Phytochemical Investigation and Antibacterial Activities of Leaves Extract of *Combretum paniculatum* Vent

By Fekadu Asefa Sukessa

A Thesis Submitted to

The Program of Applied Chemistry

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Masters of Science in Chemistry

Office of Graduate Studies

Adama Science and Technology University

September, 2017

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Advisor Yadessa Melaku (Ph.D)

Co-Advisor Milkyas Endale (Ph.D)

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APPROVAL SHEET

Adama Science and Technology University

School of Applied Natural Science

Applied Chemistry program

This is to certify that the thesis prepared by Fekadu Asefa, entitled "**Phytochemical Investigation and Antibacterial Activities of Leaves Extract of *Combretum paniculatum* Vent**". Submitted in fulfillment of the requirement for the degree of Master of Science in Chemistry complies with the regulation of the University and meets the accepted standards with respect to originality and quality.

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DECLARATION

I hereby declare that this thesis entitled "**Phytochemical Investigation and Antibacterial Activities of Leaves Extract of *Combretum paniculatum* Vent.**" submitted to Adama Science and Technology for the award of degree of master of science in Chemistry is the result of work carried out by me under the guidance of Dr. Yadessa Melaku and Dr Milkyas Endale during the period of September 2016- September 2017. It is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree or diploma in ASTU or other institutions having the same title and content.

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Signature……Date…….

Fekadu Asefa Sukessa

Program of Applied Chemistry

School of Applied Natural Sciences

ACKNOWLEDGMENT

I thank my Almighty God for the wonderful things He has done for me. I can do nothing without Him. The assistance of my advisor Dr Yadessa Melaku has been invaluable during the course of this study for his honestly advice, encouragements and support. My gratitude also goes to Dr Milkyas Endale, Mr Solomon Girmay and other staff members of the Department of Chemistry for their invaluable support and facilitating the study in various ways. My in-depth appreciation goes to the School of Natural Science and University of Adama Science and Technology University Post-Graduate Programme for financial support. I thank my colleagues and staff of the Post-Graduate Organic Chemistry laboratory for all their encouragement and support. I would also like to express my deepest gratitude to my sweet wife Tigist M. Lencho for her patience while I devote much time away from her for the study and for her constant support and pray.

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ABBREVIATION

ATCC	American Test Culture Collection
DEPT	Distortionless Enhancement by Polarization Transfer
FT- IR	Fourier Transform Infrared
MHA	Muller Hinton Agar
NMR	Nuclear magnetic resonance
Rf	Retention factor
TLC	Thin layer chromatography
US	United States
UV	Ultra violet

ABSTRACT

Combretum paniculatum (Combretaceae) is traditionally used in some parts of Ethiopia for treating ringworm, wounds, diarrhea and other infections. The leaves of C. paniculatum (300 g) were successively extracted with n-hexane, ethyl acetate and methanol to furnish 4.51 g, 8.86 g and 21.5 g, respectively. The EtOAc extract showed interesting spots on TLC and hence subjected to fractionation over repeated silica gel column chromatography which gave two compounds identified as myristic acid and 1-(2',6'-dihyroxphenyl-2-(4"-hdroxyphenyl))-1,2-ethane-diol. Structure elucidations of isolated compounds were achieved by using spectroscopic methods including IR and NMR, and in some cases by comparing spectral data with the literature value reported for the same compounds. To the best of my knowledge, the latter compound has not yet been reported in the literature. Phytochemical screening tests have shown that leaves extracts of C. paniculatum contain terpenoids, tannins, saponins, alkaloids and phenols. Furthermore the hexane, EtOAc and methanol extracts were evaluated for antibacterial activity by using paper disc diffusion method against Staphylococcus aureus, Escherichia coli, Klebsella preumanin and Proteus mirabilis. The n-hexane and ethyl acetate extracts were active against S. aureus, E. coli and P. mirabilis at concentration of 1.5mg/mL each. On the other hand, the methanol extract is not active against E. coli but showed activity against K. preumanin. 1-(2, 6'-dihyroxyphenyl-2-(4"- hdroxyphenyl))-1,2-ethane-diol was found to be active against all bacterial strains tested indicating its broad spectrum activity. The results obtained substantiate the traditional use of this plant against wound caused by bacteria.

***Key words**: Phytochemical, C. paniculatum, NMR, IR, Myristic acid and 1-(2', 6'-dihyroxyphenyl-2-(4"-hdroxyphenyl))-1,2-ethane-diol, Antibacterial*

1. INTRODUCTION

1.1. Background

The use of plants and their products for medicinal benefits has played a significant role in nearly every culture on earth. Historically all traditional remedies were obtained from plants and recent estimates have suggested that several thousands of plants have been known with medicinal applications in various cultures [1].

Medicinal plants have been used since ancient times in virtually all cultures as a source of medicines [2], and are of great importance to the health of individuals and communities [3]. Traditional medicine is used in all parts of the World and has a rapidly growing economic importance, mainly through the use of medicinal plants, especially in developing countries. Some plants are important and serve as an important source of nutrition and medicinal properties. The use of these medicinal plants is increasing and this is evident by the fact that 75 % of new anticancer drugs marketed between 1981 and 2006 have been derived from plant sources [4].

The medicinal use of plants of the family Combretaceae is widely described in the scientific literature. This family is distributed in approximately 20 genera with 600 species. The largest genera are *Combretum* and *Terminalia,* with about 370 and 200 species, respectively [5, 6]. *Combretum paniculatum* belongs to the Combretacea family. It is ascadent shrub with tailing branches. The leaves of the plants are used in folk medicine for the treatment of diseases such as stomach pain and diarrhea. The ethanol extract of the leaves of *C. paniculatum* showed significant cytotoxic activity against breast cancer cells [7]. In Ethiopia, *C. paniculatum* is used for treatment of ringworm and wounds [8]. Despite the traditional use of this plant against wide range of diseases, there is limited scientific report on the chemical constituents and antibacterial activities of the leaves of *C. paniculatum.*

1.2 Statement of the Problem

Infectious diseases caused by bacteria are among the severe diseases affecting many peoples in developing countries. The problem is worsening as a result of the fact that the pathogens are developing resistance to the currently used drugs. Hence there is an urgent need to find out effective, safe and efficient drugs that can mitigate the existing problem. In view of this it is necessary to find out bioactive compounds from natural sources that are traditionally used against bacteria and other diseases. *C. paniculatum* is among significant plants in Ethiopia traditionally used for the treatment of ring worm and wounds [8]. Hence this research attempts to explore antibacterial compounds from the leaves extract of *C. paniculatum*.

Obviously, nature has had quite an effect on the science of drug discovery, and the role of the chemist has become important for work in isolation, structure determination, and synthesis of bioactive compounds. Therefore, the focus of this study is to provide information on the structures and antibacterial activities of compounds isolated and identified from leaves of *C. paniculatum*.

1.3 Significance of the Study

The study serves as a launch pad for researchers who are interested to conduct further studies in related areas. The study on the extracts of the leaves of *C. paniculatum* may help to arrive at new natural products. The extracts and compounds to be obtained from the plant may exhibit pronounceable antibacterial activities. Furthermore, the antibacterial activities to be done may be used to approve the traditional uses of this plant.

1.4 Objectives

1.4.1 General objective

To conduct the phytochemical and antibacterial evaluations on the extracts and isolated compounds of the leaves of *C. paniculatum*.

1.4.2 Specific objectives

The specific objectives of the study were to:

1. extract the leaves of *C. paniculatum* with n-hexane, ethyl acetate and methanol successively.
2. conduct phytochemical screening of the crude extracts of leaves of *C. paniculatum*.
3. isolate compounds from the crude extracts of the leaves of *C. paniculatum*.
4. characterize the isolated compounds employing melting point and spectroscopic methods including NMR and IR
5. evaluate the antibacterial activities of the extract and isolated compounds of the leaves of *C. paniculatum* using paper disc diffusion method against *Staphylococcus aureus*, *Escherichia coli, Klebsella preumanin and Proteus mirabilis*

2. LITERATURE REVIEW

2.1 The Family-Combretaceae

Combretaceae is a large family comprising about 20 genera with 600 species [11]. Members of the Combretaceae occur mainly in tropical and subtropical areas, for example, in Africa and Brazil. The family includes the lead wood tree, *Compertum imberbe.* Three genera, *Conocarpus*, *Laquncularia,* and *Lumnitzera*, grow in mangrrove habitats (mangals). Some members of this family produce useful construction timber, such as idigbo from *Terminalia ivorensis*. The commonly cultivated *Quisqualis indica* is now placed in the genus *Compertum*[6]

2.2 The Genus *Combretum*

The genus *Combretum* belongs to the family Combretaceae. It comprises about 370 species and distributed throughout the tropics and subtropics. This genus of trees, herbs, and woody climbers and shrubs is distributed in the tropics, including southern Africa, Asia and America. The genus is well known in folk medicine for its medicinal value [6].

Some *Combretum* species are extensively used in traditional medicine against inflammation, infections, diabetes, malaria, bleeding, diarrhea and digestive disorders. In southern Africa, *Combretum* is used to treat abdominal disorders, backaches, bacterial infections, bilharzia, cancer, coughing, the urinary system, colds, conjunctivitis, diarrhea, earaches, fever, gastric ulcers, general weakness, gonorrhea, headaches, heart disease, hookworm, hypertension, leprosy, nose bleeds, pneumonia, skin diseases, sore throats, toothaches, malaria and diabetes [12]. *Combretum* species are also used for treatment of ringworm and wounds in Ethiopia [8].

2.2.1 Biological Activities of the Genus *Combretum*

Several plants of the genus *Combretum* have been reported for their biological activities. Antibacterial activity of different extracts (ethanol, chloroform, methanol or water) of *C.*

micranthum was noted against *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella species, Streptococcus species, Proteus vulgaris, Klebsiella species, Sarcina lutea, Micrococcus luteus and Bacillus subtilis* [13]. *C. molle* has demonstrated antibacterial, antifungal, anthelmintic, antiasthmatic and antitussive activities [14]. Extracts of *C. erythrophyllum* obtained with different solvents (acetone, hexane, chloroform, carbon tetrachloride and butanol) have shown antibacterial activity at different doses against *Escherichia coli, P. aeruginosa, S. aureus and Enterococcus faecalis* [15]. As referenced above, there are several studies describing the bioactivities of extracts and isolated compounds from the species of the genus *Combretum*. Although many species of *Combretum* have not been extensively investigated for their chemical constituents, various classes of secondary metabolites including terpenoids, flavonoids, stilbenoids and phenanthenes have been reported [16].

