

**ISOLATION AND CHARACTERIZATION OF
PHYTOCHEMICALS FROM THE ROOTS *OF IMPATIENS*
TINCTORIA A. RICH AND INVESTIGATING THEIR
ANTIBACTERIAL AND ANTIINFLAMMATION
PROPERTIES**



**BY
LEMA TUFA SIME
A THESIS SUBMITTED TO THE DEPARTMENT OF
CHEMISTRY
COLLEGE OF NATURAL SCIENCES
PRESENTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENT FOR THE
DEGREE OF MASTER'S IN CHEMISTRY
SPECIALIZATION: ORGANIC CHEMISTRY
SALALE UNIVERSITY**

**DECEMBER, 2023
FITCHE, ETHIOPIA**

**ISOLATION AND CHARACTERIZATION OF
PHYTOCHEMICALS FROM THE ROOTS *OF IMPATIENS*
TINCTORIA A. RICH AND INVESTIGATING THEIR
ANTIBACTERIAL AND ANTIINFLAMMATION
PROPERTIES**



By

Lema Tufa Sime

Advisor: Digafie Zeleke (Ph. D)

Co-Adviser: Tsegaye Deyou (Phd)

A Thesis Submitted To The Department Of Chemistry

College Of Natural Sciences

Presented in Partial Fulfillment of the Requirement for the

Degree of Master's In Chemistry

Specialization: Organic Chemistry

SALALE UNIVERSITY

December, 2023

Fitche, Ethiopia

APPROVAL OF BOARD OF EXAMINERS

We, the members of the Board of Examiners of the final open defense by Lema Tufa Sime, have read and evaluated his thesis titled **“Isolation and Characterization of Phytochemicals from the Roots of *Impatiens tinctoria* A. Rich and Investigating Their Antibacterial and Anti-inflammation Properties”** and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirement for the degree of Master of Science in Chemistry (Organic Chemistry).

_____	_____	_____
Advisor	Signature	Date

_____	_____	_____
Name of Co-Advisor	Signature	Date

_____	_____	_____
Chairperson	Signature	Date

_____	_____	_____
Internal Examiner	Signature	Date

_____	_____	_____
External Examiner	Signature	Date

DECLARATION

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

Name: Lema Tufa

Signature:



This MSc dissertation has been submitted for examination with my approval as thesis advisor/ dissertation Supervisor.

Name:

Signature:

Date of submission...

ADVISOR'S APPROVAL SHEET

To: chemistry department

Subject: Thesis Submission

This is to certify that the thesis entitled **“Isolation and Characterization of Phytochemicals from the Roots of *Impatiens tinctoria* A. Rich and Investigating Their Antibacterial and Anti-inflammation Properties ”** submitted in partial fulfillment of the requirements for the degree of Master's degree of Science in Chemistry (Organic Chemistry), the Graduate program of the department of Chemistry, and has been carried out by Lema Tufa Id. No RM 0078/14, under my supervision. Therefore, I recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the department.

Name of major Advisor

Signature

Date

ACKNOWLEDGEMENT

First of all, I would like to thank the Almighty God, who gave me patience, courage, good health, and potency with his blessings to complete this thesis. Next, I would like to express my deepest appreciation to my advisor, Digafie Zeleke (PhD), and co-advisor, Tsegaye Deyou (PhD), for their valuable advice, excellent comments, suggestions, kindness, and patient guidance. It is also my great pleasure to thank staff members of the Department of Chemistry and Department of Biology at Salale University for their support and cooperation throughout the study period. I want to thank Mr. Mesay Solomon in particular for his generosity and technical assistance throughout the laboratory work. I express my deepest sense of gratitude to my lovely family and friends for their unconditional, heartfelt, moral, and unreserved support at different stages of my academic life, which had a significant impact on the success of my present-day study.

TABLE OF CONTENTS

TABLE OF CONTENTS	PAGE NUMBER
APPROVAL OF BOARD OF EXAMINERS	i
DECLARATION	ii
ADVISOR'S APPROVAL SHEET	iii
ACKNOWLEDGEMENT	iv
TABLE OF CONTENTS	v
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST APPENDIX	ix
LIST OF ACRONYMS AND ABBREVIATIONS	x
ABSTRACT	xi
1. INTRODUCTION	1
1.1. Background of the Study	1
1.2. Statement of the Problem	2
1.3. Objectives of the study	3
1.3.1. General objective	3
1.3.2. Specific objectives	3
1.4. Significance of the study	3
2. LITERATURE REVIEW	4
2.1. Botanical Information	4
2.2. Ethno medicinal uses of <i>I. tinctoria</i> A. Rich	5
2.3. Phytochemistry of the genus <i>Impatiens</i>	6
2.3.1. <i>Impatiens balsamina</i>	6
2.3.2. <i>Impatiens noli-tangere</i> L.	7
2.3.3. <i>Impatiens glandulifera</i>	8
2.3.4. <i>Impatiens walleriana</i>	9
2.4. Biological Activities of the genus <i>Impatiens</i>	10
2.4.1. Antibacterial activities	10
2.4.2. Antifungal activity	10
3. MATERIALS AND METHODS	11
3.1. General experimental materials and chemicals used	11

3.2.	Plant material collection and authentication	11
3.3.	Experimental Sites.....	11
3.4.	Extraction and Isolation	12
3.4.1.	Extraction of roots of <i>I. tinctoria</i> A. Rich.....	12
3.4.2.	Isolation of roots of <i>I. tinctoria</i> A. Rich extract.	12
3.5.	Biological Assay	15
3.5.1.	Antibacterial activities of the extracts and isolates.....	15
3.6.	Molecular Docking Analysis of the Compound 21	16
4.	RESULTS AND DISCUSSION.....	17
4.1.	Structural Elucidation of the Isolated Compounds	17
4.1.1.	Characterization of Compound 20	17
4.1.2.	Characterization of Compound 21	18
4.1.3.	Characterization of Compound 22	20
4.1.4.	Characterization of Compound 23	22
4.2.	Antibacterial Activity Test of crude extract and isolated compounds	23
4.3.	Molecular Docking study	26
5.	CONCLUSION AND RECOMMENDATION	30
5.1.	Conclusion.....	30
5.2.	Recommendation.....	30
	REFERENCE.....	31
	APPENDICES	35

LIST OF TABLES

Table 1: Fractions collected from column chromatography of <i>I. tinctoria</i> A. Rich	14
Table 2: ^1H and ^{13}C NMR (400 MHz, δ ppm, CDCl_3) spectral data for compounds (20)	18
Table: 3 Chemical shifts of ^1H NMR and ^{13}C NMR of compound 21	19
Table 4: Chemical shifts of ^1H NMR and ^{13}C NMR of compound 22	21
Table 5: Chemical shifts of ^1H NMR and ^{13}C NMR of 23	23
Table 6: Results from the disc diffusion assay showing the antibacterial activity of crude chloroform/ethanol extract and the isolate compound of the roots of <i>I. tinctoria</i> A. Rich against four bacterial strains.	24
Table 7: The minimum binding energy and interacting amino acids in the molecular docking of ligands and against human Cyclooxygenase-1 ((PDB ID: 3N8Z) and Cyclooxygenase-2 (3Q7D)	27

LIST OF FIGURES

Figure 1: Picture of <i>I. tinctoria</i> A. Rich root and Powder	2
Figure 2: Classification <i>Impatiens tinctoria</i>	4
Figure 3: chemical compounds found in <i>Impatiens balsamina</i>	7
Figure 4: The chemical structures of <i>Impatiens noli-tangere</i> L. plants.....	8
Figure 5: Chemical structures of compounds (13) and (14)	8
Figure 6: Compounds isolated from <i>I. walleriana</i>	9
Figure 7: Crude extract and Fractions (SF-6) on TLC.....	12
Figure 8: General procedure of extaction and isolation of roots of <i>I. tinctoria</i> A. Rich extract.	15
Figure 9: Chemical structures of compound 20	18
Figure 10: Chemical structures of compound 21	20
Figure 11: Chemical structures of compound 22	22
Figure 12: Chemical structures of compound 23	23
Figure 13: The inhibition zone of the isolated compounds and crude extract in mm (mean \pm SD) at 250 μ g/mL.	25
Figure 14: The inhibition zone of the isolated compounds and crude extract in mm (mean \pm SD) at 150 μ g/mL.	25
Figure 15: The 3D and 2D binding interactions of 21 against human Cyclooxygenase-1 (PDB ID: 3N8Z).....	28
Figure 17: The 3D and 2D binding interactions of Flurbiprofen against human Cyclooxygenase-1 (PDB ID: 3N8Z).....	28
Figure 18: The 3D and 2D binding interactions of 21 against human Cyclooxygenase-2 (2 PDB ID: 3Q7D)	28
Figure 19: The 3D and 2D binding interactions of (R)-naproxen against human Cyclooxygenase-2 (2 PDB ID: 3Q7D)	29

LIST APPENDIX

Appendix 1: Antimicrobial activity Tests of Crude Extracts.....	35
Appendix 2: Bioassays Tests of Isolated Compound Zone of Inhibition in mm.....	35
Appendix 3: IR Spectrum of 20	36
Appendix 4: ¹ H NMR spectrum of 20	36
Appendix 5: ¹³ CNMR spectrum of 20 in CDCl ₃ as solvent.....	37
Appendix 6: DEPT-135 spectrum of 1 in CDCl ₃ as solvent.....	37
Appendix 7: IR Spectrum of 21	38
Appendix 8: ¹ H NMR spectrum of 21	38
Appendix 9: ¹³ CNMR spectrum of 21 in CDCl ₃ as solvent.....	39
Appendix 10: DEPT-135 spectrum of 21 in CDCl ₃ as solvent.....	39
Appendix 11: FT-IR Spectrum of Compound 22	40
Appendix 12: ¹ H-NMR Spectral data of compound 22	40
Appendix 13: ¹³ CNMR spectrum of 22 in CDCl ₃ as solvent.....	41
Appendix 14: DEPT-135 spectrum of 22 in CDCl ₃ as solvent.....	41
Appendix 15: IR Spectrum of 23	42
Appendix 16: ¹ H-NMR Spectral data of compound 23	42
Appendix 17: ¹³ CNMR spectrum of 23 in CDCl ₃ as solvent.....	43

LIST OF ABBREVIATIONS/ACRONYMS

^{13}C -NMR	Carbon Nuclear Magnetic Resonance
^1H NMR	Proton Nuclear Magnetic Resonance
CC	Column Chromatography
CDCl_3	Deuterated chloroform
COX	cyclooxygenase
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl Sulfoxide
DPPH	diphenyl-1-picryl-hydrazyl
FTIR	Fourier-transform infrared spectroscopy
MHA	Mueller Hinton agar
NSAIDs	nonsteroidal anti-inflammatory drugs
TLC	Thin-Layer Chromatography
UV	Ultra Violet

ABSTRACT

*Medicinal plants have been a rich source of biologically active chemicals, which are widely employed in medicine as pure or as crude extracts to treat a wide range of illnesses. Impatiens tinctoria A. Rich has been utilized for a variety of medical purposes. The rich ethnobotanical information interests us to conduct phytochemical investigation on root of Impatiens tinctoria A. Rich. In this study, four compounds were isolated from the root of Impatiens tinctoria A. Rich using silica gel chromatography. The compounds were characterized by NMR and FTIR spectroscopy. The antimicrobial activities of the compounds and crude extract were evaluated against two Gram negative and two Gram positive bacteria by paper-disc diffusion method. Compound **21** didn't show any activity against S. pyogenes. The other shows some antibacterial activities from lowest 7.43 mm by **20**, against K. pneumoniae to maximum 9.6 mm by **23** against E. coli. The molecular docking of compound **21** against two human cyclooxygenases showed stronger binding affinity than two standard anti-inflammatory drugs (Flurbiprofen and (R)-naproxen).*

Keyword: *Medicinal plants, Impatiens tinctoria A. Rich, Crude extracts, molecular docking, antibacterial activity, binding affinity.*

1. INTRODUCTION

1.1. Background of the Study

Due to their potential to help society and humanity as a whole, particularly in the fields of pharmacology and medicine, medicinal plants are receiving more attention than ever before. For millennia, medicinal plants have been a rich source of biologically active chemicals, which are widely employed in medicine as pure or as crude materials to treat a wide range of illnesses. These plants have medicinal properties because of their phytochemical components, which have specific pharmacological effects on human health (Akinmoladun *et al.*, 2007). Phytochemical components are generally classified into two groups based on the activity of their metabolism in the plant: primary constituents, which include primarily sugars, amino acids, proteins, and chlorophyll, and secondary constituents, which include alkaloids, flavonoids, saponins, tannins, phenolic compounds, and many more (Agidew, 2022).

In Ethiopia, traditional medicine serves 80% of the human population as an alternative form of primary healthcare, frequently in conjunction with modern medicine and treats 90% of livestock (Moges and Moges, 2020). Similar to other regions globally, Ethiopia's rural and urban populations heavily rely on traditional medicine, which can be attributed to its cultural acceptability, efficacy against specific diseases, accessibility, and cost-effectiveness when compared to contemporary medical practices (Shalligito and Tesfa, 2022). Natural products offer a vast variety of multi-dimensional chemical structures, and they can also be used to modify biological functions, which have garnered a lot of interest. They have been successfully used in the search for novel medications (Duke, 2019). In traditional medicine, a number of plants have long been used to treat bacterial and fungal-induced illnesses. *Impatiens tinctoria* A. Rich is one this plant that the local healer has been using because it has antimicrobial and wound-healing properties. *Impatiens tinctoria* A. Rich have been traditionally used to treat diseases caused by microorganisms like bacteria and fungi. These plants appear to be effective, even though there isn't enough scientific evidence to support their claims (Gidamo, 2023).

Historically, *impatiens tinctoria* A. Rich has been utilized for a variety of medical purposes. Ethiopian women prepared a paste from *I. tinctoria* A. Rich tubers (roots and used as a paint to give their hands and feet's nails and palms a deep reddish hue (Degu, 2019).

In an ethnobotanical study of traditional medicinal plants in the Amhara Region of Northern Ethiopia, Messay Wolde-Mariam *et al.* (2015) reported that the stem is chewed and used to treat fungal infections, mouth and throat disease, and for the aseptic cleaning of wounds. Its root decoction is drunk against abdominal pains and as a purgative (Wolde-mariam, Limenih and Umer, 2015). Phytochemical investigation and investigating their antibacterial and anti-inflammatory properties on the roots of *I. tinctoria* A. Rich were the main aims of the current research in response to the lack of sufficient research on the plant species.



Figure 1: Picture of *I. tinctoria* A. Rich root and Powder

1.2. Statement of the Problem

Impatiens tinctoria A. Rich is one of the plants used in traditional medicine. It was used to treat bacterial and fungal diseases traditional. Ethiopian women use the paste of its root tuber as traditional cosmetics and paints. They paint their palms, nails of the hands and feet to render a deep reddish hue. They believe that it helps them to control fungal infections and toughen their skin. Ethiopian women chop or mash the insides of *Impatiens tinctoria* (also known as "ensolella") tubers into a paste. It helps to control fungal infections and toughen the skin. It is regarded as a beauty treatment comparable to henna. Cloth is also dyed using the tubers as well. The juice of pounded roots is one of the ingredients for a red ink. A root infusion is consumed medicinally as a purgative and to treat stomach aches. However, irrespective of the wide traditional applications, enough phytochemical investigations were not conducted on plants in Ethiopia or elsewhere. Therefore, this study was undertaken to conduct phytochemical investigation on roots of *Impatiens tinctoria* A. Rich and also to assess the antibacterial and anti-inflammation properties of the crude extracts and the isolates

1.3. Objectives of the study

1.3.1. General objective

The main objective of the research was to conduct the isolation and characterization of phytochemicals from the roots of *Impatiens tinctoria* A. Rich and investigate their antibacterial and anti-inflammation properties in crude extract and the isolates.

1.3.2. Specific objectives

The specific objectives of the study were:

- To extract tuberous roots of *I. tinctoria* A. Rich using chloroform: Methanol (2:1 ratio)
- To isolate phytochemicals present in the roots using silica gel chromatographic techniques
- To elucidate structures of isolated compounds using FTIR and NMR spectroscopic data
- To evaluate the antibacterial activities of the crude extracts
- To investigate the anti-inflammation properties by molecular docking study.

