Phytochemical and Antibacterial Studies of the Roots of *Acokanthera schimperi*

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ABBREVIATIONS AND ACRONYMS

CC	Column Chromatography
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin layer Chromatography
IR	Infrared
LC	Liquid Chromatography
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometer
NMR	Nuclear Magnetic Resonance
R_f	Retention factor
TIA	Terpenoid Indole Alkaloid
TLC	Thin Layer Chromatography
UV-Vis	Ultra Violet and Visible Spectrometer

WHO World Health Organization

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ABSTRACT

*All through time humans have used different parts of medicinal plants to cure illness. Medicinal values of these plants lie in some chemical substances that produce a definite physiological action on the human body. A.schimperi is an important medicinal plant which belongs to the perennial family Apocynaceae and genus Acokanthera. Traditionally A.schimperi is used for treating malaria, tonsillitis, skin disorders, headache, epilepsy, amnesia, wound, syphilis, unstopped menstruation. In this study the powdered roots of A.schimperi was extracted with organic solvents of increasing polarity (n-hexane, chloroform, ethyl acetate, and methanol) successively by maceration method of extraction (72hrs each). The crude extracts were phytochemically screened using standard methods, fractionated and purified using Column chromatographic techniques. Antibacterial testing was carried out for crude extracts and fractions using disc diffusion method and tested for activity against three Gram negative bacteria; Escherichia coli, klebsiella pneumoniae, proteus mirabilis, and two Gram positive bacteria; staphylococcus aureus and Bacillus subtilis. The results of antibacterial test indicated the potential of the root of this plant in treating bacterial infections of the skin. Except ethyl acetate extract both MeOH and $CHCl_3$ fractions and crudes of the plant were found to have activity on at least two strains of bacteria with the maximum zone of inhibition 10mm. The phytochemical screening tests carried out on the roots of A.schimperi indicated the presence of tannins, cardiac glycosides, and coumarins for all crude extracts. Chemical structures of pure compounds **22**and **23** were elucidated with the help of spectroscopic techniques using NMR and IR.*

Key words: Acokanthera schimperi, Apocynaceae, antibacterial activity, structure elucidation.

1. INTRODUCTION

Before the introduction of chemical medicines, man relied on the healing properties of medicinal plants. The universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine irrespective of the underlying philosophical premise. These systems of medicine used by early-man include the Western medicine with origins in Mesopotamia and Egypt, the Unani (Islamic) and Ayurvedic (Hindu) systems centered in western Asia and the Indian subcontinent and those of the Orient (China, Japan, Tibet, etc.).

It is thought that about 80% of the 7.4 billion people of the world live in the less developed countries and the World Health Organization (WHO, 2013) estimates that about 80% of these people rely almost exclusively on traditional medicine for their primary healthcare needs (Davidson, 2004). There are nearly 2000 ethnic groups in the world, and almost every group has its own traditional medical knowledge and experiences (Liu *et al*., 2009) as indicated above. Even today, traditional medicines are used by different countries in the world. As WHO (2013) report indicated, in Europe, the use of traditional medicine ranges from 42% in Belgium to 90% in the United Kingdom; in Africa, the range is from 70% in Benin to 90% in Burundi and Ethiopia. The main reason for using traditional medicines in developing countries (WHO, 2013) is its accessibility and affordability. Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds. Many of these indigenous medicinal plants are used as spices and food plants (Harborne, 1998).

It is clear that Traditional medicine (TM) is an ancient form of health care practice before the appearance of scientific medicine. It is a part of culture of many peoples and is proven worthwhile by the long history of usage and practice. Moreover, it is accessible to the people in even the most remote areas, and it doesn't require sophisticated equipment. One of traditionally used herbal medicine for different disease treatment is *Acokanthera schimperi. A. schimperi* is an important medicinal plant which belongs to the perennial family (*Apocynaceae*) and tribe *Acokanthera*. It is a small rounded tree with short bole grows up to 5 -10m tall. Traditionally *A.*

schimperi is used for malaria, tonsillitis, skin disorders, headache, epilepsy, amnesia, wound, syphilis, and unstopped menstruation (Getahun, 1976).

1.1 Statement of the Problem

Herbal remedies have the advantage of being readily available, biodegradable and the process of isolation of active ingredients is cheaper than formulating and producing synthetic drugs (Fair, 2008). Ethiopia has over 6500 flowering plants and ferns (Getahun, 1976; Wondafrash, 2008). *Acokanthera schimperi* is one of the traditionally used herbal medicines for different disease treatment. The different parts of this plant are used for different purposes in East African countries including Ethiopia. The plant is used as food (the ripe fruits are edible), to treat tonsillitis, antifertility, skin disorder, gall bladder problem, unstopped menstruation, sexually transmitted diseases, wound, malaria, epilepsy, and as aphrodisiac (Endress & Bruyns, 2000).

In Ethiopia Local peoples use the root part for treatment of Syphilis, Gonnohrea ,and Wound effectively.

Hitherto, no detailed scientific investigation is reported on the chemical composition of the roots of *A. schimperi.* Therefore, this work is intended to investigate the phytoconstituents of the roots extracts of *A. schimperi* through chromatographic separation techniques and spectroscopic characterization methods, the compounds that could be isolated in a pure form. Furthermore the crude extracts will also be screened for their antimicrobial activities, which may explain the use of this plant by herbalists for microbial diseases treatment in Ethiopia.

1.2 Significance of the Study

Medicinal plants provide accessible and largely safe sources of primary health care to the majority of the population in the world. Poor People who are unable financially to afford formal health care systems are dependent on herbal medicine. Government is always experiencing problems with pharmaceutical companies that are regularly increasing prices of their medicines. This study is focus on the growing interest in the value and efficacy of medicinal plants based on local health systems as a means of meeting the current and future health care needs of the people. Many traditional healers use water extracts of *Acokanthera schimperi* which means there is a need to characterize its chemical components. Many Traditional healers use water extract to heal people without the knowledge of concentration measurement which means there is a need to test plant against different organisms and measure the minimum effective concentration. The

compounds isolated may have antimicrobial activity with relatively low toxicity. Different plant parts (leaves and fruits) have been shown to have antibacterial, antifungal and antiviral activity (Tadeg *et al.*, 2005) and traditional healers report the root part as an effective prophylaxis in syphilis infection.

1. 3. Objectives of the study

1.3.1 General Objective

To carryout phytochemical and antibacterial studies of organic solvents (n-hexane, chloroform, ethyl acetate and methanol) extracts of roots of *A. schimperi*.

Specific Objectives

- To perform successive solvent extraction by maceration with increasing polarity using n-hexane, $CHCl_3$, EtOAc, and MeOH solvents,
- To carry out phytochemical screening of crude extracts,
- To fractionate and isolate compounds from $CHCl_3$, EtOAc, and MeOH extracts using CC,
- To characterize the structure of the isolated compounds from $CHCl_3$, EtOAc, and MeOH extract using spectroscopic techniques (NMR& IR).
- To investigate the antibacterial activities of crude extracts and fractions using; three Gram-negative bacteria; *Escherichia coli, Klebsiella pneumoniae*, *Proteus mirabilis*, and two Gram-positive bacteria; *Staphylococcus aureus and Bacillus subtilis* by disk diffusion.

2. LITERATURE REVIEW

2.1 The Apocynaceae family

Apocynaceae is a family of flowering plants containing 375 genera and over 5100 species around the globe; that includes trees, shrubs, herbs, stem succulents, and vines, commonly called the dogbane family (Endress & Bruyns, 2000) after the American plant known as dogbane, *Apocynum cannabinum*. Several plants of the Apocynaceae family have had economic uses in the past. Many genera of this family produce cardiac glycosides that are used for many medical treatments (Heywood *et al.,* 2007). Plants of the *Apocynaceae* are often poisonous and are rich in alkaloids or glycosides, especially the seeds are valuable source of medicine, insecticides, fibers, and rubber (Nazia *et al*., 2013). Members studied and known to have such glycosides include the *Acokanthera*, *Apocynum*, *Cerbera*, *Nerium*, *Thevetia* and *Strophanthus.*

The family carries considerable importance in the field of medicine because of its extensive use in cancer chemotherapy, skin diseases, diabetes, diarrhea and malaria (Middleton, 2007). Besides its importance in economy and construction, the family serve as a potential source for vast amount of chemicals like alkaloids, steroids, triterpenoids, phenolics from *Alstonia* spp. (Singh and Singh, 2003) and terpenoids, indole alkaloids (TIA), vinblastine (**1**), vincristine (**2**), catharanthine (**3**) and vindoline (**4**) from *Catharanthus roseus* (Brossi *et al*., 1990). Similarly reports of Shazly *et al. (*2005*)* confirmed the presence of cardiotonic glycoside neriifolin (**5**), which has insecticidal property and thevetin (**6**) that work as heart stimulant from *Thevetia neriifolia* Jussieu. and *T. thevetioides* Kunth.