2.2.2 Ethnopharmacological Uses of Genus *Combretum*

Traditionally, the roots of *Combretum erythrophyllum* (regarded as poisonous) are used as a purgative and to treat venereal diseases. The bark is mixed with other herbs to make a decoction that is drunk in the morning and evening, quarter of a cup for sores. The fruit are regarded as poisonous and reputedly cause hiccups. [5]

Combretum mossambicense Engl. is used for treatment of Gonorrhoea and syphilis. Fresh leaves are crushed and soaked in water overnight; filtrate is taken orally. *Combretum hereroense* Schinz is used for treatment of Gonorrhoea. Roots are crushed and soaked in water overnight; filtrate is drunk. *Combretum elaeagnoides* Klotzsch is used for treatment of Malaria, tuberculosis and diarrhea. Fresh leaves are crushed and soaked in water overnight; filtrate is drunk. *Combretum apiculatum* Sond is used for treatment of tuberculosis. Stem bark is crushed and soaked in water overnight; filtrate is drunk. *Combretum imberbe* Wawra is used for treatment of tuberculosis. Stem bark is crushed and soaked in water overnight; filtrate is drunk. *Combretum collinum* Fresen is used for treatment of chronic diarrhea, tuberculosis, and cough. Roots are crushed and soaked in water overnight; filtrate is drunk [5].

2.2.3 Phytochemistry of the Genus *Combertum*

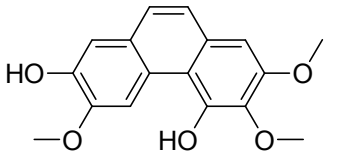
Of 370 species of the genus *Combretum*, only 31 species have been investigated for their phytochemistry. To date, at least 261 compounds, primarily terpenoids (mainly triterpenes) and phenolic compounds (flavonoids, stilbenoids, phenanthrenes) have been isolated and identified from these species [6,18].

Some compounds isolated from *C. molle,* and *C. apiculatum* were 4,7-dihydroxy-2,3,6-trimethoxyphenanthrene (**1**), 2,6-dihydroxy-3,4,7-trimethoxyphenanthrene (**2**), 4,6,7-trihydroxy-2,3-dimethoxyphenanthrene (**3**), 3,7-dihydroxy-2,4,6-trimethoxyphenanthrene (**4**), 7-hydroxy-2,4,6-trimethoxyphenanthrene (**5**), 7-hydroxy-2,3,4,6-tetramethoxyphenanthrene (**6**), 2,7-dihydroxy-3,4,6-trimethoxyphenanthrene (**7**), 2,6,7-trihydroxy-3,4-dimethoxyphenanthrene (**8**), 3,6-dihydroxy-2,4,7-trimethoxyphenanthrene (**9**), 2,6-dihydroxy-3,4,7-trimethoxy-9,10-dihydrophenanthrene (**10**), 4,7-dihydroxy-2,6-dimethoxy-9,10-dihydrophenanthrene (**11**), 2,6-dihydroxy-4,7-trimethoxy-9,10-dihydrophenanthrene (**12**), 4,7-dihydroxy-2,3,6-trimethoxy-9,10-dihydrophenanthrene (**13**), 2-hydroxy-3,4,6,7-tetramethoxy-9,10-dihydrophenanthrene (**14**), 6,7-dihydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene (**15**), 4,6,7-trihydroxy-2,3-dimethoxy-9,10-dihydrophenanthrene (**16**), 7-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene (**17**), 7-hydroxy-2,3,4,6-tetramethoxy-9,10-dihydrophenanthrene (**18**)[7].

Flavonoids such as rhamnoctrin **(19)** and quercetin-5, 3'-dimetylether (**20**) were also reported from C. *erythrophyllum* [19]

Some triterpenoids isolated from *C. laxum* were β-D-glucopyranosyl 2 α ,3 β ,24-trihydroxyolean-12-en-28-oate (**21**), Arjunolic acid (**22**), Arjunglucoside II (**23**), Bellericoside (**24**), chebuloside II (**25**), β -D-glucopyranosyl 2 α ,3 β ,*6* β -trihydroxyolean-12-en-28-oate (**26**), asiatic acid (**27**), quadranoside IV (**28**) and β -D-glucopyranosyl 2 α ,3 β ,23,24-tetrahydroxyurs-12-en-28-oate (**29**)[19]

Some structures of stilbenes and dihydrostilbene isolated from species *compertum* were (-)-combretastatin (**30**) from *C. erythrophyllum* [20], compertastin A-4 (**31**) from *C. cafrum*[18] and 3-hydroxy-2,3'-methoxybibenzyl (**32**) from *C. apiculatum* were reported [20,21]



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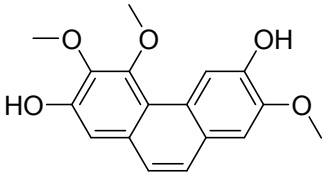

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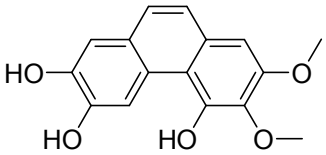

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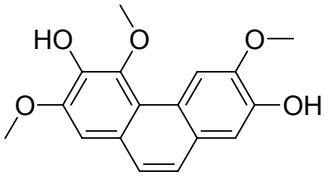

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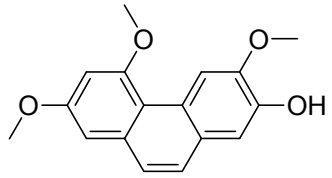

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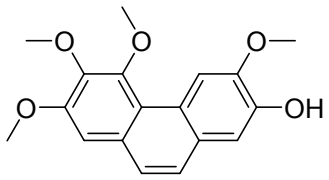

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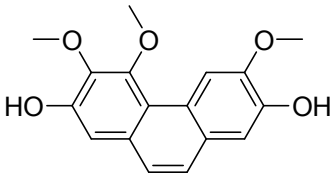

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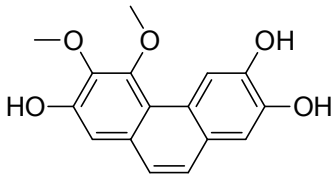

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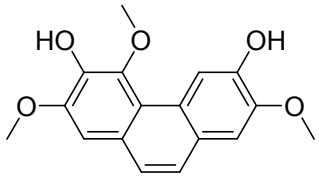

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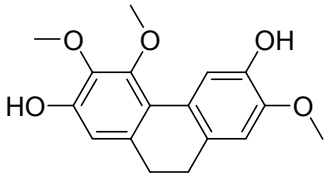

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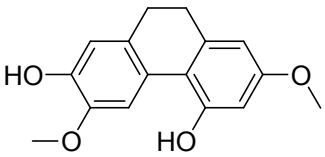

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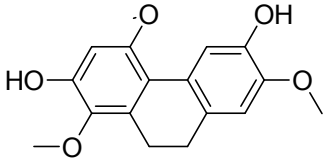

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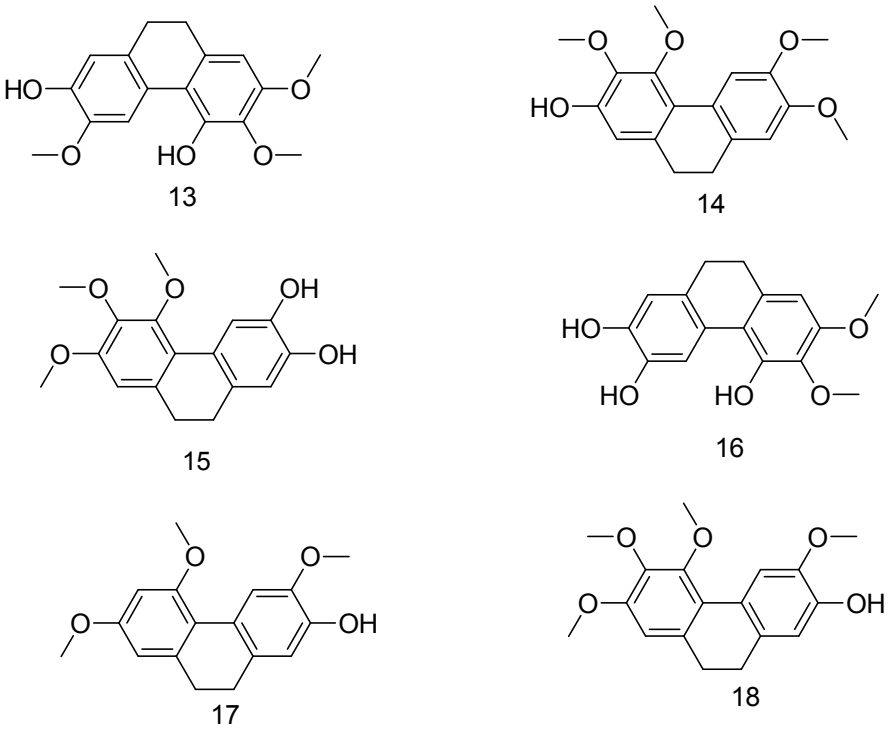


Figure 1: Some phenanthrenes and 9,10-dihydrophenanthrenes isolated from *Combretum* species

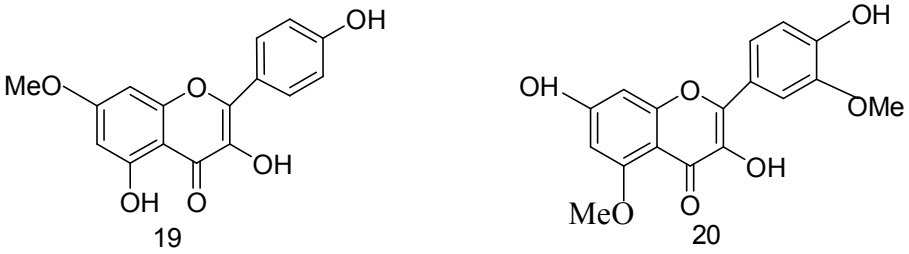
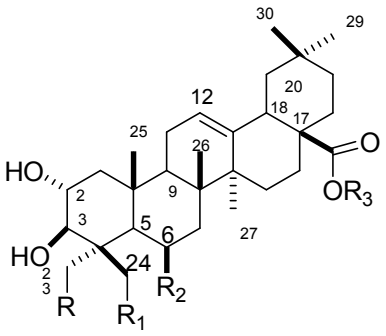
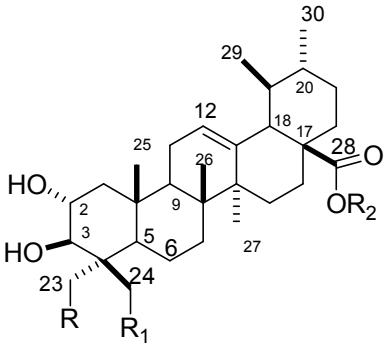


