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ON

PHYTOCHEMICAL INVESTIGATION AND ANTI-MICROBIAL ACTIVITY OF *Combretum paniculatum* STEM BARK

BY

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Abbreviations and Acronyms

TLC	Thin Layer Chromatography
DMSO	Dimethyl Sulfoxide
NMR	Nuclear Magnetic Resonance
DEPT	Distortion less Enhancement by Polarization Transfer
Uv Vis	Ultraviolet Visible
HIV	Human Immunodeficiency Virus
HIV-2	Human Immunodeficiency Virus-2
MIC	Minimum Inhibition Concentration
Rf	Retention factor

Abstract

Traditional medicine plays a significant role in the health care system. *C. paniculatum* (in afan oromo "*Baggii*") is one of the herbal medicines commonly practiced by traditional healers for the treatment of various infectious diseases. The main objective of this study was to carry out on phytochemical investigation of *C. paniculatum* stem barks and evaluation of its antimicrobial activities. The air dried plant sample was sequentially extracted with petroleum ether, chloroform, acetone and methanol. The crude extracts were tested for their antimicrobial activity against four bacterial strains (*E. coli, B. subtilis, S. aureus* and *P. aeruginosa*) and two fungal strains (*Fusarium spp*, and *S. cerevisiae*) using disc diffusion method. The extracts were observed for their number of phytochemical components by using TLC analysis and the chloroform extract was subjected to column chromatography packed with silica gel. The column was eluted with petroleum ether, with increasing gradient of ethyl acetate. The isolated compounds were characterized on the bases of NMR spectroscopic data and comparison with literature. Extraction yield of crude extracts resulted with 0.45%, 0.5%, 1.1% and 2.6% for petroleum ether, chloroform, acetone and methanol respectively. Among 125, fractions 25-41 and 64-97 provide two compounds, compound **1** (8.4 mg) and compound **2** (6.8 mg). The two isolated compounds were characterized and deduced to be cholest-5-en-3-ol and dihexyldecyl succinate respectively. In previous study compound **1** was isolated and reported from the leaves part of the plant where as, compound **2** is isolated for the first time from this species. Isolated compounds showed marginal antibacterial and little antifungal activities. Antimicrobial activity of crude extract was superior than isolated compounds. Generally, the superior activity of the crude extracts than the pure compound may be due to synergetic effects of several compounds present in the crude extract.

Key words: *C. paniculatum* stem bark, Extraction, Isolation, Antimicrobial activity, Cholest-5-en-3-ol, Dihexyldecyl succinate.

1. Introduction

1.1 Background of the study

Medicinal plants have been used since ancient times in virtually all cultures as a source of medicines [1], and are of great importance to the health of individuals and communities [2]. Traditional medicine has been used in different parts of the World and has gained tremendous importance towards the managements of diseases, especially in developing countries [3]. It is used widely as it depends on locally available plants, which are easily accessible, and capitalizes on traditional wisdom-repository of knowledge, simple to use and affordable [4]. It plays an important role in the search for new chemical bioactive agents. Isolation from plant extracts have resulted in a development of human diseases treatment and discovery of many useful drugs. In this connection, plants continue to be a rich source of therapeutic agents [5].

Combretum paniculatum is one of the medicinal plants that has been used widely in ethnomedicine in the treatment of chronic diarrhea and dysentery, flatulence, vomiting, colic, and enlarged spleen and liver [6]. The leaves of the plants are used in folk medicine for the treatment of various diseases such as stomach pain and diarrhea [7]. In Ethiopia, *C. paniculatum* is used for treatment of ringworm and wounds [8]. It was reported that *C. paniculatum* crude extract possessed antimicrobial property. The crude extract showed inhibition against the three pathogen (*Escherichia coli, Staphylococcus aureus* and *Candida albican*) [9]. Acetone, hexane and methanol extracts of *C. paniculatum* leaves showed an activity against *C. albicans* and *C. neoformans* [10]. The ethanol extract of the leaves of *C. paniculatum* showed significant activity against breast cancer cells. Phytochemicals screening tests revealed the presence of terpenoids, tannins, saponins, alkaloids and phenols [11].

Despite the Stem barks are the most abundantly used and accessible medicinal part of the plant, there were no further scientific reports on isolation of its phytochemical constituents. Hence, this study is to identify phytochemical conisituents and antimicrobial activity of the stem barks of *Combretum pariculatum.*

1.2. Statement of the problem

In Ethiopia, medicinal plants are used widely to treat different ailments caused by microorganisms. Even if they are effective in treating diseases, the phytochemical conistituents

of most of the plants are unknown. Thus, it needs to be supported by scientific experiment to identify constituents of the plant in searching for new chemically bioactive drugs. *C. paniculatum* is one of most common known medicinal plant used. However, the phytochemicals information and bioactivity of the molecules from the stem barks of *C. paniculatum,* which has been widely practiced by the local community for its medicinal role has not been sufficiently reported so far.

1.3 Objective of the study

1.3.1 General objectives

To investigate phytochemical conistituents and antimicrobial activity of the stem barks of *C. paniculatum*.

1.3.2 Specific objectives

- To isolate phytochemical constituents of *Combretum paniculatum* stem barks using chromatographic techniques (Thin Layer Chromatography and Column Chromatography).
- To elucidate the structures of the isolated compounds using NMR spectroscopic techniques (^{1}H NMR, ^{13}C NMR and DEPT-135 NMR).
- To establish the *in vitro* antibacterial (*E. coli, B. subtilis, S. aureus and P. aeruginosa*) and and antifungal (*Fusarium spp* and *S. cerevisiae*) activities of the crude extracts and isolated compounds by disk diffussion method.

1.4 Significance of the study

Today herbal medicines are more advantageous and are being credited due to their efficacy and availability from indigenous sources. It is generally accepted that plant based medicines are better than synthetic drugs as these are much safer for man and his environment. *Combretum paniculatum* is one of the medicinal plants commonly visited by traditional healers, for the treatment of different diseases. However, further studies have not been carried out on the phytochemicals and biological investigation pertaining to the stem barks of the plant. Thus, the findings of this research is useful to provide information of the chemical profile of the plant and can be used as a database and guideline for further isolation and purification of the active principles.

2. Review of Literature

2.1 Botanical description of *C. paniculatum*

The family *Combretaceae* belongs to the order Myrtales and consists of 600 species of trees and shrubs in 20 genera, which include *Anogeissus, Bucida, Combretum, Quisqualis, Terminalia*, and *Thiloa*. They are found throughout the tropics and sub-tropics. Six genera are found in southern Africa and they are as follows: *Combretum, Lumnitzera, Pteleopsis, Quisqualis, Meiostemon* and *Terminalia*. The largest genus is *Combretum*, with about 370 species, while *Terminalia* is the second largest, and has about 200 species. They occur in most parts of Africa and are often the dominant vegetation. The other genera are much smaller, including *Calopyxes* and *Buchenavia* which have 22 species each and *Quisqualis*, *Anogeissis, Conocarypis* and *Pteleopsis*, each with 16, 14, 12 and 10 species, respectively [12, 13]. *C. paniculatum* is belongs to the genus *Combretum* [14]. It is a several-stemmed liana and can climb up and over adjacent vegetation to a height of 15 m or more. The foliage is dense, dark green and rather shiny and is not shed during winter. Its brilliant scarlet flowering resembles that of *C. microphyllum*. Flowering usually takes place some time between mid August and the end of September. Inflorescences are produced in the axils both on the previous year's wood and on current extensions. With leaves mostly being opposite, inflorescences are arranged likewise [15]. The fruit is a 4-winged samara, the wing outline broadly elliptic to broadly obovate to sub-circular, with base rounded and sometimes slightly decurrent along the stripe, the apex truncate to rounded and often shallowly and widely notched. Parasitisation is minimal with the fruit ripening fast, but one has to be available to collect it as it ripens and before it is dispersed by wind. Seed can be easily extracted from the fruit and should be soaked for a few hours before sowing [16].

a) **b)**

Fig 1: Pictures of the *C. paniculatum* plant (a) and stem bark (b) taken from its natural habitats (Source: Tadasa's personal photo collection)

2.2 Traditional medicinal values of the genus *Combretum*

Traditional healers throughout Africa use species of the *Combretaceae* for medicinal purpose [10]. This includes treating fever, headaches, abdominal disorders, abdominal pains, gallstones, diarrhoea, dysentery, gastric ulcers, bilharziasis, hookworm, nosebleeds, sore throats, colds, chest coughs, pneumonia, conjunctivitis, dysmenorrhoea, venereal diseases including syphilis, earache, fattening babies, leprosy, scorpion and snake bites, swelling caused by mumps, toothache, heart diseases, cleanse the urinary system, backache, jaundice, stomach and gastric problems, blennorrhagia, constipation and general weakness [19-21].

Many species of the *Combretaceae* are used medicinally in several continents in the world [22]. *C. micranthum* is used in traditional medicine for the treatment of wounds and sores and of fever (especially malaria fever), cough and bronchitis [23-26]. *C. molle* has been widely used as a medicinal plant to treat various diseases such as parasitic, protozoan and other infectious diseases in East and West Africa [33–35]. The alcoholic extract of *C. dolichopetalum* is used in folklore medicine to relieve stomach ache, blood in the stools, diarrhea, cramps and related gastrointestinal disorders. *C. quadrangulare* seeds are used in Vietnamese traditional medicine as a remedy against round and tapeworm infections in humans [36].

C. paniculatum has been used widely in ethnomedicine in the treatment of different disease [6]. The leaves of the plants are used in folk medicine for the treatment of various diseases such as stomach pain and diarrhea [7, 8, 14]. Also it is used for the treatment of gonorrhea, flatulence, vomiting, colic, enlarge spleen, liver and also ground with salt and the paste applied to the tongue and inside the mouth of babies with stomatitis. In Ethiopia, *C. paniculatum* is used for treatment of ringworm and wounds [8].

2.3 Phytochemicals isolated from *C. paniculatum*

Phytochemical studies carried out in the genus *Combretum* have demonstrated the occurrence of many classes of constituents, including triterpenes, flavonoids, lignans and non-protein amino acids [17]. The preliminary phytochemical screening tests of *C. paniculatum* leaves has demonstrated the presence of tannins, saponins, alkaloids, terpenoids, flavonoids, anthraquinones and phenols [11].

The phytochemical investigation of the leaves of *C. paniculatum* gave compounds such as, gallocatechin **(1),** Coumaric acid **(2)**, cholest-5-en-3-ol **(3)**, beta-sitosterol **(4)**, 2,3,8-tri-Omethylellagic acid **(5)**, Cosmosiin **(6)**, apigenin **(7)**, quercetin-3-glucopyranoside **(8)** [14], stilbene derivative (1-(2',6'-dihyroxphenyl-2-(4"-hdroxyphenyl))-1,2-ethane-diol) **(9)** and myristicacid **(10)** [8], pheophorbidea (C-1) **(11)** [18], cyanidin 3,5-O-β-D-diglu-copyranoside **(12)** and pelargonidin 3,5-O-β-D-diglucopyranoside **(13)** [23].

