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M.Sc THESIS

ON

PHYTOCHEMICAL INVESTIGATION OF STEM BARKS OF *CLAUSENA ANISATA*AND EVALUTION OF ITS ANTI MICROBIAL ACTIVITY

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PHYTOCHEMICAL INVESTIGATION OF STEM BARK OF *CLAUSENA ANISATA* AND EVALUTION OF ITS ANTIMICROBIAL ACTIVITY

BY

KETEMA ABERA

THESIS SUBMITTED TO SCHOOL OF GRADUTE STUDIES JIMMA UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY

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JIMMA UNIVERSITY SCHOOL OF GRADUTE STUDIES PHYTOCHEMICAL INVESTIGATION OF STEM BARK OF *CLAUSENA ANISATA* AND EVALUTION OF ITS ANTI MICROBIAL ACTIVITY

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Abbreviation

WHO	World Health Organization
UV-vs	Ultra-visible light spectroscopy
TLC	Thin- layer chromatography
C-NMR	Carbon nuclear- magnetic resonance spectroscope
CC	Column Chromatography
δ	Chemical shift
d	Doublet
dd	Doublet of a doublet
J	Coupling constant
s	Singlet
DEPT	Distorttionless Enhancement by Polarization Transfer
ATCC	America Type culture collection
$CDCl_3$	deuterated chloroform
TMS	Tetra methyl silane

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Abstract

Clausena anisata is among the medicinal plant that has been commonly used by traditional people in some parts Africa and Ethiopia. Despite the wide usages of this plant in traditionally for the treatment of various ailments, the phytochemical investigation of the stem bark of this plant and its antimicrobial activity has not been addressed. Isolation of chemical constituents, evaluation of the antibacterial and antifungal activities are the main purpose of this research project. The air dried stem bark of *C. anisata* was sequentially extracted by petroleum ether, chloroform, acetone and methanol. The column chromatographic separation of the chloroform fraction was led to the isolation of mixture of carbazole alkaloids compound **1** and compound **2**.The structures of the compounds were characterized based on data from ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and related literature reports. Each crude extracts were evaluated on four bacteria strains *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC6633) and *Pseudomonas aeruginosa* (ATCC 27853) and two fungal strain *Fusariam spp* and *Candida albicums on* disc diffusion method. Chloroform crude extracts demonstrated significant growth inhibition comparing with standard Chloramphenicol with growth inhibition of 15 mm was observed in *B. subtilis* bacteria and more zone of inhibition was observed for *Fusarium spp* fungi 13 mm comparing to the standard Ketoconazole. In both bacteria and fungi cases chloroform extract exhibited more potency.

Keywords: bioactivity, Carbazole alkaloids, *C. anisata*, ,isolation

CHAPTER ONE

1. 1Background

Medicinal plants play a key role in human health care system. According to the World Health Organization 80% of people still rely on plant-based traditional medicines for primary healthcare. .These traditional medicinal plants are widely used in different part of the world for curing diseases. For instance, in china, about 30 to 50 % of the total medicinal consumption was obtained from traditional herbal preparations. In Africa, such as Ghana, Mali ,Nigeria and Zambia, Ethiopia rural population uses traditional medicine due to the cultural acceptability of healers and local pharmacopeias, the relatively low cost of traditional medicine and difficult access to modern health facilities [1].In addition to the traditional uses plants have been also a source of modern drug in the treatment of malaria such as quinine (**1)** extracted from *Cinchona officinalis* bark and artemisinin (**2**) from *Artemisia annual* [2].Breast cancer drug Paclitaxel (**3**) from*Taxus brevifolia* [3] and Anti bacterial ditrepine from *Plectranthus hereoensis* (**4**) [4]

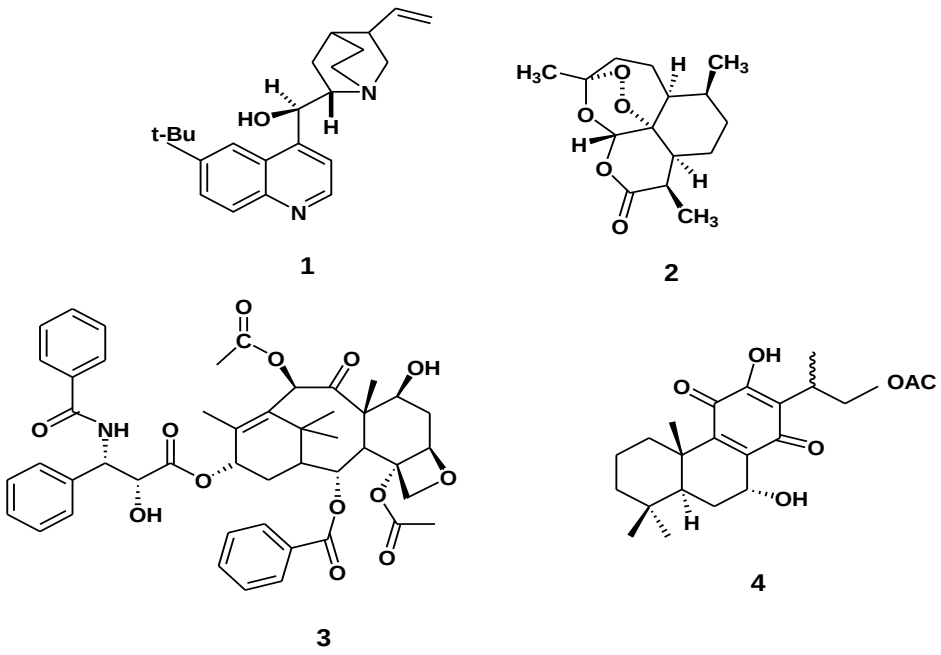


Figure 1 . Structure of some drug isolated from medicinal plant

Ethno medicinal studies play a vital role to discover new drugs from indigenous medicinal plants. A number of modern drugs have been discovered since the history of Ethno botany paying a distinct importance to the documentation of traditional information of medicinal plants [5].The recent reports have indicated the modern drugs have been derived from the extract of medicinal plants [6].The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents [7].