Figure 2: Structures of flavonoids isolated from *C. erythrophyllum*

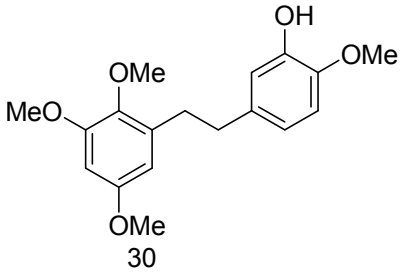


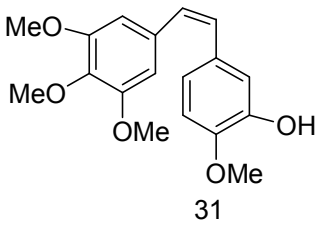
	R	R1	R2	R3
21	H	OH	H	β-D-Glc
22	OH	H	H	H
23	OH	H	H	*β-D*-Glc
24	OH	OH	H	*β -D*-Glc
25	OH	H	*OH*	*β -D-Glc*
26	H	H	OH	*β -D*-Glc



	R	R1	R2
27	OH	H	H
28	OH	H	*β-D*-Glc
29	OH	OH	*β-D*-Glc

Figure 3: Structures of the triterpenoids isolated from *C. laxum.*





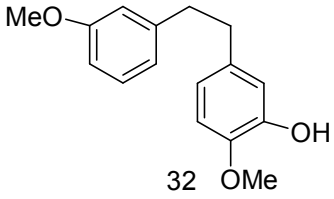


Figure 4: Structures stilbenes and dihydrostilbene isolated from species of *Compertum*

2.3 The Species *C. paniculatum*

Combretum, the genus of the family Combretaceae comprises about 370 species of trees and shrubs**.** A review study of thirty-six of *Combretum* species revealed that many of them possess biological activities and they are used in traditional medicine against infections, diabetics, bleeding, malaria, diarrhoea, inflammation, digestive disorder. [6, 13]

C. paniculatum is a scan dent shrub or robust line attaining 15 meters length. In Ethiopia it is found in different part of the country including West Oromia. It is locally called *baggee* in Afan Oromo. The plant is harvested from the wild for local use as a food, medicine and source of materials. It reduces vivid scarlet flowers during the dry season; they are very fantastic and the plant is worthy of cultivation [8]

It is known by its hazards properties. Fruits eaten by children at Ikire, South Nigeria, made them very ill and were found to have a paralyzing action on test animals, an effect analogous to that of acetylcholine. It also uses for medicinal purpose. An infusion of roots, leaves and stems is used as a garli medicine [6].

2.3 .1 Biological Activities of Species *C. paniculatum*

C. paniculatum extracts displayed good antibacterial activity and some anti-inflammatory activity in other studies. Different plant parts, namely stem bark, root bark and leaves screened for antiviral and antibacterial activity and showed good activity. Ethanoic extract of leaves of *C. paniculatum* is active agains human pathogens *E. coli, S. aureus, S. typhi and P. aurinosaI* [18].

Some compounds isolated from *C. paniculatum* showed antibacterial activity against Gram-positive and Gram-negative pathogens, as well as some antifungal activity. Cholest-5-en-3-ol, 2-phyten-1-ol, gallocatechin and apigenin were active against *Escherichia coli* (Gram-negative) and *Mycobacterium vaccae*, and against the fungi *Sporobolomyces salmonicolor* and *Penicillium notatum*. Cholest-5-en-3-ol and 2-phyten-1-ol were also active against *Bacillus subtilis* (Gram-positive) [22]

In Ethiopia, *C. paniculatum* is traditionally used for treatment of ring worms and wounds. The sap expressed from freshly ground-up flowers is also used to treat conjunctivitis and other eye-troubles. The aqueous extract of the inflorescence has shown some action against carcinomous tumours. As literature indicates, in West Wollega zone Bark latex of *C. paniculatum* is pounded and mixed with soda and creamed on affected skin [8]

2.3.2 Ethnopharmacological Uses of species *C. paniculatum*

An infusion of roots, leaves and stems is used as a garli medicine. Garli (Fula) is a condition of 'stomach staggers' affecting all cattle in Nigeria. The leaf-sap is applied externally in the treatment of gonorrhoea. An aqueous decoction of the roots is used as a treatment for diarrhoea, stomach-ache, gonorrhoea and fever. The sap expressed from freshly ground-up flowers is used to treat conjunctivitis and other eye-troubles. The aqueous extract of the inflorescence has shown some action against carcinomous tumours [6].

The dried leaves of *C. paniculatum* are also used to treat diarrhea, malaria and fevers. Leaves are pounded and soaked in water; infusion is drunk. Bark latex of *C. paniculatum* is pounded and mixed with soda and creamed on affected skin to treat wounds in Ethiopia [8].

a) Photo taken on July, 2016 *b) photo taken on February, 2017*

(Picture taken by Fekadu ASefa from Debeso, Sayo Nole, West Wollega, Oromia)

Figure 5: Aerial parts of *C. paniculatum*

2.3.3 Phytochemistry of *C. paniculatum*

Preliminary phytochemical screening of different extracts of *C. paniculatum* was reported that it contains terpenoids, tannins, saponins, alkaloids and phenols. It has been reported that leave extract of *C. paniculatum* contains apigenin **(33)**, apigenin-7-glucoside (**34**), cholest 5-en-3-ol (**35**), quercetin-3-glucopyranoside (**36**) [22].

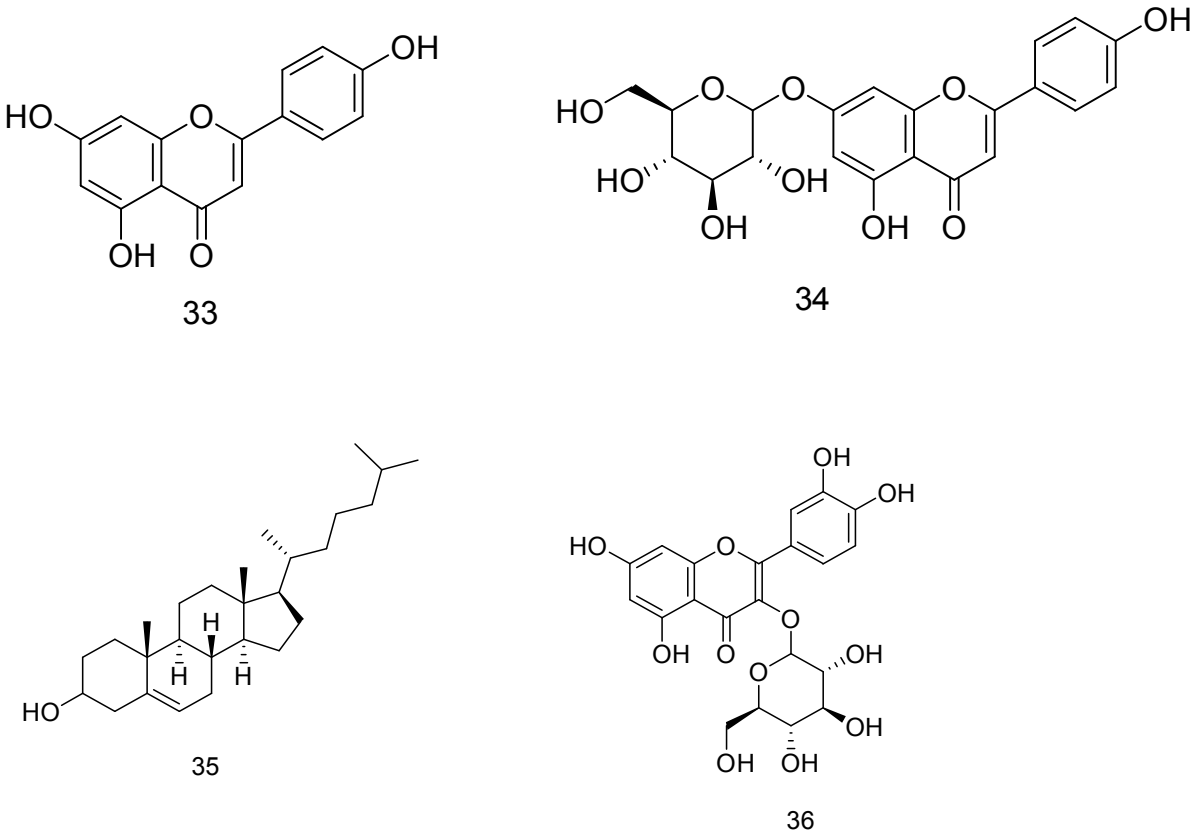


Figure 6: Structures of compounds isolated from *C. paniculatum*

3. MATERIALS AND METHODS

3.1 Chemicals

Chemicals used in the study include n-hexane (Ran chem PLC, Hind), ethyl acetate (EtOAc) (Carlo Erba, France), and methanol (MeOH) (Ranchem PLC, Hind). All these chemicals were of analytical grade and all of them were bought from Ran Chem General Trading PLC, Addis Ababa, Ethiopia.

3.2. Instruments

Analytical thin layer chromatograms were run on a readymade 0.2mm thick layer of silica gel GF254 (Merck) coated on aluminium plate. Column chromatography was performed using silica gel (230-400 mesh) Merck. ^{1}H-, ^{13}C- and DEPT-135 NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz in $CDCl_3$ and acetone. Infrared (IR) spectra were obtained on Perkin-Elmer 65FT ((IR ν_{max}KBr (4000-400) cm^{-1}) infrared spectrometer using KBr pellets.

3.3. Collection, Authentication and Preparation of Plant Material

The leaves of *C. paniculatum* were collected in February, 2017 from Debeso, Sayo Nole Woreda, WesternWollega zone, Oromia region, Ethiopia, Which is 569 km from Addis Ababa found to Western part of the country. The plant material was authenticated by Mr Shambel Alemu and voucher specimen (Voucher no- CP 001) was deposited in the National Herbarium of Addis Ababa University. The freshly collected plant leaves were washed with tap water, taken and then dried in air under shade. The dried leaves of the plant were grinded using mortar and pestle.