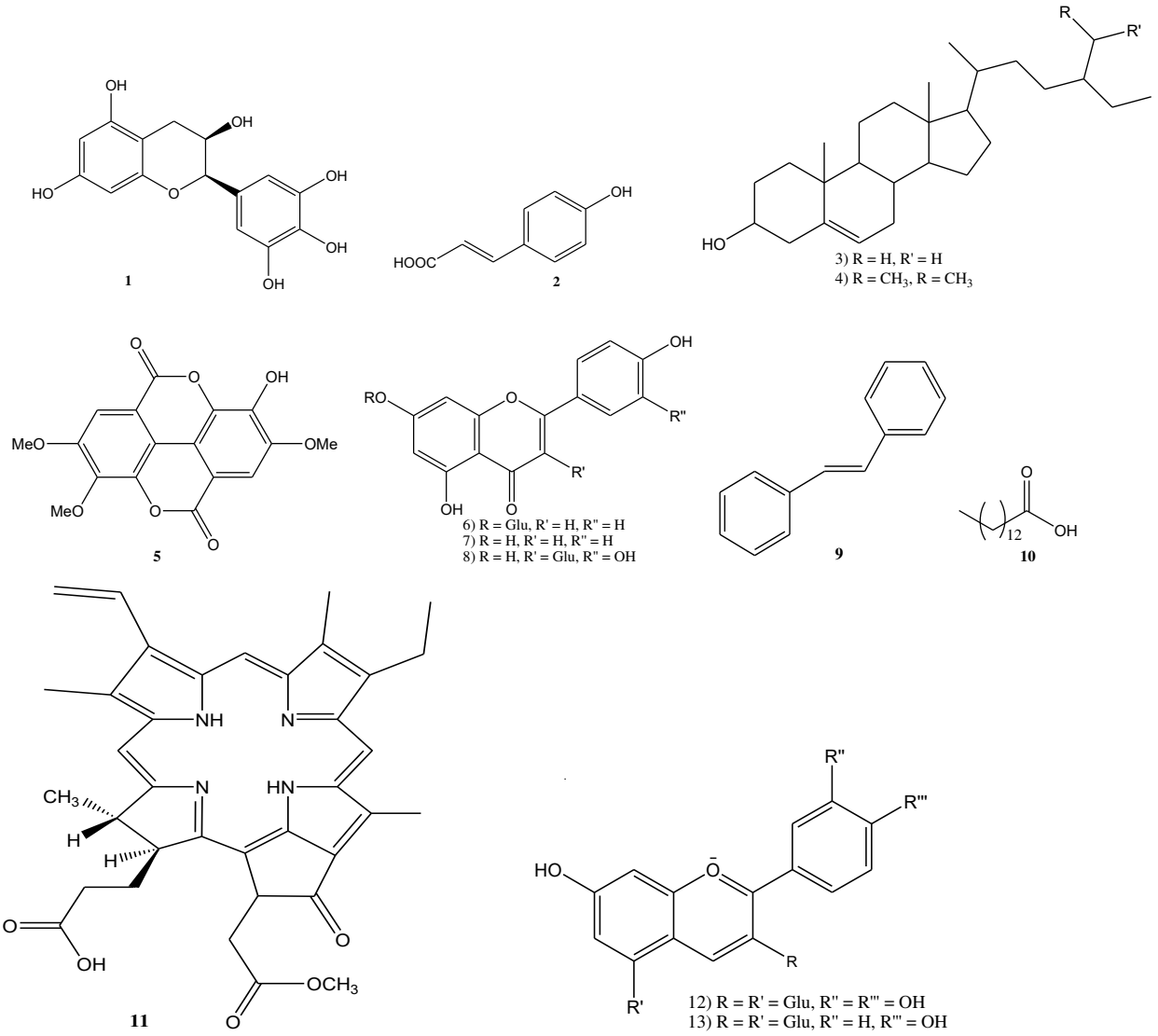


Fig 2: Chemical structure of some of the compounds reported from *C. paniculatum*

2.4 Biological activities of genus *Combretum*

C. micranthum extracts obtained with different solvents (ethanol, chloroform, methanol or water) showed antibacterial activity against the following bacterial species: *Pseudomonas aeruginosa,*

Staphylococcus aureus, Salmonella species, Streptococcus species, Proteus vulgaris, Klebsiella species, Sarcina lutea, Micrococcus luteus and *Bacillus subtilis* [27–29]. Also it was reported that, it has anti-Malarial activity against *Plasmodium falciparum* [30, 31]. Another research demonstrated an antidiabetic effect for the aqueous leaf extract of *C. micranthum* [32].

In studies *C. molle* have demonstrated antibacterial activity against *Staphylococcus aureus* and *Helicobacter pylori* at different extract concentrations [37–39]. Antifungal activity was reported in models that used *Epidermophyton floccosum, Microsporum gypseum, Trichophyton mentagrophytes, T. rubrum, Candida albicans, C. neoformans, Aspergillus fumigatus, Sporothrix schenckii* and *Microsporum canis* [40]. *C. molle* was also able to inhibit the growth of *Mycobacterium tuberculosis* [41]. Antitrypansomal and anthelmintic activities of different extracts have also been reported [42, 43]. Molluscicidal effect of aqueous extract against *Biomphalaria pfeifferi* was also observed [43].

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, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecalis* [44, 45]. Moreover, in studies evaluating antifungal activity, extracts obtained with different solvents (acetone, hexane, dichloromethane and methanol) were active against the following species: *C. albicans, C. neoformans, A. fumigatus, S. schenckii* and *M. canis*. Toxicity studies have shown that the aqueous extract of *C. erythrophyllum* has mutagenic activity against *Salmonella typhimurium*. The methanol, dichloromethane and acetate extracts of *C. erythrophyllum* showed bioactivity in a yeast-based microtiter assay for DNA-damaging agents [46].

The ethanolic extract of *C. dolichopetalum* has shown a gastroprotective effect in stress-induced and non-steroidal antiinflammatory (indomethacin)-induced ulcer models. The hepatoprotective effects of the ethanolic extract of *C. dolichopetalum* root bark were evaluated on paracetamol-induced liver intoxication in rats. It was demonstrated that the methanol and chloroform extracts obtained with dried roots of *C. dolichopetalum* have antiinflammatory activity [48].

The ether and ethanolic extracts of dried root bark or dried seed of *C. quadrangulare* are effective against earthworms when tested in vitro. Acetone, MeOH, and aqueous extracts of *C.*

quadrangulare were tested for their trypanocidal activity against epimastigotes of Trypanosoma cruzi, the causative agent of Chagas disease. Strong trypanocidal activity was found in the acetone extract of *C. quadrangulare* [49].

C. paniculatum has been noticed to inhibits the growth of enteric bacteria [9]. Antibacterial analysis of the hexane, EtOAc and methanol extracts showed inhibition against *Staphylococcus aureus, Escherichia coli, Klebsella preumanin* and *Proteus mirabilis* [11]. The ethanol extract of the leaves of *C. paniculatum* showed significant cytotoxic activity against breast cancer cells [50]. Water and EtOAc extract of *C. paniculatum* leaves showed an activity against *S. haematobium* and *C. elegans* of *in vitro*-Worms [51]. It is reported that, *Escherichia coli* bacteria were observed to be most susceptible organism to the crude extract of leaves of *C. paniculatum* [6]. For water extracts of the leaves, anti-inflammatory activity showed 54% inhibition, there was no anthelmintic activity and the MIC for antischistosomal activity was 25 mg/ml. The acetone extract had a better anti-inflammatory inhibition of 73% and for the anthelmintic activity, 80-90% of the nematodes were alive after exposure to the extract. The ethyl acetate extract had the best anti-inflammatory activity of 76%, and no anthelmintic activity [22]. The plant extracts also displayed good antifungal and some anti-inflammatory activity in other studies. The antimicrobial, anti-inflammatory, antishistosomal, and central nervous system stimulation activities of *C. paniculatum* have been documented [10]. Antimicrobial compounds such as cholest-5-en-3-ol **(3)**, gallocatechin **(1)** and apigenin **(7)** have been reported from the plant [52].

3. Methods and Materials

3.1 Chemicals and apparatus

Chemicals used were analytical reagent: petroleum ether, chloroform, acetone and methanol solvents were used for extraction and ethyl acetate is used for column elution. Silica gel (60-120 mesh) was used to pack Column chromatography. DMSO was used to prepare stock solution and antibiotic drug (Gentamicine) and antifungal drug (miconazole) as standard drugs, nutrient agar, and Muller Hinton agar were used in performing antimicrobial test. The materials used were NMR spectroscopy (400 MHz Bruker ultra shield TM) (^{1}H NMR, ^{13}C NMR and DEPT-135 NMR), rotary evaporator (Heidolph Laborata 4000) for concentration of extract and UV chamber (Uvitec) to visualize the spots on TLC were used.

3.2 Plant material collection and preparation

The stem bark of *C. paniculatum*, was collected in June, 2018 from Sadi chanka District, Kellem Wollega Zone, Oromia region, Ethiopia. The collected plant material was washed with tap water to remove any dust and cut in to small size, allowed to drain off the water from the surface. Then, it was allowed to dry at room temperature in an open air protected from direct exposure of sun light. The air dried plant material was ground to small size with mortar and pestle.

3.3 Extraction of plant material

Powdered plant material was soaked sequentially with petroleum ether, chloroform, acetone and methanol three times each with 2.5 L for 24 h. The extracts were filtered using Whatman No.1 and concentrated using rotary evaporator under reduced pressure at 40°C. The resulting semisolid extracts were stored in desiccators. Percentage yield for each extract was calculated as:

$$\text{Percentage yield} = \frac{\text{Weight of crude extracts (g)}}{\text{Weight of draid sample used (g)}} \times 100$$

3.4 Isolation of compounds

The crude extracts were observed for their number of phytochemical components by using TLC analysis. The chloroform crude extract (5 g) was adsorbed on silica gel and applied to column chromatography already packed with silica gel. The column was eluted with petroleum ether

with increasing gradient of ethyl acetate and mixed fractions were purified using sephadex (with 1:1 ratio of chloroform to methanol).

3.5 Characterization of isolated compounds

The pure isolated compounds were characterized by NMR spectroscopic techniques and depending on the obtained spectrum the structures of the compounds were elucidated.