Plants yields prima two classes of metabolite and are broadly divides in to primary and secondary Metabolites. Primary metabolites includes proteins, carbohydrates, lipids and nuclic acids which are responsible for growth and development i.e direct roles in the processes of photosynthesis, respiration and major metabolic process of plants whereas, secondary metabolites are usually of relative to limited occurrences, unique to particular species produced for the purposes of defense. These metabolites are refereed as natural products once served humankind as the source of all drugs, and higher plants provided most of these therapeutic agents. They are responsible for protecting the plant against microbial infection, viral or infestation by pests [8].These phytochemical may be derived from different parts of plant including the bark, leaves, flowers, roots, seeds, fruits, rhizomes and others. Based on their structural Varity and composition, the major groups of phytochemical are (alkaloids, tannins, flavonoids, polyphenols, anthraquinones, etc) are now widely used to treat degenerative diseases, such as bacteria, malaria, cancers, diabetes etc [9]. They can be derived from plants depends on the geographical location, type of plants used and their parts as (root, leave, steam bark and seed) and mostly the type methods of extraction and isolation.

Nowadays exciting antibiotics drug are endanger of losing their efficacy because of the increase in microbial resistance. Currently its impact is considerable with treatment failures associated with multidrug resistance bacteria and it has become a global concern to public health. For this reason discovery of new antibiotic is an exclusively important. Natural products are still one of the major source of new drug molecules today. Plant products occupy the major parts of the anti microbial compounds discovered until now [10].*C. anisata* is among the medicinal plant that has been commonly used by traditional people in some parts Africa and Ethiopia. Despite the wide usages of

this plant in traditionally for the treatment of various ailments, the phytochemical investigation of the stem bark of this plant and its antimicrobial activity has not been addressed. Isolation of chemical constituents, evaluation of the antibacterial and antifungal activities are the main purpose of this research project.

1.2 Statement of the problem

Plants are traditionally used for medicinal purpose and usually they are safe for human use because, they have long history of use by humans without major adverse effect [11].In most of the cases, these practices are handed down from generation to generation empirically without knowing the plausible mechanisms, safety, and efficacy of herbal treatments [12].In Jimma Zone Chora Botor woreda traditionally people use *C. anisata* to repel mosquito and for teeth cleaning. But they do not know what chemical constituent present in this plant species. Hence, this study was conducted to isolate and characterize compounds from *C. anisata* and to evaluate antimicrobial activity in order to know bioactive constituents of the plant to provide a rationale for the use of the plant in ethno-medicine or to provide some information on its constituents.

1.3 Objective of the study

1.3.1 General Objective

The main objective of the study was to identify secondary metabolites in the stem bark of *Clausena anisata* and to evaluate its antimicrobial activity.

1.3.2 Specific Objectives

1. To isolate secondary metabolites from the stem bark of *Clausena anisata* using chromatographic techniques.
2. To elucidate the structures of the isolated compounds using spectroscopic techniques, ^{1}H-NMR ,DEPT-135 and ^{13}C-NMR
3. To evaluate the antibacterial activity of crude extracts and isolated compounds against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis* bacterial strains and two fungal strain *Fusariam spp* and *Candida albicums*

1.4 Significance of the steady

To give information about the compounds (constituents) in the stem bark of *C. anisata* and to serve as a base line for the development of antimicrobial drug from these plant.

CHAPTER TWO

2. Literature Review

2.1The Botanical information

Rutaceae family, consisting of about 150 genera and over 1500 species, are distributed in both the tropical and temperate regions, especially in Australia and South Africa [13].These family dividedinto6subfamilies:Aurantioideae,Dictyolomoideae,,Rutoideae,Spathelioideae,andToddalioideae and a total of 25 tribes [14].Currently, about 30 species of the genus *Clausena* have been identified and are widely distributed over tropical and subtropical areas .Occurring mostly in India, tropical Asia and Africa [15] There are wide differences in the character of the growth and the height of the species; shrubs of 20 to 40 cm high in indo-china and reaching a height of 20 m in Africa. One of the most advantageous features of the species of this genus is their availability in the different parts of the world but few species of the genus *Clausena* have been explored and identified for their chemical and biological studies [16]

2.2 Description and Distribution Clausena anisata

Clausena anisata (willd) Hook (Family Rutaceae) is a small tree , about 10 m in height belonging to the Rutaceae family of plants in figure 2. The more descriptive Africans common name perdepis meaning horse urine because the crushed leaves has an unpleasant smell, characteristic of horse, urine),

Figure 2. *C. anisata* A and stem bark B taken from its natural habitat(pictured by ketema Abera)

It is abundant in forests and forest margins, riverine thickets and bushveld, from Western cape up the eastern coast of KwaZulu-Natal, eastern Zimbabwe, Mozambique northwards to Ethiopia and the Sudan and Westwards to Sierra Leone. The main stem is smooth, grey to light brown and is without thorns, the leaves alternate and crowded towards the ends of the branches in figure 3 each

leaf has 4-10 pairs of leaflets. The flowers have petals with numerous yellow stamens and the fruit are berries, initially are green , then reddish- black when fully ripe providing a source of nourishment to many bird and insect species [17]