3.4. Extraction and Isolation of Compounds

The powdered leaves of *C. paniculatum* (300 g) were first soaked with 1500 mL n-hexane (1.5 L) for 72 hours at room temperature, filtered and concentrated to give 4.51 g of dark yellow solid material. The marc was then extracted with ethyl acetate (1.5 L) after soaking for 72 hours, filtered and concentrated to furnish 8.86 g of dark green solid substance. The marc left after EtOAc extraction was finally extracted with methanol (1.5 L) for 3 days at room temperature, filtered and concentrated under reduced pressure to furnish 21.5 g of black solid material. The TLC of ach extract were analyzed and depicted in Fig 7. The ethyl acetate and methanol extract were showed promising spot. Ethyl acetate extract (8 g) was adsorbed on equal amount of silica gel and fractionated over silica gel (150 g) column chromatography using n-hexane for column packing. The column was eluted with n-hexane:EtOAc:MeOH of increasing polarities to give 41 fractions each 100 mL (Table 1)

Table 1: Silica gel column chromatographic fractionation of ethyl acetate crude extract

Fractions	Eluent	Ratio	Volume collected (mL)	Yield (mg)
1	n-Hexane	100%	100	-
2	n-Hexane:EtOAc	9:1	100	-
3	n-Hexane:EtOAc	4:1	100	-
4	n-Hexane:EtOAc	7:3	100	123.8
5-9	n-Hexane:EtOAc	1:1	500	125
10-12	n-Hexane:EtOAc	2:3	300	-
13-14	n-Hexane:EtOAc	3:7	200	-
15-19	n-Hexane:EtOAc	1:4	500	174.4
20	n-Hexane:EtOAc	1:9	100	-
21-22	EtOAc	100%	200	360.6
23	EtOAc:MeOH	9:1	100	-
24-25	EtOAc:MeOH	4:1	200	-
26-28	EtOAc:MeOH	7:3	300	-
29-30	EtOAc:MeOH	3:2	200	236
31	EtOAc:MeOH	1:1	100	105.4
33-34	EtOAc:MeOH	2:3	200	-
35-36	EtOAc:MeOH	3:7	200	-
37-38	EtOAc:MeOH	1:4	200	103
39	EtOAc:MeOH	1:9	100	-
40-41	MeOH	100%	200	-

Fractions 1-3 showed no visible spots on TLC. Fractions showing similar TLC profile were merged together (fractions 5-9, 10-12, 13-14, 15-19, 21-22, 24-25, 29-30, 33-34, 35-36, 37-38 and 40-41).

Fraction 4 (123.8 mg) showed about 4 spots having different R_f values on TLC which was developed using n-hexane:EtOAc (7:3) as mobile phase. The spots were visualized after dipping in iodine vapour. It was then rechromatographed over silica gel column chromatography using n-hexane:EtOAc:MeOH as an eluent to furnish 31 fractions each 10mL(Table 2). Fraction 20 (8 mg), 21 (6 mg), 22 (7 mg), 23 (4mg), 25 (8 mg) and 26 (8 mg) showed a single spot with different Rf value on TLC developed in solvent system n-hexane:EtOAc (9:1). Fractions 25 and 26 were labelled as FCP-01and FCP-02, respectively.

Table 2: Silica gel column chromatographic fractionation of fraction 4

Fractions	Eluent	Ratio	Volume collected (mL)	Yield (mg)
1-3	Hexane	100%	30	-
4-6	hexane:EtOAc	9:1	30	-
7-15	hexane:EtOAc	4:1	90	-
16-19	hexane:EtOAc	7:3	40	-
20	hexane:EtOAc	3:2	10	8
21	hexane:EtOAc	1:1	10	6
22	hexane:EtOAc	2:3	90	7
23	hexane:EtOAc	3:7	10	4
24	hexane:EtOAc	1:4	10	-
25	hexane:EtOAc	1:9	10	8
26	EtOAc	100%	10	8
27	EtOAc:MeOH	9:1	10	
28	EtOAc:MeOH	4:1	10	-
29	EtOAc:MeOH	7:3	10	-
30	EtOAc:MeOH	1:1	10	-
31	EtOAc:MeOH	100%	10	-

Fraction 21-22 (360.6 mg) of the ethyl acetate extract, showed four coloured spot on TLC using EtOAc:MeOH (9:1) as a mobile phase, was rechromatographed over silica gel column chromatography with n-hexane:ethyl acetate:MeOH of increasing polarities as an eluent to give 10 fractions. Fraction 4 (35.5 mg) was found to have one spot on TLC which labelled as FCP 03 and hence analyzed with NMR.

Table 3: Silica gel column chromatographic fractionation of fractions 21-22.

Fractions	Eluent	Ratio	Volume collected (mL)	Yield (mg)
1	n-hexane	100%	10	-
2	n-hexane:EtOAc	1:1	10	-
3	EtOAc	100%	10	-
4	EtOAc:MeOH	9:1	10	35.5
5-6	EtOAc:MeOH	4:1	20	33
7	EtOAc:MeOH	7:3	10	-
8	EtOAc:MeOH	3:2	10	18
9	EtOAc:MeOH	1:1	10	-
10	MeOH	100%	10	-

3.5 Phytochemical Screening of Crude Extracts

The preliminary phytochemical tests were performed for testing different chemical groups present in the three successive extract of the leaves of *C. paniculatum.* Determination of the presence of tannins, saponins, alkaloids, terpenoids, flavonoids, anthraquinones and phenols was done qualitatively using protocols reported in the literature [23]

3.5.1 Tannins

Ferric chloride test: 0.5 g of each extract was dissolved in 10 mL of distilled water, then a few drops of 10% ferric chloride solution was added and a brownish green was observed [24].

3.5.2 Saponins

Froth test: 0.5 g of the extracts were dissolved in 5 ml distilled water. The mixture was shaken vigorously and stable persistent froth was obtained [25].

3.5.3 Alkaloids

Wagner's Reagent: Potassium iodide (2 g) and iodine (1.27 g) were dissolved in distilled water (5 mL) and the solution was diluted to 100 mL with distilled water. Few drops of this solution were added to the extracts and reddish colored precipitate was observed [26]

3.5.4 Terpenoids

Salkowski test: The crude extracts (about 0.5 mg) were shaken with chloroform (2 mL) in the test tube followed by the addition of concentrated sulfuric acid (3 mL) along the side of the test tube using dropper, and a reddish brown coloration of the interface was observed [27]

3.5.5 Flavonoids

Sodium hydroxide test: About 1 g of the extracts were weighed in the first beaker, completely detained with acetone. The residue extracted in warm water after evaporating the acetone in water bath. Mixture was filtered while still hot in the second beaker. Filtrate was cooled and then 5mL 20% of sodium hydroxide was added to equal volume of the detained water extract, to obtain yellow solution [28].

Alternatively, 5 mL dilute ammonia was added to 5 mL ethyl acetate extract and then 5 mL concentrated sulfuric acid was added, to obtain yellow colour [28].

3.5.6 Anthraquinones

Bontrager's test: About 0.5 g of the extracts were taken in to the first test tube and added 5 ml chloroform and shaken for 5 min. The extract was filtered to the second test tube and shaken with an equal volume of 100% ammonia solution, to obtain a pink violet or red colour in the ammoniacal layer (lower layer) [29]

3.5.7 Phenols

1 mL of each extracts was dissolved in 2 mL of distilled water and added few drops of 10% $FeCl_3$, to obtain blue or green colour [26]

3.6. Evaluating Antibacterial Activities

The anti-bacterial activity of the three extracts of the leaves of *C. paniculatum* was done *in vitro* using the American Test Culture Collection (ATCC) bacterial strains. Four bacterial strains were obtained from Oromia Public Health Research, Capacity Building and Quality Assurance Laboratory Center, Adama, Ethiopia. Three Gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Klebsella preumanin* ATCC 35032, *Proteus mirabilis* ATCC 25923) and one Gram–positive (*Staphylococcus aureus* ATCC 25923) human bacterial pathogens were selected for the study. The antibacterial activities were determined using well diffusion method against different strains of bacteria [27]. Ciprofloxacin and chloroform were used as a positive and negative control, respectively.

3.6.1 Inoculation and Incubation

Antibacterial activity was done on Mueller Hinton agar. 1 mL of bacteria suspension was uniformly spread on the sterile Mueller Hinton Agar Petri dish. Standard solutions of 1.5 mg/mL concentration of the extracts and isolated compounds were prepared and 10 µL solutions from the concentration were loaded to the discs in different replications. 6 mm-diameter wells were cut from the agar using a sterile cork-borer and the sample were placed in the wells. The Petri dish was then placed in an incubator for 24 hours at 37^0C. At the end of incubation period, the inhibition diameter was measured and expressed in millimeters. 3 drops of each bacterial suspension were applied on the Petri dish to compare with 1 drop of chloroform applied on it. Ciprofloxacin antibiotic standards were used as a positive control group reference drug. Chloroform was used as a negative control group. Antibacterial activity was determined by measuring the inhibition zone diameter (mm) against each test organism.

4. RESULTS AND DISCUSSION

4.1 Extraction Yield

The leaves of *C. paniculatum* were successively extracted with n-hexane, EtOAc, and MeOH to give 4.51 g (1.5%), 8.86 g (2.9%) and 21.5 g (7.2%), respectively. The amount extracted using MeOH was much higher than the EtOAc and hexane extract. This indicates that the secondary metabolites present in the plant material are mainly polar.

4.2. TLC Profile of the Extracts

The hexane, EtOAc and MeOH extracts were analyzed with TLC and the results were depicted in Figure 7. The mobile phases used for n-hexane, EtOAc and MeOH extracts were n-hexane: ethyl acetate (1:1), n- hexane: ethyl acetate (1:1), and ethyl acetate: methanol (1:1), respectively. The spots in each case were visualized with after dipping in iodine vapour.

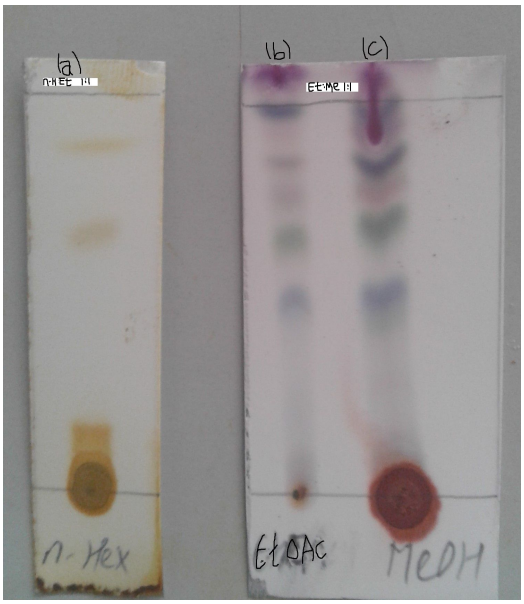


Figure 7: TLC profile of n-hexane extract, Ethyl acetate extract, Methanol extract

4.3. TLCAnalysis of the Fraction 4

Fraction 4(123.8 mg) of the ethyl acetate extract showed about 4 spots and analyzed with TLC and the results were depicted in Figure 8. The mobile phase used was n-hexane: ethyl acetate (7:3). TLC was visualized after dipping in vanillin-H_2SO_4 followed by heating on heating mantle.