3.6 Antimicrobial activity of crude extracts and isolated compounds

The crude extracts and isolated compounds were evaluated for *in vitro* anti-microbial activities. They were tested against four bacterial strains (*Escherichia coli* (ATTC 25922), *Bacillus subtilis* (ATTC 6633), *Staphylococcus aureus* (ATTC 25923) *and Pseudomonas aeruginosa* (ATTC 27853)) and two fungal strains (*Fusarium spp* and *Saccharomyces cerevisiae*) by disc diffusion method. Both bacterial and fungal strains were obtained from Microbiology laboratory, Biology Department, Jimma University. Stocked microbial strains were sub-cultured on Muller Hinton agar. Incubation was done for 16 and 24 h to obtain freshly growing bacterial and fungal strains respectively [53]. The test solutions were prepared by dissolving 0.2 g of crude extracts in 1 mL of DMSO to achieve final stock concentrations of 200 mg/mL. For isolated compounds 5.25 mg was dissolved in 0.15 mL of DMSO to get 35 mg/ml stock concentrations. Sterile Whatman filter paper discs (6 mm) were soaked with stock solution of the extract, then placed over incubated plate. Positive controls standard antibiotic (Gentamicine) and antifungal (Micanazole) drugs and negative controls DMSO were also included. The plates were then inverted and incubated at 37°C for 24 and 72 h for bacterial and fungal tests respectively. Inhibition zone diameter was measured in millimeter from clearance of zone around the disks [54]. All antimicrobial activity of crude extracts and isolated compounds were evaluated in triplicate and mean zone of inhibition was recorded.

4. Results and Discussion

4.1 Extraction Yield

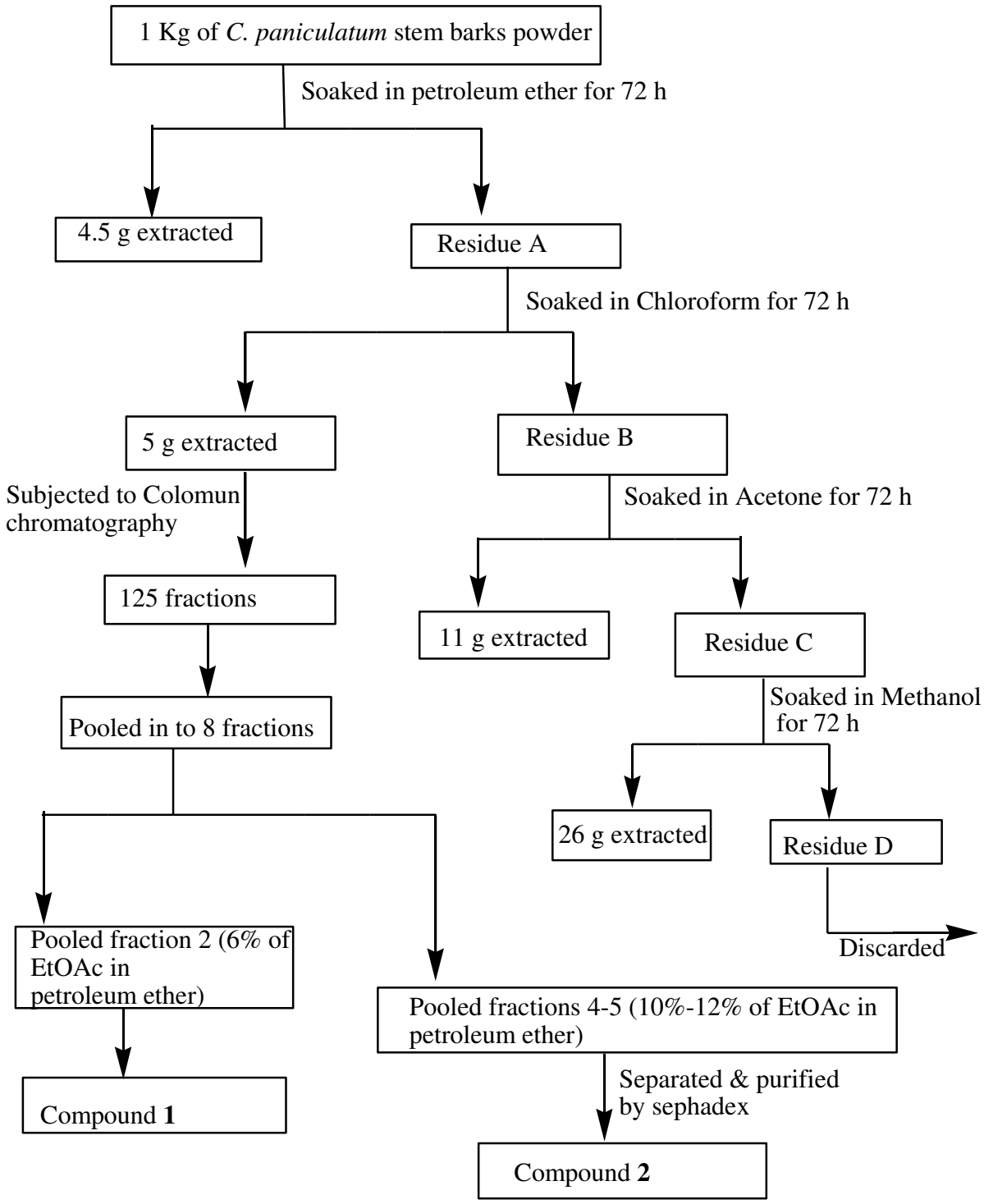
Percentage yield of the extract obtained from extraction of powdered plant material (1.0 Kg) with petroleum ether, chloroform, acetone and methanol was given in Table 1 below. Thus, by the principle like dissolves like non polar and less polar substance are soluble in nonpolar and moderate polar solvent and polar substance are soluble in a polar solvent. As the polarity of the extract solvent increase gradually the selectivity of the solvent toward the solute increase. This result showed that most secondary metabolites in a *C. Paniculatum* stem bark are polar. Percentage yield of crude extracts were increased with the increasing polarity of extracting solvent.

Table 1: Percentage yield of each crude extracts.

Solvents	**Mass in gram**	**Percentage yield in %**
Petroleum ether	4.5	0.45
Chloroform	5	0.5
Acetone	11	1.1
Methanol	26	2.6

4.2 Compounds isolated

The chloroform crude extract showed rich in chemical components. The column elution provide a total of 125 fractions each with 25 mL which were pooled together to 8 major fractions (8-24, 25-41, 42-63, 64-83, 84-97, 98-101, 102-111 and 112-125). Among those fractions, fraction 2 (6% EtOAc in petroleum ether) provide white amorphous solid (8.4 mg) considered as compound **1**. Fractions 4-5 (10%-12% EtOAc in petroleum ether) provide brown amorphous solid (6.8 mg) considered as compound **2** (scheme **1**).



Scheme 1: Extraction and isolation process of *C. paniculatum* stem barks

4.3 Structural elucidation of isolated compounds

Isolation of compound from chloroform crude extract was resulted with two compounds.

Characterization of compound 1

Compound **1** was isolated as a white amorphous solid. The ^{1}H NMR spectrum of compound **1** (Appendix **1**) exhibited signals of methyl groups at δ_H 0.7 ppm (H-27), 0.84 ppm (H-25), 0.90 ppm (H-21), 0.93 ppm (H-18), and 0.95 ppm (H-19). These peaks correspond to H-atoms attached to the external CH_3 groups on cholesterol. One methine proton multiplet exhibited at δ_H 3.55 ppm, the position indicated the presence of a hydroxymethine group and characteristic steroid signals. The signal of methine proton revealed at δ_H 5.37 ppm belongs to olefinic proton and was evident for steroidal skeleton (Table **2**).

The ^{13}C NMR spectrum exhibited 27 carbon signals that accounted for five methyl groups, eleven methylene groups, eight methine groups and three quaternary carbons. The signals of five methyl groups were revealed at chemical shift value of δ_C 11.9 ppm (C-27), δ_C 18.7 ppm (C-25), δ_C 19.0 ppm (C-21), δ_C 19.4 ppm (C-18) and δ_C 19.8 ppm (C-19). Eleven methylene groups were exhibited at δ_C 21.1 to 42.3 ppm and the eight methine groups were exhibited at δ_C 29.2 (C-24), 31.9 (C-20), 45.8 (C-8), 50.1 (C-9), 56.0 (C-17), 56.7 (C-14), 71.8 (C-3) and 121.7 (C-6) ppm. The three quaternary carbons were revealed at δ_C 36.1 (C-13), 36.5 (C-10) and 140.7 ppm (C-5).

Position of five methyl groups, δ_H 0.7, 0.84, 0.90, 0.93 and 0.95 ppm were placed at C-27, C-25, C-21, C-18 and C-19 respectively. Two downfield signals exhibited at δ_C 140.7 and 121.7 ppm belongs to carbon-carbon double bond, C-5 and C-6 respectively, in which proton of methine group, δ_H 5.37 ppm is placed at C-6. Position of methine group, δ_H 3.55 ppm is established at C-3 (δ_C 71.8 ppm) that bonded to the hydroxyl group (Table **2**). The DEPT-135 NMR spectrum (Appendix **1**) showed thirteen carbon signals positively, comprising five methyl carbons and eight methine carbons chemical shift. The absence of signals at δ_C 36.1 ppm (C-13), 36.5 ppm (C-10) and 140.7 ppm (C-5) in DEPT-135 NMR spectrum which is revealed in ^{13}C NMR spectrum also confirmed the presence of quaternary carbon (Table **2**). Therefore, based on the spectroscopic data and comparison of this data with the literature [15], the compound was identified to be cholest-5-en-3-ol (cholestrol) (Figure **3**).

Cholest-5-en-3-ol is a characteristic sterol of higher animals. It occurs either free or as esters, of fish liver oils, egg yolk, bile, bran, and gallstones. It is also used as a pharmaceutical aid (emulsifying agent). Exposure to very high doses has teratogenic effects. The compound is also found in virtually all plant oils, for example rapeseed oil (*Brassica napa*), soybean oil (*Glycine max*) and wheatgerm oil (*Triticum spp.*). It is also reported from the leaves part of the *C. paniculatum* [15].