2.3 Traditional uses and biological activity of *C. anisata*

C.anisata is used in treating an uncommonly wide range of ailments and conditions. Various parts of the plant are used alone or in association with other plants in folk medicine. The essential oils extracted from the fresh leaves of *C.anisata* has showed antibacterial activity [18]. In Nigeria, the mixture of *C.anisata*, *Afraegle paniculata*, and *Azadirachtha indica* are taken against gut disturbance [19]. In Tanzania, traditional healers are used *C. anisata* against oral candidiasis is and fungal infections of the skin [20].Crushed leaves are applied to open wounds, mouth infection south Africa [21].In some parts of Africa and in the Philippine the burning of fresh leaves are utilized to repel mosquitoes. The tree is used in African traditional medicine in the treatment of epilepsy, convulsions, arthritis, rheumatism, hypertension, heart failure, taeniasis, impotence sterility and the smoke from burning dried plants is used to repel mosquitoes [22].The boiled roots and leaves is used to wash mouth to alleviate toothache and to treat oral infection in India [23].The presence of essential oil in the *Clausena anisata* has been reported to be responsible for its several pharmacological activities such as antimicrobial, anti-diabetics properties which is also affirmed its folkloric usage [24].The acetone extract of leaf of *C. anisata* might be a potential control agent for flies encountered in cutaneous myiasis [25].Leaf extract of *C.anisata* has exhibited antioxidant, anti-diabetic and antibacterial activity [26].An aqueous leaf extract of *C.anisata* is used to reduce blood pressure [27]. *C. anisata* widely used in West Africa for the treatment of bacterial and fungal infections of the skin including boils ringworm and eczema. Presence of bioactive compounds in Ethanolic stem bark extract of *C.anisata* possesses antioxidant, anti-inflammatory, antimicrobial, insetifuge and cancer preventive [28]

2.4 Phytochemical constituents of the Genus *Clausena*

The genus *Clausena* has been reported to produce divers group of secondary metabolites including carbazole alkaloid, coumarins, limonoids , few phytosteriods and amine derivatives. The major secondary metabolites so far reported are carbazole alkaloids and coumarins.

2.4.1 Carbazole alkaloids

Phytochemical investigation of different species *Clausena* has led to the isolation of different classes of carbazole alkaloids. For instance from the roots of *C. guillauminii* carbazole alkaloids: fluroclausine(**1**) 7-hydroxyheptaphylline (**2)**, mukonal (**3**),7- methoxyl komal (**4**), and clauszoline (**5**) have been reported [29].Clausenal (**6**) from the leaves of *C. heptaphylla* [30] and two carbazole alkaloids mafaicheenaimne D(**7**), mafaicheen amine E(**8**) from the root of *C. lansium* are also reported [31].There are also different classes of dimeric carbazole alkaloids which are reported from the genus *Clausena*. For instance the girinimbine carbazole alkaloids (**9**) isolated from the roots of *C. anisata* [32], heptayline(**10**) also from roots *C. anisata* [33], dunnine A,(**11**) and others also isolated from the stems and leaves of *C. dunniana* [34] are some examples

2.4.2 Coumarins

Coumarins are also considered as the main constituents of the genus *Clausena* xanthyletin (**12**), dentatine (**13**) and nordentatin (**14**) are among reported from C. excavate [35].New monterpnoid coumarins from C. anisumolens (**15**) [36] and two coumarins xanthotoxin, (**16**) 3,10-bis(1,1-dimethyl prop-2-en-1-yl) -5,6,7-trihyroxy-8,8-dimetyl-7,8, 8-dihydro and pyrano chromen-2-one (**17**) [37] and furanocoumarins from stem of (**18**) [38] are also reported from C.anisata and *C.lansium*

