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COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

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MASTER'S THESIS

TITLE: PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITY EVALUATION OF THE ROOT EXTRACTS OF *OTOSTEGIA INTEGRIFOLIA*

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ADVISOR: SALAH HAMZA (Ph.D.)

HAWASSA, ETHIOPIA

MAY, 2023

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A THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY OF HAWASSA UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY (ORGANIC CHEMISTRY)

HAWASSA, ETHIOPIA

MAY, 2023

DECLARATION

I, the undersigned, declare that this thesis entitled "phytochemical investigation and antibacterial activity evaluation of the root extracts of *Otostegia integrifolia*" is my original work and has not been presented for a degree or diploma in any University and that all sources of materials used for this thesis have been duly acknowledged.

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This thesis has been submitted for examination with my approval as University advisor.

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ADVISOR’S APPROVAL SHEET-I

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(Submission Sheet-1)

This is to certify that the thesis entitled ‘‘Phytochemical investigation and antibacterial activity evaluation of the root extracts of *Otostegia integrifolia*” submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry with specialization in Organic Chemistry of the graduate program of the Department of Chemistry, Hawassa University, and is a record of original research carried out by Feleke Tigabieneh Moges (ID. No. GPOrgCR/0003/12), under my supervision, and no part of the thesis has been submitted for any other degree or diploma. The assistance and help received during this investigation have been duly acknowledged. Therefore, we recommend that it be accepted as fulfilling the thesis requirements.

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We, the undersigned, members of the board of Examiners of the final open defense by Feleke Tigabieneh Moges have read and evaluated his thesis entitled **"**Phytochemical investigation and antibacterial activity evaluation of the root extracts of *Otostegia integrifolia*" examined the candidate. This is therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree.

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DEDICATION

I dedicated this thesis to my mother, Dejitinu Agmas Asfaw, and my little brother, Abraham Tigabieneh. It is also dedicated to my memory at Hawassa University.

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ACRONYMS

O. integrifolia	*Otostegia integrifolia*
O. fruticosa	*Otostegia fruticosa*
O. limbata	*Otostegia limbata*
O. persica	*Otostegia persica*
A. baumannii	*Acinetobacter baumannii*
B. subtilis	*Bacillus subtilis*
E. aerogenes	*Enterobacter aerogenes*
E. coli	*Escherichia coli.*
E. fecalis	*Enterococcus fecalis*
K. aerogenes	*Klebsiella aerogenes*
K. pneumonia	*Klebsiella pneumonia*
L. monocytogens	*Listeria monocytogens*
P. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus.*
S. epidermidis	*Staphylcoccus epidermidis*
S. faecalis	*Staphylococcus faecalis*
S. pyogenes	*Streptococcus pyogenes*
S. typhi	*Salmonella typhi*
V. cholerae	*Vibrio cholerae*
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO-d6	Deutrated Dimethyl Sulfoxide
IC_{50}	Half-maximal inhibitory concentration
L.A.	Lead acetate
MIC	Minimal inhibitory concentration
TLC	Thin- layer chromatography

ABSTRACT

Medicinal plants have been the oldest and most widely used system of medicine to treat diseases caused by different pathogens and fight infections worldwide since ancient times. Recently, including in Europe and America, there has been a growing interest in medicinal plants. Traditionally, *O. integrifolia* leaves have been used in Ethiopia & Eritrea as insecticides, to treat tonsillitis, uvulitis, hypertension, etc. Its roots have also been used as an anthelmintic and for the treatment of lung conditions. Thus, this study was aimed at phytochemical investigation and antibacterial activity evaluation of the root extracts of *Otostegia integrifolia*. The roots were collected, washed, chopped into pieces, air-dried, ground and extracted successively with $CHCl_3$, $CHCl_3/CH_3OH$ (1:1 v/v), and CH_3OH to obtain 0.4, 3.77, and 1.27 % of crude extracts, respectively. The phytochemical screening test revealed the absence of alkaloids and anthraquinones and the presence of terpenoids, phenols, flavonoids, saponins, tannins, steroids, coumarins, carbohydrates, and glycosides in all extracts except phenols, which is absent in $CHCl_3$ extracts. The $CHCl_3/CH_3OH$ (1:1 v/v) extract was subjected to gradient elution of silica gel column chromatography in n-hexane/EtOAc solvent system. The elution was result in a total of 122 fractions, and fractions 48-51 (FT1) and 59-65 (FT2) had single spots on TLC. Based on the spectral data obtained from IR, ^{1}H NMR, ^{13}C NMR, and DEPT-135, the compounds were proposed to be hystroxene-I for FT1 and Angustanoic acid E for FT2. The extracts and isolated compounds were evaluated for their antibacterial activities against two gram positive bacterial strains (S. pyogen (ATCC19615), S. aureus (ATCC25923)) and two gram negative bacterial strains (P. aeruginosa (ATCC27853), E. coli (ATCC25922)) using ampicillin as a positive control, and all showed positive effects. Among FT1 and all the extracts FT2 exhibited the most effective antibacterial activity and the $CHCl_3$ extract showed the least. The 400 µg/ml of isolate FT2 showed comparible effects (11.5, 12, 11, and 10.5 mm) with Ampicillin (13, 14, 12.5, and 12 mm) on *S. aureus, S. pyogen, E. coli,* and *P. aeruginosa* respectively.

Keywords: *Otostegia integrifolia,* phytochemical screening, hystroxene-I, angustanoic acid E, antibacterial activity.

1. INTRODUCTION

1.1. Background the study

Medicinal plants include plants that are used as traditional medicines; plants that are used as sources of pure pharmaceutical molecules such as quinine and quinidine (from *Cinchona species*), reserpine (from *Rauvolfia* species), vincristine and vinblastine (from *Catharanthus roseus*), and artemisinin (from *Artemisia annua*); plants that are used as sources of nutraceuticals such as ginseng, *Ginger officinalis, Moringa oleifera,* and so on; and plants that are used as sources of essential oils that are used for cosmetics, food additives/flavors, and carminatives [1]. Medicinal plants play a vital role in providing ecological, economic, and cultural services. In addition to this, they are very important in their uses for medical purposes [2].

Until today medicinal plants are the oldest and most widely used system of medicine in the world [3]. Since ancient times, they have been the primary means of treating diseases caused by different pathogens and fighting infections worldwide [2]. According to the World Health Organization (WHO), 70-95% of the population in developing countries, including Ethiopia rely on traditional medicines, especially herbal medicines, for their daily healthcare needs [2, 4, 5], and over 25 % of prescribed medicines in developed countries are derived from wild plant species [6]. Recently, including in Europe and America, there has been a growth of interest in medicinal plants and traditional knowledge about plant uses to explore new commercial medical products in the international pharmaceutical industry since they are noticed as a source of "qualified leads" in the identification of bioactive agents in the production of synthetic modern drugs so that all the major herbal-based pharmaceutical companies are showing constant growth [7]. Medicinal plants have been used as remedies for human and animal diseases because they contain components with therapeutic value [8]. The main phytochemical components present in medicinal plants are tannins, alkaloids, saponins, cardiac glycosides, steroids, terpenoids, flavonoids, phlobatannins, anthraquinones, essential oils, fatty acids, phenolic compounds, and reducing sugars [4].

Genus *Otostegia* belongs to the family *Lamiaceae* [9], which is one of the largest and most distinguished families of flowering plants, with about 220 genera and almost 4000 species worldwide [10]. The *Otostegia* genus comprises about 33 species, which grow mainly in the

Mediterranean region and adjoining Asia Minor [10]. In Pakistan, just 3 species have been discovered namely, *O. limbata, O. aucheri,* and *O. persica* [11]; In Iran, only 3 species are available; *O. aucheri*, *O. michauxi*, and *O. persica*, of which the last two are endemic to Iran [10]; while 15 species are endemic to the Northern part of tropical Africa and Southwestern and Central Asia; and 5 species have been reported in the flora of Ethiopia including *O. integrifolia, O. fruticosa,* and *O. tomentosa* [9, 12, 13]. Medicinally, the species of *Otostegia* are very crucial and have been widely used traditionally against various diseases, and its constituents have shown to possess antiulcer, antispasmodic, antidepressant, anxiolytic, and sedative activities [11].

O. integrifolia (Figure 1 below) is an indigenous plant in Ethiopia, Eritrea, and Yemen [13, 14, 15]. It is locally called 'tinjute' in Amharic and 'cheindog' in Tigray provenance of Ethiopia and Eritrea [13]. It is a 1-3 m high, highly branched, spiny at the nodes, and an erect perennial shrub [14, 15]. In Ethiopia, it is distributed in different parts of the dry and moist agro-climatic zones of the district called Dega, at altitudes of 1,300-2,800 m above sea level [16]. The species *O. integrifolia* is well known for its pleasant odour and omnipotent medicinal values. Traditionally, *O. integrifolia* has insecticidal properties (mosquito repellent) and has been used as a fumigant for pots and houses [9, 12]. Its roots are also used for treating lung diseases [9]. Several studies have been done on biological activities and phytochemical screening tests of the leaves extracts of this plant. Furthermore, about 40 pure compounds were isolated from its leaves. However, to the best of our knowledge, until today no phytochemical studies and investigations, or antibacterial activity tests have been reported from the roots of this plant. Therefore, this study aimed to conduct the phytochemical investigation and antibacterial activity test from the root extracts of *O. integrifolia* and isolated compounds

1.2. Statement of the problem

The current antibiotics are resistant to overcome diseases, so this study looks for medication from medicinal plants. Traditionally, different parts of *O. integrifolia* have been utilized in the management of various ailments in many Eritrean & Ethiopian communities. For instance, the juice of the leaves of this plant is used for the treatment of stomach aches and its root is used to treat lung diseases [9]. It is also used as a fumigant for pots, houses, and reservoirs [9, 12]. The therapeutic ability of this medicinal plant is something that herbalists are aware of from their

daily activities, cultures, and societal customs, even if they are unaware of the scientific basis for it. Due to this reason, the knowledge and practice of using it are transferred orally from generation to generation, and this limits the importance and popularity of this plant. The majority of chemical components found in medicinal plants can operate alone or in synergy to promote health. Several researchers reported biological activities and phytochemical screening tests from the leave extracts of *O. integrifolia,* and about 40 compounds were isolated. I have done a repeated search and found that until today no phytochemical studies and investigations have been reported from the roots of this plant. Thus, the important concern of this study is to find bioactive phytochemicals that have significant effect on bacterial strain which support the traditional use of the plant.

Figure 1: *O. integrifolia* leave part (Photo taken by Feleke Tigabieneh on January 25, 2014 E.C.)

1.3. Objective of the study

1.3.1. General objective

The general objective of this study was to investigate phytochemicals and antibacterial activity evaluation of the root extracts of *O. integrifolia***.**

1.3.2. Specific objectives

- To extract the crudes from the roots of *O. integrifolia* using $CHCl_3$, $CHCl_3/CH_3OH$ (1:1), and CH_3OH.
- To assess phytochemical screening tests on each crude extracts of the plant.
- To isolate compounds from $CHCl_3/CH_3OH$ (1:1) root extract of *O. integrifolia,* which showed the best TLC profile, using silica gel column chromatography.
- To evaluate the antibacterial activities of the root extracts of *O. integrifolia* and isolated compounds using two gram positive bacterial strains (S. pyogen (ATCC19615), S. aureus (ATCC25923)) and two gram negative bacterial strains (P. aeruginosa (ATCC27853), E. coli (ATCC25922)).
- To elucidate the structure of isolated compounds using spectroscopic data such as FTIR, ^{1}H NMR, ^{13}C NMR, and DEPT-135.

1.4. Significance of the study

To the best of our knowledge, until today nothing was done on the phytochemical screening tests, isolation of compounds, and antibacterial activity tests from the root extracts of *O. integrifolia*. The results of this study provided information about the class of secondary metabolites present in the root extracts of *O. integrifolia.* This also leads researchers from related fields to have information about the nature of the chemical constituents present in the root of the plant by identifying the structure of isolated chemical constituents. The antibacterial effects of the crude extracts and isolated compounds also contributed to support the existing plant's traditional usage against bacterial infection and used as a template to develop structurally similar synthetic pharmaceuticals to overcome diseases. Moreover, this study fill some gaps in the previous studies on *O. integrifolia,* and it may serve as a reference to look for further in phytochemical studies and studies in the area of antimicrobial agents from the species.

2. LITERATURE REVIEW

2.1. The Genus *Otostegia*

Genus *Otostegia* belongs *Lamiaceae* family [9] and comprises about 33 species, namely *O. ambigens, O. kaiseri, O. olgae, O. arabica, O. kotschyi, O. persica, O. aucheri, O. limbata, O. repanda, O. benthamiana, O. ongipetiolata, O. scariosa, O. bucharica, O. megastegia, O. sinaitica, O. ellenbeckii, O. michauxii, O. somala, O. erlangeri, O. migiurtiana, O. steudneri, O. fruticosa, O. minuccii, O. schimperi, O. glabricalyx, O. modesta, O. tomentosa, O. hildebrandtii, O. moluccoides, O. schennikovii Scharasch, O. integrifolia, O. nikitinae Scharasch,* and *O. sogdiana Kudr,* which grow mainly in the Mediterranean region and adjoining Asia Minor [10]. Since ancient times, naturally, the species of *Otostegia* are medicinally decisive, and they have been traditionally used as an ophthalmia, antihyperglycemic, antioxidant, antimicrobial, anti-inflammatory, antispasmodic, antiulcer, insecticidal, anti-malaria, antidepressant, anxiolytic and sedative activities to prevent different kinds of sickness and disorders [9, 11, 13].

2.2. Ethnobotanical uses of the genus *Otostegia*

The market is full of a wide range of allopathic drugs to provide relief to patients suffering from infections [17]. It is estimated that up to 80% of the world's population living in the developing world rely on herbal medicinal products and supplements as a primary source of healthcare and traditional remedial practice [18]. Plants have provided medicinally valuable substances since ancient times and are the cheapest as well as a vital source of pharmacologically active chemical compounds used for the treatment of various diseases; and local herbs based remedies are being remarkably consumed by a large number of people living in rural areas for several purposes [19]. The species of the genus *Otostegia* are widely used by traditional practitioners against various diseases, and their constituents have shown to possess anti-ulcer, antispasmodic, antidepressant, anxiolytic, and sedative activities [20]. The ethnobotanical uses of the four species of *Otostegia* are explained well in the following subsections.

2.2.1. Ethnobotanical uses of *Otostegia limbata*

The species *O. limbata* (Benth.) Boiss. (Syn. *Ballota limbata* Benth.; *Labiatae*) is vernacularly known as "spin aghzai", "pishkand", "Spin azghay", "chiti booti", Chitti jharri, "chitta jand", "Bui" and "Phut kanda" [13, 19, 21]. It is widely distributed in the North-West frontier provinces and lower hills of West Punjab in Pakistan as well as in diverse parts of Northern Pakistan together with the Siran Valley of Mansehra, Malam Jaba, along the Lahore to Islamabad motorway, and in Northern West of Pakistan in the hilly part near Abbottabad [19, 21]. It is a tiny bush, greatly branched, slender in shape, and a 2 feet tall spiny shrub, and has a yellowish or brownish stem, which is quadrangular on new shoots with short eglandular hairs [19]. The Main branches are erect, divided, spiny, woody, and white or maybe gray-colored bark, while the leaves are oblong oblanceolate, crenate or dentate, clustered, attenuate at base, obtuse at apex, small with short petiole and spiny bracts, and it has pale yellow, clusters and long axillary flowers bloom in its flowering period April to May [19].

Ethnomedicinally, *O. limbata* has been used as an antiseptic, antibacterial, antiulcer, antispasmodic, antidepressant, anticancer, and treatment of goiter, boils, scabies, jaundice, dental problems, wounds, cuts, narcotic, tonic, hypertension, eye infections, children's gums diseases and remedial purposes in cases of ophthalmia, as well as it used to improve eye vision in many provinces of Pakistan such as Punjab, Malakand, Azad Jammu, and Kashmir [13]. It is also used for wound healing in the Himalayas [13, 19-21]. In Pakistan, the juice of the fresh leaves of *O. limbata* (Benth.) is taken orally to treat gums and scabies, and powder of dried leaves is mixed with butter and layered on wounds and boils [22], while an aqueous extract of the herb is locally used for the treatment of eye inflammations and infections [17]. In the high mountainous region of Chail valley, the dried, grounded, and powdered leaves of *O. limbata* (Benth.) is mixed with honey and a tablespoon is taken once a day for wound healing and gum disease [23].

2.2.2. Ethnobotanical uses of *Otostegia persica*

O. persica (Burm.) Boiss. is locally known as "Golder", in Baluchistan, Iran [13]. It is a 1-2 m high shrub that is widely distributed in the arid and subtropical regions of the south and southeastern parts of Iran, particularly in Fars between Shiraz and Jahrum (southeast of Iran), Kerman, Sistan, and Baluchestan provinces (east region of Iran), and southwest Pakistan [9, 14,

24, 25]. It is a spiny shrub plant with rectangular woody stems. Its leaves are opposite on stems with short petioles and obovate blades and covered with dense white hairs. The flowers of this plant have funnel-shaped calyx with longitudinal ridges and a bilabiate white corolla with a hairy upper lip [9].