Table 2: ^{1}H-NMR, ^{13}C NMR and DEPT-135 NMR data of compound **1** and comparison with the Reported Literature [15]:

Position	δ^{1}H Observed	δ^{13}C Observed	DEPT-135 Observed	δ^{1}H Reported	δ^{13}C Reported	DEPT-135 Reported
1	-	33.9	CH_2	-	33.9	CH_2
2	-	31.7	CH_2	-	31.7	CH_2
3	3.55(1H,*m*)	71.8	CH	3.50(1H,*m*)	71.8	CH
4	-	42.3	CH_2	-	42.3	CH_2
5	-	140.7	C	-	140.8	C
6	5.37(1H,*t*)	121.7	CH	5.34(1H,*t*)	121.7	CH
7	-	42.2	CH_2	-	42.2	CH_2
8	-	45.8	CH	-	45.9	CH
9	-	50.1	CH	-	50.2	CH
10	-	36.5	C	-	36.5	C
11	-	39.8	CH_2	-	39.8	CH_2
12	-	37.2	CH_2	-	37.2	CH_2
13	-	36.1	C	-	36.1	C
14	-	56.7	CH	-	56.8	CH
15	-	24.3	CH_2	-	24.3	CH_2
16	-	23.0	CH_2	-	23.1	CH_2

17	-	56.0	CH	-	56.1	CH
18	0.93(3H,*s*)	19.4	CH_3	0.92(3H,*s*)	19.4	CH_3
19	0.95(3H,*s*)	19.8	CH_3	0.95(3H,*s*)	19.8	CH_3
20	-	31.9	CH	-	31.9	CH
21	0.90(3H,*d)*	19.0	CH_3	0.90(3H,*d)*	19.0	CH_3
22	-	26.0	CH_2	-	26.2	CH_2
23	-	28.2	CH_2	-	28.2	CH_2
24	-	29.2	CH	-	29.2	CH
25	0.84(3H,*d)*	18.7	CH_3	0.85(3H,*d)*	18.7	CH_3
26	-	21.1	CH_2	-	21.1	CH_2
27	0.7(3H,*t*)	11.9	CH_3	0.66(3H,*t*)	11.9	CH_3

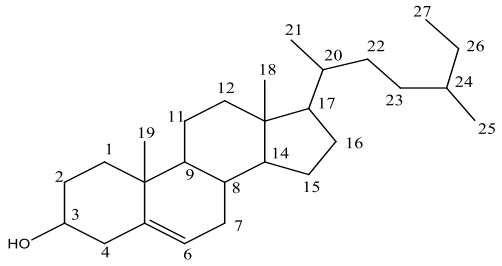


Fig 3: Proposed structure of compound 1 (Cholest-5-en-3-ol)

Characterization of compound 2

Compound **2** was obtained as brown amorphous solid. The ^{1}H NMR spectrum of Compound **2** (Appendix **2**) showed signals for one methyl group at δ_H 0.90 ppm and two alpha methylene protons at δ_H 2.36 and δ_H 3.66 ppm for acid and alcohol portion of an ester respectively (Table **3**). The ^{13}C NMR spectrum exhibited 18 carbon signals, accounted for one methyl group, sixteen methylene groups and one quaternary carbon. The one methyl carbon is exhibited at δ_C 14.12 ppm (C-16). Five methylene group were revealed at chemical shift value of δ_C 21.04 to 35.63 ppm and one oxymethylene carbon exhbited at δ_C 63.09 ppm (C-1). One downfield quaternary carbon was exhibited at δ_C 179.72 ppm (C-1'). The existence of downfield signals, carbonyl

carbon at δ_C 179.72 ppm and an alpha methylene carbon at δ_C 63.09 ppm showed the compound to be an ester (Table **3**).

The placement of one methyl group, δ_H 0.90 ppm is established at C-16 (δ_C 14.12 ppm). Position of alpha methylene groups, δ_H 3.66 ppm is placed at C-1 (δ_C 63.09 ppm) for alcohol portion and δ_H 2.36 ppm is established at C-2' (δ_C 35.63 ppm) of an acid portion (Table **3**). The DEPT-135 NMR spectrum (Appendix **2**) showed one carbon signal positively, comprising one methyl carbon chemical shift and this indicates the structure of compound is symmetric. The absence of downfield signal at δ_C 179.72 ppm (C-1') in DEPT-135 NMR spectrum which is revealed in ^{13}C NMR spectrum also confirmed the presence of one quaternary carbon (Table **3**). Thus, based on the above evidence the structure of the compound was deduced to be dihexadecyl succinate (Figure **4**).

Table 3: ^{1}H-NMR, ^{13}C NMR and DEPT-135 NMR data of compound **2**

Position	δ ^{1}H Observed	$\delta^{13}C$ observed	DEPT-135 observed
1	3.66 (2H,*t)*	63.09	CH_2
2	-	34.04	CH_2
3	-	32.73	CH_2
4	-	31.94	CH_2
5	-	29.71	CH_2
6	-	29.68	CH_2
7	-	29.61	CH_2
8	-	29.45	CH_2
9	-	29.38	CH_2
10	-	29.26	CH_2
11	-	29.08	CH_2
12	-	25.73	CH_2
13	-	24.70	CH2

14	-	22.70	CH_2
15	-	21.04	CH_2
16	0.90 (3H,*t*)	14.12	CH_3
C1'	2.36 (2H,*t*)	179.72	C
C2'	-	35.63	CH_2

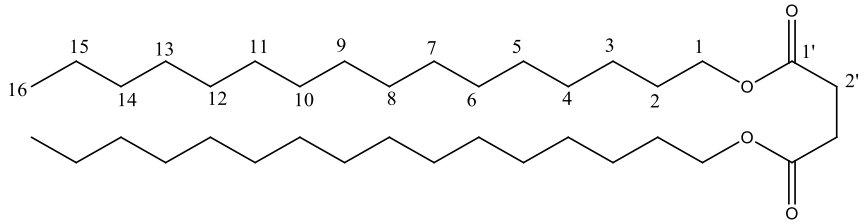


Fig 4: Proposed structure of compound **1** (dihexadecyl succinate)

4.4 Antimicrobial activity results

An *in vitro* antimicrobial activity results of crude extracts against four bacterial strains (*E. coli. S. aureus, B. subtiles* and *P. aeruginosa*) and two fungal strains (*S. cerevisiae and Fuzarium spp.*) were measured in millimeter and mean zone of inhibition was recorded as follows (Table **4**).

Table 4: *In vitro* antibacterial and antifungal activities of the crude extracts and isolated compounds and references

Strains	Diameter zone of inhibition (mm)								
	Crude extracts				Isolated Compounds		Controls		
	PE	CE	AE	ME	Cpd1	Cpd2	G	M	DMSO
P. aeruginosa	11	10	11	9.5	10.5	11	19.5	-	NI
E.coli	15	10	12	11	10.5	8.5	19	-	NI
B. subtilis	11	12	11	10	9	8	24	-	NI
S. aureus	12	13	11.5	9	9	10.5	21.5	-	NI
S. cerevisiae	11.5	9.5	10	9	8	8	-	21	NI
Fusarium spp	14	10	12	11.5	10	8.5	-	22	NI

*(Key: PE: Petroleum ether Extract, CE: Chloroform Extract, AE: Acetone Extract, ME: Methanol Extract, Cpd 1: Compound **1**, Cpd 2: Compound **2**, G: Gentamycin, M: Mucagonaza, and NI: No Inhibition).*

As shown in Table **4**, relatively both crude extracts and isolated compounds shows moderate activity aganist bacterial and fungal strains when compaired to control groups. The chloroform crude extract showed an activity against four bacterial strains (*E. coli, S. aureus, B. subtilis* and *P. aeruginosa*) with inhibition zone diameter 13, 10, 12, and 10 mm respectively. Methanol exract shows less activity against the four bacterial strains with mean zone of inhibition 9.8 mm. Acetone (11 mm) and petroleum ether (12 mm) extracts have moderate activity against the four bacterial strains. *Fusarium spp* is more susceptible than *S. cerevisiae* to crude extracts with mean inhibited zone of 12 mm and 10 mm respectively. In previous study compound 1 was reported for its antimicrobial and anti-inflammatory activity [15]. In present study compound **1** and Compound **2** showed relatively marginal activity against the four bacterial strains with mean inhibition zone diameter of 9.75 mm and 9.5 mm and little activities against fungal strains with mean inhibition zone of 8.5 and 8.25 mm respectively.

5. Conclusion and Recommendation

5.1 Conclusion

This study was aimed to phytochemical investigation of *C. paniculatum* stem barks and evaluation of antimicrobial activities. Plant material was extracted using sequential method of extraction with solvent such as petroleum ether, chloroform, acetone and methanol. The study has characterized two compounds; compound **1** (cholest-5-en-3-ol) and compound **2** (dihexadecyl succinate). Compound **2** was isolated for the first time from this species. Four crude extracts and compounds isolated were analyzed for their antimicrobial activity against four bacterial and two fungal strains. The antimicrobial activity analysis of crude extracts showed moderate activity against both bacterial and fungal strains when compaired to reference drugs, gentamycin and micanazole. Antibacterial activity analysis of compound **1** and compound **2** showed marginal activity against bacterial strains with mean zone inhibition of 9.75 mm and 9.5 mm and little activity against fungal strains with mean zone of inhibition of 8.5 and 8.25 mm respectively. Generally, the superior activity of the crude extracts than the pure compound was exhibited and this may be due to synergetic effects of several compounds present in the crude extract.

5.2 Recommendation

Based on the above results the following recommendations were forwarded.

i. In different study it was reported that, the crude extracts and isolated compounds from the leaves of *C. Paniculatum* showed significant antimicrobial activity and recommended to investigate bioactive compounds from other parts of the plant.

ii. The present study carried out on isolation of phytochemicals and evaluation of antimicrobial activity of stem bark of the plant. Therefore, isolation of phytochemicals from root part is recommended.

iii. In this study only chloroform extract was used and further study in isolating phytochemical and elucidating their structure from petroleum ether, acetone and methanol extracts is recommended.