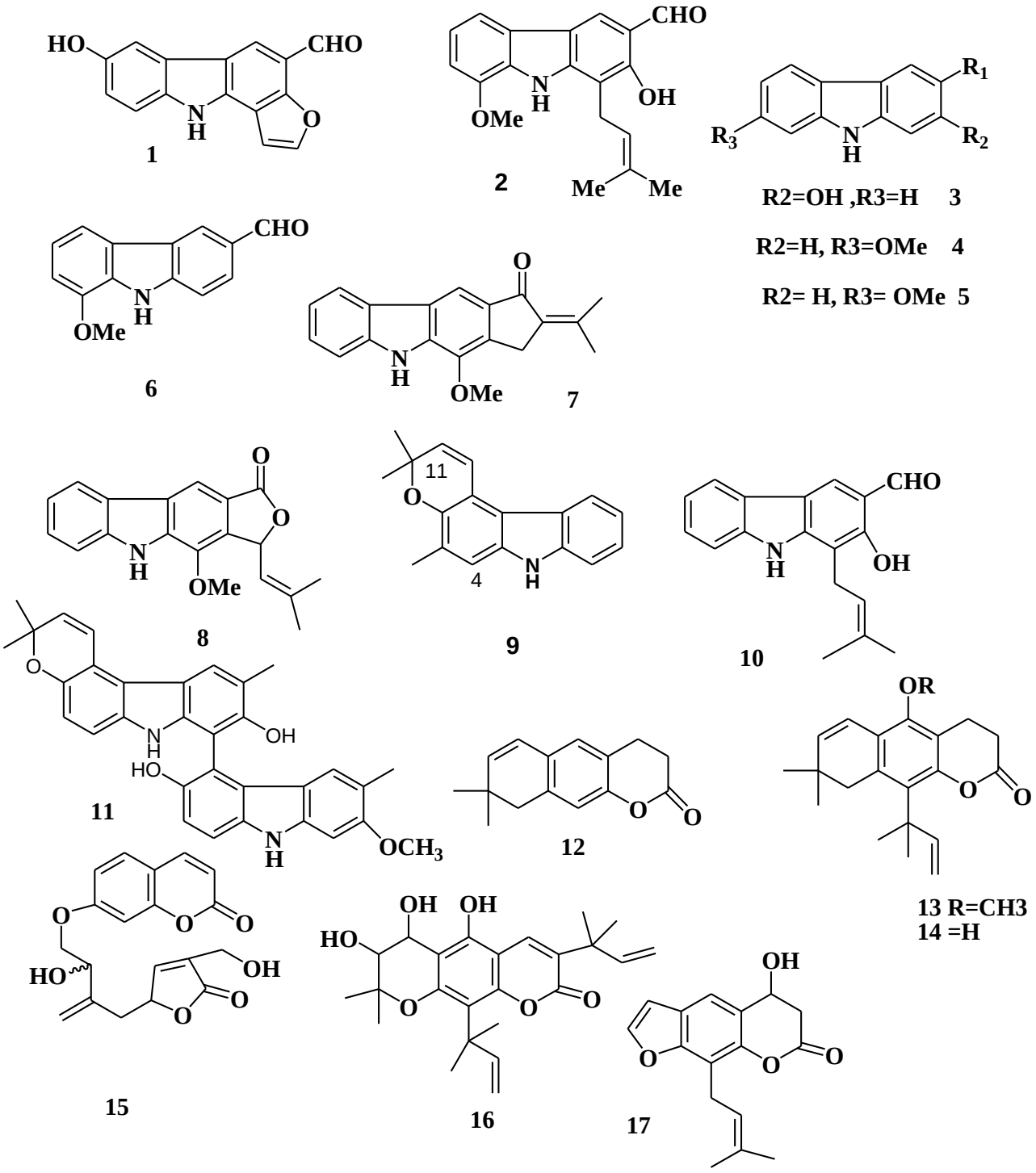


Figure 3. Structures of compounds isolated from genus Clausena

CHAPTER THREE

3. Materials and methods

3.1 Chemicals

The chemical used for this work were as analytical grade petroleum ether, chloroform, acetone , methanol , hexane and ethyl acetate (Loba Chemie Pvt Ltd , India), silica gel 60-120 mm mesh size(Loba Chemie, India) , Mueller Hinton agar and nutrient broth as culture media and DMSO were used for antimicrobial activity test.

3.2 Apparatus and Equipment

Rotary evaporator (Heidolph, Germany, laboratory 4000, No, 519-0000-00-2) for solvent evaporation, Uv-Tech (254 and 365nm)chamber for detection of spots on TLC plate, glass column chromatography (500mm, B-34/35) and Sephadex LH-20 for separation and purification techniques, Mortar and pestle for grinding round bottom flask of size 250,500, and 1000 mL measuring cylinder different volumes, falter papers, cotton swab, weighing balance (model NWT100001X) oven (N5OC GENLAB WIDNES, England) for drying purpose. Analytical TLC was performed on pre- coated silica gel 60 F_{254} plate (Sigma-Aldrich), ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Bruker ultra shield TM 400 to process NMR data at Addis Abeba University for characterization. The spectra were processed using MestReNova software, Incubator (Gene lab incubator), Hood (CLB-201-04, vertical laminar cabinet) for antimicrobial activity study.

3.3 Plant materials collection and Preparation

The stem bark *C.anisata* was collected from Oromia Region, Chora Botor Woreda 110 Km far from Jimma town. Identification of the plant was done by Botanist Dr. Dereje Denu and voucher specimenswas deposited at Botanical Science laboratory Herbarium biology department Jimma University. The stem bark *C.anisata* was air dried under shade and powdered to suitable size to improve the subsequent extraction by rendering the sample more homogenous, increasing the surface area, and facilitating the penetration of the solvent in to the cells. The study was carried out at Jimma University, Jimma Ethiopia.

3.4 Extraction

The air-dried 1 kg stem bark of *C.anisata* were grounded using mortar and pestle. It was extracted by sequential methods at room temperature. The extraction was carried out by using solvents of increasing polarity starting from petroleum ether, chloroform, acetone and methanol with 2.5 L of each solvent for period of 24 h. The extracts were filtered first with cotton plug followed by what man N0̲1 filter paper. The petroleum ether extract was dark green where as the rest extract were dark brown in color. The solvents were evaporated in Rotary evaporator from each of the extract to give semi solid crude extracts. The extracts were weighed after concentrated to give 4.97, 7.6, 4.05 and 4.54 gram of petroleum ether, chloroform, acetone and methanol, respectively.