Traditionally, in Iran, the decoction of the leaves and flowers of *O. persica* is used for the treatment of malaria, rheumatism, diabetes, fever, cardiac distress, reducing palpitation, hypertension, antipyretic, cold, hyperlipidemia, gastric discomfort, inflammatory conditions, oral infections, headache, parasite repellent, sedative, laxative and carminative [13, 26-29]. It has also been used as an analgesic in toothache, and the aqueous extract of the aerial parts of this species is traditionally used as antihistaminic, antispasmodic, anti-arthritis, and antipyretic [29].

2.2.3. Ethnobotanical uses of *Otostegia fruticosa*

O. fruticosa (Forssk.) Schweinf. ex Penzig is commonly known as 'sasa' in the Tigray region, 'geram tungut' in Amharic in Central Ethiopia, ‘‘Fesi hadima” in Eritrea, and 'shakab and sharm' in Saudi Arabia [13, 30]. It is a shrub widely distributed in West and Eastern Africa and Middle Eastern countries including Ethiopia, Eritrea, Djibouti, Sudan, Cameroon, Saudi Arabia, Yemen, Israel, and Palestine [31]. It is also growing in its natural habitat on the stony ground in the Red Sea coastal region, Sinai, and El-Tih desert in Egypt [32]. The branches of this plant are more or less densely hairy; the leaves are oval to rounded and are 5-12 cm long; the flowers are cream in colour [30].

O. fruticosa has been used in the treatment of tonsillitis, stomach ache, asthma, arthritis, febrile illness, sun-stroke, paralysis, eye diseases, and as a mosquito repellent and gynaecological problems in different parts of Ethiopia and other countries. [31, 33]. In Eritrea, this plant's leaf and stem are used for treating arthritis, tonsillitis, and gynaecological problems [34], while in Saudi Arabia infusion of the flowering branches of *O. fruticosa* is used to treat sun-stroke [13, 35]. It is also used for the treatment of eye diseases and anti-paralytic in Yemen; as a remedy for diarrhea, mosquito repellent, and tonsillitis in North and central Ethiopia [13].

2.2.4. Ethnobotanical uses of *Otostegia integrifolia*

O. integrifolia Benth. (syn: *Rydingia integrifolia* (Benth.) Scheen and V.A. Albert) is commonly known as Abyssinian rose, and it is also known by its vernacular name 'Tinjut' (Amharic) [9]. It is an erect perennial shrub, much branched and spiny at the nodes, 1-3 m high, and is endemic to Eritrea, Ethiopia, and Yemen [14, 15]. In Ethiopia, it is distributed in the dry evergreen woodlands of the Bale Mountains, Tigray, Gondar, Wollo, Gojjam regions, North Shewa, Kaffa, and Hararghe, as well as in the dry and moist agro-climatic zones of the district called Dega, at altitudes of 1,300-2,800m above sea-level [16]. It is a very common shrub in overgrazed hillsides or old and abandoned farms in the wild, and cultivated in gardens [16, 36]. This species is easily recognized and stands out due to the grayish color of the leaves, the yellow flowers that are showy when in full bloom, and the fruits that are small nutlets within the calyx [9, 12, 36].

Traditionally, the leaves of *O. integrifolia* are used to treat tonsillitis, uvulitis, hypertension, and insecticides in Eritrea [34]. It is used as an anticough, antirheumatic, antiabortifacient, and application against infertility [36], insecticides, antimalarial, and in the treatment of type 2 diabetes mellitus, stomach ache, evil eye, fever, vomiting, nausea, diarrhea, dysentery, as well as sterilization and ritual custom in Ethiopia, especially in the northern part of Ethiopia. It is also used in the treatment of ectoparasites in livestock in Tigray, Ethiopia [13, 37]. It is also reported that the juice of the leaves of *O. integrifolia* with water has been taken orally as a single (alone) or multiple (combined with other plants) prescription to treat stomach ache and 'Megagna'; inhaling the steam of boiled leaves in water to treat 'Mich'; inhaling the smoke of the leaves and stems to treat 'evil eye' [38].

In addition to this, the juice of dried powdered roots is taken as anthelmintic in the Asendabo district of southwest Ethiopia (Jimma zone) [39]. It is also reported that the roots of *O. integrifolia* has been used for treating lung diseases [9].

2.3. Biological activity of *Otostegia*

It is well known that plants provide a diverse range of secondary metabolites and many of which have different biological activities [17]. The plants in the genus *Otostegia* is the imperative source of different biologically active natural products for pharmacological use, the crude form

as well as different fractions or extracts of the genus *Otostegia* have exhibited good anti-inflammatory, antimicrobial, antioxidant activities and other biological activities [19]. In addition to this, the compound kaempferol pentaglycosides isolated from the genus *Otostegia* have been used for the authentication and standardization of medicinal interests as well as possessing great chemotaxonomic value. It is also reported that the flavonoid 4-hydroxy (E)-cinnamoyl glucosides in *Otostegia* species have been viewed as valuable markers from a chemotaxonomic point of view [19]. Different concentrations and different solvent extracts of the plant species of the genus have different biological activities for example the methanol extract of aerial parts of *O. persica* has been shown antibacterial activities against gram-positive strains of bacteria [40], and hepatoprotective activity against the CCl_4-induced acute hepatotoxicity, while the ethyl acetate extract exhibited the highest inhibition against a pathogenic fungus [10]. Some of the biological activities of the four species of this genus are explained briefly in the following subsections.

2.3.1. Biological activity of *Otostegia limbata*

O. limbata provides varieties of biologically active secondary metabolites that have good antioxidant, anti-inflammatory and antimicrobial activities. This species provides varieties of biologically active secondary metabolites which have good anti-inflammatory and antimicrobial activities [41]. The free radical scavenging potential of methanol extract from the leaves of this plant was reported in terms of radical scavenging or hydrogen donating ability through the stable radical 1, 1-diphenyl-2-picrylhydrazyl [41]. The aqueous leaves extract of *O. limbata* contains almost 4.600 mg/100 g of Zinc so it is used for the treatment of eye inflammation and eye infection in Abbottabad (Pakistan) [19]. Methanol extract of the leaves of *O. limbata* also possessed the best antibacterial activities against six strains of bacteria (two were gram-positive i.e. *B. subtilis* and *S. aureus* and four were gram-negative i.e. *V. cholerae*, *E. aerogenes*, *E. coli*, and *K. pneumonia*) and two strains of fungi (*Aspergillus niger* and *Aspergillus fumigatus*) [41].

2.3.2. Biological activity of *Otostegia persica*

The aqueous extract of the aerial parts of *O. persica* has been used as antispasmodic, antihistaminic, and antiarthritic [42]. The methanol extract of the aerial parts of this species displayed a strong antioxidant activity due to active flavonoids identified as kampferol, morin,

quercetin, and C-glucoflavone (isovitexin), and the major mode of action of these compounds is related to their ability to scavenge free radicals through the phenolic moiety of their structure [42, 43]. Besides this, the methanol extract of aerial parts (shoot) of this plant exhibited hepatoprotective activity against the CCl_4-induced acute hepatotoxicity in male rats, while the ethyl acetate extract of the aerial parts exhibited the highest inhibition against a pathogenic fungus *Candida albicans* [10]. The methanol extract of aerial parts of *O. persica* was active against gram-positive strains of bacteria such as *S. aureus*, *S. epidermidis*, *L. monocytogens*, and *E. fecalis,* while $CHCl_3$ and n-hexane extracts were effective against *S. aureus*, *S. epidermidis* and *E. fecalis* with MIC values from 0.62 to 20 mg/ml [40]. It also showed positive activity in the brine shrimp lethality assay, whereas its hydro-alcoholic extract is effective on morphine withdrawal syndrome [24]. β -sitosterol was identified in methanolic extract of the aerial parts of *O. persica.* β-sitosterol alone and in combination with similar phytosterols decreases blood cholesterol levels and is sometimes used in treating hypercholesterolemia. It also inhibits cholesterol absorption in the intestine [10]. The CH_2Cl_2 fraction of the 80% ethanol extract of air-dried flowering aerial parts of *O. persica* exhibited a very good antiglycation activity which is attributed to the presence of 3´, 7-dihydroxy-4´, 6, 8- trimethoxy-flavone [27]. The aqueous extract *O. persica* exhibited anti-diabetic effect in streptozotocin (STZ)-induced diabetic rats and suggested as a candidate drug for treating diabetes [44]. In addition to this, an aqueous extract of the root of *O. persica* had shown a significant effect on blood glucose levels in diabetic rats [45]. On the other hand, the fruits and leaves of *O. persica* were found to exhibit in vitro antimalarial activity with an IC_{50} value of 31.1 µg/mL in chloroquine-resistant *Plasmodium falciparum* [26].

2.3.3. Biological activity of *Otostegia fruticosa* Forssk.

Compared with rifamycinn (antibacterial), the essential oil obtained from the hydro distillation of fresh aerial parts of this species exhibited higher antibacterial activity against tested microorganisms (*B. subtilis, S. aureus, S. epidermidis, S. faecalis, E. coli,* and *K. aerogenes*), and it showed comparable antifungal activity with Amphotericin B (antifungal) against *Candida albicans* [32]. The 70% ethanol extract and organic solvent fractions of the leaves of *O. fruticosa* exhibited a potent analgesic, anti-inflammatory, and enzyme-inhibitory activities [31].

2.3.4. Biological activity of *Otostegia integrifolia*

The methanol and ethyl acetate leave extracts *O. integrifolia* exhibited several pharmacological properties, including anti-diabetic [12], antibacterial, and antioxidant [16] as well as acaricidal [46] activities. In comparison with ciprofloxacin (antibacterial) and griseofulvin (antifungal), the essential oil obtained from hydro-distillation extract of the fresh leaves of this plant has shown that a very strong broad-spectrum antibacterial and antifungal activity against both gram-positive bacteria as well as fungi respectively [36, 47]. In the same research report, it was explained that the essential oil also exhibited significant antioxidant activity due to the free radical scavenging activity of the components such as eugenol, α-pinene, β-pinene, β-terpinolene and terpinen-4-ol [36]. Besides this, the 80% methanol leaf extract of *O. integrifolia* revealed significant antihypertensive activity in rats [15]. Due to the presence of otostegindiol, the 80% methanol leaf extract of *O. integrifolia* possesses a significant in vivo antimalarial activity against *Plasmodium berghei* in a dose-dependent manner [48]. In addition to this, the 80% methanol leaf extract of *O. integrifolia* exhibited significant analgesic and anti-inflammatory activity [12].

2.4. Phytochemical studies of the genus *Otostegia*

Different studies have shown the presence of different bioactive compounds in different solvent extracts of the genus *Otostegia* which have been used in the pharmaceutical industry, and they are marked as definable physiology upon which the therapeutic potential of the body is dependent [19]. Phytochemical studies of this genus have shown the presence of terpenoids, flavonoids, phenolic compounds, saponins, reducing sugars, and other secondary metabolites. Diterpenoids are the main constituents of the genera [13, 16]. In addition to this, phytochemical studies of the genus *Otostegia* have demonstrated the presence of thymol, γ -terpinene, labdane diterpenes, and *p*-cymene as major constituents in the essential oils of the aerial parts [36]. It is also reported that the compound kaempferol pentaglycosides have been found in the genus *Otostegia* [19]. Some of the phytochemicals isolated from some of the species of this genus are discussed briefly in the following subsections.

2.4.1. Phytochemicals in *Otostegia limbata*

The chloroform fraction of the methanolic root extract of *O. limbata* was subjected to silica gel chromatography using n-hexane with a gradient on chloroform up to 100% and followed by methanol. Eleven fractions were collected, and then fraction 9 and fraction 11 were loaded on silica gel with elution solvent systems n-hexane/EtOAc (7:3) and (6:4) to give two new tricyclic clerodane-type diterpenoids limbatolide D **(1)** and limbatolide E **(2)** respectively [49]. Similarlly, eleven fractions were collected from the chloroform fraction of the methanolic root extract of *O. limbata* that subjected to silica gel chromatography using n-hexane with a gradient on chloroform up to 100% and followed by methanol. Fraction 7, 8 and 10 with n-hexane/EtOAc (78:22), (73:27), and (65:35); while 5 and 4 with (82:18) were eluted again to give three new cis-clerodane type tricyclic diterpenoids trivially named as limbatolide A **(3)**, limbatolide B **(4)** and limbatolide C **(5)** along with two known compounds; oleanic acid and β-sitosterol respectively [50]. In addition to this, two new acylated flavonol pentaglycosides, (**6)** and (**7)** were isolated from the methanol root extract of the BuOH partition along with two known flavones cirsimaritin **(8)** and 3'-O-methyleupatorin **(9)** from the $CHCl_3$ partition [51]. On the other study, a BuOH-soluble part of the 57 from the roots of *O. limbata* yielded two new flavonol glycosides; compound **(10)** and compound **(11)** in figure 2 below [21].

On the other hand, the chloroform extract of the whole plant was subjected to silica gel chromatography using n-hexane with a gradient of chloroform up to 100% and followed by methanol. Twelve fractions were also collected, and then fraction 6 was loaded on silica gel and eluted with n-hexane/EtOAc (80:20) and (72:28) to purify compounds (**12)** and **(13)** respectively in figure 2 below [20].

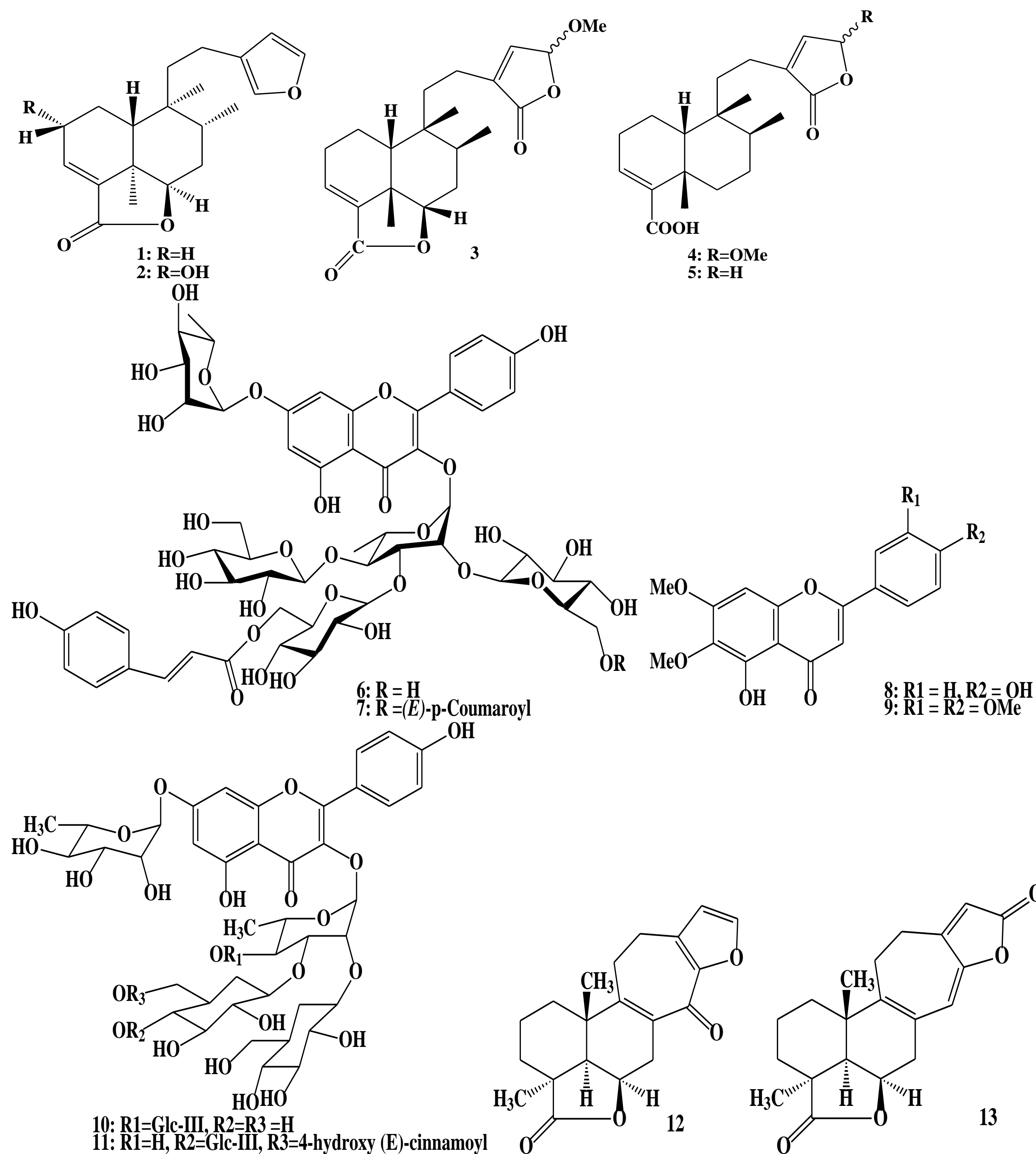


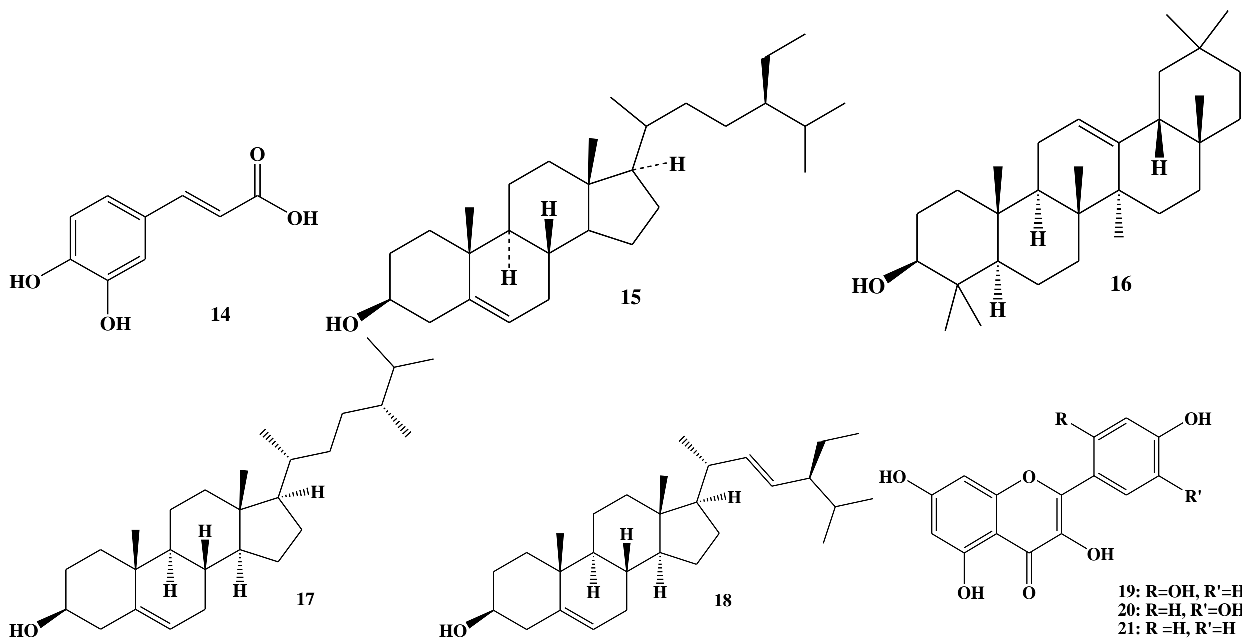
Figure 2: Structure of compounds from **1** to **13.**

