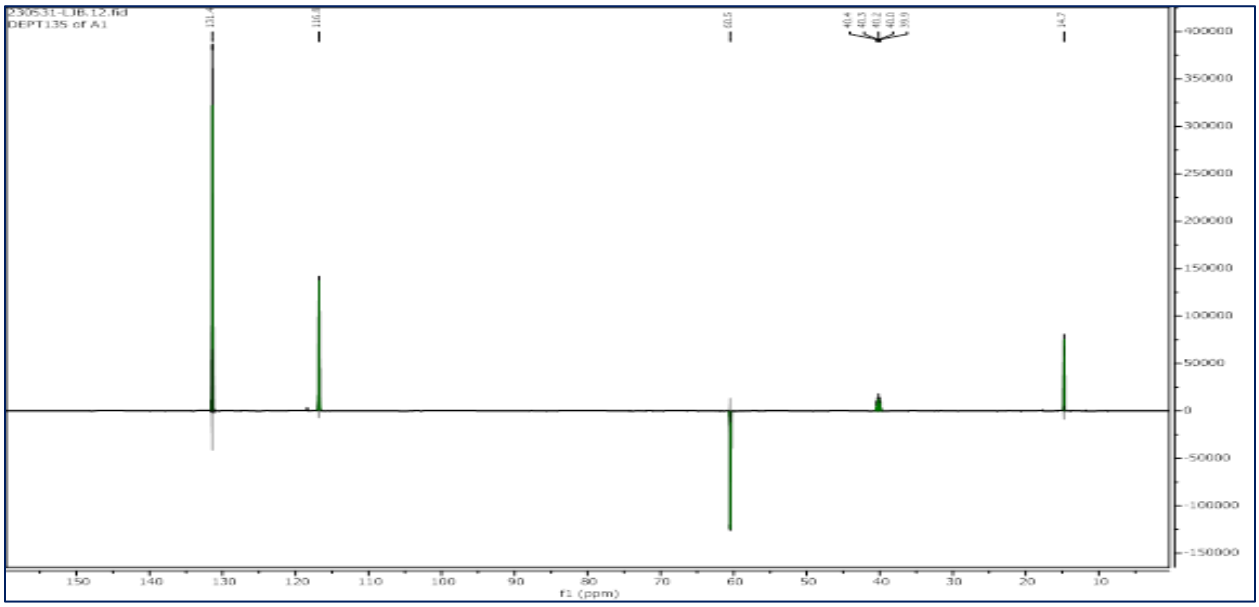
Appendix -3.4: ^{1}H NMR spectrum of compound 6.



Appendix -3.5: ^{13}C NMR spectrum of co mpound 6(Sr-102)..

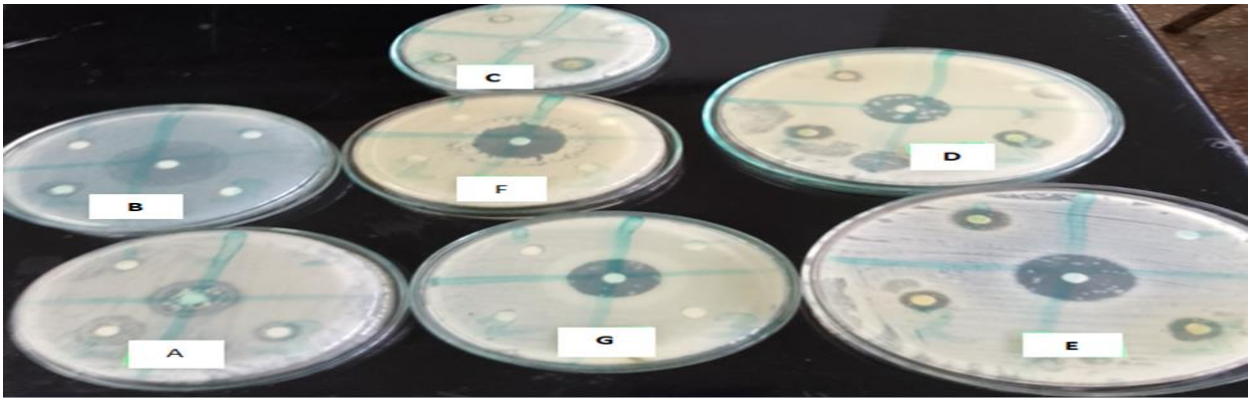


Appendix -3.6: DEPT-135 NMR spectrum of compound 6.



