Phytochemical Investigation, Biological Evaluation and Computational Studies of Isolated Compounds from the Root Extracts of *Acanthus polystachius*

Hailemikael Gebremariam Baye

A Thesis Submitted to

The Department of Applied Chemistry

School of Applied Natural Sciences

Presented in Partial Fulfillment of the Requirement for the Degree of Masters in Applied Chemistry (Natural Product Chemistry)

Office of Graduate Studies

Adama Science and Technology University

February 2024

Adama, Ethiopia

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Advisor: Hailemichael Tesso (Ph.D.)

Co-Advisor: Worku Dinku (Ph.D.)

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DECLARATION

I hereby declare that this Master Thesis entitled **"Phytochemical Investigation, Biological Evaluation, and Computational Studies of the Isolated Compounds from Root Extracts of *Acanthus polystachius*"** is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Hailemikael Gebremaraim _____ _____

Name of student Signature Date

RECOMMENDATION OF ADVISORS

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Phytochemical Investigation**, **Biological Evaluation and Computational Studies of Isolated Compounds from the Root Extracts of *Acanthus polystachius***" prepared under our guidance by Hailemikael Gebremariam Baye. Submitted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Chemistry (Natural Product Chemistry). Therefore, we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

Hailemichael Tesso (PhD)	_____	_____
Major Advisor	Signature	Date
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Co-advisor	Signature	Date

APROVAL PAGE

we, the advisors of the thesis entitled**" Phytochemical Investigation, Biological evolution and Computational studies of Isolated compounds from the root extracts of *Acanthus polystachius"*** by Hailemikael Gebramariam, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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APPROVAL OF BOARD OF REVIEWERS

We, the undersigned, members of the Board of Examiners of the thesis by Hailemikael Gebremariam Baye have read and evaluated the thesis entitled "**Phytochemical Investigation, Biological Evaluation and Computational studies of isolated compounds from the root extracts of *Acanthus polystachius***" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Chemistry (Natural Product Chemistry).

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LIST OF ABBREVIATIONSL AND ACRONYMS

ASTU	Adama Science and Technology University
ATCC	American-type culture collection
br, s	Broad singlet
^{13}C-NMR	Carbon13-Nuclear Magnetic Resonance
DEPT	Distortion less enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
DPPH	2-2- diphenyl-2-picrylhydrazyl
d	Doublet
dd	Doublet of doublet
DEPT	Distortionless Enhancement by Polarization Transfer
FT-IR	Fourier Transform-Infra-Red
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
IZ	Inhibition zones
MHA	Muller Hinton Agar
m	Multiplet
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatograph
R*f*.	Retention factor
s	Singlet
t	Triplet

ABSTRACT

Acanthus polystachius (Acanthaceae) is a medicinal plant traditionally used for treatment of diseases in livestock and humans; it alleviates bleeding and stabbing pain, scorpion stings,, malaria, and vomiting. In view of its traditional uses and the absence of scientific reports, an attempt was made to explore its phytochemicals, evaluate their biological activities, and perform computational studies on the isolated compounds from the solvent extracts of the roots of A. polystachius. In this regard, the dried and pulverized roots of the plant (800g) were extracted with n-hexane, chloroform, EtOAc, and MeOH, resulting in extract yields of 1 g, 1.25 g, 2.1 g, and 27.65 g, respectively. Phytochemical screening of the n-hexane, chloroform, EtOAc, and MeOH extracts revealed the presence of flavonoids, saponins, terpenoids, alkaloids, phenols, coumarins, and steroids. The essential oil obtained by hydrodistillation was analyzed using GC-MS and showed the presence of 37 components, with n-hexadecanoic acid identified as the major constituent (34.89%) of the total components. EtOAc and chloroform extracts were subjected to repeated silica gel column chromatography and four compounds were isolated and identified as benzoxazol-2(3H) -one, 2, 4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one, 2-hydroxy-2H-1,4-benzoxazin from EtOAc, and β-stigma sterol from chloroform. Structural elucidation of these compounds was performed using UV-Vis, IR, and NMR spectroscopy techniques, and the obtained data were compared with literature. The radical scavenging activities of the n-hexane, chloroform, EtOAc, MeOH extracts, as well as EO and isolated compounds were assessed using the DPPH assay, and found to inhibit 93.72% with IC_{50} 10.20±4 for MeoH extract and 85.99% with IC_{50} 33.16±4 for APF-42 isolated compound. The extracts and isolated compounds were also evaluated for their antimicrobial activities by disc diffusion method against four bacterial strains viz., E. coli, P. aeruginosa, E. faecalis and S. pyogenes and one fungi C. albicans. IZ of 21.66±0.57 mm by n-hexane extract and 22.33±0.57 mm by APR-29 against E. faecalis. APF-170 displayed 26.67±00mm against C. albicans. These were promising antibacterial and antifungal activities compared to the standard drugs. The results obtained from the study support the traditional uses of plant against mos and provide the scientific basis for future research.

***Key words**: - Acanthus polystachius, Biological activity, Medicinal plants, Phytochemical*

1. INTRODUCTION

1.1. Background of the study

Antimicrobial resistance (AMR) is a well-known, growing international problem, with an estimated 4·95 million deaths associated with bacterial AMR in 2019, including 1·27 million deaths attributable to bacterial AMR (Murray *et al*., 2022). As a result, it is necessary to search for novel chemical compounds to treat AMR microorganisms unless progress will cost approximately 10 million lives and approximately US$100 trillion per year by 2050 (Guevara, Santos *et al.,* 2023). AMR in bacterial pathogens is a global challenge leading to high morbidity and mortality in clinical settings (Clifford *et al.,* 2018). *Escherichia coli, Staphylococcus aureus, Clostridioides difficile, and Pseudomonas aeruginosa* are the most common pathogens that develop antibiotic resistance and predominantly cause the majority of nosocomial infections in healthcare settings (Palmer & Smaldone, 2014).

Various methods, such as creating new antibiotics in a laboratory (Stanton, 2013), adding adjuvants to existing antibiotics (Yu *et al.,* 2022), implementing policies and guidelines on antibiotic use (Allen *et al.,* 2014) and creating awareness among the public regarding the appropriate use of antibiotics, have been used to reduce antibiotic resistance (Roca *et al.,* 2015). However, combating antibiotic-resistant infections and other bacterial diseases has become a challenge due to the shortage of effective drugs and the limited number of new antibiotics in the clinical pipeline (Frieri *et al.,* 2017). As a result, there is a pressing need to develop novel compounds derived from natural products that can function as drugs or lead compounds or act as scaffolds for antibacterial treatment.

Novel drugs and lead compounds can be discovered through various sources, such as chemical libraries (collections of different compounds having drug-like properties), computational medicinal chemistry (combining computational methods and medicinal chemistry principles) and natural product discovery (isolating bioactive compounds from biological sources), especially plants and microorganisms (Kohli, 2018).

Natural products have served as a major source of drugs, agrochemicals, flavor, fragrance ingredients, food additives and pesticides (Ogunnupebi *et al.,* 2020; Siahsar *et al.,* 2011). Throughout human history, plants have been one of the natural products that have been utilized traditionally for treating a wide array of diseases and illnesses still now (Pan *et al.*, 2014). The reason behind this enduring use is that plants are easily accessible and affordable, and the chemicals found in them often exhibit minimal side effects as well as have better efficacy compared to their synthetic counterparts (El-Saber *et al.,* 2020; Mazid *et al.*, 2012).

A medicinal plant is any plant one or more of its parts contains substances that can be used for therapeutic purposes or that are precursors for the synthesis of useful drugs (Sofowora *et al.,* 2013). They are used by the vast majority of people in the world (87.5%) to treat health issues in both developed and developing nations (Sánchez *et al.,* 2020). Nowadays, nearly 9,000 plant species are actively used as traditional medicine for therapeutic purposes worldwide (Mazid *et al*., 2012). Africa has a rich biodiversity of plant species with a significant portion used in traditional medicine (Brendler *et al.,* 2010). In Ethiopia, about 80% of the population is dependent on traditional medicine, essentially plants (Alebie *et al.,* 2017)

The medicinal value of plant is due to active compounds produced during secondary metabolism, which are often called secondary metabolites (R. Singh, 2015). These metabolites are synthesized and concentrated in specific plant species and organs (Dudareva *et al.,* 2013). They serve in plants as defending agents, attraction of pollinators, signaling and UV radiation protectors (Makkar *et al.,* 2007). The major classes of secondary metabolites are tannins, glycosides, flavonoids, alkaloids, terpenoids, steroids, quinones and saponins (Deyab *et al.,* 2016). These metabolites exhibit a wide of biological and pharmacological properties (Devi & Krishnakumari, 2015). Due to these activities, compounds isolated from them have been used to treat infections and health disorders (Wink, 2015). In recent years, a large number of studies have been conducted to explore the antimicrobial activity of natural products with a particular focus on plants, herbs, and spices (Tajkarimi *et al.,* 2010). Currently, over 1,300 plants have been identified with defined antimicrobial activities, and over 30,000 antimicrobial compounds have been isolated from plants (Joshi, 2018)

Acanthus (A) polystachius is a medicinal plant in the *Acanthaceae* family and is native to East African countries (Demilew *et al.,* 2018). Traditionally, in Rwanda, a decoction of dried leaves of *A. polystachius* has been used to treat scabies (Moshi *et al*., 2010). It is also reported as one of the medicinal plants used in managing diseases of the respiratory system in Kenya (Mailu *et al.*, 2020). In Ethiopia, it is used for treating malaria, scorpion stings, eye infections, bleeding, and relief of stabbing pain in different parts of the country (Teklehaymanot *et al.,* 2007). The root and leaf crude extracts with methanol have been shown to have strong antimalarial activity *in vitro* (Derebe & Wubetu, 2019; Kifle & Atnafie, 2020). The root methanol extract has also exhibited an antihyperglycemic effect on diabetic rats (Derebe *et al.*, 2020). Although *A. polystachius* is widely used in traditional medicine, scientific studies on this plant have focused only on crude extracts. Therefore, studies are still lacking on the chemical constituents of the extracts along with their biological tests; therefore, it is important to conduct studies on phytochemical investigations, biological activities, and computational studies to identify promising new compounds that can potentially treat human diseases, including drug-resistant infections.

1.2. Statement of the problem

Now day the prevalence of antibiotic-resistant infections and pharmacological side effects drugs is a major issue. Reports showed that 4.95 million people died associated with antimicrobial resistance globally in 2019 (Murray *et al.,* 2022). Infection with antimicrobial resistance leads to serious illnesses, prolonged hospital admissions, increases in healthcare costs and treatment failures (Dadgostar, 2019). The Centres for Disease Control and Prevention (CDC) reported that more than three million people in the United States become ill, and a minimum of 48,000 people die due to antibiotic-resistant diseases every year (Control & Prevention, 2019). Bacterial strains causing common and severe infections, such as *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*, have rapidly developed resistance to both old and recently developed antibiotics (Prestinaci *et al.,* 2015).

Therefore, it is crucial to search new drug from natural products to address these issues. *A. polystachius* is one of the most widely used traditional medicines, especially in Ethiopia *(*Derebe & Wubetu, 2019*)*. However, despite its traditional uses, there is a lack of prior studies on the phytochemical constituents, biological evaluation and computational properties of compounds from the root extracts of *A. polystachius*. Thus, conducting such study is significant as it can contribute findings to treat infectious diseases, resolving antibiotic-resistant infections and potentially lead to the discovery of novel compounds for drug development.

1.3. Justification of the research

To the best of our knowledge, *A. polystachius* is widely distributed in different parts of Ethiopia and has been traditionally used to treat various diseases (Demilew *et al.*, 2018). The scientific evaluation of crude extracts from the leaves of *A. polystachius* has assured anti-malarial effects and wound-healing potential (Kifle & Atnafie, 2020). The methanol root extract of the plant has also been reported for its significant antihyperlipidemic and antihyperglycemic properties and can be a potential source of valuable and effective antidiabetic drugs (Derebe *et al.,* 2020). *Acanthus* species are mainly used for treatment of ailments such as, respiratory, nervous, reproductive, gastrointestinal systems, urinary tract and skin infections. Scientific investigations also showed that the aforementioned genus contains several secondary metabolites and approximately 125 chemical compounds from different chemical classes that were isolated and characterized. The crude and isolated compounds also possess remarkable pharmacological activities (Patrícia *et al.,* 2022). However, the phytochemical profile of *A. polystachius* has been the least studied species of the genus. Thus, inspired by its traditional medicinal value, we set out to conduct the photochemistry and *in vitro* antibacterial and antioxidant activities along with an *in silico* molecular docking study of isolated compounds from the roots of the plant.

1.4. Scope of the study

Although *A. polystachius* is used for the treatment of malaria, vomiting, scorpion stings, bleeding, and wound care in different places in Ethiopia, no phytochemical and molecular docking analyses have been carried out on the crude root extract constituents of the plant. Therefore, this study aimed to conduct extraction, isolation, characterization, biological evaluation and computational studies of isolated compounds from the root extracts of *A. polystachius.*

1.5. Significance of the study

Identifying the phytochemical and biological activities of plant constituents in traditional medicinal plants helps in drug discovery and development. Therefore, this study is expected to reveal the following benefits:

- Provides scientific support for the traditional uses by characterizing the chemical constituents, and availing biological activity of *A. polystachius*.
- Offers additional knowledge to the medicinal applications of plants.
- Delivers information on molecular docking analysis.
- Establish a scientific basis for the use of these plants.
- Provides baseline information for researchers who are interested in further studies.

1.6. Objectives

1.6.1. General objective

The overall objective of this study was to extract, isolate and characterize the chemical constituents from the roots of *A. polystachius* and evaluate biological activities along with *in silico* molecular docking interactions of the isolated compound with selected protein domains.

1.6.2. Specific objectives

- To extract dried and pulverized roots of *A. polystachius* by maceration technique using Methanol followed by partitioning with organic solvents such ethyl acetate, chloroform and hexane
- To extract essential oils from the roots of *A. polystachius* by hydrodistillation
- To identify the chemical constituents of the essential oil using GC-MS
- To conduct phytochemical screening tests of the crude extracts using standard experimental protocols
- To isolate compounds from the ethyl acetate and chloroform extracts via silica gel column chromatography
- To characterize the structures of isolated compounds using spectroscopic techniques (UV-Vis, IR, and NMR)
- To assess the antioxidant activity of crude extracts and isolated compounds using the DPPH assay method
- To evaluate the *in vitro* antibacterial and antifungal activities of crude extracts and isolated compounds against two gram-positive bacteria (*Streptococcus pyogenes* and *Enterococcus faecalis*), two gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) and one fungal strain (*Candida albicans*) by the disc diffusion method;
- To study the molecular docking analysis of the isolated compounds using AutoDock version 4.2 software;
- To evaluate the *In silico* pharmacokinetic parameters, such as drug-likeness, ADME, and toxicity profile of the isolated compounds

2. LITERATURE REVIEW

Natural products have played a vital role in modern medicine and folklore for the treatment of many diseases and illnesses since ancient times (Dias *et al.,* 2012). Due to their distinctive chemical diversity, they exhibit a variety of biological actions and drug-like properties (Lautie *et al.,* 2020). Plant-derived medicinal products have attracted the attention of researchers around the world for many years due to their minimal side effects and effective efficacy in treating human health problems (Aye *et al.,* 2019). Furthermore, these products may become important resources for developing new lead compounds and scaffolds (Galm & Shen, 2007).

2.1. The Family *Acanthaceae* and the Genus *Acanthus*

The family *Acanthaceae* is a large family of dicotyledonous herbs, shrubs, and twinning vines, and some are epiphyte-only flowering plants. Consisting of 4300 species in 346 genera, (Khan *et al.,* 2017). It is among the top 12 most diverse families of flowering plants worldwide (Manzitto *et al.,* 2022). The four main centres of distribution families of *Acanthaceae* were Indonesia and Malaysia, Africa, Brazil, and Central America (Xu *et al.,* 2017). Some of this family's pharmacological properties include hepatoprotective, immunomodulatory, antiplatelet, antiviral, antifungal, anti-inflammatory, and antipyretic effects (Patrícia *et al.,* 2022). The genus *Acanthus* is a robust perennial herb distributed mostly in tropical and subtropical regions of the world and consists of 30 species (K. Tan *et al.,* 2023)..

2.2. Ethnobotanical Uses of the Genus *Acanthus.*

The different species in the genus *Acanthus* have been used by traditional practitioners for different ailments (Zohora *et al.,* 2023). The leaves, roots, stems, and seeds of the genus *Acanthus* are active for treating different diseases (Khan *et al*., 2017) *Acanthus species* are commonly used in decoctions, infusions, and poultices to treat conditions affecting the nervous, reproductive, and respiratory systems, including cough, asthma, skin, gastrointestinal, and urinary tract infection (Patrícia *et al.,* 2022). Among the 29 species identified by World Flora Online, approximately nine were reported for their usual uses below (*A*: abbreviated for *Acanthu*s). *A. arboreus, A. mollis, A. montanus, A. spinosus, A. hirsutus, A. ilicifolius, A.*

leucostachyus, and *A. ebracteatus* are among the species well known by the traditional (WFO 2021). Some common species in the genus are well known for their therapeutic properties all over the world, as seen in the table below.

Table 1**:** Lists of some traditional uses and parts of *Acanthus species*

Botanical Name of *Species*	Illness/Symptoms Claimed to Be Treated Traditionally	Parts Used	References
A .arboreus	For snake bite	ND	(Dharmadasa *et al.,* 2016)
A. Mollis	For treating wounds, psoriasis, and swollen legs	Leaves roots	(Adeyemi *et al.,* 2005)
A. ilicifolius	For asthma, diabetes, ringworm, rheumatism	Leaves	(Saranya *et al.,* 2015)
A. eminens	For backache, cough, eye infections, diarrhea, and dema.	Leaves	(Woldemariam *et al.,* 2016)
A. hirsutus	For wound healing, conception and dry cough	Stems and leaves	(Uysal *et al.*, 2018)
A.leucostachyus	Treating toothache, fever, burns, and wounds	Leaves	(Dev *et al.,* 2022)
A. sennii	Treating scorpion sting	Root	(Assefa *et al.,* 2016)
A. ebracteatus	Treating conditions associated with inflammations	entire plant	(Hokputsa *et al*., 2004)
A. polystachius	Treat malaria, cough and wounds	Leaves	(Demilew *et al.,* 2018)

ND: information not described in the work

2.2.1. *Acanthus polystachius* Delile

A. polystachius was named by French botanist Alire Raffeneau Delile. In Ethiopia, it is known by the vernacular names *Kosoru* in Afan Oromo, *Nech Koshashile* in Amharic, *Jawkala* in Gumze, and *Dendero* in Shinasha (Asnake *et al.,* 2016; Chekole *et al.,* 2015; Giday, Teklehaymanot *et al*., 2007). It is a shrub or small tree up to 7 metres that is ecologically widespread and locally cultivated from medium to high altitudes (1000–3200 m) in the agro-climatic zones of Ethiopia (Getaneh, 2011). This species grows slightly larger and has pink blooms and silky, hairy leaves (Mailu *et al.,* 2021)

A. polystachius grows in the wild and is native to several East African countries, including Kenya, Uganda, Tanzania, Burundi, Sudan, Rwanda, and Ethiopia. In Ethiopia, it was found in the north western part of Ethiopia, around Bahir dar City and Dilla (Alebie *et al.,* 2017; Demilew *et al.,* 2018).

Several times, the roots and leaves of *A. polystachius* have been highly used for different diseases in livestock and humans in various ethnobotanical groups (Chekole *et al.,* 2015; Patrícia Matos *et al.,* 2022). Pounded fresh roots diluted with water are used to treat livestock attacked by rabies (Chekole *et al.*, 2015). In Kenya, the root part is used to relieve cough (Mailu *et al*., 2021). Leaf and root decoctions have been employed as a medication with butter to alleviate bleeding and stabbing pain, as well as scorpion stings (Asnake *et al*., 2016). Malaria and vomiting have also been treated using the leaf portion (Asnake *et al.,* 2016; Meragiaw & Asfaw, 2014). The roots of *A. polystachius* have also been used to treat intestinal worms (Giday *et al*., 2007). *In vivo,* leaf and root methanol extracts show promising antimalarial effects (Derebe & Wubetu, 2019; Kifle & Atnafie, 2020). Additionally, studies indicate that methanol crude extracts of leaves can heal wounds (Demilew *et al*., 2018). The root showed significantly reduced hyperlipidaemia and hyperglycaemia with methanol extracts (Derebe *et al*., 2020). Due to its varied ethnobotanical and biological activities, the plant was selected for investigation to identify its active principles that may act as a lead to develop clinical drugs to fight diseases and drug resistance infections.

Taxonomy

Table 2: Taxonomical Classification of *A. Polystachius* Delile.

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopside
Subclass	Asterida
Order	Lamiales
Family	*Acanthaceae*
Genus	*Acanthus*
Species	*Polystachius*

Figure 1: Aerial and Root parts of *A. polystachius*

Photo was taken by Hailemikael Gebremariam at Dilla University Botanical and Ecotourism garden on 04/12/2022.

2.3. Pharmacological study of the genus *Acanthus*

In various scientific studies, some extracts and compounds from *Acanthus species* showed different biological and pharmacological activities, supporting some of its ethnomedicinal uses (Saranya *et al.,* 2015) The three species that are most commonly utilized for treatments are *A. montanus, A. ilicifolius*, and *A. ebracteate* (Patrícia *et al.,* 2022). Among the species in this genus, *A. ilicifolius* has been the subject of in-depth research on its pharmacological properties. The investigation revealed that acanthus ilicifolius possesses a variety of bioactivities, such as anti-inflammatory, anti-leishmanial, antiulcer, antibacterial, antinociceptive, and osteoblastic activities (Firdaus *et al.,* 2013). It has been stated that several portions of this plant have anti-inflammatory and anti-osteoporotic properties (Van Kiem *et al.,* 2008) Because A. ilicifolius contains alkaloid components, it is utilized as a sedative, skeletal muscle relaxant, and anticonvulsant. (Amer *et al.,* 2004). *A. montanus* leaves are believed to have antibacterial and immunologic properties, as well as anthelmintic, anti-inflammatory, and antipyretic properties. Additionally, it exerts peripheral analgesic and antipyretic effects on both short-term and long-term inflammatory processes (Asongalem *et al.,* 2004). *Acanthus sennii* has antiviral, antifungal, cytotoxic, anti-inflammatory, antipyretic, antioxidant, insecticidal, hepatoprotective, and immunomodulatory properties (Assefa *et al.,* 2016). Antioxidant, antihepatotoxic, antibacterial, antiviral, anticancer, anti-feed ant, analgesic, anti-inflammatory and antifertility activities have been discovered in several *Acanthus* species (Çapanlar *et al.,* 2010).