Figure 8 TLC profile of fraction 4

4.4. TLC Analysis of the Fractions of Fraction 4

Fractions of the fraction 4 above were analyzed with TLC and the results were depicted in Figure 9. The mobile phase used was n-hexane: ethyl acetate (9:1). TLC was visualized after dipping in vanillin-H_2SO_4 followed by heating on heating mantle.

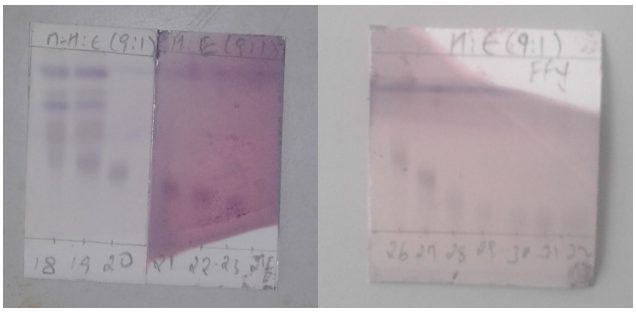


Figure 9 : TLC Profile of the fractions of fraction 4

4.5. TLC Profile of the Fractions 21-22

Fraction 21-22(123.8 mg) showed about 4 spots and analyzed with TLC and the results were depicted in Figure 10. The mobile phase used was n-hexane: ethyl acetate (7:3). TLC was visualized after dipping in vanillin-H_2SO_4 followed by heating on heating mantle.

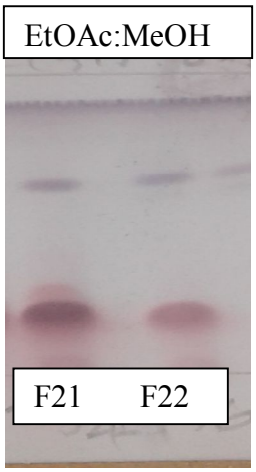


Figure 10 : TLC profile of the fractions 21-22

4.6. TLCAnalysis of the Fractions of Fraction 21-22

The TLC of fractions of fractions 21-22 were analyzed and the results were depicted in Figure 11. Fraction 4 of fraction 21-22 showed a single spot using EtOAc:MeOH(9:1) as solvent system. TLC was visualized after dipping in vanillin-H_2SO_4 followed by heating on heating mantle.

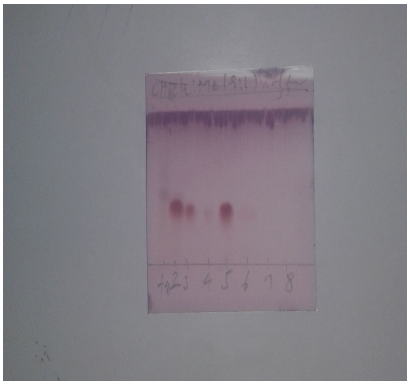


Figure 11: TLCprofile of the fractions of fraction 21-22

4.7 Phytochemical Screening of Crude Extracts

Phytochemical screening of n-hexane, ethyl acetate and methanol extracts of the leaves of *C. paniculatum* revealed the presence of chemical components shown in Table 4. Secondary metabolites such as saponins, flavonoids, alkaloid, steroids, tannins, anthraquinones and phenols were detected in the extracts of the leaves of *C. paniculatum*. The present study revealed that n-hexane leaves extract of *C. paniculatum* contains terpenoids, whereas alkaloids, tannins, saponins, anthraquinones and phenols were not detected. Ethyl acetate extract was found containing alkaloids, tannins, phenols, flavonoids, terpenoids and anthraquinones. Conversely, it was found that ethyl acetate extract does not contain saponins. On the other hand, the methanol leaves extract of *C. paniculatum* contains alkaloids, terpenoids, tannins, anthraquinones, saponins and phenols (Table 4). The medicinal value of plants lies in some chemical substances or secondary products that have definite physiological functions in the human body and which may help in protection against various diseases.

Table 4: Phytochemical analysis of n-hexane, ethyl acetate and methanol extracts of *C. paniculatum*

S. No.	Phytochemical	Tests/Reagents	n-hexane Extract	EtOAc Extract	MeOH extract
1	Saponins	Froth test	-	-	-
2	Flavonoids	NaOH Test	-	+	+
3	Phenols	10% $FeCl_3$	-	+	+
4	Tannins	Ferric chloride test	-	-	+
5	Alkaloid	Wagner's test	-	+	+
6	Anthraquinones	Bontrager's test	-	+	+
7	Terpenoids	Salkowski Test	+	+	+

(+ means present; - means absent)

The use of some plants for medicinal purpose is due to the presence of secondary metabolites [28], which supports the use of *C. paniculatum* for the treatment of several diseases by local herbalists or traditional healers.

Literature reported the biological properties of secondary metabolite. Among which phenolic compounds possesses anticarcinogenic, anti-oxidant, anti-malarial, anti-microbial activities. They have also been investigated as drug. For instance, crofelemer is a drug for treatment of diarrhea associated with anti- HIV drugs and combretastin A-4 compound (**31**) (Figure 4), is an anti cancer molecule, which have a potent target in cancer chemotherapy in cell division. Thus, the presence of phenolics in the leaves extract of *C. paniculatum* supports the traditional uses of this plant.

4.8 Structural Elucidation of Isolated Compounds

In the course the present study, two compounds were isolated and characterized. Herein are the characterizations of these compounds.

4.8.1 Characterization of FCP 01

FCP 01 was obtained as white solid (8 mg) with melting point of 53-54°C. Similar value was reported in the literature for myristic acid. TLC profile showed R_f. 0.53 with n-hexane:ethyl acetate (9:1) solvent system as a mobile phase. After re crystallization a single spot was observed on TLC (Figure 12).

Figure 12: TLC profile of FCP 01

In the IR (KBr) spectrum of FCP 01 (Appendix 1), the absorption band at 3430 cm^{-1} showed the O-H stretching of carboxyl group. The strong absorption band at 2915 cm^{-1} showed the presence

of the C-H stretching of alkyl groups. The absorption band at 1730 cm^{-1} showed the presence of the C=O stretching of carboxyl. The absorption band at 1381 cm^{-1} showed C-O stretching carboxylic acids.

The ^{1}H-NMR spectrum (Appendix 2) showed the characteristic of aliphatic carboxylic acid at region between δ 0.90 to 2.40 ppm. The triplet signal at δ 2.36 integrating for two protons is accounted to methylene protons adjacent to a carbonyl. The multiplet signal observed at δ 1.69 is ascribed to methylene protons on carbon flanked between two methylene carbons. Another multiplet signal was also observed at δ 1.65. The intense broad singlet signal δ 1.27 integrating for eighteen protons were evident for protons on many overlapping methylene carbons. The presence of terminal methyl group is evident at δ 0.9.

The proton decoupled ^{13}C-NMR spectrum (Appendix 3) with the aid of DEPT-135 revealed the presence of eleven well resolved carbon resonances. The signal at δ 177.9 (C-1) is accounted to a carbonyl group. The terminal methyl group is evident at δ 14.1 (C-14). An intense signal at δ 29.6 is characteristics of the presence of many overlapping methylene group. The remaining carbon signals were observed at δ 33.6, 31.9, 24.7 and 22.6. The data generated was found in agreement with myristic acid (Table 5).

Table 5: ^{1}H-NMR (400MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data of FCP 01

Carbon position	^{1}H-NMR δ (ppm) (*J*-Hz)	^{13}C-NMR data δ ppm)	DEPT-135 data δ (ppm)	Literature[30] δ (ppm)
1	-	177.9	C=O	179.2
2	2.36(2H, *t*, *J* = 7.6)	33.6	CH_2	34
3	1.69 (2H, *m*)	24.7	CH_2	22.7
4-11	1.27 (18H, *m*)	29.06-29.69	CH_2	29.0-29.7
12		31.9	CH_2	31.9
13	1.65(2H, *m*)	22.6	CH_2	22.7
14	0.9 (3H, *t*, *J*= 6.8)	14.1	CH_3	14.1

The NMR spectral data of FCP 01 was compared and found in agreement with the literature reported for myristic acid (Table 5) [30], whose structure is shown in (Figure 13).

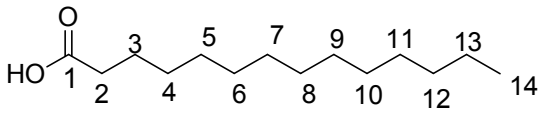


Figure 13: Proposed structure of FCP 01

4.8.2 Characterization of FCP 03

FCP 03 (35.5 mg) was obtained as solid crystal with golden color and a melting of 183ºC-184 ºC. Its TLC showed one spot at R_f 0.54 with ethyl acetate:methanol (9:1) as a mobile phase. FCP 03 was soluble in ethyl acetate that showed a characteristic colour change from colourless to dark red on TLC plate upon dipping in vanillin-H_2SO_4 and heating for a few minutes.

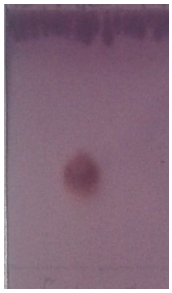


Figure 14: The TLC Profile of FCP 03

The IR (KBr) spectrum of the FCP 03 (Appendix 5) showed broad absorption band due the O-H stretching at 3430 cm^{-1}. The strong absorption band at 2925 cm^{-1} showed the presence of the C-H stretching. The absorption band at 1633 cm^{-1} showed the presence of the C=C stretching. The absorption band at 1381 cm^{-1} showed the presence of C-C stretching.

The ^{1}H-NMR spectrum (Appendix 6) showed the presence of nine proton signals of which seven are on the aromatic ring and the remaining two are on the oxygenated aliphatic carbons. The peak at δ 7.32 (2H, H-2'', *d*, J = 8.8 Hz) and δ 6.82 (2H, H-3'', *d,* J = 8.8 Hz) are due to symmetrically placed protons on unsymmetrically *p*-substituted aromatic ring. The other signal observed in the aromatic region at δ 6.12 (2H, *d,* J = 2) is accounted to the proton on C-3',5'. The triplet signal at δ 6.08 (1H, *t*,) is due to aromatic proton on C-4'. The peaks at δ 5.37 and 3.61 with J = 1.6 Hz each are due to protons on methine carbons.