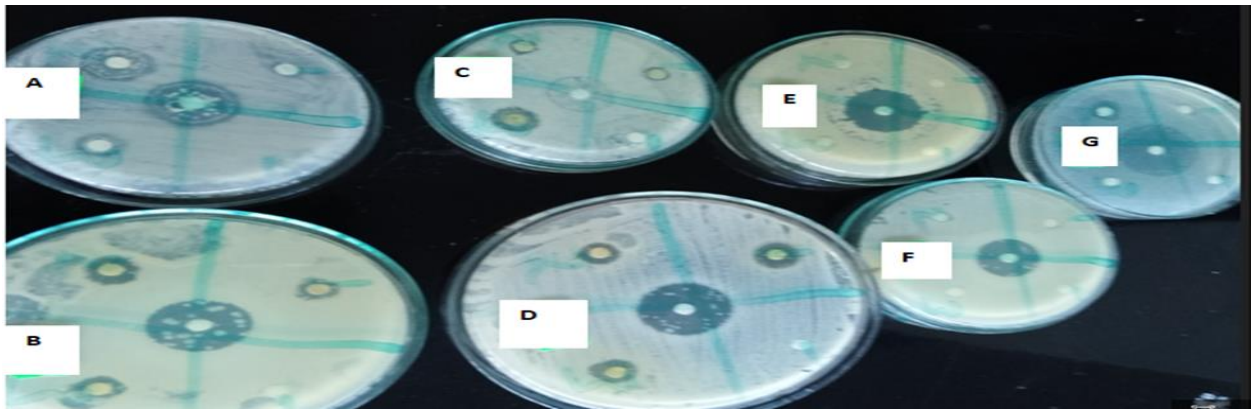
APPENDIX 4: The antimicrobial activity of various extracts from the three studied plants was reported.

Appendix -4.1: Antimicrobial activity of petroleum ether, chloroform/methanol (1:1) and methanol extracts from *Vernonia amygdalina*



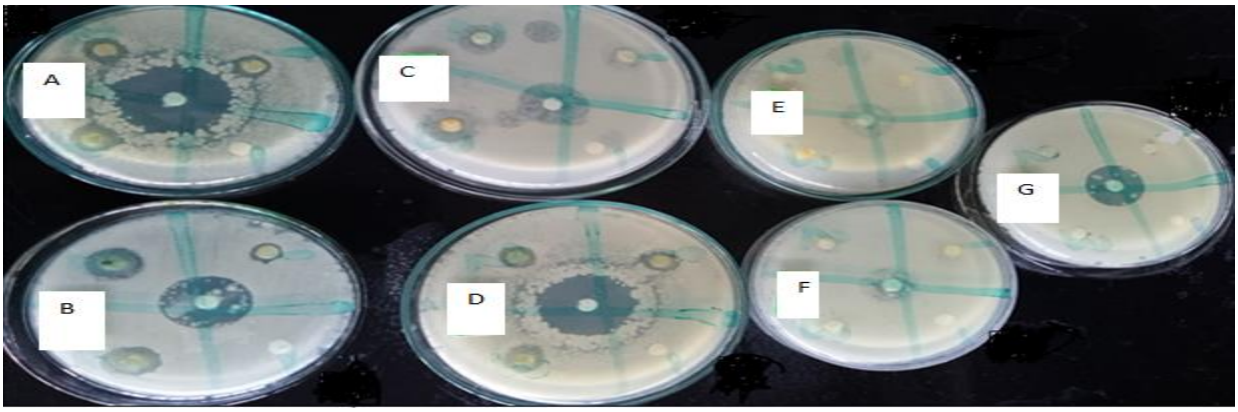
The showing data appendix -4.1 represents understudy of three extracts derived from Vernonia amygdalina. The extracts are as follows: A-Petroleum ether extracts tested against *Pseudomonas aeruginosa.* B & C - Chloroform/Methanol (1:1) extracts tested against *Staphylococcus aureus and Candida albicans,* respectively. D, E, F & G - Methanol extracts tested against *Staphylococcus epidermidis, Klebsiella pneumonia, Aspergillus niger and Candida albicans,* respectively.

Appendix -4.2: Antimicrobial activity of petroleum ether, chloroform/methanol (1:1) and methanol extracts from *Otostegia integrifolia*



The showing data appendix -4.2 represents understudy of three extracts derived from *Otostegia integrifolia*. The extracts are as follows: A & B - Methanol extracts tested against *Staphylococcus epidermidis and Staphylococcus aureus*, and, respectively. C - Petroleum ether extracts tested against *Candida albicans.*D, E, F & G - Chloroform/Methanol (1:1) extracts tested against *Aspergillus niger, Staphylococcus aureus, Pseudomonas aeruginosa* and *Staphylococcus aureus,* respectively.