2.4. Phytochemical studies

Phytochemicals are naturally occurring compounds found in medicinal plants in different parts of plants, such as leaves, flowers, stems, bark, and roots. They are grouped as primary and secondary metabolites based on their function in plant metabolism (Theis & Lerdau, 2003). Secondary metabolites have no direct role in the growth and development of plants but have evolved in defense against biotic and abiotic stresses (Jan *et al.*, 2021). Bases on chemical structure and function classified into polyphenols, flavonoids, terpenoids, alkaloids, and plant

sterols, which contribute much to the bioactivity exhibited in the beneficial of human health evolved in combating and preventing diseases (Vasanthi *et al.,* 2012).

Phytochemical screening was performed on the genus *Acanthus* using various extractive solvents, which has indicated the presence of diverse and novel phytochemical constituents, such as triterpenoids, alkaloids, saponins glycosides, flavonoids, and steroids (Wöstmann & Liebezeit, 2008). Leaves and roots of *A. ilicifolius* contain alkaloids, phenol, steroids, protein, resins, steroids, tannins, terpenoids, cardiac glycosides, carbohydrates, saponins, glycosides, and catechol by extraction using different solvents, such as aqueous, hexane, ethanol, methanol, acetone, and chloroform (Aiyer & Manju, 2019; Govindasamy & Arulpriya, 2013; Poorna *et al.,* 2011)**.** The study also revealed the occurrence of alkaloids, terpenoids, saponins, phenolics, flavonoids, and tannins but the absence of steroids from flowers extracted by methanol (Firdaus *et al.,* 2013). Another study revealed that the plant possesses alkaloids, steroids, tannins, flavonoids, and reducing sugars, but saponins and gums are absent in crude methanol extract (Avijit *et al.,* 2012).

A study performed on the qualitative and quantitative determination of the chemical composition of *A. polystachius* reported the presence of glycosides, phenols, saponins, and tannins in the aqueous root extracts of *A. polystachius,* but alkaloids, flavonoids, and terpenoids were not present, while the acetone and methanol root extracts were reported to contain alkaloids, flavonoids, glycosides, and phenols, along with the presence of saponins and terpenoids in the methanol root extracts; however, saponins, tannins, and terpenoids were not shown in acetone extracts. (Mailu *et al.*, 2021). *Studies on A. polystachius* leaves were found to be positive for the presence of tannins, flavonoids, saponins, polyphenols, terpenoids, glycosides, and anthraquinones, whereas alkaloids and steroids were absent (Demilew *et al.,* 2018).

2.4.1. Chemical constituents of *Acanthus* species

Abundant secondary metabolites were found in the genus *Acanthus,* and over 125 chemical compounds from various chemical classes were identified and isolated, primarily from *A. ilicifolius* (approximately 80), followed *by A. ebracteatus* and *A. montanus* species from

various regions of the species. Among these alkaloids, benzoxazinoids, glycosides, flavonoids, and triterpenoids are major compounds that are isolated and possess remarkable pharmacological activities (Patrícia *et al.,* 2022; Singh & Aeri, 2013)

Table 3: Parts and compounds isolated from the *Genus Acanthus*

Alkaloids

S.no	Plants Source	Parts of Extracts	Isolated Compounds	References
1	*A. arboreus*	Entire plant	4-Hydroxy acanthamin	(Amer *et al*., 2004)
2	*A. arboreus*	Entire plant	Acanthaminoside	
3	*A. ilicifolius,* *A.mollis*	Arial parts	Acanthicifoline	(Wu *et al*., 2003)
4	*A. ilicifolius*	Arial parts	Trigonellin	(Tan *et al.,* 2016)
5	*A. ilicifolius*	Leaves	Acanthiline A	(Cai *et al.,* 2018)
6	*A. ilicifolius (*var.*xiamenensis)*	Roots	2-benzoxazolinone	(Huang *et al.,* 2014)
7	*A.ilicifolius (*var.*xiamenensis)*	Root	2-hydroxy-2H-1,4-benzoxazin-3(4H)-one	
8	*A. ilicifolius (*var.*xiamenensis)*	Root	(2R)-2-O-*β*-d-glucopyranosyl-2H-1,4-benzox azin-3(4H)-one	(Huang *et al*., 2014)
9	*A. ilicifolius*	Root	2R)-2-O-*β*-d-glucopyranosyl-4-hydroxy-2H-1,4-benzoxazin-3(4H)-one	(Kanchanapoom *et al.,* 2001)
10	*A.ilicifolius*	Root	2R)-2-O-*β*-d-glucopyranosyl-7-	

			hydroxy-2H-1,4-benzoxazin-3(4H)-one	

Flavonoids

11	*A. ilicifolius*	Leaves	Quercetin	(Tan *et al*., 2016)
12	*A. ilicifolius*	Leaves	Quercetin-3-O-*β*-D glucopyranoside	
13	*A. ilicifolius*	Aerial parts	Vitexin	(Wu *et al.,* 2003)
14	*A. ilicifolius*	Aerial parts	Apigenin-7-O-*β*-D glucuronide	
15	*A. spinosus*	Aerial parts	Apigenin-7-O galactoside	(Amer *et al.,* 2004; Huo *et al.,* 2005)
16	*A. spinosus*	Aerial parts	Apigenin-7-O glucuronide	(Huo *et al.,* 2005)
17	*A. hirsutus boiss*	Aerial parts	Luteolin-7-O-*β*-gluco pyranoside	(Çapanlar *et al.*, 2010)
18	*A. volubilis*	Aerial parts	Schaftoside	(Kanchanapoom *et al.,* 2006)

Lignans

19	*A. ilicifolius,*	Aerial parts	(+) - Lyoniresinol 3α-O-*β* glucopyranoside	(Kanchanapoom *et al.,* 2001)
20	*A. ilicifolius* *A. ebracteatus*	Aerial parts	(-) - Lyoniresinol 3α-O-*β* glucopyranosid	(Patrícia *et al.,* 2022)
21	*A. ilicifolius*	Leaves	Acancifoliuside	(Van *et al.,* 2008)
22	*A. ilicifolius*	Leaves	Acteoside	

23	*A. ilicifolius*	Leaves	Isoacteosid	
24	*A. ilicifolius*	Leaves	Acanthaminoside	

Phenols

25	*A.ilicifolius*	Aerial parts	2,6-Dimethoxyp-hydroquinone-1-O-*β*-glucopyranosyl ester	(Sardar *et al.*, 2018)
26	*A. volubilis*	Aerial parts	Canthoside B	(Li *et al.,* 2009)
27	*A. ilicifolius*	Aerial parts	Cistanoside F	(Wu *et al.*, 2003)
28	*A. volubilis*	Aerial parts	Verbascoside	(Noiarsa *et al.,* 2010)
29	*A. ebracteatus*	Aerial parts	Leucosceptoside A	(Noiarsa *et al.*, 2010)
30	*A. ebracteatus.*	Aerial parts	Martynoside	
31	*A. ilicfolius*	Aerial parts	Campneoside I	(Saranya *et al.*, 2015a)
32	*A. ilicfolius*	Aerial parts	Ilicifolioside A	(Wu, et al., 2003b)

Steroids

33	*A. montanus*	leaves	Stigmasterol	(Jonathan & Okieimen, 2020)
34	*A. montanus*	leaves	*β*-sitosterol	
35	*A. montanus*	leaves	cholesterol	
36	*A. ilicifolius*	Leaves	Campesterol	(Patel *et al.*, 2020)
37	*A. ilicifolius*		*β*-amyrin	(Patrícia *et al.,* 2022)

Megastigmane glycosides

38	*A. ebracteatus*	Aerialparts	Plucheoside B	(Kanchanapoom *et al.*, 2001)
39	*A. ebracteatus*	Aerial parts	Ebracteatoside A	

40	*A. ebracteatus*	Aerial parts	Premnaionoside C	
Simple aliphatic compounds				
41	*A. ebracteatus*	Aerial parts	Ebracteatoside D	(Li *et al.*, 2009)
42	*A. ilicifolius*	Aerial parts	Ilicifolioside C	(Kanchanapoom, *et al.,* 2001)
43	*A. ilicifolius*	Root	Octacosyl alcohol	(Kokpol *et al.,* 1986)
Miscellaneous compoussnds				
44	*A. volubilis*	Aerial parts	Adenosin	(Kanchanapoom,*et al.,* 2001)

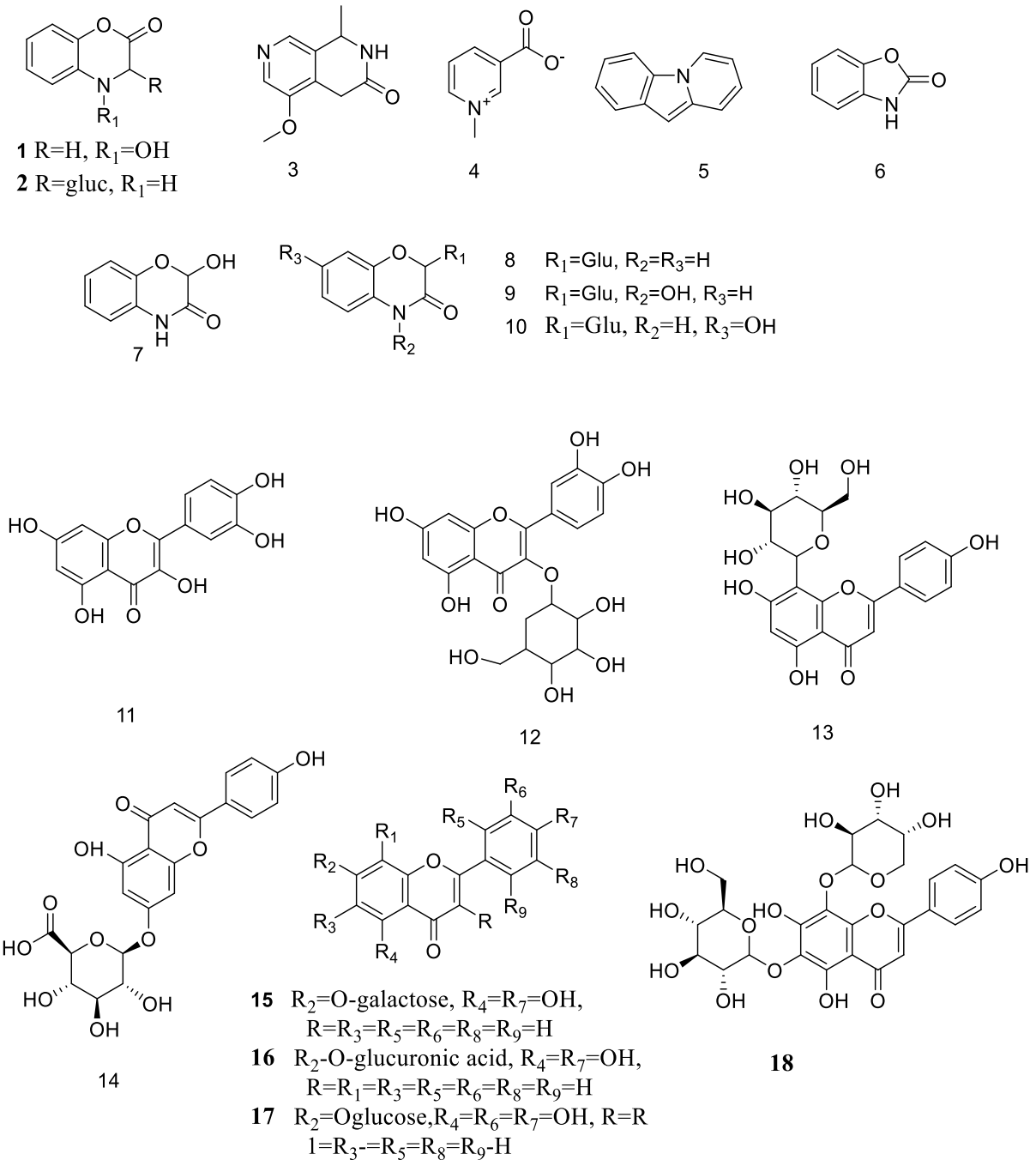


Figure 2: Alkaloids 1-10 and Flavanoids 11-18 isolated from genus Acanthus

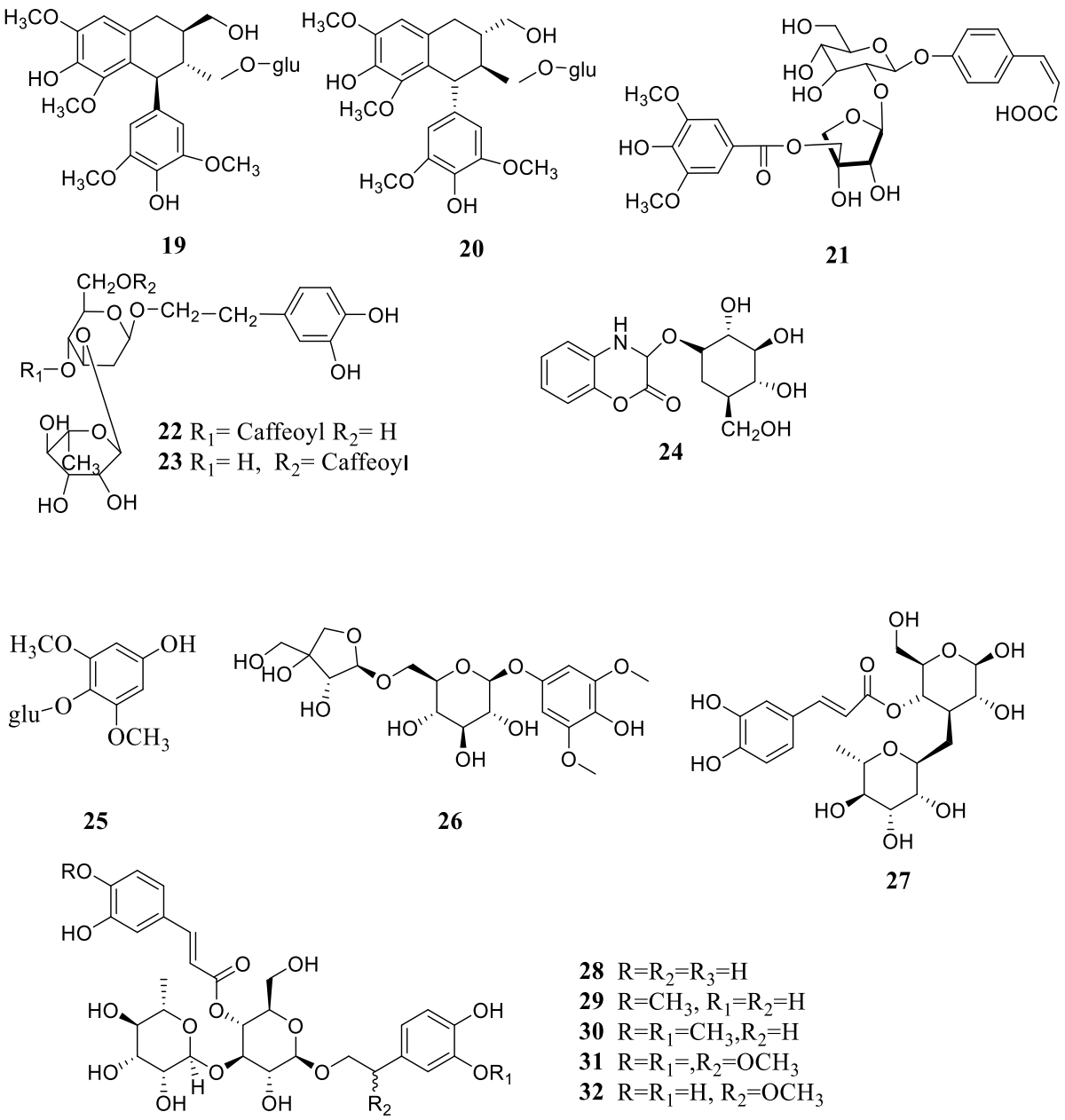


Figure 3: Lignans 19-24 and Phenols 25-32 isolated from genus of *Acanthus*

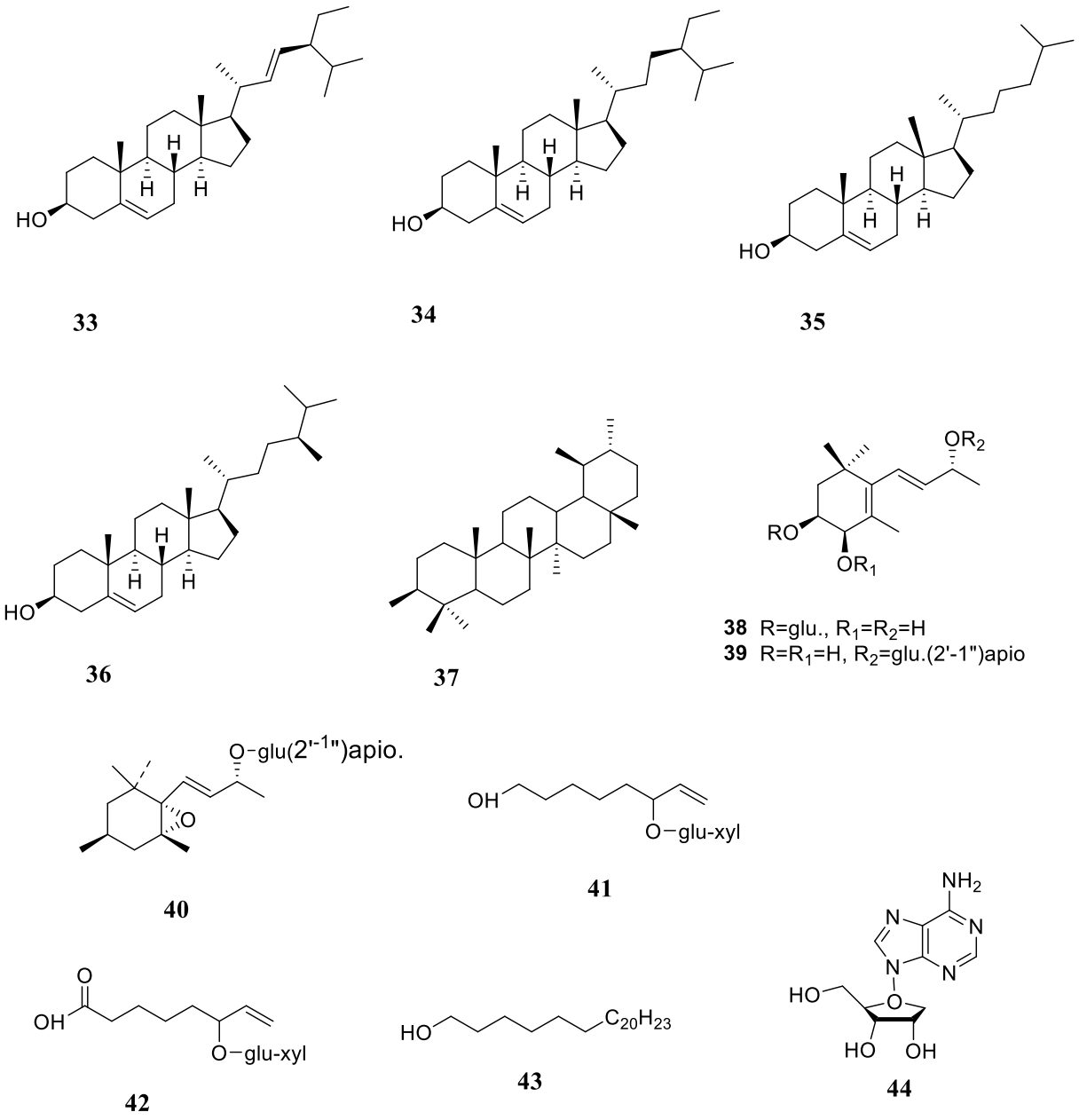


Figure 4: Sterols, Triterpeniods, and others isolated compounds from genus of Acanthus

2.5. Biological activities of the *Genus Acanthus*

The *genus Acanthus* shows various biological activities. Thus, the antimicrobial and antioxidant extracts and isolated compounds of the *Acanthus genus* were reviewed in this literature.

2.5.2. Antimicrobial activity

A review of the literature suggests that the *Acanthus genus*, which was gathered in several places, exhibits varying biological activity. *Acanthus species* are mainly used for diseases of the respiratory, reproductive, gastrointestinal, urinary tract, and skin infections (Patrícia *et al*., 2022). Alcoholic and chloroform extracts of leaves of *A.ilicifolius* showed strong inhibitory action against *B. subtilis, S. aureus, C. albicans, A. fumigatus, and A. niger* and moderate inhibitory action against *P. aeruginosa and P. vulgaris.* The MBC and MIC values was between 0.5 to 3 mg/mL and 2 mg to 4 mg/mL respectively for chloroform extract (Bose & Bose, 2008). According to Okoli's report, furuncles were effectively treated with the crude root of *A. montanus* (Okoli *et al*., 2008). The n-butanol fraction of *A.ilicifolius* leaves inhibits *V. harveyi in vitro* and is potentially active against *Vibrio cholera, K. pneumonia,* and *E. coli* (Saranya *et al.,* 2015). The chloroform extract showed a minimum concentration (MIC) value of 2-4 mg/ml against skin pathogens (Govindasamy & Arulpriya, 2013).

A study performed on the crude extracts of leaves of *A. sanni* by ethanol extracts revealed high antibacterial activity against standard strains of *S. aureus* with an inhibition zone of 14±0.6 mm at 25 mg/ml and 17±0.7 mm at 50 mg/ml, and the bud parts also showed high antibacterial activity against *S. aureus* strains with an inhibition zone of 25.7±0.7. The mean minimum inhibitory concentrations of 5.2±1.8 and 2.6±0.5 mg/ml were reported for the ethanol extract of leaves against standard strains of *E. coli* and clinical isolates of *S. aureus* (Geta & Kibret, 2020). 2-Benzoxazolinone and benzoxazinium derivatives of *A.ilicifolius* exhibited phytotoxic, antimicrobial, antifeedant, antifungal, and insecticidal activity (Wu *et al*., 2003). 6-Hydroxybenzoxazolinone,(Z)-4-coumaric acid 4-O- -D-glucopyranoside and 3,5-dimethoxy-4-hydroxy methyl benzoate were identified as the antibacterial compounds of *A.ilicifolius* (Ravikumar *et al.,* 2012).

A study performed on leaves of *A. polystachius* compared with a simple ointment base revealed a significant wound-healing effect in mice infected with *S. aureus*. The minimum inhibitory concentration (MIC) of the extracts against gram-positive *Bacillus cereus* ranged from 12.5 to 100 mg/mL, and the minimum inhibitory concentration (MIC) of the extracts against gram-negative *Staphylococcus aureus* ranged from 12.5 to 200 mg/mL (Demilew et al., 2018). Even though the genus *Acanthus* has such attractive and remarkable study findings, there has been no study done on the roots of *A. polystachius* for phytochemical investigation, biological activities, and molecular docking properties. Thus, it is important to conduct studies on the extraction, isolation, characterization, biological evaluation and molecular docking of isolated compounds from root extracts.