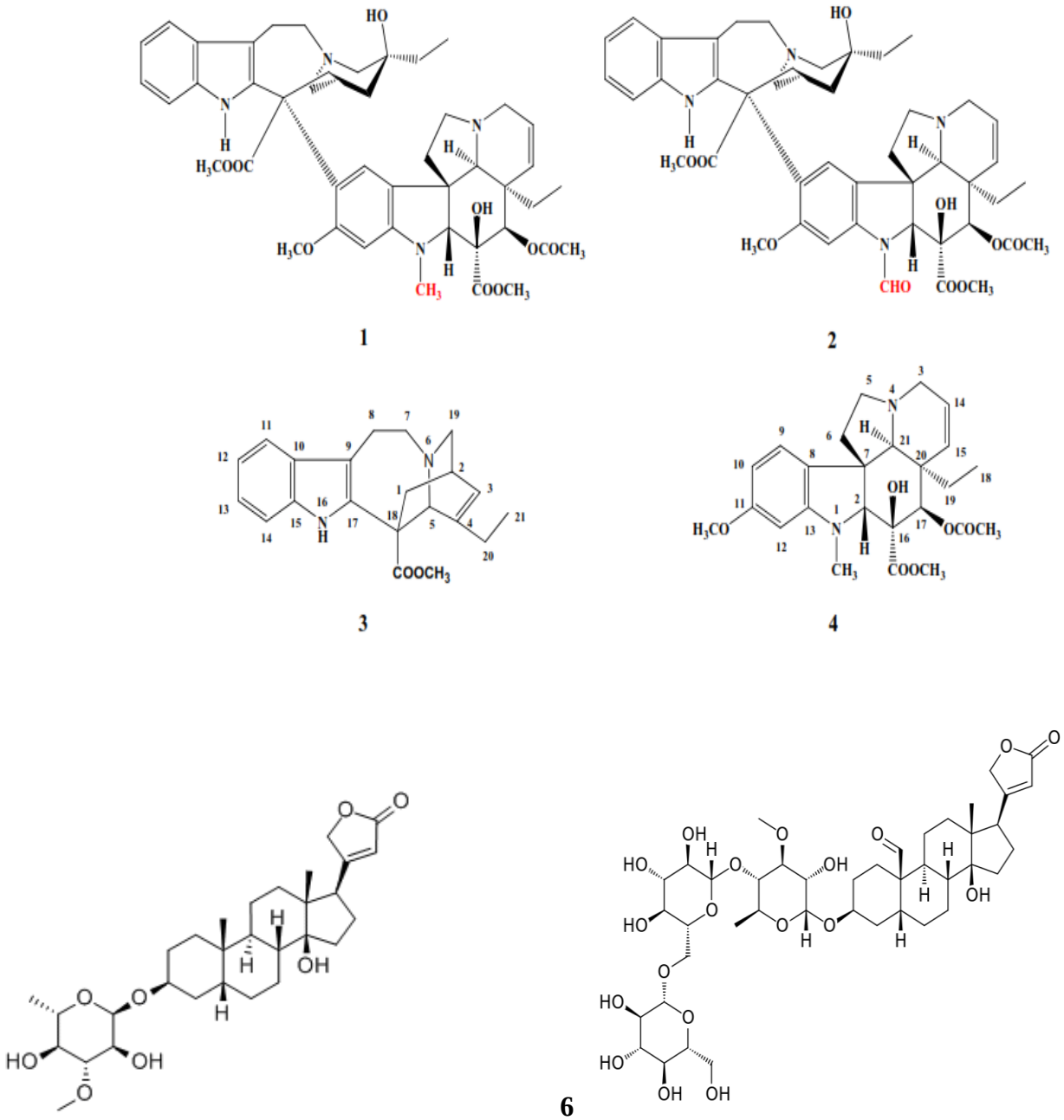


Fig. 1 Some of the compounds reported from the *Apocynaceae* family

2.2. The genus *Acokanthera*

Acokanthera is a genus of flowering plants in the family *Apocynaceae*. It comprises 5 species and is generally restricted to Africa, although *Acokanthera schimperi* also occurs in Yemen. The sap of *A. schimperi* contains the deadly cardio toxic glycoside *ouabain* (7). The sap is among the most commonly used in arrow poisons, including those used for poaching elephant (Schmelzer & Gurib, 2008). *Acokanthera* species are among the most commonly used plant species for the preparation of poison in East Africa. It is either used on its own or mixed with other plant or animal parts. The bark, wood and roots are the usual ingredients for arrow poison, and they are also used for suicide and homicide (Endress & Bruyns, 2000).The only treatment against the poison is immediate excision of the flesh around the wound, or sucking the blood from the wound. The poison is also used in killing wild animals and stray dogs from fields and homes. The five species belonging to this genus are : *A. laevigata*, native to Tanzania and Malawi, *A oblongifolia* , native to Mozambique and South Africa and is also found in Honduras and cultivated in Pakistan, *A. oppositifolia*, widespread in southern and central Africa from Zaire to Tanzania, *A. rotundata* is native to Zimbabwe, Swaziland and South Africa, and *A. schimperi*, native to Eritrea, Ethiopia, Somalia, Kenya, Uganda, Tanzania, Rwanda and DR Congo. (Schmeltzer & Gurib, 2008).

2.2.1. Some of the Secondary Metabolites Reported from the Genus *Acokanthera*

Five known triterpenes, α-amyrin, (**8**), lupeol acetate (**9**), betulinaldehyde (**10**), lupeol (**11**), and betulinic acid (**12**) were isolated from *A. oblongifolia* leaves (Rasmia *et al.*, 2015). The structures of these compounds are shown in Fig.2 below. One cardiotonic glycoside, three triterpenes and one steroidal glycoside were isolated from *A. oblongifolia* fruits (pericarp) growing in Libya (13-17). Lupeol (**13**), oleanolic acid (**14**) ursolic acid (**15**), acovenoside A, (**16**), ß-sitosterol -3-O- ß-D-glucoside (**17**) shown in Fig.3.