Appendix -4.3: Antimicrobial activity of petroleum ether, chloroform/methanol (1:1) and methanol extracts from *Salvia rosmarinus*

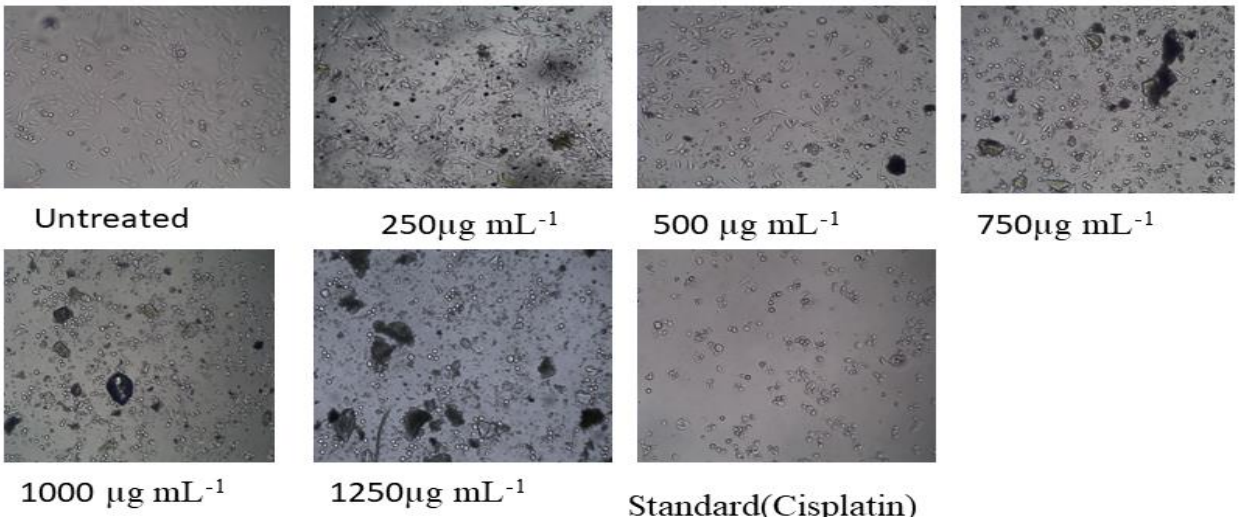


The showing data appendix -4.3 represents understudy of three extracts derived from *Salvia rosmarinus*. The extracts are as follows: A, B & C - Petroleum ether extracts tested against *Staphylococcus aureus, Staphylococcus epidermidis,* and *Candida albicans,* respectively. D, E & F - Chloroform/Methanol (1:1) extracts tested against *Escherichia coli*, *Pseudomonas aubergines,* and *Klebsiella pneumoniae,* respectively. Lastly, G - Methanol extract tested against *Aspergillus niger*.

APPENDIX 5: Cell proliferation and cytotoxicity assay

Appedix-5.1: *Vernonia amygdalina* leaves against human cervical cancer cell lines (*HeLa*) at different Concentrations.

A



B

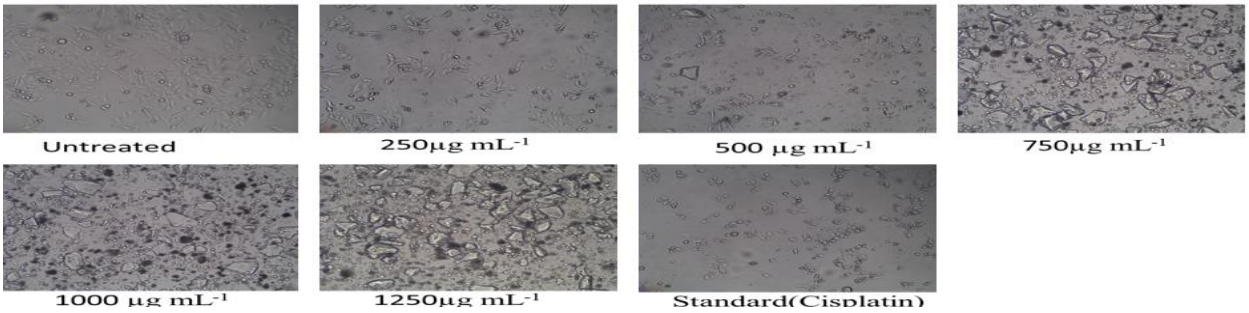


Figure inverted biological microscope images showing the cytotoxicity of Petroleum ether (Figure A), and Chloroform/Methanol (1:1) (Figure B) extract *of Vernonia amygdalina* leaves against human cervical cancer cell lines (*HeLa*) at different Concentrations(250,500,750,1000 and 1250µg mL^{-1})