The proton decoupled ^{13}C-NMR spectrum (Appendix 7, Table 6) with the aid of DEPT-135 showed the presence of ten well resolved carbon resonances of which four are due to quaternary

carbons. Among these, the signals observed δ 107.7 (C-3',5'), 114.9 (C-3'',5''), 127.7 (C-2''6'') and 157.7 (C-2',6') indicating the number of carbons in the structure of the compound to be fourteen. The signals observed at δ 157.7 and 156.7 are due to an oxygenated aromatic carbons. On the other hand the signals characteristics of oxygenated aliphatic carbons were observed at δ 58.0 and 84.5. Also observed signals are at δ 100.7 (C-4'), 133.2 (C-1''), 141.2 (C-1'), 84.5 (C-2) and 58.0 (C-1).

Table 6: ^{1}H-NMR (400MHz, Acetone-d6) and ^{13}C-NMR (100 MHz, Acetone-d6) spectral data of FCP 03

Carbon position	^{1}H-NMR δ (ppm) and (J in HZ)	^{13}C-NMR data δ (ppm)	Multiplicity δ(ppm)
1''	-	133.2	Q
2",6"	7.32 (2H, *d*, J = 8.8)	127.7	CH
3'',5"	6.82 (2H, *d*, J = 8.8)	114.93	CH
4"	-	156.7	Q
1	3.61 (1H, *d,* J = 1.6)	58.0	CH
2	5.37 (1H, *d,* J = 1.6)	84.5	CH
1'	-	141.2	Q
2',6'	-	157.8	Q
3',5'	6.12 (2H, *d*, J = 2)	107.9	CH
4'	6.08 (1H, *t*)	100.7	CH

The data generated showed that FCP 03 is in Table 6, whose structure is as shown in Figure 11. To the best of my knowledge there is no prior report in the literature.

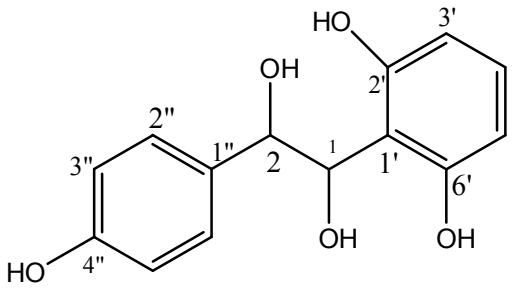
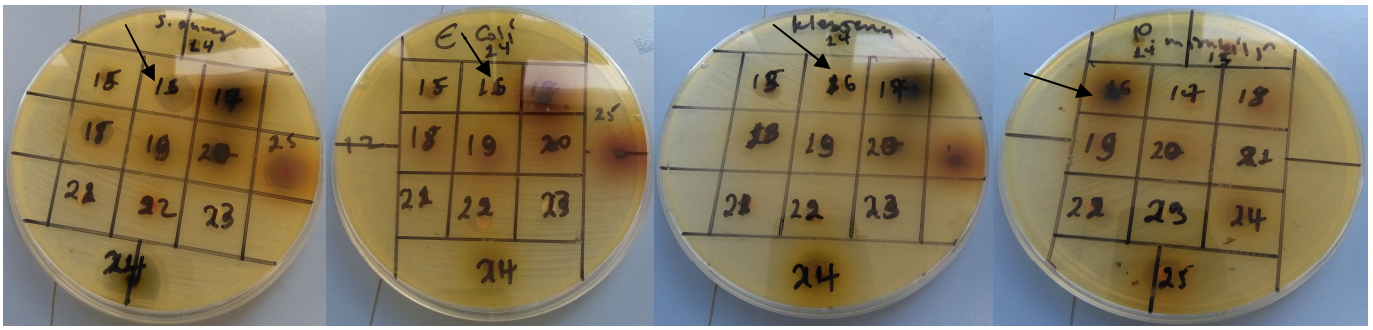


Figure 15: Proposed structure of FCP 03

4.5. Antibacterial activities

The aim of performing antibacterial activity was to identify the zone of inhibition at which the three successive extracts of *C. paniculatum* leaves and FCP 03 were active against the selected bacterial strains. The inhibitory effects of *C. paniculatum* extract against *S. aureus*, *E. coli*, *K. pneumonin* and *P. mirabilis* were determined with the results shown in Figure 12 and Table 7.



a) *S. aureus* b) *E. coli* c) *K. pneumonin* d) *P. mirabilis*

Figure 16: Antibacterial activities of ethyl acetate, methanol extracts and FCP 03.

The n-hexane and EtOAc extract were inactive against *K. preumanin* and *C. paniculatum* while the methanol extract was inactive against *E.coli.* The extracts demonstrated significant difference in antibacterial activity against *S. aureus* and other bacterial strains with zone of inhibition ranging from 6-12 mm. FCP 03 demonstrated zone of inhibition of 19 mm against *S. aureus* which was turned out to be comparable with ciprofloxacin which inhibit the pathogens by 23 mm.

Table 7: Inhibition zone diameter of the three successive plant extract, FCP 03, antibiotics and chloroform

Extract/control	Zone of inhibition (mm)			
	S. aureus	*E. coli*	*K. preumanin*	*P. mirabilis*
n-hexane extract	8	10	-	9
Ethyl acetate extract	15	12	-	14
Methanol extract	7	-	10	8
FCP 03	19	12	11	10
Ciprofloxacin (positive control)	23	21	19	24
Chloroform (negative control)	0	0	0	0

(-) no zone of inhibition

According to Abera B. [12] *C. paniculatum* is traditionally used to treat wound caused by bacteria. Hence, based on the relationship between traditional use and bioactivity test result of *C. paniculatum*, one can decided that leaves extracts of this plant may be recommended as a remedy against bacteria causing wound.

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The dried powdered leaves of *C. paniculatum* were successively extracted with n-hexane, ethyl acetate and methanol which gave 4.51 g, 8.86 g and 21.5 g, respectively. The TLC analysis of each extracts showed the presence of several compounds as observed under UV lamp (265nm). Phytochemical screening tests has shown that *C. paniculatum* leaves extract contain terpenoids, tannins, saponins, alkaloids and phenols. The ethyl acetate extract was subjected to column chromatography and yielded two compounds namely myristic acid and 1-(2',6'-dihyroxphenyl-2-(4"-hdroxyphenyl))-1,2-ethane-diol. The latter compound was isolated for the first time. The antibacterial activity of the ethyl acetate extract showed moderate activity against *S. aureus* compared with the standard drug. *However,* 1-(2',6'-dihyroxphenyl-2-(4"-hdroxyphenyl))-1,2-ethane-diol significantly inhibit *S. aureus* with inhibition zone of 19 mm. Therefore the antibacterial activities displayed by the leaves of *C. paniculatum* substantiate the traditional use of this plant against bacteria.

5.2 Recommendations

i. Although, the compounds isolated are not the only compounds present in the plant material as evidenced from TLC analysis, future work is needed to isolate other compounds not isolated in this study and determine their antimicrobial activity. The structure of FCP 03 was conducted by IR, ^{1}H- NMR, ^{13}C-NMR and DEPT-135. It is recommended to carry out further works by using UV, 2D-NMR, HPLC-MS, MS for the extracts of the plant leaves.

ii. Methanol extract from *C. paniculatum* leaves showed biological activity against *S. aureus, K. preumanin* and *P. mirabilis* and it is recommended to isolate compounds of this extract using HPLC.

iii. It is also recommended that further *in vivo* anti-bacterial efficiency tests with the bacteria used in mice or other animals so as to validate the *in vitro* results. Future research should be extended to cover other biological activities including antifungal, anti-cancer, antiviral, antimalarial, and antioxidant activity.