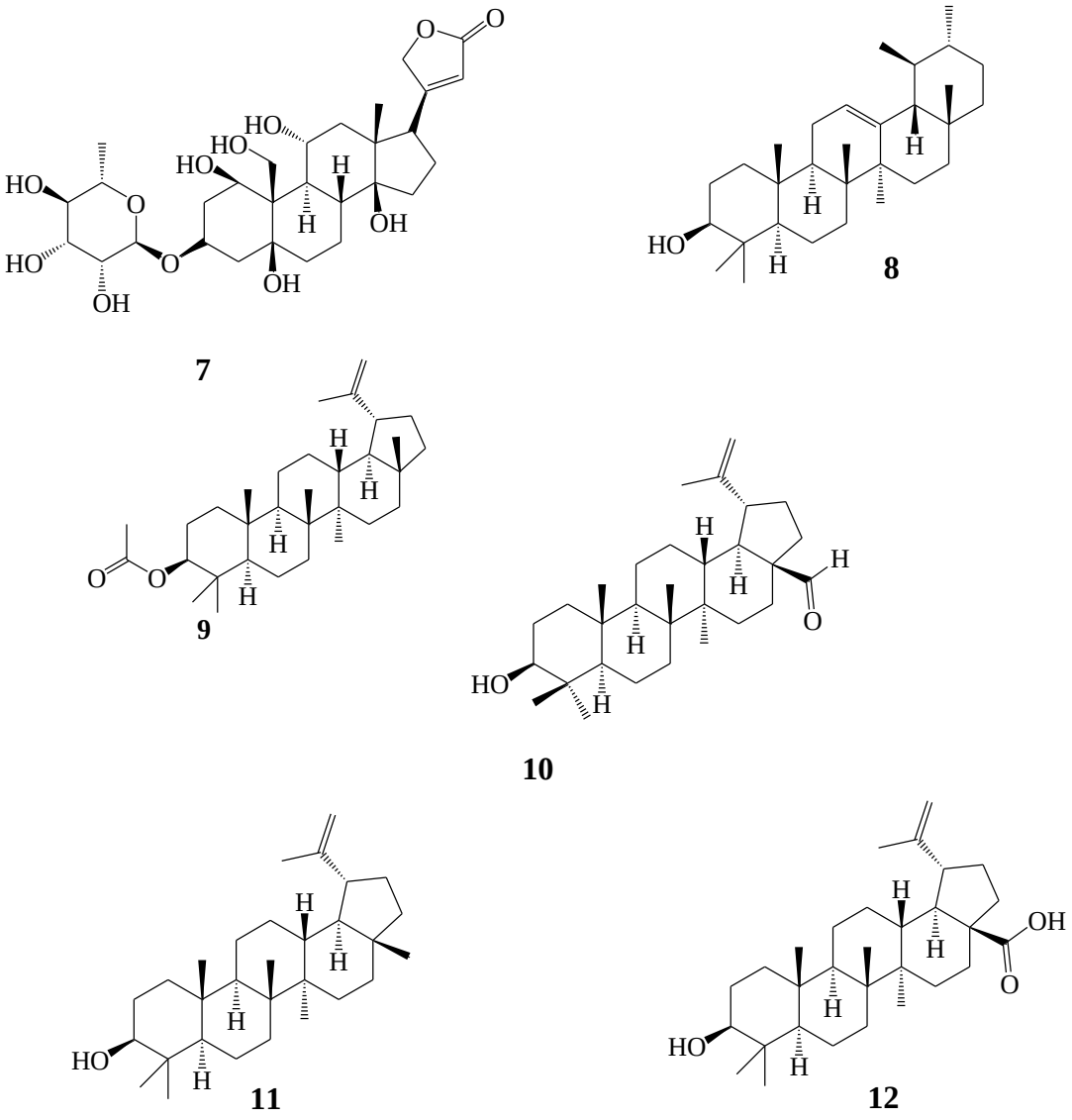


Fig. 2 Ouabain and triterpenes reported from *A. oblongifolia* leaves

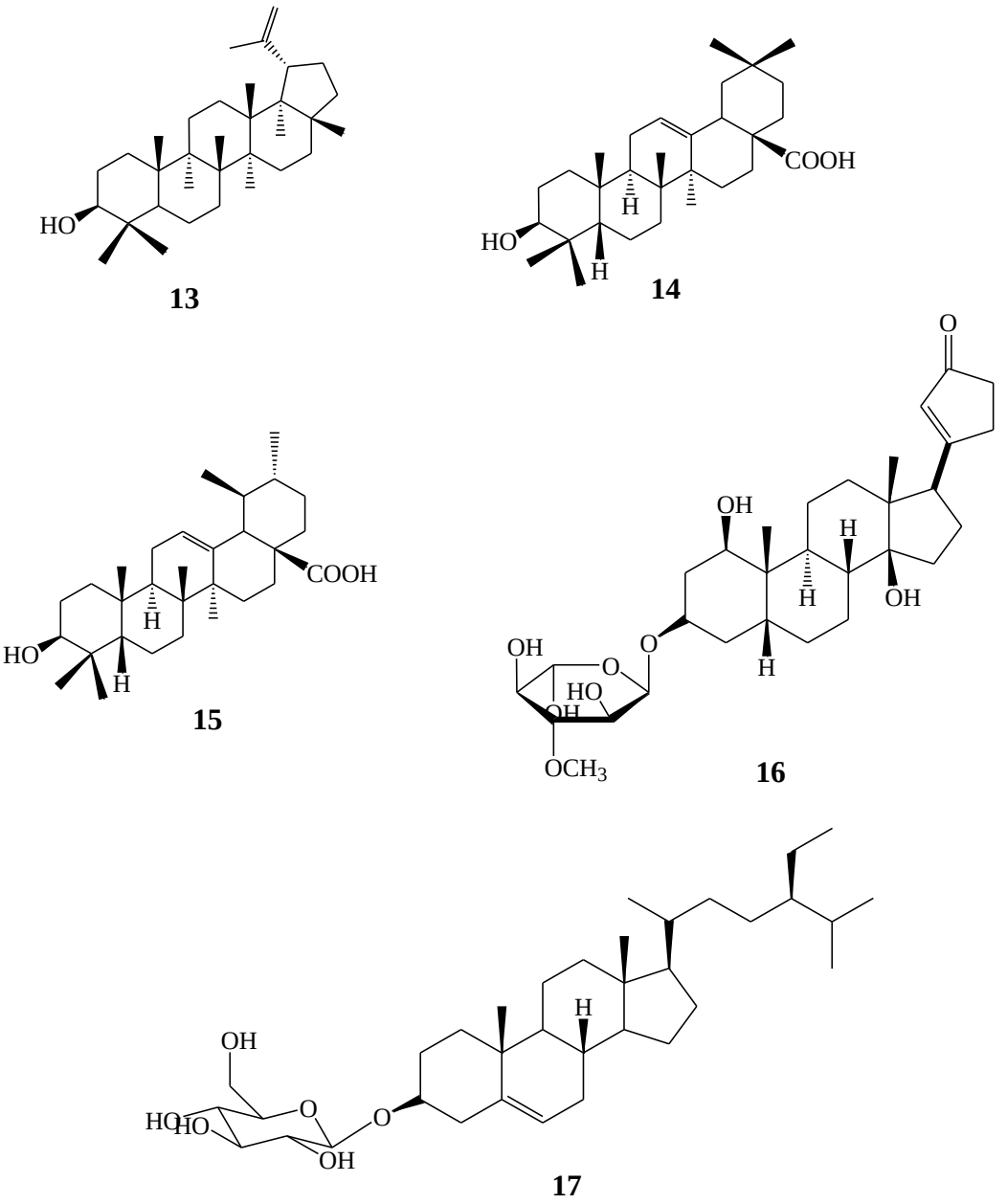


Fig.3. Reported structures (**13-17**) from the fruits of *A. oblongifolia*

2.3 *Acokanthera schimperi*

2.3.1 Botanical Description of *A.schimperi*

A. schimperi, (*syn.Carissa schimperi*) and local name *Keraru* (Oromiffa), *Ye merz enchet* (Amharic) is a shrub or small rounded tree, with short bole, to 5 m, sometimes 10 m belonging to

the family *Apocynaceae*. It prefers rich well-drained forest soil but also grows on black cotton and poor soils in dry sites, used as tools (spear shafts), food (ripe fruit), medicine (roots), ornamental, arrow poison (white latex from roots, leaf, bark) (Cassels, 1985). The bark is dark brown, grooved with age, young twigs flattened. The leaves are opposite, dark shiny green above, stiff and leathery, oval to rounded 4-7 cm, the tip pointed and sharp. The flowers are appearing with early rains, in dense, fragrant clusters**,** almost stalk less, white-pink, tubular. Fruits are oval berries to 2.0 cm, red, becoming purple when ripe, edible (Gebre-Mariam *et al.*, 2006).

2.3.2 Traditional and Medicinal Use of *A. schimperi*

The fruit is edible, and is eaten as a famine food. In Ethiopia the leaves and bark are applied to the skin to treat skin disorders, for the treatment of headache, epilepsy, amnesia, eye disease, syphilis, rheumatism, schistosomiasis, and an infusion of the leaves is gargled to treat tonsillitis (Abebe & Ayehu, 1993). Dried pulverized leaves with honey are taken as an anti fertility medicine. A hot infusion of the pounded root is drunk in small quantities to treat sexually transmitted diseases, and also as an aphrodisiac (Gebre-Mariam *et al*., 2006). A mixture made from the leaves, bark and butter is used for gall-bladder problems. The smoke of dried roots and twigs is insect repellent; too much smoke is harmful for humans as well. In Kenya Samburu women drink a bark decoction when their menstruation does not stop. In Uganda a leaf decoction is given to cattle that have a cold (Getahun, 1976).

2.3.3. Reported Anti microbial Activity of *A. schimperi*

A methanol extract from the leaves of *A. schimperi* showed significant antiviral activity against influenza virus Coxsackie virus by inhibiting their replication (bacteriostatic) for which no antiviral agents or vaccines are currently available. The extract also exhibited significant antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and significant antifungal activity against *Trichophyton mentagrophytes*. Both methanol and aqueous extract of the leaf showed anti plasmodial activity (Hedberg *et al*, 2006).

2.3.4. Chemical Constituents of *A. schimperi*

Reported phytochemical analysis of *A. schimperi* leaves collected from Ghinda, Eritrea showed that the extracts of the plant contained steroids, triterpenoids, phenols, tannins, phloroglucocides, coumarins, anthranoids, and flavonoids (Abebe *et al.*,2001). All plant parts of *A. schimperi*,

except the pulp of the ripe fruit, contain large amounts of cardiac glycosides (cardenolides) (Hart *et al.*, 2007). The main compounds are acovenoside (**18**) (0.3–1.8%), with acovenosigenin as aglycone, followed by ouabain (0.1–5%) with ouabagenin as aglycone, and traces of acolongifloroside (**19**). Ouabain and acolongifloroside are the most cardio active compounds; they are highly toxic and can cause death even in minute doses (Abebe *et al.*, 2001). Two coumarins were reported from *A. schimperi* leaves. The compounds were identified as 8-hydroxy-2H -chromen -2- one (**20)** and (*E*)-methyl-4- hydroxyl -7- oxo-5- (2-oxo-2H-Chromen-8-yloxy) oct-2- enoate **(21)** Fig. 4.

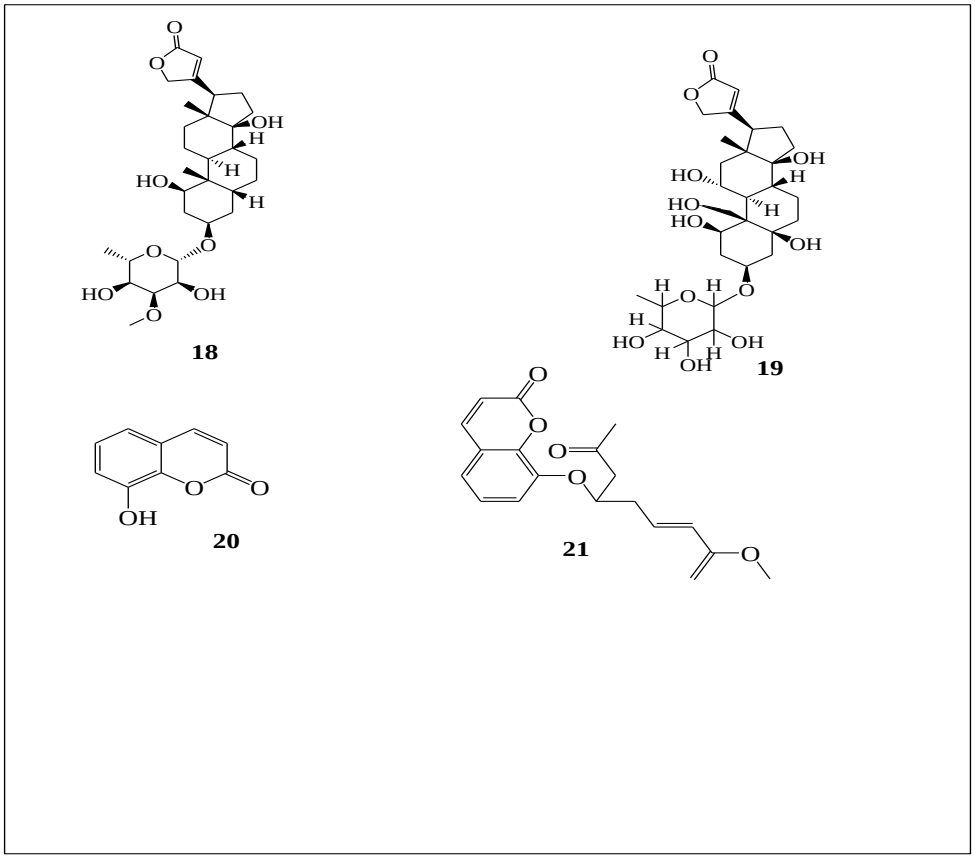


Fig. 4 Chemical structures of compounds reported from EtOAc Extracts of *A. schimperi* Leaves

3. MATERIALS AND METHODS

3.1 Chemicals, Reagents, Apparatus and Instruments

3.1.1 Chemicals and reagents

Chemicals used in this research were n-hexane, chloroform, ethyl acetate, methanol, silica gel, Wagner's reagent, Keller's reagent, Keller Kiliani reagent, Salkowski reagent, distilled water, ferric chloride, potassium hydroxide, sodium hydroxide, vanillin (spraying reagent), Iodine, and Borntrager's reagent.