Appedix-5.2: *Otostegia integrifolia* leaves against human cervical cancer cell lines (*HeLa*) at different Concentrations.

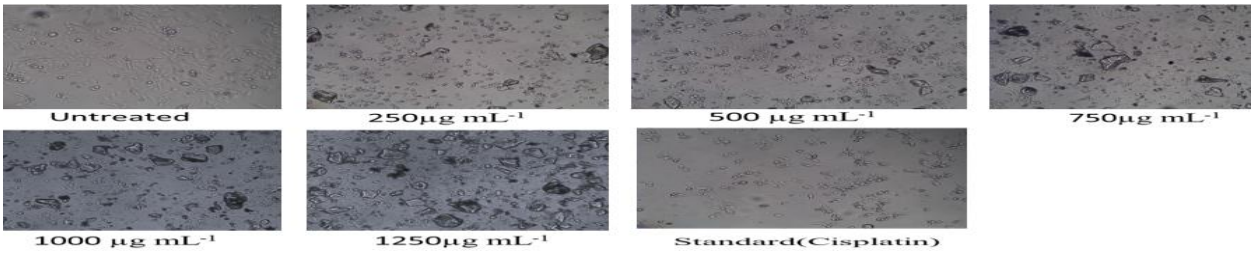
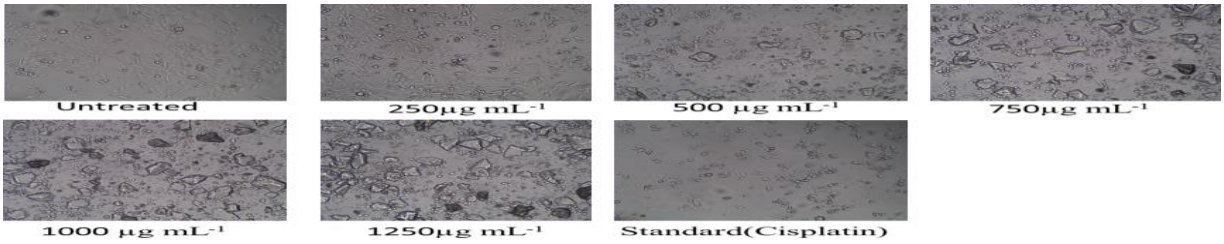


Figure inverted biological microscope images showing the cytotoxicity of Chloroform/Methanol (1:1) extract of *Otostegia integrifolia* leaves against human cervical cancer cell lines (*HeLa*) at different Concentrations(250,500,750,1000 and 1250µg mL^{-1})

Appedix-5.3: *Salvia rosmarinus* leaves against human cervical cancer cell lines (*HeLa*) at different Concentrations.

A



B

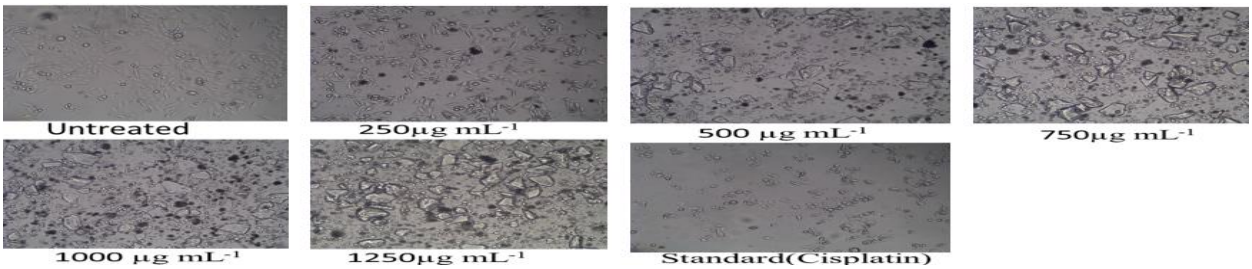


Figure inverted biological microscopic images showing the cytotoxicity of Petroleum ether (Figure A) and Chloroform/Methanol (1:1) (Figure B) extract of *Salvia rosmarinus* leaves against human cervical cancer cell lines (*HeLa*) at different Concentrations (250, 500, 750, 1000 and 1250µg mL^{-1})