2.4.2. Phytochemicals in *Otostegia persica*

Preliminary phytochemical screening of the hydroalcoholic extract of this species revealed the presence of flavonoids, steroids, tannins, and triterpenoids [52]. It was reported that Geraniol, eugenol, ceryl alcohol, and hentriacontane have been isolated from n-hexane soluble fraction;

and caffeic acid **(14)**, p-hydroxybenzoic acid, β-sitosterol **(15)**, β-sitosteryl acetate, β-amyrin **(16)**, campesterol **(17)** and stigmasterol **(18)** are isolated from ethyl acetate fraction of the methanolic extract of the aerial part of *O. persica* [25]. The other important flavonoids identified from the methanolic aerial part extract of *O. persica* are morin **(19)**, quercetin **(20)** [42, 43], kaempferol **(21)**, isovitexin (C-glucoflavone) **(22)** and trans-Cinnamic acid **(23)** using UV, IR, ^{1}H and ^{13}C NMR, and MS spectroscopies [42]. On the other study, the phytochemical investigations of the extract of the aerial parts of *O. persica* led to the isolation of 14 labdane-type diterpenoids **(24-37)**, which are depicted in figure 3, of which compounds **24-28, 31**, and **35-37** were new natural products, while the remaining compounds were isolated from the genus *Otostegia* for the first time [14].

Besides this four known diterpenoids (**38, 39, 40,** and **41)** in figure 3 belonging to the clerodane and tetracyclic diterpene types were isolated from dichloromethane fraction of 80% ethanol extracts of aerial parts of *O. persica* [24], while 3´, 7-dihydroxy-4´, 6, 8- trimethoxy-flavone (**42**) were isolated from dichloromethane fraction of 80% ethanol extract of air-dried flowering aerial parts of *O. persica* [27].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15
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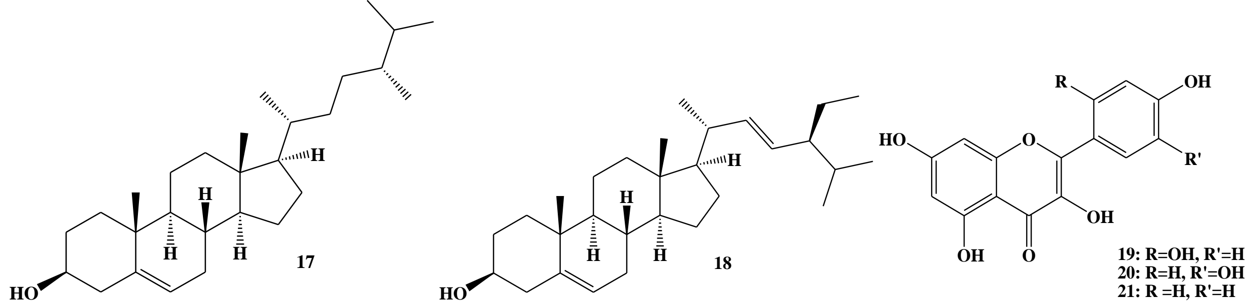
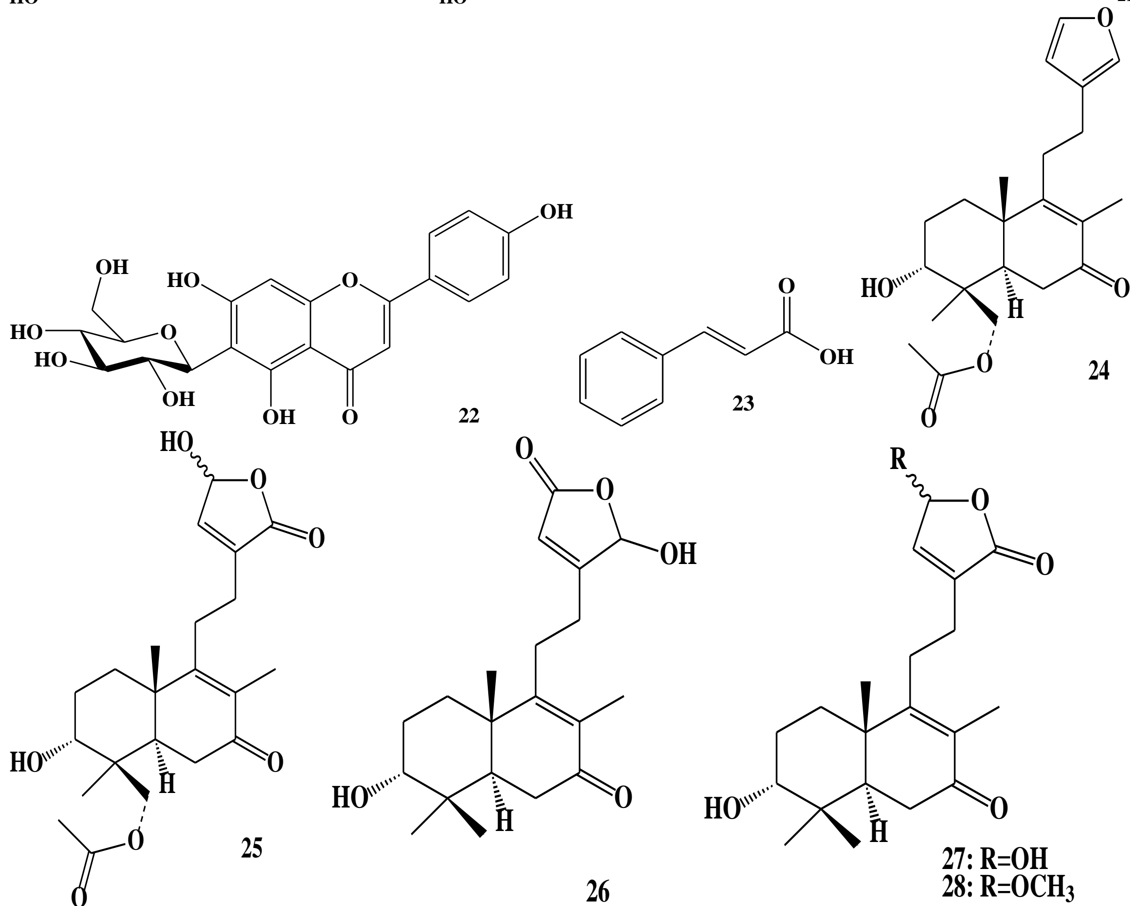

17
18
19: R=OH, R'=H
20: R=H, R'=OH
21: R =H, R'=H

22
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27: R=OH
28: R=OCH3

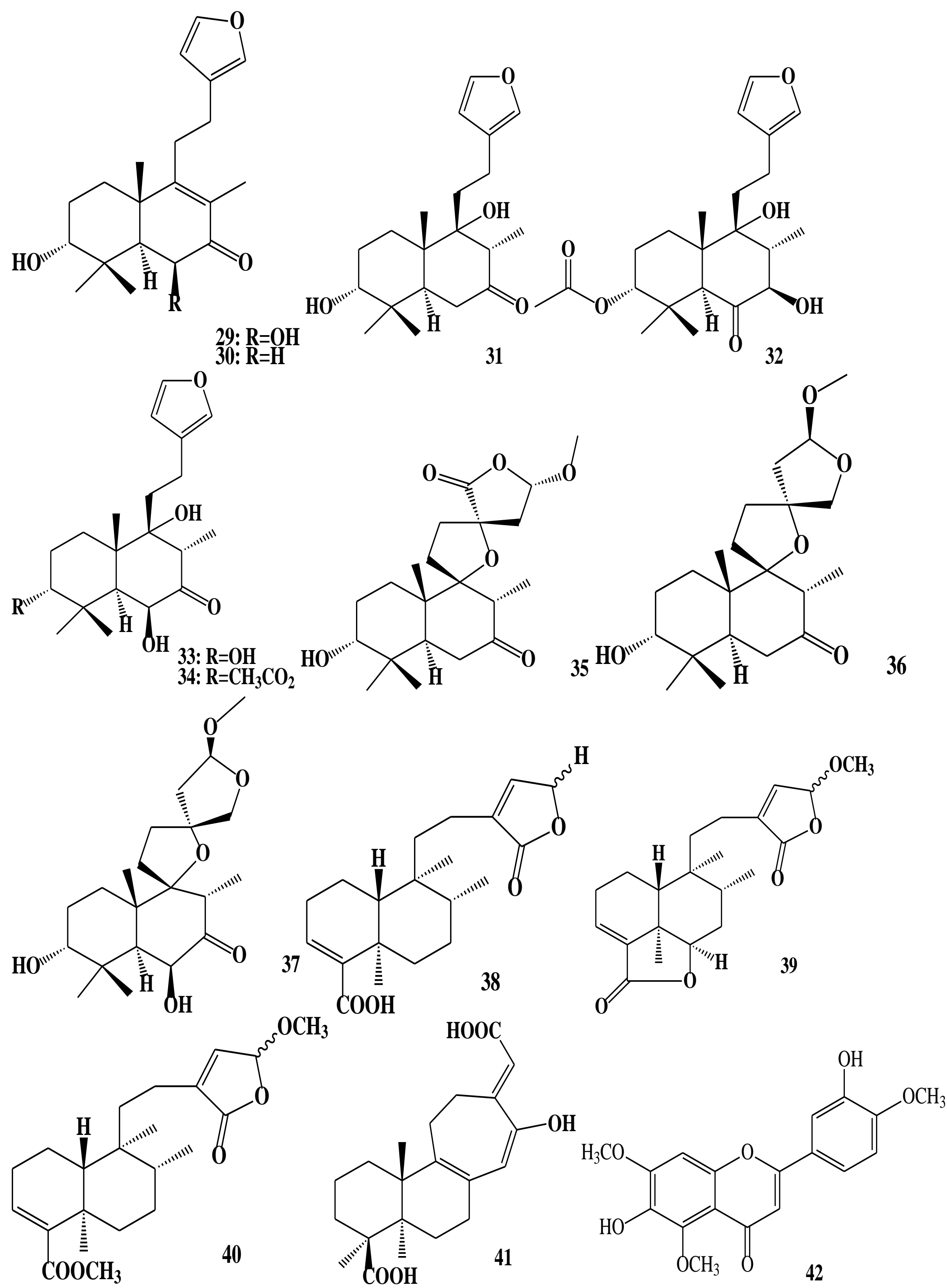


Figure 3: Structure of compounds from **14** to **42.**

2.4.3. Phytochemicals in *Otostegia fruticosa*

Analyzed by the GC-MS instrument, thirty-six compounds were identified in the hydro-distilled essential oil of fresh aerial parts of *O. fruticosa,* in which thymol, γ-terpinene, and p-cymene were reported as the main components [32]. The new labdane diterpenes; otostegin A **(43)**, otostegin B **(44),** and 15-epi-otostegin B **(45)** along with the previously known labdanes preleoheterin (**46**), leoheterin (**47**), leopersin C, 15-epi-leopersin C (**48, 49**), ballonigrin (**50**), vulgarol **(51),** and the iridoid glucoside 8-O-acetylharpagide (**52**), which are shown in figure 4, were isolated from acetonitrile and n-hexane partition of neutral and acetic acid-free ethyl acetate extract of the aerial parts of *O. fruticosa* [53].

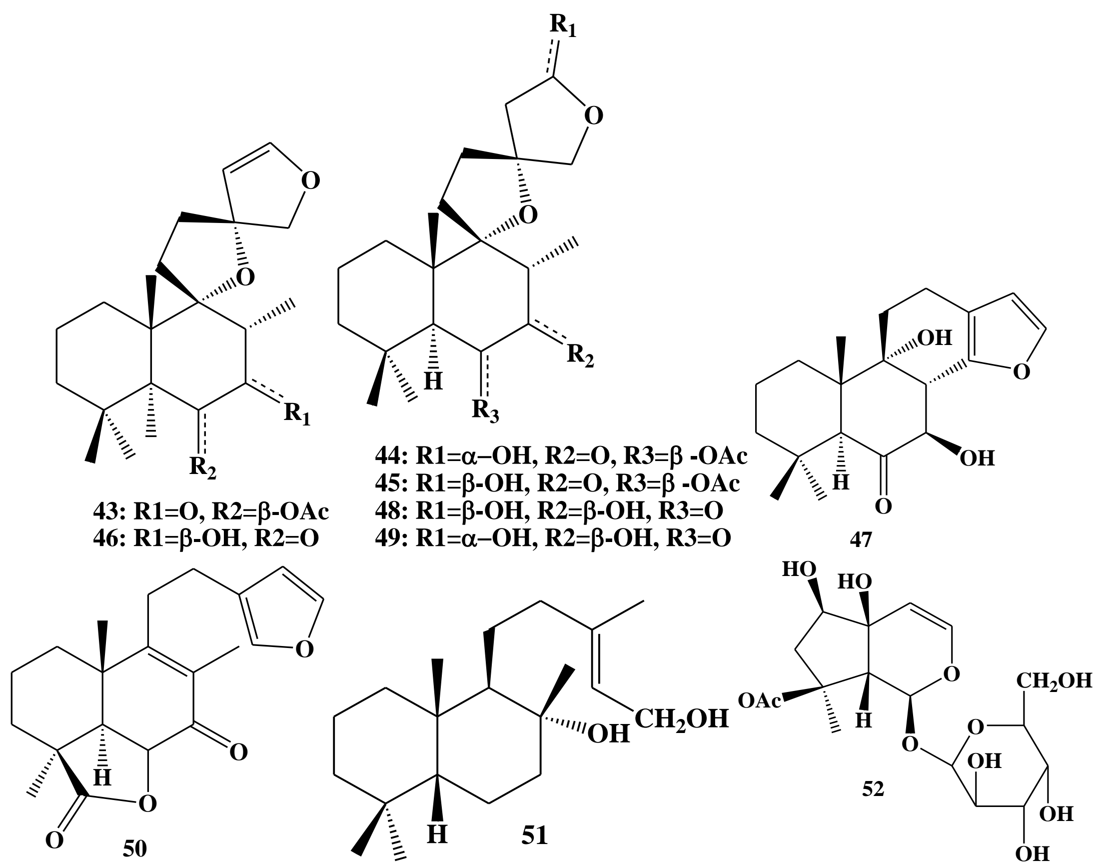


Figure 4: Structure of compounds from **43** to **52.**

2.4.4. Phytochemicals in *Otostegia integrifolia*

Preliminary phytochemical screening investigations of the 80% methanol leaf extract of *O. integrifolia* contains terpenes, phenolic compounds, saponins, reducing sugars, and flavonoids, while alkaloids, tannins, and steroidal compounds were absent [12, 15, 54]. On the other hand, the methanol and the ethyl acetate extracts were found to have a wide range of bioactive compounds including flavonoids, phenols, terpenoids, saponins, steroids, and glycosides because of the high polarity of methanol and medium polarity of ethyl acetate, whereas the petroleum ether was able to extract very limited compounds such as steroids and glycosides due to very low polarity of the solvent. However, alkaloids and tannins were absent in methanol, ethyl acetate, and petroleum ether extracts of the leaf parts of this species [16].