2.5.2. Antioxidant activity

Antioxidants are a class of chemical substances naturally found in plants that can prevent or reduce the oxidative stress of the physiological system of humans (Gulcin, 2020). An *ilicifolius* flower extract by methanol showed strong antioxidant activity (IC_{50}=17.51 µg/mL) (Andriani *et al.,* 2020). *A. ilicfolius* ethanol extracts of leaves exhibited very strong potential as an antioxidant IC_{50} of 49.73 ± 1.14 µg/ml with ascorbic acid at 29.84 ± 0.34 µg/ml and had medium antioxidant effects (Aisiah *et al*., 2022)**.** The methanol extract of the *A. ilicifolius* flower showed the highest antiradical efficiency (AE=1.41×10^{-3}) against DPPH radicals (Firdaus *et al.,* 2013*)*. Stem extract displayed significant free radical scavenging activity comparable to that of ascorbic acid (Asha *et al.,* 2012). The roots also showed a medium IC_{50} of 59.85 µg/ml for antioxidant activity (Paul & Ramasubbu, 2017). Flavonoids and phenolic compounds that were isolated from *A.ilicifolius* also showed high antioxidant activity in rats (Asha *et al*., 2012). The *A. polytchius* leaves revealed that the crude methanol extracts had very strong activity with IC_{50} values of 9.37 µg/mL (Kifle & Atnafie, 2020).

2.6 Molecular docking

Molecular docking is a kind of computational modeling that facilitates the prediction of the preferred binding orientation of one molecule (e.g., a ligand (compound)) to another (e.g., a receptor) when both interact with each other in order to form a stable complex (Agarwal &

Mehrotra, 2016). It is an important component of many drug discovery projects when the structure of the protein is available. Although it is primarily used as a virtual screening tool, and subsequently for lead optimization purposes, there are also applications in target identification (Cases & Mestres, 2009). There are various kinds of molecular docking procedures involving either ligand/target flexible or rigid based upon the objectives of docking simulations. Flexible ligand docking, which incorporates target as rigid molecule. This is the most commonly used in docking. Rigid body docking, where both the target and ligand molecules are kept as rigid molecules. Flexible docking that involves both interacting molecules as flexible (Mukesh & Rakesh, 2011). Molecular docking has two stages: docking molecules into the target's binding site (pose identification), and predicting how strongly the docked conformation binds to the target (scoring) (Kitchen *et al.,* 2004). Molecular docking can predict the optimized orientation of a small molecule or ligand on its target. It can predict different binding modes of ligand in the groove of the target molecule (Shoichet *et al.,* 2002). Additional this approaches have also been applied to the study of pharmacokinetic features (ADMET: Absorption, Distribution, Metabolism, Excretion, and Toxicity) and pharmacodynamics data (e.g., potency, affinity, efficacy, and selectivity) (Bernardi *et al.*, 2023). Knowledge gained from such investigations may be employed to develop more potent, selective, and efficient drugs before real experimentation, which decreases resource and cost.

3. MATERIALS AND METHODS

3.1. Chemicals and Solvents

Analytical grade solvents, chemicals, and reagents used in this study included methanol 99% (Follium pharmaceuticals), n-hexane (99%), dichloromethane (98%), Chloroform (99%), ethyl acetate (98%) (EtOAc), Petroleum ether (PE), Dimethyl sulfoxide (DMSO), Deuterated DMSO (DMSO-d_6), Deuterated acetone ($(CD_3)_2CO$), and Deuterated chloroform ($CDCl_3$). The chemicals used were ciprofloxacin, ketoconazole, vanillin, Na_2SO_4, DPPH, silica gel (200-400 mesh), $FeCl_3$, NaOH, HCl, H_2SO_4, Wagner's reagent (KI and I_2), and Mayer's reagents ($HgCl_2$ + KI).

3.2. Apparatus and Instruments

Mortar and pestle, electrical grinder (Willy), reagent bottle, rotary evaporator (Lasec), Clevenger-type apparatus, Melting apparatus (Japson lab melting point apparatus), orbital shaker (JEIOTECH), thin layer chromatography (TLC) plate, TLC chamber, capillary tubes, round bottom flasks, digital electronic balance, UV lamp, beakers, vials, test tubes, measuring cylinders, ruler, pencil, pipette, Whatman no:1 filter paper, separatory funnel, and conical flasks, UV-Vis Spectrophotometry (Optizen2120uv). Spectroscopies like UV/Visible, (SYSTRONIC AU-2701), infrared (IR) (Agilent Technologies ATR-FT 5500), NMR Bruker BioSpin GmbH (600 MHZ) and Bruker Avance 400 MHz. GC-MS (Agilent 7890B GC),

3.3. Description of the sample collection Site

The plant was collected from the Dilla University botanical and ecotourism garden at longitude and latitude of 6° 24' 3" N 38° 18'30" E with an elevation of 1570 meters above sea level, which is located in the Southern Nations, Nationalities and Peoples Region (SNNP), Gedio Zone. The place is 356 km from the capital of Ethiopia on the main road to Nairobi Kenya.

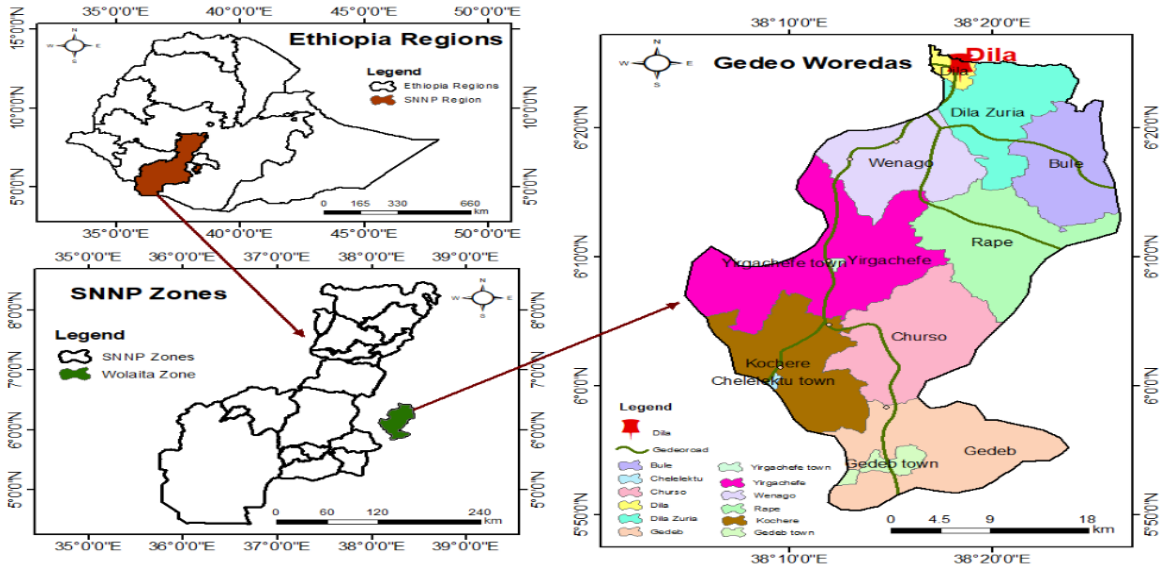


Figure 5: Map of plant collection site

3.4. Collection and Identification of the plant material

Fresh roots of *A. polystachius* were collected and washed with tap water on 04/12/2022. Then, the samples were packed in a black plastic bag to protect them from direct sunlight contact and transported from Dilla University Botanical Garden to the ASTU department of applied chemistry laboratory. The plant material was authenticated by botanist Ato Melaku Wondafrash, and a voucher specimen (Voucher No:HG001) was deposited in the National Herbarium, Department of Botany/Biology, Addis Ababa University, Addis Ababa, Ethiopia.

3.5. Plant preparation and Extraction

The collected fresh roots of *A. polystachius* were dried under shaded open air for one month. The dried plant materials were powdered with an electrical grinder to smaller particle sizes. A total of 800 grams of dried powdered plant material was macerated with 4 L of 99% methanol and shaken using an orbital shaker with 100 rotations per minute (RPM) for 72 h three times at room temperature. The macerated sample was filtered by using Whatman no-1 filter paper and concentrated at reduced pressure in a rotary evaporator at 40°C at 90-120 RPM. The concentrated methanol extracts were dried at room temperature and successively partitioned

with 500 ml of n-hexane, chloroform, and ethyl acetate until the solvents in each crude extract were almost colourless. Each concentrated extract resolution was analysed by using thin layer chromatography (0.25 mm thick layer of silica gel GF254 on an aluminum plate). Spots were visualized under an ultraviolet lamp at 254 and 365 nm wavelengths. Based on TLC resolution and yield, the extracts were selected for column chromatography.

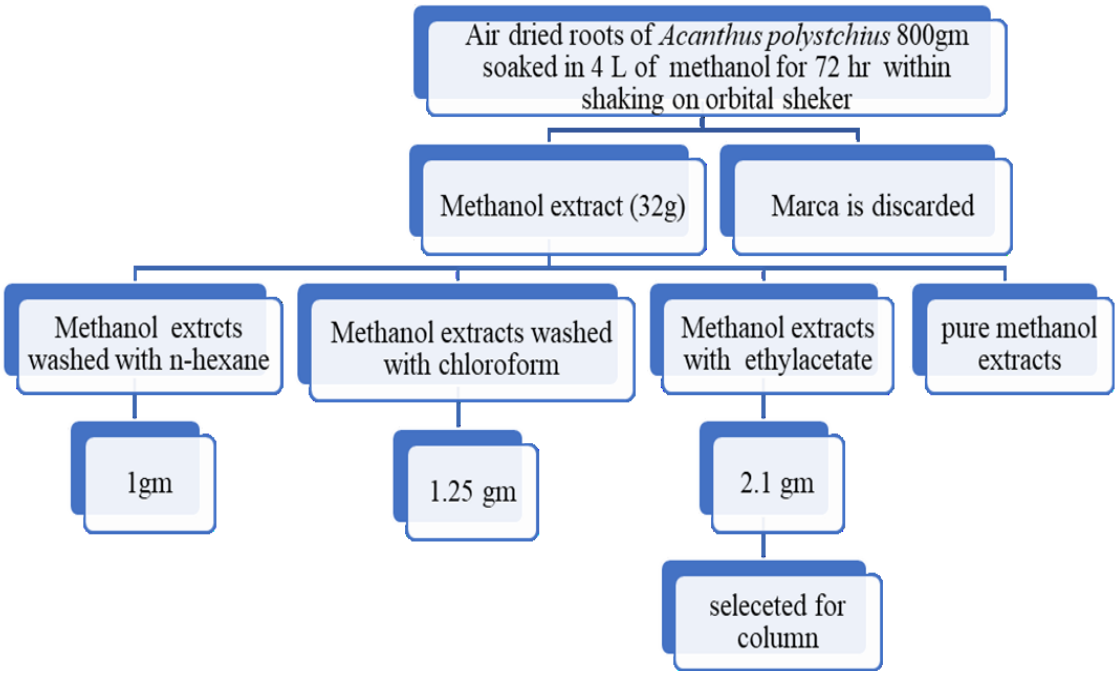


Figure 6 : Scheme flow chart diagram showing extraction procedure.

3.6. Hydrodistillation

Air-dried powder roots of *A. polystachius* 300 gm were subjected to hydrodistillation for 3 hours using a Clevenger-type apparatus according to (Dorsaf *et al.,* 2016). The condensate was collected and extracted with 150 ml dichloromethane (3×) using a separatory funnel. To the combined organic solvent extract, approximately 10 gm of Na_2SO_4 was added to remove moisture and then filtered using Whatman no 1 filter paper. The organic solvent was concentrated evaporated in a vacuum rotary evaporator, and the yields were kept in an amber glass vial in the refrigerator until analysis.

3.6.1. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The essential oil was analyzed by GC-MS using an Agilent 7890B GC with Agilent 5977B mass selective detector [MSD operated in the EI mode (70 eV). Scan range 40-400 amu and scan rate 3.99 scan/sec and an Agilent Chem Station data system. The GC column was HP-5 Fused silica capillary with a 5% phenyl 95% poly methyl siloxane stationary phase, length of 30m, internal diameter of 0.25 mm, and film thickness of 0.25 µm and the carrier gas was helium at a flow rate of 1.0 mL/min. The detector and injector temperatures were 280 and 250ºC respectively. The oven temperature was programmed from 100 to 300ºC at 10 ºC/min, held for 5 minutes and the total running time was 25 min. The volume injected was 0.1 µL of chloroform solution with a split ratio of 1:20.

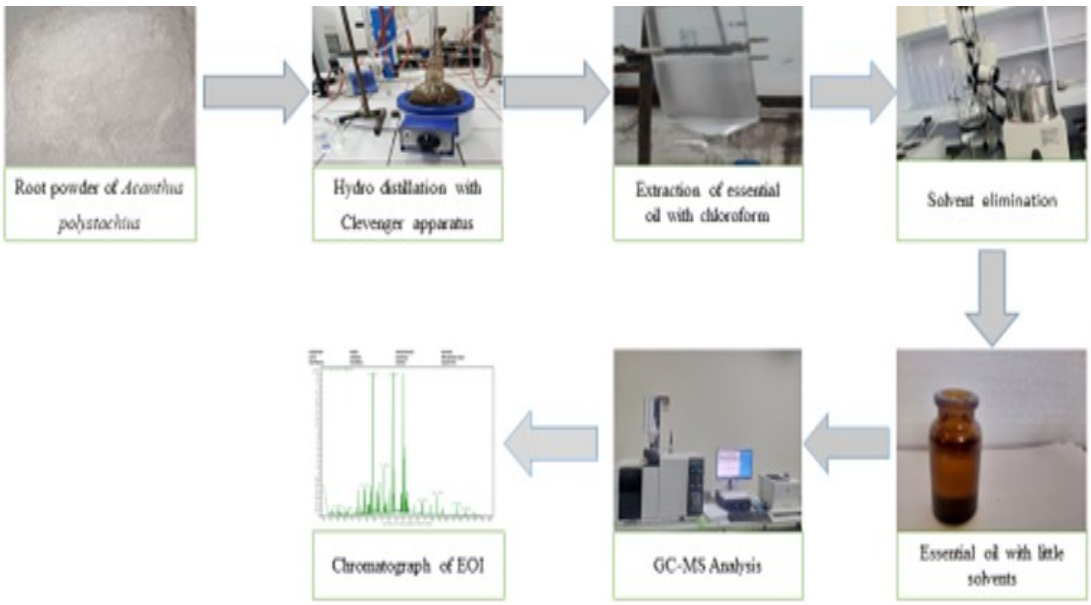


Figure 7: Scheme diagram of essential oil extraction from *A. polystachius* root

3.6.2. Compound Identification

An essential oil (EO) constituent was identified based on a comparison of the name of the compound, the Confirmation of Acceptance for Studies (CAS) registry number, and the GC–MS analysis procedure used with the National Institute of Standards and Technology

(NIST), the Chemistry Webbook (NIST Chemistry WebBook, SRD 69), and the previously reported literature.

3.7. Phytochemical Screening Tests

Preliminary phytochemical screening for each crude extract of the roots of *A. polystachius* was carried out to analyse the presence of flavonoids, saponins, tannins, terpenoids, phenolic, alkaloids, coumarins, and steroids. It was performed qualitatively using colour-forming and precipitating chemical reagents on the crude extracts of the plant according to the standard method on Shaikh & Patil (Shaikh & Patil, 2020), and the values are tabulated in **Table 8.**

Test for Flavonoids ($Fecl_3$ test): Each extract (2mL) was treated with a few drops of 10% $Fecl_3$ solution and showed a green precipitate presence of flavonoids.

Test for Saponins (Froth formation test): To 1mg of each crude extract, 5mL of distilled water was added and shaken vigorously. The formation of persistent foam indicates the presence of saponins.

Test for Tannins (Braymer's test): Each 1mg of crude boiled in 10 mL distilled water for 3 min and then filtered. To each filtrate, 3-4 drops of 10% $FeCl_3$ were added to give a blue–green colour, which confirms the presence of tannins.

Test for Terpenoids: (Salkowski test): Each crude extract (0.5mg) was slackened with chloroform (2 mL) in the test tube, followed by the addition of concentrated sulfuric acid (3 mL) along the side of the test tube using a dropper. A reddish brown colouration of the interface indicates the presence of terpenoids.

Test for Phenolic ($Fecl_3$ test): Each crude extract (1 gm) was mixed with 5 mL of distilled water and 5% FeC_{l3} successively. The formation of a dark green colour indicates the presence of phenolic compounds.

Test for Alkaloids (Wagner's test): Each extract (1 mg) was mixed with 5 ml of 1% HCl for 5 min in a water bath for 5 min and filtered. Two to five drops of Wagner's reagent (iodine in

potassium iodide) were added. The formation of a brown/reddish precipitate indicates the presence of alkaloids.

Test for Coumarins (NaOH test): Each crude extract (1 mg**)** was mixed with 2 ml of 10% NaOH to form a yellow colour, indicating the presence of Coumarins.

Test for Steroids (Salkowski's test): Each extract (1 mg) was combined with 3 mL of chloroform and 3 mL of H_2SO_4, and the formation of the bilayer (red top layer and greenish bottom layer) revealed the presence of steroids.

3.8. Isolation of Compounds

Isolation of compounds was carried out using column chromatography that was packed with silica gel (mesh size 200-400) as stationary phase. Petroleum ether, ethyl acetate and their mixture were used as mobile phase. Visualization of the spot was achieved by UV at λ max 254 nm and 365 nm and spraying of appropriate reagents such as iodine and vanillin.

3.8.1. Column Chromatography Technique

The TLC profiles of the hexane, chloroform ($CHCl_3$) and ethyl acetate (EtOAc) extracts were found were analysed. Depending on the extraction yields, the ethyl acetate (1g) and chloroform (1 g) extracts were selected for further purification. Accordingly, 1g of the EtOAc extract was adsorbed on silica gel 1g and subjected to gravity column chromatography with an increasing gradient of ethyl acetate in petroleum ether as eluents. A total of 177 fractions (10 mL each) were collected, their compositions were analysed using TLC, and invisible compounds were visualized using vanillin spray. In 30% EtOAc in the PE solvent system, fractions 41, 42 and 43 had a single spot with a minor impurity. After separate the impurity by chloroform decantation, fraction 42 with an R_f value of 0.53 in 30% EtoAc in PE and a yield of 30 mg was labelled as compound APF-42. Fraction F-170 also displayed a single spot on TLC in 60% EtoAc in PE with an R_f value of 0.48. (21mg) and afforded compound APF-170. Fractions 61-86 (220 mg) were combined and further using silica gel column chromatography (30g) via an increasing gradient solvent system starting from 30% EtOAc in PE and eluted 57

fractions. Fraction 29 (55% PE in EtOAc) yielded 24 mg with an R_f value of 0.5, showed a single spot on TLC and gave compound APR-29.

Table 4: Fractionation of the EtOAc extract of the root of *A. polystachius*

Fraction	Solvent System	Ration	Remark	Fraction	Solvent System	Ration	Remark
F_1-F_{12}	PE: EtOAc	90:10		F_{160}-F_{170}	PE:EtOAc	5:95	
F_{13}-F_{23}	PE: EtOAc	85:15		F_{171}-F_{177}	PE:EtOAc	100	
F_{24}-F_{29}	PE: EtOAc	80:20		F_{61}-F_{86}	*rechromatographed*		
				R_1-R_6	PE:EtoAc	70:30	
F_{30}-F_{37}	PE: EtOAc	75:25		R_7-R_{16}	PE: EtOAc	65:35	
F_{38}-F_{44}	PE: EtOAc	70:30	*F-41,42, 43 coded as APF-42*				
F_{45}-F_{56}	PE: EtOAc	65:35		$R_{17}R_{26}$	PE : EtOAc	60:40	
F_{57}-F_{65}	PE: EtOAc	60:40		R_{27}-R_{38}	PE : EtOAc	55:45	*APR-29*
F_{66}-F_{74}	PE: EtOAc	55:45		R_{39}-R_{46}	PE : EtOAc	50:50	
F_{75}-F_{84}	PE: EtOAc	50:50		R_{47}-R_{56}	PE : EtOAc	*45:55*	
F_{85}-F_{94}	PE: EtOAc	45:55	*Single spot coded APF-170*				
F_{95}-F_{101}	PE: EtOAc	40:60					
F_{102}-F_{109}	PE: EtOAc	35:65					
F_{110}-F_{120}	PE: EtOAc	30:70					
F_{121}-F_{131}	PE: EtOAc	25:75					
F_{132}-F_{141}	PE: EtOAc	20:80					
F_{142}-F_{151}	PE: EtOAc	15:85					
F_{152}-F_{159}	PE: EtOAc	10:90					

The chloroform extract (1g) was also subjected to silica gel (50 g) column chromatography following the above similar procedures. A total of 194 fractions were collected. Fractions 41-55 (72 mg) with two spots on TLC were combined and rechromatographed over silica gel (20 g) using the isocratic solvent system PE: EtOAc (9:1) as eluent 40 Fractions each 5 mL and

fractions 29-31 afforded a single spot on TLC (8:2, R_f value of 0.6, 35 mg) and coded as compound APR-31.

Table 5: Fractions of the chloroform extracts from roots *A. polystachius*

Fraction	Solvent System	Ration	Remark	Fraction	Solvent System	Ration	Remark
F_1-F_{15}	PE: EtOAc	90:10		F_{41}-F_{55}	*PE:EtOAc*		*Rematographed*
F_{16}-F_{28}	PE: EtOAc	85:15		R_1-R_{40}	*PE: EtoAc*	90:10	$F_{29\text{-}31}$ *single spot labeled* ***(APR-31)***
F_{29}-F_{42}	PE: EtOAc	80:20					
F_{44}-F_{69}	PE: EtOAc	75:25		F_{127}-F_{136}	PE: EtOAc	35-65	
F_{70}-F_{80}	PE: EtOAc	70:30		F_{137}-F_{144}	PE: EtOAc	30-70	
F_{81}-F_{90}	PE: EtOAc	65:35		F_{145}-F_{151}	PE: EtOAc	25-75	
F_{91}-F_{97}	PE: EtOAc	60:40		F_{152}-F_{157}	PE: EtOAc	20-80	
F_{98}-F_{103}	PE: EtOAc	55:45		F_{158}-F_{166}	PE: EtOAc	15-85	
F_{104}-F_{112}	PE: EtOAc	50:50		F_{167}-F_{175}	PE: EtOAc	10-90	
F_{113}-F_{118}	PE: EtOAc	45:55		F_{176}-F_{185}	PE: EtOAc	05-95	
F_{119}-F_{126}	PE: EtOAc	40-60		F_{186}:F_{194}	EtOAc	100	

3.8.2 Structural Elucidations

The structures of the isolated compounds were elucidated based on data obtained from physical properties, such as melting points, R*f* value colour on TLC and interpretation of data generated from spectroscopic methods including UV/Visible, Infrared (IR) and 1D NMR spectra. The ^1H NMR and ^{13}C NMR spectra of compounds APF-170 and APR-31 were obtained in $CDCl_3$ on a Bruker Avance 400 MHz spectrometer from the NMR laboratory, Addis Ababa University (AAU), Ethiopia. However, in acetone-d_6 was also used for the characterization of compounds APF-42 and APR-29. After all, the chemical structures were elucidated to match the closest NMR results from the literature.