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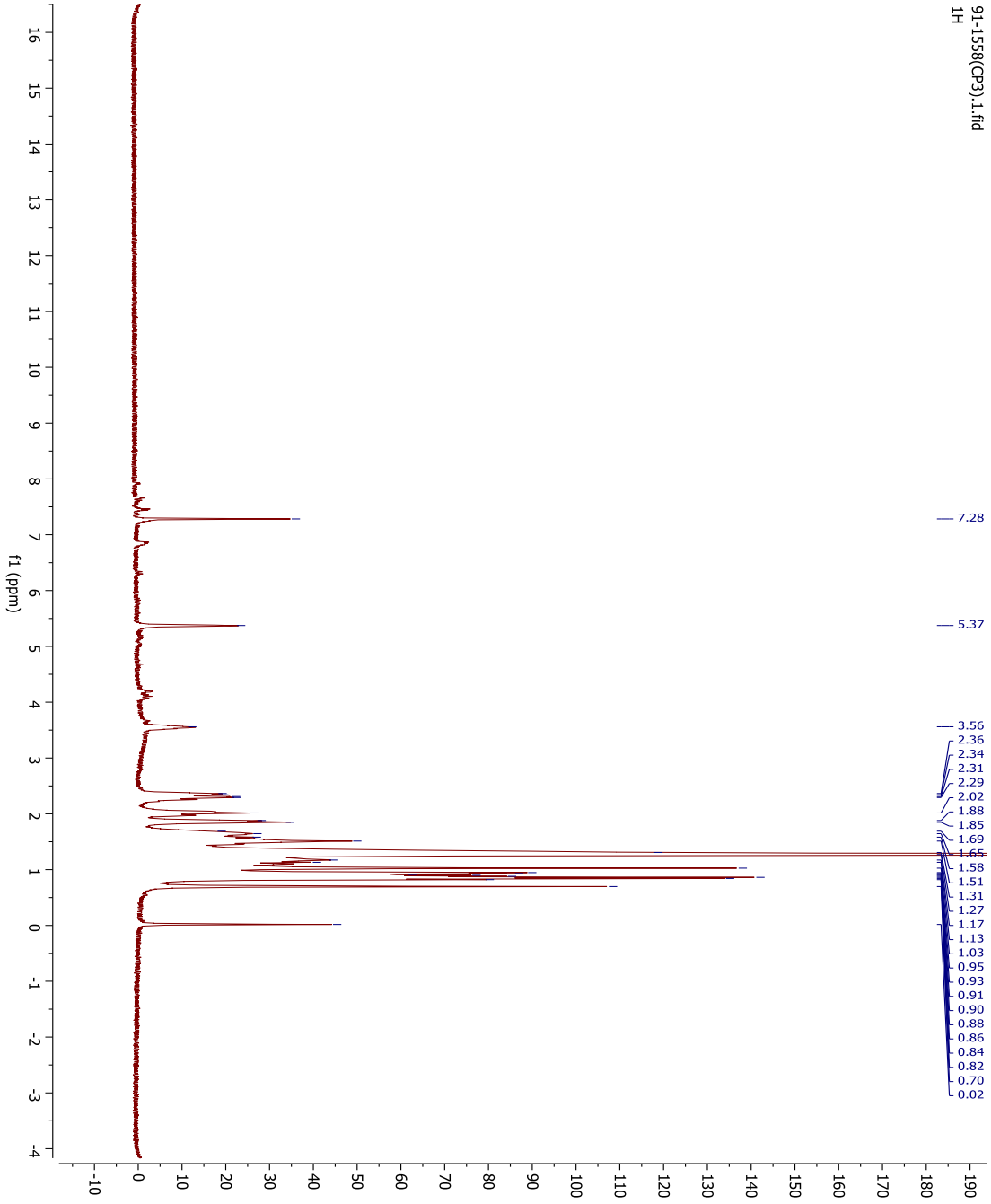
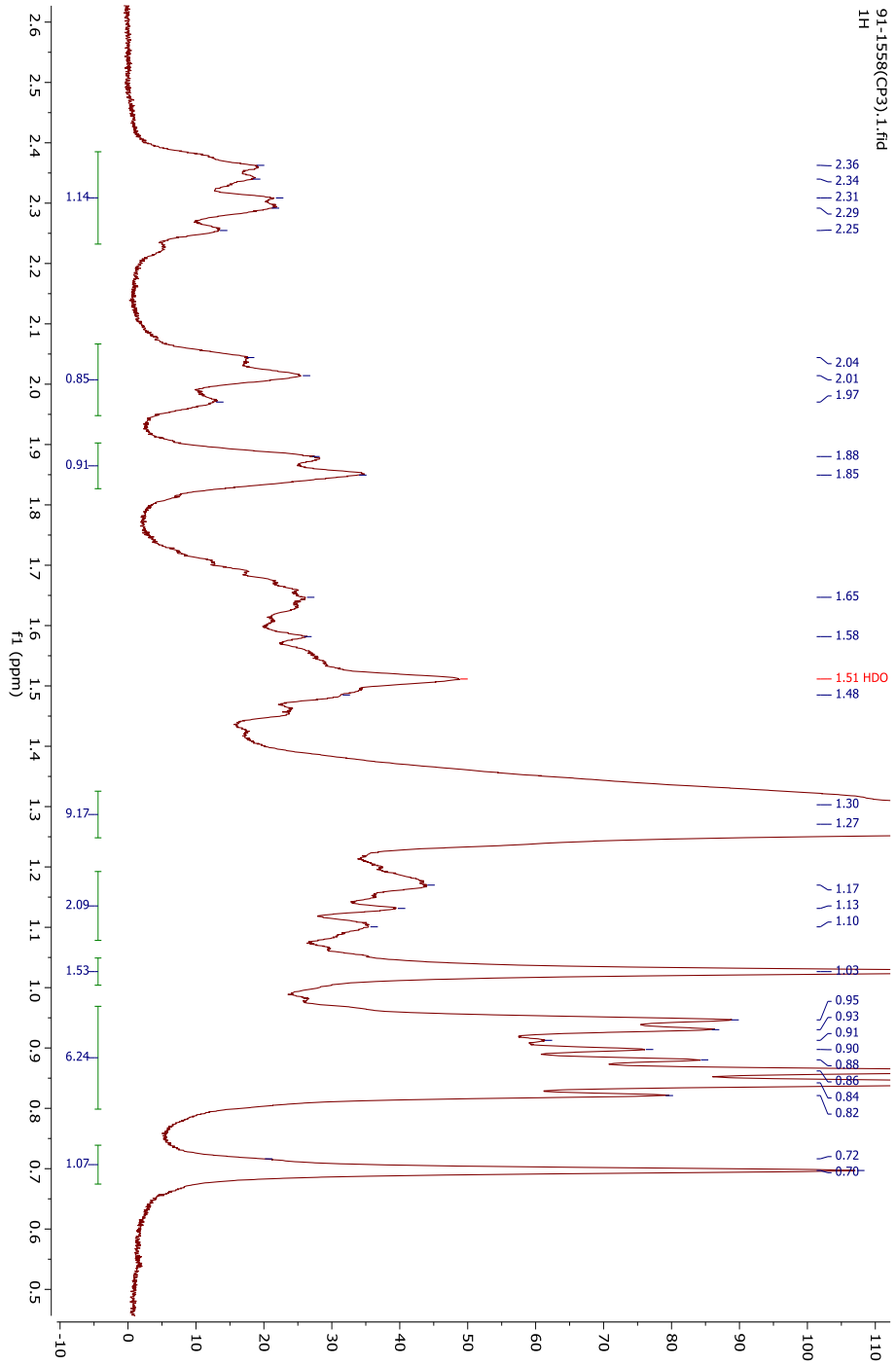
APPENDIXES

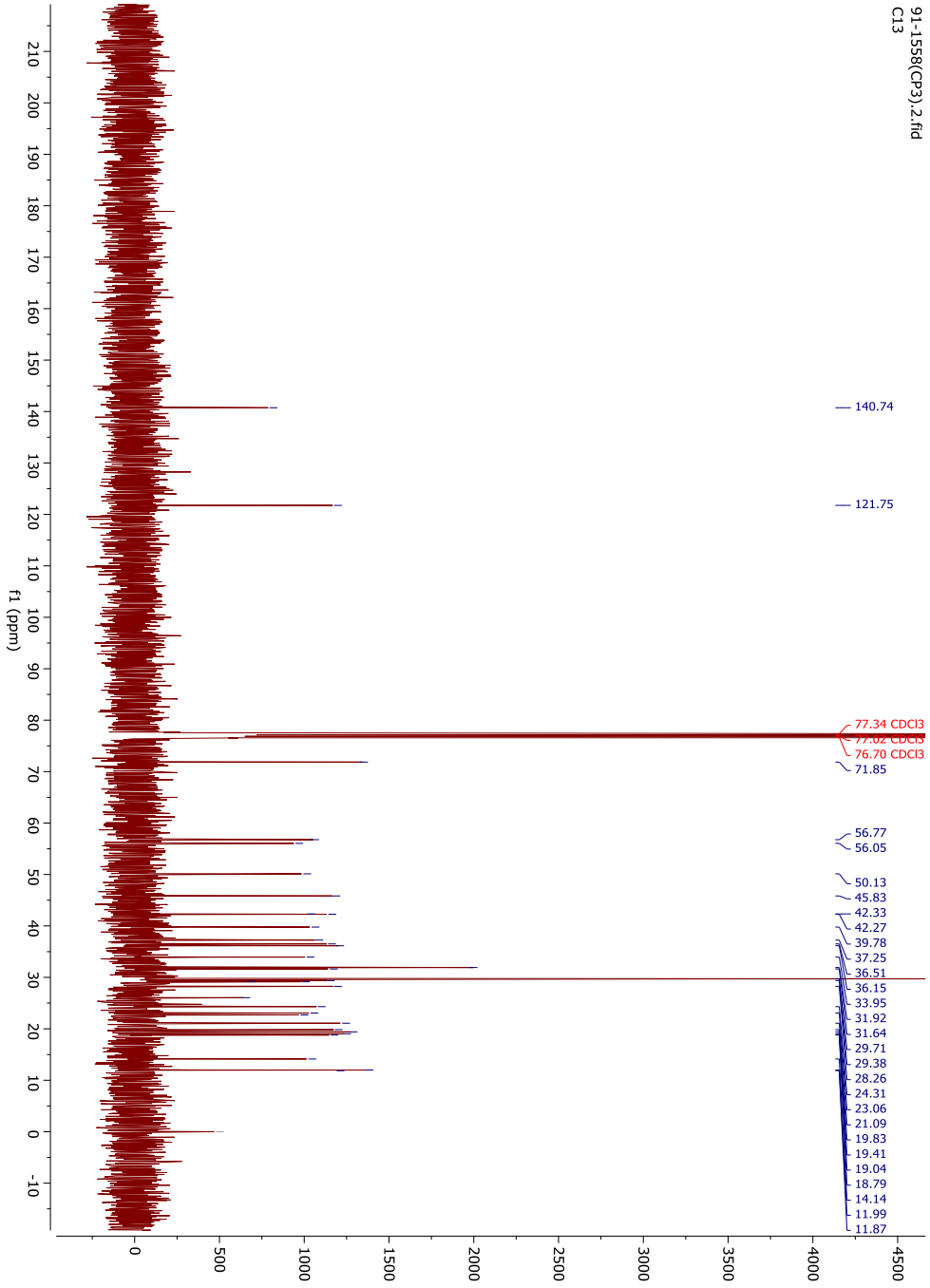
Appendix 1: ^{1}H, ^{13}C and DEPT-135 NMR Spectrum of compound **1**