Some compounds have been identified and isolated from the essential oil and the chloroform extract of air-dried leaves of *O. integrifolia* and consist of terpenes in the form of monoterpenes, sesquiterpenes, and diterpenes [9]. Trans-2-hexenal, trans-hex-3-ene-1-ol, 1-hexanol, α-thujene, α-pinene, thuja-2,4(10)-diene, 1-octene-3-ol, β-pinene, 3-octanol, phenylacetaldehyde, limonene, (Z)- β-ocimene, linalool, trans-sabinol **(53)**, mentha-1,5-diene-8-ol, terpinene-4-ol, α -terpineol, β-cyclocitral **(54)**, nerol, geraniol, vinylguajacol, dihydroedulan **(55)**, theaspirane **(56)**, eugenol, α-ylangene, β-bourbonene, E-β-caryophyllene, geranylacetone, α-humulene, β-ionone, c-muurolene, germacrene D, 4,5-di-*epi*-aristolochene, α-muurolene, δ-amorphene, E-nerolidol, spathulenol, caryophyllenoxide, and β-eudesmol were identified from the essential oil of air-dried leaves of *O. integrifolia,* which was analyzed by GC and GC–MS, Whereas (+)-1-methyl-4-(5,9-dimethyl-1-methylene-deca-4,8-dienyl)-cyclohexene (axinyssene) **(57)** was isolated by preparative GC and its structure was established from its MS, 1D and 2D NMR data [9]. Besides this, two new furanolabdane type diterpenes, otostegindiol (**58)** and preotostegindiol (**59)**, a saturated hydrocarbon pentatriacontane and stigmasterol were also isolated from the chloroform extract of the dried and pulverized leaves of the plant [9]. The structure of these compounds are shown in figure 5 below.

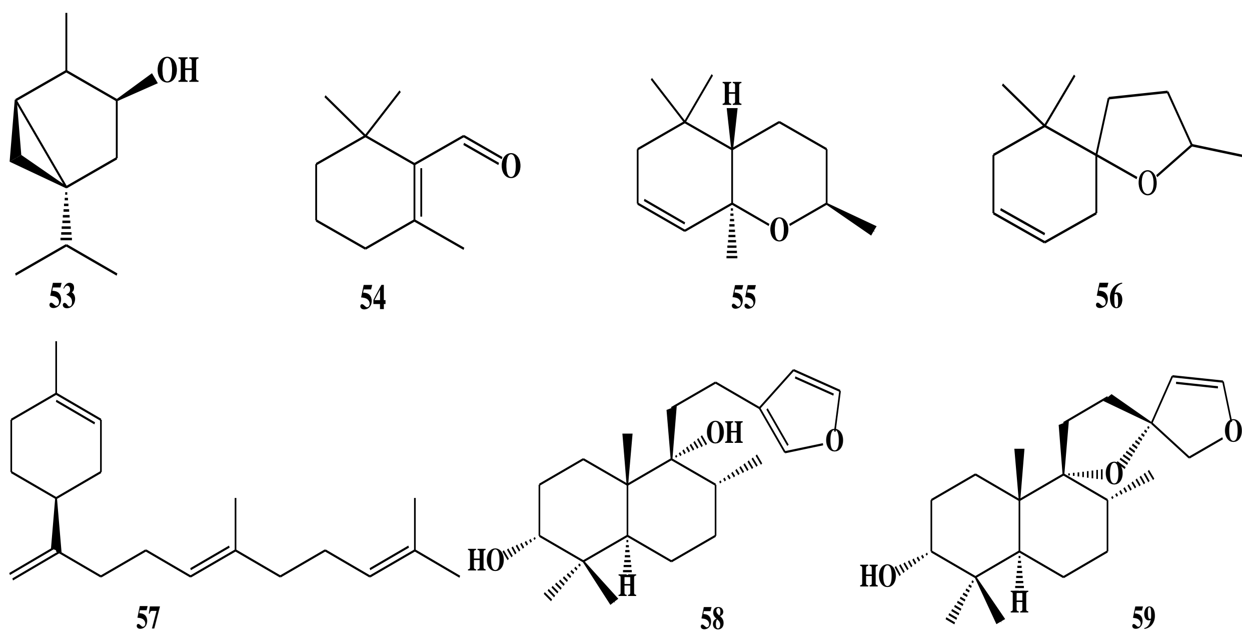


Figure 5: Structure of compounds from **53** to **59.**

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Methanol (99.9%) (Carlo Erba, France) and chloroform (99.9%) (Carlo Erba, France) were used for gradient extraction, whereas ethyl acetate (99.9%) (Mumbai, India) and n-hexane (99.9%) (pentokey organy, India) were used for column chromatography elution, and TLC, aluminum sheets coated with silica gel 60 F_{254} (Darmstadt, Germany) as a stationary phase. Column chromatography was performed using silica gel (60-120 mesh size) as a stationary phase. Conc. H_2SO_4 (98%) (Alphachemika, India), Conc. HCl (37%) (UNI-CHEM), Potassium Iodide, Iodine, Lead acetate, $FeCl_3$, ammonia, Benedict's reagent, NaOH (99.8%) (Turkey), and distilled water were also used for preliminary phytochemical screening tests. In addition to this, Mueller Hinton (MHA) agar were used for culturing bacterial strains. All of them are analytical-grade reagents.

3.1.2. Instruments

The instruments used in this study were Analytical balance (Scout Pro SPU2001, China) for measuring mass, Thomas-Willey laboratory mill model 4 miller for grinding the plant material, oven (OV 150 SS, GENLAB, WIDNES, ENGLAND) for drying apparatus, Erlenmeyer flasks and thermostatic bath shaker (Grant GLS400, Cambridge, UK) for maceration of plant materials, filter paper, 6 mm filter paper disk, beakers, Petri dish, test tubes, graduated cylinder, water bath for heating, rotary evaporator (ROTALABO 4000, Heidolph, Germany) for concentrating crude extracts, capillary tubes, Column chromatograph, TLC for checking the separation of spots, 254/365 nm UVitec chamber (CAMBRIDGE CB4 0WS - ENGLAND) for the detection of spots under UV light, An incubator to grow and maintain bacterial strains, Bruker Avance 400 MHz Nuclear Magnetic Resonance (NMR) spectrometer (Germany) in $CDCl_3$ with TMS as internal standard for ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral data, infrared spectrometer (PerkinElmer FTIR, using KBr) for IR spectral data to characterize the isolated compounds.

The NMR spectra were done at Addis Ababa University department of chemistry, Addis Ababa, Ethiopia and except antibacterial test the rest activities were done at Hawassa University department of chemistry, Organic chemistry research laboratory, Hawassa, Ethiopia.

3.1.3. Bacterial strains

The antimicrobial activities were done at Adama Science and Technology University department of biology, microbiology laboratory, Adama, Ethiopia. It was carried out using gram-positive bacteria (*S. aureus* ATCC25923 & *S. pyogenes* ATCC19615) and gram-negative bacteria (*E. coli* ATCC25922 & *P. aeruginosa* ATCC27853), which were obtained from the microbiology laboratory.

3.1.4. Plant material collection and identification

The roots of *O. integrifolia* were collected in January 2014 E.C. at Tara-Gedam kebele, Libo Kemkem woreda, South Gondar zone, Amhara regional state, Ethiopia. It is located about 655 km north of Addis Ababa and about 928 km away from Hawassa. It is also sited very close to Addis Zemen town, northeast of Lake Tana, northwestern Ethiopia (Figure 6) [55]. Addis Zemen town is located at 12°06'59" N – 12°07'25" N and 37°46'14" E – 37°47'02" E, on the Addis Ababa - Gondar main road, about 82 km north of Bahir Dar and 93 km south of Gondar town [55]. The altitude of Tara-Gedam ranges from 2217 to 2457 m above sea level [55]. The plant species was identified and authenticated by botanist Mr.   Regassa, department of biology at Hawassa College of teachers' education and a voucher specimen (AD001) was deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University.

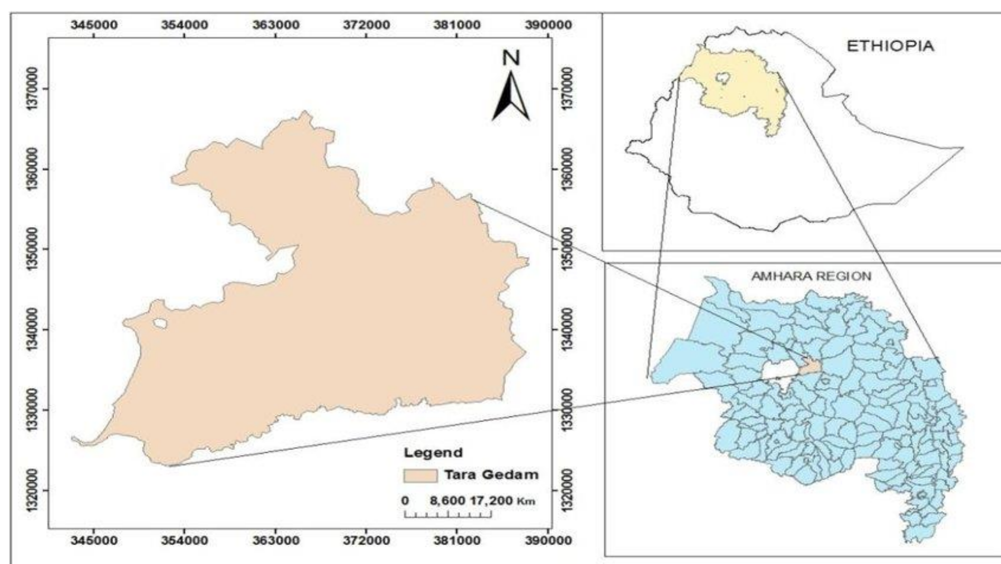


Figure 6: Geographical location of Tara-Gedam forest (site where sample harvested) [55].

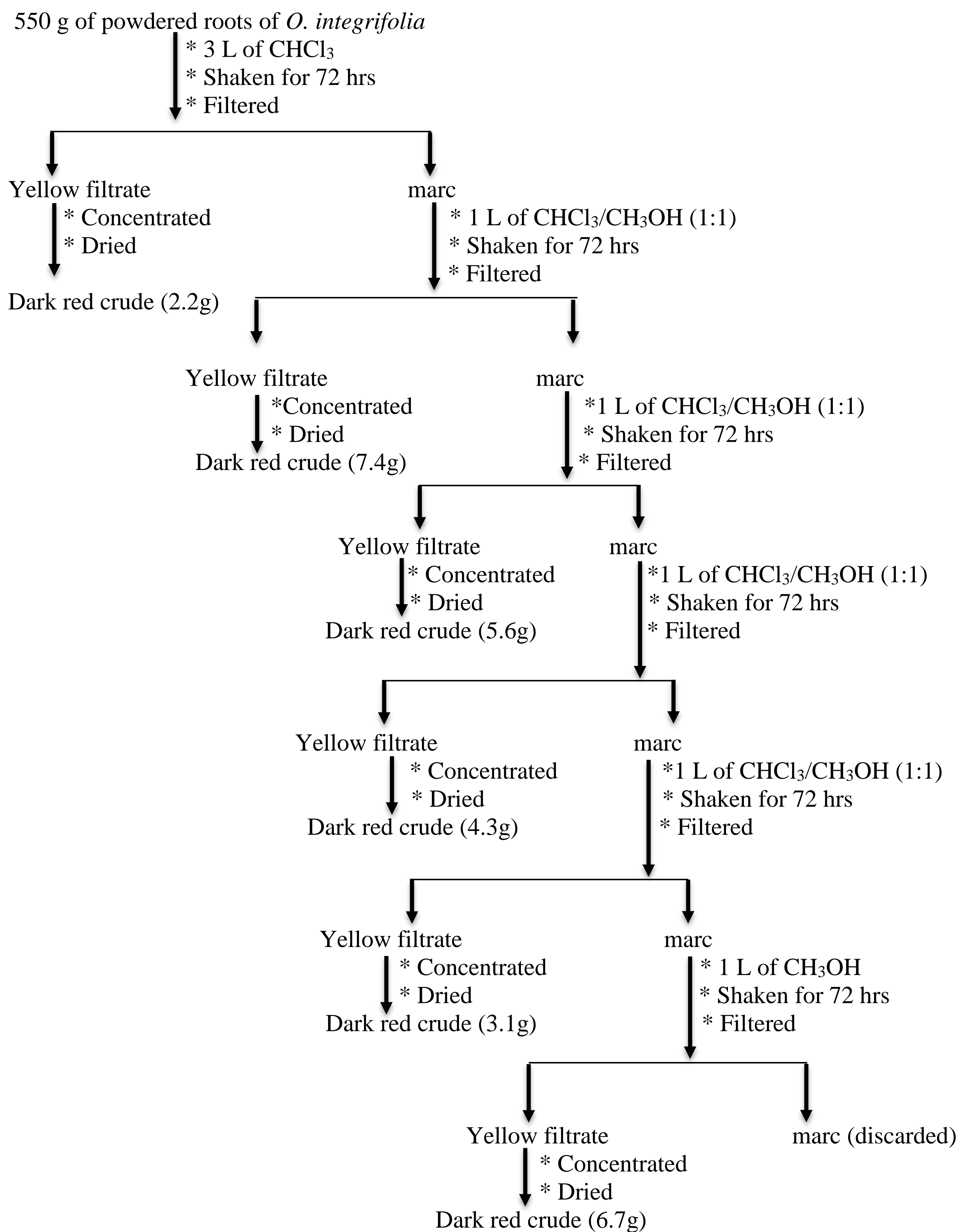
3.2. Methods

3.2.1. Preparation of the plant material

The collected fresh roots of the plant (*O. integrifolia*) were washed with tap water gently and then with distilled water. The barks were peeled off and chopped into small pieces, then dried for three weeks under shade at room temperature. After it was well dried, it was grounded into powder using Thomas-Willey laboratory mill model 4 miller and sealed. Then it was stored in a cool and dried place (Organic chemistry research laboratory) until it was used up for extraction.

3.2.2. Extraction

The 550 g of pulverized roots of *O. integrifolia* was soaked in 3L $CHCl_3$ and kept on water bath shaker for 72 hrs at room temperature. The extract was filtered using Whatman filter paper, and the filtrate was concentrated using a rotary evaporator under reduced pressure at a temperature of 40 ^{o}C with a speed of 90-120 rotations per minute (rpm). Then the marc was re-subjected to four successive extractions for 72 hrs using 1 L of $CHCl_3/CH_3OH$ (1:1 v/v) (to maximize the amount of the crude extract yield). Finally, it was re-extracted using 1 L of pure CH_3OH for 72 hrs (Scheme 1). The extracts were filtered and concentrated under the same conditions as the first one. Then the crude extracts were safely placed in a beaker until dried in the air, and the profiles of the crude extracts were monitored by TLC. The dried crude extracts were collected and kept in the deep freezer until needed for further analyses (secondary metabolite tests, isolation of compounds, and antibacterial activity tests).



Scheme 1: Flow chart showing the extraction process

3.2.3. Thin Layer Chromatography (TLC) Analysis of the crude extract

The TLC profile of the crude extracts were analyzed in various solvent systems to identify the appropriate solvent system for the separation of pure compounds in column chromatography. The TLC profile of $CHCl_3$, $CHCl_3/CH_3OH$ (1:1), and CH_3OH crude extracts were checked in varying gradients of n-hexane/EtOAc, $CH_2Cl_2/CHCl_3$, n-hexane/CH_3OH, and $CHCl_3/CH_3OH$ solvent systems. The n-hexane/EtOAc combination showed a relatively good TLC profile for $CHCl_3$ and $CHCl_3/CH_3OH$ (1:1) crude extract, but the CH_3OH extract didn't show good separated spots. The $CHCl_3/CH_3OH$ (1:1) crude extract was used for column chromatography in n-hexane/EtOAc solvent system with increasing gradient of EtOAc in n-hexane as an appropriate mobile phase.

3.2.4. Preliminary phytochemical screening tests

The qualitative preliminary phytochemical screening tests (color reactions) were carried out on all crude extracts ($CHCl_3$, $CHCl_3/CH_3OH$ (1:1), and CH_3OH extracts) using standard procedures reported in the literature to identify the presence/absence of constituents of secondary metabolites, namely alkaloids, terpenoids, phenols, flavonoids, saponins, tannins, steroids, anthraquinones, coumarins, carbohydrates, and glycosides [8, 56-62].

1. Test for alkaloids

Alkaloids are one of the main and largest components that are derived from the metabolic by-product of amino acids in plants [4]. 0.5 g of each extract was mixed in 2 ml of 1% HCl, heated in water bath, and filtered. Then 1 ml of each the filtrates was taken into each separate test tube and treated with 1 mL of Wagner's reagents (Iodine in Potassium Iodide). The formation of a brown/reddish precipitate was taken as an indicator of the presence of alkaloids [8, 56-58].

2. Test for terpenoids

Terpenoids are small molecular products synthesized by plants and are probably the most widespread group of natural products. Terpenoids show important pharmacological activities, such as antiviral, antibacterial, antimalarial, anti-inflammatory, inhibition of cholesterol synthesis, and anti-cancer activities [4]. 0.5 g of each of the extracts was added to 2 mL of

$CHCl_3$ and shaken in the test tube. Then 3 mL of concentrated H_2SO_4 was added carefully along the side of the test tube to form a layer. The formation of a reddish-brown colour at the interface was indicated the presence of terpenoids [59, 61].