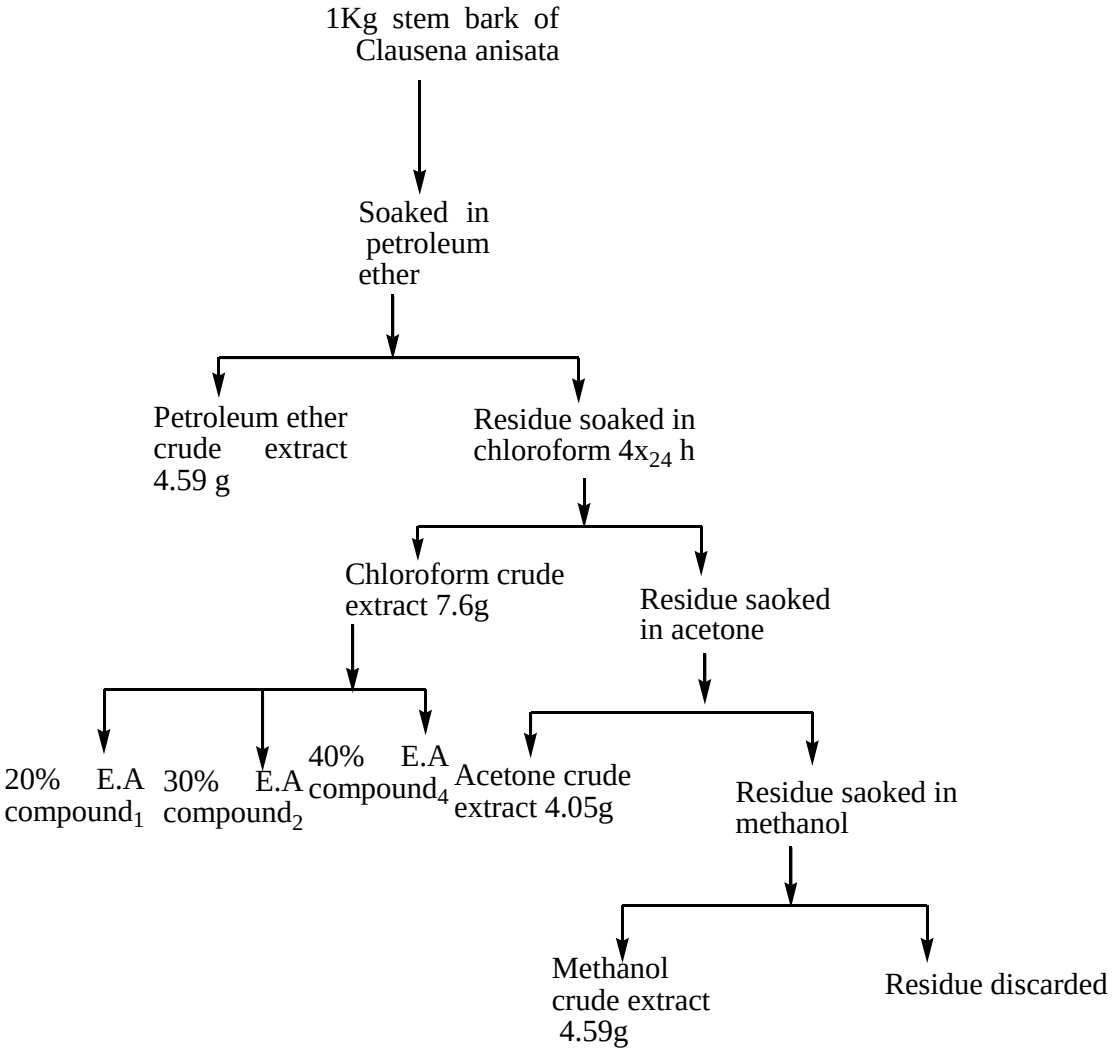


Figure 4 .Flow chart for extraction and isolation of compounds from stem bark of C. anisata

3.5 Isolation of compounds

Solvent selection was done for column chromatography by spotting dissolved sample solutions of the crude extract on TLC plate. Hexane and ethyl acetate combinations of different polarities were selected eluent solvents for Column chromatography .The packed column was stay for 24 h before loading the sample in order to attain uniform settlement. The chloroform crude extract 7.6 g was adsorbed on 100 g of silica gel and loaded on to packed column chromatography (500 mm). The column was first eluted with hexane as mobile phase with increasing polarity ethyl acetate in hexane from 0 to 100 % to provide 200 fraction, fraction similar Rf value were combined. Fractions 65-76 were combined to give 6.1mg. This was further purified by Sephadex LH-20 followed by washed with n- hexane to give afford compound **1** (4.5mg). Similarly fraction 120-122 (5 mg) were combined concentrated and washed repeatedly with n-hexane to afford compound **2** (3.5 mg) .Fraction 160-169 (3 mg) were combined concentrated and washed repeatedly with n-hexane to afford compound **4**. After isolated compounds were dried their TLC profile were re-checked and sent for characterization to Addis Abeba university. Characterization of isolated compounds were carried out using ^{1}H-NMR,^{13}C-NMR and DEPT-135

3.6 Antimicrobial assay

The test solutions of plant were prepared by dissolving 200 mg of each crude extracts and in 1mL of dimethyl sulfoxide (DMSO) to prepare 0.2 mg/mL stock solution of the test samples. The standard drug for antibacterial taste Chloramphenicol and antifungal taste Ketoconazole .The culture media was prepared by dissolving Muller Hinton Agar in distilled water and boiled to dissolve the media completely.

3.6.1 Antibacterial assay

Agar disk diffusion method was used to evaluate the antibacterial activity of both crude extract and isolated compounds on nutrient agar. The stock cultures were maintained on the nutrient agar slants which were stored at 4℃. Agar cultures of the test microorganisms were prepared according to manufacture instruction. The test solutions were prepared by dissolving 200 mg ratio of plant extracts to achieve final stock concentrations of 0.2 mg/ml in DMSO .Freshly prepared grown liquid culture of the test pathogens solutions were added over the Mueller-Hinton Agar medium with sterile swab. Filter paper pieces containing the test samples were put on Petri dish covered and incubated at 37 ℃ for 24 h.DMSO was used as a negative control and Chloramphenicol as

appositive control for each Petri dish. After the incubation clear zones were formed around each disk and measured in millimeter using ruler [39].

3.6.2 Antifungal assay

A disc diffusion method was applied to test the plants extracts against the test fungus using standard antifungal agent Ketoconazole as a positive control. The prepared culture media was autoclaved for 2 h at 121 °C temperature. After the culture media was solidified, 1 mL of the fungal solutions was uniformly added to it. Filter paper pieces containing the test sample were put on Petri dish and covered and incubated at 27 °C for 72 h. DMSO solvent was used as a negative control. Finally, the results were taken on the third day by measuring the diameter of zone of inhibition [40].