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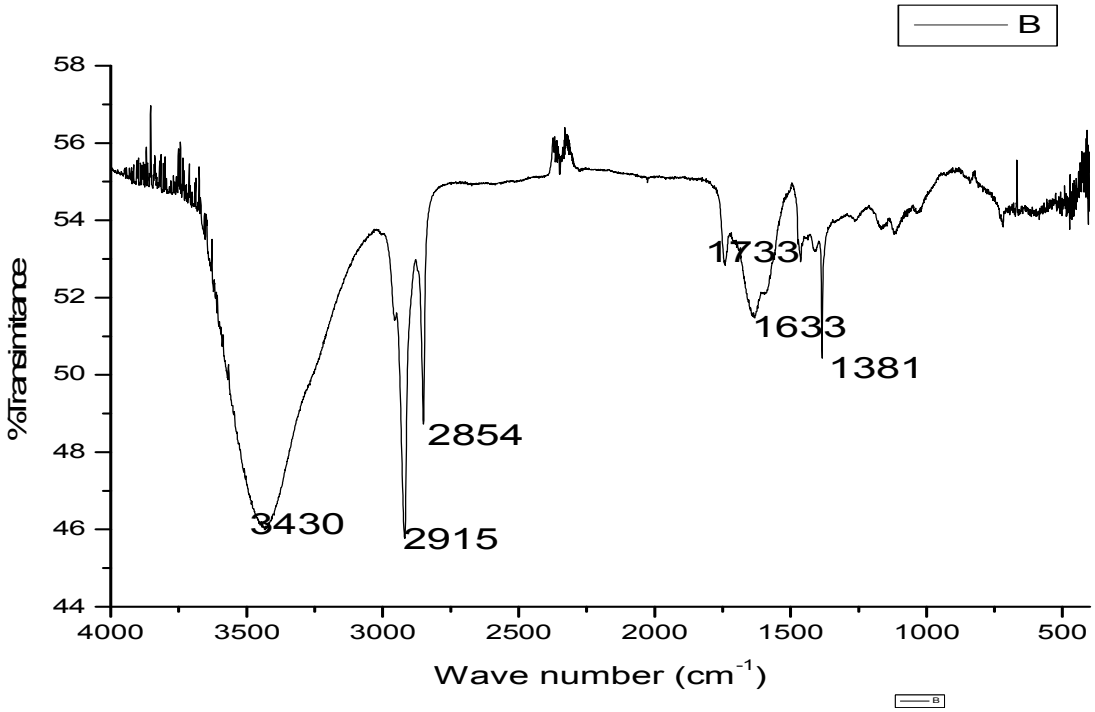
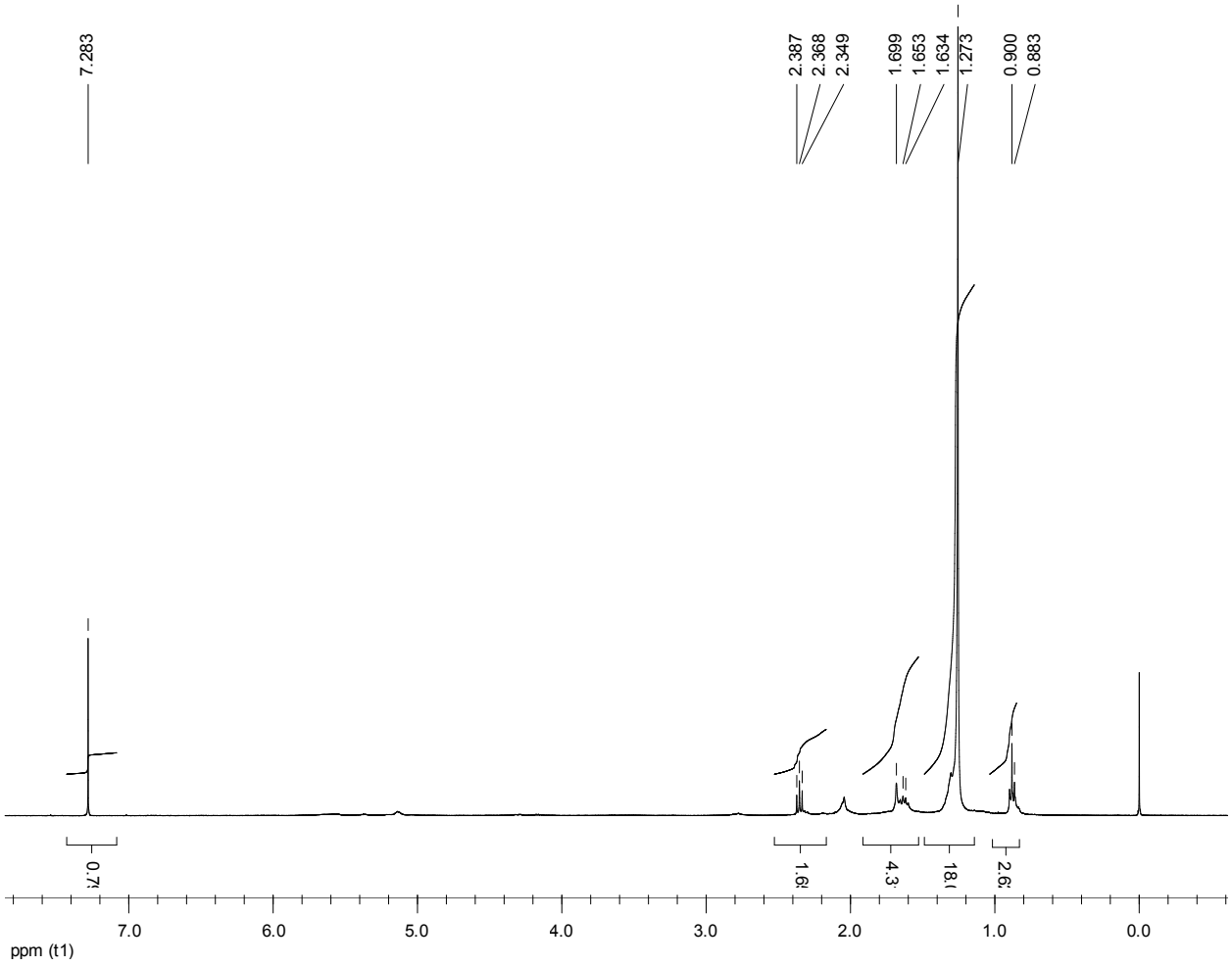
APPENDIX

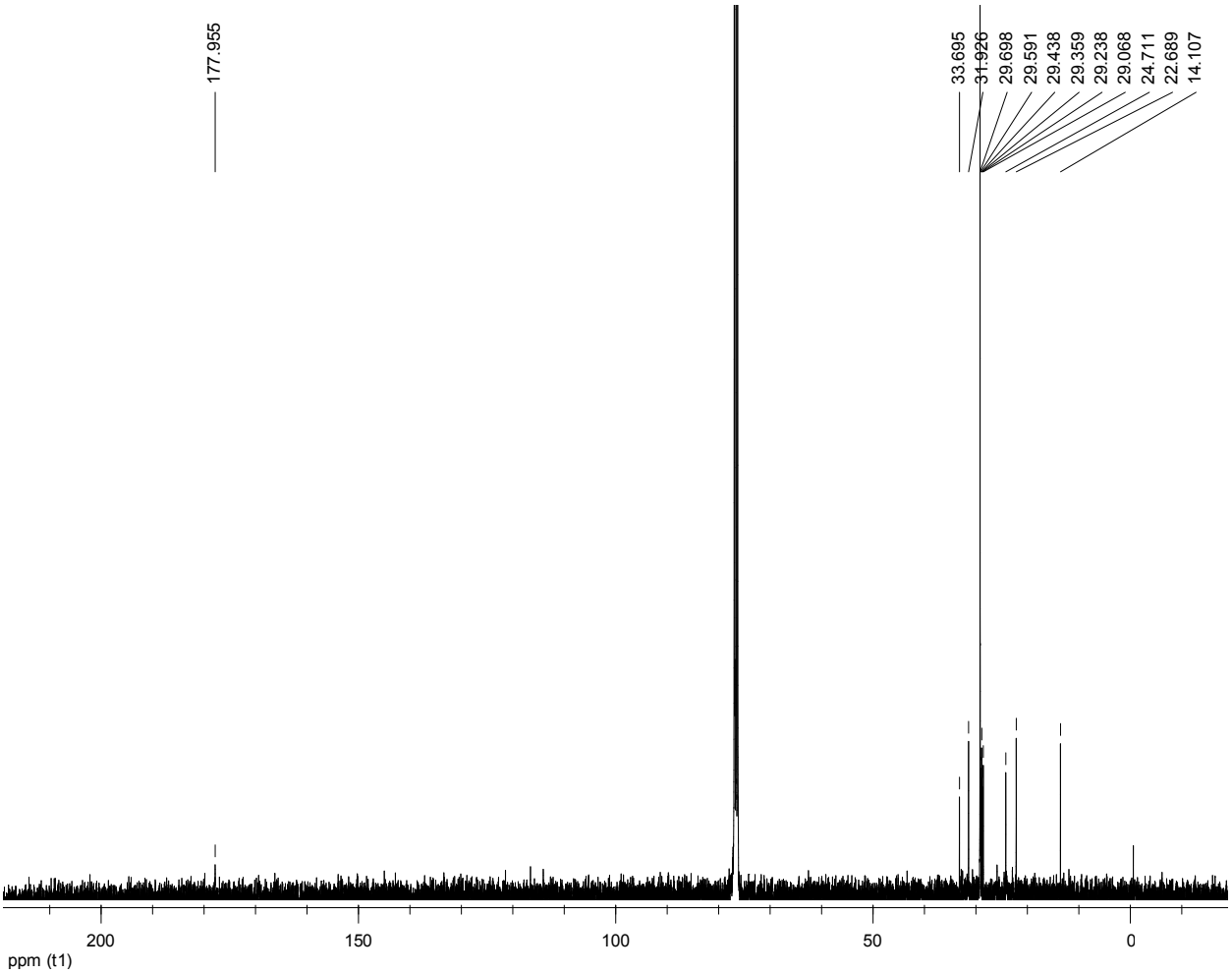
Appendix 1: IR spectrum of FCP 01



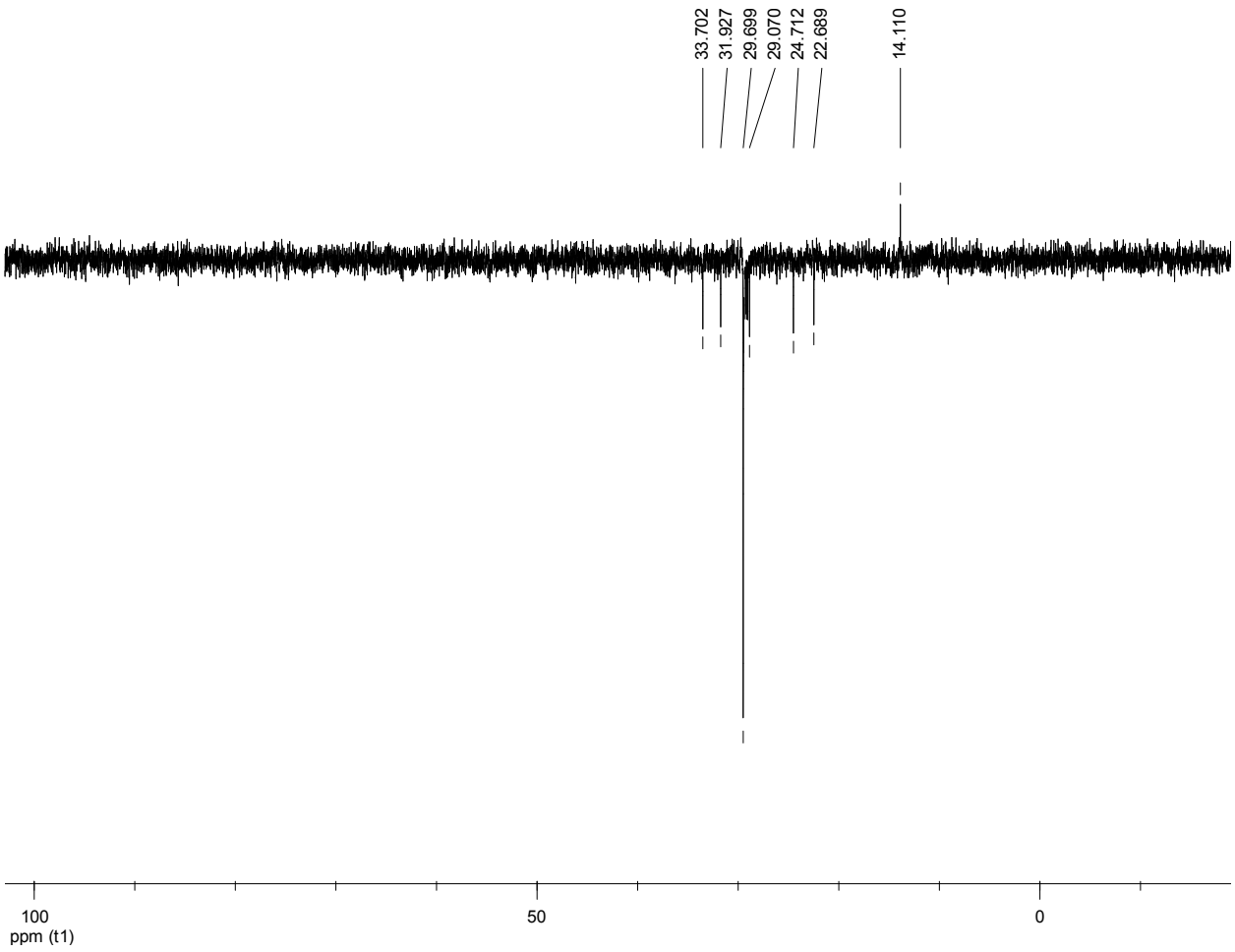
Appendix 2: ^{1}H-NMR spectrum of FCP 01