3. Test for phenols

Phenols are widely found in most plants, and they are the highest group of secondary metabolites in plants [4]. About 0.5 g of each crude extract was dissolved in 2 mL of distilled water and taken into separate test tubes then heated at 45-50 ^{0}C followed by addition of 2 mL of 0.3% $FeCl_3$. The development of a bluish-black or green or blue color was shown the presence of phenols [57, 58].

4. Test for flavonoids

Flavonoids are polyphenol antioxidants found naturally in plants [4]. The following methods were used to test the presence of flavonoids in the plant.

a) **Alkaline reagent test:** 0.5 g of each of the extracts was treated with a few drops of 10% NaOH solution. The formation of an intense yellow colour that becomes colourless with addition of 1% HCl indicated the presence of flavonoids [58].
b) **Lead acetate test:** 0.5 g of each of the extracts was treated with a few drops of 10% lead acetate solution. The formation of a yellow colour precipitate indicated the presence of flavonoids [58].

5. Test for saponins

Saponins contain polycyclic aglycones. The name was derived from the Latin word ‘sapo’, which means the plant consisting of the frothing agent. They are important neutral and water-soluble group of glycosides that are widely distributed as plant constituents [4].

Froth test: 0.5 g of each of the extracts was diluted with 10 mL of distilled water and shaken in a test tube for 10 minutes. The formation of about a 1 cm layer of foam that persists for 10 minutes indicated the presence of saponins [58, 62].

6. Test for tannins

Tannins are the complex organic, non-nitrogenous derivatives of polyhydroxy benzoic acids which are widely distributed in the plant kingdom, and most of the true tannins have a molecular weight between 1000 and 5000 [4].

Ferric chloride test: About 0.5g of each of the dried extracts was boiled in 20 mL of distilled water in a test tube and then filtered. A few drops of 0.1% aqueous solution of $FeCl_3$ were added to a filtrate. Appearance of intense green, brownish green-black, purple, or blue-black coloration indicated the presence of tannins [8, 57, 59].

7. Test for steroids

Steroids are organic compounds with four rings. These steroidal compounds have been used to cut stress, reduce cholesterol levels, activate the immune system, enhance memory and learning, and to treat tumor cells in cancer cases [4].

Salkowski's test: 2 mL $CHCl_3$ and 2 mL concentrated H_2SO_4 was added to 0.5 g of each of the extracts in test tubes and shaken well. The formation of a greenish fluorescence layer of $CHCl_3$ and the acid was shown the presence of sterols [60, 63].

8. Test for anthraquinones

Borntrager's test: 0.5 g of each of the extracts was boiled with 10 mL of H_2SO_4 in separate test tubes and filtered while hot. The filtrate was shaken with 5 mL of $CHCl_3$. The $CHCl_3$ layer was transferred into another test tube and treated with 1 mL of dilute ammonia. The resulting solution was observed for colour changes. The colour changes/the development of pink/violet/red colour/ on the ammoniacal layer was taken as indicator of the presence of anthraquinone [56, 59].

9. Test for coumarins

0.5 g of each of the extracts was taken into separate test tubes and covered with filter paper that was moistened with dilute NaOH. Then, the contents of the test tubes were heated in a water bath for a few minutes. The filter paper will be examined under UV light. The yellow fluorescence was indicated the presence of coumarins [56].

10. Test for carbohydrates

0.5 g of each of the extracts was dissolved individually in 5 mL of distilled water and filtered. The filtrates were used to test for the presence of carbohydrates [35, 56, 58].

Benedict's test: 1 mL of each of the filtrates was mixed with 5 mL of Benedict's reagent and heated gently in a boiling water bath for 2 minutes and cooled. The development of an orange-red precipitate was considered as an indicator of the presence of reducing sugars [35, 56, 58].

11. Test for glycosides

Glycosides are molecules that sugar is bounded to a non-carbohydrate moiety, usually a small organic molecule [57].

Aqueous NaOH test: 1 mL distilled water was added into a 0.5 g of each of the extracts in test tubes and shaken well. Then 10% aqueous solution of NaOH was added. Appearance of yellow colour was indicated the presence of glycosides [60].

3.2.5. Compound isolation and structural elucidation

About 10 g of dried $CHCl_3/CH_3OH$ (1:1) crude extract of the root of *O. integrifolia* was taken, dissolved in to $CHCl_3/CH_3OH$ (1:1), and adsorbed onto 10 g of silica gel (60-120 mesh size) by meshing using a rotary evaporator until all solvents evaporated. After meshing and dried well, it was loaded on to chromatography column, which was packed in dry packing using 100 g of silica gel (60-120 mesh size) as a stationary phase. The elution was begun with 100 % n-hexane and progressed through an increasing polarity gradient of n-hexane with an increasing proportion of EtOAc. The elution resulted in a total of 122 fractions, each of which had a volume of 50 mL. The profile of each fraction was checked by TLC using a 254/365 nm UVitec chamber and iodine vapor. Then those successive fractions that showed similar R_f values of TLC profile were combined. Thus, combined fractions having single spot of similar R_f values of the TLC profile were dried and subjected to spectroscopic analyses such as FTIR, ^{1}H NMR, ^{13}C NMR, and DEPT-135 for structural elucidation. The FTIR was done at Hawassa University; and the ^{1}H NMR, ^{13}C NMR, and DEPT-135 were done at Addis Ababa University. Then, spectroscopic data

were interpreted using theoretical knowledge of the spectra generated and compared with literature to elucidate the structure of the compounds.

3.2.6 Antibacterial activity test

The antibacterial activity of the plant extracts and isolated compounds were determined using the disc diffusion method [64-66]. A broad-spectrum antibiotic (Ampicillin) was used as a positive control. 400 and 350 µg/ml concentrations of the plant extracts and isolated compounds were prepared. The 6 mm diameter filter paper discs containing crude extracts were placed on the surface of solidified nutrient medium in petri dishes and incubated at 37 ^{0}C for 24 hours. The antibacterial activity was assessed by measuring the diameter of the colonization zone and compared with the standard antibiotic used. The inhibition zone diameter (IZD) was measured using a ruler to the nearest mm to the diameter of the filter paper disc. The assay was done in triplicate and antibacterial activity was expressed as the mean diameter of the inhibition zone (mm).

4. RESULTS AND DISCUSSIONS

In this section, the yields of extracts, results of qualitative phytochemical tests, structural elucidation of isolated compounds, and the results of antimicrobial activity tests were discussed.

4.1. Yields of the crude extracts

The crude extracts obtained from the organic solvent root extracts of *O. integrifolia* were figured out in table 1 below.

Table 1: The yields of crude extracts obtained from the root of *O. integrifolia*

Nº.	Solvent used	Mass of dry sample (g)	Mass of crude extract (g)	% Yield
1	$CHCl_3$	550	2.2	0.40
2	$CHCl_3/CH_3OH$ (1:1)	547.8	20.4	3.72
3	CH_3OH	527.4	6.7	1.27

As shown in scheme 1 above, the yields of crude extracts obtained from the extraction of the root of *O. integrifolia* increases as the polarity of the solvent increases. This showed that the roots of this plant contains more polar components than non-polar components. However, 16.6% (w/w) of crude was obtained from the 80% methanol leave extract of this plant [15]. Therefore, it showed that the root of *O. integrifolia* has lesser polar components than its leave.

4.2. Phytochemical screening test

Preliminary phytochemical screening is a very important step, in the detection of the bioactive compounds present in medicinal plants and subsequently may lead to drug discovery and development [67]. The preliminary phytochemical screening tests were carried out following standard procedures described in Section 3.2.4. Thus, the results of these tests (Table 2) and the developed colors in each test were depicted in Appendix 1.

Table 2: Phytochemical constituents of the root extracts of *O. integrifolia.*

Nº	Phytochemical constituents	Type of test	Extracts		
			$CHCl_3$	$CHCl_3/CH_3OH$(1:1)	CH_3OH
1	Alkaloids	Wagner's	_	_	_
2	Terpenoids	Salkowski's	+	+	+
3	Phenols	$FeCl_3$	_	+	+
4	Flavonoids	Alkaline/L.A.	+	+	+
5	Saponins	Froth	+	+	+
6	Tannins	$FeCl_3$	+	+	+
7	Steroid	Salkowski's	+	+	+
8	Anthraquinone	Borntrager's	-	-	-
9	Coumerins	dilute NaOH	+	+	+
10	Carbohydrates	Benedict's	+	+	+
11	Glycosides	Aqueous NaOH	+	+	+

(+) stands for presence and (-) stands for absence.

The phytochemical screening of this study was revealed that alkaloids and anthraquinones are absent in all extracts and phenols in $CHCl_3$ extract whereas the rest secondary metabolites are present in all extracts. On the other hand, the preliminary phytochemical screening investigations of the 80% methanol leave extract of *O. integrifolia* contains terpenes, phenolic compounds, saponins, reducing sugars, and flavonoids [12, 15, 54], while the petroleum ether extract contains very limited compounds such as steroids and glycosides due to very low polarity of the solvent [16]. However, alkaloids and tannins were absent in methanol extract, ethyl acetate extract, and petroleum ether extract of the leave parts of this species [12, 15, 16, 54]. Therefore, terpenes, phenolic compounds, saponins, reducing sugars, steroids, glycosides, and flavonoids are present in both roots and leaves of *O. integrifolia* whereas alkaloids are most probably absent in both roots and leaves of this plant.

4.3. Compound isolation

The elution resulted in a total of 122 fractions. Out of these fractions collected, fractions 1, 2, 6-27 were colorless with no spots, fractions 3-5 were red with a spot that didn't move on TLC with any solvent system, fraction 28 was colorless, fractions 29-33 were intense yellow, fractions 48-51 were colorless, and the rest fractions were colorless and became light yellow when dried. As presented in section 3.2.5 above and table 3 below, these all fractions were analyzed by TLC, and fractions having similar R_f values were combined. Among these, fractions 48-51 (**FT1**) and fractions 59-65 (**FT2**) were shown single spots each with R_f values of 0.68 and 0.67 respectively in n-hexane/EtOAc (95:5) solvent system on TLC. When combined and dried, 16 mg of **FT1** and 21 mg of **FT2** were obtained. The compounds were analyzed by spectrometers (FTIR, ^{1}H NMR, and ^{13}C NMR) and their antibacterial activities were assessed. The FTIR, ^{1}H NMR, and ^{13}C NMR spectroscopic data were obtained, analyzed, and compared with literature to elucidate the chemical structures of isolated compounds (**FT1** and **FT2**).

Table 3: The solvent systems of the column chromatographic fractionation of the $CHCl_3/CH_3OH$ (1:1) root extract of *O. integrifolia*.

Fractions	Column solvent system (n-hexane/EtOAc)	TLC profile (Nº of spot) of fractions
1-2	100:0	No spot observed
3-5	100:0	Blue spot that didn't move on TLC in any solvent system
6-15	99:1	No spot observed
16-27	98:2	
28	98:2	More than one spots
29- 33	98:2	More than four spots
34-35	98:2	More than three spots
36-41	95:5	More than three spots
42-47	95:5	Two spots
48-51	95:5	Single spot
52-58	95:5	Two spots
59-65	95:5	Single spot
66-79	90:10	Two spots
80-87	90:10	More than one spots
88-97	90:10	Two spots
98-101	90:10	More than two
102-122	90:10	More than three

4.4. Structural elucidation of isolated compounds

As discussed in the previous section (section 4.3), the two compounds (**FT1** and **FT2**) were isolated from the $CHCl_3/CH_3OH$ (1:1) root extract of *O. integrifolia* using n-hexane/EtOAc (95:5) as eluent in column chromatography. To elucidate their structures, the spectroscopic data of these compounds (FTIR, ^1H NMR, ^{13}C NMR, and DEPT-135) were interpreted and compared with the literatures reports. The structural elucidation of **FT1** and **FT2** was discussed briefly in the following subsections (Section 4.4.1 and 4.4.2, respectively).

4.4.1. Structural elucidation of compound FT1

A colorless viscous liquid, **FT1** was analyzed using PerkinElmer FTIR spectrometer. In the FTIR spectrum (Appendix 2), the strong absorption band at 2925 cm^{-1} and 2855 cm^{-1} were suggested as the C-H vibrational stretching of CH_3 and CH_2, which is confirmed by the appearance of an absorption band around 1378 cm^{-1} (bending). The strong absorption band at 1740 cm^{-1} indicated the presence of ester carbonyl group (-C=O) stretching. The absorption peak around 1460 cm^{-1} was suggested due to C=C stretching at the aromatic ring. The three strong bands in the range 1300-1050 cm^{-1} were suggested as C-O-C stretching. The absorption band around 1649 cm^{-1} was suggested as olefinic C=C stretching. Furthermore, the two strong bands around 820 cm^{-1} and 780 cm^{-1} indicated the compound to be para-substituted benzene.

The ^1H NMR spectrum (400 MHz, $CDCl_3$, TMS) in (Appendix 3), the two doublet peaks at δ_H 7.73 and 7.55 which were suggestive peaks of aromatic protons; one triplet peak at δ_H 5.37 showed the presence of olefinic proton; one triplet peak at δ_H 4.18 and one doublet peak at δ_H 4.32 were suggested the presence of deshielded protons due to the attachment of the protons to the carbon that has directly attached to the electronegative atom oxygen and $-CH_2-$; one triplet peak at δ_H 2.83 also suggested as the deshielded proton attached to the carbon that has a direct attachment with $-CH_2-$; two doublet peaks at δ_H 2.24 and 0.91 proposed the presence of protons attached to carbons that are directly attached to -CH- group; one multiplet peak at 2.08 was suggested the proton attached to a carbon that directly attached to more than one alkyl group; two singlet peaks at 1.74 and 1.76 (Table 4) were suggested as protons attached to a carbon that is directly attached to quaternary carbon.

The ^{13}C-NMR (Appendix 4) in combination with DEPT-135 (Appendix 5) have shown the presence of 18 carbons. The spectra displayed quaternary carbons at δ_C 173.2, which was suggestive of the presence of carbonyl carbon (C-1''); 157.7, which was proposed as oxygenated quaternary carbon (C-4); 130.9 (C-1); and 138.1 (C-3'''). The remaining carbons of the aromatic methine carbons were observed at δ_C 130.2 (C-2 & C-6) and 114.8 (C-3 & C-5). The spectra also displayed methine carbons at δ_C 25.7 and 120.2 which were proposed to be C-3'' and C-2''' respectively. Furthermore, the spectra displayed methylene peaks at δ_C 34.0 (C-1'); 43.7 (C-2''); and 65.5, which showed the presence of oxygenated methylene (C-1''' & C-2'). The methyl group carbons were also displayed at δ_C 22.6 (C-4'' & C-5''); 25.8 (C-4'''); and 19.1 (C-5''') (Table 4).

Thus, based on the above spectral data analysis and comparison with literature reported spectroscopic data, the structure of compound **FT1** was proposed to be 4-(3'''-methyl-2'''-butenyloxy)-2'-(3''-methylbutanoxy) ethyl benzene which is known by the common name of hystroxene-I **(60)** (Figure 7) [68]. This compound was isolated from the acetone crude extract of the roots of *Citrus hystrix* [68]. However, to the best of our knowledge, it has not been reported from *O. integrifolia*. The observed ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 spectral data of compound **FT1** from *O. integrifolia* was compared with the spectroscopic data of hystroxene-I and found consistent with reported data [68].

Table 4: δ -values (ppm) of ^{1}H NMR (400 MHz, $CDCl_3$, TMS), ^{13}C NMR and DEPT-135 (101 MHz, $CDCl_3$, TMS) spectral data of compound **FT1** and reported in the literature.

Position	^{1}H NMR δ & J-values of **FT1**	^{1}H NMR δ & J-values (300 MHz, $CDCl_3$) in the literature [68]	^{13}C NMR δ-values of **FT1**	^{13}C NMR δ-values (75 MHz, $CDCl_3$) in literature [68]	Carbon type
1	-	-	130.9	131.0	C
2	7.73 (d, 8.4)	7.12 (d, 8.4)	130.2	129.8	CH
3	7.55 (d, 8.4)	6.84 (d, 8.4)	114.8	114.7	CH
4	-	-	157.7	157.6	C
5	7.55 (d, 8.4)	6.84 (d, 8.4)	114.8	114.7	CH
6	7.73 (d, 8.4)	7.12 (d, 8.4)	130.2	129.8	CH
1’	2.83 (t, 8)	2.86 (t, 7.2)	34.0	34.3	CH_2
2’	4.18 (t, 8)	4.24 (t, 7.2)	65.5	64.8	CH_2
1”	-	-	173.2	173.0	C
2”	2.24 (d, 8)	2.16 (d, 6.6)	43.7	43.4	CH_2
3”	2.08 (m)	2.08 (m)	25.7	25.7	CH
4”,5”	0.91 (d, 8)	0.92 (d, 6.3)	22.6	22.4	CH_3
1”’	4.32 (d, 8)	4.48 (d, 6.6)	65.5	64.8	CH_2
2”’	5.37 (m, 6.6)	5.49 (m, 6.6)	120.2	119.8	CH_3
3”’	-	-	138.1	138.0	C
4”’	1.76 (s)	1.79 (s)	25.8	25.8	CH_3
5”’	1.74 (s)	1.74 (s)	19.1	18.1	CH_3
Solvent	7.28	-	-	-	$CDCl_3$

Coupling constants (J) in the ^{1}H NMR spectrum indicated in parentheses is in Hz.