1.4. Significance of the study

As a solution of stated problems scientific studies have to be conducted on the traditional medicinal plants to develop new, effective and safe antimicrobial drugs. In Africa, the use of Medicinal plants still plays an important role in microbial treatment. Taking into account that plants were potential sources of the existing first line antimicrobial drugs, there is still great potential of identifying antimicrobial drugs from plant-based sources. Thus, screening and identifying the bioactive constituents of traditional medicinal plants empirically used to treat infectious diseases will have a great contribution in addressing this global problem. Locally, Ethiopian women chop or mash the inside of the roots of *I. tinctoria* A. Rich in to a paste to dye the palms and nails of the hands and feet as a beauty treatment, to control fungal infections and to toughen the skin. Therefore, the findings of this research would be useful:

- ✓ To give information about the chemical constituents of roots of *I. tinctoria* A. Rich.
- ✓ To provide the obtained result for further study on this plant.

2. LITERATURE REVIEW

2.1. Botanical Information

The family Balsaminaceae has two genera: the monotypic genus *Hydrocera* and the vast genus *Impatiens*, which have roughly 1000 species. Plants of the genus *Impatiens* are characterized as perennial and succulent plants that are often found in tropical and subtropical humid forests, especially near river slopes. They usually have simple foliage, are medium-sized, and have attractively coloured flowers (Pires *et al.*, 2021).

The *Impatiens tinctoria* taxonomy, or scientific categorization, is

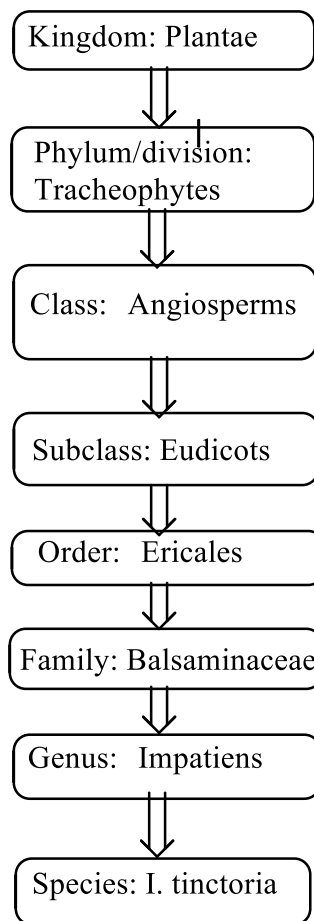


Figure 2: Classification *Impatiens tinctoria*

Impatiens tinctoria A. Rich is called Balsamine in English and “Insosila” in Amharic language. In Africa, *impatiens* has been recognized as more than 100 different species, with *I. tinctoria* being the most widely distributed. Based on variations in the size of their

leaves, flowers, and spurs, *Impatiens tinctoria* includes five geographically distinct subspecies (Access, Directorate and Seboka, 2017).

- ❖ *I. tinctoria* subsp. *tinctoria*: Ethiopia, Sudan, Uganda, Zaire
- ❖ *I. tinctoria* subsp. *elegantissima*: Kenya, Uganda
- ❖ *I. tinctoria* subsp. *abyssinica*: Ethiopia
- ❖ *I. tinctoria* subsp. *latifolia*: Tanzania, Malawi, Zambia
- ❖ *I. tinctoria* subsp. *songeana*: Tanzania

Study showed is the large concentration of *I. tinctoria* found in East Tropical African nations such Northern Malawi, Western and Southern Kenya, Uganda, the Democratic Republic of the Congo, Southern Tanzania, and Southeast Sudan (Access, Directorate and Seboka, 2017). This perennial herb is upright and reaches a height of around two meters. At or slightly below the soil's surface, it grows a sizable tuberous rootstock (**Figure 2**). The flowers of *Impatiens tinctoria* (pink to purple) are most strongly scented in the evening and attract moths. It grows over several regions of Ethiopia and is indigenous to East Africa. According to studies on the assessment of flora biodiversity, *I. tinctoria* A. Rich is one of the most common herb species in Ethiopia's Bonga Forest, Oromia Region, and it is the most common cash herb in Mahoney and Maichew, Southern Tigray Region (Degu, 2019).

2.2. Ethno medicinal uses of *I. tinctoria* A. Rich

Medicinal plants represent the most ancient form of medication, used for thousands of years in traditional medicine in many countries around the world. The empirical knowledge about their beneficial effects was transmitted over the centuries within human communities (Plants, 2021). Traditional medicine refers to any ancient and culturally based health care practice differing from scientific medicine and is largely transmitted orally by communities of different cultures. The traditional system of medicine is a centuries-old practice and a long-serving companion to humankind in the fight against disease and in leading a healthy life. Medicinal plants contain bioactive organic chemical compounds, often referred to as phytochemicals, which play a defensive role against major chronic diseases in both host metabolic or genetic dysfunctional disease and infectious disease and are found in grains, vegetables, fruits, and other plant products (Ugboko *et al.*, 2020). The bioactive substances

presented in medicinal plants are tannins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids, and phenols (Mandal *et al.*, 2013).

Due to the cost effectiveness, safety, increasing failure of chemotherapy and antibiotic resistance exhibited by pathogenic microbial agents, search for plant products has increased for their potential antimicrobial activity. Therefore, scientific studies must be conducted on traditional medicinal plants to develop new, effective and safe antimicrobial drugs. Locally, Ethiopian women chop or mash the tuberous roots of *I. tinctoria* A. Rich into a paste to dye the palms and nails of the hands and feet as a beauty treatment as well as to control fungal infections and to toughen the skin (Degu *et al.*, 2021). Also root decoction is drunk as a purgative against abdominal pains. The stem is chewed to treat mouth and throat diseases. For instance, in an ethnobotanical study of traditional medicinal plants in Amhara Region of Northern Ethiopia, (Wolde-mariam, Limenih and Umer, 2015) reported that the crushed roots of *Insosila* being mixed with water are drunk once or twice for abortion purpose; and similarly, crushed and boiled roots are drunk to treat arthritis. Treatment by *Insosila* helps to control fungal infections as well as to toughen the skin (Hedberg *et al.*, 2006).

2.3. Phytochemistry of the genus *Impatiens*

Current studies affirm the existence of more than 300 distinct compounds present in several studied species, among them flavonoids, phenolic acids, coumarins, quinones, terpenes, and saponins stand out. The following kinds of compounds have been found in phytochemical studies of *Impatiens* species: triterpenes, which are composed of triterpenoid saponins; quinones, which include naphthoquinones and anthraquinones; and phenolic compounds, which include flavonoids, phenols, and coumarins (Szewczyk, 2018). Some of the compounds isolated from the genus *Impatiens* are given below.

2.3.1. *Impatiens balsamina*

Impatiens balsamina L., known as garden balsam or rose balsam, is an annual plant belonging to the family Balsaminaceae and is widely distributed in Korea, Japan, and mainland China. Diverse parts of *I. balsamina*, including flowers, stems, and leaves, have long been used as traditional medicines to treat various diseases. The flowers of *I. balsamina* have been used as remedies for lumbago, burns, and scalds, whereas its aerial parts have been used to treat articular rheumatism, abscesses, and tumors (Lee *et al.*, 2020). As a several

reports shows *Impatiens balsamina* have diverse Pharmacological activities including Antibacterial activity, Antifungal activity, Antioxidant activity, anticancer activity antitumor activity and Anti-inflammatory activity. The groups of compounds commonly found in this plant are naphthoquinones, coumarins, phenolic acids, flavonoids, anthocyanidins and steroids (D, Rejimon and Varghese, 2015). Kim and coworkers identified twelve compounds for inhibitory effects on the production of nitric oxide (NO) in lipopolysaccharide (LPS) activated. From that, some compounds were listed in **Figure 3**, namely tetrahydronaphthalene (**1**), (Dihydrodehydrodiconiferyl alcohol-9- β -O-Dglucopyranoside (**2**), Kaempferol-3-O- β -D glucopyranoside (**3**), p Hydroxybenzoic acid (**4**), β -Amyrin (**5**), Lupenone (**6**) and Lupeol (**7**) isolated from the leaves of *Impatiens balsamina* (Kim *et al.*, 2019).

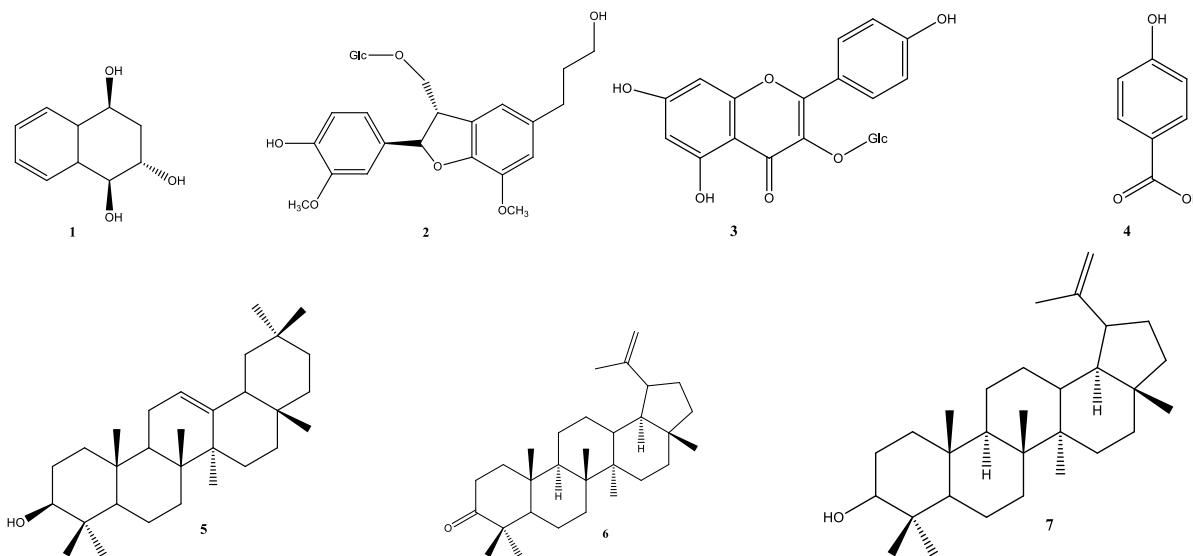


Figure 3: chemical compounds found in *Impatiens balsamina*

2.3.2. *Impatiens noli-tangere* L.

Impatiens noli-tangere L., Balsaminaceae, commonly known as touch-me-not balsam, is an annual herbaceous plant found in damp places and forests in Europe, Asia, and North America. Plants belonging to the genus *Impatiens* are rich in organic acids, anthraquinones, flavonoids, and phenolic acids. Also, *Impatiens noli-tangere* L. has antioxidative, anti-inflammatory, astringent, hemostatic, and wound-healing properties (Paun *et al.*, 2018).

In Poland, five phytochemicals were isolated from the leaves of *I. noli-tangere*, which includes gentisic acid (**8**), syringic acid (**9**), p-hydroxybenzoic acid (**10**), caffeic acid (**11**), and sinapic acid (**12**) (**Figure 4**).

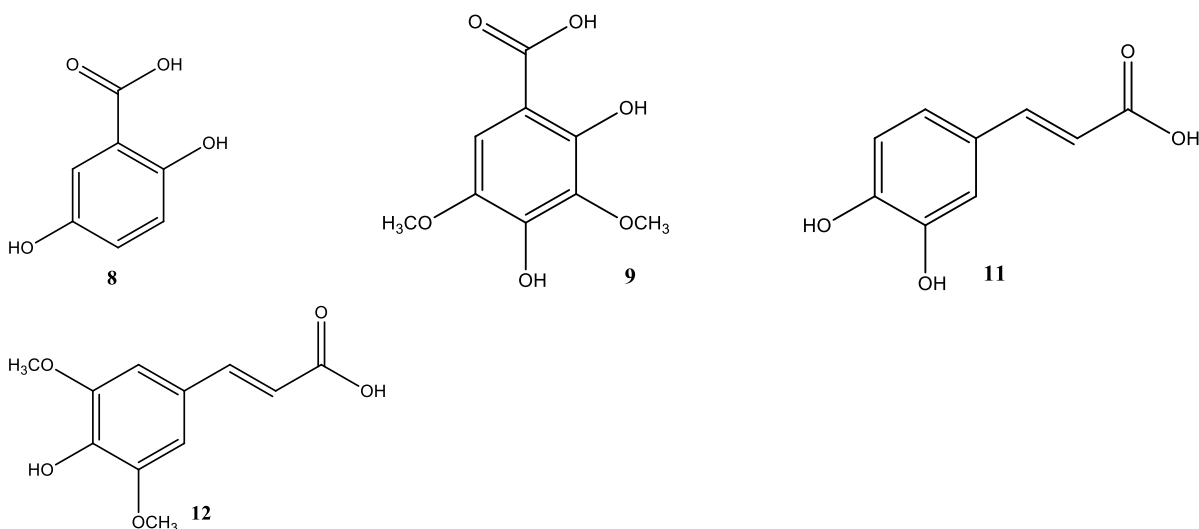


Figure 4: The chemical structures of *Impatiens noli-tangere* L. plants.

2.3.3. *Impatiens glandulifera*

Impatiens glandulifera Royle is known for its thin pin-shaped extra-floral nectaries (EFNs) covering its shoot nodes, leaf petioles, and basal leaf teeth. Native to the western Himalaya, *I. glandulifera* is a tall annual plant that was introduced as an ornamental in the 19th century. It can grow rapidly up to 4 m tall, making it the tallest annual herb in Europe (Ab Razak *et al.*, 2023). Recent evidence indicates that for nectaries inhibit the growth of fungal nectar microbes 2-methoxy-1, 4-naphthoquinone (2-MNQ) (**13**), and 2-hydroxy-1,4 naphthoquinone (**14**) (**Figure 5**) from its leaves and roots (Block, Yakubova and Widhalm, 2019).

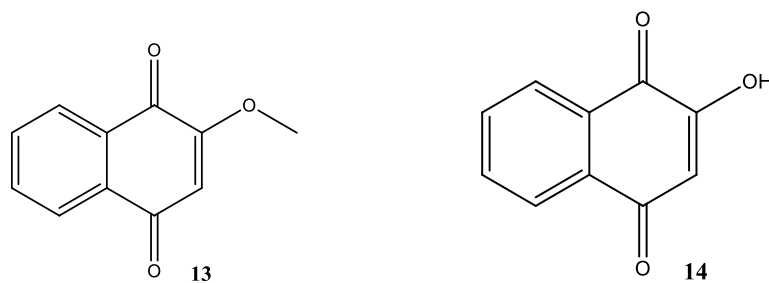


Figure 5: Chemical structures of compounds (**13**) and (**14**)

2.3.4. *Impatiens walleriana*

Impatiens walleriana Hook. f., popularly known as balsam, is an ornamental plant belonging to the plant family Balsaminaceae and is native to East Africa. This species is widely cultivated for its ornamental value in many parts of the world and has naturalized in many of these areas. In addition to its ornamental value, the flowers of *Impatiens walleriana* are edible and the decoction prepared from the dried leaves and roots is used as an abortifacient (Thangavelu and Arumugam, 2020). Furthermore, the whole plant of these species can be used as a source of antioxidant and antimicrobial agents, and these plants have been widely exploited by the horticulture industry around the world since they have been used as ornamental or decorative plants (Sharma, 2022). More than 300 distinct compounds exist in the broad genus *Impatiens*, with mainly flavonoids, phenolic acids, coumarins, quinines, terpenes, steroids, and saponins (D, Rejimon and Varghese, 2015). Latest studies have revealed that *I. walleriana* contains non-anthocyanin and anthocyanin compound. Anthocyanin flavonoids Cyanidin ($C_{15}H_{11}O_6$) and Malvidin ($C_{17}H_{15}O_7$) and non-anthocyanin compounds *p*-coumaric acid hexoside (**17**) and Eryodictiol-O-hexoside (**18**) (**figure 6**) are reported to be present in *I. walleriana* (Sokovi *et al.*, 2021).