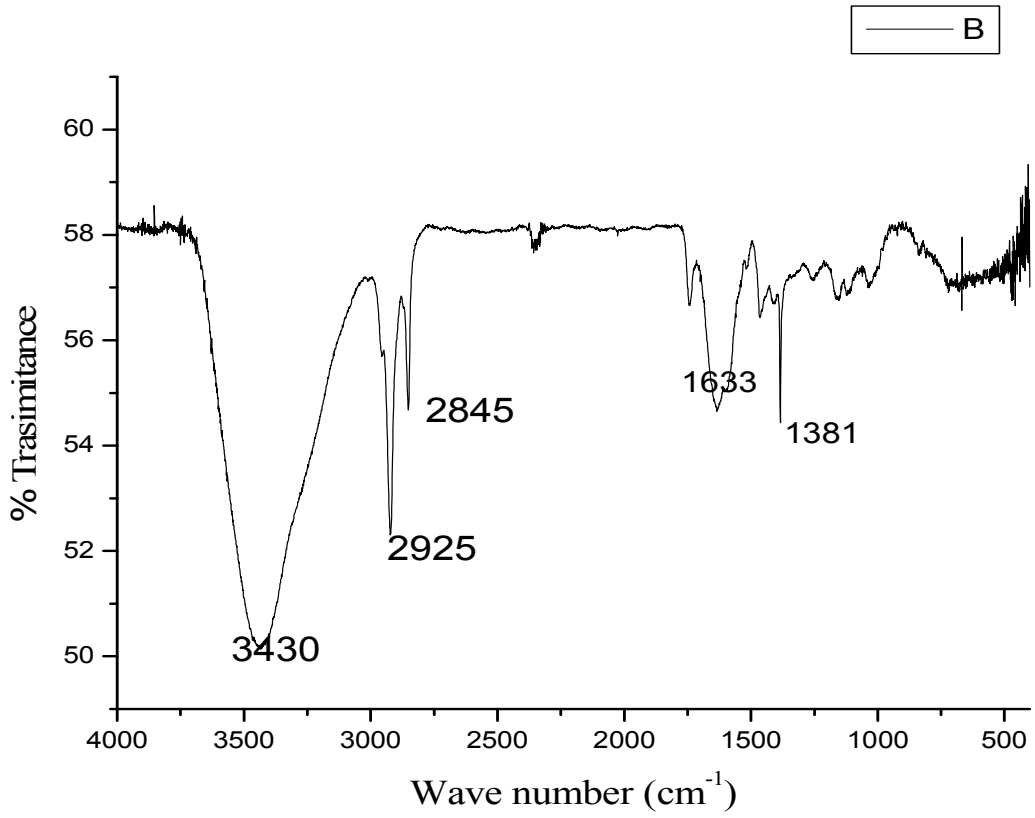
Appendix 3: ^{13}C- NMR spectrum of FCP 01



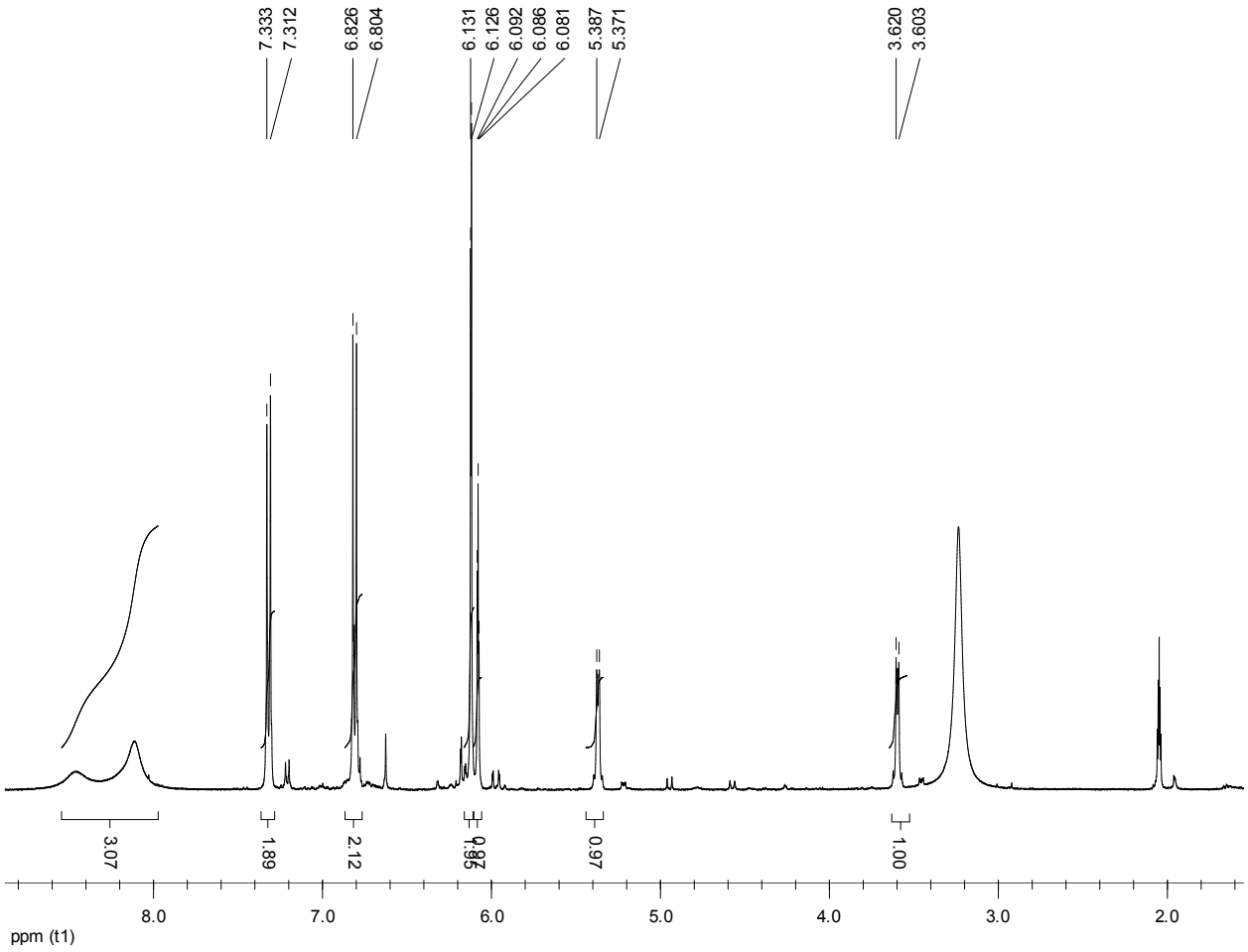
Appendix 4: DEPT-135 of FCP 01



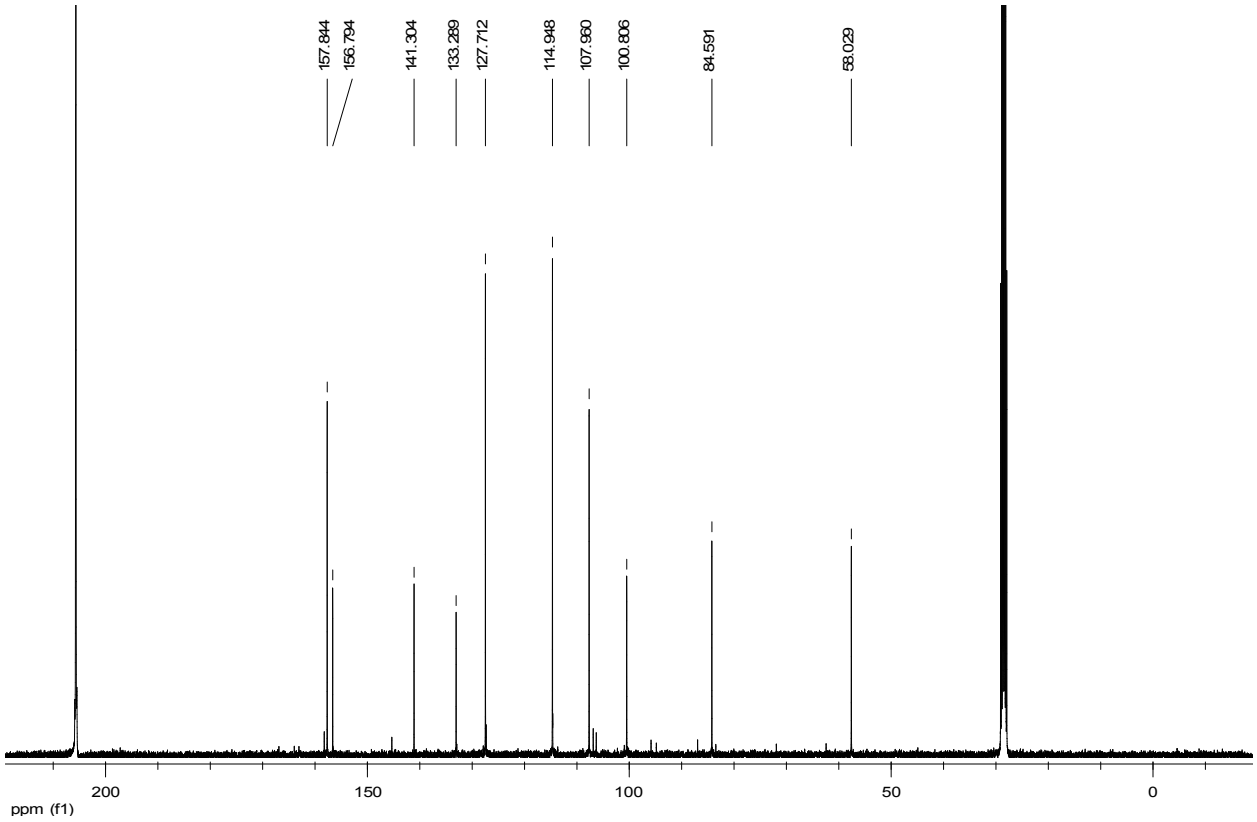
Appendix 5: IR spectrum of FCP 01



Appendix 6: ^{1}H -NMR spectrum of FCP 03



Appendix 7: ^{13}C -NMR spectrum of FCP 03



Appendix 8: Dept-135 spectrum of FCP 03