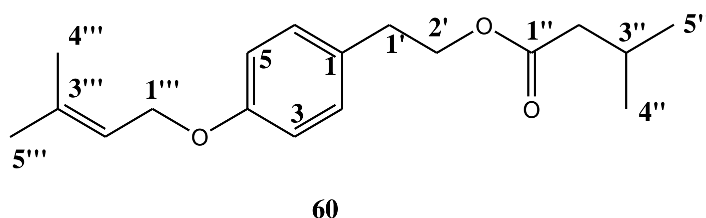


Figure 7: The proposed chemical structure of compound **FT1** (Hystroxene-I)

4.4.2. Structural elucidation of compound FT2

In the FTIR spectrum (Appendix 6), a broad absorption band at the range 3400-2500 cm^{-1} indicated the presence of carboxylic acid O-H stretching. A very strong absorption band that overlapped on O-H stretching absorption band at 2930 cm^{-1} and 2858 cm^{-1} showed C-H stretching of CH_3 and CH_2, which was confirmed by the appearance of an absorption band around 1377 cm^{-1}. A very strong absorption band around 1717 cm^{-1} suggested the presence of a

carbonyl group (-C=O), and the three medium absorption bands in the range 1300-1050 suggested the presence of C-O stretching. Furthermore, the absorption band peaks around 1650 cm^{-1} and 1460 cm^{-1} is due to C=C stretching in the aromatic ring, and the very weak intensity around 1650 cm^{-1}, which is overlapped on C=C aromatic ring stretching, indicated the presence of olefinic C=C stretching. The absorption bands at 949 cm^{-1} and 725 cm^{-1} also suggested the C-H out-of-plane bending of the aromatic ring.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$, TMS) in (Appendix 7) of **FT2** displayed a broad weak peak at δ_H 9.86, which was suggested as a labile acidic proton; the three doublet peaks at δ_H 7.06, 7.20, and 7.22 were suggestive peaks of aromatic protons; the two peaks at δ_H 5.14 and 5.36 showed the presence of olefinic protons; the three singlet proton peaks at δ_H 1.12, 1.32, and 2.07 were supposed to be attached to quaternary carbons. Moreover, the ^{1}H NMR spectral data analysis and comparison of most of the chemical shifts in **FT2** ^{1}H NMR spectrum with the literature spectroscopic data was found consistent with reported data (Table 5) [69].

The ^{13}C-NMR (101 MHz, $CDCl_3$, TMS) (Appendix 7) in combination with DEPT-135 (101 MHz, $CDCl_3$, TMS) (Appendix 8) showed the presence of 30 carbons. The spectra displayed quaternary carbons at δ_C 180.2, which was suggestive of the presence of a carboxylic group (C-19); 147.8 (C-9); 143.8 (C-15); 138.3 (C-13); 135.1 (C-8); 43.9 (C-4), and 39.7 (C-10). Methine carbons were observed at δ_C 126.4 (C-11 & C-14), 123.2 (C-12), and 52.1 (C-5). The spectra also displayed methylene carbons at δ_C 111.7, which showed the presence of terminal olefinic group (C-16); 39.3 (C-1), 37.4 (C-3); 31.9 (C-7); 21.1 (C-6); and 20.4 (C-2). Besides this, the spectra displayed methyl group peaks at δ_C 28.7 (C-18); 24.6 (C-20); and 22.7 (C-17). Moreover, the ^{13}C NMR spectral analysis and comparison of most of the chemical shifts in **FT2** ^{13}C NMR spectrum with the literature spectroscopic data was found consistent with reported data (Table 5) [69].

Thus, based on the above spectral data analysis and comparison with literature spectroscopic data, the structure of compound **FT2** was suggested to be a proposed structure of abietane diterpenoid, 8,11,13,15-abietatetraen-19-oic acid (Angustanoic acid E) **(61)** (Figure 8) [69]. It was reported that compound **61** has been isolated from CH_2Cl_2 extract of the aerial parts of *Illicium angustisepalum* [69]; 95% EtOH extract of the roots of *Illicium jiadifengpi* [70]; and 95% EtOH extract of the twigs and leaves of *Illicium majus* [71]. However, to the best of our

knowledge, it has not been reported from *O. integrifolia*. The observed ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 spectral data of compound **FT2** from *O. integrifolia* was compared with the literature spectroscopic data of **61** [69], and found consistent with reported data (Table 5 below).

Table 5: δ -values (ppm) of ^{1}H NMR (400 MHz, $CDCl_3$, TMS), ^{13}C NMR and DEPT-135 (101 MHz, $CDCl_3$, TMS) spectral data of compound **FT2** and reported in the literature.

Position	^{1}H NMR δ & J-values of **FT2**	^{1}H NMR δ & J-values in the literature [69]	^{13}C NMR δ-values of **FT2**	^{13}C NMR δ-values in the literature [69]	Carbon type
1	1.40 (α) 2.34 (β)	1.38 (α) 2.28 (β)	39.3	39.3	CH_2
2	1.65 (α) 2.03 (β)	1.62 (α) 2.03 (β)	20.4	19.3	CH_2
3	1.04 (α) 2.25 (β)	1.09 (α) 2.26 (β)	37.4	37.4	CH_2
4	-	-	43.9	43.9	C
5	1.63	1.57	52.1	52.9	CH
6	2.21 (α) 2.06 (β)	2.19 (α) 2.06 (β)	21.1	20.9	CH_2
7	2.80	2.80	31.9	32.1	CH_2
8	-	-	135.1	135.1	C
9	-	-	147.8	147.4	C
10	-	-	39.7	38.5	C
11	7.20 (d, 8.4)	7.20 (d, 8.4)	126.4	125.4	CH
12	7.22 (dd, 8.4, 1.7)	7.25 (dd, 8.4, 1.7)	123.2	123.1	CH
13	-	-	138.3	138.3	C
14	7.06 (d, 8)	7.14 (br d)	126.4	126.1	CH
15	-	-	143.8	143.0	C
16	5.36 (s) 5.14 (t, 1.4)	5.32 (s) 5.02 (t, 1.4)	111.7	111.7	CH_2
17	2.07 (s)	2.12 (3H, s)	22.7	21.7	CH_3
18	1.32 (s)	1.33 (3H, s)	28.7	28.7	CH_3
19	-	-	180.2	180.9	C
	9.86	-	-	-	-OH
20	1.12 (3H, s)	1.12 (3H, s)	24.6	23.1	CH_3

Coupling constants (J) in the ^{1}H NMR spectrum indicated in parentheses is in Hz.

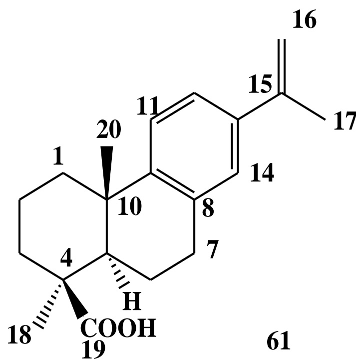


Figure 8: The proposed chemical structure of one of the isolated mixture compounds in **FT2**

4.5. Antibacterial Activity Evaluation

In this study, the antibacterial activity of crude extracts of *O. integrifolia* and isolated compounds were assessed using the disc diffusion method at concentrations of 350 and 400 µg/ml against gram positive-bacteria *(S. aureus* and *S. pyogenes),* and gram negative-bacteria (*E. coli* and *P. aeruginosa*) in comparison with positive control of 10 µg/ml Ampicillin (Table 6). Those activities were done at the microbiology laboratory, department of biology, Adama Science and Technology University, Adama, Ethiopia. All extracts exhibited the lowest antibacterial activity as compared to the standard (Ampicillin). However, the antibacterial activity test of crude extracts and isolated compounds showed positive effects for both labels of concentration and higher effects for 400 µg/ml at all bacterial strains. Among the extracts, CH_3OH extract has shown the highest effect, which suggests it contains more bioactive phytochemicals than the others, and $CHCl_3$ extract displayed the least effect on all strains. On the other hand, compound **FT2** has exhibited the most effective antibacterial activity than **FT1** and all the extracts. The 400 µg/ml of compound **FT2**, which is the most effective of all the tests, showed comparable effects (11.5, 12, 11, and 10.5 mm) with the 10 µg/ml of positive control (Ampicillin) (13, 14, 12.5, and 12 mm) on *S. aureus, S. pyogenes, E. coli,* and *P. aeruginosa* respectively. In addition to this, as a positive control Ampicillin, all the extracts, and isolated compounds have displayed higher antibacterial effects on gram-positive bacteria than gram-negative bacteria, which is shown below (Table 6).

Table 6: The effects of different solvent root extracts of *O. integrifolia* and isolated compounds on selected bacterial strains.

Tasted extracts, isolated compounds, and positive control with concentration (µg/ml)		Zone of inhibitions (mm) of bacterial strains			
		Gram positive		Gram negative	
		S. aureus ATCC25923	*S. pyogenes* ATCC19615	*E. coli* ATCC25922	*P. aeruginosa* ATCC27853
$CHCl_3$	A1=350	7	7.5	7	6.5
	A2= 400	8	8.5	7.5	7.5
$CHCl_3/CH_3OH$	B1= 350	8	8	7.5	7
(1:1)	B2= 400	9	9	8	8
CH_3OH	C1= 350	8.5	9	8	7.5
	C2= 400	9.5	10	9	9
FT1	D1= 350	9	10	8.5	8
	D2= 400	10	10.5	10	9.5
FT2	E1= 350	10	11	9	8.5
	E2=400	11.5	12	11	10.5
Ampicillin	10	13	14	12.5	12

Hystroxene-I **(60)** was evaluated for antioxidant, anti-HIV, and antibacterial activity (MDR *A. baumannii* JVC 1053 and *E. coli* ATCC 25922) at a concentration of 100 µg/ml and 50 µg/ml and has revealed no significant effect [68]. Angustanoic acid E **(61)** has been found in the roots of *Illicium jiadifengpi,* and the twigs and leaves of *Illicium majus.* It has exhibited important antiviral properties against the Coxsackie B virus as well as good anti-inflammatory activity (IC_{50} = 2.47 ± 0.43 µM) [71, 72]. Therefore, the antiviral and anti-inflammatory activity of this plant species were supported by the presence of compound **61**. The methanol and ethyl acetate extracts of the leaves *O. integrifolia* exhibited a significant antibacterial activity against some bacterial strains such as gram-positive (*S. aureus,* and *S. pyogenes*) and gram-negative (*E. coli*, *S. typhi,* and *K. pneumonia*) with minimum zone of inhibition (13.5+0.40 and 17.1+0.14) and (13.9+0.16, 10.1+0.04, and 16.8+0.41) respectively [16]. As shown in table 6 and discussed above, the antibacterial activities of the root of this plant against bacterial strains such as gram-positive (*S. aureus* and *S. pyogenes*) and gram-negative (*E. coli* and *P. aeruginosa*) bacteria were studied and confirmed by this study. Ophthalmia neonatorum is caused by *S. aureus, E. coli,* and

others [73]. Since it is effective on these bacterial strains, by analyzing the cytotoxicity of the $CHCl_3$, $CHCl_3/CH_3OH$ (1:1), and CH_3OH root extracts and isolated compounds (**60** and **61**), it can be used in the treatment and prevention of the bacterially induced condition ophthalmia neonatorum (caused by *S. aureus, E. coli,* and others). The current research generally supported the local community's traditional use of *O. integrifolia* for the treatment of various bacterial infections and as anti-inflammatory, antiviral, and anti-diabetic medications.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Generally, to the best of our knowledge, this study was the first work in isolating the phytochemical constituents from the root of *O. integrifolia.* Phytochemical screening of the root extracts of *O. integrifolia* revealed the absence of alkaloids and anthraquinones in all extracts and the presence of terpenoids, phenols, flavonoids, saponins, tannins, steroids, coumarins, carbohydrates, and glycosides in all extracts except phenols, which is absent in $CHCl_3$ extracts. **FT1** (Hystroxene-I) and **FT2** (Angustanoic acid E) were isolated from the $CHCl_3$/CH_3OH (1:1) root extract of *O. integrifolia* using n-hexane/EtOAc (95:5) as eluent in column chromatography. The structures of the compounds were identified based on spectral data such as IR, ^1H NMR, ^{13}C NMR, and DEPT-135 as well as in comparison with previously reported literatures. To the best of our knowledge, these compounds were isolated from this plant species for the first time. Even if compared to the dose of Ampicillin, all extracts and isolated compounds exhibited lower antibacterial activities on *S. aureus, S. pyogenes, E. coli,* and *P. aeruginosa*, *O. integrifolia* can be used as an antibacterial agent in a dose-dependent manner. This study also revealed scientific details regarding the plant's phytochemical composition and ethnomedicinal uses. The present study largely supported the traditional usage of *O. integrifolia* by the local population for the treatment of several bacterial illnesses including ophthalmia. Furthermore in other research, the isolated compound Angustanoic acid E exhibited antiviral and anti-inflammatory activity. Therefore, the presence of these compounds supported the usage of *O. integrifolia* as an anti-inflammatory, and antiviral treatment.

5.2. Recommendations

According to the present study the following recommendations were forwarded:

- Further phytochemical work on each root extract of *O. integrifolia* and eluting the column with very higher non-polar solvent systems than used in this study such as isocratic elution with n-hexane/EtOAc of 99:1, 98:2, 97:3, and 96:4 proportion could lead to the isolation and characterization of more compounds with antimicrobial activities.
- Antioxidant, antiviral, antiprotozoal, antifungal, and anti-diabetic activities of each extract and isolated compounds should be done and evaluated in future studies.
- It is recommended to examine the cytotoxicity and appropriate dosage of each extract and isolated compounds to implement as a folk medicine in traditional practitioners as well as to develop pharmaceuticals in future studies.
- It is also recommended to use the root extracts of this plant as anti-diabetic, anti-inflammatory, and antiviral, and for the treatment and prevention of various bacterial infections including ophthalmia neonatorum that induced by *S. aureus* and *E. coli*.
- 2D-NMR and MS spectroscopic techniques should also be used to fully characterize the isolated compounds.