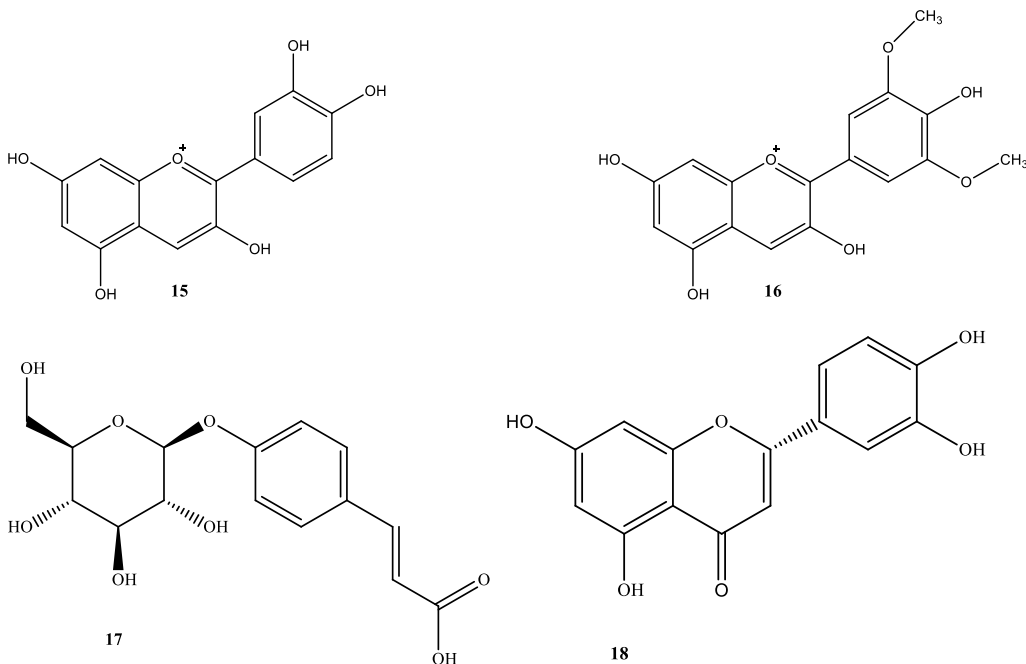


Figure 6: Compounds isolated from *I. walleriana*

2.4. Biological Activities of the genus *Impatiens*

Various pharmacological activities and therapeutic effects are ascribed to genus *Impatiens*. Some of the effects are; antimicrobial effect, anti-inflammatory effect, antidiabetic effect, antioxidant effect, gastro intestinal protective effect, and wound healing effects. Paun *et al.* reported that *Impatiens noli-tangere* L. is rich in organic acids, anthraquinones, flavonoids, and phenolic acids that show antioxidative, anti-inflammatory, astringent, hemostatic, and wound-healing properties (Paun *et al.*, 2018). In 2022 sharma reported antioxidant and antimicrobial activities of the ethanolic extract from whole plant of *I. balsamina*, *I. hawkeri* and *I. walleriana* (Sharma, 2022). Also in Ethiopia degu *et al* reported Evaluation of Antibacterial and Acute Oral Toxicity of *Impatiens tinctoria* A. Rich Root Extracts (Degu *et al.*, 2021) and Gidamo reported Antioxidant activity and mineral content of *Impatiens tinctoria* A. Rich (Gidamo, 2023).

2.4.1. Antibacterial activities

Antimicrobials come in a variety of forms, including antibiotics, antivirals, antifungals, and anti-protozoans. Antibiotic (antibacterial) resistance is a major issue that affects the entire world, and new therapies are desperately needed. The rate at which new drugs are being discovered under the current antibiotic discovery methodology is insufficient to counteract the current state of antibiotic resistance (Jackson, Czaplewski and Piddock, 2018).

The antibacterial activity of aqueous, ethanol and ethyl acetate extracts of the roots of *I. tinctoria* A. Rich was screened against 13 bacteria and showed highest activities against Gram-negative bacteria: *S. flexneri*, *S. soni*, and *P. mirabilis* (Degu *et al.*, 2021).

2.4.2. Antifungal activity

According to (Murtaza and Mukhtar, 2015), the crushed tuberous extract helps to control fungal infections like ringworm that cause athletes foot (tinea pedis). Recently, the antifungal activity of *I. tinctoria* A. Rich root was evaluated by using different solvents (ethyl acetate, ethanol and water) against *C. albicans*, *T. rubrum*, *T. mentagrophytes*, *A. niger*, and *A. flavus* fungal species. The dermatophytes, *T. rubrum* and *T. mentagrophytes*, were more susceptible compared to the other fungal species at all tested extract types and concentrations. Ethyl acetate extract showed a higher inhibition of the growth of tested fungi when compared to aqueous and ethanol extracts (Degu *et al.*, 2020).

3. MATERIALS AND METHODS

3.1. General experimental materials and chemicals used

Analytical grades of n-hexane, chloroform, methanol, and ethyl acetate were purchased from Loba Chemie and used for extraction and isolation. Another chemicals and apparatus including dimethylsulfoxide, vanillin, Analytical TLC (pre-coated silica gel 60 F254 20cm x 20cm size plates, silica gel 60–120 mm mesh size for packing column chromatography were purchased used throughout the experiment. Apparatus such as a rotary evaporator (Heidolph, Germany, Laborota 4000, No. 519-0000-00-2), Uv-lamp (254 and 365 nm), FTIR and ^1H NMR (400MHz) and ^{13}C NMR (100MHz) spectroscopy were conducted on a Bruker Avance 400 spectrometer in Addis Ababa University. Genlab incubator was used for antimicrobial activity assay.

3.2. Plant material collection and authentication

The roots of *I. tinctoria* A. Rich were collected in November 2023 from Werxu Kebele, Gerar Jarso Woreda, North Shoa Zone, Oromia Region, which is 114 km from Addis Ababa. The collected plant materials were identified by a botanist, Dr. Zewdu Kasa, from the Department of Biology at Salale University. After collection, the roots were washed in clean water and dried in an open area for two weeks at room temperature protected from direct sunlight. 600 g dried tuberous root of the plant was ground using a laboratory grinder mill. The powdered sample was weighted using an electrical weighing balance in the laboratory. The resulting powder was kept in a polyethylene bag to protect it from certain environmental conditions (moisture, air, and other surrounding dust) until used for further analysis.

3.3. Experimental Sites

The crude extracts were done at Salale University, Organic Chemistry laboratory, whereas antibacterial activity test was done in Biology Department (Microbiology laboratory) at the same University. FTIR and NMR spectroscopy were done in Addis Ababa University.

3.4. Extraction and Isolation

3.4.1. Extraction of roots of *I. tinctoria* A. Rich

600 g of powdered roots of *I. tinctoria* A. Rich were soaked in a 2:1 ratio of chloroform and methanol for 72 hours. The mark was macerated three times with the same solvent system. The filtrate was concentrated using a rotary evaporator at 45 °C under reduced pressure to yield 36 g, a brown crude extract (**figure 7A**), which was stored at refrigerator. TLC analysis of the crude extract showed six spots (**Figure 7b**), using n-hexane: ethyl acetate (9:1) and chloroform: methanol (4.5:0.5) as eluent.

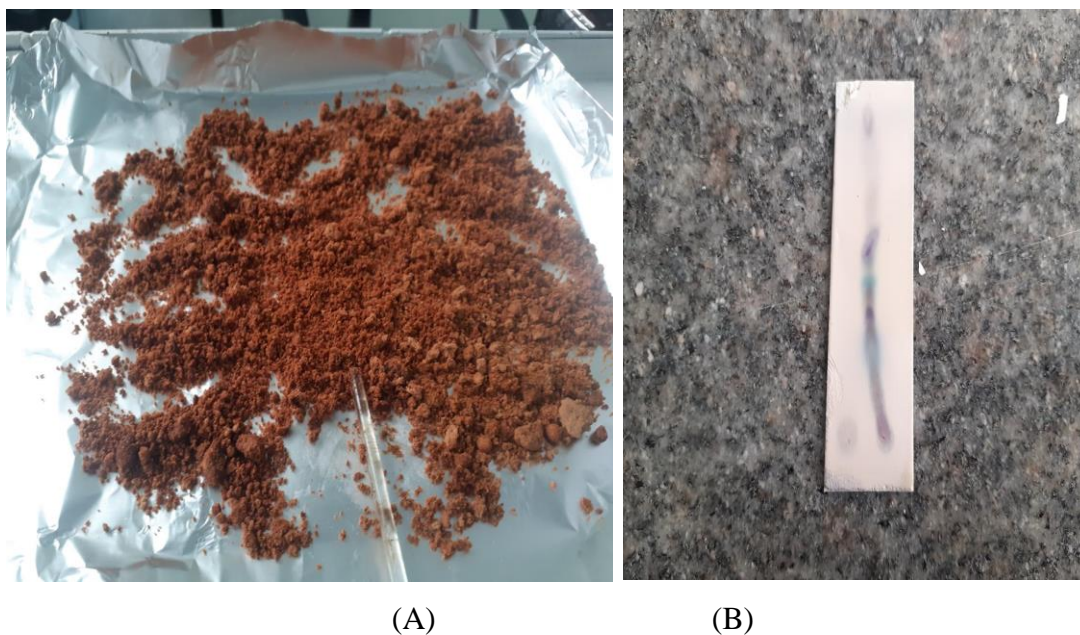


Figure 7: Crude extract and their spot on TLC

3.4.2. Isolation of roots of *I. tinctoria* A. Rich extract.

The 25 g of crude extract was adsorbed on 25 g of silica gel and loaded onto a column packed with 300 g of silica gel. After packing the column with the sample, elution of the column was using a gradient solvent system containing ethyl acetate in n-hexane (00:100 to 90:10), MeOH in CHCl_3 (5:95 to 50:50). A total of 385 fractions each with 20 ml were collected and pooled together according to their TLC profile combined fractions into ten subfractions (SF-1 to SF-10). From such ten subfractions only three sub-fractions were selected, such as SF-2, SF-4, and SF-7. But other than three were discarded because their TLC results did not show attractive spots. The sub-fraction SF-2 (90 mg) from ethyl acetate: n-hexane was loaded on 45 g silica gel repacked column chromatography using isocratic

solvent systems (1:9) and afforded compound **20** (41 mg) and compound **21** (32.5mg) (**figure 9**). Subfraction SF-4 (2.5 g) for purification subjected to column chromatography over silica gel (45g) using an isocratic solvent system of ethyl acetate in n-hexane (3:7) as eluent to give compound **22** (26.4 mg) and fraction SF-7 (865 mg) from MeOH in CHCl_3 (2:8) was repacked in column chromatography over silica gel (45 g). The collected fractions were concentrated to give compound **23** (45.2 mg) (**figure 9**). The level of separation was determined by TLC analyses, and detection was done using vanillin reagent and UV lamp (254 and 365 nm). After TLC analysis, fractions with similar R_f values were collected together as follows (**Table 1**):



Figure 8: The isolation of compound

The number of fractions, solvent system, and volume of 10 subfractions collected were shown as follows (**Table 1**).

Table 1: Fractions collected from column chromatography of I. tinctoria A. Rich

F.No.	Solvent system	Ratio	Volume of fraction collected (ml),
SF1	n-hexane/ethyl acetate	100:00	160ml
SF2	n-hexane/ethyl acetate	9:1	180ml
SF3	n-hexane/ethyl acetate	8:2	100ml
SF4	n-hexane/ethyl acetate	7:3	160ml
SF5	n-hexane/ethyl acetate	6:4	120ml
SF6	n-hexane/ethyl acetate	5:5	80ml
SF7	n-hexane/ethyl acetate	6:4	100ml
SF8	n-hexane/ethyl acetate	7:3	60ml
SF9	n-hexane/ethyl acetate	8:2	140ml
SF10	n-hexane/ethyl acetate	9:1	80ml

Purification of SF-2, by repacking afforded two compound **20** and **21** (41 mg and 32.5 mg). Compound **20** was pale red in color with $R_f = 0.4$ in n-hexane: ethylacetate (9:1), whereas compound **21** was white powder ($R_f = 0.3$, in above solvent system. Also similar procedures used for Purification of SF4 isolated compound **22** (26 mg). Compound **22** was Peach in color with $R_f = 0.5$ in n-hexane: ethylacetate (8:2). Again, by a similar method, subfraction SF-7 golden -colored compound **23** was isolated with an R_f value of 0.73 in MeOH : CHCl_3 (2:8) solvent system. Then four compounds (**20**, **21**, **22** and **23**) were isolated and submitted for spectral analyses (**figure 9**).

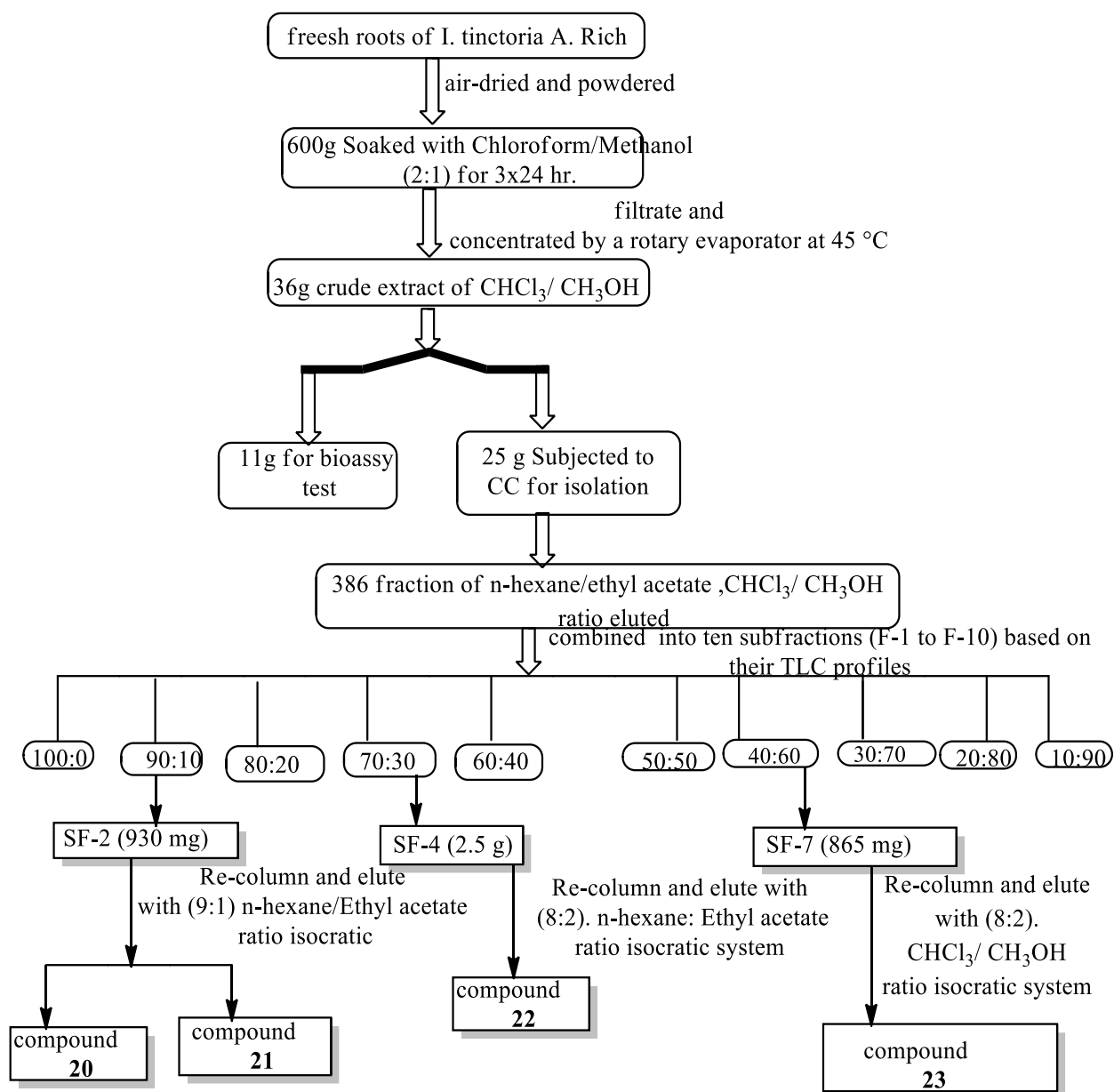


Figure 9: General procedure of extraction and isolation of roots of *I. tinctoria* A. Rich extract.

3.5. Biological Assay

3.5.1. Antibacterial activities of the extracts and isolates

Antibacterial activity of (chloroform /ethanol) extracts of the crude extract and isolated compounds from the roots of *I. tinctoria* A. Rich were evaluated by using the paper disc diffusion method against four bacterial species (two Gram positive and two Grams negative) (Table 6 and Appendix 1) namely; *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus*

aureus, and *Streptococcus pyogenes*. Experiments were done in collaboration with the Microbiology Laboratory of Biology Department of Salale University.