3.1.2 Apparatus and Instruments

Apparatus and instruments used in this research are electrical grinder, drying oven, measuring cylinders, reagent bottles, ruler, filter papers, funnels, round bottom flasks, rotary evaporator, water bath, test tubes, vials, TLC Chamber, TLC plates, capillary tubes, UV-Vis cabinet (for TLC and PTLC visualization) , glass columns (different sizes), PTLC plates, PTLC Chamber, IR- spectrometer, and NMR- Spectrometer.

3.2. Plant Materials Collection and Identification

The roots of *A. schimperi* was collected from East Arsi Assela Woreda which is 175 Km from Addis Ababa, Ethiopia in the month of January, 2017. The plant material was taxonomically identified by a Botanist Mr. Shambel Alemu with voucher specimen number 001 in National Herbarium Biology Department, Addis Ababa University, Ethiopia. The plant materials for chemical work was stored in post graduate chemistry laboratory of Adama Science and Technology University, air dried at room temperature under shade. The dried root was ground to fine powder using electrical disintegrator Model FT 80 and kept in glass container for further work.

3.3. Method of Extraction

The powdered plant material (500g) was extracted using maceration method successively with increasing solvent polarity i.e. n- hexane, Chloroform, ethyl acetate, and methanol for three days each (72 hrs.) with frequent agitation. This means, first the powdered root was soaked in n-hexane and then filtered. The extract was concentrated under a reduced Pressure using a Rota vapor. The marc obtained from the hexane extract was dried and soaked in chloroform for three days, filtered and the extract was concentrated. The dried residue from the chloroform extract was then soaked in ethyl acetate also for three days, filtered and the extract was concentrated. Finally the dried marc from ethyl acetate was soaked in methanol for three days filtered, and concentrated under a reduced pressure using a Rota vapor. Each crude extracts were stored in refrigerator till isolation and phytochemical analysis. For each extract, the percentage yields were calculated.

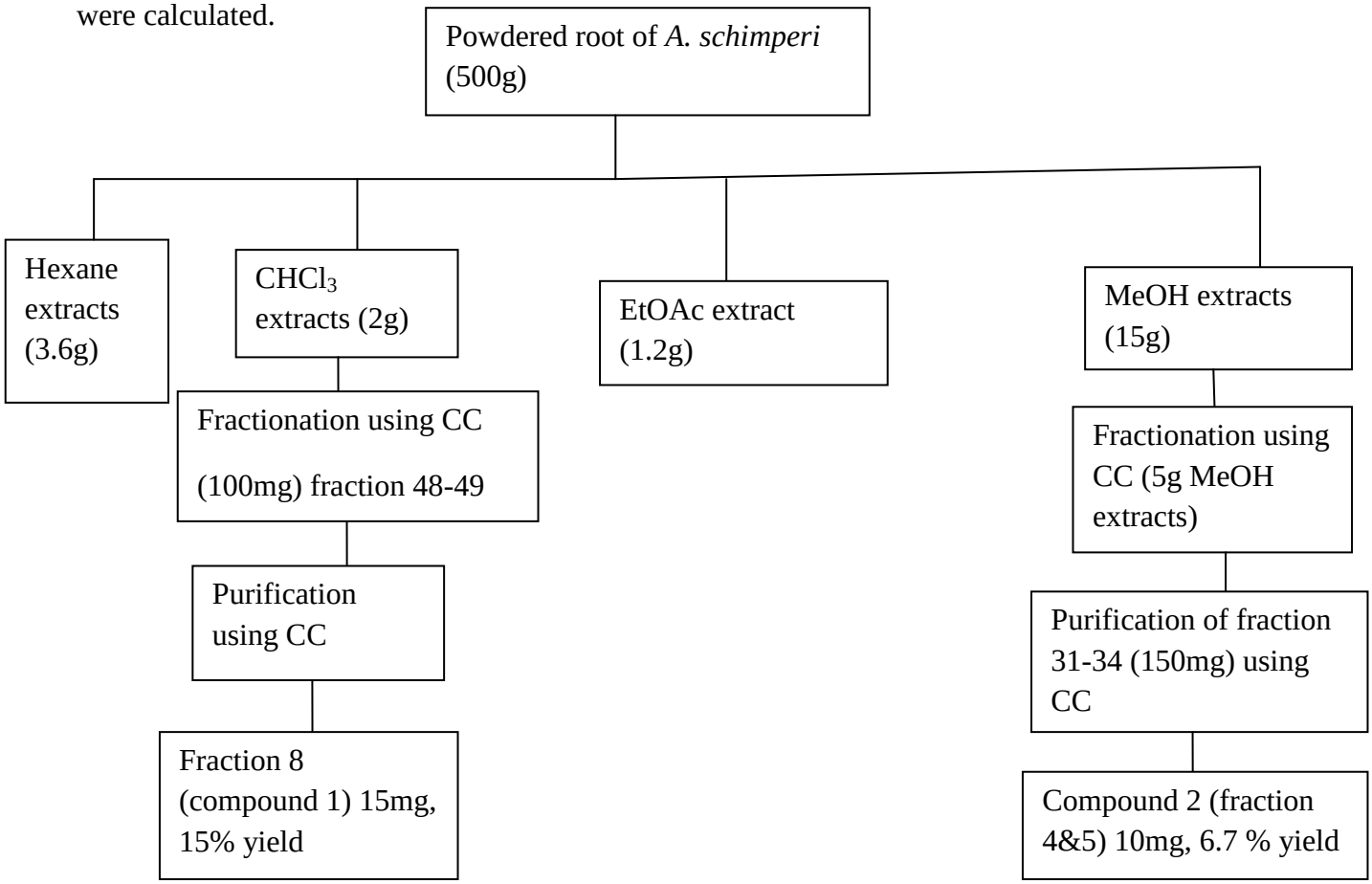


Figure 5: A schematic flow chart of extraction procedure of roots of *A. schimperi*.

3.4 Phytochemical Screening of Crude Extracts from Roots of *A. schimperi*

Standard screening test of the extracts were carried out for various plant constituents. Four crude extracts were screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins and anthraquinones using standard procedures (Marston, 2011).

a) **Test for alkaloids:** The extracts (15 mg) were stirred separately with 1% HCl (6 mL) in the beaker on a water bath holding by tong for 5 min and filtered in to test tubes. Few drops of Wagner's reagent were added; a brown/reddish colored precipitate indicates the presence of alkaloids (Iqbal et al., 2015).
b) **Detection of Tannin:** 30mg of extract was added in to 5ml of freshly prepared 10% potassium hydroxide (KOH) solution and shaken to dissolve. A dirty precipitate indicates the presence of tannin (Falodun et al., 2006).
c) **Detection for flavonoid by Shinoda test:** Four pieces of magnesium fillings (ribbon) were added to the extracts in the Test tubes followed by few drops of concentrated hydrochloric acid. A pink or red color indicates the presence of flavonoid. Colors varying from orange to red indicate flavones, red to crimson indicates flavonoids, and crimson to magenta indicates flavonones.
d) **Detection of terpenoids by Salkowiski:** The crude extract (about 100 mg) was shaken with chloroform (2 mL) in the test tube followed by the addition of concentrated H_2SO_4 (2 mL) along the side of the test tube using dropper, a reddish brown coloration of the interface indicates the presence of terpenoid (Iqbal et al., 2015).
e) **Detection of Anthraquinone Glycoside by Born trager's:** To the extract solution (1mL) in the test tube, 5% H_2SO_4 (1mL) was added. The mixture was boiled in a water bath and then filtered. Filtrate was shaken with equal volume of chloroform and kept to stand for 5 min. Then the lower layer of chloroform was shaken with half of its volume with diluted ammonia. The formation of rose pink to red color of the ammoniacal layer gives indication of anthraquinone glycosides (Iqbal, et al., 2015).
f) **Detection of Cardiac Glycoside by Keller-Killiani:** 50mg of Extracts were shaken with distilled water (5 mL). To this, glacial acetic acid (2 mL) containing a few drops of ferric chloride was added, followed by H_2SO_4 (1 mL) along the side of the test tube. The

formation of brown ring at the interface gives positive indication for cardiac glycoside and a violet ring may appear below the brown ring (Iqbal et al., 2015) or a brown ring obtained at the interface indicates the presence of de-oxysugar characteristics of cardenolides (Falodun et al., 2006).

g) **Detection of Anthranol Glycoside by Modified Borntrager's Test:** Extracts were hydrolyzed with dil. HCl in the test tube and treated with Ferric Chloride solution, immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink color in the ammonical layer indicates the presence of anthranol glycosides.

h) **Detection of Saponins by Froth Test:** About 50mg of the plant extract was shaken with distilled water (10 mL) in test tube, frothing which persists on warming using water bath for 5 min considered as preliminary evidence for the presence of Saponins (Iqbal et al., 2015).

i) **Test for Coumarins:** 3 ml of 10% NaOH was added to 2 ml of aqueous extract formation of yellow color indicates the presence of coumarins (Falodun et al., 2006).

3.5 Isolation of compounds from crude extracts

For this research, first each extracts were analyzed using TLC with different solvent mixtures to select the best mobile phase for column chromatography. The crude extracts which showed better TLC was adsorbed on silica gel and the sample was loaded on column. The column was eluted with increasing polarities of selected solvent systems. Composition of each fraction collected from column chromatography was monitored by TLC. Spots were visualized using UV lamp at 254nm, short-wavelength UV light, and at 366 nm, long-wavelength UV light, I_2 vapors and spraying reagents. Further purification was carried out by using PTLC. The preliminary TLC analysis were done for the n-hexane, $CHCl_3$, EtOAc, and MeOH extracts using different mixtures of solvents to observe the best separation of components for isolation of selected extract by column chromatography. In this way two extracts (the chloroform extract which had better separation with solvent system of 9:1 $CHCl_3$: EtOAc, and methanol extract which showed better separation with solvent system 9:1 $CHCl_3$: EtOAc detected under UV lamp) were selected to carry out the column chromatography.