3.9. Biological activity tests

In the present study, the antioxidant, antibacterial and antifungal activities of the crude extracts and isolated compounds were tested to determine the biological activity of the roots of *A. polystachius* using different standard methods.

3.9.1. Antimicrobial activity

In vitro, antimicrobial susceptibility tests of essential oils, crude extracts and compounds isolated from roots of *A. polystachius* were evaluated by the agar medium disk diffusion method (Ghasemzadeh *et al.,* 2015). Antibacterial and antifungal activities were evaluated by measuring the diameter of clear inhibition zones surrounding the wells according to the standards of Clinical and Laboratory Standards. The standard bacterial and fungus cultures were obtained from Oromia Regional Laboratory Adama.

3.9.1.1. Bacterial and fungal Strains A medium (Mueller Hinton Agar) was used for bacterial and fungal growth, and two gram-positive bacteria (*Streptococcus pyogenes* ATCC 19615) and *Enterococcus faecalis* ATCC25923), two gram-negative bacteria (*Escherichia coli,* ATCC 25922 and *Pseudomonas aeruginosa,* ATCC 27853) and a fungal strain (*Candida albicans*, ATCC10231) were selected based on their pathogenicity. Ciprofloxacin and ketoconazole were used as the positive controls, and DMSO was used for the reconstitution of the sample as well as the negative control.

3.9.1.2. Media preparation and inoculation

Mueller Hinton Agar and Potato Dextrose Agar (PDA) medium were used for bacteria and separately by dissolving the solid media (38g) in 1000 mL of distilled water. The dissolved fungus. Each medium was autoclaved at 121°C for 15 minutes. After autoclaving, the medium was cooled to approximately 50°C. The medium was then poured into sterile Petri dishes and allowed to solidify. Bacteria and fungus cell suspensions were adjusted to 0.5 McFarland turbidity standards to prepare 1×10^8 CFU/mL and inoculated. 6 mm diameter of sterile filter Paper discs were soaked in 1 mL DMSO solution of the crude extracts containing 250 mg/ml, 125 mg/ml,62.5 mg/ml 31.25, essential oils, and isolated compounds 0.4 mg/ml, 0.2 mg/ml,

0.1 mg/ml and 0.05 mg/ml concentrations. Then, the saturated paper discs were placed on the center of each MHA and PDA plate and incubated in ambient air at 37°C for 16 h for bacteria and at 27°C for up to 72 h for fungi. After the incubation period, the plates were examined for inhibition zones. The inhibition zones were compared with those produced by the reference ciprofloxacin (30 µg/disk) for antibiotics and ketoconazole for antifungal agents (30µg/disk). The resulting diameters of zones of inhibition produced by the plant extracts, isolated compounds, and standard antimicrobials were measured using a ruler and reported in millimetres. Each experiment was performed in triplicate, and the average value of inhibition and standard deviation were calculated. The results are expressed as M ± SEM.

3.9.2. Antioxidant activity

3.9.2.1. DPPH assay: The free radical scavenging power of the crude extracts and isolated compounds were measured based on the scavenging activity of stable 2,2-diphenyl-1-picrylhydra (DPPH) according to the standard protocols adopted by Ugye *et al.,* (Ugye *et al.,* 2018). Serial dilutions were carried out with 1 mg/mL stock solutions of the plant extract and isolated compounds to obtain concentrations of 1000, 800, 600, 400, 200, and 100 µg/ml. The solutions were prepared using methanol as the solvent. Two millilitres from each of the six diluted concentrations of the samples was mixed with 2 freshly prepared 0.04% DPPH solutions in methanol.

The mixture was shaken and incubated in an oven at 37°C for 30 minutes. The absorbance of the resultant solution was measured at 517 nm using UV-Vis spectrophotometry. Ascorbic acid with a similar concentration of test sample was used as the positive control. The DPPH radical scavenging activity of each of the tested crude extracts and isolated compounds was reported as a percentage inhibition using the following formula:

$$\%\ \text{radical scavenging activity} = \frac{Ab\ control - Ab\ sample}{Ab\ control} \text{x } 100$$

Where $Ab_{control}$ is the absorbance of DPPH solution and $Ab_{samples}$ is the absorbance of the test sample (DPPH solution plus sample). The DPPH radical scavenging activities of the crude extracts and compounds were also expressed as IC_{50}, the concentration of the test samples to

give a 50% decrease in the absorbance from that of the control solution calculated by using Graph Pad Prism.

3.10. Molecular docking study of the isolated compound for antibacterial activity

Auto Dock Vina was used to dock the selected proteins and compounds into the active site of proteins. To investigate the mode of interaction between the microbial DNA gyrase enzymes and isolated compounds in a 3D fashion, the compounds were docked within the binding site of the protein. The 2D chemical structures of the compounds were drawn using the Chem Draw 16.0 and assigned with proper orientation. The crystal structure of the receptor molecules, complex with standard drug and DNA was downloaded from the protein data bank. The preparation of the protein and target protein file was carried out as per standard protocols. Then, the best-docked conformation between the compounds and the protein was explored with the docking algorithm provided by Auto Dock Vina.

3.10.1. *In silico* Drug Likeness and Toxicity Predictions

Pharmacokinetics (PK) and pharmacodynamics (PD) evaluations play a crucial role in drug development (Garg, Tadesse, & Eswaramoorthy, 2021). This is one mechanism of knowing the physicochemical and biopharmaceutical nature of the isolated lead compound, which can be supported by the Lipinski rule (rule of five). The evaluation of pharmacological parameters such as drug-likeness, ADME, and toxicity is paramount in identifying lead compounds of interest (Anza *et al*., 2021). Drug likeness is a prediction that determines whether a particular pharmacological agent has properties consistent with being an orally active drug (Garg *et al*., 2021). Recently, *in silico* methods that include molecular docking analysis and online ADMET predictions (SwissADME and ProTox-II) have facilitated the process of identifying potential molecules (Garg *et al.*, 2021). The isolated compound was isolated from *Acanthus polystachius* root extract, and *the in silico* antibacterial molecular docking analysis and pharmacological property predictions, such as drug-likeness, ADME, and toxicity, were performed. The drug likeness of isolated compounds was predicted based on an already established concept by Lipinski's rule (Franc *et al*., 1997). The structures of isolated compounds were converted to their canonical simplified molecular-input line-entry system

(SMILE). They were submitted to the SwissADME tools to estimate *Insilco* pharmacokinetics, such as the number of hydrogen bond donors, hydrogen bond acceptors, rotatable bonds, and total polar surface area of a compound. The selection of compounds as drug candidates was determined by the parameter called the drug score. The higher the drug score value is, the higher the compound's chance of being considered a drug candidate (Behrouz *et al*., 2019).

3.11. Statistical data analysis

Antibacterial and antioxidant data obtained by triplicate measurements are reported as the mean ± standard error of the mean (SEM). Graph Pad Prism version 8.0.2 for Windows was used to perform the analysis. Auto Dock Tools were used for the docking analyses.

4. RESULTS AND DISCUSSION

4.1. Extraction Yields

The powdered root of *A. polystachius,* which were extracted by the solvent extraction method, yielded 1 g (3.12%), 1.25 g (3.9%) and 2.1 g (6.56%) of n-hexane, chloroform and ethyl acetate extracts, respectively (Table 6). Furthermore, the hydrodistillation of the dried and powdered roots of the plant generated approximately 0.68 g (0.22%) of essential oils.

Table 6: Extracts yields of the roots of *A. Polystachius*

Type of extract	Color fresh	Amount (g)	Yield (%)
n-Hexane	Reddish	1.00	3.12
Chloroform	Yellow	1.25	3.90
Ethyl acetate	Reddish	2.1	6.560
Essential oil	Colorless	0.68	0.22

4.2. GC-MS Analysis of the Essential Oil

The hydrodistillation of the air-dried roots of *A. polystachius* yielded colourless essential oil with a pleasant odour. The GC-MS analysis of the essential oils revealed a total of 37 compounds comprising approximately 97.6% of the oil. Esters and aliphatic alkanes constitute 23.68% of the total composition each. Alcohols account for 21% of the composition, while fatty acids make up 18.42%, ranking second and third in terms of relative abundance, respectively. n-hexadecanoic acid (34.89%) (**45**), nonadecane (15.45%) (**46**), phytol (8.20%) (**47**), and 9, 12-octadecadienoic acid (Z, Z) (4.72%) (**48)** were among the major identified components.

n-hexadecanoic acid, which is the major compound, is commonly utilized in the production of soaps, cosmetics, and release agents. In recent developments, the oily palmitate ester of hexadecanoic acid has been employed as a long-acting release carrier medium in the synthesis of paliperidone palmitate, a long-acting antipsychotic medication used for the treatment of

schizophrenia (Antonisamy *et al., 2011)*. Nanodacane has revealed mutagenic activities (Lawal *et al.,* 2015). Phytol reported to possess anti-inflammatory, antifungal and antiplasmodial properties (McNeil *et al.,* 2012). Anti-inflammatory,hepatoprotective, insectcide and Nematocidal activities were exhibit from 9, 12-Octadecadienoic acid-(z,z) (McNeil *et al*., 2012).

The GC-MS chromatogram was shown in (**Appendix 1**) and its components were identified as shown in Table 7.

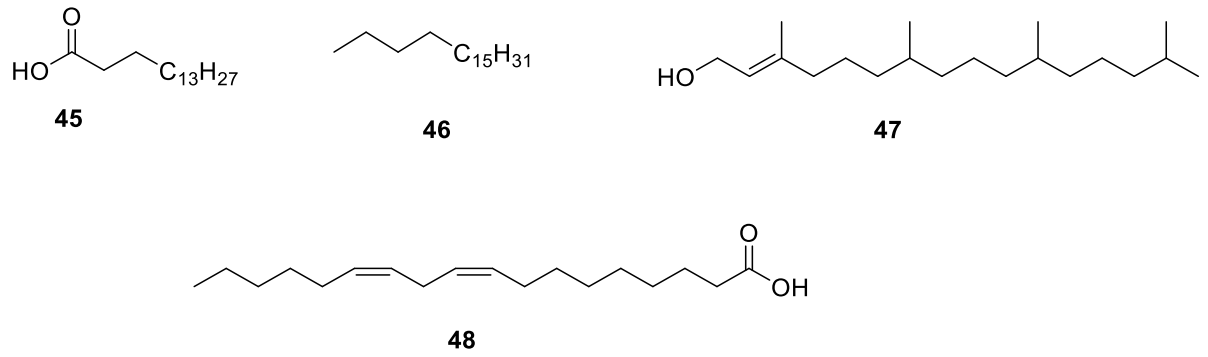


Figure 8: Structures of the major constituents of the EO from roots of A. polystachius

Nonadecane, phytol and γ-eudesmol were previously reported from the leaf parts of *A. dioscoridis* grown in Iraq (Abdullah, 2021). 9,12-Octadecadienoic acid (Z,Z), nonadecane, linoelaidic acid, n-hexadecanoic acid, phytol and hexadecanoic acid methyl ester were also identified from leaves of *Phlogacanthus pubinervius* and *Phlogacanthus thyrsifloru* by GC–MS analysis (Kripasana & Xavier, 2020). Myristic acid, hexahydrofarnesyl acetone and palmitic acid were extracted by hydrodistillation from the leaves of *A. schaueriana (Acanthaceae)* (Machado *et al.*, 2017). Even though the chemical composition has been reported in different *Acanthaceae* family members, this is the first report of the constituents of *A. polystachius* essential oils from the root. Further characterizations are necessary to identify the constituents of the EO of *A. polystachius* using techniques such as high-performance liquid chromatography and NMR, which will contribute to a more comprehensive understanding of its overall chemical composition.

Table 7: Essential oil composition of the roots of *A. polystachius*.

S.N	Compound name	Molecular formula	RT	RI	% Area	References
1.	E-2-Hexenyl benzoate	$C_{13}H_{16}O_2$	4.18	1590	**2.40**	(Andriamaharavo, 2014)
2.	n- Capric acid	$C_{10}H_{20}O_2$	4.98	1368	1.67	(Benkaci *et al.,* 2007)
3.	trans-Carvone	$C_{10}H_{14}O$	5.39	1242	0.72	(Benkaci *et al.*, 2007)
4.	Cyclotetradecane	$C_{14}H_{28}$	6.38	1673	0.75	(Liu *et al.*, 2006)
5.	Acorenone B	$C_{15}H_{24}O$	7.12	1700	0.23	(Liu *et al.,* 2006)
6.	Pentadecane	$C_{15}H_{32}$	7.60	1500	0.57	(Pino *et al.,* 2011)
7.	Bis(tert-butyl)phenol	$C_{14}H_{22}O$	7.78	1513	**2.00**	(C.-x *et al.*, 2006)
8.	Theaspirane B	$C_{13}H_{22}O$	8.15	1305	0.27	(C.-X *et al.,* 2005)
9.	Lauric acid	$C_{12}H_{24}O_2$	8.26	1565	0.49	(Zhao *et al.*, 2009)
10.	Shyobunol	$C_{15}H_{26}O$	8.343		0.49	(Figuérédo *et al.,* 2006)

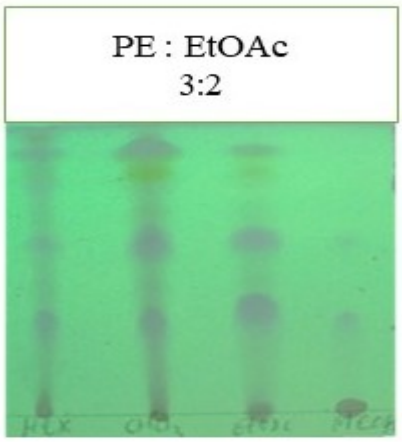
11.	Cyclohexadecane	$C_{16}H_{32}$	8.64	1709	1.66	(Andriamaharavo, 2014)
12.	Cetane	$C1_6H_{34}$	8.72	1883	**1.69**	(Zeng *et al.*, 2007)
13.	Eudesma-4,11-dien-2-ol	$C_{15}H_{24}O$	9.08	1691	0.76	(Vujisić *et al.*, 2006)
14.	Isolongifolene	$C_{15}H_{24}$	9.24	1391	0.71	(Andriamaharavo, 2014)
15.	γ-Eudesmol	$C_{15}H_{26}O$	9.30	1629	0.66	(Xu *et al.,* 2009)
16.	τ-Cadinol	$C_{15}H_{26}O$	9.38	1647	0.68	(Zeng *et al.*, 2007)
17.	β.-Eudesmol	$C_{15}H_{26}O$	9.54	1645	**2.30**	(Hazzit *et al.,* 2006)
18.	Nonadecane	$C_{19}H_{40}$	9.85	**1898**	**15.45**	(Javidnia *et al.,* 2005)
19.	Aristol-1(10)-en-9-yl isovalerate	$C_{20}H_{32}O_2$	10.00		0.82	(Hazzit *et al.,* 2006)
20.	5-Cyclodecen-1-ol	$C_{15}H_{24}O$	10.32	1694	**1.29**	(Andriamaharavo, 2014)
21.	Myristic acid	$C_{14}H_{28}O_2$	10.47	1767	1.59	(Zeng *et al.*, 2007)

22.	Phenylmethyl benzoate	$C_{14}H_{12}O_2$	10.69	1753	1.02	(Kundakovic *et al,* 2007)
23.	cis-9,cis-12-Octadecadienoic acid	$C_{18}H_{32}O_2$	11.31	2095	0.34	(Zeng *et al.*, 2007)
24.	Hexahydrofarnesyl acetone	$C_{18}H_{36}O$	11.38	1839	**2.25**	(Qin *et al.,* 2022)
25.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	12.17	1927	1.17	(C.-X. *et al.*, 2005)
26.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	12.63	1958	**34.89**	(Liu *et al.,* 2006)
27.	Methyl (Z,Z)-9,12-octadecadienoate;	$C_{19}H_{34}O_2$	13.82	2098	0.98	(Pripdeevech & Saansoomchai, 2013)
28.	[Z,Z,Z]-9,12,15-Octadecadienoic acid methyl ester	$C_{19}H_{32}O_2$	13.88	2100	1.18	(C. Zhao *et al*., 2009)
29.	Phytol	$C_{20}H_{40}O$	14.00	2113.9	**8.20**	(Andriamaharavo, 2014)
30.	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	14.20	2113	**4.72**	(Xu *et al.,* 2009)
31.	Tricosane	$C_{23}H_{48}$	15.62	2296	0.42	(Ogunwande *et al.,* 2010)

32.	9-Octadecenoic acid,(E)-	$C_{18}H_{34}O_2$	16.35	-	0.18	(Obi & Okwute, 2023)
33.	Pentacosane	$C_{25}H_{52}$	17.91	2497	0.79	(Ogunwande *et al*., 2010)
34.	Diisooctylphthalate	$C_{24}H_{38}O_4$	18.75	-	1.82	(Zhou *et al.,* 2010)
35.	Hexacosane	$C_{26}H_{54}$	19.47	2606	0.72	(Moronkola *et al,* 2015)
36.	Eicosane	$C_{20}H_{42}$	21.44	1994	1.07	(Ogunwande *et al.*, 2010)
37.	Bis(2-ethylhexyl) isophthalate	$C_{24}H_{38}O_4$	22.61	-	0.53	(Obi & Okwute, 2023)

4.3. TLC Analysis of the Extracts of the Roots of *A. Polystachius*

The methanol extract was washed successively with n-hexane, chloroform, and ethyl acetate. The extracts were analysed with TLC in different solvent systems by using petroleum ether and ethyl acetate as mobile phases. Spots were visualized using an ultraviolet lamp at 254 and 365 nm wavelength and vanillin/H_2SO_4 reagents. The results of the TLC profile of the crude extract demonstrated the presence of different spots which confirmed the existence of different compounds in the crude extract. The TLC profile (Figure 8) revealed similarities between hexane, chloroform and EtOAc extracts at 40% of EtoAc in PE. However, there is a slight difference with that of n-hexane, which had a less intense spot compared to the chloroform and EtoAc extracts.The chloroform extract exhibits an intense spot attached to the first compound. Based on the TLC profile and yield, the EtOAc extracts were subjected to silica gel column chromatography for further isolation of the compound.



Figue 9: TLC profile of the crude extracts of the roots of *A. polystchius.*

4.4. Phytochemical Screening

Phytochemicals are the active constituents naturally found in plants that are responsible for combating and preventing diseases (kumar *et al.,* 2015; Velu *et al.,* 2018). In the present study, owed the presence of major phytochemicals, such as flavonoids, saponins, tannins, terpenoids, alkaloids, phenols, coumarins, and steroids, in the roots of *A. polystachius* (Table 8).

Table 8: Phytochemical screening of the various crude extracts of *A. polystachius*

S.No	Phytoconstituents	Method applied	Extract type			
			n- hexane	$CHCl_3$	EtOAC	MeOH
1.	Flavonoids	Ferric chloride	+	-	+	+
2.	Saponins	Froth formation	+	+	+	+
3.	Tannins	Braymer's	-	-	+	+
4.	Terpenoids	Salkowski	+	+	+	+
5.	Alkaloids	Wagner's	+	+	+	+
6.	Phenolic	Ferric chloride	+	+	-	+
7.	Coumarins	NaOH paper	+	-	+	+
8.	Steroids	Salkowski's	+	-	+	+

The present study revealed that the methanol extract of the roots of *A. polystchius* showed the presence of flavonoids, saponins, tannins, terpenoids, alkaloids, phenolic, coumarins and steroids and the absence of phenols in the ethyl acetate extract. The chloroform extract also exhibited the presence of saponins, terpenoids, alkaloids and phenols. However, tannins, flavonoids, and coumarins showed no indication in the extract. The results of the present study for the methanol extract were in good agreement with a related report from the roots of *A. polystchius* in Kenya, which displayed comparable results except for the absence of tannins (Mailu *et al.,* 2021). Another report on the leaves and roots of *A. polystchius* from Ethiopia showed the presence of all the screened phytochemicals except alkaloids and steroids, which was also in good agreement with the findings of this study. Some differences arose due to certain factors, such as geographical location, climate, biotic interactions, growth conditions, harvesting time and extraction techniques employed (Iloki-Assanga *et al.*, 2015; Pandey,

Irulappan *et al.,* 2017). The phytochemical screening of the present work also revealed that the plant contains major compounds possessing pharmacological activities. Therefore, the screening results support the traditional and social claims of the plant.

4.5. Characterization of the Isolated Compounds

In this work total four compounds were isolated three from the ethyl acetate and one from chloroform extracts and their chemical structures were elucidated using UV Vis, IR and NMR spectroscopic techniques. The structural elucidation of the compounds were also supported with reported NMR data of relevant literatures.

4.5.1. Compound APF-42

Compound APF-42 (30 mg, R*f*: 0.53 in 30% EtOAc in PE), was isolated as a white crystal from the EtOAc extract using 30% EtOAc in PE. A vanillin-H_2SO_4 reagent was applied on the TLC plate followed by heating at 110 °C for a complete visualization of the spot. It showed colorless color. The melting point of the compound was found to be in the range of 138 up to 140 ºC, which matches with the reported values of 2-benzoxazolinone (Murty *et al.*, 1984).

Figure 10: TLC profile of compound APF-42

The UV–Vis spectrum (λ_{max}/nm in MeOH) of compound APF-42 (**Appendix 2**) displayed an absorption band at 264 nm, revealing that the molecule has a carbonyl (C=O) group with chromophores ($\pi \rightarrow \pi^*$) and $n \rightarrow \pi^*$ transitions. The **IR** spectrum (ν_{max}, cm^{-1}) in KBr (**Appendix**

3) showed an absorption band at 3438 $cm^{-1,}$ which indicates N-H stretching; a sharp band of medium intensity at 1738 and 1638 showed the presence of carbonyl and amide groups, respectively. An absorption band at 1481 revealed the presence of an aromatic ring. Bands at 1256 and 1111 cm^{-1} are due to the (C-O-C) absorption of the oxazole group. The data closely parallel those of benzoxazol-2(3H)-one. (Reddy *et al.,* 2014).