APPENDIX 6: *S. rosmarinus* extracts against protozoa

Appendix- 6.1: *S. rosmarinus* extracts on *Acanthamoeba* Trophozoites

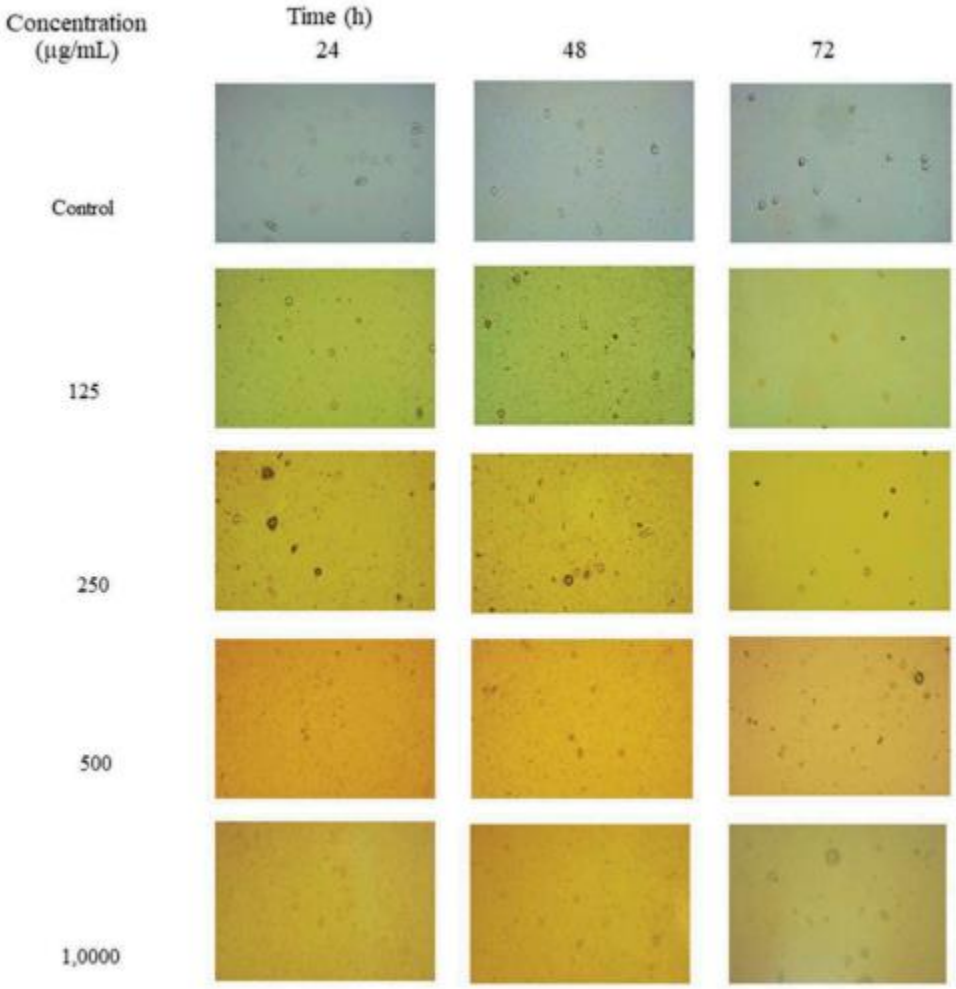


Figure of the effected of *S. rosmarinus* leaves of the Chloroform: Methanol (1:1) extracts against *Acanthamoeba* trophozoites. The cells were treated with the extract at different concentrations, incubated for 24, 48, and 72 h. One percent DMSO was included as negative control. Images of the treated cysts were observed by inverted microscope (200X). After 24 h, trophozoite form of *A. triangularis* and *A. castellanii* were observed in the negative control while the cyst form was detected in the treatment groups.