CHAPTER FOUR

4 .Result and Discussion

4.1 Extraction

About 1 kg of the dried stem bark of *Clausena anisata* was sequentially extracted on increasing solvent polarity for petroleum ether, chloroform, acetone and methanol for each 24 h and the amount calculated as percentage yield. Percentage yield is the ratio of mass of crude extract to mass of drained sample multiplied by 100 (equation 1). The highest amount of crude extracted was obtained from chloroform. After evaporating the solvent 4.97, 7.6, 4.05 and 4.54 g were obtained respectively for petroleum ether, chloroform, and acetone and methanol. Percentages yield of crude extracts can calculated and presented in Table 1.

$$\% \text{ yield} = \frac{weight\,of\,crude}{weight\,of\,sample} \times 100 \text{----------(1)}$$

Table 1 Percentage yields of each crude extracts

Types of solvents	Amount of the extract(g)	Percentage yields
Petroleum ether	4.97	0.497
Chloroform	7.6	0.76
Acetone	4.05	0.405
Methanol	4.54	0.454

4.2 Isolation of the Compound

The chloroform crude extract (7.6 g) was adsorbed on 100 g silica gel and applied on column chromatography already packed with silica gel. The column was eluted with hexane, with increasing gradient of ethyl acetate to afford 200 major fractions. Fractions 65-75 (20% EtOAc in hexane) were combined and concentrated to yields 6.1 mg which has yellow color and further purified in Sephadex LH-20 and final washed with hexane and afford compound **1 (** 4.5 mg) compound **2** (3 mg) whereas, compound **4** (2 mg) was isolated from fractions 120-122 (30%) and 160-169(40%)ethyl acetate in hexane respectively.

4.3 Structural elucidation of compound 1

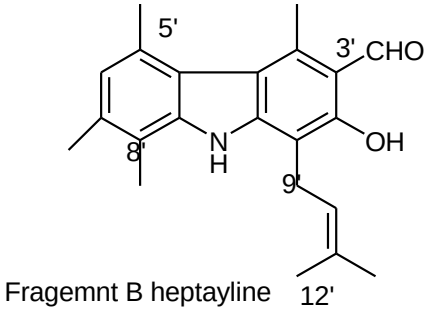
The ^{1}HNMR and ^{13}C NMR, spectral data of compound **1** established in Table 2 showed the presence of mixture of carbazole alkaloids . The ^{1}HNMR data spectrum indicated the presence of two , carbazole moieties, fragment A and B are showed in Figure 5. The data revealed and from literature, fragment B, has similar chemical environment with heptaphylline as showed by

resonance for a singlet hydroxyl (OH) proton at δ11.71 ppm (1H, s, OH). This hydroxyl protons shifted far downfield to δH 11.70 ppm indicated that it is adjacent to a very polar group resulting in inter hydrogen bonding with , aldehyde proton resonated at δH 9.93(1H, s, CHO). The broad singlet proton resonated at δH 8.22 for N-H (1H, br, s, N-H) was observed. The two aliphatic methyl protons at δH 1.93 (3H, s, H-12`and δH 1.80 ppm (3H, H-13`), the olefinic proton at δH 5.35 (H-10`, t ) and benzylicmethylene at δH 3.64(1H, t, H-9`) suggested the presence of a prenyl group in the compound. The single proton resonated at δH 8.04 (1H, s, H-4`) deshielded proton was an indication that the formyl (CHO) group is attached to the carbon atom adjacent to the carbon bearing the H-4` proton. The singlet proton δH 7.70 (1H, d, H-5`),δH6 (1H,s, 7.41)with two other strongly coupled *ortho* protons δH 7.95 (1H, d, J=8 Hz, H-7) and δH 8.00 (1H, d, J=8 Hz, H-8). This chemical environment is similar to that for heptaphylline [33]

The ^{13}C-NMR spectrum ($CDCl_3$,100 MHz) Table 2 in combination with DEPT-135 showed the presence of 18 carbons for the structures which is similar to heptaphyline. Those are six methine carbons, eight quaternary carbons, one benzylic methylene carbon, two methyl carbons, and one carbonyl carbon see structure (18). The carbonyl carbon at δC 195.4 (CHO at C-3`) and quaternary oxygenated sp2 hybridized carbon was observed at δ 157.8 (C-2`) whereas the aromatic methine carbons were observed at δ 125.9 (C-4`), 125.4 (C-7`), 119.8 (C-5`) (120.9 C-6') and 110.9 (C-8`) were revealed. Furthermore, the spectrum displayed signals due to quaternary carbons at δ 109.1 (C-1`), 115.5 (C-3), 117.4 (C-4a`), 123.7 (C-4b`) , 134,2 (C-11`), 140.2(C-8a`), and 145.1 (C-8b`) confirms the main heptaphyline carbazole alkaloids . The prenyl moiety attached to C-1` appeared at $\delta_C$ 22.9 (C-9), 121.2 (C-10), 134.2(C-11), 18.2 (C-14`) and δ 25.8 (C-13`), revealed from the $^{13}$C-NMR data (Table 2).The DEPT-135 spectra data revealed the methine (CH) carbons of heptaphyline resonated at δ (125.9 , C-4`), (125.4, C-7`), (119.8, C-5`), (110.9, C-8`). Thus, based on the above spectral data and comparison with literature report the structure of fragment B was found to be a of carbazole alkaloids known by the common name heptaphylline which is previously isolated from the root of *C. anisata* and *Clausena willichii* (figure 5, Table 2).