The ^{1}H NMR spectrum **(600 MHz, $(CD_3)_2CO$) (Appendix 4)** revealed the presence of signals of four aromatic methine protons at δ 7.21 (*d, H-7*), 7.14**,** (*dd,* H-5), 7.13 (*dd,* H-4) and 7.10 (*d* J= 2.1 Hz, H-6). The ^{13}C NMR **(151 MHz, $(CD_3)_2CO$)** spectrum (**Appendix 5, Table 9**) showed a total of seven well-resolved carbon signals. Of them, four aromatic methines carbons observed at δ 124.40, 110.31, 110.10, and 122.69 were assignable to C-7, C-6, C-5 and C-4, respectively. Two aromatic quaternary carbons, at δ 130.9 and 144.63, were attributed to C-9 and C-8, respectively, and the third carbon at δ 154.83 (C-2) was assignable to a carbamate (urethane) carbonyl. In its DEPT-135 spectrum, the compound showed four aromatic methines signals (=CH) at δ 122.69, 110.10, 110.31, and 124.40 corresponding to C-4, C-5, C-6 and C-7, respectively, which were consistent with the ^{13}C NMR spectral data. The remaining carbon signals that did not appear in the DEPT-135 spectrum belong to quaternary carbons. Overall, the spectral analyses were in close agreement with the reported values of 2-benzoxazolinone (Hui-zhen *et al.*, 2022).

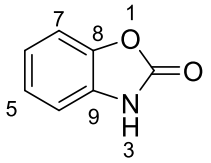


Figure 11: Proposed structure of compound APF-42

Table: 9 ^{1}H, ^{13}C and DEPT-135 NMR spectral data of compound APF-42

Position	^{1}HNMR Experimental values	^{13}C NMR Experimental values	DEPT-135	^{1}H NMR (Hui-zhen *et al.*, 2022)	^{13}C NMR (Hui-zhen *et al.*, 2022)	Nature of Carbon
2		154.83			157	-C-
4	7.14, (*d* H)	122.69	122.69	7.14	123.4	-CH-
5	7.13, (*dd*, H)	110.10	110.10	7.06	110.9	-CH-
6	7.10, (*dd*, H)	110.31	110.31	7.10	110.1	-CH-
7	7.21, (*d*, J = 8.0 Hz, H)	124.63	124.63	7.21-7.18	125.1	-CH-
8		130.90			131.9	-C-
9		144.65			145.4	-C-

4.5.2. Compound APF-170

Compound APF-170 (21 mg, R*f* = 0.48 in 60% EtOAc in PE) was obtained as brown amorphous substance from EtOAc extract, using 55% EtOAc in PE. The melting point of the compound was obtained in range 138-142 ºC.

Figure 12: TLC profile of compound 170

UV (λ_{max}/nm, MeOH) (Appendix 7) showed absorption bands at 255 nm, indicating characteristics of aromatic absorption. The absorption peaks at 283 showed the presence of π-

π* and n-π* electronic transitions of the carbonyl group (C=O). The **IR** spectrum (ν_{max}, cm^{-1}) in KBr (**Appendix 8**) displayed broad and sharp absorption bands at 3439 $cm^{-1,}$ which are characteristic of OH stretching frequencies, respectively. The medium absorption band at 1637 cm^{-1} indicates the presence of a carbonyl group. Moreover, the weak absorption bands at 1431 and 1121 cm^{-1} indicate C=C stretching and C-O absorption for aromatics and cyclic ethers, respectively.

The ^{1}H NMR spectrum (((CD3)2S=O) 400 MHz) (**Appendix 9,** Table 10) displayed four aromatic proton signals at δ 7.26 (d, J = 7.6 Hz, 1H, H-5), 7.10 (m, H-6), 7.10 (m, H-7) and 7.04 (*d,* d, J = 4.3 Hz, 1H-8), one oxymethine proton at δ 5.68 (H, *s*). The **^{13}C-NMR spectrum (Appendix 10**) (((CD3)2S=O)400 MHz) of the compound revealed eight carbons, of which four are aromatic methines (δ 113.45 (C-5), 123.00 (C-6), 124.27 (C-7) and 117.54 (C-8), three are quaternary carbons (δ 158.07, C-3), two are quaternary of aromatic carbons at δ 141.10 (C-9) and 129.23 (C-10) and one is an oxygenated aliphatic carbon at δ 92.46 (C-2).

The NMR spectral data of compound APF-170 were consistent with previous findings reported for 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (Macías *et al.*, 2006).

Table 10: ^{1}H, ^{13}C and DEPT-135 NMR spectral data of compound APF-170 with literature

Position	^{1}H NMR Experimental values	^{13}CNMR Experimental values	DEPT-135	^{1}H NMR (Matos *et al.*, 2018)	^{13}C NMR (Matos *et al.*, 2018)	Nature of Carbon
2	5.68 (s H)	92.49	92.49	5.72 (*s)*	92.29	-CH-
3	--	158.07	--	--	158.74	-C-
4		--	--	--	--	
5	7.27 *(d, J = 7.6* Hz, 1H)	113.45	113.45	7.38	113.02	-CH-
6	7.10 (*m,* 1H)	124.27	124.27	7.12	122.39	-CH-
7	7.07 (*m,* 1H)	123.00	123.00	7.02	124.20	-CH-

8	7.04 *(d, J = 4.3* Hz, 1H)	117.54	117.59	7.09	117.02	-CH-
9	--	141.10	--		141.11	-C-
10	---	129.23			128.24	

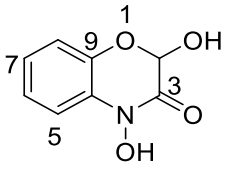


Figure 13: Proposed structure of compound APR-170

4.5.3. Compound APR-29

Compound APR-29 (24 mg, R*f* = 0.50 in 50% EtOAc in PE) was obtained as white powders with a melting point range of 175-180 ºC. The compound was soluble in chloroform and spraying it in vanillin-H_2SO_4 followed by heating at 110 ºC for a few minutes showed a characteristic color change from colorless to slightly yellow.

Figure 14: TLC profile of compound APR-29

The UV–Vis spectrum (MeOH) (**Appendix 12**) displayed absorption bands at λ_{max} 250 nm, which showed the π-π* transition of aromatic C=C bonds. The absorption band at 290 nm revealed π-π* and n-π* electronic transitions of the carbonyl group (C=O). The broad absorption band at 405 nm showed the presence of conjugation in the compounds. The IR (KBr) spectrum of the compound (**Appendix 13**) showed broad and strong sharp absorption

bands at 3373 cm-[1,] indicating O-H and N-H stretching frequencies in alcohols and secondary amides, respectively. Furthermore, the absorption bands at 1660 and 1450 cm^{-1} and the strong sharp band at 1030 cm^{-1} indicated the presence of C=O stretching, aromatic C-H bending and C-O stretching, respectively.

The ^{1}H-NMR (600 MHz, $(CD_3)_2CO$) (**Appendix 14**) spectrum showed the presence of six different proton signals. Two of them were singlets, one deshielded proton at δ 8.13 (H-4) due to the electronegive group of nitrogen and the other singlet at δ 5.59 (H-2) because they were flanked by two electronegative atoms. The aromatic parts contain four protons, which are 7.03 (m, H-8), 7.02 (m, H-7), 7.00 (m, H) and 6.99 (m, H-5). The ^{13}C NMR spectrum (**Appendix 15) (Table 11**) of compound **APR-29** revealed the presence of eight well-resolved carbon signals, of which four were aromatic methane carbons observed at δ 118.85, 127.83, 124.00, 116.43 assignable to C-5, C-6 C,7 and C-8. Two aromatic quaternary carbons and one carbonyl carbon were also observed at δ 142.00, 127.83 and 163.07 corresponding to C-9, C-10 and C-3, respectively. An oxygenated aliphatic hydroxyl group bearing carbon was also observed at δ 91.89, which was assignable to C-2. The NMR spectral data were in close agreement with the NMR values reported in the literature for 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one, also known as HBOA (Macías *et al*., 2006). This compound was reported from the genus *Acanthaceae* in the species *A. ilicifolius* and *A. mollis*. (Bhattarai, Steffensen, Staerk, Laursen, & Fomsgaard, 2022; Ma *et al.*, 2022).

Table 11: ^{1}H, ^{13}C and DEPT-135 NMR spectral data of compound APR-29

Position	^{1}H NMR Experimental values	^{13}C NMR Experimental values	DEPT-135	^{1}H NMR (Macías *et al*., 2006)	^{13}C NMR (Macías *et al*., 2006)	Nature of Carbon
2	5.59 (*s*, H)	91.89	91.98	5.77 (*s,* H-3)	96.5	-CH-
3		163.07			163.2	-C-
4	8.14 (s, H)					
5	6.98 (*m*, H)	116.43	116.13	6.95 (*m,* H-5)	116.9	-CH-

6	7.00 (*m*, H)	124.10	123.79	7.05 (*m,* H-6)	125.10	-CH-
7	7.02 (*m*, H)	123.51	123.21	7.05 (*m*, H-7)	124.2	-CH-
8	7.03 (*m*, H)	118.55	118.24	7.11 (*m,* H-8)	119.1	-CH-
9		142.00			142.2	-C-
10		127.83			127.2	-C-

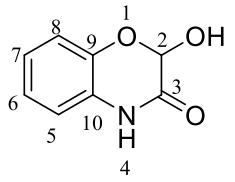


Figure 15: The proposed structure of compound APR-29

4.5.4. Compound APR-31

A compound APF-31 (25mg, R*f* 0.67 20 % EtOAc in PE) was isolated as white powder from chloroform extracts with 10% EtOAc in PE with melting point 154-156 ºC. It displayed blue color when immersed in a mixture of vanillin and H_2SO_4 reagents.

The UV spectrum (MeOH) (**Appendix 17**) of compound **APR-31** showed absorption at λ_{max} 271 nm, indicating the presence of non-conjugated olefinic (C=C) groups (π-π^* electronic transition). The IR spectrum (ν_{max}, cm^{-1}) in **KBr** (**Appendix 18**) showed a broad absorption band observed at 3413 $cm^{-1,}$ which is characteristic of O-H stretching and indicates the presence of a hydroxyl group. The strong absorption band at 2929 cm^{-1} and the medium absorption band at 2856 cm^{-1} indicate the presence of asymmetric and symmetric C-H stretching and aliphatic side chains. A weak absorption band at approximately 1661 cm^{-1} revealed the presence of C=C stretching. The absorption band at 1458 cm^{-1} was assignable to the cyclic $(CH_2)_n$ bending absorption frequency, while the band at 1374 cm-^{1} is attributable to OH deforming absorption. The absorption band at 1164 cm^{-1} is due to C-O (secondary alcohol) stretching, the cyclohexane ring vibration at 1050 cm^{-1} and the C-H bending of the alkene at 963 cm-1.

The ^{1}H-NMR (400 MHz, CDC_{l3}) spectrum of compound APR-31 (**Appendix 19, Table 12**) revealed the existence of three olefinic methines proton signals at δ 5.35 (H-6), 5

15 (H-22), 5.01 (H-23). A broad integration for one proton suggested the presence of a methine olefinic proton on a carbon bonded to one quaternary and one methylene carbon. Doublets of doublet signals at δ 5.14 (J =, 15.2, 8.7 Hz 1H) and 5.02 (J =12, 6 Hz 1H) for H-22 and H-23, respectively, indicating the presence of double bond protons on the aliphatic chain. An oxymethine proton was observed at δ 3.52 (H-3) broad with multiplet peak integrating for one proton. Two angular methyl singlet signals at δ 1.25 (H-19) and 0.69 (H-18). Over all six methyl, nine methylene and eleven methines were observed in between 0.69-5.35, which is characteristic of steroid skeleton signals.

The ^{13}C-NMR spectrum in $CDCl_3$ 400MHZ (**Appendix 20**) Compound APR-31 showed four olefinic carbons at δ 140.59 (C-5) and 121.73 (C-6), which indicate the presence of olefinic carbon with a more deshielded signal assignable to the quaternary carbon at the bridge and at δ 138.18 (C-22) and 129.11 (C-23), assignable for the olefinic side chain carbon. There was also the presence of one oxygenated carbon atom at δ 71.66 (C-3) common for the hydroxyl carbon group and six methyl carbons, with two carbons at δ 19.25, (C-19) and 11.89, (C-18), 20.90 (C-21), 20.91, (C-26), 18.82, (C-27), and 12.11. (C-29) were side chains.

Generally, the spectrum of compound APR-29 shows good agreement with the literature data reported on the isolation of 3*β*, 22E-stigmasta-5, 22-dien-3-ol, also known as *β*-stigmasterol. (Cayme & Ragasa, 2004; Hazimah *et al.,* 2023)

Table: 12 ^{1}H and ^{13}C-NMR spectral data of compound APR-31 and reported literature.

Position	^{13}C NMR Experimental	DEPT-135	^{1}HNMR Experimental	^{13}C NMR	^{1}H NMR	Nature of Carbon
				(Cayme & Ragasa, 2004)		
1	37.09	37.25	1.83 *(m, 2H)*	37.2	1.83	-

						CH_2-
2	31.73	31.8	1.49 (*m,* 2H)	31.6	1.49	- CH_2-
3	71.66	71.8	3.52 (*t*, 1H)	71.8	3.53	-CH-
4	42.14	42.23	2.23 (*m*, 2H)	42.27	2.28	- CH_2-
5	140.59		-	140.7	-	-C-
6	121.58		5.35 (*br*, *s*, H)	121.7	5.35	-CH-
7	31.73	31.83	2.00 (*m*, 2H)	31.9	1.98	- CH_2-
8	31.50	31.6	1.45 (*m,* H)	31.9	1.46	-CH-
9	49.96	50.14	0.93 (*m*, H)	50.1	0.94	-CH-
10	36.36	-	-	36.5	-	-C-
11	21.06	21.23	1.49 (*m*, 2H)	21.6	1.45	- CH_2-
12	39.50	40.52	1.96 *(m*, H)	39.7	1.97	- CH_2-
13	42.14	--	----	42.19	----	-C-
14	56.71	56.87	0.93 (m, H)	56.8	1.03	-CH-
15	24.20		1.08 (m, 2H)	24.4	1.05	- CH_2-
16	28.78		1.69 (m 2H)	28.9	1.71	CH_2-
17	55.78		1.13 (m, H)	55.9	1.13	-CH-
18	12.11	12.11	0.69 (s, 3H)	12.04	0.70	$-CH_3$
19	19.25		1.25 (s 3H)	19.40	1.01	$-CH_3$
20	40.36		2.00 (d, H)	40.50	2.04	-CH-
21	20.91	21.13	1.01 (d, J 8 Hz 3H)	21.09	1.02	$-CH_3$
22	138.18	138.97	5.14 (*dd,* J =	138.34	5.15	-CH-

			15.2, 8.7 Hz, 1H)			
23	129.11	129.06	5.02 (dd, J=15.2 8.7 Hz H)	129.20	5.02	-CH-
24	51.08	50.19	1.59 (m, H)	51.2	1.53	-CH-
25	31.73	31.	1.45	31.9	1.44	-CH-
26	20.91		0.80 (m,3H)	21.2	0.84	$-CH_3$
27	18.82		0.82 (3H)	18.97	0.82	$-CH_3$
28	25.26	25.43	1.15 (d, 2H	25.4	1.15	$-CH_2-$
29	12.11	12.	0. 84 (*t,* 3H)	12.3	0.80	$-CH_3$

Stigmasterol is a steroid belonging to the class of tetracyclic triterpenes. It is one of the most common plant sterols and is found in a variety of natural sources, including vegetable fats or oils from many plants. It is an important component of the cell membrane(Du *et al*., 2022). The human body cannot naturally produce this plant sterol; therefore, it is only available through foods and diets such as vegetables (Vanmierlo *et a*l., 2015). Stigmasterol-rich plant extracts exhibited effective anti-inflammatory and immunomodulatory activities in vivo(Wen *et al*., 2021). The findings showed that stigma sterol holds promise as a potential therapeutic agent for the treatment or management of diabetes (Nualkaew *et al.,* 2015). It also showed potential as a natural compound for development of anticancer therapy (Antwi *et al*., 2018).

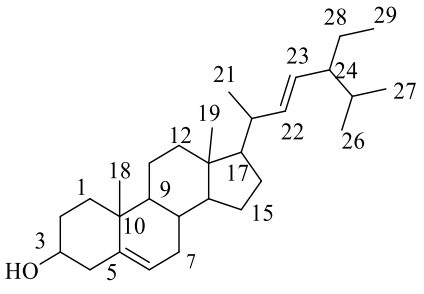


Figure 16: The proposed structure of compound APR-31

4.6. Biological Activities

Crude extracts and isolated compounds were evaluated the ability of free radical scavenging activity, potency and its antimicrobial activity against pathogenic microbial.

4.6.1. Antimicrobial Activity

The *in vitro* antibacterial and antifungal activities of crude extracts, essential oils and isolated compounds were evaluated using a disc diffusion assay against the bacteria E. *coli,P. aeruginosa, S. pyogenes, E. faecalis* and fungus *C. albicans* at different concentrations. All the extracts and isolated compounds showed different degrees of activity against the bacterial and fungal pathogens. The results tabulated in tables, 13, 14, 15 showed that all of the test samples clearly indicate considerable antimicrobial activities against the bacterial species used in the study. In principle, it is believed that any tested analyte with an inhibition zone less than or equal to 10 mm is considered to have low antibacterial activity, an inhibition zone between 10 and 15 mm has moderate antibacterial activity, an inhibition zone between 15 and 20 mm has strong antibacterial activity and an inhibition zone greater than or equal to 20 mm is extremely strong (Paudel *et al.,* 2014). In this study, the antimicrobial activity of the tested samples was also described on the basis of the aforementioned principle. The antimicrobial activity results showed that the examined samples exhibited low to extreme antibacterial activities. The maximum antibacterial activity was recorded for the n-hexane extract, with a mean inhibition zone of 21.66±0.57 mm, followed by the EtOAc extracts (20.33±0.57) against *E. faecalis* compared to ciprofloxacin (26.33±0.57). Chloroform and methanol extracts also displayed strong activity against *E. faecalis* with inhibition zones of 18.67± 0.57 and 19.33 ± 0.57 mm, respectively. n-Hexane and chloroform, EtOAc showed inhibition in the range of 14.66±0.57-17.66±0.57 against *P. aeruginosa* at a concentration of 250 mg/ml. The n-hexane extracts exhibited a strong inhibition zone against *C. albicans* (19.66±0.57), followed by the chloroform and EtOAc extracts (16.33±0.57, 15.33±0.57) at a concentration of 250 mg/m. A low inhibition zone was recorded in all extracts against the *E. coli* pathogen, where most of the extracts showed effective activities against *E. faecalis* at a concentration of 250 mg/ml. The chloroform extract inhibition zone against *E. coli* agreed with a study performed on the leaf *A.ilicifolius* but differed against *P. aeruginosa* (Ravikumar *et al.,* 2012). Essential oils showed

maximum antibacterial activity against *S. pyogenes* within an inhibition zone of 20.33±0.57 *C. albicans* (19.*67±0.57) E. faecalis* 18.33±0.57 at a concentration of 0.3 mg/ml.

Compound **APR-29** exhibited maximum antibacterial activity against *E. faecalis* with a zone inhibition 22.33±0.57 at 0.4 mg/ml. Compound APR-31 revealed strong inhibitory effects against *P. aeruginosa* (21.3±0.57 at a concentration of 0.4 mg/ml). *S. pyogenes* was strongly inhibited by compound APR-29 at a concentration of 0.4 mg/ml 19±00 compared with the standard drug. All compounds revealed a maximum inhibitory effect against *C. albicans* at the highest concentration. Crude extract and isolated compounds showed dose-dependent activities against all pathogens. *Crudes and* compounds exhibited significant activities on gram-positive and *C. albincans.* Throughout, *A. polystachius* has been found to be a rich source of constituents with potential antimicrobial activities.

Table 13: Anti-microbial activities of crude extracts of *A. polytachius*

Bacterial Stains	[Cont] Mg/ml	n-Hexane	Chloroform	EtOAc	MeOH	Cipro30 µg/Dis
E. coli	250	8.67±0.57	9±00	11.66±0.57	11.66± 0.57	16.58±2.13
	125	7±00	7±00	9.66±0.57	8.33 ± 0.57	
	62.5	NG	NG	7 ±00	7±00	
	31.25	NG	NG	NG	NG	
P.aeruginosa	250	17±00	14.66±0.57	15.33±0.57	17.66±0.57	19.33±1.07
	125	15.33 ±0.57	12.33±.57	12.66±0.57	14.6±0.57	
	62.5	12±00	8.33±00	8±00	9±00	
	31.25	9±.57	7.33±00	7±00	7±00	
	250	20.33±0.57	17.0±1	14±00	12±00	27±00
	125	19.66±66	14 ±00	10.33±0.57	9±00	
	62.5	12.33±33	11.33±00	8±00	7±00	
	31.25	10±00	11.00±00	7±00	NG	

S. pyogenes						
E. faecalis	250	21.66±0.57	18.66±00	20.33±0.57	19.3±0.57	26.33±0.57
	125	15.33±0.57	15.33±0.57	18.66±0.57	14.3±1.5	
	62.5	13.33±0.57	NG	1±00	8±00	
	31.25	8.33±0.57	NG	13.66±0.57	NG	
C. albicans	250	19.66±0.57	16.33±0.57	15.33±0.57	8.33±0.57	Keto30µg/ disk
	125	13.66±0.57	14±00	12 ±00	7 ±00	25.33±0.57
	62.5	11.66±0.57	8±00	7±00	NG	
	31.25	13.33±1.15	7.67±0.57	NG	NG	

Table 14: Antibacterial and Antifungal activities of isolated compounds

Bacteria strains	Concmg /ml	APF- 42	APF- 170	APR-29	APR-31	control
E. coli	0.4	8.67±00	**15.33 ± 0.57**	8±00	10.33±0.57	19.3±0.57
	0.2	7±00	10 ±0.57	NG	8±00	
	0.1	NG	NG	NG	NG	
	0.05	NG	NG	2.3±4.12	NG	
P.aeruginosa	0.4	20.67± 0.57	8.33 ±0.57	17±1	**21.3±0.57**	26.33±1.07
	0.2	17.33±0.57	NG	15.3±0.57	13±00	
	0.1	8.3±0.57	NG	2.33±4	4±4	
	0.05	2.3±4	NG	NG	NG	
S. pyogenes	0.4	18±00	18±00	**19±00**	18±00	25
	0.2	15.3± 0.57	15.3± 0.57	16.3±0.57	14.3±0.57	
	0.1	15.3±0.57	15.3±0.57	8±00	7±00	
	0.05	8±00	8±00	NG	NG	

E. faecalis	0.4	17±00	17±1	**22.33±0.57**	17.67±0.57	27.67±1.57
	0.2	15±00	15.33±0.57	16	11.67±0.57	
	0.1	7±00	8±00	7±00	7±00	
	0.05	NG	NG	NG	NG	
C. albicans	0.4	20.67±0.57	26.67±00	22.33±0.57	20.33±0.5	Keto30µg/disc
	0.2	16.67±0.57	15.33±00	16±00	15.67±0.57	29.67±0.57
	0.1	8±00	8.67±00	7±00	2.33±4	
	0.05	NG	7.33±00	NG	NG	

Table15: Anti-microbial activities of essential oils from the roots of *A. polystachius*

Conc µg	*E. coli*	*P. aeruginosa*	*S. pyogenes*	*E. faecalis*	*C. albicans*
0.3	9±00	10.3±0.57	**20.33±0.57**	**18.33±0.57**	**19.67±0.57**
0.15	7±00	8.3 ±0.57	16.3±0.57	12.33±00	14.67±0.57
0.075	NG	NG	9.3±0.57	10.33±0.57	11.33±0.57
0.0375	NG	NG	7±00	NG	4 ± 4
Cipro30µg/disk	15±100	21.6 ±0.57	25±00	27.3±0.57	-
Keto30µg/disk	-	-	-	-	29.33±0.57

NG: not growth

4.6.2. Antioxidant Activity

The antioxidant activity of the sample was tested against DPPH free radicals. Free radicals are known to cause oxidative stress, which pathological manifestations (Phaniendra, Jestadi, & Periyasamy, 2015). Antioxidants have been reported to have the ability to inhibit these compounds and prevent the occurrence of diseases through scavenging activities (Rahman *et al*., 2021). The DPPH assay was used to assess the electron transfer reaction to the sample (Platzer *et al.,* 2021). Furthermore, the antioxidant activity of natural extracts and isolated

compounds can be categorized based on their IC_{50} values, namely, very strong (< 50 µg/mL), strong (50-100 µg/mL), moderate (101-150 µg/mL), and weak (>150 µg/mL) (Priyanto *et al.*, 2023).