Appendix- 6.2: ***S. rosmarinus*** **extract on** ***Acanthamoeba*** **cysts**

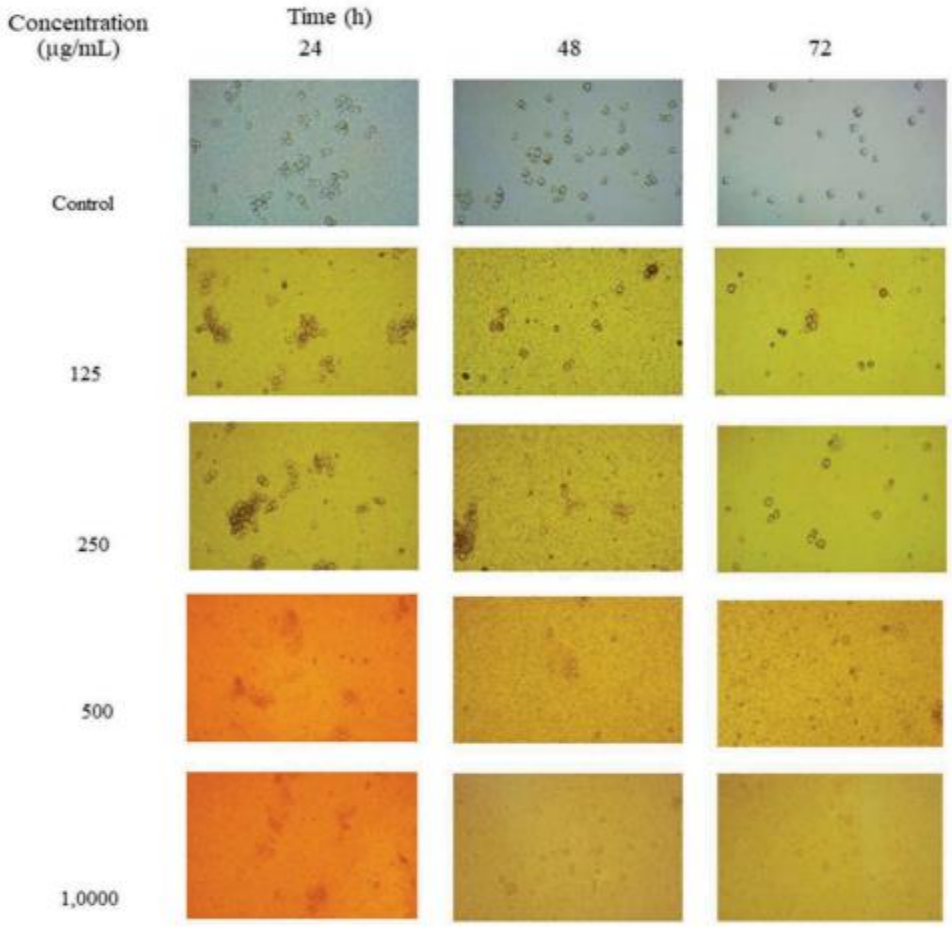
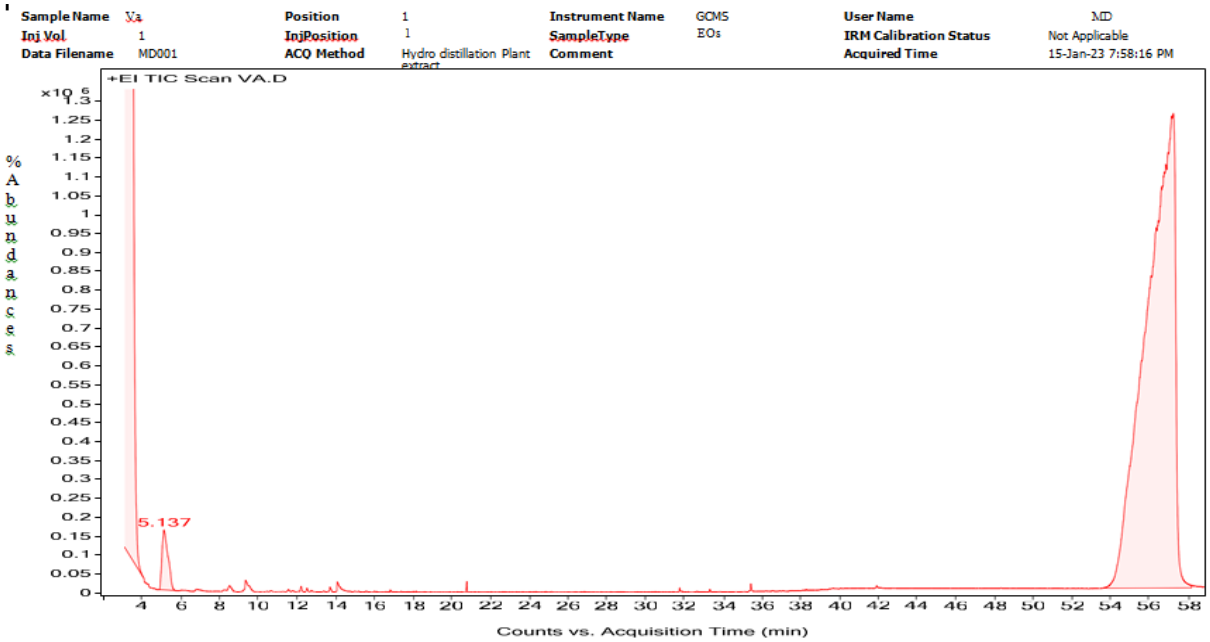