3.5.1 Column Chromatography (CC)

i) Chloroform Extract

Column was packed with a silica gel adsorbent (60-120 mesh) using n- hexane as solvent; n-hexane: $CHCl_3$, $CHCl_3$:EtOAc, and $CHCl_3$:MeOH were used as solvent for elution and 66 fractions were collected. Using 1:1 $CHCl_3$: hexane fraction 1-3, fractions 4-6 (6:4), 7-8 (7:3), 9-21 (8:2), 22-23 (9:1), 24-31(100% $CHCl_3$), 32 (9:1 $CHCl_3$:EtOAc), 33-40 (8:2), 41(7:3), 42(6:4), 43(1:1), 44-51(9:1 $CHCl_3$:MeOH), 52(8:2), 53-55(7:3), 56-60(6:4), 61(1:1), 62(4:6), 63(3:7), 64(8:2), 65(9:1), 66(100% MeOH).

ii) Methanol Extract

Column was packed with a silica gel adsorbent (60-120 mesh) using n-hexane as solvent; n-hexane: $CHCl_3$, n-hexane: EtOAc, $CHCl_3$: EtOAc, and $CHCl_3$: MeOH was used as solvent for elution and 61 fractions were collected. Using 3:7 $CHCl_3$: hexane fraction 1-5, fractions 6-7 (4:6), 8-10 (1:1), using 9:1 hexane: EtOAc fractions 11-15, (8:2) 16-18, (7:3) 19-21, (6:4) 22, (1:1)23, using 9:1 $CHCl_3$: EtOAc 24-25, (8:2) 26, (7:3) 27, (6:4) 28, (1:1) 29, (9:1 $CHCl_3$: MeOH) 30-39, (8:2) 40-41, (7:3) 42, (6:4) 43-49, (1:1) 50, (4:6) 51-52, (3:7) 53, (8:2) 54-56, (9:1) 57-59, (100% MeOH) 60-61.

The TLC analysis was done for each fraction to combine similar fractions and to investigate pure compounds. 40 fractions from MeOH extract and 31 different fractions from $CHCl_3$ extract after combining similar fractions using TLC analysis but, there was no any pure compound isolated from both chromatography columns. Therefore, the fraction with good amount and better TLC profile was selected [(Fr 31-34) from MeOH extract and (48-49) from $CHCl_3$ extract]; Fr 48-49 was loaded on to small column packed with silica gel using hexane as solvent for isolation and compound **22** was isolated with 7:3 hexane: EtOAc solvent system. Fr 31-34 was isolated using CC with the solvent ratio of 1:1 $CHCl_3$: EtOAc; compound **23** was obtained.

3.6 Structural Elucidation of Isolated Compounds

The most important tools for structure elucidation of natural products are nuclear magnetic resonance (NMR) and mass spectroscopic (MS) techniques. In addition, infrared (IR) and ultraviolet-visible spectrophotometer (UV-Vis) methods are of importance (Harborne, 1998; Cseke *et al*., 2010). Therefore, in this research, the isolated compounds were submitted for IR and NMR analysis. The spectral data of compounds generated were used to elucidate structures of the compounds.

3.7. Antibacterial Activity Testing of the Crude Extracts and Isolated Compounds

The antibacterial activity of the roots extracts and purified fractions of *A. schimperi* was determined using disk diffusion method which is commonly used for screening of the antimicrobial activities of herbal drugs. Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk. For the antibacterial screening, three Gram-negative bacteria; *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and two Gram-positive bacteria; *Staphylococcus aureus and Bacillus subtilis* were used. In this way bacterial strains were grown on Mueller-Hinton Agar (MHA) plates. Two to three colonies of bacteria were transferred into a tube containing 15mL nutrient broth and grown overnight at 37 °C. The turbidity of the culture was adjusted with sterile saline solution. Sensitivity test was carried out using the disc diffusion assay. Bacterial strains of standardized cultures were evenly spread on to the surface of the agar plates using sterile swab sticks. Test plates were then incubated at 37 °C for 18-24 hrs. The diameter of resulting zone was then measured in millimeter (Tadeg et al., 2005).

4. RESULTS AND DISCUSSION

4.1 Percentage yield of plant extracts

From 500g of powdered root of *A. schimperi*, methanol extraction yielded 15g extract (3%), ethyl acetate afforded 1.2g extract (0.24%), chloroform gave 2g extract (0.4%), and n-hexane extracted 3.6g extract (0.72%). The selected solvents showed variation in percentage yield of extract for the same plant and the highest yield was recorded for methanol extract. The high extraction yield obtained with methanol may be related to the possible presence of a large quantity of more polar compounds in this plant.

4.2 Phytochemical screening of plant extracts

The result of phytochemical study show that from the investigated extracts MeOH was found to show positive results for 8 phytoconstituents (alkaloid, tannins, flavonoids, terpenoids, anthraquinone glycosides, cardiac glycosides, saponins, and coumarins), EtOAc was positive for 7 phytoconstituents (alkaloid, tannins, flavonoids, terpenoids, anthraquinone glycosides, cardiac glycosides,and coumarins), $CHCl_3$ was show positive result for 5 phytoconstituents (coumarins, tannins, terpenoids, cardiac glycosides, and Anthranol glycosides), and n-hexane showed positive test for 4 constituents (tannins, cardiac glycosides, anthranol glycosides, and coumarins). All extracts showed positive result for tannins, coumarins and cardiac glycosides. From literature reports the leave part shows the presence of terpenoids, phenols,tannins, phloroglucosides, coumarins, anthranoids, and flavonoids(Gebrehiwot *et al*.,2009). The results of the extracts from roots of A. schimperi are summarized under the table below.

Table 1: Summary of Phytochemical Results of Root Extracts

Class of compounds	**Reagents used**	**Extracts**			
		n-hexane	**$CHCl_3$**	**EtOAc**	**MeOH**
Alkaloids	Wagner's reagent	-ve	-ve	+ve	+ve
Tannins	KOH	+ve	+ve	+ve	+ve
Flavonoids	Shinoda test	-ve	-ve	+ve	+ve
Terpenoids	Salkowiski reagent	-ve	+ve	+ve	+ve
Anthraquinone glycosides	Born trager's reagent	-ve	-ve	+ve	+ve
Cardiac glycosides	Keller killiani's reagent	+ve	+ve	+ve	+ve
Anthranol glycosides	Modified born trager test	+ve	+ve	-ve	-ve
Saponins	Foam test	-ve	-ve	-ve	+ve
Coumarins	NaOH test	+ve	+ve	+ve	+ve

+ve: presence, -ve: absence

4.3 Characterization of compounds 22and23

The two isolated compounds were analyzed by spectroscopic techniques (1-D NMR and IR) for characterization.

4.3.1 Characterization of compound 22

Compound **22** was obtained as a bright yellow amorphous solid. This compound was isolated using hexane ethyl acetate (7:3) as solvent system. The structure of compound **22** has been characterized as given in figure 6 based on its ^{1}H and ^{13}C NMR data as well as DEPT-135 experiments and IR. The FT-IR (Fourier Transform Infra red spectrometer) result of Compound **22** showed the functional group of C-C and C-O-C single bond in the region 1460 and 1188 cm^{-1}. The peak at 1621cm^{-1} correspond to C=C (alkenes), 1732cm^{-1} and 1719cm^{-1}indicates the presence of carbonyl carbon strong stretching of esters and ketones. The Absorption at 2923cm^{-1} show that Sp^{3}C-H stretching for alkanes and the sharp peak at 3488cm^{-1} correspond to free OH group.

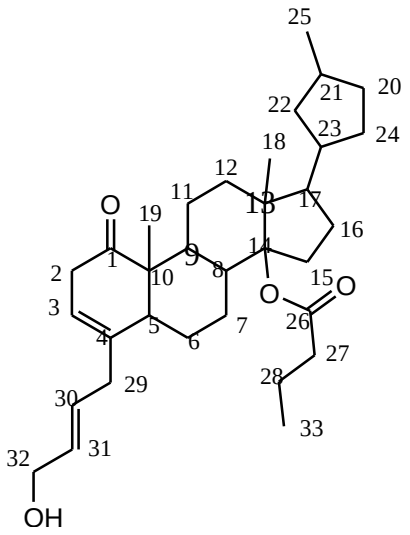
The proton NMR data contained an intense singlet peak at δ 0.95 (6H- C-18 and C- 19) integrated for six protons due to two overlapping methyl's; amethyl doublet at δ 0.75 (3H-25), and methyl triplet at δ 0.89(3H-33). The proton NMR spectrum also showed seven methine multiplets at δ1.6 (1H- 21), δ1.45 (1H- 23), δ1.58 (1H-17), δ1.87 (1H-8), δ2.1 (1H-9), two signals at δ 5.63 (1H- 31), and δ 5.9 (1H- 30) which indicates olefin proton, methine triplet at δ 5.45(C-3),and δ 2.44 (C-5). The spectrum also showed overlapped multiplet signals from 1.4-1.9,

which includes eight methylene signals at δ 1.42(8H-C_{16}, $C_{24,}$ C_{20}, C_{22}), δ 1.57 (2H-C_{11}), δ 1.74 (2H- C_6), δ 1.82(2H- C_7), δ 2.36 (2H- C_{28}). The proton NMR spectrum also indicates three methylene signals at δ 2.2 (2H, C_2 attached to carbonyl carbon of ketone), a doublet at δ 2.9 (J= 6.5, 2H- C_{29}), δ 4.8 (J= 7.3, 2H- C_{32}), and three methylene triplets at δ 1.58 (2H-C_{12}), δ 1.89 (2H-C_{15}), and δ 2.45 (2H- C_{27}) which is attached to carbonyl carbon of ester. For hydroxyl proton it is concluded that the peak is overlapped in the region from δ 2- 2.9 ppm.