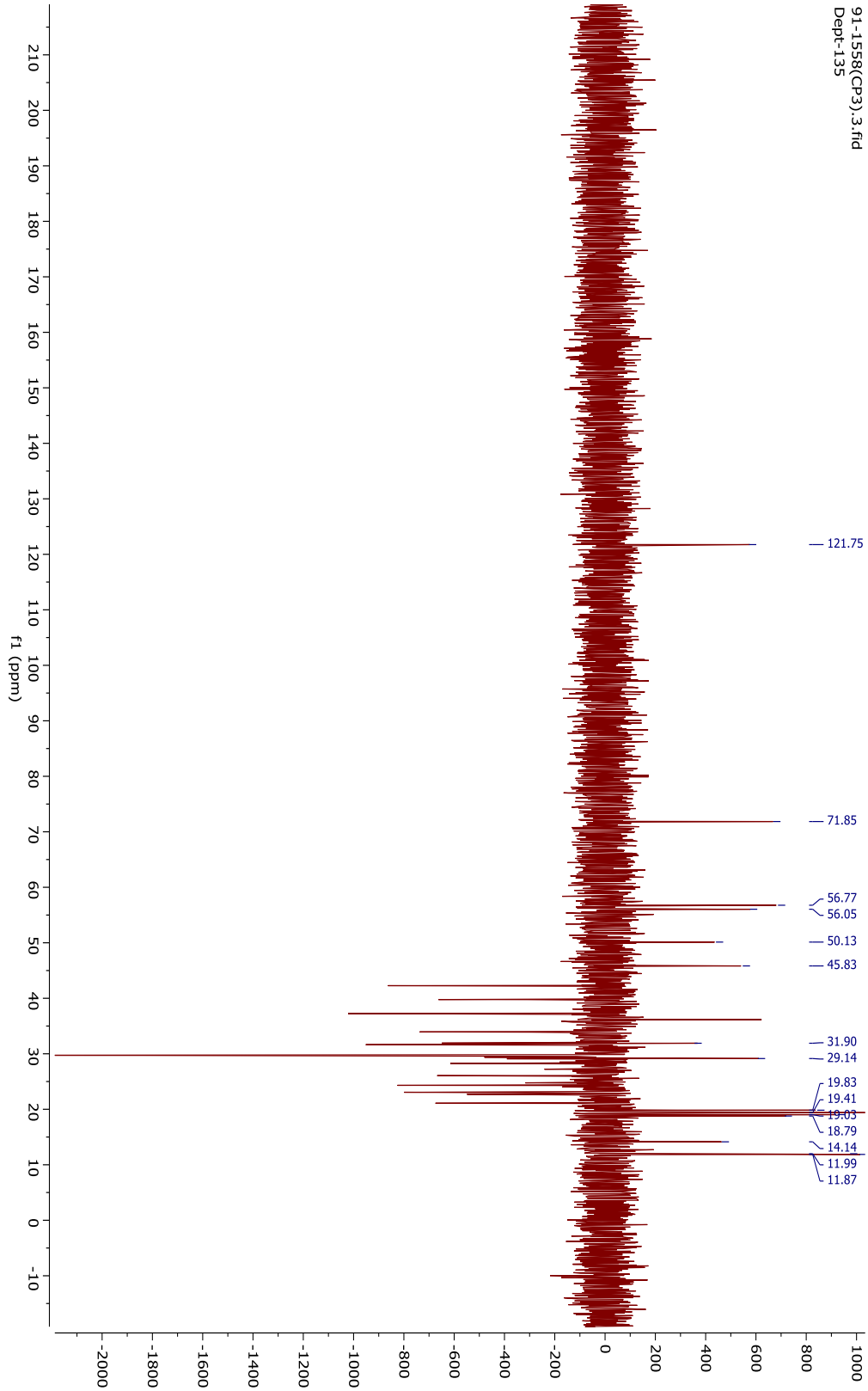
^{1}H NMR spectrum of compound **1**



Expanded ^{1}H NMR spectrum of compound **1**

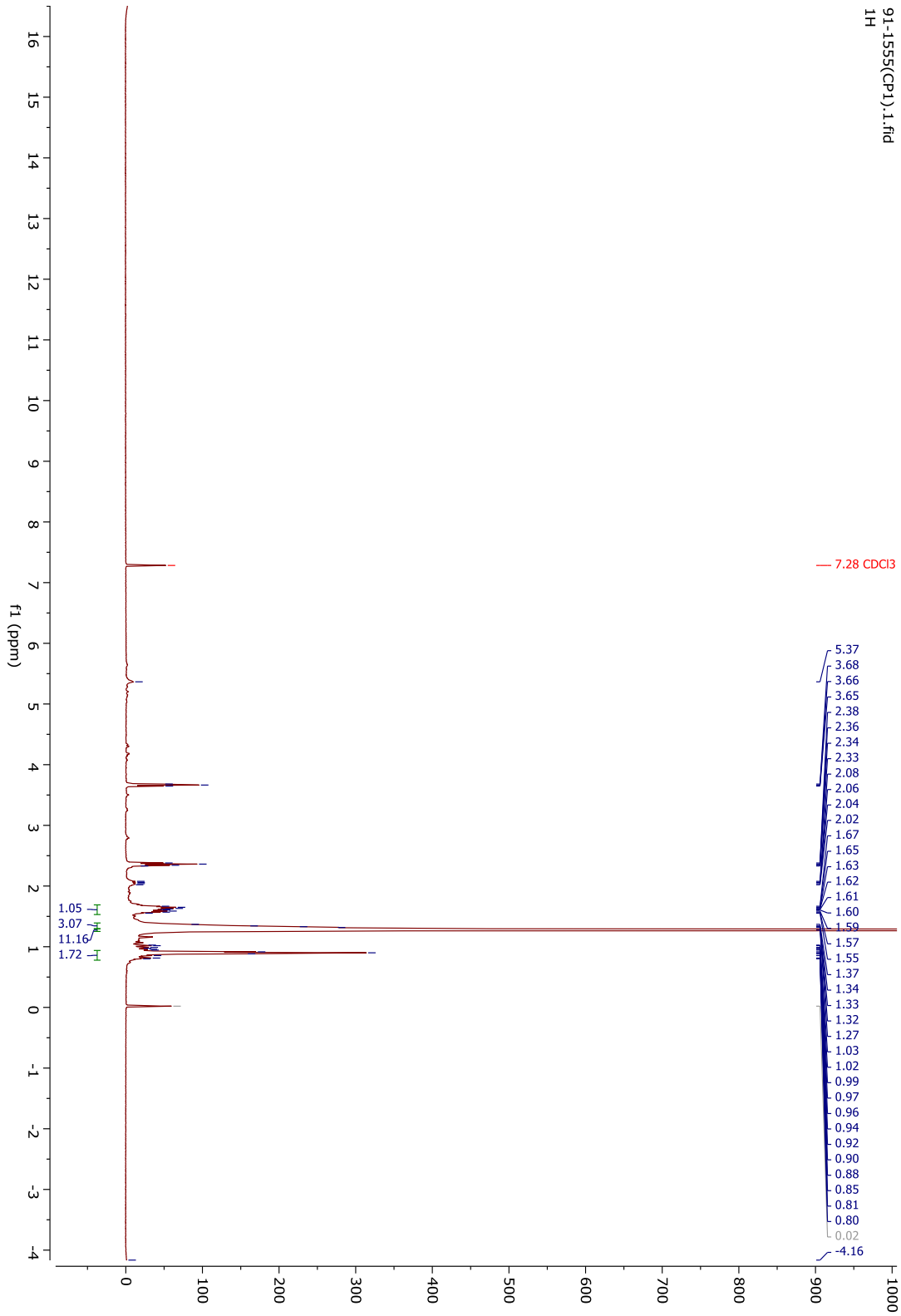


^{13}C NMR spectrum of compound **1**

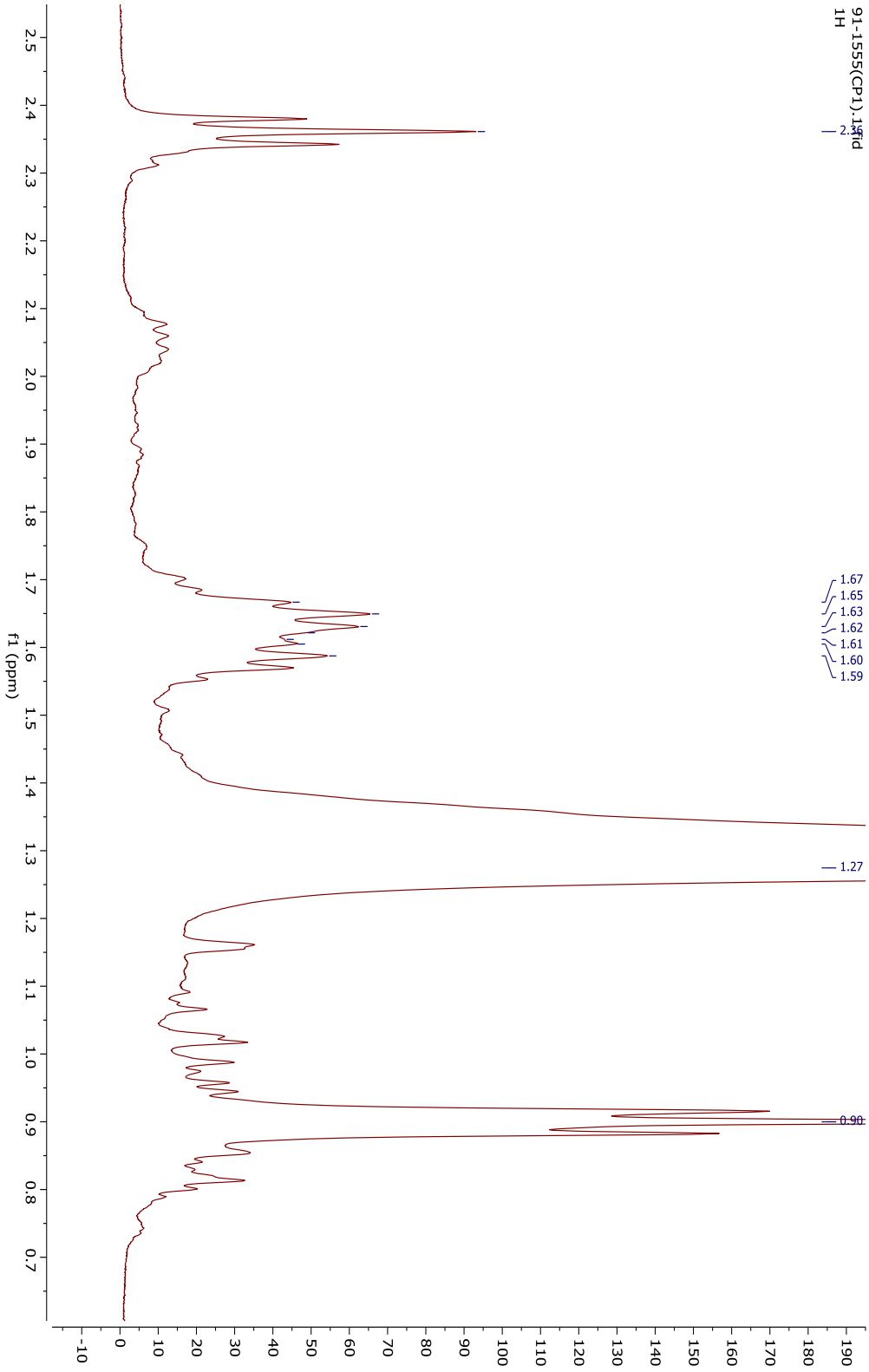


DEPT-135 NMR spectrum of compound **1**