Figure of the effected of *S. rosmarinus* leaves of the chloroform: methanol (1:1) extracts against *Acanthamoeba* cysts. The cells were treated with the extract at different concentrations, incubated for 24, 48, and 72 h. One percent DMSO was included as negative control. Images of the treated cysts were observed by inverted microscope (200X). Triangle shape of *A. triangularis* and *A. castellanii* cysts were observed in non-treated cells. It was found that the cysts treated with the extract at high concentration demonstrated abnormal shape after 24 h

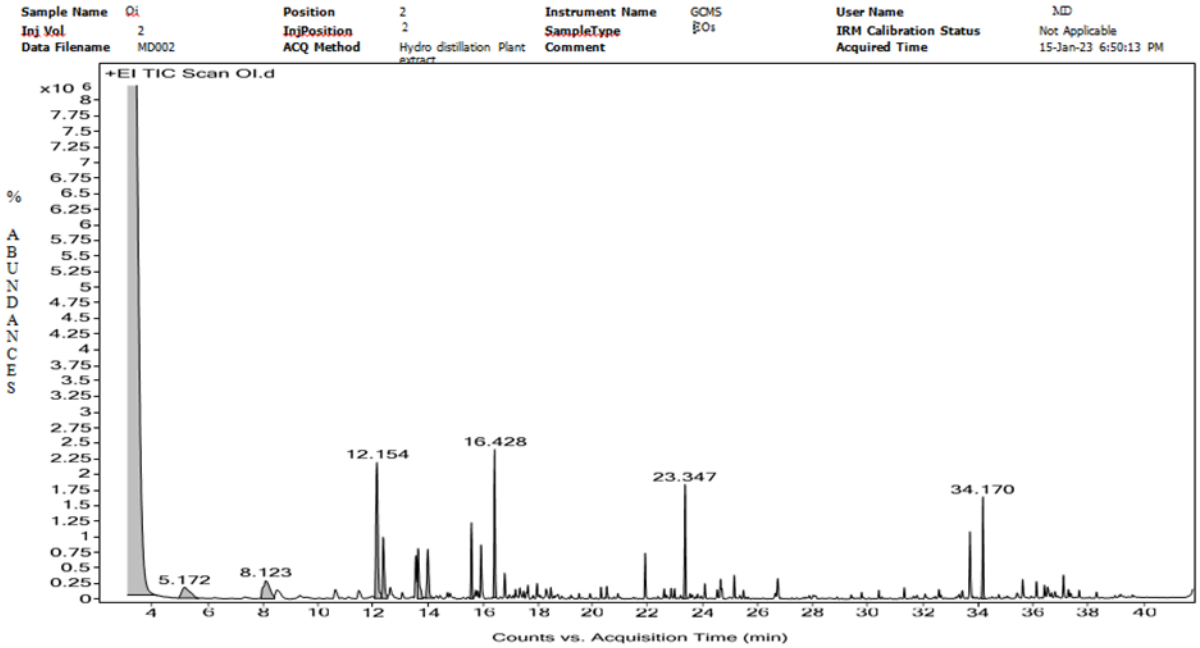
APPENDIX 7: Chemical composition of essential oils

The demonstration of the Chemical composition of essential oils of *V. amygdalina, Otostegia integrifolia*, and *S. rosmarinus* leaves via GC-MS in Appedix-7.1, Appedix-7.2,and Appedix-7.3,respectively