The radical scavenging ability of the crude extracts and isolated compounds of *A. polystachius* was assessed using the DPPH radical scavenging experiment. The DPPH assay measures the decrease in the absorbance of DPPH at 517. It is a stable free radical that, when acted upon by an antioxidant, is converted into 2, 2-diphenyl-1-picrylhydrazine, resulting in a colour change from purple to pale yellow. The extent of DPPH scavenging activity by the plant extract and compounds can be quantified spectrophotometrically. The percentage of radical scavenging activity was calculated using the formula mentioned in Section 3.8.1.

The various crude extracts essential oils and isolated compounds of *A. polystachius* significantly reduced the DPPH. In **Table 16,** the DPPH radical scavenging activities (%) of the crude extracts using different solvents, such as n-hexane, chloroform, ethyl acetate, and methanol, at a concentration of 1000 µg/ml were found to be 89.27, 90.45, 91.10, and 93.72, respectively. In **Table 17,** Compounds, APF-42, APF-170, APR-29 and APR-31 showed 85.99, 84.04, 81.11 and 66.44 radical scavenging activities and essential oils revealed 78.83, at a concentration of 1000 µg/ml respectively. The positive control, ascorbic acid, exhibited a maximum scavenging effect at both low and high concentrations. The scavenging percentages were 90.84, 91.62, 92.93, 93.98, 94.50, and 95.16 at concentrations of 100, 200, 400, 600, 800, and 1000 µg/ml, respectively.

These results indicate that the DPPH scavenging activity increased with increasing concentration of the samples, and the plant was rich in secondary metabolites that are capable of reducing the DPPH radical. These secondary metabolites include phenolic groups, alkaloids, flavonoids, and terpenoids. The presence of these constituents was also confirmed by phytochemical screening. The percentage of scavenging activity was highest in the methanol extracts, followed by the ethyl acetate and chloroform extracts. The methanol extracts have comparable scavenging activity with ascorbic acid with the highest concentration, and this result is also akin to a study performed on *A .ilicifolius* (Poorna *et al.*,

2011).This suggests that extracts are rich in phenolic groups that exhibit strong DPPH-reducing activity. Studies have shown that many polyphenols and flavonoids contribute to better antioxidant activities in plant extracts *(*Barhé & Tchouya, 2016*)*.The observed antioxidant capacity was not solely attributed to the phenolic contents but could be attributed to the presence of other phytochemicals, such as aromatic amines. These compounds possess the ability to donate hydrogen and electrons, and their synergistic effects may also contribute to the overall antioxidant activity. All sample scavenging activities ranged from 10.20±4-33.16±4, which showed very strong antioxidant activities. However, the compound exhibited lower antioxidant activity than the crude extracts. This may be due to different compounds having synergistic effects with antioxidant activities. Among the samples, the root methanol extract exhibited very strong antiradical activity with an IC_{50} of 10.20±4, which is in fair proximity to the IC_{50} value of the experimental standard, ascorbic acid (4.98 µg/ml). Thus, it has good antioxidant power. In comparison to a previous study, the IC_{50} of the methanol extract from the leaves showed a scavenging activity of 82.54% at 1000 µg/mL, and the IC_{50} value was determined to be 9.37 µg/mL. According to reported studies, the species *A. ilicifolius* has shown the highest antioxidant activity in its flower extracts by using a methanol extraction method (Firdaus *et al.,* 2013). The obtained results could be attributed to the presence of tannins, saponins, alkaloids, phenolic compounds, and other bioactive compounds with lone pairs and free hydroxyl groups. Therefore, the results show that the plant containing these bioactive compounds with lone pairs and free hydroxyl groups in the crude extracts has medicinal value and may be utilized for its antioxidant properties.

Table: 16 % Scavenging activity of crude extracts from roots of *A. polystachius*

Conc (µg/ml)	Ascorbic acid		n-Hexane		Chloroform		Ethyl acetate		Methanol	
	A	%	A	%	A	%	A	%	A	%
1000	0.037	95.16	0.08	89.27	0.07	90.45	0.07	91.10	0.05	93.72
800	0.042	94.5	0.10	86.91	0.09	88.87	0.08	90.05	0.06	92.28
600	0.044	93.98	0.15	80.76	0.12	84.29	0.09	88.35	0.07	91.49
400	0.043	92.93	0.17	78.93	0.15	80.63	0.10	86.78	0.07	90.31
200	0.053	91.62	0.19	77.49	0.17	78.14	0.13	83.51	0.09	87.96
100	0.037	95.16	0.22	75.13	0.18	76.44	0.14	81.54	0.11	85.34
IC_{50}	4.98±4		18.10±4		19.58±4		12.83±4		10.20±4	

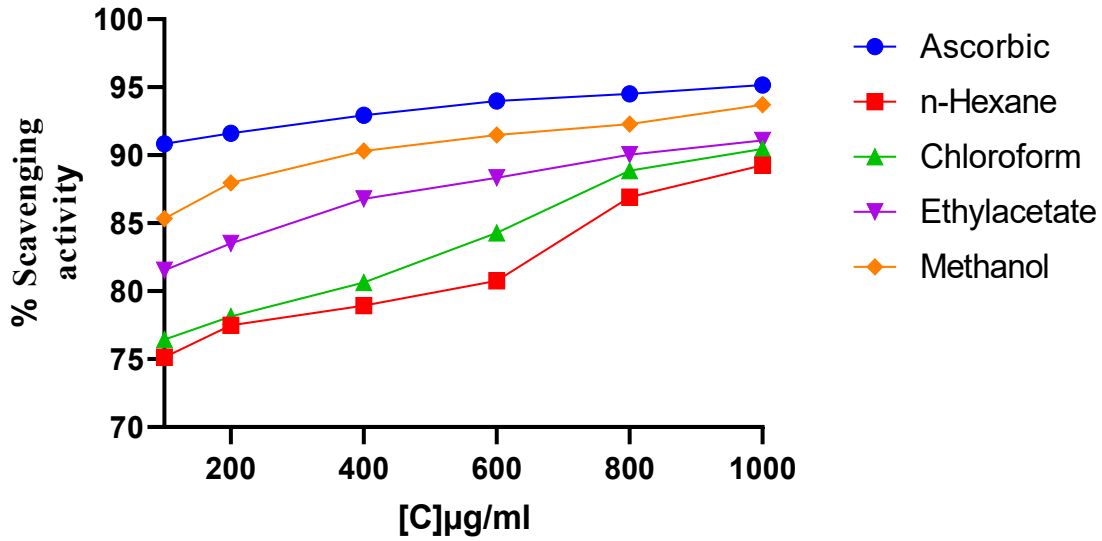


Figure 17: DPPH scavenging activity (%) of A. polystachius extracts and positive reference

Table 17: % Scavenging activity of isolated compounds and Essential oil from the roots of *A. polystachius.*

Conc (µg/ml)	Ascorbic acid		APF- 42		APF -170		APF-29		APF- 31		EO	
	A	%	A	%	A	%	A	%	A	%	A	%
1000	0.037	95.16	0.04	85.99	0.05	84.04	0.06	81.11	0.103	66.44	0.07	78.83
800	0.042	94.5	0.05	84.36	0.07	77.85	0.06	80.46	0.108	64.82	0.07	76.90
600	0.044	93.98	0.06	81.11	0.08	74.59	0.07	78.18	0.13	56.35	0.08	74.59
400	0.043	92.93	0.07	76.87	0.09	71.01	0.07	77.85	0.15	51.79	0.08	72.64
200	0.053	91.62	0.09	71.34	0.10	68.08	0.08	73.62	0.16	48.2	0.09	70.68
100	0.059	90.84	0.10	67.10	0.11	64.17	0.10	67.75	0.20	34.52	0.12	60.91
IC_{50}	4.98±4		33.16±4		30.53±4		21.84±4		98.1±4		30.11±4	

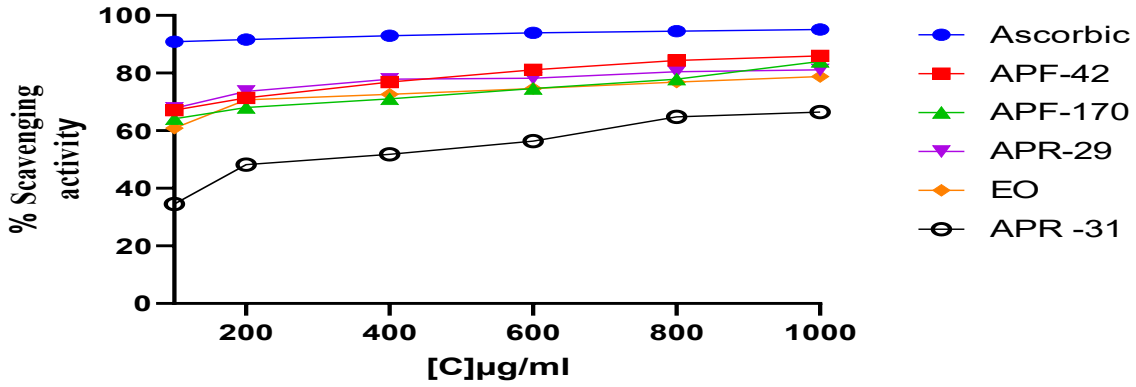


Figure 18: DPPH scavenging activity (%) of isolated compounds and positive reference

4.7. Molecular docking study of the isolated compound for antibacterial activity

In this study, molecular docking was employed to investigate the binding energy and validate the molecular mechanisms of ligands at the active site of a protein. The selected compounds were docked against the *E. coli* DNA gyrase B protein (PDB ID: 7P2W) using AutoDock Vina 4.2.6 software. Ciprofloxacin was used as a reference drug for the docking studies. By analyzing the binding affinity, interactions and binding modes of the ligands. Such information can contribute to understanding the molecular mechanisms of the ligands and guide further drug discovery and development efforts. Binding affinity and interactions with different amino acids were presented in the (Table 18), and 3D and 2D binding interactions was depicted in the Figure 18.

The results obtained from the molecular docking study revealed that the isolated compounds showed smaller binding affinities towards *E. coli* DNA gyrase B (PDB ID: 7P2W) with binding energy values of -5.8 kcal/mol, -6.0 kcal/mol, and -6.2 kcal/mol for compounds APR-29, APF-170, and APF-42, respectively. However, compound APR-31 exhibited a binding affinity (-7.2 kcal/mol) that was nearly equal to the standard drug ciprofloxacin (-7.3 kcal/mol). Compound APF-42 formed three hydrogen bonds with amino acid functional groups Arg-76, Gly-77, and Asp-73. Compound APF-170 and APR-29 showed two hydrogen bonds each, with compound APF-170 interacting with Gly-77 and Arg-76 through an acid functional group, and compound APR-29 interacting with Arg-76 and Glu-50. Compound APR-31 formed one hydrogen bond with the amino acid functional group Val-47. Compound APR-31 demonstrated a binding affinity similar to that of ciprofloxacin, forming hydrogen bonds with residual amino acids Gly-71, Arg-136, and Arg-76, as well as hydrophobic or pi-cation interactions with residual amino acids Thr-165, Asp-73, Ala-47, and Asn-46.

Table 18: Molecular Docking Scores and Residual Amino Acid Interactions of Compounds against *E. coli* DNA gyrase B (PDB ID (7P2W).

S.No	Compounds	Affinity (kcal/mol)	H-bond	Residual Amino acid Interactions	
				Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions	Van-der Walls interactions
1	APF-42	-6.2	Arg-76,Gly-77,Asp-73	Glu-50, Ale-78	Val-118, Gly-75, Gly-164, Ala-47
2	APF-170	-6.0	Gly-77,Arg-76	Val-118,Ile78	Apr-79, Glu-50 Gly-75, Asp-73, Thr-165
3	APR-29	-5.8	Arg-76,Glu-50	Ile-50	Asp-46, Ala-47, Asp-73, Thr-165, Pro-76, Glly-77
4	APR-31	-7.1	Asn-47	Pro-79,Ile-94, Val-118,Ile-75	Arg-136, Glu-50, Thr-165, Asp-73, Ala-47, Arg-76
5	Ciprofloxacin	-7.3	Gly-71, Arg-136, Arg-76	Glu-50, Arg-76, Ile-94, Val-118, Ile-78, Pro-79	Thr-165, Asp-73, Ala-47, Asn-46

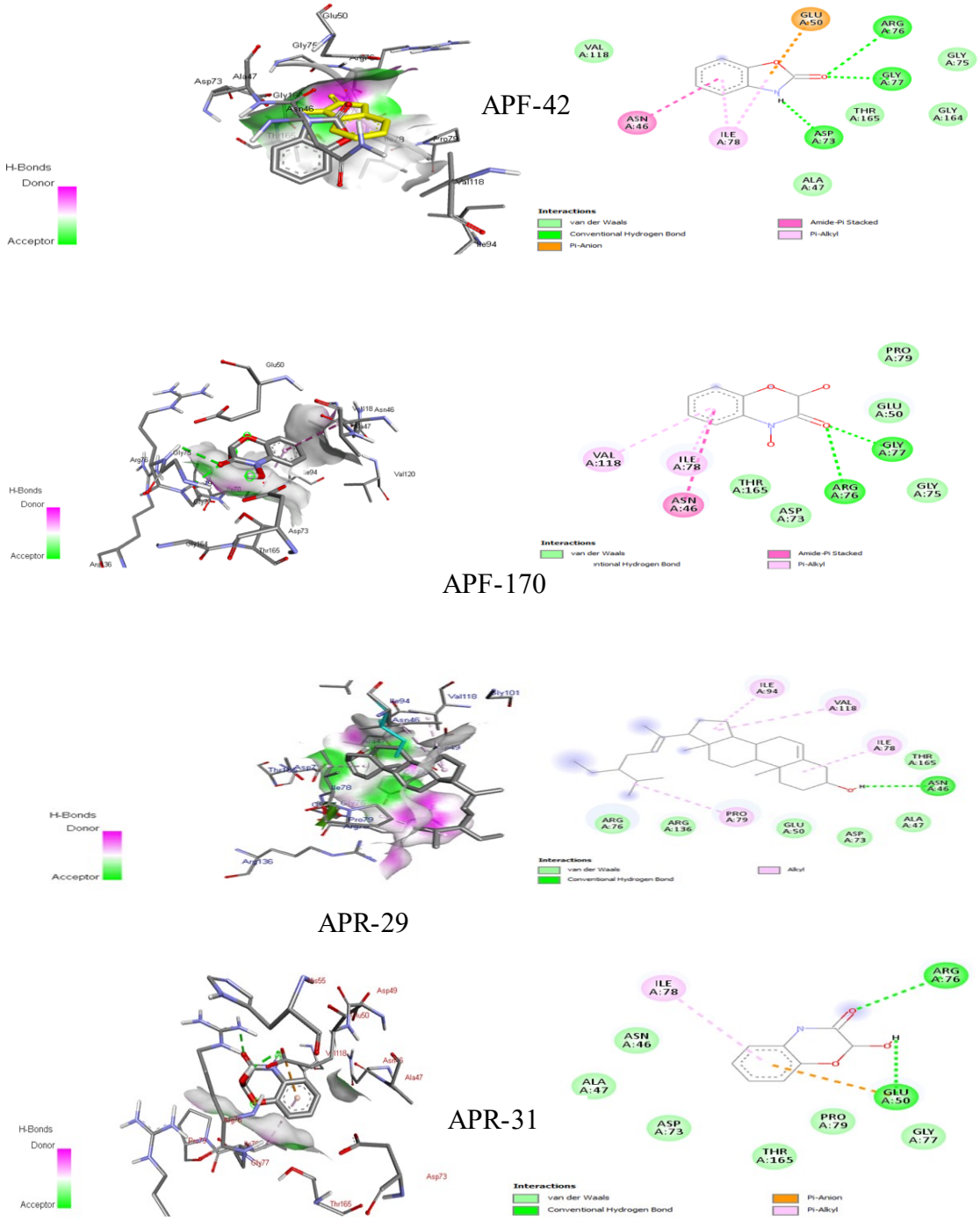


Figure : 19 The 3D and 2D binding interactions of compounds against *E. coli* DNA gyrase B (PDB ID: 7P2W)

4.7.1. *In silico* Pharmacokinetics (DrugLikeness) and Toxicity Analysis

The *in silico* drug-likeness of the isolated compounds were determined based on the concept of Lipinski's rule of five. Lipinski's rule of five states that a compound with molecular weight (MW) < 500 Daltons, H-bond donors (NHD) < 5, H-bond, acceptors (NHA) < 10, alog P of < 5, could be a good drug candidate. Compound with a fewer or preferably no violations are likely to be considered as a potential drug candidate. The SwissADME computed results showed that three isolated compounds(APF-42,APF-170 and APR-31) satisfy Lipinski's rule of five with zero violations excpet compound APR-31 which violet lipinskirule five iLogP < 5. The pharmacokentics prediction parameters showed that all the compounds exhibit low skin pentreion and compounds APF-42,APF-170 and APR-29 have high gastrointestinal (GI) absorption expcept compound APR-31 the compound APF-42 is the only one that has the ability to pass through the blood-brain barrier and reach the brain, which is beneficial for its therapeutic effects on the central nervous system. All compounds are not substrates of P-glycoprotein and do not undergo transport by this efflux transporter. Two compounds were (APF-42) and (APR-31) has intarction with enzyme CYP1A2 and CYP2C9 respectively. Toxicological prediction results suggested that compound APF-170 has ability to couse Mutagenicity and compounds APF-42, APF-170 and APR-29 have the potential to cause carcinogenicity and hepatotoxicity. All the compounds were found to be noncytotoxic, and the Lethal Dose 50 predictions for APF-42 and APR-31 were 890mg/kg, while for compounds APF-170 and APR-29, it was 1500mg/kg. These values indicate that compounds APF-170 and APR-29 are more toxic than ciprofloxacin.

Table 19: Pharmacokenetics, ADME, and Toxcicity Profile of Isolated Compounds.

Parameters		Compounds				
		APF-42	APF-170	APR-29	APR-31	Cipro
Formula		$C_7H_5NO_2$	$C_8H_7NO_4$	$C_8H_7NO_3$	$C_{29}H_{48}O$	$C_{17}H_{18}FN_3O_3$
Mol.Wt. (g/mol)		135.12	181.15	165.15	412.69	331.34
NHD		1	2	2	1	3
NHA		2	4	3	1	2
NRB		0	0	0	5	5
TPSA (A°2)		46.00	70.00	58.56	20.23	74.57
iLogP		1.33	0.86	0.99	5.01	2.24
Lipinski's Rule of Five Violation		0	0	0	1	0
Pharmacokinetics						
Log Kp (skin permeation)cm/s		- 6.30	-7.40	-7.08	-2.74	-9.09
Gastrointestinal absorption		High	High	High	Low	High
blood-brain barrier permeant		yes	No	No	No	No
Inhibitors	P-gp substrat	No	No	No	No	Yes
Interction	CYP1A2	Yes	No	No	No	No
	CYP2C19	No	No	No	No	No
	CYP2C9	No	No	No	Yes	No
	CYP2D6	No	No	No	No	No
	CYP3A4	No	No	No	No	No
Toxicity	Hepatotoxicity	Yes	Yes	Yes	No	No
	Carcinogenicity	Yes	Yes	Yes	No	No
	Immunotoxicity	No	No	No	yes	No
	Mutagenicity	No	Yes	No	No	Yes
	Cytotoxicity	No	No	No	No	No
LD_{50}(mg/kg)		890	1500	1500	890	2000
Toxicity class		4	4	4	4	4

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

In the present study, extraction, isolation, characterization, biological evaluation, and molecular docking of the isolated compounds were performed on the root extracts of *Acanthus polystachius* to identify bioactive compounds that exhibit activity against infectious microorganisms. The dried and powdered roots of *A. polystachius* were partitioned extracted with organic solvents, such as n-hexane, chloroform, EtOAc and Methanol. Phytochemical analyses revealed the presence of flavonoids, saponins, tannins, alkaloids, phenols, steroids, terpenoids, and coumarins in all the root extracts. Furthermore, the oils extracted from the plant roots through hydrodistillation were analyzed by GC-MS, and major compounds such as hexadecanoic acid (34.89%), nonadecane (15.45%), phytol (8.20%), and 12-octadecadienoic acid (Z, Z) (4.72%) were identified as major compounds. Through silica gel column chromatography, the crude root extracts of EtOAc and chloroform yielded four compounds:benzoxazol-2(3H)-one,2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one,2-hydroxy-2H-1,4-benzoxazin-3(4H)-one from EtOAc, and β-stigmasterol from chloroform extracts. The identification of these compounds was performed using spectroscopic methods, and they were isolated from this plant for the first time. The DPPH radical scavenging activities (%) of methanol crude extracts (93.72%) and isolated compound APF-42 (85.99%) showed the highest radical scavenging activity with IC_{50} values of 10.20±4 and 33.16±4, respectively. The crude extracts, oil, and isolated pure compounds from the roots of the plant were active against the tested bacteria *E. coli, P. aeruginosa, E. faecalis,* and *S. pyogenes*. n-hexane extract (21.66±0.57 mm) and the isolated compound APR-29 (22.33±0.57 mm) showed the highest inhibition zone against *E. faecalis*. Molecular docking study was done using Autodock and it was found that among isolated compounds, compound APR-31 shows the highest and best scoring pose (lowest energy) which was -7.1 Kcal/mol towards DNA gyrase. The SwissADME prediction results showed that only β-stigmasterol violated one of Lipinski's rules of five among the isolated compounds. Furthermore, this study provided the scientific basis for the use of *A. polystachius* in future research aimed at the discovery of the Chemical compounds responsible for the therapeutic action, as well as the mechanisms involved.