Appendix 2: ^{1}H, ^{13}C and DEPT-135 NMR Spectrum of compound **2**



^{1}H NMR spectrum of compound **2**



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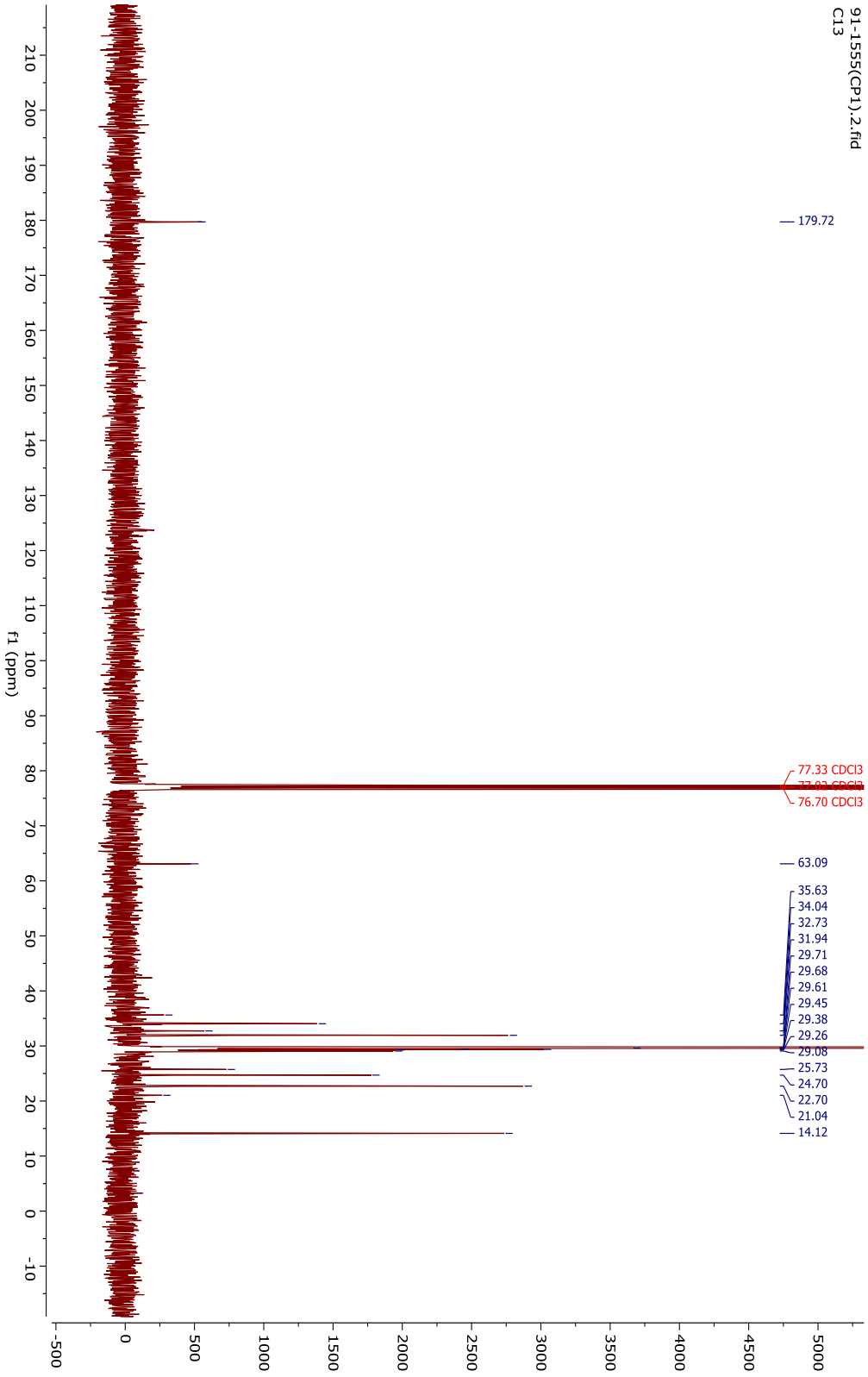
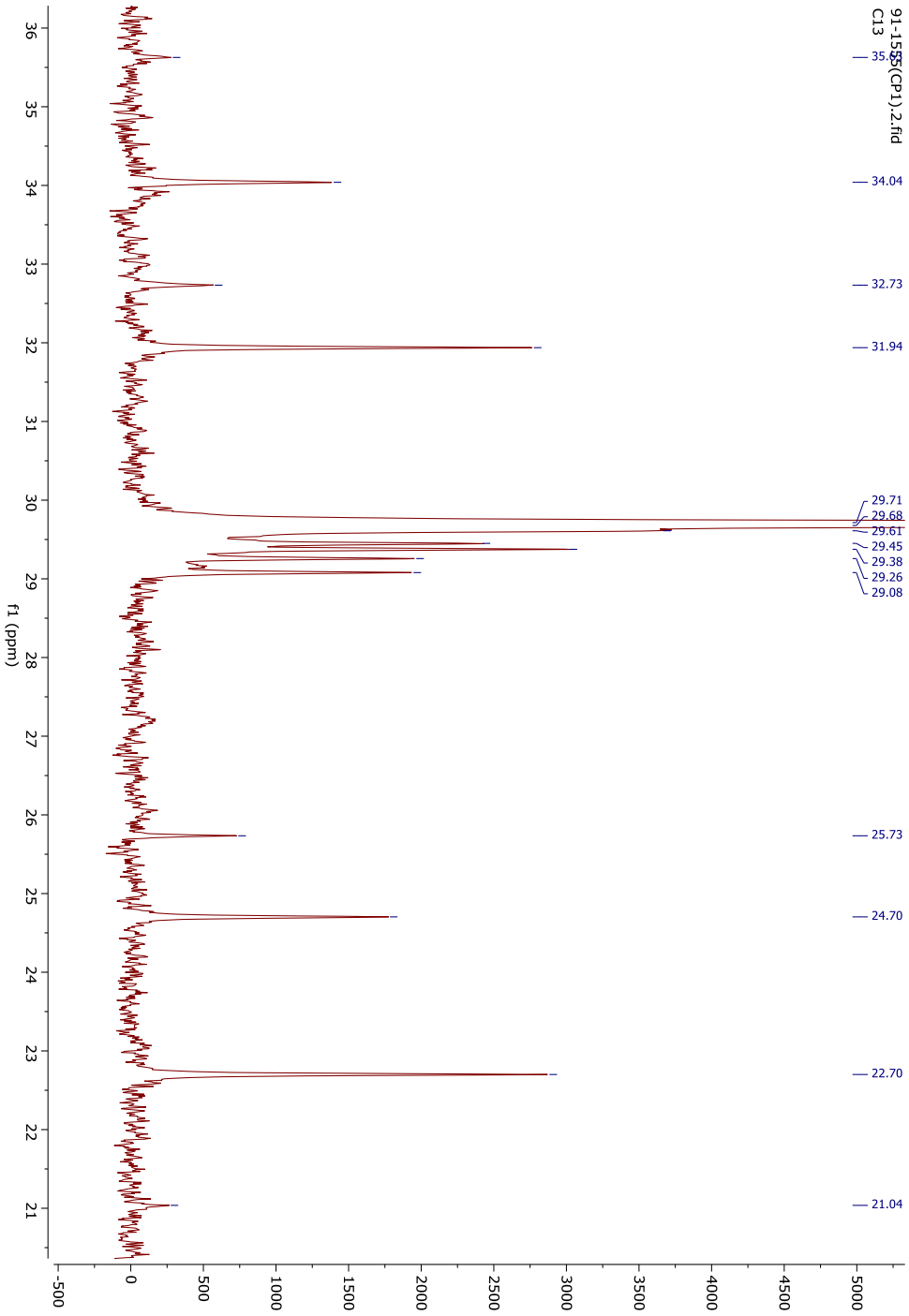
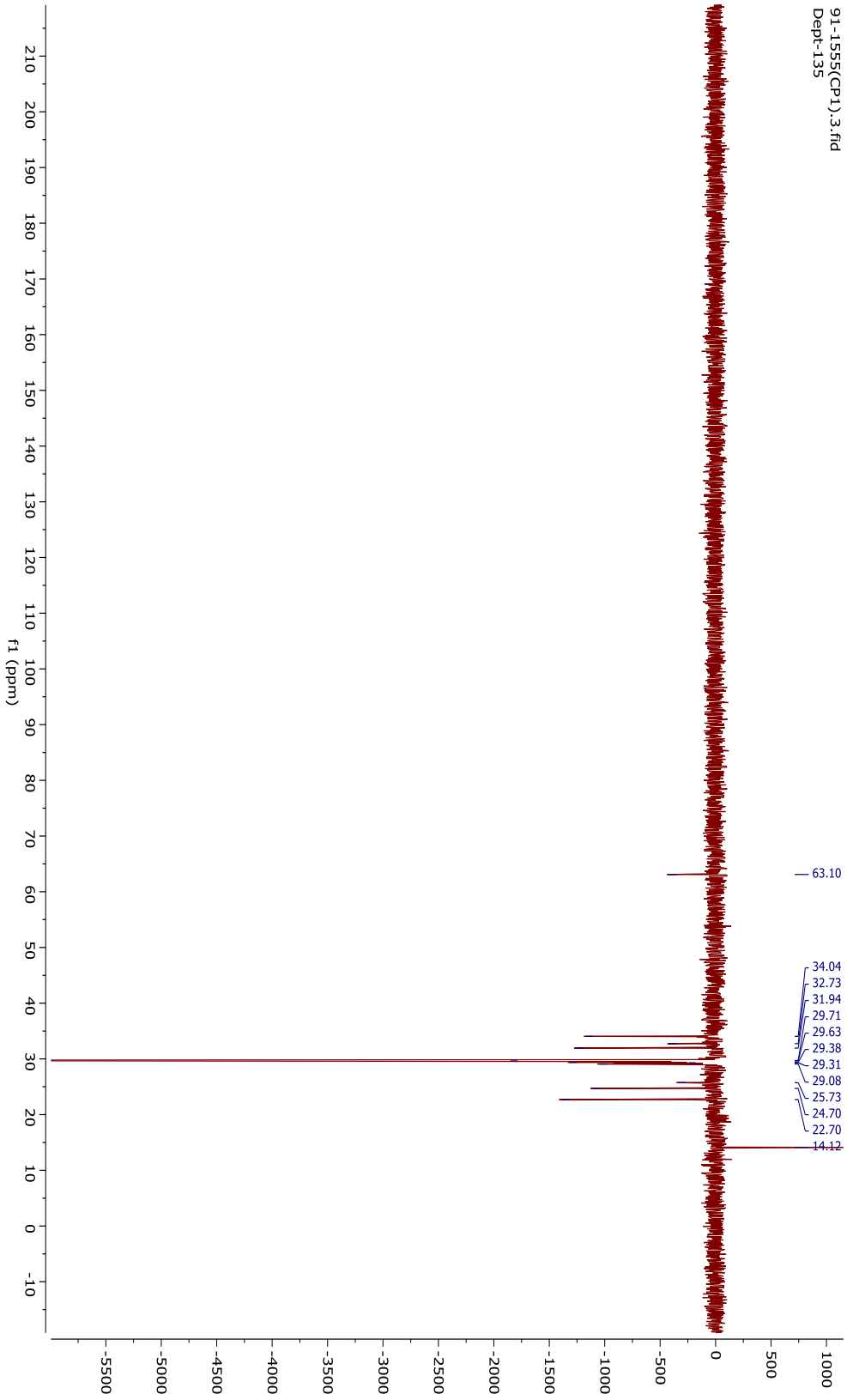