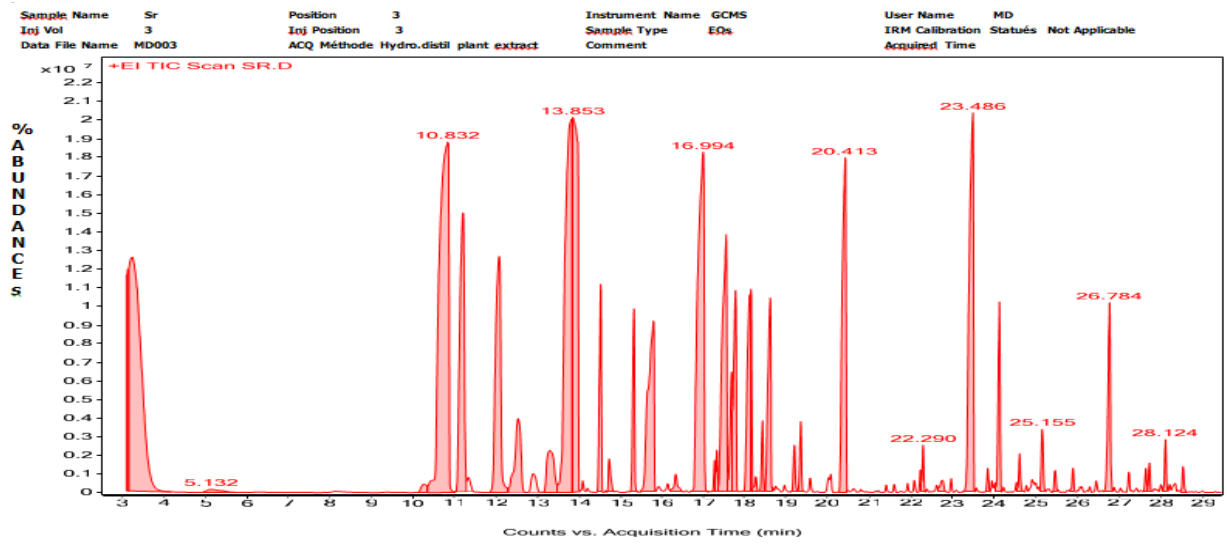
Appedix-7.1: Gas chromatogram of essential oil of *V. amygdalina* leaves.



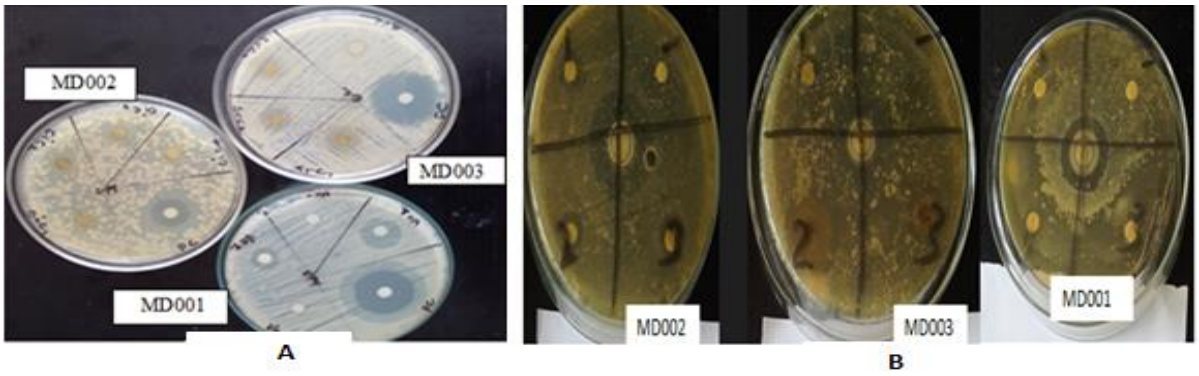
Appedix-7.2: Gas chromatogram of essential oil of *Otostegia integrifolia* leaves.



Appedix-7.3: Gas chromatogram of essential oil of *S. rosmarinus* leaves.



Appedix-7.4: Antimicrobial agent of Essential oils diffuses into the agar and inhibits germination and growth of the test microorganism



The showing data appendix -7.4 represents understudy of three EO extracts : **A**= Antibacterial activity: Agar well diffusion method of essential oil of *V.amygdalina and O.integrifolia* using both *S. pyogens* and *S. rosmarinus using E. coli* as test microorganisms. **B**= Antifungal activity*:* Agar diffusion method of essential oil of *V. amygdalina, O. integrifolia, and S. rosmarinus* using *C. albicans* as test microorganisms. MD001 used sample plate for *V. amygdalina;* MD002 used sample plate for *O. integrifolia,* and MD003 used sample plate for *S. rosmarinus.*