5.2. Recommendation

- More phytochemical analysis and biological activities of the extracts and isolated compounds of the roots need to be carried out to support the use of these plants in traditional medicine.
- It is recommended that further *in vivo* anti-bacterial and anti-fungal efficacy tests validate the in vitro results.
- Furtherer research should be extended to cover other biological activities such as: anti-cancer, antiviral and anti-plasmodiam for all the extracts and other organic solvent extracts of the roots of *A. polystachius*

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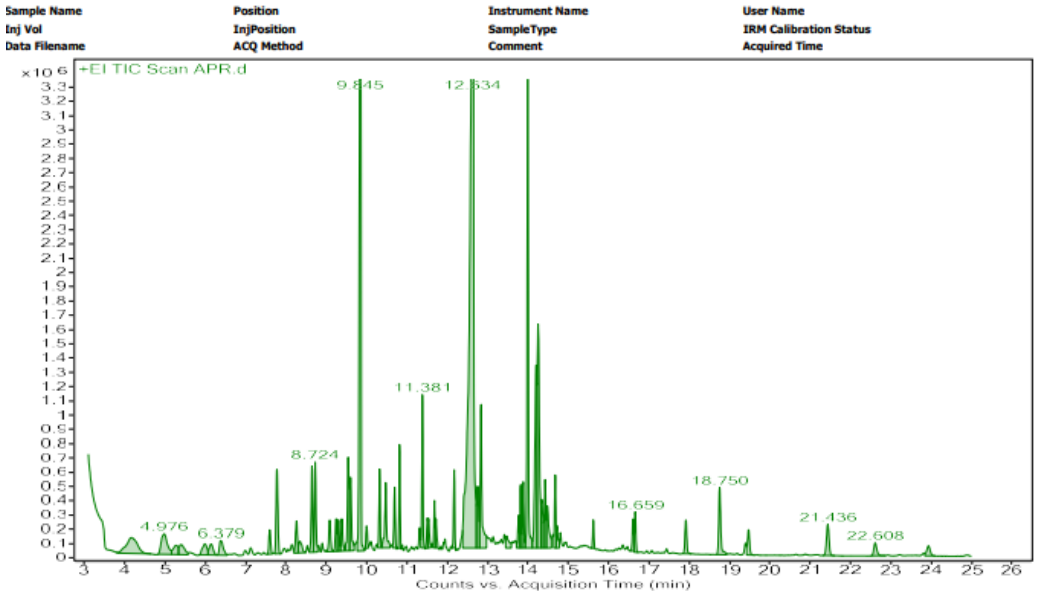
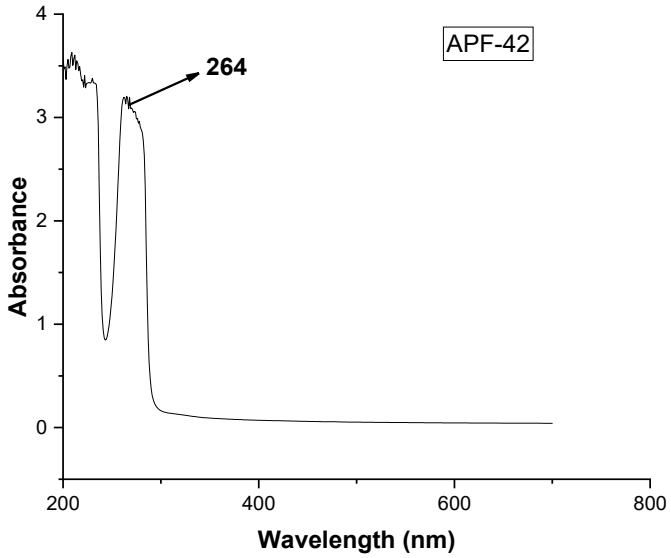
7. APPENDIXES

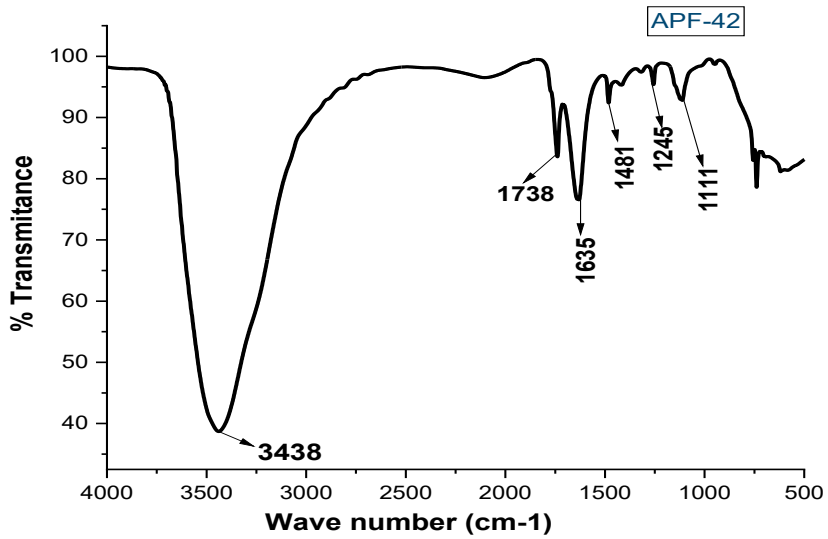
Appendix 1: GC-MS chromatogram of essential oils of the root of *A. polystachius*



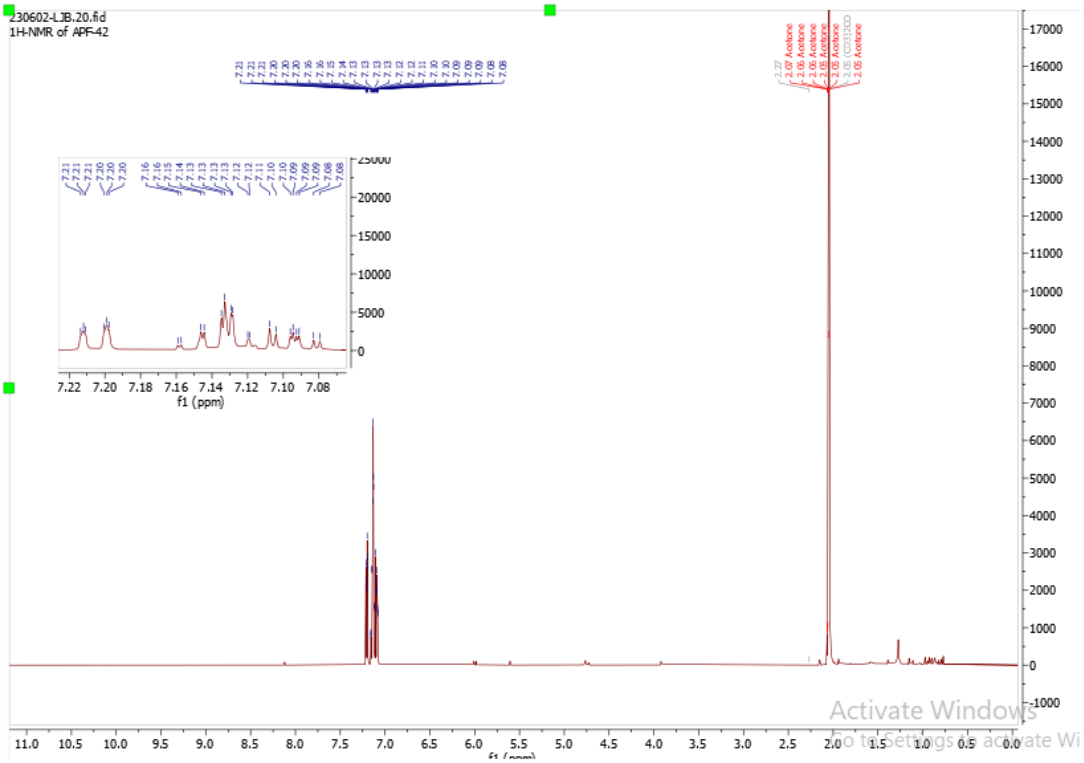
Appendix 2: UV/vis spectrum of compound APF-42



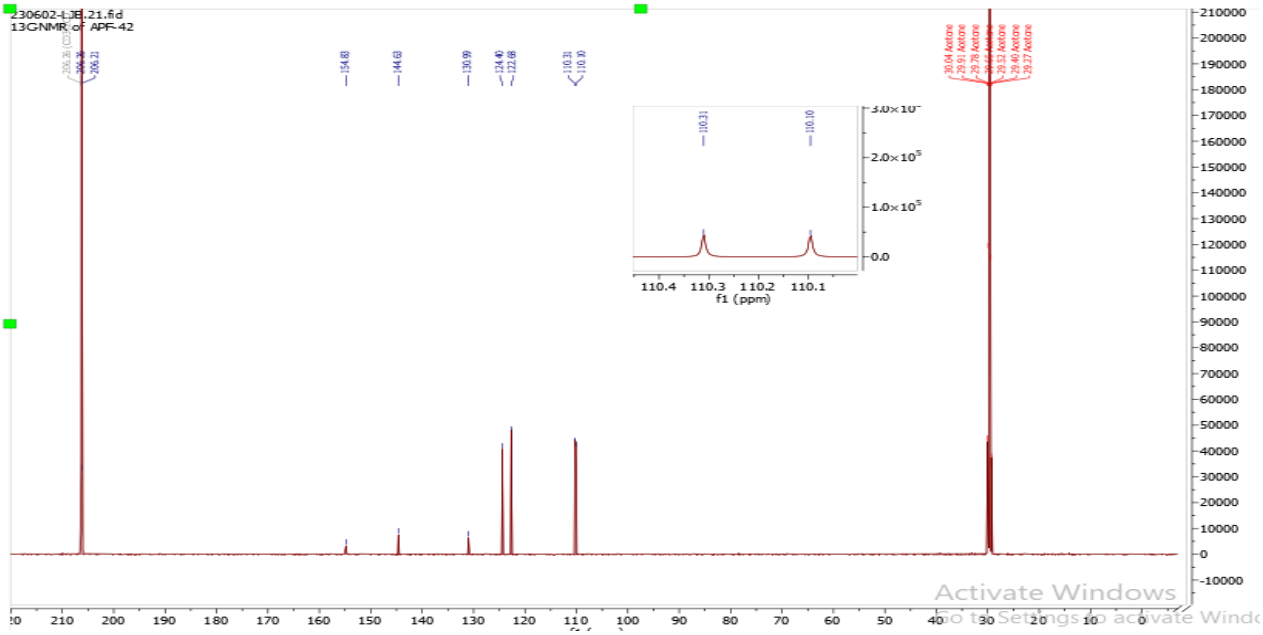
Appendix 3: IR spectrum of compound APF-42



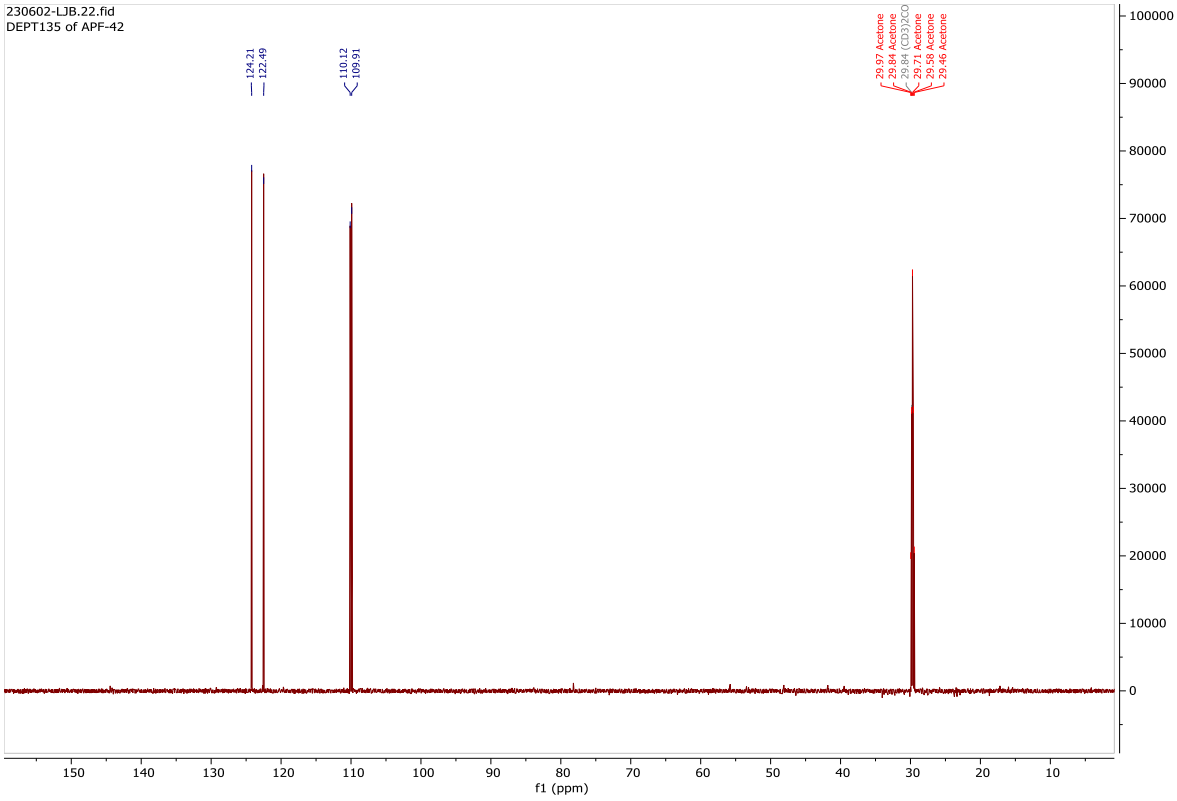
Appendix 4: ^{1}H-NMR spectrum of compound F-42 in $(CD_3)_2CO$



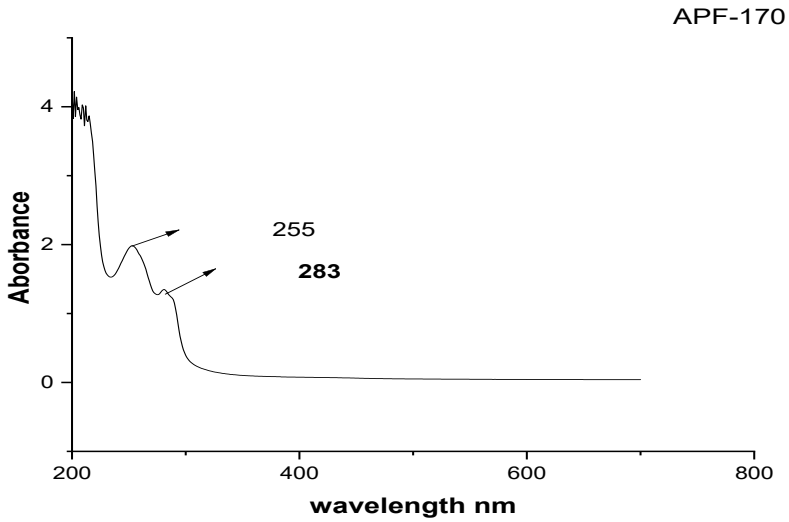
Appendix 5: ^{13}C-NMR spectrum of compound APF-42 in $(CD_3)_2$ CO



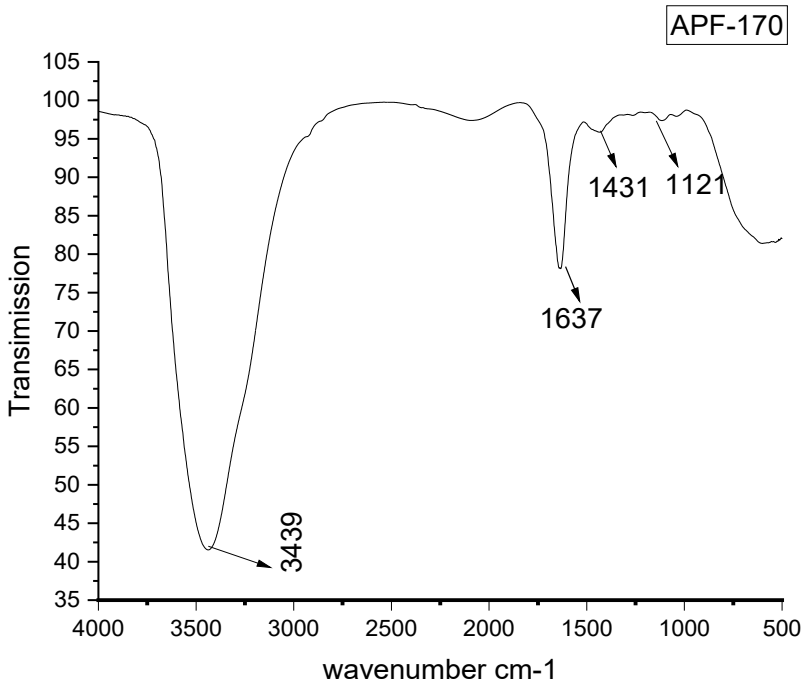
Appendix 6: DEPT-135 spectrum of compound APF-42 in $(CD_3)_2CO$



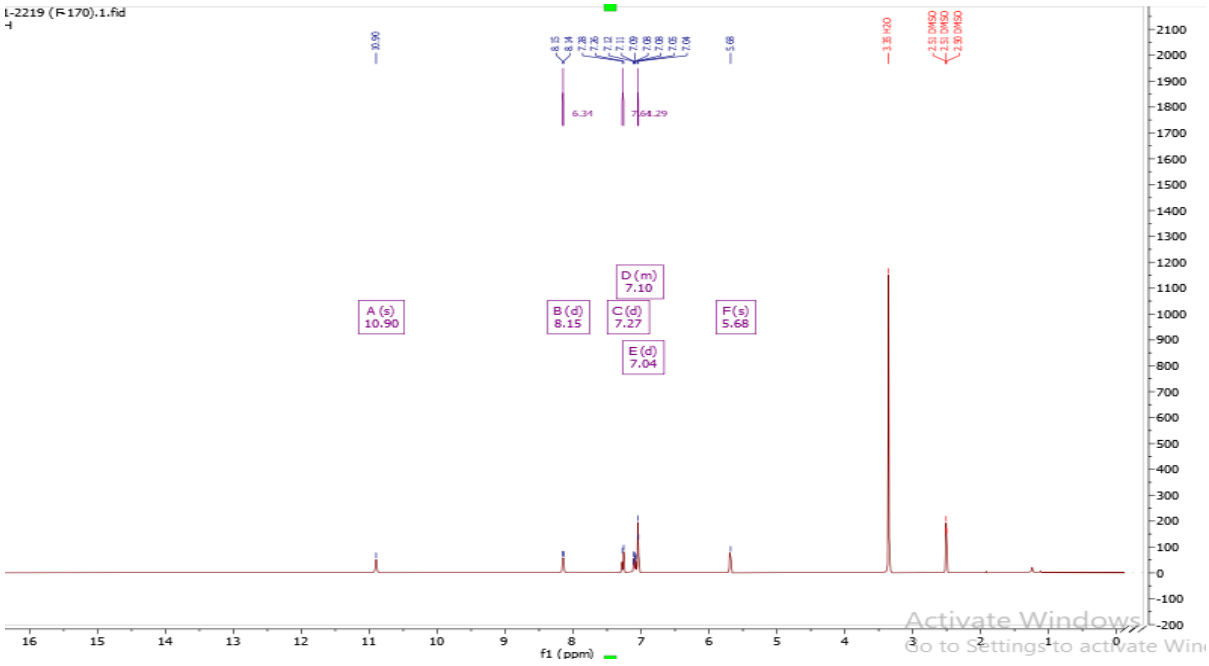
Appendix 7: UV/vis spectrum of compound APF-170



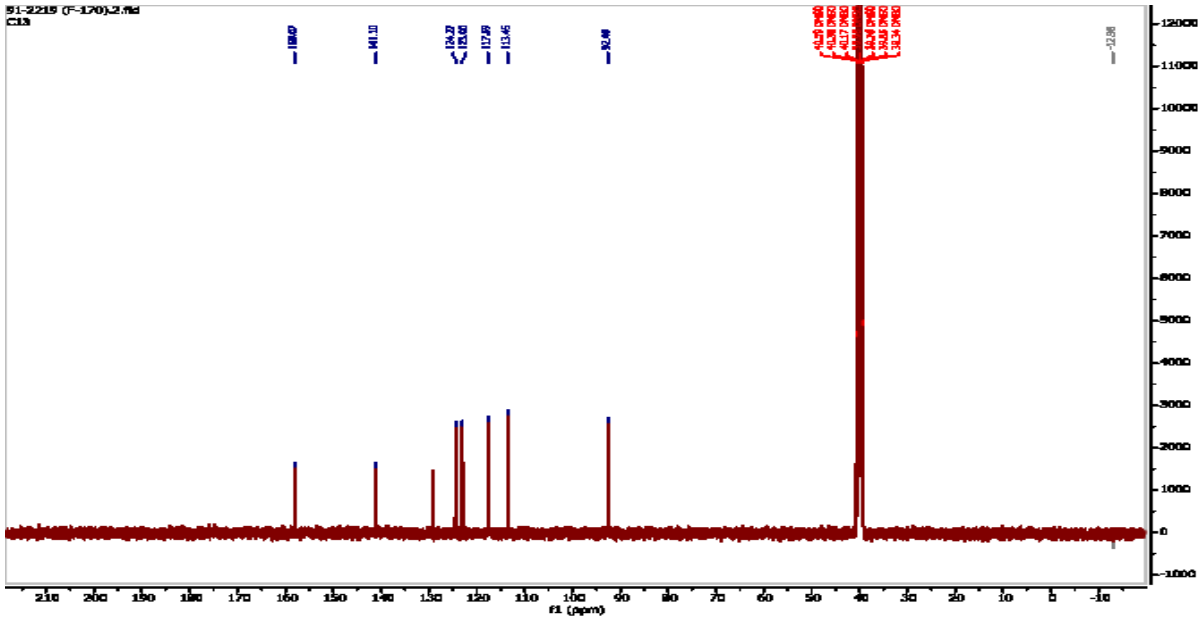
Appendix 8: IR spectrum of compound APF-170