The medium was prepared by dissolving 38 g of Mueller Hinton agar in 1000 mL of distilled water, then heated on a hotplate and autoclaved at 121 °C for 15 min. The isolated compounds at the concentrations of 250µg/ml and 500µg/ml were prepared by dissolving in DMSO (Alaa *et al.*, 2024). 6.0 mm paper discs were prepared by cutting Whatman filter paper no. 1. The sterile discs were infused with the isolated compounds and placed on the surface of the medium with sterile forceps, and gently pressed down to ensure its contact with the MHA (Shalligito and Tesfa, 2022).

Gentamicin was used as positive controls and Dimethylsulfoxide solution was used as a negative control and then plates were inverted and incubated at 37 °C for 24 hrs (Bate *et al.*, 2020). After incubation, clear zones formed around the discs indicated the presence of antibacterial activity. The diameter of the zones of complete inhibition (including the diameter of the disk) was measured and recorded in millimeters. The assay was done in triplicates.

3.6. Molecular Docking Analysis of the Compound 2

Energy minimized ligands were prepared using Chem Draw Ultra 12.0 (both 2D and 3D) and the two proteins (PDB ID: 3Q7D and PDB ID: 3N8Z) were downloaded from protein data base (RCSB PDB) (Sidhu *et al.*, 2010). The proteins were prepared by Autodock Tool-1.5.7 (Autodock4 and autogrid4) following the standard procedure which include deleting water molecules, co-crystallized ligand and unbonded heteroatoms as well as by adding polar hydrogen, Kollman charge and by computing Gasteirger charge (Agu *et al.*, 2023)(Azam and Abbasi, 2013). The grid box was set using grid dimension X, Y, Z (60,60, 60) and a grid point spacing of 0.5 °A. The center of the grid box was adjusted from attributes of the co-crystalized ligand which were X, Y, Z = -33.212977, 56.742126, -8.577015740771 for Cyclooxygenase-1 with Flurbiprofen (Id, Title and Taz, 2017) and X,Y, Z = -36.673964, 40.375931, 40.740771 for Cyclooxygenase-2 with (R)-naproxen (Wurzberg, Tarchevskaya and Jardtetzky, 2006) respectively. Then, the docking analysis was performed for choosing the most stable conformers (least binding energy) and the 3D and 2D ligand-protein interaction for most stable conformers were drawn using Discovery Studio Visualizer.

4. RESULTS AND DISCUSSION

4.1. Structural Elucidation of the Isolated Compounds

36 g crude extract was extracted from 600 g dried powder roots of *I. tinctoria* A. Rich using chloroform-methanol (2:1) mixture and 25 g of the crude extract was subject to silica gel chromatography isolation using polarity gradient solvents systems. Further purification by repacking and isocratic chromatography isolation afforded four compounds **20**, **21**, **22** and **23**. The isolates were characterized by NMR and FTIR spectroscopy as follows.

4.1.1. Characterization of Compound **20**

Compound **20** was a pale red and its melting point is 60°C-64°C. The IR spectrum (Appendix 3) of compound **20** absorption bands of 2929 cm⁻¹ and 2842 cm⁻¹ revealed the presence of C-H asymmetric stretch for CH₃ and CH₂ stretches, respectively. The overtone band (multiple fundamental absorption frequency) of 1697-1644 cm⁻¹ indicated the existence of alkenes of C=C stretch.

The ¹H-NMR spectrum (400 MHz, CDCl₃) of compound **20** showed the presence of two methyls at δ 0.87 (3H,t,H-15) and 0.98 (3H, t, H-1. The other four olefinic protons appeared at δ 5.36 which were assigned for the olefinic protons at H-3, H-4, H-6 and H-7 respectively. From the ¹³C-NMR and DEPT-135 spectra of compound **20**, the spectrum gave 15 signals, containing two methyl (CH₃) groups, nine methylene (CH₂) groups, and four olefinic. The olefinic carbons of compound **20** appeared at δ 131.9 (C-3), 127.1 (C-6), 128.3 (C-4), and 129.9 for C-7 carbons (**Table 2**) (**Appendix 5**). Dept-135 spectrum further showed the presence nine methylene carbons δ C at 27.24 (C-2), 34.1 (C-5), 31.96 (C-8), 29.76 (C-9), 29.4 (C-10), 27.2 (C-11), 25.6 (C-12), 24.9 (C-13), 20.6 (C-14), 22.7 (C-15) (**Table 2**). The spectroscopic data suggest that the most probable structure of compound **20** is (3E, 6E)-pentadeca-3, 6-diene.

Table 2: ^1H and ^{13}C NMR (400 MHz, δ ppm, CDCl_3) spectral data for compounds (**20**)

Carbon position	Experimental ^{13}C -NMR δ (ppm)	Experimental ^1H -NMR δ (ppm)	Experimental DEPT-135 δ (ppm)	Nature of carbon
1	15.8	0.98		CH_3
2	22.7	2.03	27.24	CH_2
3	131.9	5.36		CH
4	128.3	5.36		CH
5	34.1	2.8	34.1	CH_2
6	127.1	5.36		CH
7	129.9	5.36		CH
8	31.96	2.3	31.96	CH_2
9	29.6		29.76	CH_2
10	29.4		29.4	CH_2
11	27.2		27.2	CH_2
12	25.6	1.01	25.6	CH_2
13	24.9	1.27	24.9	CH_2
14	20.6	1.31	20.6	CH_2
15	14.1	0.89	22.7	CH_3

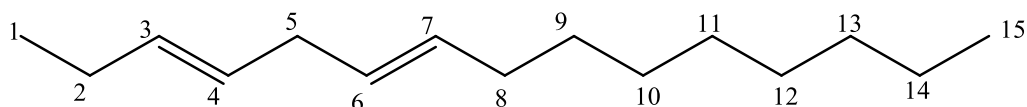


Figure 10: Chemical structures of compound **20**

4.1.2. Characterization of Compound **21**

Compound **21** (*Spinasterol*) was white powdered and its melting $164\text{--}166^\circ\text{C}$. The structure of the compound was determined based on spectroscopic data (IR, ^1H -NMR and ^{13}C -NMR). The IR spectrum (Appendix 7) of compound **21** showed absorption bands characteristic for the hydroxyl group (3382cm^{-1}) and absorption at 2935 cm^{-1} and 2867 cm^{-1} , due to aliphatic C-H stretching. Other absorption frequencies include 1695 cm^{-1} as a result C=C stretching

and weak band, at 1462 cm^{-1} is a bending frequency for cyclic $(\text{CH}_2)_n$, and 1371 cm^{-1} for $-\text{CH}(\text{CH}_3)_2$. The absorption frequency at 1050 cm^{-1} signifies cycloalkane.

The ^1H -NMR (400 MHz, CDCl_3) spectrum of compound **21** varied between 0.56 to 5.37 ppm. This spectrum showed three olefinic protons at δ 5.37, δ 5.13, and δ 5.0; a carbonyl proton at δ 3.6; and there are also eighteen protons on six methyl at δ 0.87, 0.85, 0.84, 0.83, 0.81 (s), and 0.55 (s). Which were assigned to the H-21, H-27, H-26, H-29, H-19 and H-18 protons respectively. One proton appeared at δ 5.37 as the doublet represents the endocyclic double bond proton H-7 of compound **21**. The other two olefinic protons appeared as two doublets of doublets at δ 5.04 and δ 5.16 which were assigned for the other olefinic protons at H-23 and H-22 respectively. Compound (**21**) was identified as stigmasta-7,22-diene-3 β -ol (spinasterol) by comparison of the spectroscopic data with those of the known compound (Wang *et al.*, 2011).

The ^{13}C -NMR spectrum of compound **21** showed the presence of 29 carbons. From the ^{13}C -NMR and DEPT-135 spectrum of compound **21** it was revealed that the compound has six methyl, 9 methylene, 11 methine and three quaternary carbons. The presence of an endocyclic carbon-carbon double bond of compound **21** was represented by two signals at δ 117.5 and 139.5 of C-7 and C-8. Other olefinic carbons of compound **21** appeared at δ 138.2 and 129.4 for C-22 and C-23 carbons.

Table: 3 Chemical shifts of ^1H NMR and ^{13}C NMR of compound **21**

Carbon position	Experimental ^{13}C -NMR δ (ppm)	^{13}C NMR Literature	Experimental ^1H -NMR δ (ppm)	^1H NMR Literature	Nature of carbon
1	37.1	37.1	1.39		CH_2
2	31.4	31.5	1.64		CH_2
3	71.05	71.0	3.6	3.6 (3H, m)	CH
4	37.9	38	2.5		CH_2
5	40.9	40.2	1.26		CH
6	29.7	29.7	1.71		CH_2
7	117.5	117.4	5.37	5.15 (3H, t)	CH
8	139.5	139.5	-	-	C
9	49.4	49.4	2.81		CH
10	34.2	34.2	-		C
11	23	21.5	1.2		CH_2
12	39.5	39.4	1.05		CH_2
13	43.3	43.3	-		CH
14	55.1	55.1	2.33		CH
15	25.4	23	1.5		CH_2
16	29.6	28.5	1.42		CH_2
17	51.3	55.8	1.3		CH
18	12.1	12	0.56	0.55 (3H,s)	CH_3

19	13.1	13	0.81	0.79 (3H,s)	CH ₃
20	40.3	40.8	2.82		CH
21	19	21.1	0.87	1.02 (3H, s)	CH ₃
22	138.2	138.2	5.16	5.15 (3H,dd)	CH
23	129.4	129.4	5.04	5.02 (3H,dd)	CH
24	49.44	51.2	2.15		CH
25	31.9	31.9	1.78		CH
26	21.4	19	0.83	0.8 (3H, d)	CH ₃
27	21.6	21.4	0.85	0.85 (3H, d)	CH ₃
28	28.5	25.4	1.03		CH ₂
29	12.3	12.3	0.82	0.80 (3H, t)	CH ₃

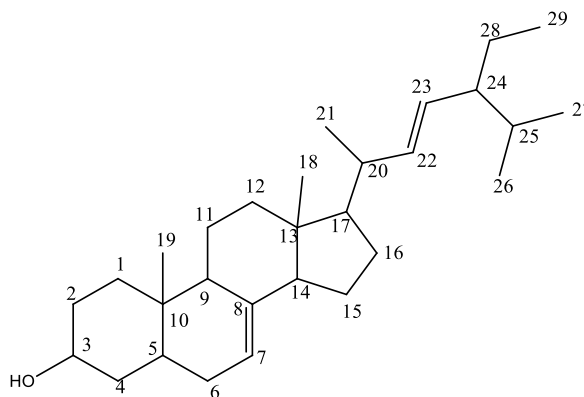


Figure 11: Chemical structures of compound **21**

4.1.3. Characterization of Compound **22**

Compound **22** was isolated as yellow semi solid with melting point 75°C -77°C. The FT-IR spectrum (Appendix 11) of this compound displayed a broad and strong absorption band at 3226-2479 cm⁻¹ which indicated the presence of hydroxyl functional group, The intense and sharp peak at 1705 cm⁻¹ confirms the presence of carbonyl carbon (C=O group of Carboxylic acid chelated with OH). the strong sharp peaks at 2926 cm⁻¹ and 2850 cm⁻¹ showed the presence of methyl and methylene C-H stretching respectively.

Compound **22** was eluted 30 % ethyl acetate with n-Hexane. The proton NMR spectrum of compound **22** showed the presence of one methyl peaks at δ 0.81 which was assigned to the H-27 proton. It also shows olefinic protons at δ 5.36, 5.38, 5.39 and 5.3781 which were assigned to the H-21, H-22, H-24 and H-25 protons respectively.

The ¹³C-NMR spectrum of compound **22** showed the presence of 28 carbons. From the ¹³C NMR and DEPT-135 spectrum of compound **22** it was revealed that the compound has one methyl, 22 methylene, 4 methine and one quaternary carbon. The four olefinic methine carbons or sp² hybridized carbon atoms resonating at δ 130 ppm, 127.7 ppm, 129.7 ppm, and

131.9ppm. Furthermore, the ^{13}C NMR spectrum also revealed the presence of one sp^3 hybridized (aliphatic) carbon, resonating at δ 14.1 and one carbonyl group resonating at δ 180.5 consistent with DEPT spectra and assigned as C-1. The DEPT-135 spectrum reveals the presence of one CH_3 resonating the peak at δ 14.1 (CH and CH_3 give positive peaks in the DEPT spectrum whereas CH_2 gives negative peaks in the DEPT spectrum) and there is no peak for quaternary carbons in the DEPT-135 spectrum. The spectroscopic data suggest that the most probable structure of compound **22** to be (22E, 25E)-octacos-22, 25-dienoate.

Table 4: Chemical shifts of ^1H NMR and ^{13}C NMR of compound **22**

Carbon Position	Experimental ^{13}C -NMR $\delta(\text{ppm})$	Experimental ^1H -NMR $\delta(\text{ppm})$	Nature of carbon
1	180.5	-	C
2	32	2.38	CH_2
3	22.6	2.08	CH_2
4	25.7	1.65	CH_2
5	25.5	0.88	CH_2
6	25.6	0.9	CH_2
7	27.1	0.91	CH_2
8	27.2	0.97	CH_2
9	27.2	0.99	CH_2
10	29	1.01	CH_2
11	29.1	1.27	CH_2
12	29.1	1.3	CH_2
13	29.2	1.32	CH_2
14	29.3	1.33	CH_2
15	29.4	1.34	CH_2
16	29.5	1.63	CH_2
17	29.6	1.66	CH_2
18	29.7	2.02	CH_2
19	29.7	2.04	CH_2
20	31.6	2.06	CH_2

21	31.8	2.36	CH ₂
22	130	5.36	CH
23	127.7	5.38	CH
24	34.2	2.8	CH ₂
25	129.7	5.39	CH
26	131.9	5.37	CH
27	24.7	2.34	CH ₂
28	14.1	0.81	CH ₃

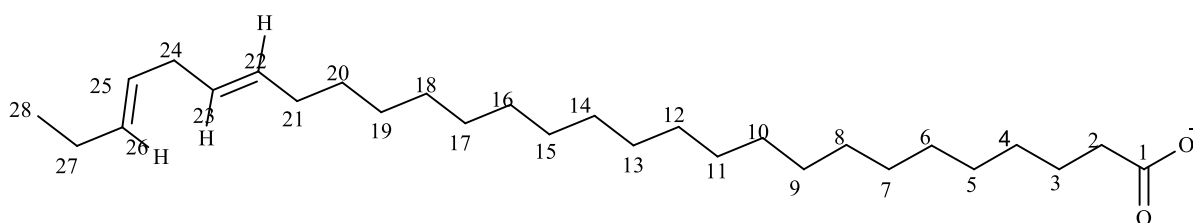


Figure 12: Chemical structures of compound **22**

4.1.4. Characterization of Compound **23**

Compound **23** was isolated as a brown color with melting point 57°C -59°C. The IR spectrum (Appendix 15, 16, 17) of compound **23** exhibited absorptions at 3368 cm⁻¹, indicating the presence of the hydroxyl group (-OH), 2921 cm⁻¹, and 2851 cm⁻¹, indicating the presence of -C-H stretching vibrations. The absorption frequency at 1076 cm⁻¹ is due to C-O. The ¹H-NMR spectra (**Table 5 and Appendices 16, 17, and 18**) showed three methyl groups at δ 0.89 (d, 3H, Me-13), δ 0.87 (t, 3H, Me-12) and δ 1.12 (t, 3H, Me-1), and the hydroxyl methine proton signal at δ 3.81 (1H, m) was attributed to H-4. Thirteen carbon atoms were found in the ¹³C-NMR and DEPT spectra, which corresponded to three methyl, eight methylenes, and one carbon atom attached to a hydroxy group (Table 5). The signals at δ 14.1, 16.8, and 20.6 indicate the presence of three methyl carbons. The signal at δ 70.9 shows the presence of a carbon atom connected to a hydroxy group. The hydroxyl group is placed in the C-4. The signals at 33.8, 31.9, 29.7, 29.5, 29.4, 29.2, 29.7, and 22.7 indicated the presence of eight methylene groups. The spectroscopic data suggest that the most probable structure of compound **23** to be 2-methyldodecan-4-ol.