The ^{13}C NMR of compound **22** contained signals due to a total of 33 carbon atoms. These are four primary, fourteen secondary, nine tertiary, and six quaternary carbons. Two carbonyl carbons at δ 211ppm (C-1) correspond to ketone and δ 174ppm (C-26) for ester. Oxygenated quaternary at δ 86ppm(C-14), olefinic quaternary at δ 140ppm (C-4), aliphatic quaternaries C-10 (δ 49.5ppm), and C-13(δ 35.5ppm) oxygenated methylene attached to hydroxyl group at δ 73ppm (C-32).

The DEPT-135 spectrum of compound **22** indicates the presence of nine methine carbons at (δ (117(C-3), 125 (C-30),128(C-31) olefinic carbons), 24 (C-17), 44(C-23), 26(C-9), 25(C-21), 38 (C-5), and 38 (C-8); fourteen methylene carbons at δ 20.9 (C-11), δ 22.9 (C-7), δ 26.8 (C-6), δ 33.6 (C-2), δ 26.5 (C-12), δ 29.7 (C- 15), δ 20.9 (C-16), δ 29.3 (C-24), δ 29.7 (C-20), δ 31.2 (C-22), δ 33.2 (C-27), δ 22.6 (C-28), δ 39.2 (C-29), δ 73 (C-32 hydroxyl CH_2). The DEPT-135 spectrum also showed four methyl carbon signals at δ 15.6 (C-18, and C-19), δ 14.1 (C-33), δ 18.6 (C-25).

Compound **22** belongs to a cardiac glycoside class of compounds without sugar portion having steroid ring with ketone, alcohol, and ester functional groups. The structure of compound **22** was proposed using the structure of Acovenoside A(**16**) as reference.



17- methyl cyclopentyl-3, 30- diene- 26-oate, cardene-1-one-32-ol

Figure6: Proposed structure of compound **22**

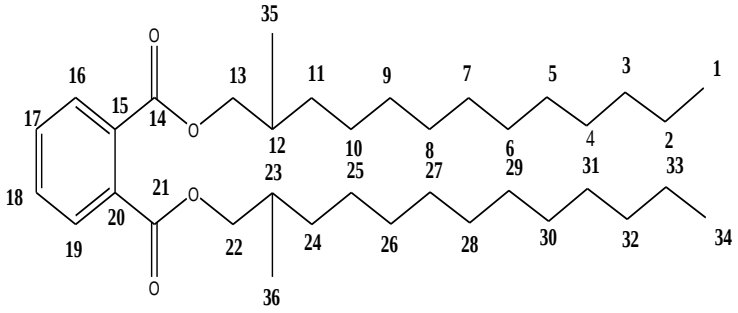
Table: 3 ^{1}HNMR, ^{13}C NMR and DEPT-135 spectroscopic data of compound **22**

Position of carbon	1H data (δ in ppm)	13C data (δ in ppm)	DEPT 135 data (in ppm)
C-1		211.9	-
C-2	2.2	33.6	33.6
C-3	5.45	122.2	122.2
C-4	-	140	-
C-5	2.44	38.2	38.2
C-6	1.74	26.8	26.8
C-7	1.82	22.9	22.9
C-8	1.87	38.1	38.1
C-9	2.1	26.7	26.7
C-10	-	49.5	-
C-11	1.57	20.9	20.9
C-12	1.58	26.5	26.5
C-13	-	35.5	-
C-14	-	86.1	-
C-15	1.89	29.7	29.7
C-16	1.42	20.9	20.9
C-17	1.58	24.2	24.2
C-18	1.45	44.5	44.5
C-19	1.42	29.3	29.3
C-20	1.42	29.7	29.7
C-21	1.6	25.1	25.1

C-22	1.4	31.2	31.2
C-23	0.75	18.6	18.6
C-24	0.95	15.6	15.6
C-25	0.95	15.6	15.6
C-26	-	174.5	
C-27	2.45	33.2	33.2
C-28	2.36	22.6	22.6
C-29	2.9	39.2	39.2
C-30	5.9	125.4	125.4
C-31	5.63	128.5	128.5
C-32	4.8	73.5	73.5
C-33	0.89	14.1	14.1

4.3.2 Characterizations of Compound 23

The chemical structure of compound **23** was proposed as phthalate ester that was masked by a pollutant which interfered during the analytical process. This pollutant may have contaminated the roots. According to Kirchmann et al (1991), phthalates released to environment can be deposited on or taken up by crops; meaning that there is a possibility that *A. schimperi* may have absorbed di-n-octylphthalate from soil. The FT-IR (Fourier Transform Infra red spectrometer) result of Compound **23** showed the functional group of C-O-C single bond in the region 1098 cm^{-1} and 1262 cm^{-1} and 1464cm^{-1} indicates the presence of benzene ring. The peak at 1709cm^{-1} correspond to C=O of esters. The Absorption at 2918cm^{-1} shows that Sp^3C-H stretching for alkanes (medium stretching).



Phthalic acid bis-(2-methyl-tridecyl) ester

Fig.7 Proposed Structure of Compound **23**

Table: 4 ^{1}HNMR^{13}C NMR and DEPT-135 spectroscopic data of Compound **23**

Position of carbon	1H data (δ in ppm)	13C data (δ in ppm)	DEPT 135 data (in ppm)	Remark
C-1&C-34	0.9triplet	14.1	CH_3	
C-2&C-33	1.27multiplet	24.7	CH_2	
C-3&C-32	1.3multiplet	24.7	CH_2	
C-4&C-31 C-5&C-30 C-6&C-29 C-7&C-28 C-8&C-27 C-9&C-26	δ 1.64ppm, multiplet	δ 29.4ppm intense peak	CH_2	six equivalent carbons
C-10&C-25	δ 1.83 multiplet	31.9	CH_2	
C-11&C-24	2.37multiplet	33.7	**CH_2**	
C-12&C-23	2.1multiplet	27.7	CH	
C-13&C-22	4.12 doublet	71.8	CH_2-O	
C-14&C-21	-	174	-	quaternary
C-15&C-20	-	132.4	-	quaternary
C-16&C-19	7.75	128.8	Ar-CH	
C-17&C-18	7.56	130.9	Ar-CH	
C-35&C-36	1.01	19.2	CH_3	

4.4. Antibacterial Activity of the Crude Extracts and Isolated Compounds

Results of antibacterial activity of the plant are shown in the table2. Three extracts and two fractions were screened for their antibacterial activity. The $CHCl_3$ root extracts of *A. schimperi* had inhibitory activity against two gram positive bacteria (*B. subtilis & S.aureus*) and one gram negative bacteria (*K. pneumonia*). MeOH extract showed activity against *B. subtilis* & *K. pneumoniae*, EtOAc extract had no activity. Purified fraction (8) of chloroform extract had inhibitory activity against *B.subtilis, S.aureus & K. pneumonia*. Fraction 31-34 of MeOH showed activity against *B.subtilis* & *K.pneumonia*. Gentamycin and oxacillin as positive control and DMSO was used as negative control. The anti bacterial activity was more pronounced on the gram positive bacteria (*B.subtilis*) than the gram negative bacteria (*E.coli, K.pneumonia* and *P.mirabilis).*

Table 2: Anti bacterial activities of crude extracts and fractions of *A.schimperi* against different bacterial strains

Extracts	Inhibition Zones (mm) against					Concentration *µg/ml*
	B.subtilis	*S. aureus*	*E.coli*	*K.pneumonia*	*P.mirabilis*	
Chloroform	10	10	0	8	0	30
Ethyl acetate	0	0	0	0	0	30
Methanol	8	0	0	8	0	30
Fractions						
Fr 8 of $CHCl_3$	10mm	10mm	0mm	10mm	7mm	30
Fr 31-34 of MeOH	10mm	0mm	5mm	8mm	0mm	30
Gentamycin	NT	NT	11mm	13mm	12mm	30
Oxaxillin	15mm	12mm	NT	NT	NT	30

Sensitive $\geq$ 6, NT: not tested

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

A. schimperi is an important medicinal plant which belongs to the perennial family (*Apocynaceae*) and tribe *Acokanthera*. Traditionally *A. schimperi* is used for malaria, tonsillitis, skin disorders, headache, epilepsy, amnesia, wound, syphilis, and unstopped menstruation (Getahun, 1976). In Ethiopia Local peoples use the root part for treatment of Syphilis, Gonnohrea , and Wound effectively. All extracts showed positive result for tannins, coumarins and cardiac glycosides. From literature reports the leave part shows the presence of terpenoids, phenols,tannins, phloroglucosides, coumarins, anthranoids, and flavonoids(Gebrehiwot *et al*.,2009). Compound **22** was isolated with 7:3 hexane: EtOAc solvent system. Compound **23** was isolated using CC with the solvent ratio of 1:1 $CHCl_3$: EtOAc. The two isolated compounds were analyzed by spectroscopic techniques (1-D NMR and IR) for characterization. The $CHCl_3$ root extracts of *A. schimperi* had inhibitory activity against two gram positive bacteria (*B. subtilis & S.aureus*) and one gram negative bacteria (*K. pneumonia*). MeOH extract showed activity against *B. subtilis* & *K. pneumoniae,* EtOAc extract had no activity. Isolated fraction (8) of chloroform extract had inhibitory activity against *B.subtilis*, *S.aureus & K. pneumonia*. Fraction 31-34 of MeOH showed activity against *B.subtilis* & *K.pneumonia*. Gentamycin and oxacillin as positive control and DMSO was used as negative control.

5.2 Recommendation

Further additional compounds were isolated by chromatographic methods but their structure could not be elucidated because of their very small quantities. Therefore, to get enough amount additional samples should be collected and applying the same procedure is better.