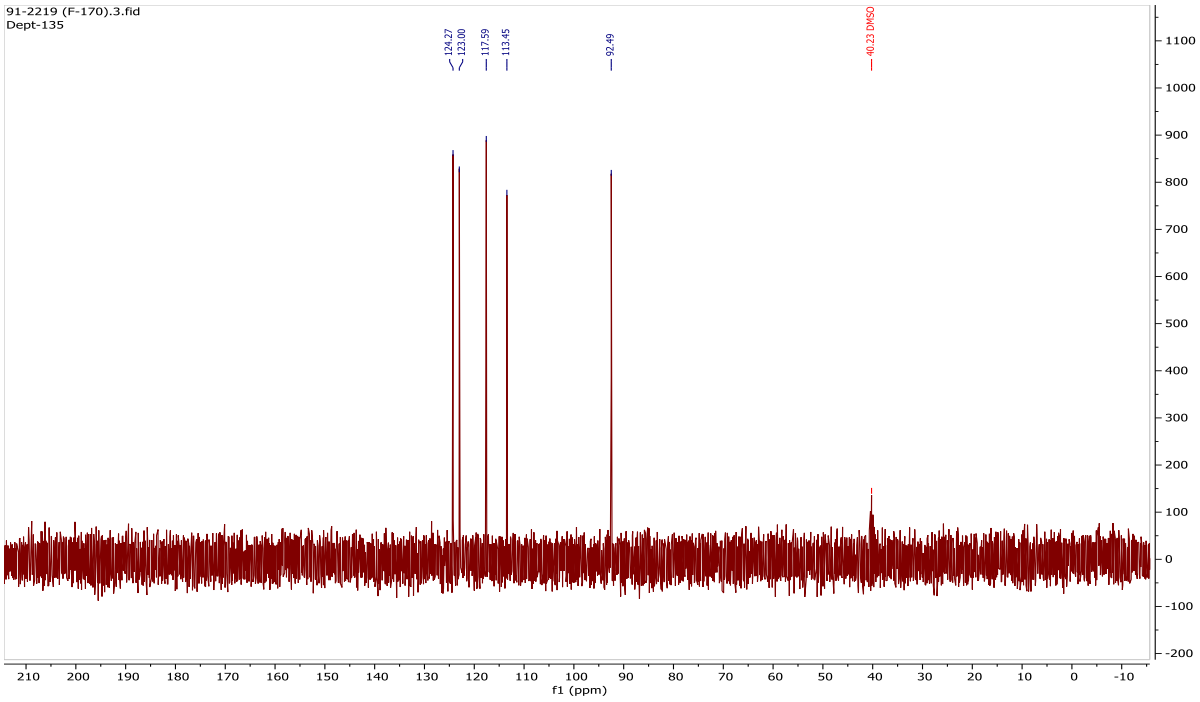
Appendix 9: ^{1}H-NMR spectrum of compound F-170 in $(CD_3)_2SO$



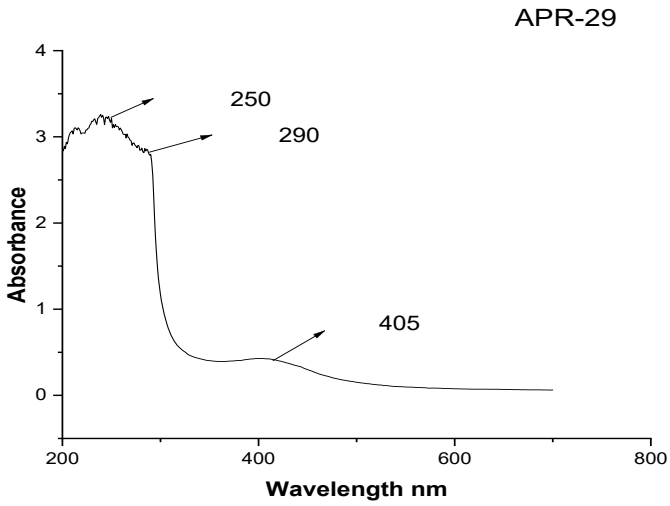
Appendix 10: ^{13}C-NMR spectrum of compound APF-170 in $(CD_3)_2SO$



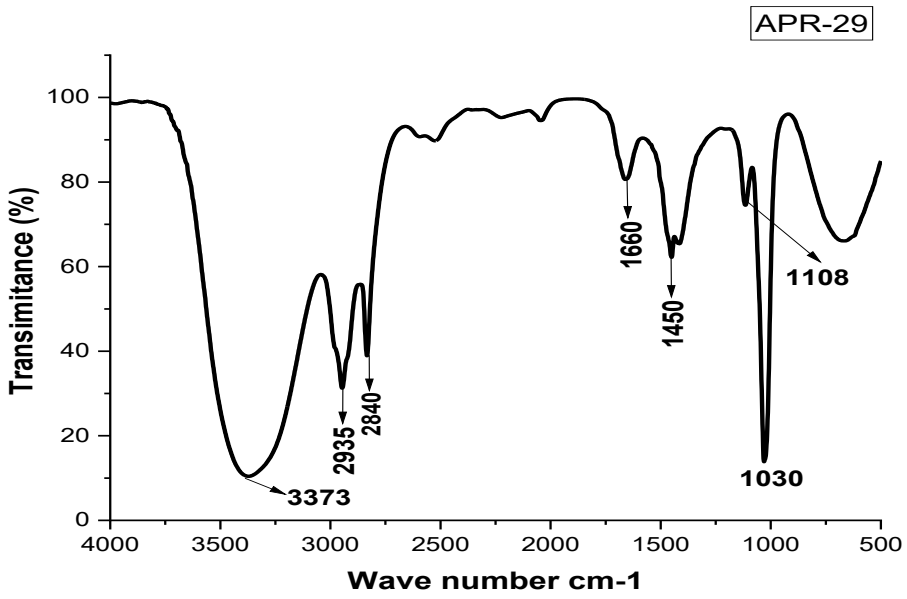
Appendix 11: DEPT-135 spectrum of compound APR-170 in $(CD_3)_2SO$



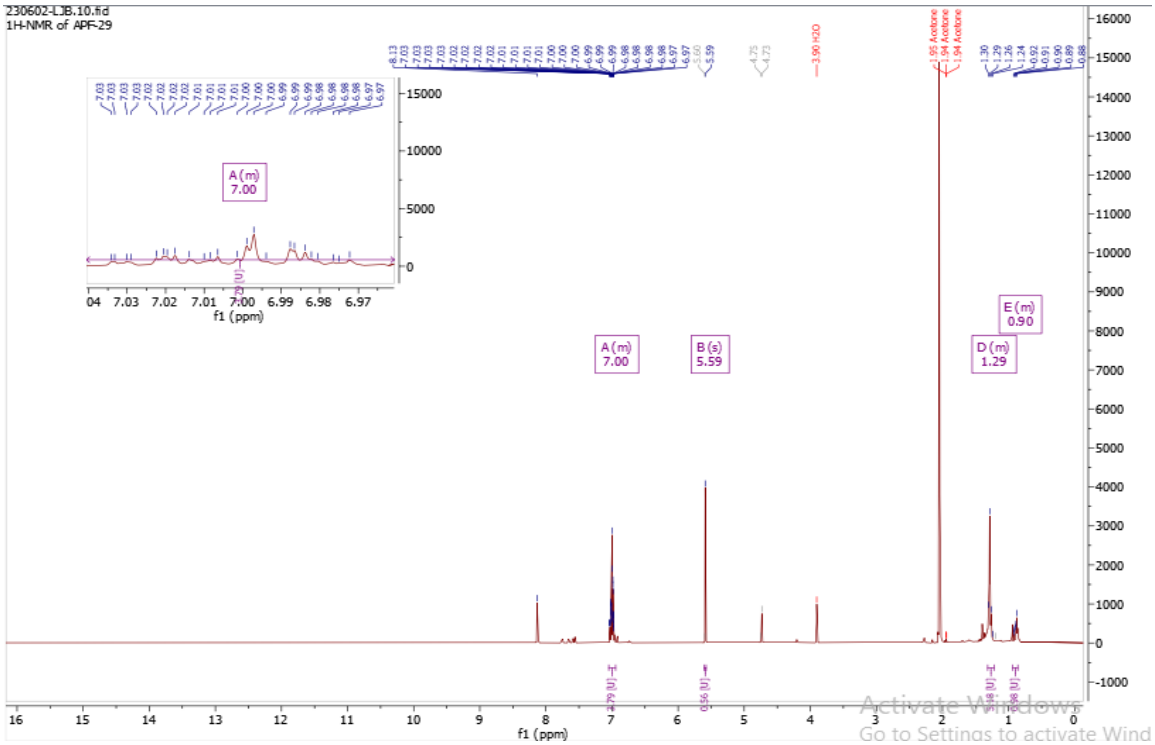
Appendix 12: UV/vis spectrum of compound APR-29



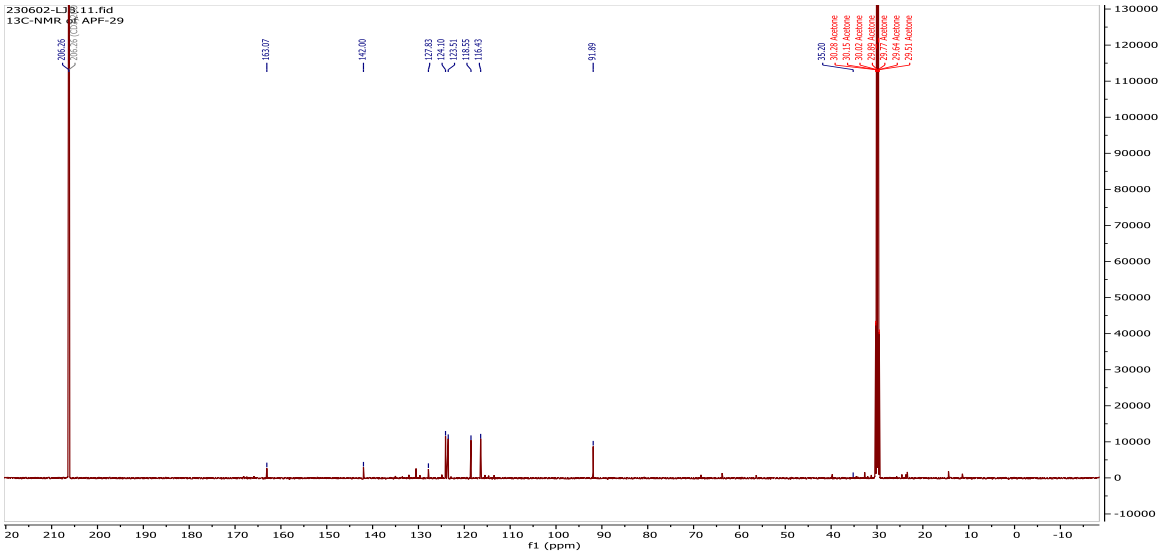
Appendix 13: IR spectrum of compound APF-29



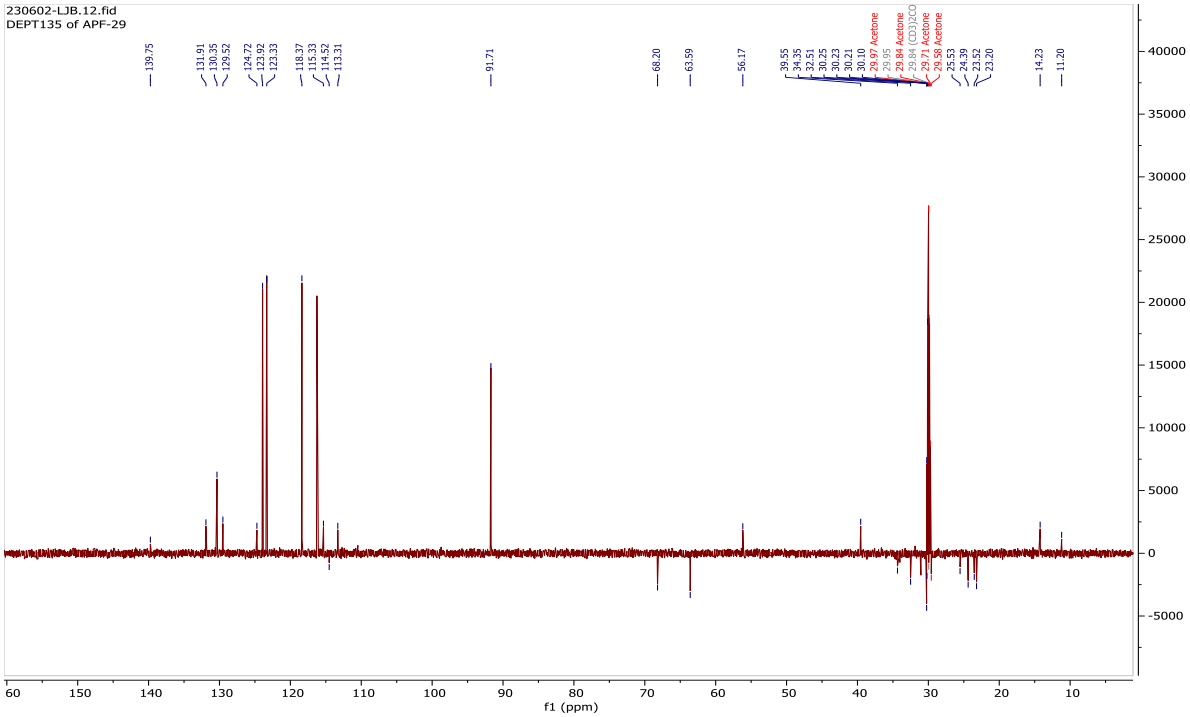
Appendix 14: ^{1}H-NMR spectrum of compound APR-29 in $(CD_3)_2CO$



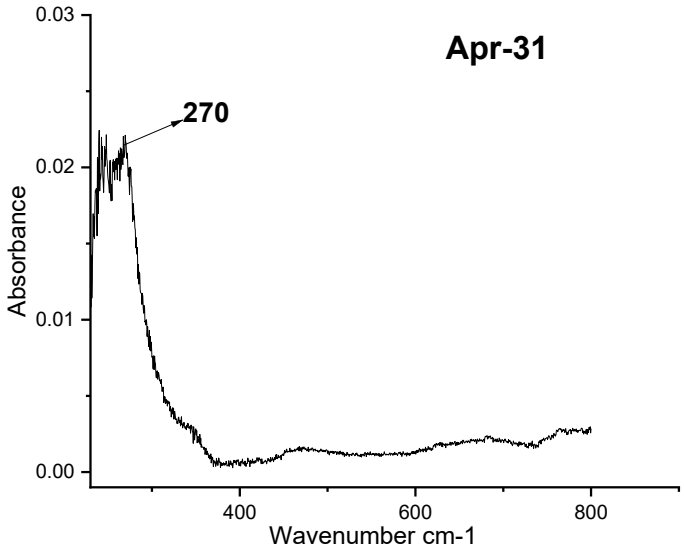
Appendix 15: ^{13}C-NMR spectrum of compound APR-29 in $(CD_3)_2CO$



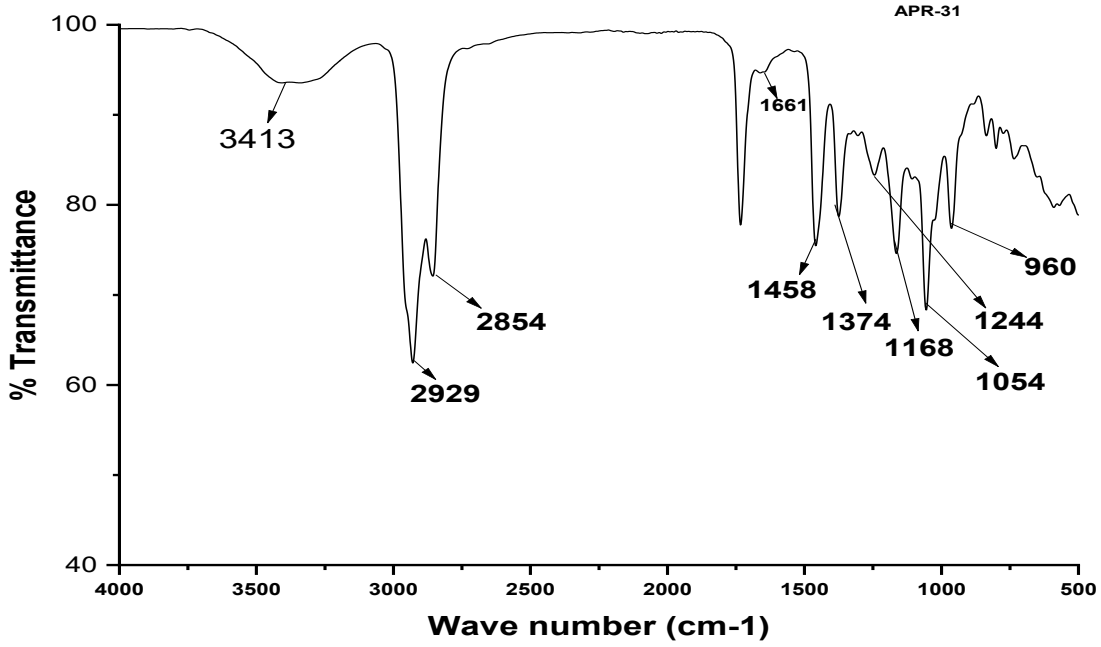
Appendix 16: DEPT-135 spectrum of compound APR-29 in (CD3)2CO



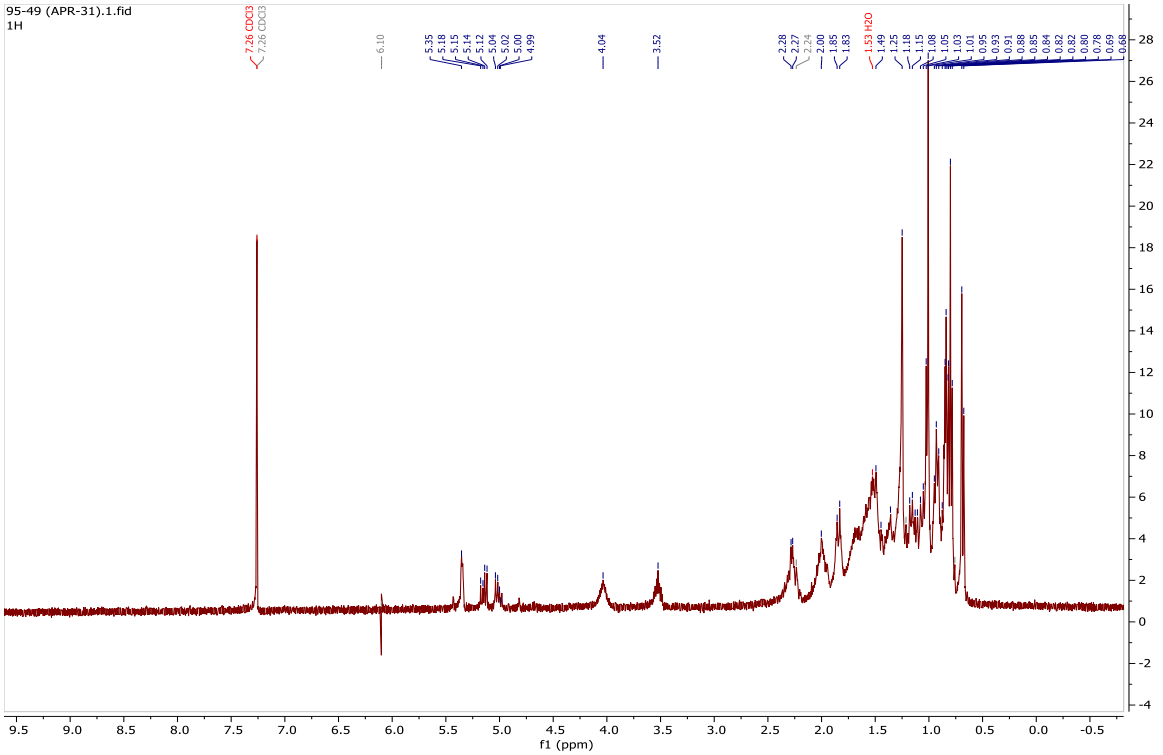
Appendix 17: UV/vis spectrum of compound APR-31



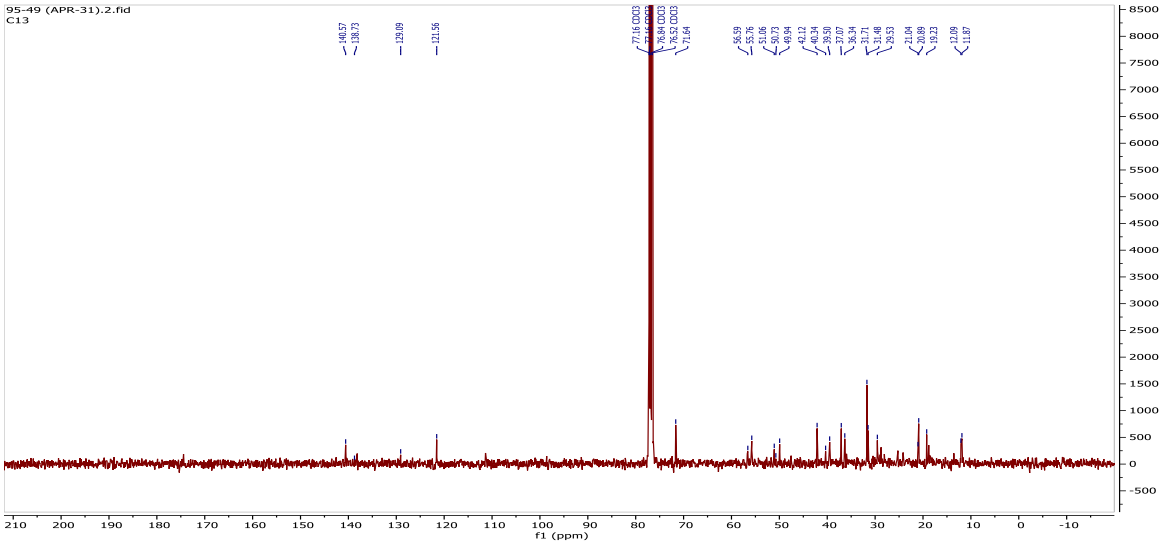
Appendix 18: IR spectrum of compound APR-31



Appendix19: ^{1}H-NMR spectrum of compound APR-31 in $CDCl_3$



Appendix 20: ^{13}C-NMR spectrum of compound APR-31 in $CDCl_3$



Appendix 21: DEPT-135 spectrum of compound APR-29 in (CD3)2CO