Table 5: Chemical shifts of ^1H NMR and ^{13}C NMR of 23

Carbon atom	^1H NMR Experimental	^{13}C NMR Experimental	Nature of carbon
1	1.12	20.6	CH_3
2	2.65	28.8	CH
3	2.05	33.8	CH_2
4	3.81	70.9	CH
5	2.31	31.9	CH_2
6	1.62	29.7	CH_2
7	1.34	29.5	CH_2
8	1.31	29.4	CH_2
9	1.29	29.2	CH_2
10	1.26	29.7	CH_2
11	1.14	22.7	CH_2
12	0.87	14.1	CH_3
13	0.89	16.8	CH_3

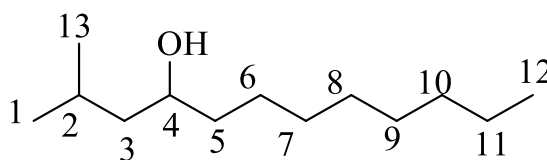


Figure 13: Chemical structures of compound 23

4.2. Antibacterial Activity Test of crude extract and isolated compounds

The results of antibacterial assay for the crude Chloroform/ethanol (2:1) extract and the isolated compounds of the roots of *I. tinctoria* A. Rich were described expressed-as mean value \pm standard Deviation (SD) of the diameter of inhibition zone. All cases, the antibacterial assay were conducted in triplicate. Gentamicin and DMSO were used as positive and the negative control respectively (**Table 6**).

Table 6: Results from the disc diffusion assay showing the antibacterial activity of crude chloroform/ethanol extract and the isolate compound of the roots of *I. tinctoria* A. Rich against four bacterial strains.

Compound	Conc. (µg/ml)	Bacterial strains			
		<i>E. coli</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>K. pnemounia</i>
20	250	7.8±0.26 mm	7.7±0.35 mm	7.83±0.46mm	7.43±0.21mm
	500	8.27±0.31 mm	8.26±0.21mm	8.5±0.2 mm	7.93±0.29 mm
21	250	8.17±0.15 mm	8.17±0.21mm	NI	7.67±0.29 mm
	500	8.5±0.1 mm	8.7±0.2 mm	NI	8.37±0.21 mm
22	250	9.1±0.12 mm	8.5±0.4 mm	7.9±0.66 mm	7.63±0.55 mm
	500	9.6±0.36 mm	8.97±0.25 mm	8.23±0.25 mm	8.57±0.12 mm
23	250	9.5±0.3 mm	8.57±0.12 mm	9.2±0.29 mm	8.3±0.36 mm
	500	9.9±0.36 mm	9.13±0.15 mm	9.87±0.36 mm	8.9±0.36 mm
Crude extract	250	7.9± 0.26 mm	8± 0.3 mm	7.77± 0.15 mm	8± 0.1 mm
	500	8.49± 0.22 mm	8.2± 0.16 mm	8.3± 0.27 mm	8.2± 0.3 mm
Gentamycin	10	22	19	21	24

The zones of inhibition were measured and found to fall in the range of 7.77–8.49 mm for chloroform/methanol (2:1) crude extract. The inhibition zone for the four isolated compounds against *E. coli* ranges from 7.8 to 9.5 mm at a concentration of 250 µg/mL compared to standard Gentamycin (22 mm). On average, compound **23** showed the largest inhibition zone of all the four bacterial strains. Compound **23** exhibited the maximum inhibition zone, which was 9.9 mm against *E. coli* and the least against *S. pyogenes* (8.87 mm) at 500 µg/mL. Except for compound **20**, all three compounds showed their maximum inhibition against *E. coli*. Compound **20** showed the highest inhibition zone (8.50 mm) against *S. pyogenes* and the lowest inhibition zone (7.93 mm) against *K. pnemounia* at 500 µg/mL. The highest activity was recorded for **23** (9.2 mm), followed by **22** (7.9 mm) at a concentration of 250 µg/mL, and **21** didn't show any activity against *S. pyogenes*.

Generally, at 500µg/mL concentration, compounds **20** and **22** displayed moderate to good activity against all tested bacterial species.

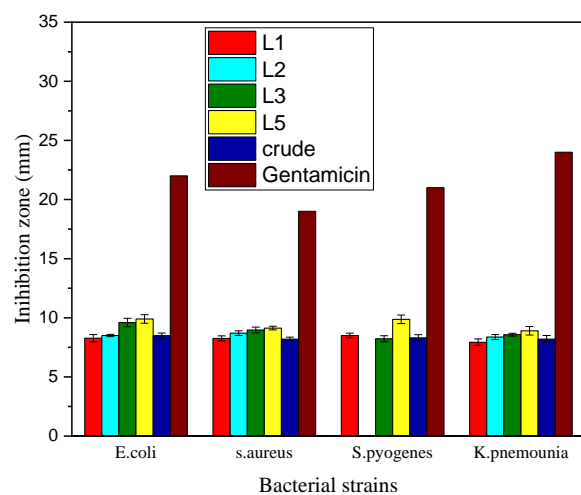


Figure 14: The inhibition zone of the isolated compounds and crude extract in mm (mean \pm SD) at 250 μ g/mL.

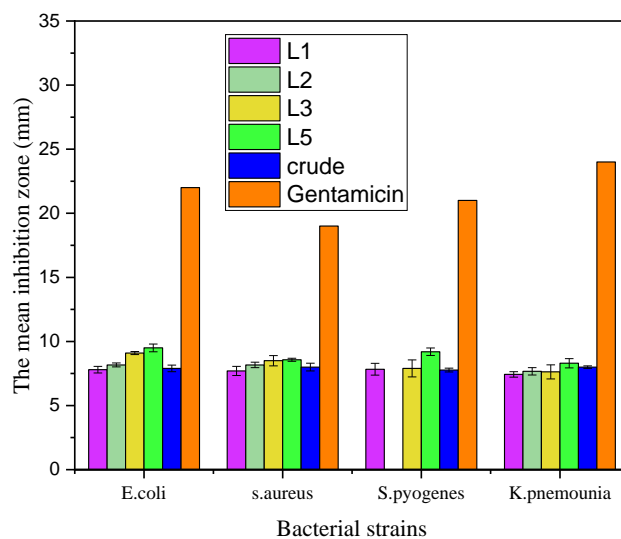


Figure 15: The inhibition zone of the isolated compounds and crude extract in mm (mean \pm SD) at 150 μ g/mL.

4.3. Molecular Docking study

Molecular Docking is an essential aspect of *in-silico* drug development. This technique involves predicting the interaction between a small molecule and a protein at the atomic level (Agu *et al.*, 2023). This enables researchers to study the behavior of small bioactive molecules within the binding site of a target protein and appreciate the basic biochemical process underlying this interaction. It has become a progressively essential instrument for drug development (Bartuzi *et al.*, 2017). The process of inflammation causes the initiation and progression of several disorders, including asthma, rheumatoid arthritis, cardiovascular diseases, diabetes, Crohn's disease, multiple sclerosis, neurodegenerative conditions (Alzheimer's disease), and even cancer (Redzicka *et al.*, 2023). The activation of cyclooxygenases (COX) and lipoxygenases (LOX) produce inflammatory mediators from arachidonic acid (Redzicka *et al.*, 2023). Three isoforms of COX enzymes, COX-1, COX-2, and COX-3 were identified (Faki and Er, 2021). COX-2 is produced during inflammation, pain, and on cogenesis and can be found in many cell types, including brain, kidney, endothelial cells, reproductive tissues, inflamed tissues, and tumor cells. In addition to inflammation, overexpression of cyclooxygenase-2 (COX-2) is linked with breast cancer. Several studies have shown that COX-2-derived metabolites involved in the development of tumor survival, pre-cancerous hyperproliferation, tumor development, transformation, invasion, and metastatic spread (Selvaraj, 2020)(Jones Lipinski *et al.*, 2021). Thus, compounds expected to inhibit the synthesis of leukotriene are pharmaceutically significant to treat inflammation and some form of cancers. With anticipation and ethno botanical study, one of the isolated compounds (**21**) and two NSAIDs (Flurbiprofen and (R)-naproxen) were docked against two cyclooxygenase (COX-1 and COX-2) and the docking results were compared. The docking result showed very nice result. Compound **21** has strong binding affinity for above two enzymes than the above NSAIDs. Thus, **21** is strong inhibitor of cyclooxygenases, which appeared to have antiinflammation and anti-breast cancer properties (Table 7). This finding strongly supports ethnobotanical and the traditional uses of tuberous root of *Impatiens tinctoria* A. Rich for inflammation and cultural cosmetics.

Table 7: The minimum binding energy and interacting amino acids in the molecular docking of ligands and against human Cyclooxygenase-1 ((PDB ID: 3N8Z) and Cyclooxygenase-2 (3Q7D)

Enzymes	Compound No	Binding energy (kcal/mol)	H-bond	Residual interactions	
				Hydrophobic/ Pi –sigma/ anion	Van der Waals
Cyclooxygenase -1 (PDB ID: 3N8Z)	21	-8.20	Ser-541		His-226, Pro-538, Tyr-544
	Flurbiprofen	-8.14	Arg-374, Arg-376, Asn-375, Pro-538	Gly-227, Tyr-373, Gly-536	
Cyclooxygenase-2 PDB ID: 3Q7D)	21	-9.59	Gly-533, Asp-537		Phe-142, Leu-145, Pro-538
	(R)-naproxen	-6.15	Gly-227, Val-228, GAsn-375, Gly-533, Asp-537	Gln-374, Pro-538	

There is also addition attractive trend with compound **21** its binding energy was -8.20 kcal/mol with Cyclooxygenase-1, while -9.59 kcal/mol for Cyclooxygenase-2, indicating it is more selective for cyclooxygenase-2 than cyclooxygenase-1. COX-1 is continuously synthesized and is involved in the maintenance of physiological events and expressed mainly in the kidneys, lung mucosa, gastric, and, as well as on platelets. When it is also induced by inflammatory factors, COX-1 is expressed in all tissues. Under normal condition, it is used for the synthesis of prostaglandins, which protect the gastric mucosa and regulate platelet aggregation and renal blood flow. Thus it is among important enzymes (Redzicka *et al.*, 2023). Whereas COX-2 is induced during inflammation, pain, and oncogenesis and can be found in many cell types including brain and breast. Thus, selective inhibitors of COX-2 are among candidate drugs needed to treat inflammation and breast cancer (Faki and Er, 2021)(Selvaraj, 2020). The 3D and 2D binding interactions of compound **21** and two standard NSAIDs against human Cyclooxygenase-1 and Cyclooxygenase-2 displayed below (Figure 15-19)

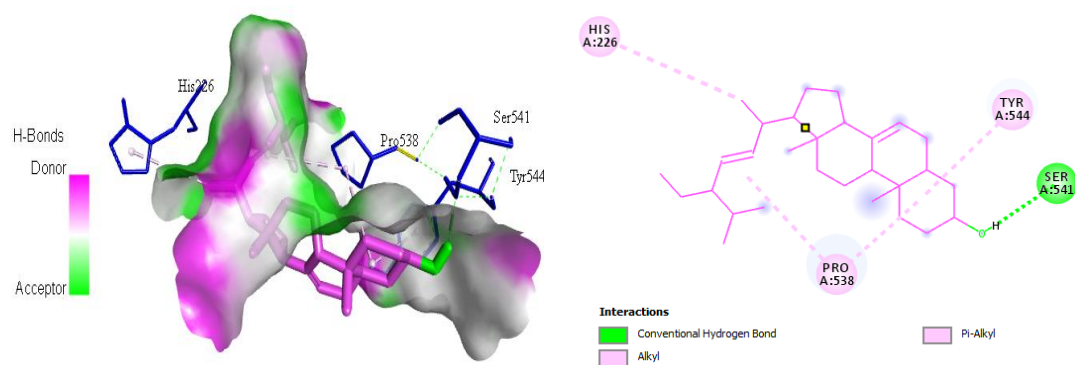


Figure 16: The 3D and 2D binding interactions of **21** against human Cyclooxygenase-1 (PDB ID: 3N8Z)

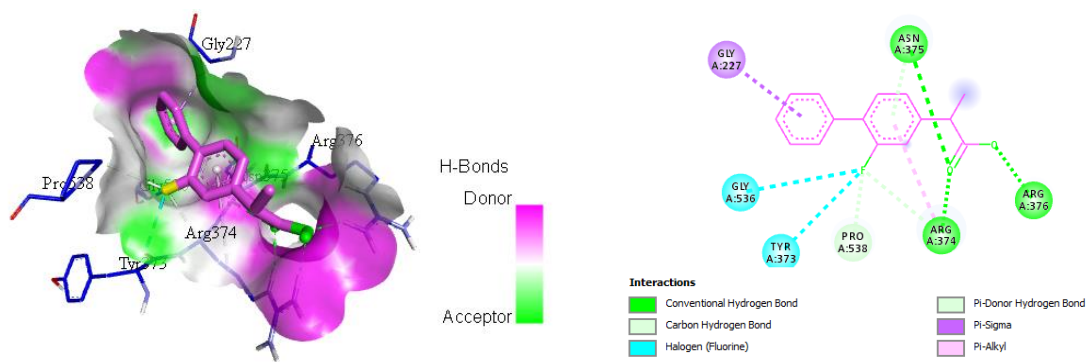


Figure 17: The 3D and 2D binding interactions of Flurbiprofen against human Cyclooxygenase-1 (PDB ID: 3N8Z)

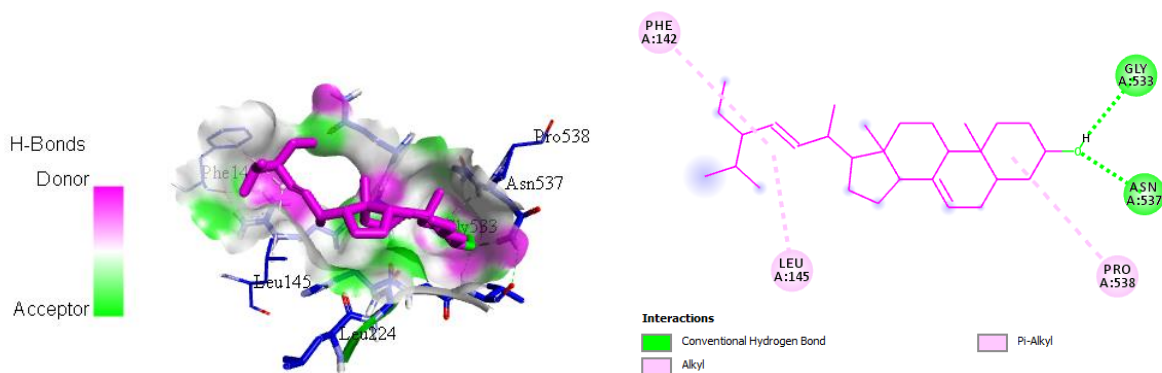


Figure 18: The 3D and 2D binding interactions of **21** against human Cyclooxygenase-2 (2 PDB ID: 3Q7D)

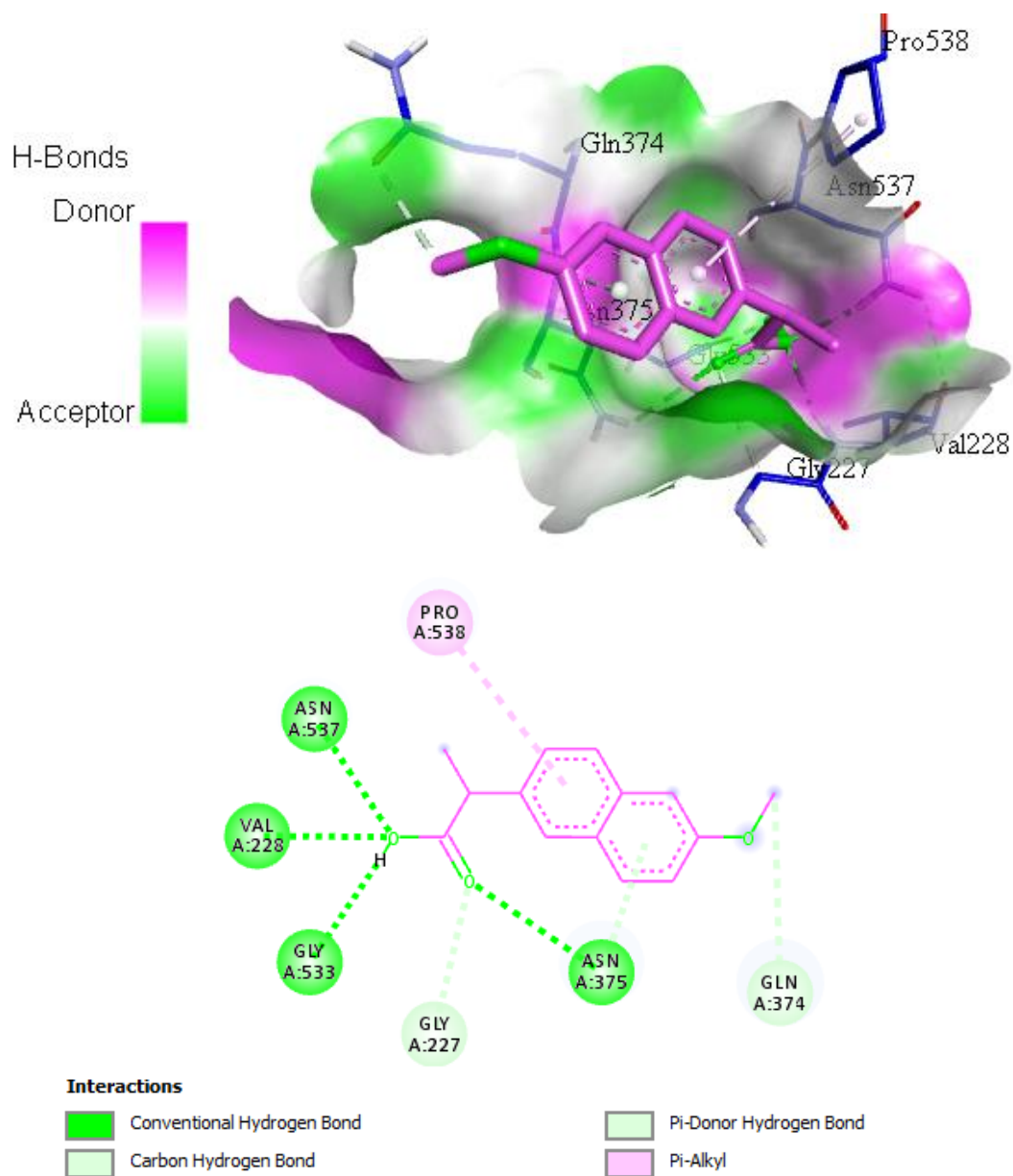


Figure 19: The 3D and 2D binding interactions of (R)-naproxen against human Cyclooxygenase-2 (2 PDB ID: 3Q7D)

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

Plant secondary metabolites have got wide application in pharmaceutical and nonpharmaceutical industries including dye, food processing and cosmetic industries. Traditionally *I. tinctoria* A. Rich widely used as antiinflammation and home-made cosmetics. Driven by this study, phytochemical investigation and antibacterial assay were carried out on root *I. tinctoria* A. Rich. Four phytochemicals were isolated and characterized by NMR and FTIR spectroscopy data. Both the crude extract as well as the isolates showed moderate activity against both Gram-negative and Gram-positive bacterial strains.