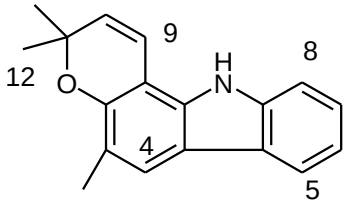
Thus, the 1H-NMR spectral data of carbazole A demonstrated the presence broad singlet at 7.89 indicate the presence of (1H, br, s N-H), and the four ABCD type protons δH 7.43 (1H, m, H-5), 7.22 (2H, t, H-7) and 7.38 (1H, m, H-8) with each two *ortho*-coupled protons resonated at δH 6.63 (1H, *d*, J=9.72, H-9) and 5.72 (1H, *d*, J=7.92, H-10) with two singlet methyl protons resonated at,

δH 1.56 (3H, s, 12-Me) and 1.48 (3H, s, 13-Me) indicated that the structure of the fragment A was girinimbine. The presence of chromene ring was confirmed by ^{13}C-NMR resonance at δC 149.8 (C-2), 129.4 (C-10), 117.3 (C-9), 75.9 (C-11), 27.6 (C-13) and 27.6 (C-12). Further, analysis of carbazole A from ^{13}C-NMR and DEPT-135 showed the presence of 18 carbons which consisted of eight quaternary carbons δC 104.4, C-1), (149.8, C-2), (123.9, C-3), (116.8, C-4a), (118.6, C-4b), (134.8, C-8a), (139.4, C-8b) and δ (75.9, C-11) with three methyl carbons δC (27.6, C-12), (27.6, C-13), (16.1, C-14) and seven methine carbons (119.3, C-5), (119.5, C-6), (123.9, C-7), (124.2, C-8), (129.4, C-9), (117.2, C-10) was observed from DEPT. Based on the above analysis and comparison with literature reports the structure of carbazole A was girinimbine group [45] Thus, the proposed structure of isolated compound 1 is mixture of two type of carbazole alkaloids heptayline and girinimbine which is previously isolated from *C. anisata*



Fragemnt B heptayline

(18)



Fragment A, girinimbine

(19)

Figure 5 Proposed structure of compound 1 is mixture of the two fragment girinimbine and heptayline.

Table 2 ^{1}HNMR and ^{13}CNMR spectra data of compound **1**

Position	^{1}H-NMR compound **1**	^{13}C-NMR	^{1}H-NMR Literature	^{13}C-NMR Literature	
1	-	104.5	-	104.5	[32]
2	-	149.8	-	149.8	
3		123.9	-	123.9	
4	-	121.3	7.66 (1H, s)	121.1	
4a	-	116.8	-	116.7	
4b	-	118.6	-	118.6	
5	7.43 (1H, m)	193.3	7.92 (1H, d, J= 8.28)	193.3	
6	7.22 (1H, t, J=7.36,	119.5	7.19 (1H, t, J=8.82)	119.4	
7	7.22 (1H, t, J=7.36,	124.3	7.31 (1H, t, J=8.24)	124.2	
8	7.38 (1H, m)	110.4	7.36 (1H, d, J= 8.28)	110.4	
8a	-	134.8	-	134.8	
8b	-	139.	-	139.4	
8-NH	7.89 (1H, brs, N-H)		7.84 (1H, br, s)	-	
9	6.63 (1H, d, J=9.72)	117.3	6.60 (1H, d, J=9.2)	117.2	
10	5.72 (1H, d, J= 9.72)	129.4	5.69 (1H, d, J= 9.2)	129.4	
11	-	75.8		75.8	
12-CH3	2.38 (3H, s)	27.6	2.33 (3H, s)	27.6	
13-CH3	1.18 (3H, s)	27.6	1.56 (3H, s)	27.6	
14-CH3	1. 53 (3H, s)	16.1	1.48 (3H, s)	16.1	
1`	-		-	109.0	[33]
2`	-	157.9	-	157.9	
3`	-	115.5	-	115.4	
4`	8.04 (1H, s)	129.9	8.04 (1H, s)	125.9	
4`a	-	117.4	-	117.3	
4`b	-	123.7	-	123.6	
5`	7.70 (1H, s)	119.8	7.97 (1H, d, J=7.8)	119.8	
6`	7.41(1H, s)	120.9	7.41(1H, s)	120.9	
7`	7.95 (11H, d, J=8 Hz)	125.4	7.37-7.41 (1H, m)	125.3	
8`	8.00 (1H, d, J=8Hz)	110.9	7.37-7.41 (1H, m)	110.9	
8`a	-	140.2	-	140.1	
8`b	-	145.1	-	145.0	
8`-NH	8.22 (1H, brs, N-H)	-	8.23 (1H, brs, N-H)	-	
9`	3.66 (2H, d, J= 8Hz)	22.9	3.64 (1H, d, J=6.9 Hz)		
10`	5.35 (1H, t, J=8, 6 Hz)	121.2	5.31 (1H, t, J= 6.9)	121.2	
12`-CH3	1.81 (3H, s)	25.8	1.77 (3H, s)	25.7	
13`-CH3	1.93 (3H, s)	18.2	1.90 (3H, s)	18.1	
14`-CHO	9. 92 (1H, s, H-CHO	195.5	9.91 (1H, s, H-CHO)	195.4	
2`-OH	11.71 (1H, s, OH)	-	11.65 (1H, s, OH)	-	

4.4 Structural elucidation of compound 2

The characterization of compound 2 is similar to that of compound girinimbine. Thus, the ^{1}H-NMR spectral data of compound 2 demonstrated the presence broad singlet at 7.89 indicate the presence of (1H, br, s N-H), and the four ABCD type protons δH 7.43 (1H, m, H-5), 7.22 (2H, t, J 7.36, 7.36) and 7.38 (1H, m, H-8) with each two *ortho*-coupled protons resonated at δH 6.63 (1H, *d*, J=9.72, H-9) and 5.72 (1H, *d*, J=7.92, H-10) with two singlet methyl protons resonated at, δH 1.56 (3H, s, 12-Me) and 1.48 (3H, s, 13-Me) indicated that the structure of the compound **2** was girinimbine.