On the basis of this investigation and literature review this plant is recommended for further studies. More over further analysis of the isolated compound on mass spectroscopy, 2D NMR (to further confirm the structure), and antimicrobial activity might be necessary Thus, I would recommend these further investigations to be continued.

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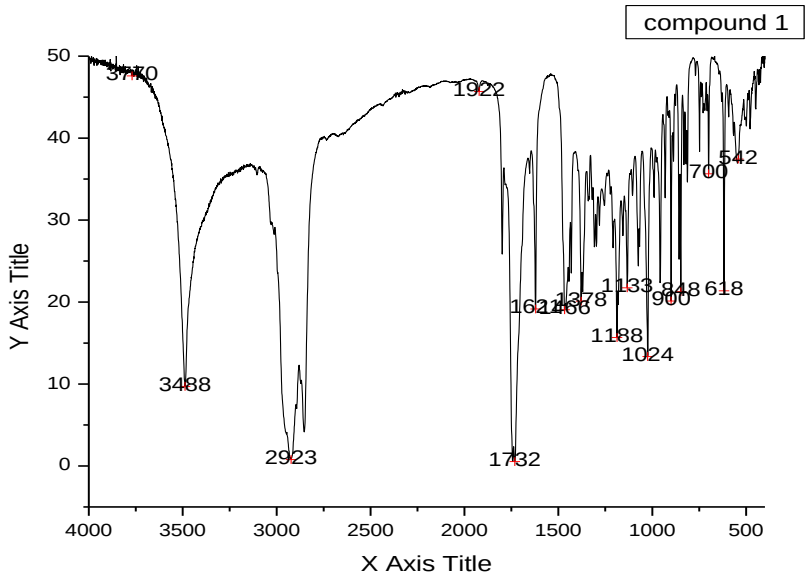
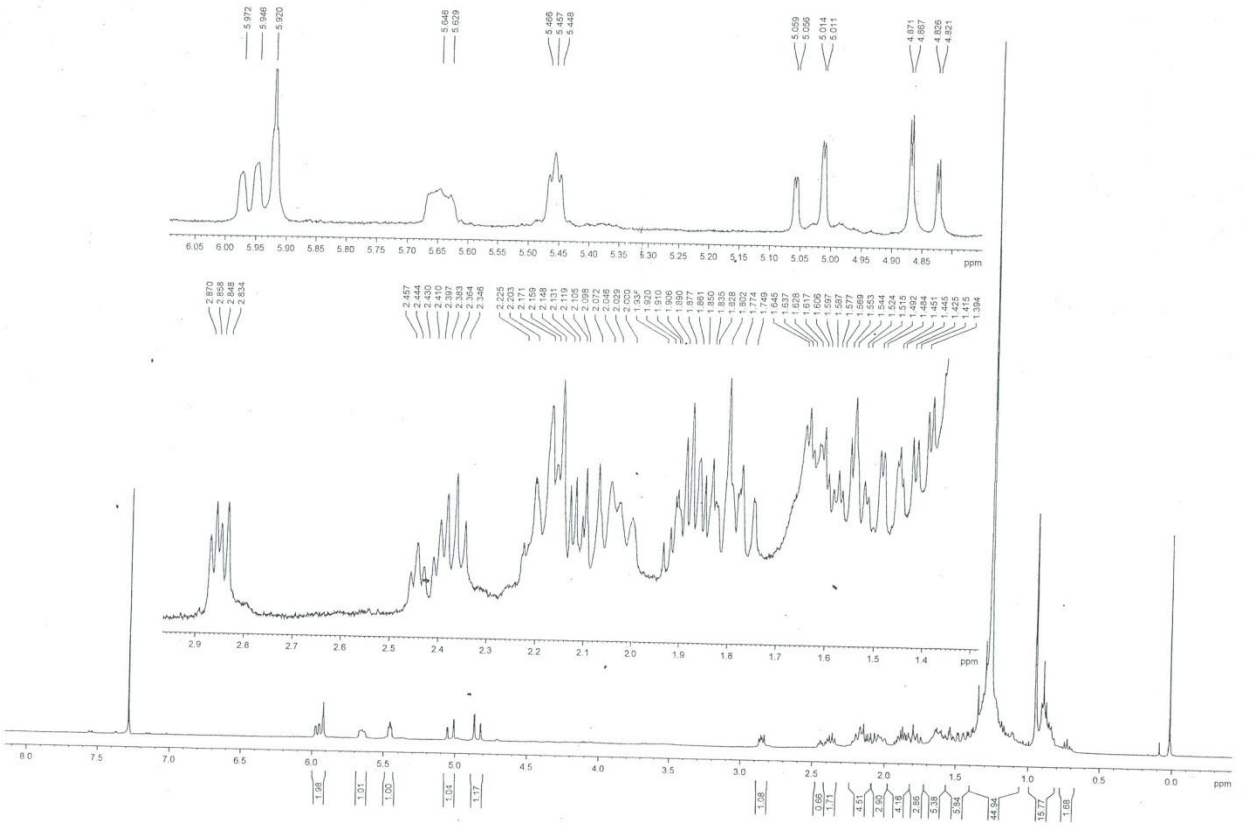
7. APPENDIX