The isolated compounds displayed better *in vitro* antibacterial activity against four bacteria strains (*Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Streptococcus pyogenes*) than the crude extract. Also, the molecular docking result of Compound **21** against human cyclooxygenases, showed very nice result. So far, the molecular docking of results of **21** appeared as strong inhibitor of cyclooxygenases, which contribute to have anti-inflammation and anti-breast cancer properties. Thus, present study indicated the plant root tuber possessed moderate antibacterial activity and nice anti-inflammation properties because of its phytochemical constituents.

5.2. Recommendation

Based on the current findings, the following are recommended for the study:

- ❖ 2D NMR and Mass spectroscopy should be used to confirm the structure of the compounds.
- ❖ Further phytochemical investigation and antimicrobial activity assay needs to be conducted.
- ❖ More biological assays such as antioxidant, antiplasmodial, and anti-fungal activities need to be conducted on various extracts and their isolates of the plant so as to validate the traditional use of the plant.

REFERENCE

- Ab Razak, N. *et al.* (2023) 'The Invasive Plant *Impatiens glandulifera* Manipulates Microbial Associates of Competing Native Species', *Plants*, 12(7), pp. 1–18. Available at: <https://doi.org/10.3390/plants12071552>.
- Access, G.R., Directorate, B.S. and Seboka, N. (2017) 'Bioprospecting Potential of *Impatiens tinctoria* for Access and Benefit Sharing', pp. 1–6.
- Agidew, M.G. (2022) 'Phytochemical analysis of some selected traditional medicinal plants in Ethiopia', *Bulletin of the National Research Centre*, 46(1). Available at: <https://doi.org/10.1186/s42269-022-00770-8>.
- Agu, P.C. *et al.* (2023) 'Molecular docking as a tool for the discovery of molecular targets of nutraceuticals in diseases management', *Scientific Reports*, 13(1), pp. 1–18. Available at: <https://doi.org/10.1038/s41598-023-40160-2>.
- Akinmoladun, A.C. *et al.* (2007) 'Phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum*', *Scientific Research and Essay*, 2(May), pp. 163–166.
- Alaa, H. *et al.* (2024) 'Bacteriostatic and antidiabetic activities of various extracts from the mixture leaves and stems of *Suaeda maritima*', *Journal of Umm Al-Qura University for Applied Sciences* [Preprint], (0123456789). Available at: <https://doi.org/10.1007/s43994-023-00110-0>.
- Azam, S.S. and Abbasi, S.W. (2013) 'Molecular docking studies for the identification of novel melatonergic inhibitors for acetylserotonin-O-methyltransferase using different docking routines', *Theoretical Biology and Medical Modelling*, 10(1), pp. 1–16. Available at: <https://doi.org/10.1186/1742-4682-10-63>.
- Bartuzi, D. *et al.* (2017) 'Recent advances and applications of molecular docking to G protein-coupled receptors', *Molecules*, 22(2), pp. 1–23. Available at: <https://doi.org/10.3390/molecules22020340>.
- Bate, P.N.N. *et al.* (2020) 'In vitro activity against multi-drug resistant bacteria and cytotoxicity of lichens collected from Mount Cameroon', *Journal of King Saud University - Science*, 32(1), pp. 614–619. Available at: <https://doi.org/10.1016/j.jksus.2018.09.001>.

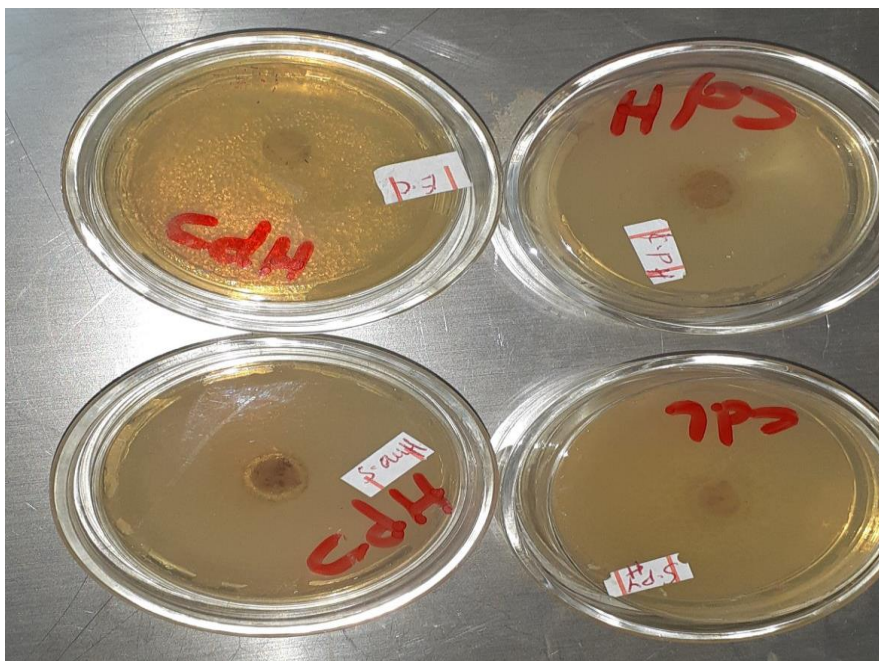
- Block, A.K., Yakubova, E. and Widhalm, J.R. (2019) ‘Specialized naphthoquinones present in *Impatiens glandulifera* nectaries inhibit the growth of fungal nectar microbes’, *Plant Direct*, 3(5), pp. 1–7. Available at: <https://doi.org/10.1002/pld3.132>.
- D, N.E., Rejimon, G. and Varghese, A. (2015) ‘*Impatiens balsamina*: An overview’, *Journal of Chemical and Pharmaceutical Research*, 7(9), pp. 16–21. Available at: www.jocpr.com.
- Degu, S. (2019) ‘Evaluation of antibacterial and antifungal activity , phytochemical content and acute oral toxicity of *Impatiens tinctoria* A . Rich root extracts By : Sileshi Degu (BSc , MSc candidate) Advisors : Adane Bitew (MSc , PhD) Negero Gemedo (MSc , PhD fell’.
- Degu, S. *et al.* (2021) ‘Evaluation of antibacterial and acute oral toxicity of *Impatiens tinctoria* A. Rich root extracts’, *PLoS ONE*, 16(8 August), pp. 1–13. Available at: <https://doi.org/10.1371/journal.pone.0255932>.
- Duke, T.N. (2019) ‘Phenolic and Glycoside Compounds Obtained from *Brucea antidysenterica*’, *Chemical and Process Engineering Research* [Preprint], (March). Available at: <https://doi.org/10.7176/cper/60-01>.
- Faki, Y. and Er, A. (2021) ‘Different chemical structures and physiological/pathological roles of cyclooxygenases’, *Rambam Maimonides Medical Journal*, 12(1), pp. 1–13. Available at: <https://doi.org/10.5041/RMMJ.10426>.
- Gidamo, G.H. (2023) ‘Antioxidant activity and mineral content of *Impatiens tinctoria* A. Rich (Ensosila) tuber, an Ethiopian medicinal plant’, *Scientific Reports*, 13(1), pp. 1–7. Available at: <https://doi.org/10.1038/s41598-023-41824-9>.
- Id, P.D.B., Title, K.J.E. and Taz, C.B.P. (2017) ‘Full wwPDB NMR Structure Validation Report o’, 01(2010), pp. 1–20.
- Jackson, N., Czaplewski, L. and Piddock, L.J.V. (2018) ‘Discovery and development of new antibacterial drugs: Learning from experience?’, *Journal of Antimicrobial Chemotherapy*, 73(6), pp. 1452–1459. Available at: <https://doi.org/10.1093/jac/dky019>.
- Jones Lipinski, R.A. *et al.* (2021) ‘Molecular docking-guided synthesis of NSAID–glucosamine bioconjugates and their evaluation as COX-1/COX-2 inhibitors with potentially reduced gastric toxicity’, *Chemical Biology and Drug Design*, 98(1), pp.

- 102–113. Available at: <https://doi.org/10.1111/cbdd.13855>.
- Kim, D.H. *et al.* (2019) ‘Chemical constituents of *Impatiens balsamina* stems and their biological activities’, *Natural Product Sciences*, 25(2), pp. 130–135. Available at: <https://doi.org/10.20307/nps.2019.25.2.130>.
- Lee, T.H., Suh, W.S., Subedi, L., Kim, S.Y., Choi, S.U., Lee, K.R. and Kim, C.S., 2020. Three new oleanane-type triterpenoidal glycosides from *Impatiens balsamina* and their biological activity. *Plants*, 9(9), p.1083.
- Moges, A. and Moges, Y. (2020) ‘Ethiopian Common Medicinal Plants: Their Parts and Uses in Traditional Medicine - Ecology and Quality Control’, *Plant Science - Structure, Anatomy and Physiology in Plants Cultured in Vivo and in Vitro*, pp. 1–20. Available at: <https://doi.org/10.5772/intechopen.86202>.
- Paun, G. *et al.* (2018) ‘Anti-inflammatory and antioxidant activities of the *Impatiens noli-tangere* and *Stachys officinalis* polyphenolic-rich extracts’, 28, pp. 57–64.
- Pires, E.O. *et al.* (2021) ‘Current status of genus *Impatiens*: Bioactive compounds and natural pigments with health benefits’, *Trends in Food Science and Technology*, 117(February), pp. 106–124. Available at: <https://doi.org/10.1016/j.tifs.2021.01.074>.
- Redzicka, A. *et al.* (2023) ‘Design, Synthesis, Biological Evaluation, and Molecular Docking Study of 4,6-Dimethyl-5-aryl/alkyl-2-[2-hydroxy-3-(4-substituted-1-piperazinyl)propyl]pyrrolo[3,4-c]pyrrole-1,3(2H,5H)-diones as Anti-Inflammatory Agents with Dual Inhibition of COX and LOX’, *Pharmaceuticals*, 16(6). Available at: <https://doi.org/10.3390/ph16060804>.
- Selvaraj, J. (2020) ‘Molecular docking analysis of COX-2 for potential inhibitors’, *Bioinformation*, 16(10), pp. 753–758. Available at: <https://doi.org/10.6026/97320630016753>.
- Shalligito, H.A. and Tesfa, K.H. (2022) ‘Phytochemical Investigation and Antibacterial Activity Assessment of *Zehneria scabra* (L . f) Sond (ሀረግ ሬሃ) Leaves Extract Department of Chemistry , Jinka University , E - mail : habtamuabebe20@gmail.com Department of Biochemistry , College of Medicine’, 2(1), pp. 247–257.
- Sharma, V.R. (2022) ‘ ta in Chronic Unpredictable Mild Stress Induced Behavioral Alterations in Rats’. Available at: <https://doi.org/10.4103/asl.ASL>.
- Sidhu, R.S. *et al.* (2010) ‘Comparison of cyclooxygenase-1 crystal structures: cross-talk

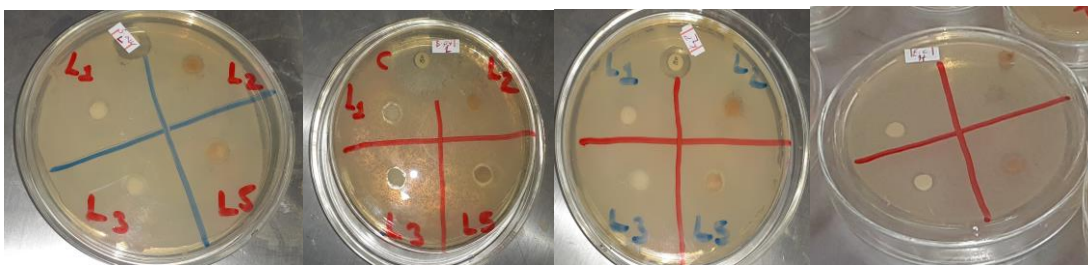
- between monomers comprising cyclooxygenase-1 homodimers.’, *Biochemistry*, 49(33), pp. 7069–7079. Available at: <https://doi.org/10.1021/bi1003298>.
- Pires Jr, E.D.O., Pereira, E., Pereira, C., Dias, M.I., Calhelha, R.C., Ćirić, A., Soković, M., Hassemer, G., Garcia, C.C., Caleja, C. and Barros, L., 2021. Chemical composition and bioactive characterisation of *Impatiens walleriana*. *Molecules*, 26(5), p.1347.
- Szewczyk, K. (2018) ‘Phytochemistry of the genus *impatiens* (Balsaminaceae): A review’, *Biochemical Systematics and Ecology*, 80(April), pp. 94–121. Available at: <https://doi.org/10.1016/j.bse.2018.07.001>.
- Thangavelu, M. and Arumugam, P. (2020) ‘Influence of an Arbuscular Mycorrhizal Fungus and Phosphate-Solubilizing Bacterium Inoculation at Stem Cutting Stage on P Uptake and Growth of *Impatiens walleriana* Plants in an Unsterile Field Soil’, *Journal of Horticultural Research*, 27(2), pp. 11–22. Available at: <https://doi.org/10.2478/johr-2019-0015>.
- Ugboko, H.U., Nwinyi, O.C., Oranusi, S.U., Fatoki, T.H. and Omonhinmin, C.A., 2020. Antimicrobial importance of medicinal plants in Nigeria. *The Scientific World Journal*, 2020.
- Wang, Y.C. *et al.* (2011) ‘In vitro activity of 2-methoxy-1,4-naphthoquinone and stigmasta-7,22-diene- 3 β -ol from *Impatiens balsamina* L. against multiple antibiotic-resistant *Helicobacter pylori*’, *Evidence-based Complementary and Alternative Medicine*, 2011. Available at: <https://doi.org/10.1093/ecam/nep147>.
- Wolde-mariam, M., Limenih, Y. and Umer, S. (2015) ‘Ethnobotanical study on traditional medicinal plants in Dega Damot woreda, Amhara region, North Ethiopia’, *International Journal of Research in Pharmacy and Chemistry*, 5(2), pp. 258–273.
- Wurzburg, B.A., Tarchevskaya, S.S. and Jardetzky, T.S. (2006) ‘Structural Changes in the Lectin Domain of CD23, the Low-Affinity IgE Receptor, upon Calcium Binding’, *Structure*, 14(6), pp. 1049–1058. Available at: <https://doi.org/10.1016/j.str.2006.03.017>.