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APPENDICES

APPENDIX 1: Phytochemical Screening Test Shown Color

Anthraquinones' test

Carbohydrates' test

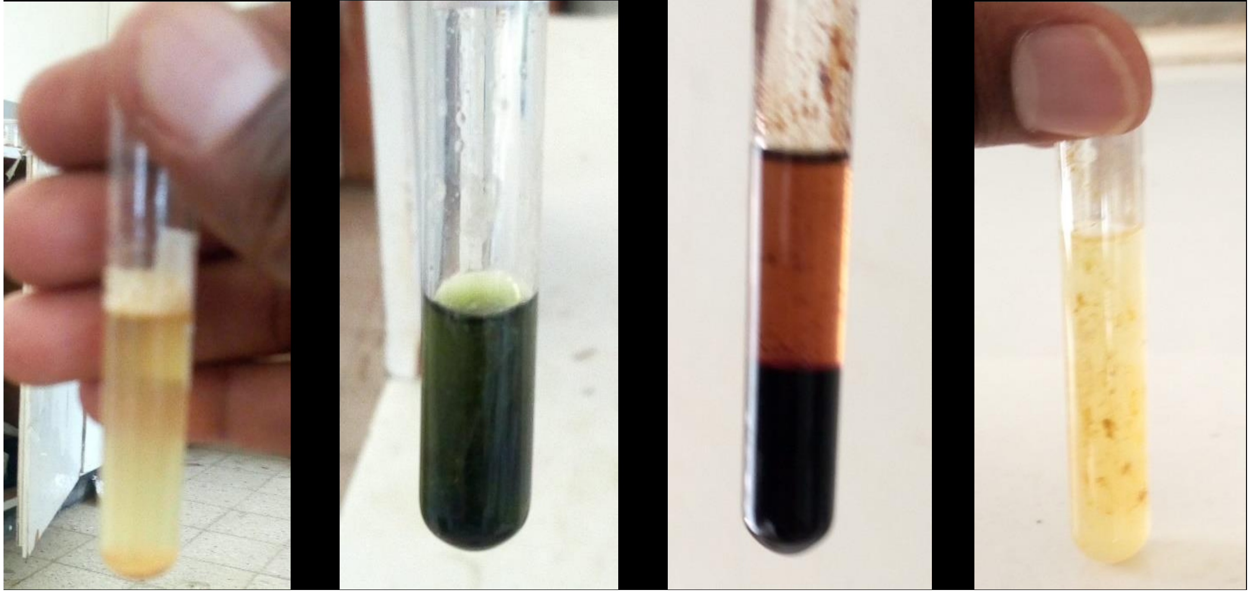
Coumarins' test

Glycosides' test

Flavonoids' test

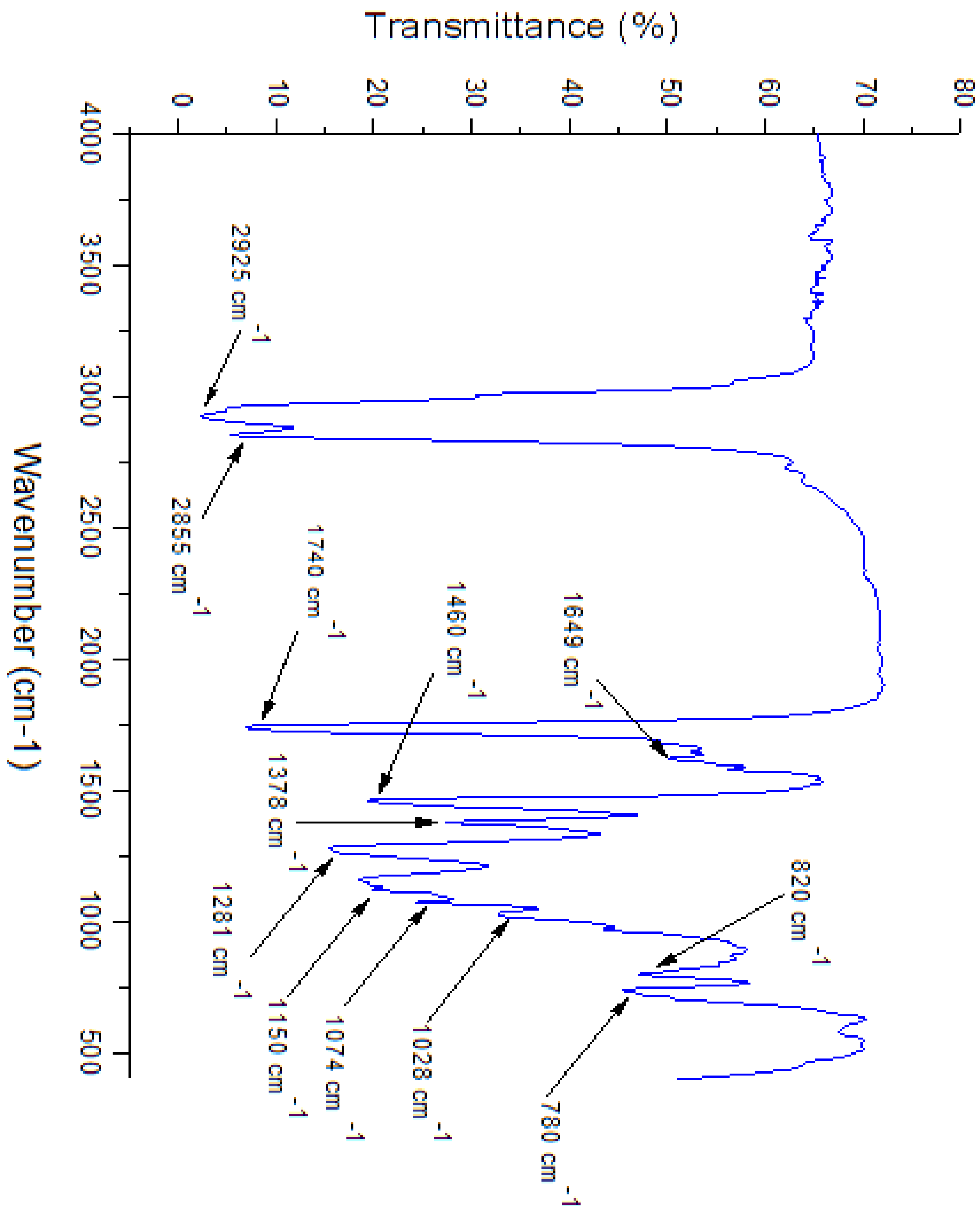
Phenols' test

Steroids' test

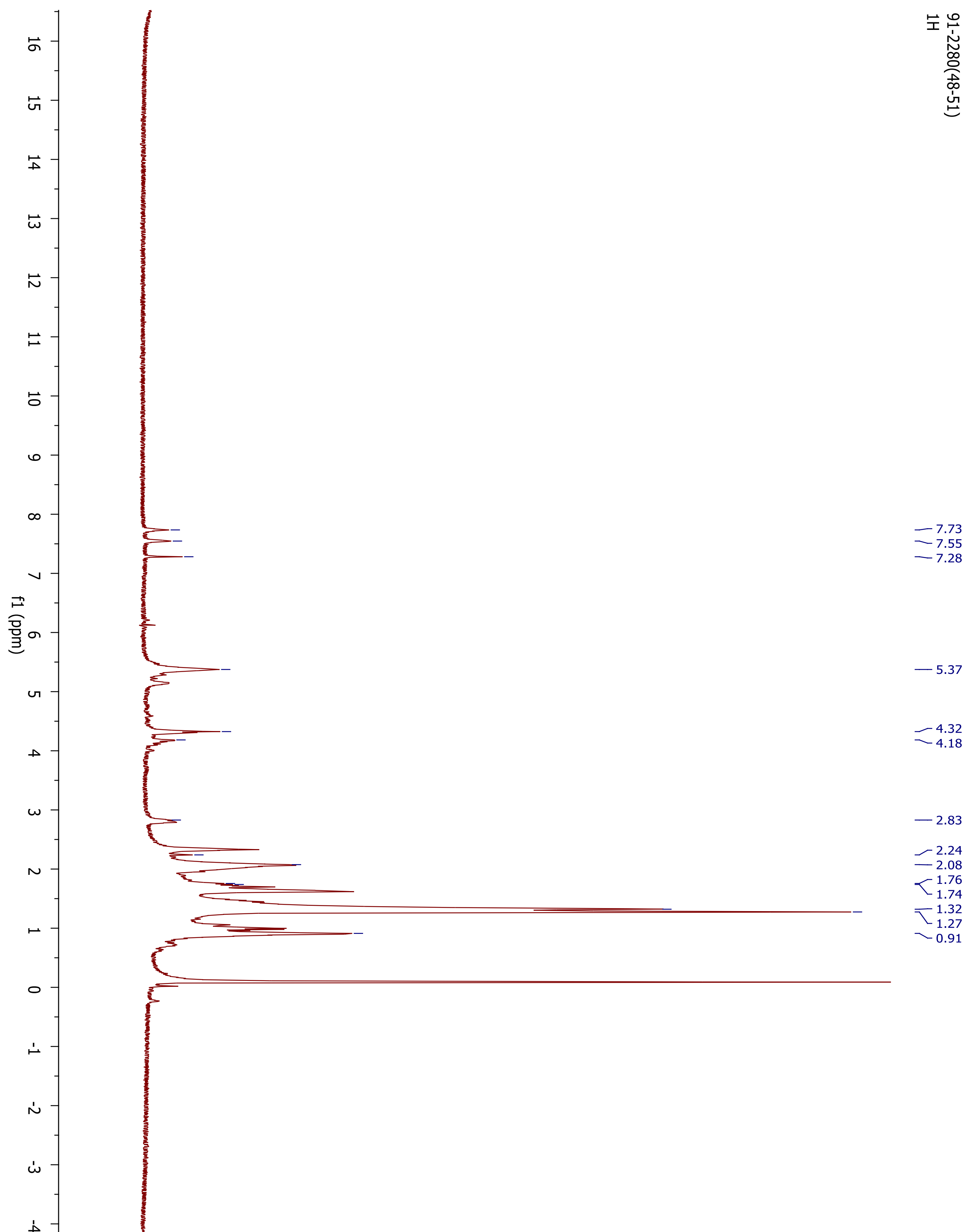


Saponins’ test Tannins’ test Terpenoids’ test Alkaloids’ test

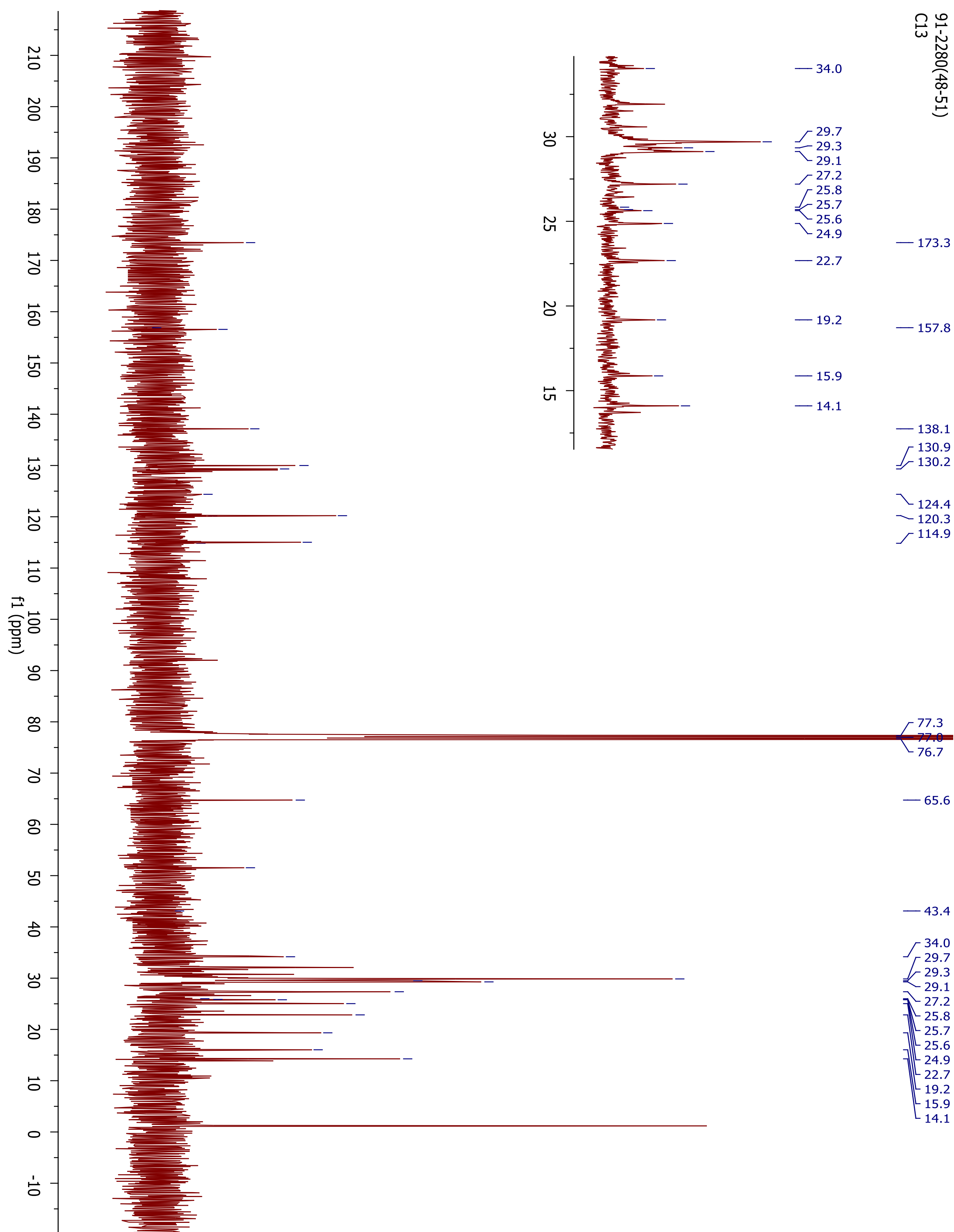
APPENDIX 2: The FTIR spectrum of compound **FT1**



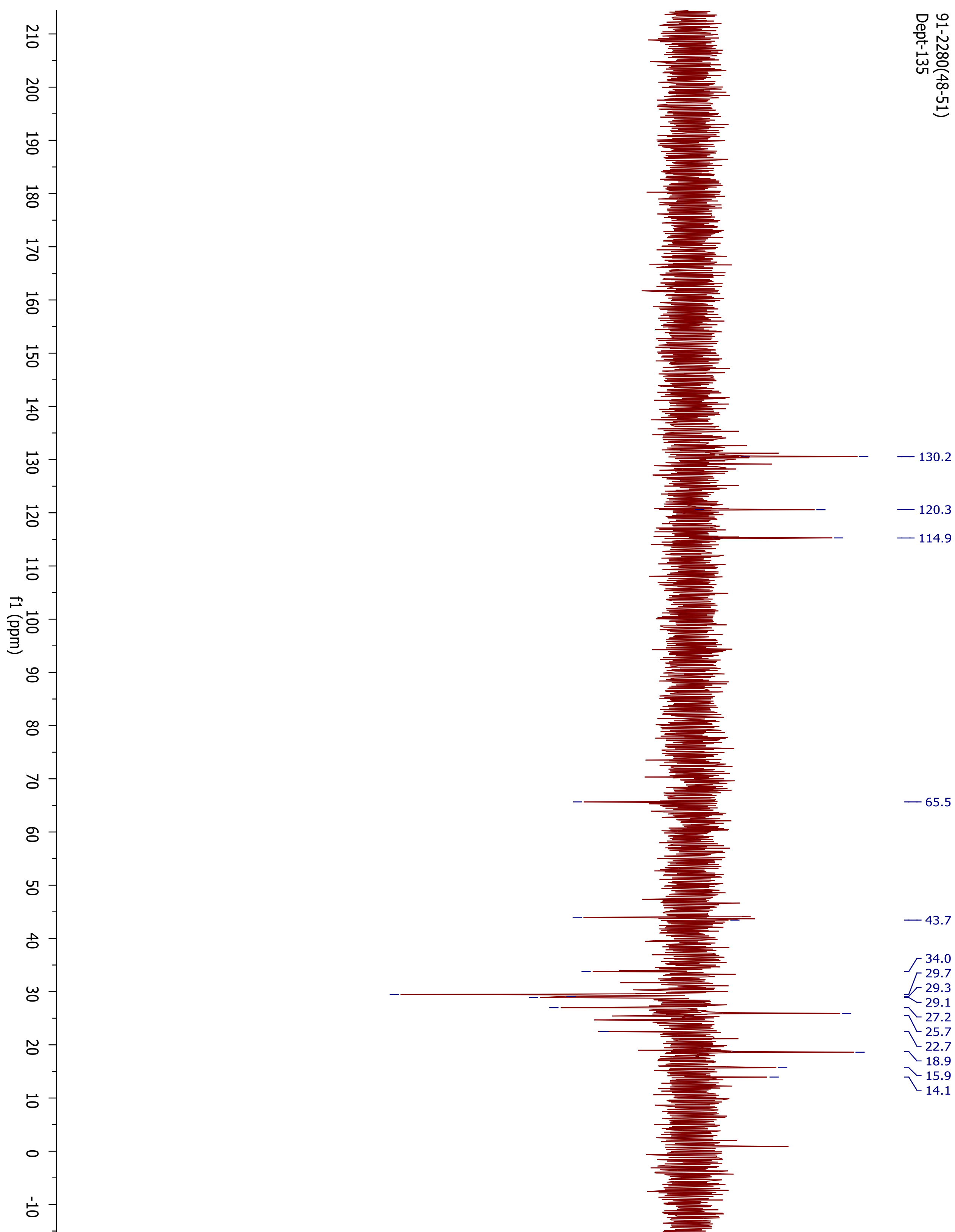
APPENDIX 3: The ^{1}H NMR (400 MHz, $CDCl_3$, TMS) spectrum of compound FT1



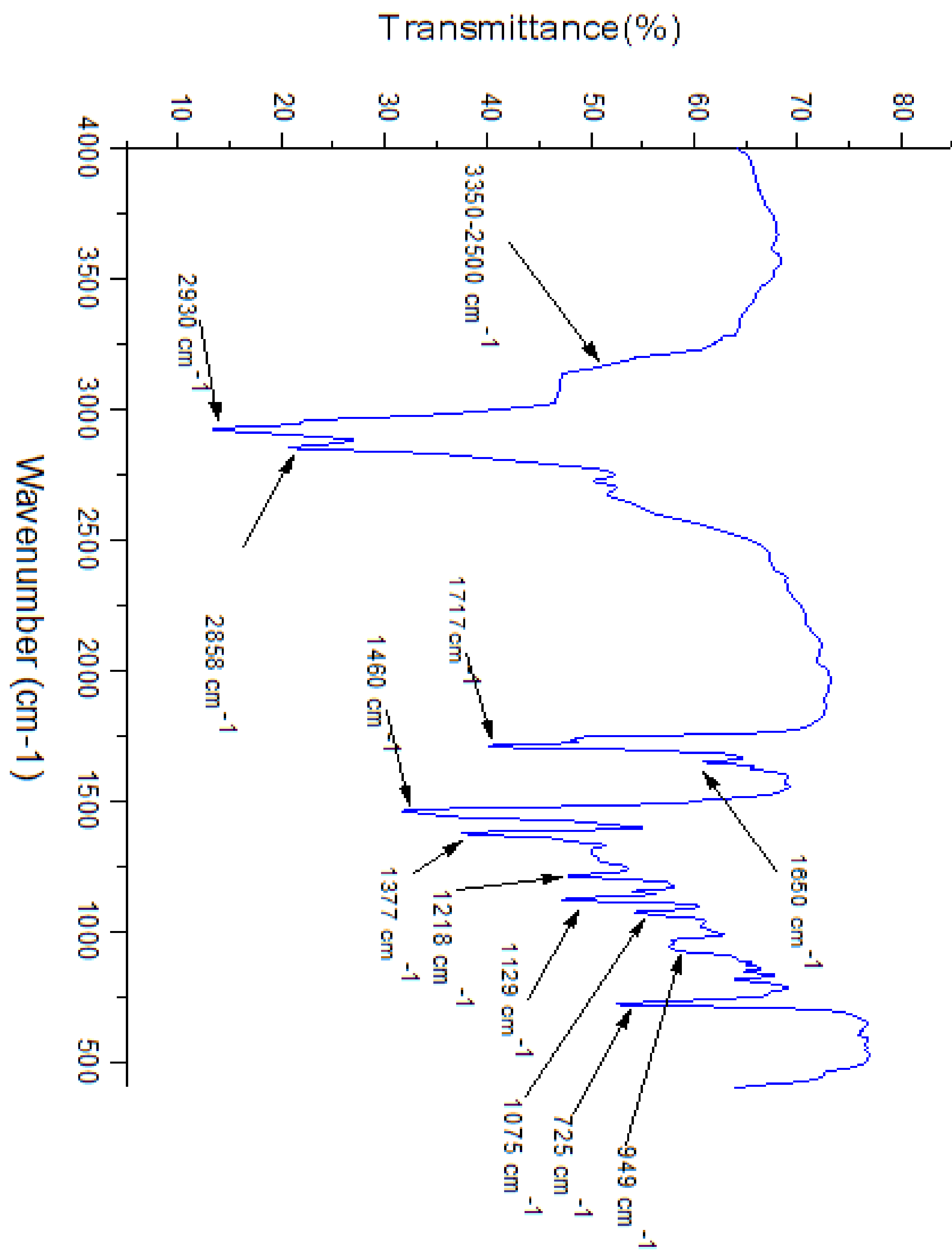
APPENDIX 4: The ^{13}C NMR (101 MHz, $CDCl_3$, TMS) spectrum of compound **FT1**