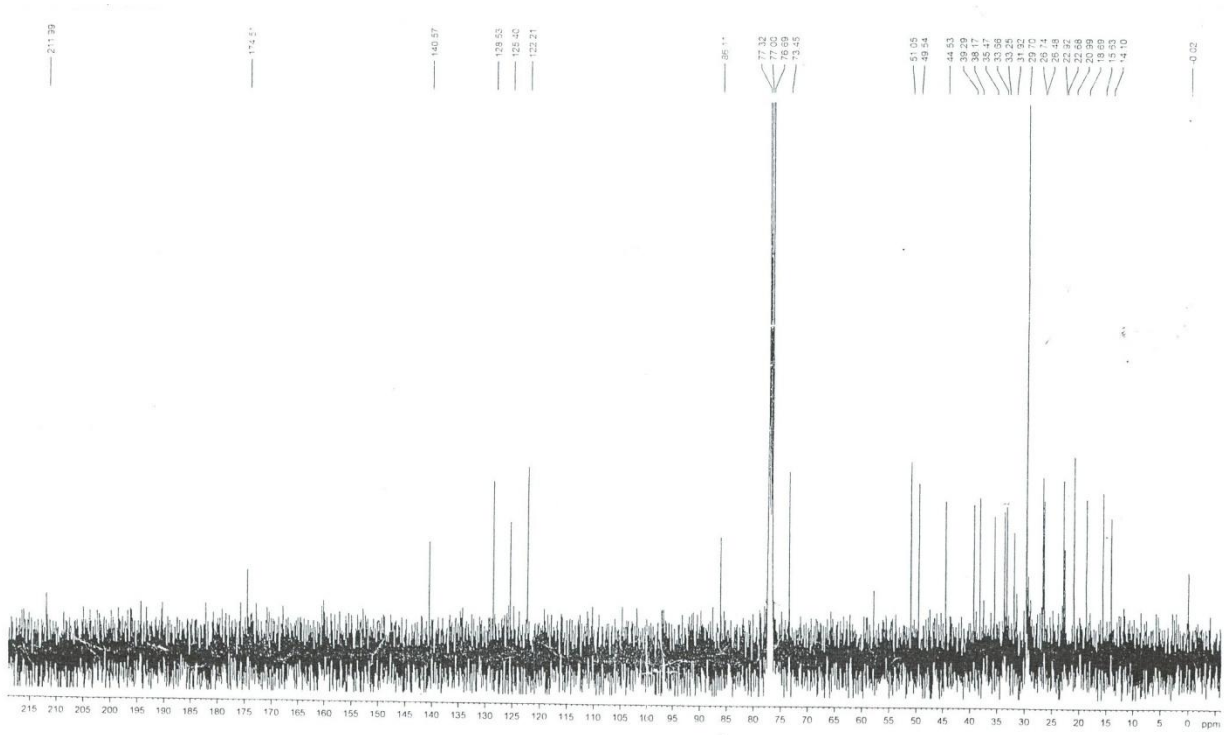
APPENDIX 1.1 IR Spectrum for Compound 1



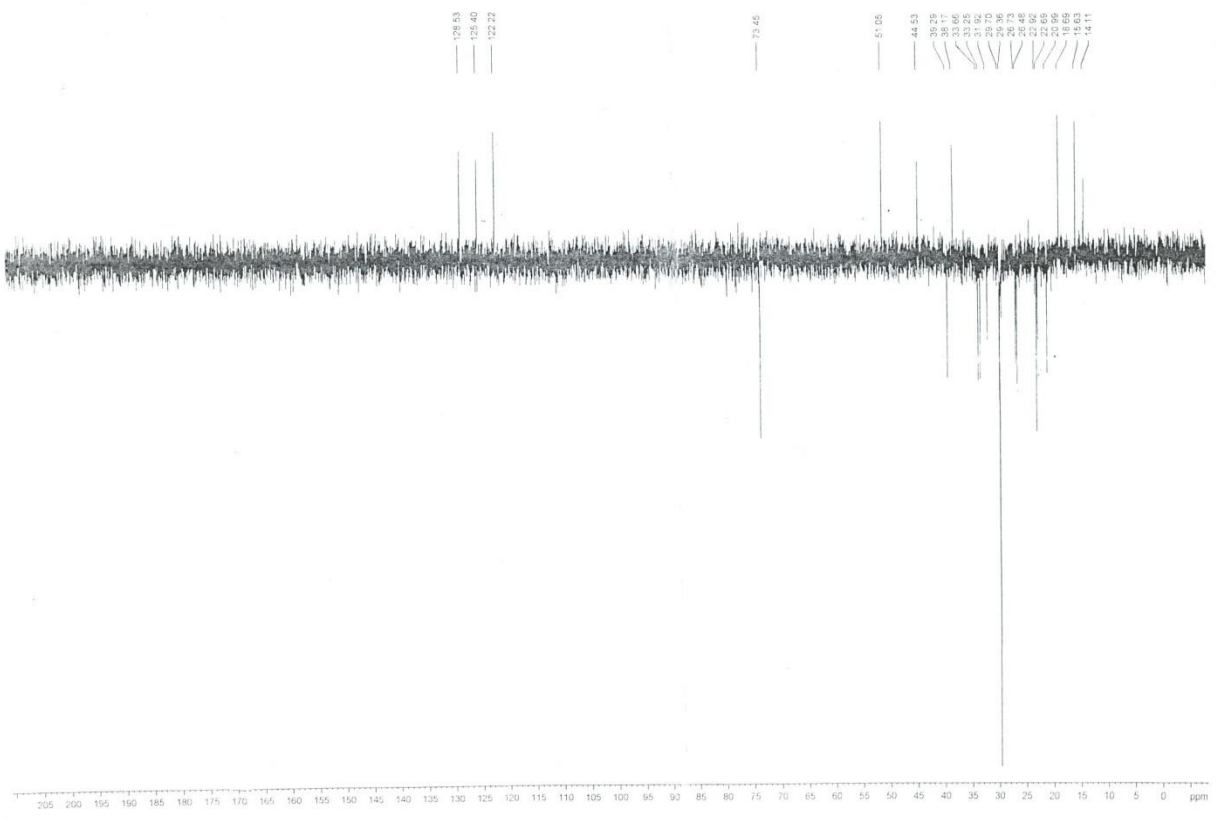
APPENDIX 1.2 ^{1}H NMR for Compound 1



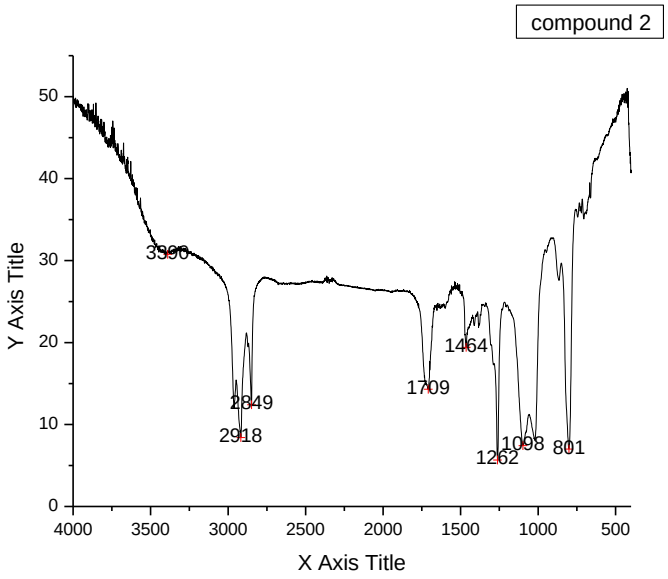
APPENDIX 1.3 ^{13}C -NMR for Compound 1



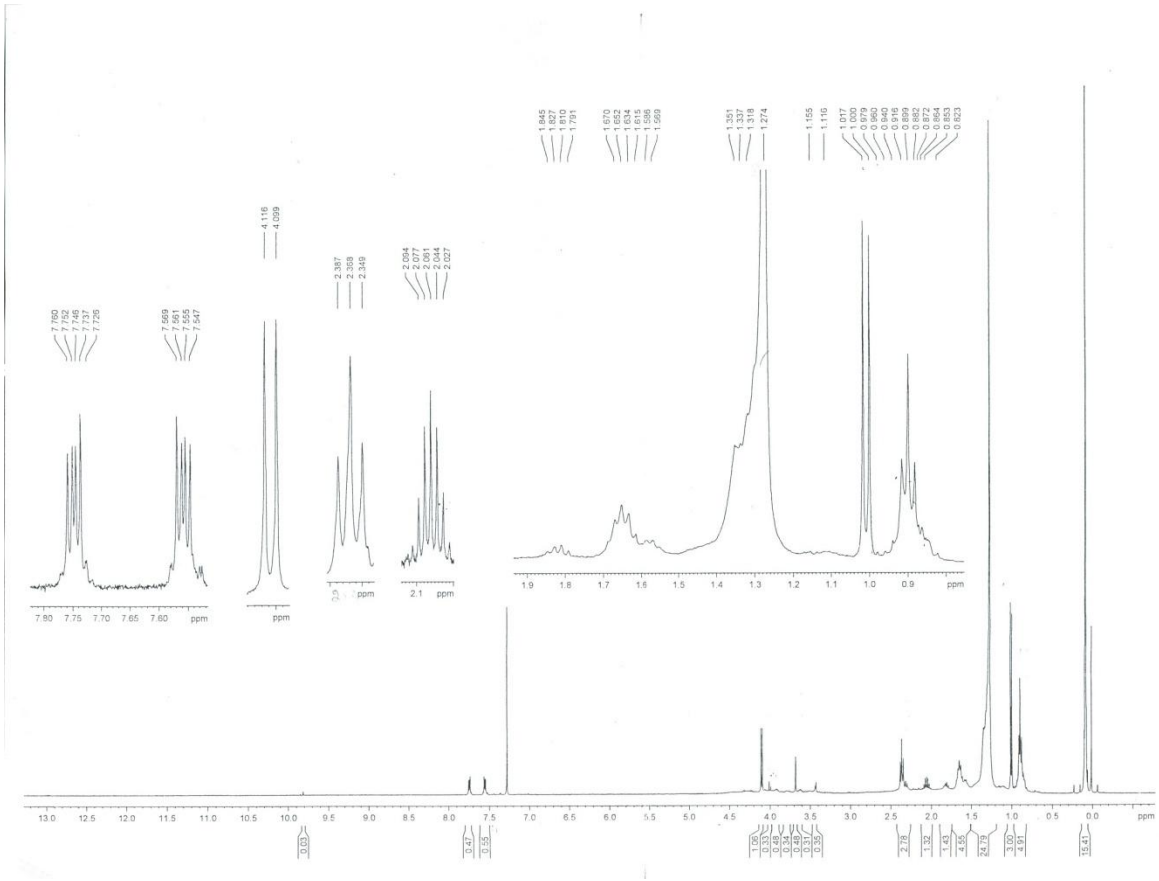
APPENDIX 1.4 DEPT -135 NMR for Compound 1



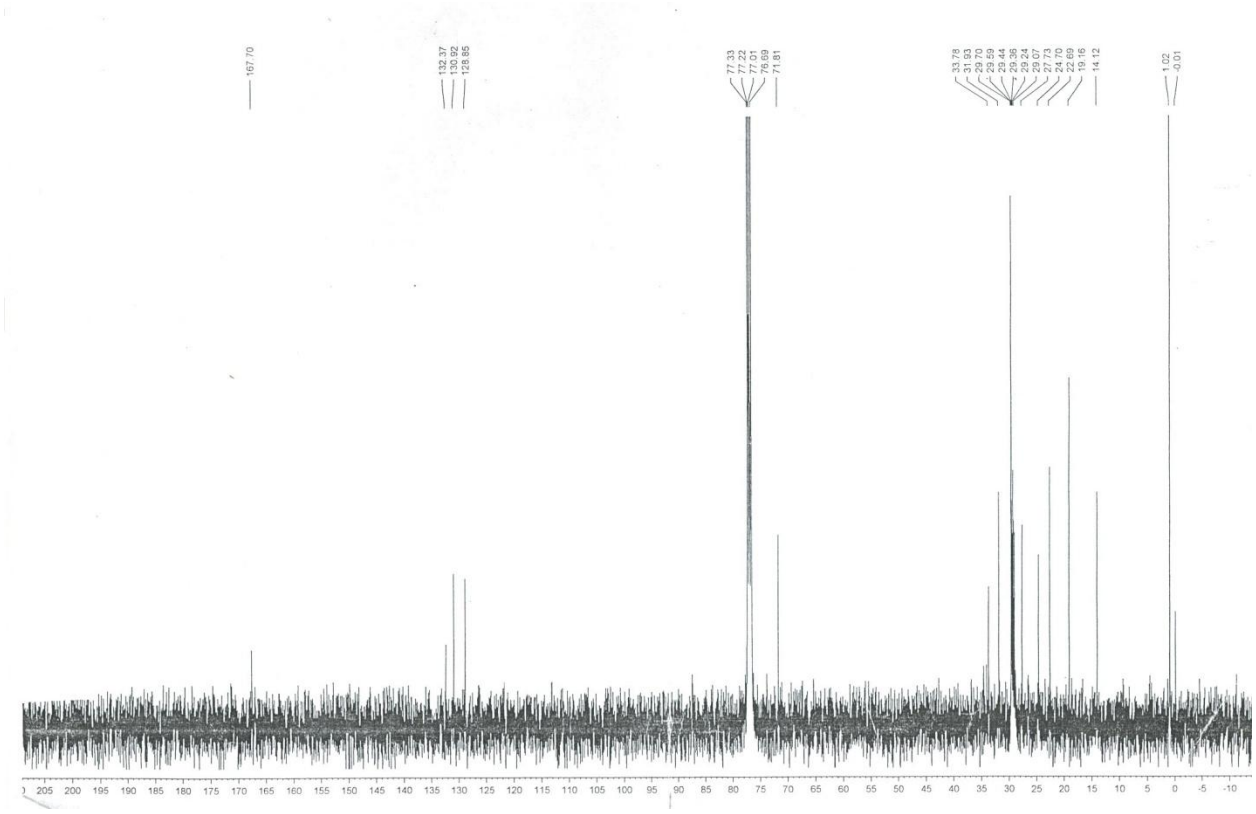
APPENDIX 2.1 IR Spectrum for Compound 2



APPENDIX 2.2 ^{1}H NMR for Compound 2



APPENDIX 2.3 ^{13}C -NMR for Compound 2



APPENDIX 2.4 DEPT -135 NMR for Compound 2