APPENDICES

Appendix 1: Antimicrobial activity Tests of Crude Extracts



Appendix 2: Bioassays Tests of Isolated Compound Zone of Inhibition in mm



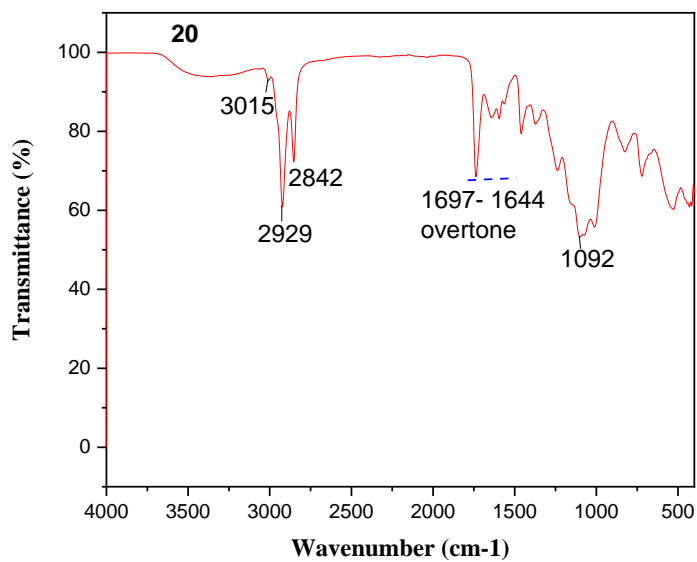
S.aureus

S. pyogenes

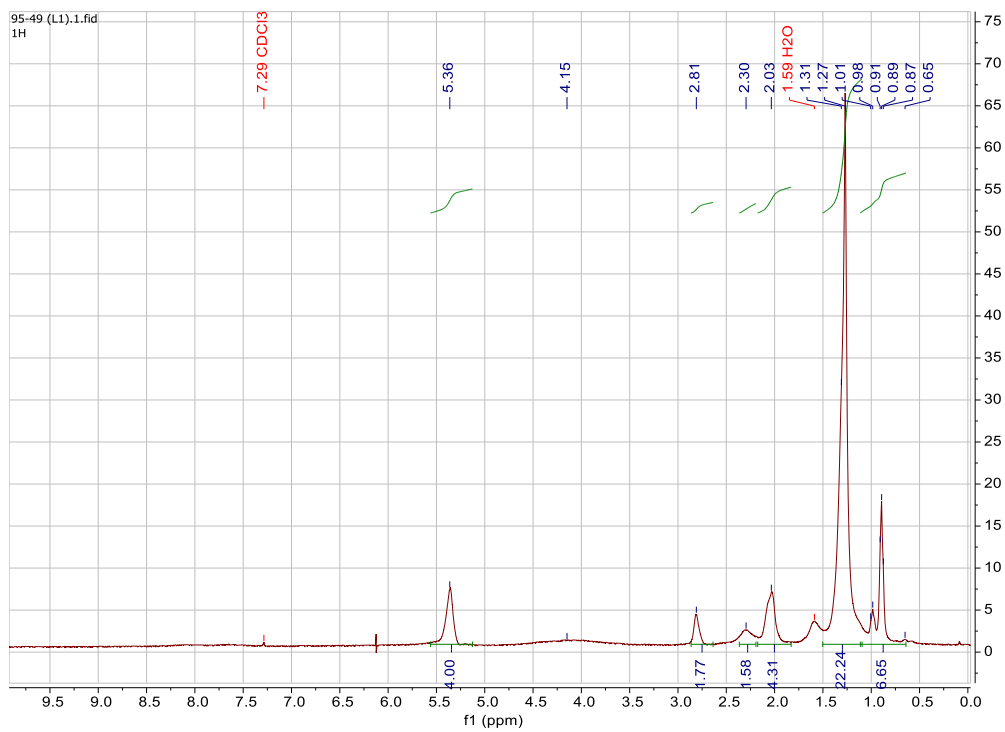
K.pnemounia

E.coli

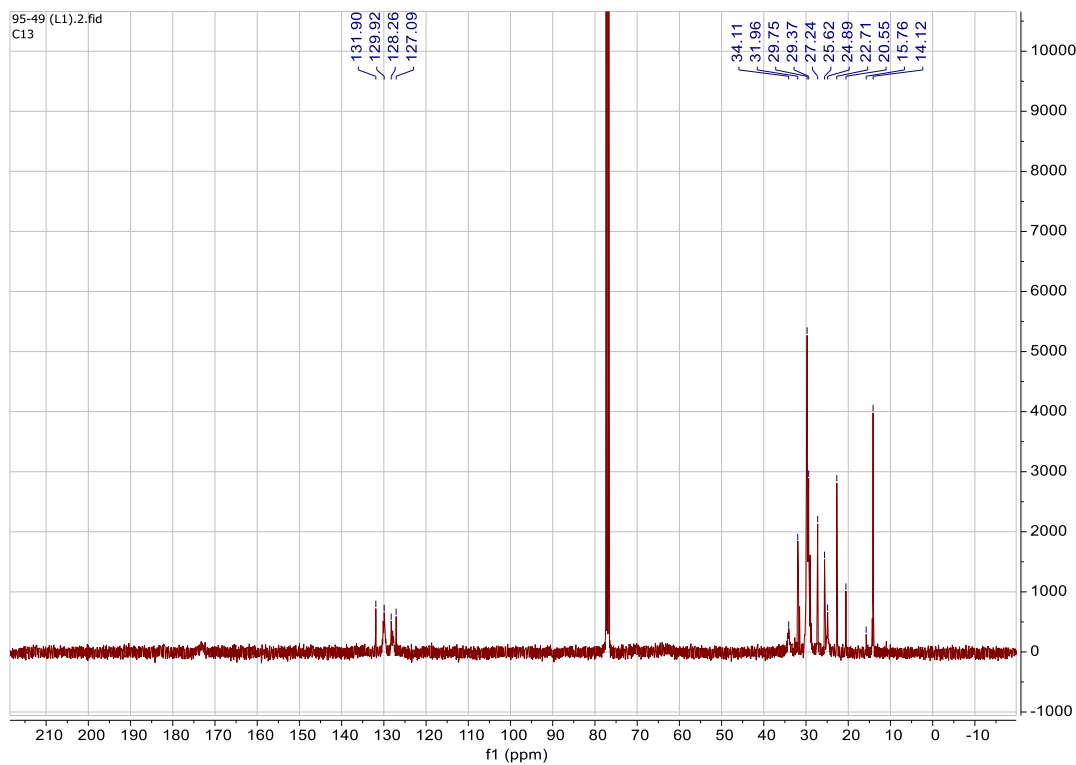
Appendix 3: IR Spectrum of 20



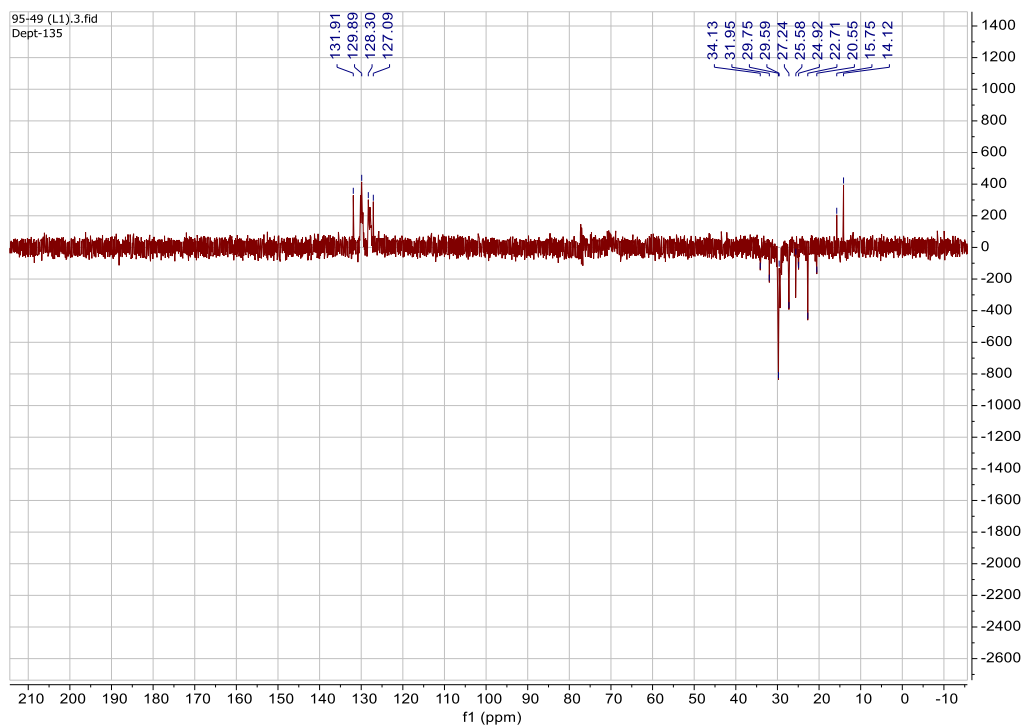
Appendix 4: ^1H NMR spectrum of 20



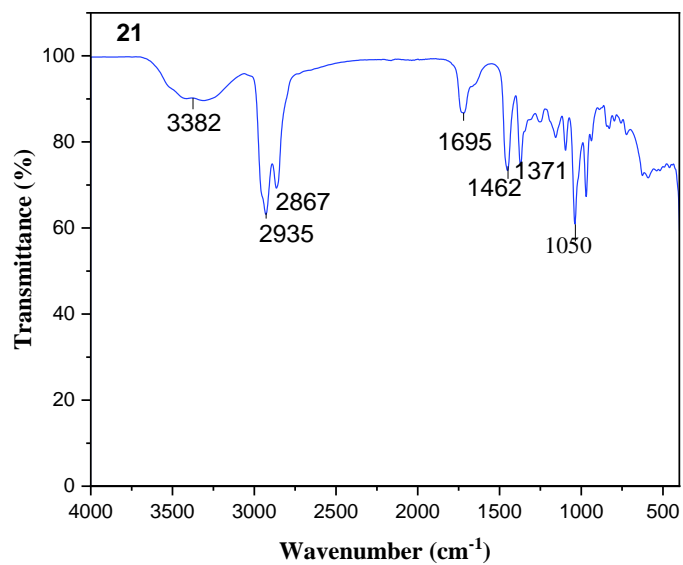
Appendix 5: ^{13}C NMR spectrum of **20** in CDCl_3 as solvent.



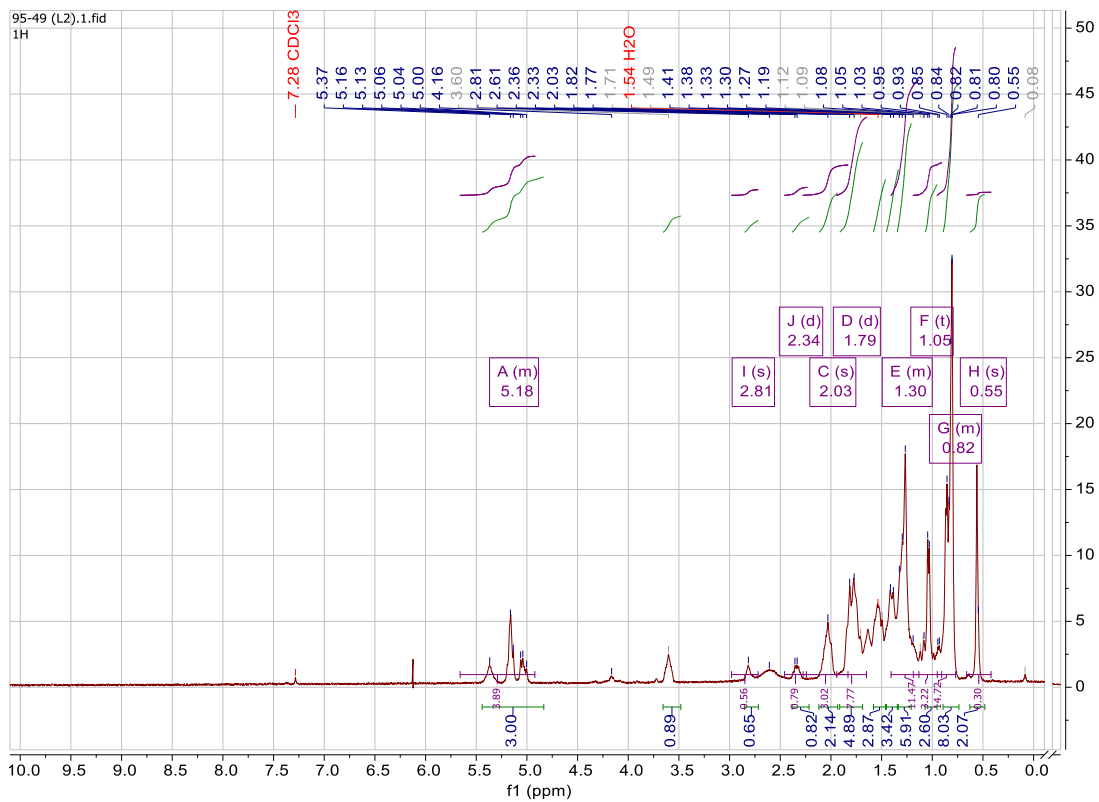
Appendix 6: DEPT-135 spectrum of **20** in CDCl_3 as solvent.