^{13}CNMR spectrum of compound **2**



Expanded ^{13}C NMR spectrum of compound **1**

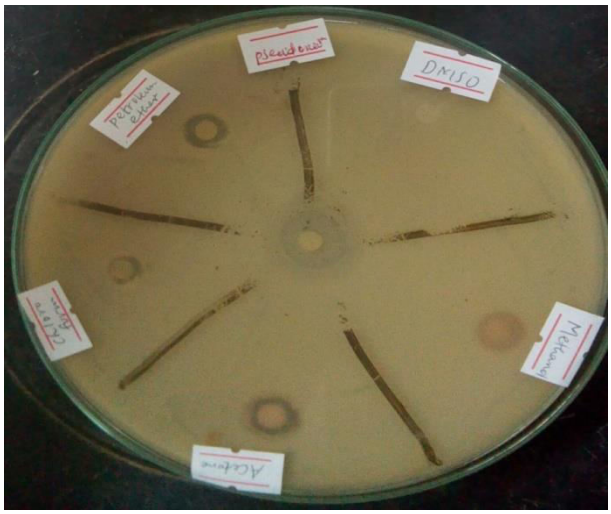


DEPT-135 NMR spectrum of compound **2**

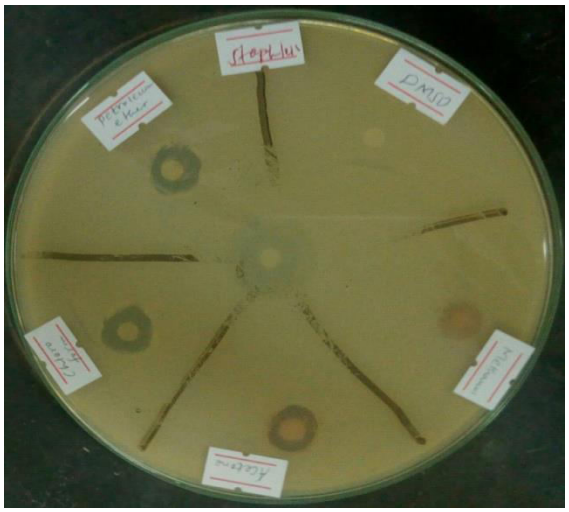
Appendix 3: Antimicrobial activity of crude extracts and isolated compounds

Antibacterial activities of crude extracts

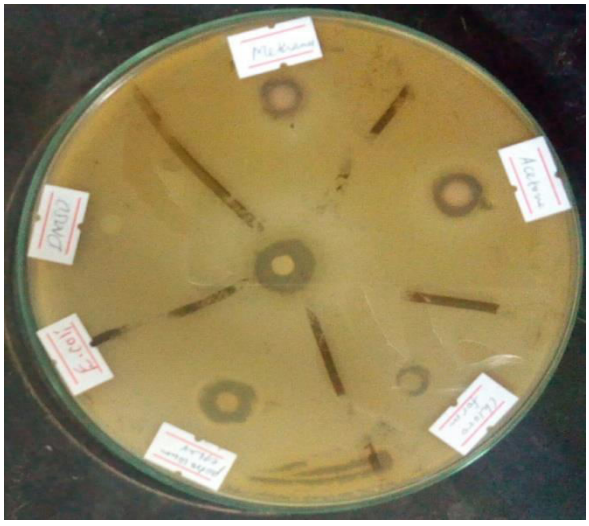
P. eurogenosa



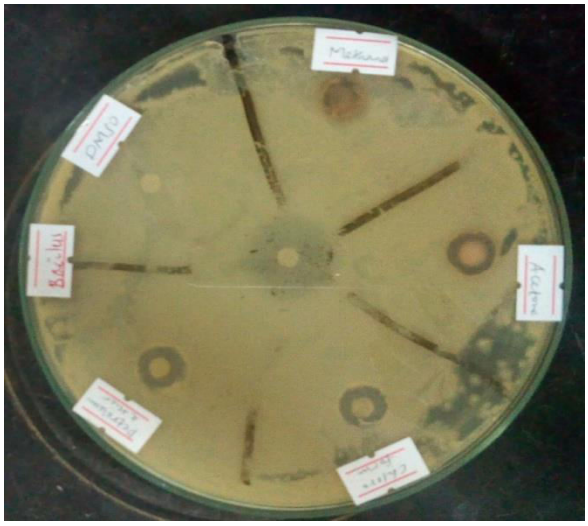
S. aureus



E. coli



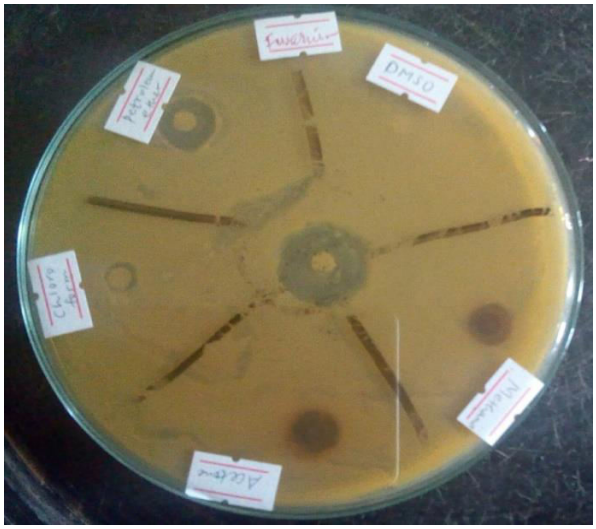
B. subtilis



Antifungal activity of crude extracts

S. cerevisiae

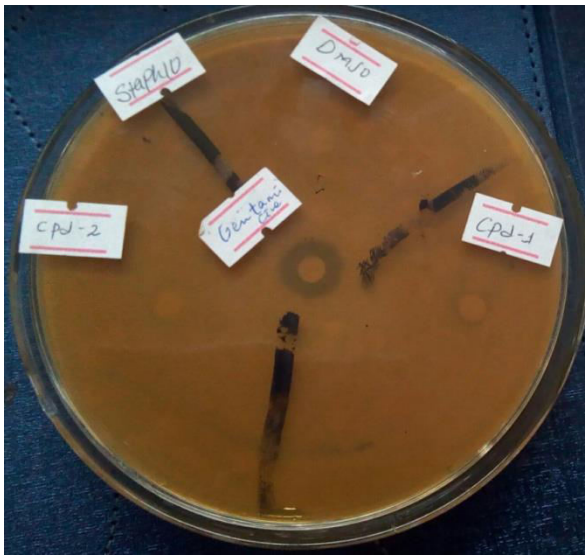
Fusarium spp.



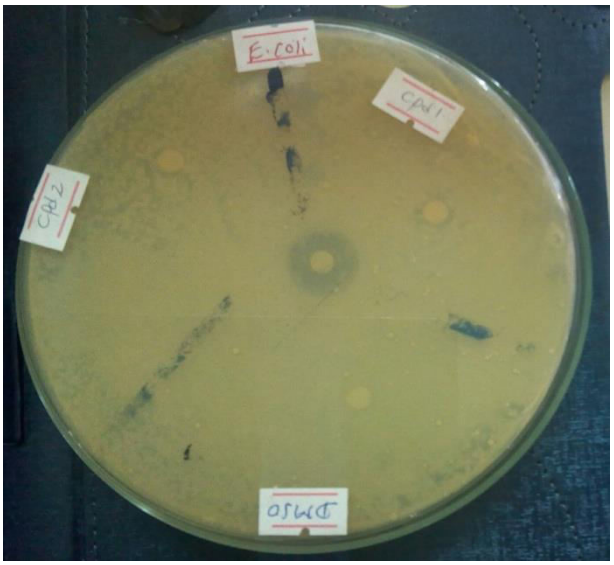
Antibacterial activity of isolated compounds

P. eurogenosa

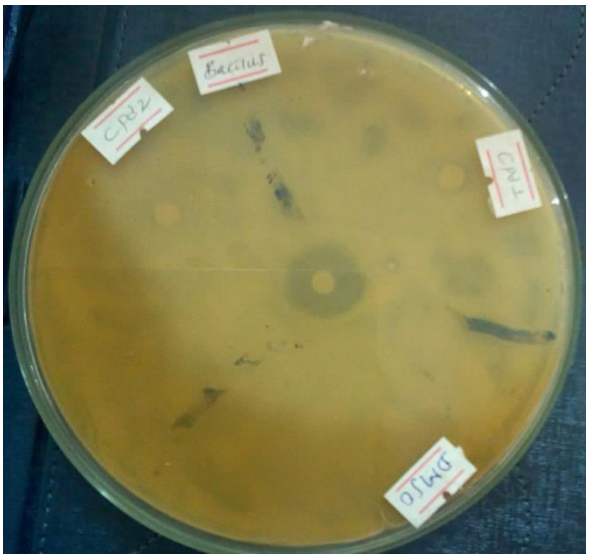
S. aureus



E. coli

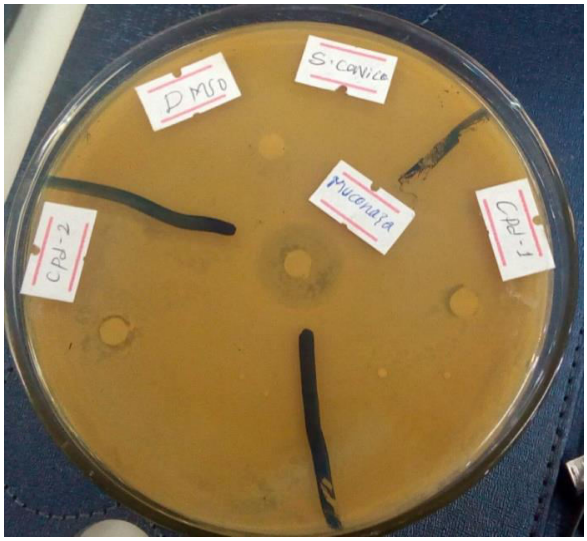


B. subtilis



Antifungal activity of isolated compounds

S. cerevisiae



Fusarium spp.