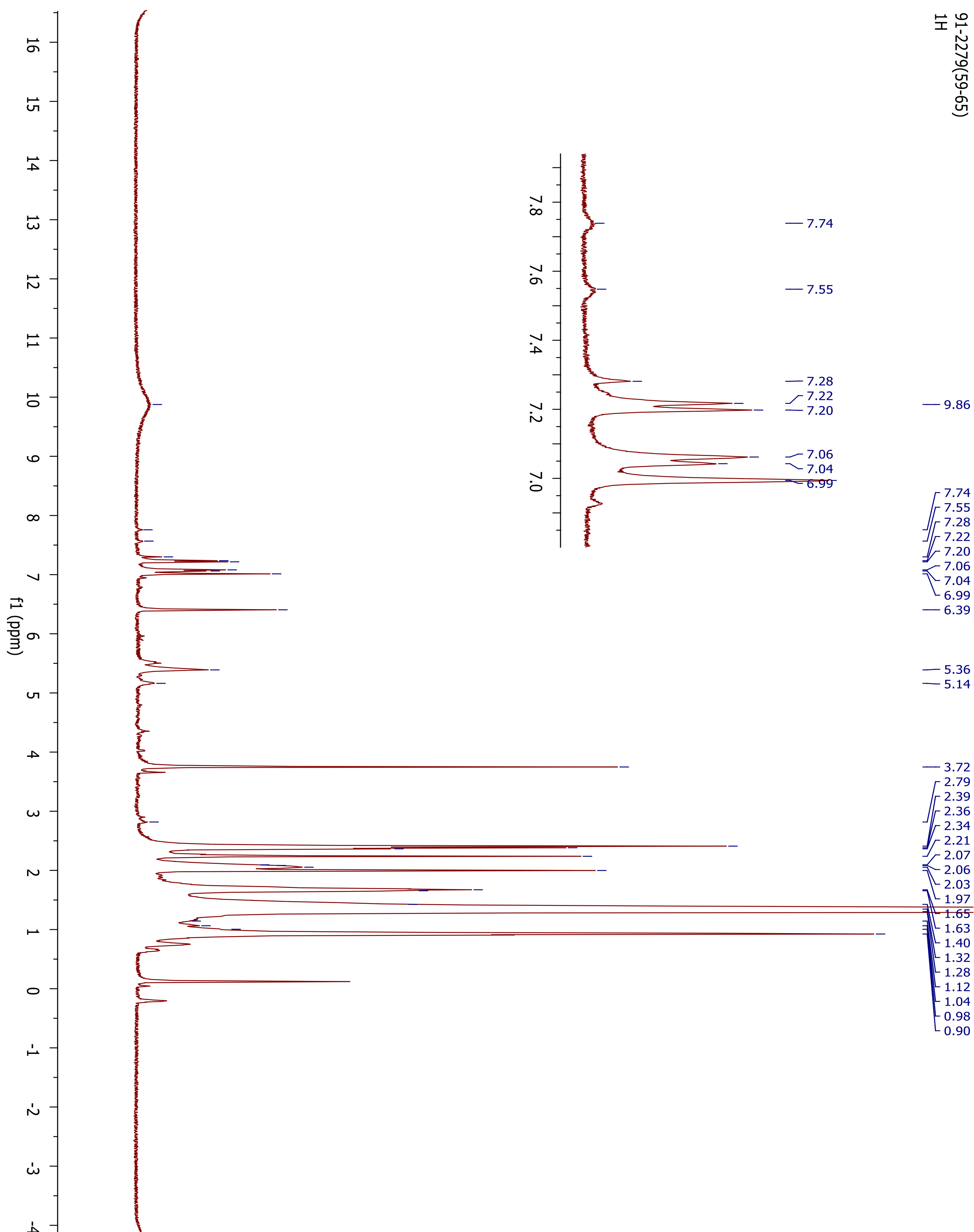
APPENDIX 5: The DEPT-135 NMR (101 MHz, $CDCl_3$, TMS) spectrum of compound **FT1**



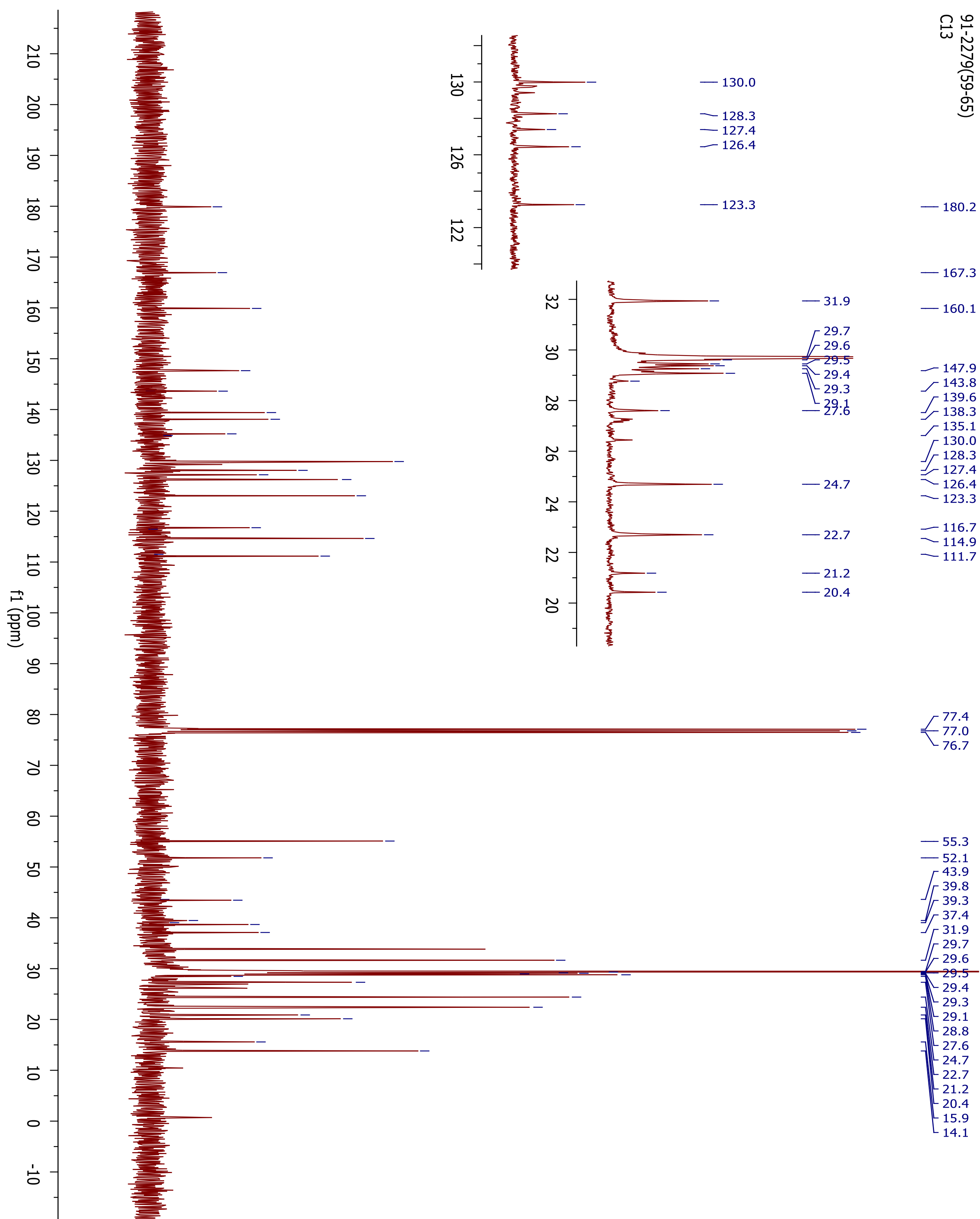
APPENDIX 6: The FTIR spectrum of compound **FT2**



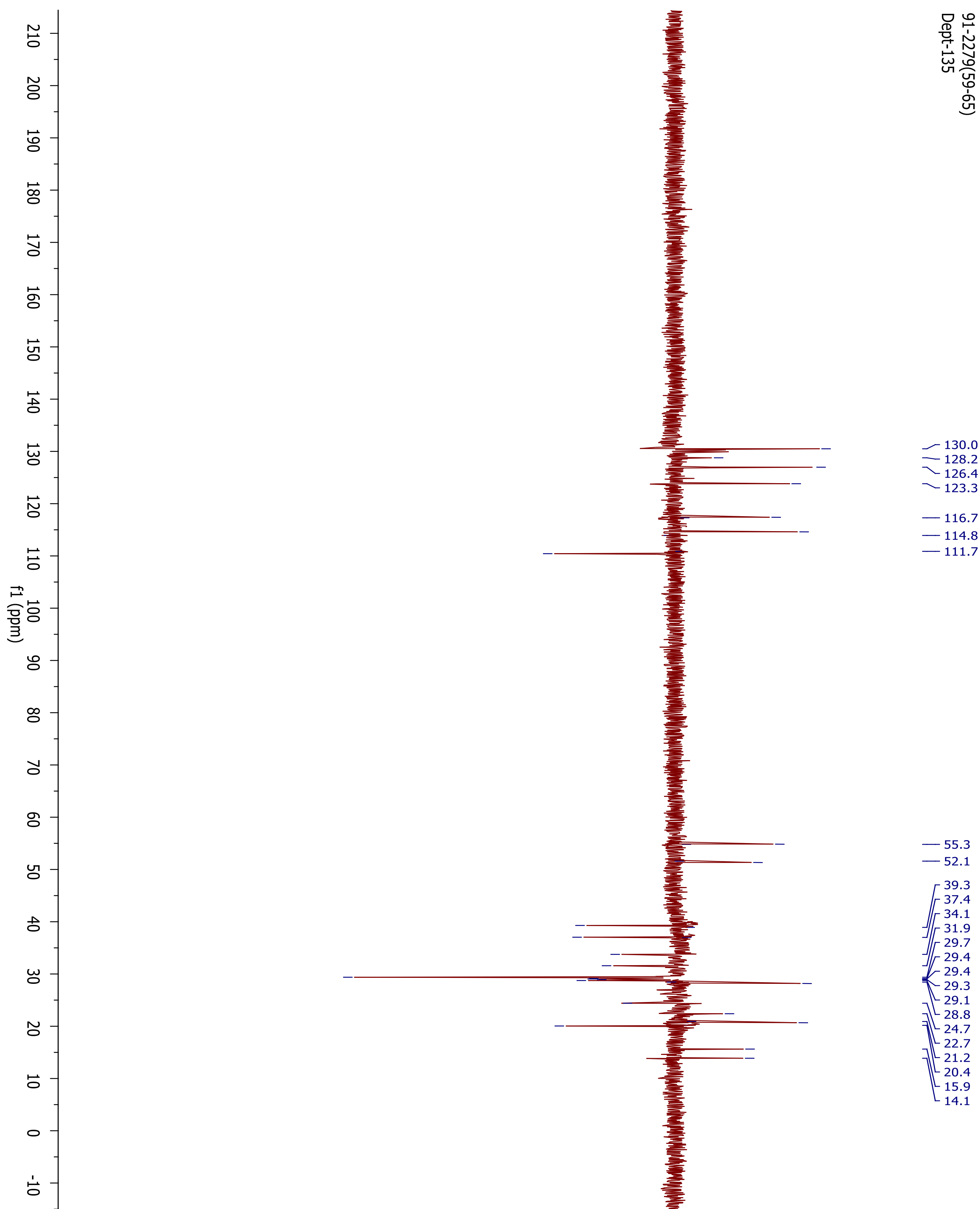
APPENDIX 7: The ^{1}H NMR (400 MHz, $CDCl_3$, TMS) spectrum of compound **FT2**



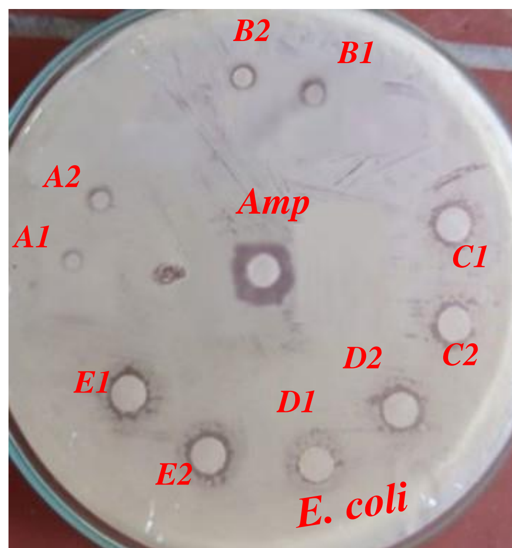
APPENDIX 8: The ^{13}C NMR (101 MHz, $CDCl_3$, TMS) spectrum of compound **FT2**

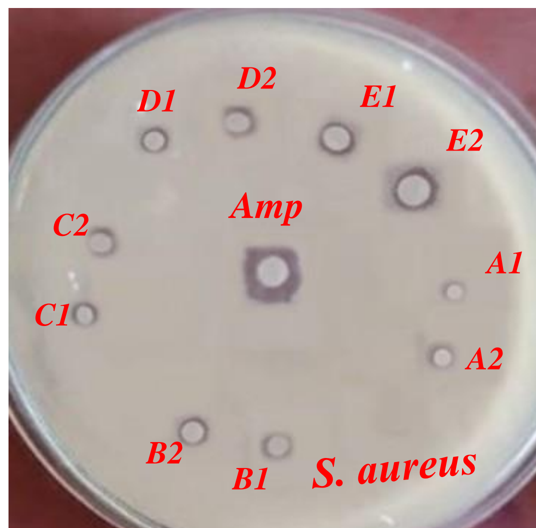


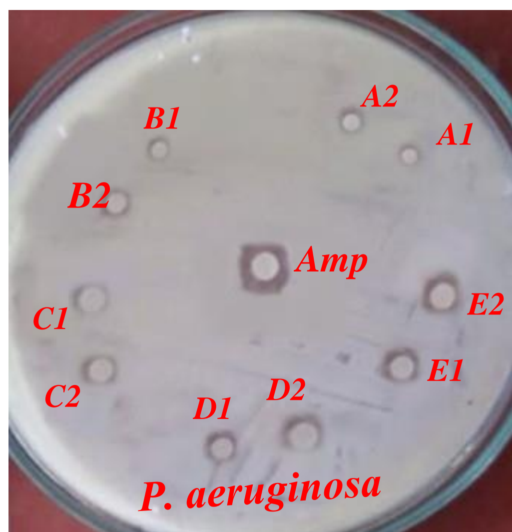
APPENDIX 9: The DEPT-135 NMR (101 MHz, $CDCl_3$, TMS) spectrum of compound **FT2**

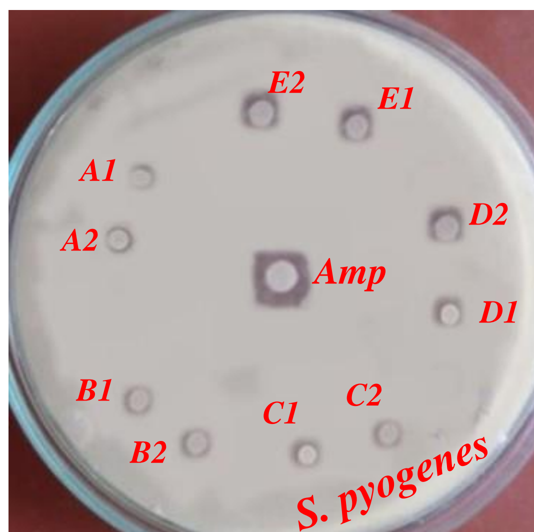


APPENDIX 10: Antibacterial Activity for Extracts and Isolated Compounds









Remarks:

A1 and A2 = $CHCl_3$ extract

B1 and B2 = $CHCl_3/CH_3OH$ (1:1) extract

C1 and C2 = CH_3OH extract

D1 and D2 = 48-51 = **FT1**

E1 and E2 = 59-65 = **FT2**