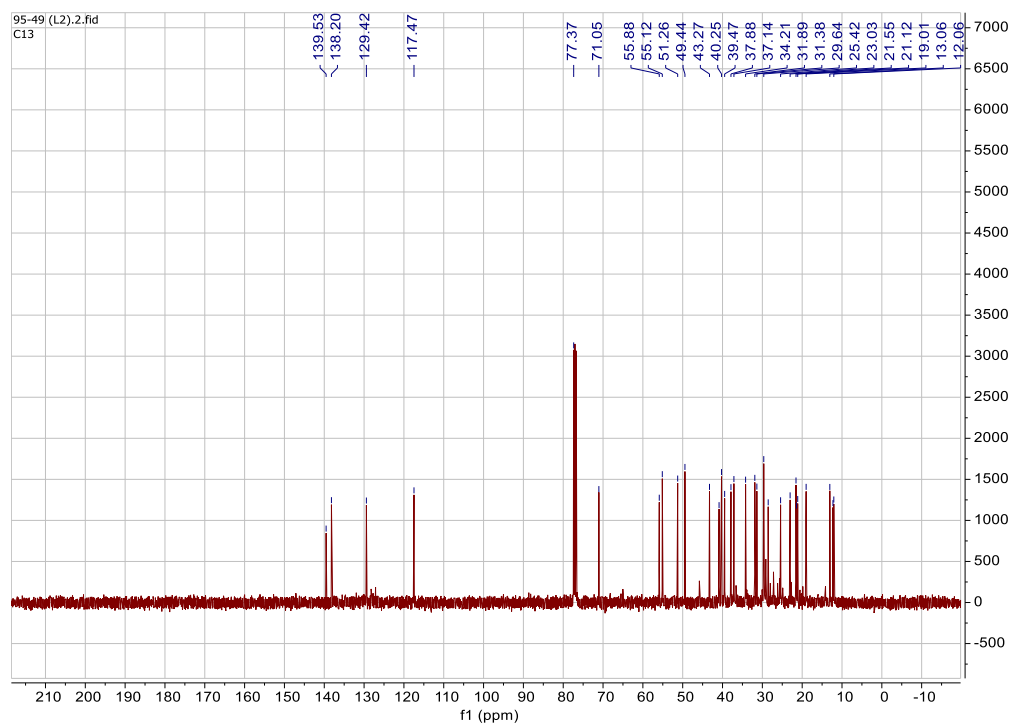
Appendix 7: IR Spectrum of **21**



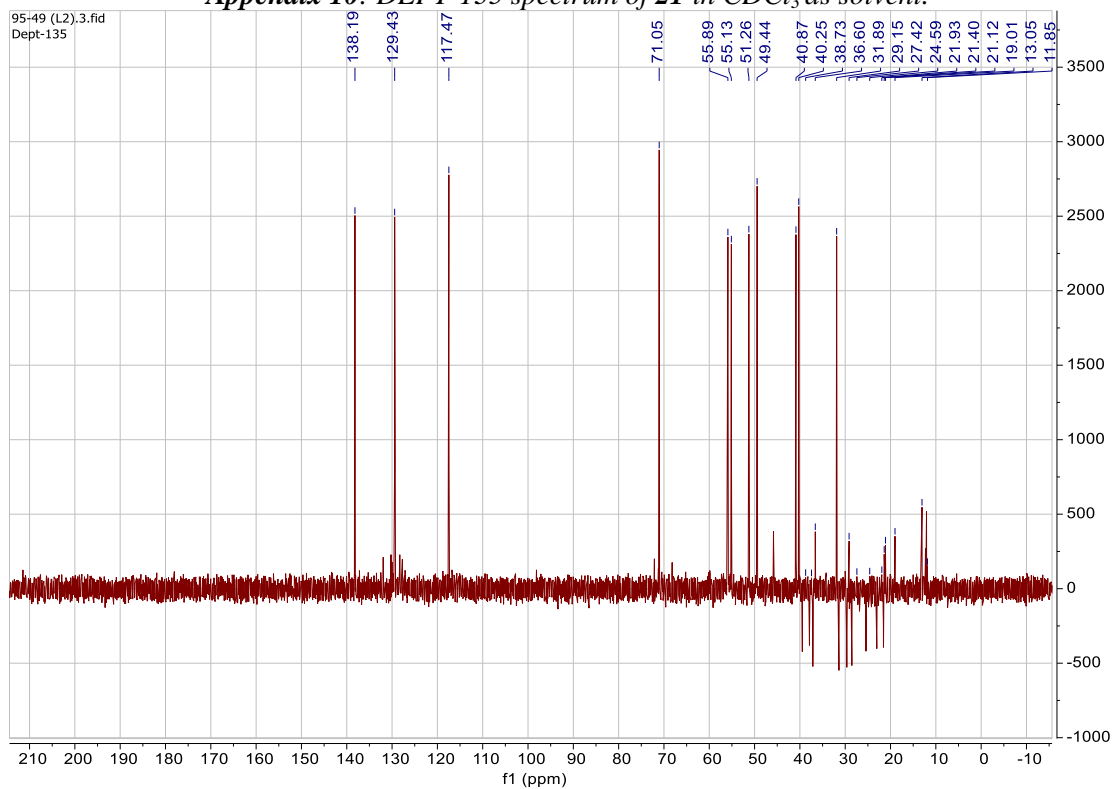
Appendix 8: ¹H NMR spectrum of **21**



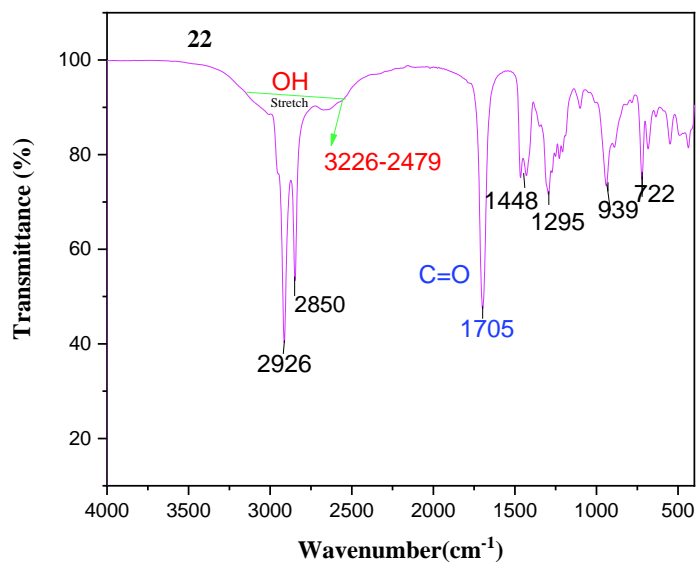
Appendix 9: ^{13}C NMR spectrum of **21** in CDCl_3 as solvent.



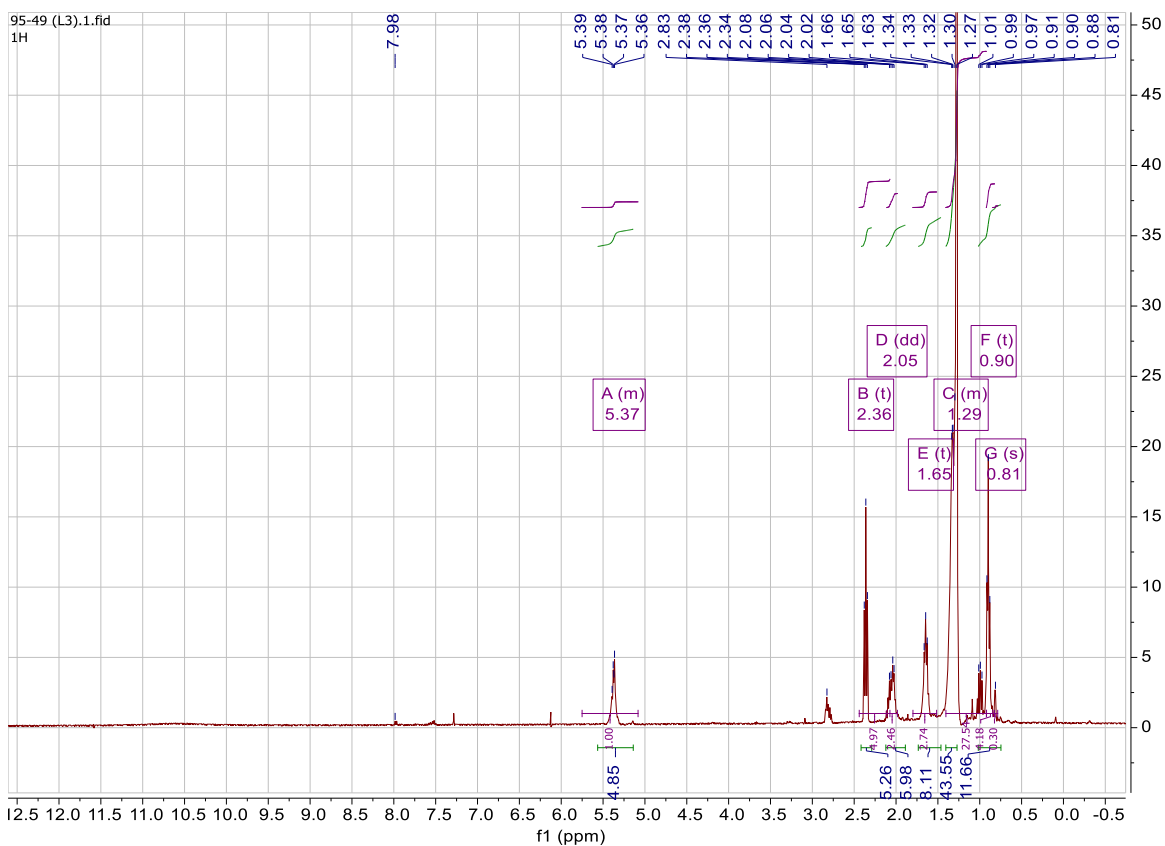
Appendix 10: DEPT-135 spectrum of **21** in CDCl_3 as solvent.



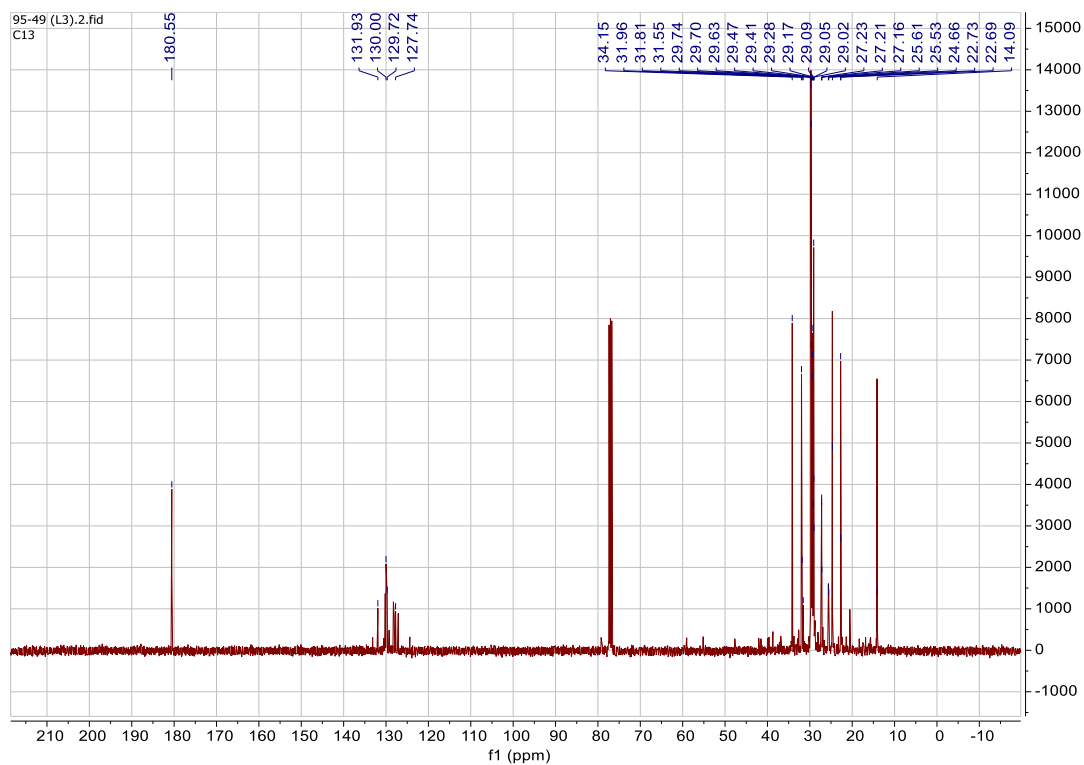
Appendix 11: FT-IR Spectrum of Compound 22



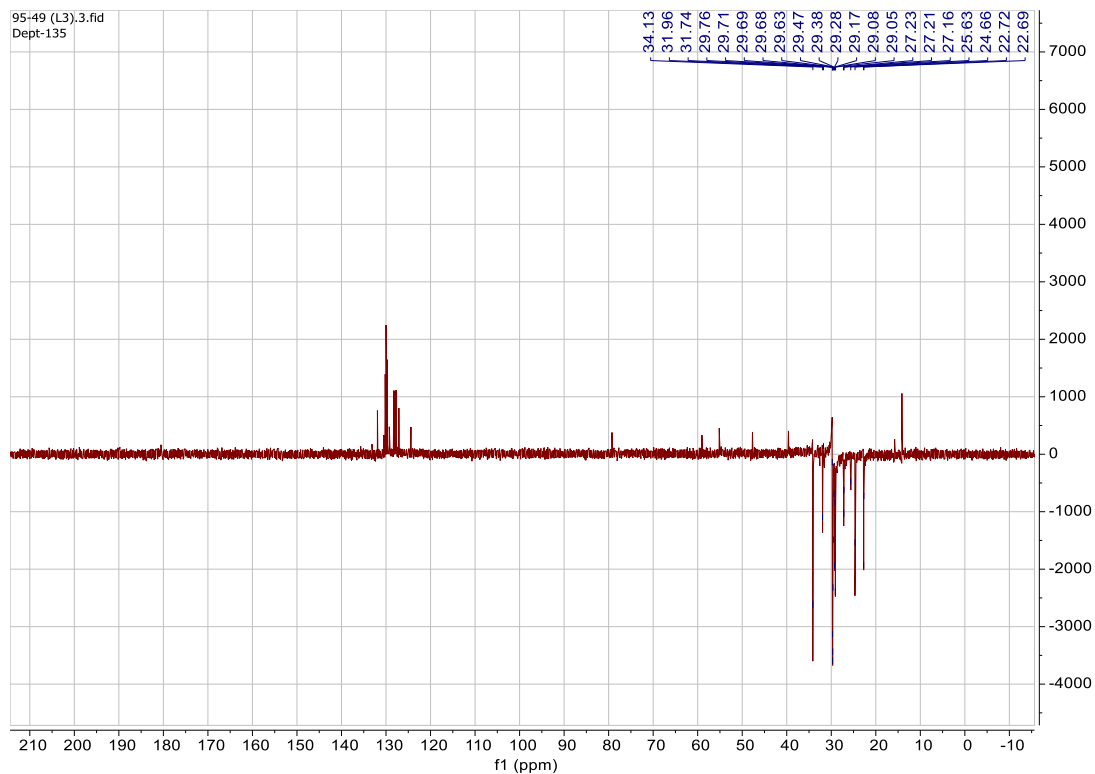
Appendix 12: ¹H-NMR Spectral data of compound 22



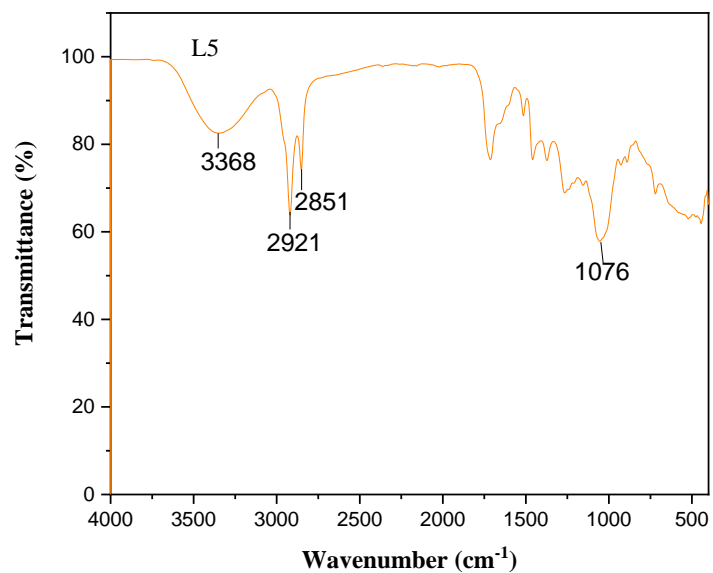
*Appendix 13: ^{13}C NMR spectrum of **22** in CDCl_3 as solvent.*



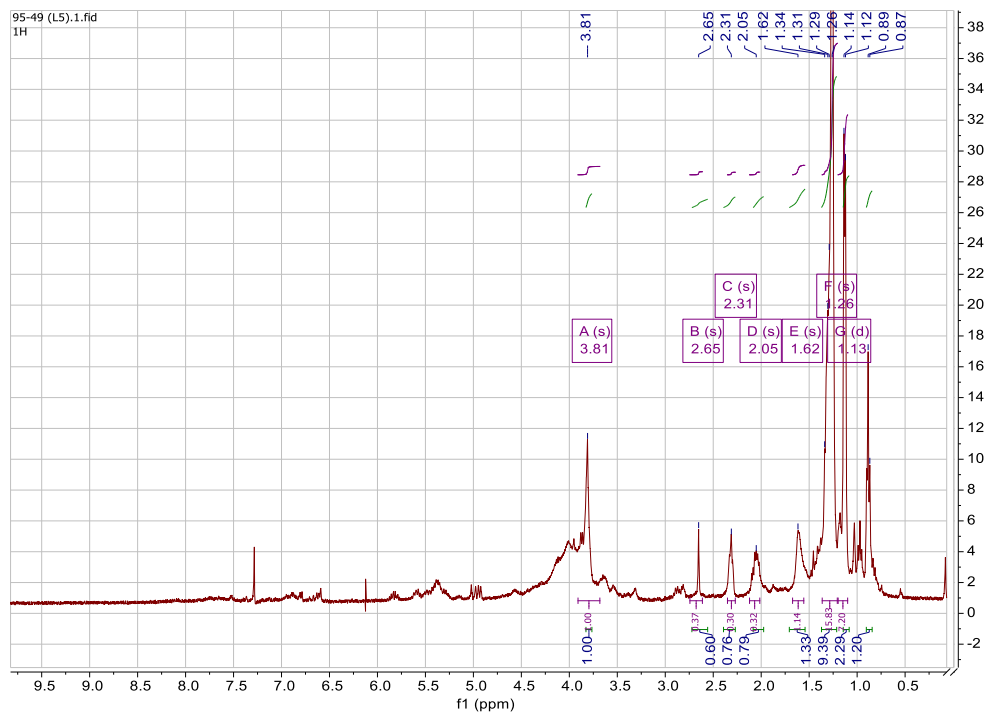
*Appendix 14: DEPT-135 spectrum of **22** in CDCl_3 as solvent.*



Appendix 15: IR Spectrum of 23



Appendix 16: ¹H-NMR Spectral data of compound 23



Appendix 17: ^{13}C NMR spectrum of **23** in CDCl_3 as solvent.