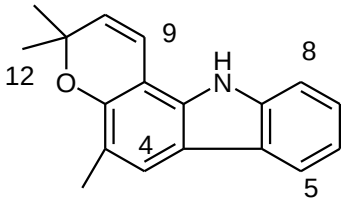


Figure. 6 Proposed structure of compound

4.5 Antibacterial activity

The petroleum ether, chloroform, acetone and methanol crude extracts were evaluated for antibacterial activity on four bacterial strains; *S. aureus* (ATCC25923),*B. subtilis* (ATCC6633),*E. coli* (ATCC25922) and *P. aeruginosa* (ATCC27853).The disk diffusion method was used and the zone of growth inhibition of each crude extracts were measured in millimeter (mm) compared to the standard positive control, Chloramphenicol and negative control DMSO.

Table 3.Antibacterial activity of crude extracts from *C. anisata*.

	Diameter zone of inhibition in (mm)			
Test sample	*E. coli* (-)	*P. aeruginosa* (-)	*B.subtilis* (+)	*S.aureus* (+)
Petroleum crude	7	7	8	11
Chloroform	8	9	15	12
Acetone	NI	NI	NI	10
Methanol	8	8	8	10
Chloramphenicol	31	20	16	18
DMSO	NI	NI	NI	NI

4.6 Antifungal activity

The activity of the plant extracts were tested against two fungus strains *Candida Albicums* and *Fusariam spp*. These standard fungal strains were obtained from Department of Biology, Jimma University.

Table 4 .Antifungal activity of crude extracts

Test sample	Diameter of zone inhibition of in mm	
	Fusariam spp	*Candia Albicums*
Petroleum ether	11	8
Chloroform	13	12
Acetone	NI	NI
Methanol	NI	NI
Ketoconazole	10	8
DMSO	NI	NI

The chloroform crude and petroleum ether crude extract is active against the tested fungus *Fusariam spp* and *C.albicums* but the methanol and acetone crude extract were not active

4.7 Spectroscopic data of the isolated Compound 1

^{1}H-NMR (400MHz, $CDCl_3$) δ(ppm): fragment B δH 8.22(1H, s, 9-H), δH 11.71 (2-OH ,s), δH 9.93 (3-CHO,s), δH 8.04 (H-4) δH 5.35 (1H, t , H-2'), δH 3.64 (2H, d, H-1'), δH1.91 (3H, s, H-4') and δH1.80 (3H, s, H-5'), δH 7.24-7.28 (1H, m, H-6) and δH7.37-7.41(2H, m, H-7 and H-8) ^{13}C-NMR (100 MHz 400 $CDCl_3$), δ(ppm) : 110.9 ,157.8, 115.4, 117.4 , 125.4, 119.8 , 140.1 , and 145.0 which were assigned to C-1,(C-2), C-3, C-4a, C-4b, C-5, C-8a, and C-9a respectively, δC-4 (125.9) , δC-6 (123.7) , δC-7, (120.9) , δC-8 (110.4) , δC-1' (25.8) , δC-2' (121.2) , δC-3' (134.2), δC-4'(22.9) , δ C-5'(18.2) and δ CHO (195.4) .^{1}H-NMR (400 MHz, $CDCl_3$) δ(ppm): fragment A δH 7.84 (1H, s, N-H), δH 2.33 (3H, s, 12-Me) ,δH1.48 (3H, s,14-Me), δH1.56 (3H, s,13-Me), δH 7.36 (H-8',d), δH7.66 (1H,s,H-4),δH 6.63(H-9, d,) ,δH 5.72 (H-10 , d) , δH 7.31 (t, H-7'), and δH7 .19 (t, H-6') ^{13}C-NMR (100MHz 400 $CDCl_3$), δ(ppm) : δC-1'(104.5), δC-2'(149.8), δC-3'(123.9), δC-4'(121.3), δC-4a'(116.8), δC-4b'(118.6),δC-5'(119.3) ,δC-6'(δ119.4), δC-7'(124.3), δC-8'(110,4), δC-8a'(134.8), δC-9a(δ139.4),δc-10(117.3), δC-11(129.4), δC-12(75.9), δC-12 (27.6), δc-13(27.6) δC-14(δ 16.11

CHAPTER FIVE

5. Conclusion and Recommendation

5.1 Conclusion

Chromatography separation of crude extract of chloroform led to isolation of mixtures of two carbazole alkaloid. The structures of these compounds are determined from ^{1}H-NMR ^{13}C-NMR with standard literature reports. Antimicrobial activity of the stem bark of *C.anisata* crude extracts were tested based on the four bacterial and two fungal strains. The two crude extracts of chloroform and petroleum ether extracts showed considerable bacteria growth inhibition compared to the positive control Chloramphenicol for antibacterial and Ketoconazole for antifungal.

5.2 Recommendation

- In this work isolation was done form chloroform extract, thus isolation should be done form other fraction in order to conform the presence of other phytochemicals from stem part of this plant.
- Further phytochemical investigation should be take place in order to confirm the diverse structures of Clausenlines compounds.
- Further studies should be conducted on fruit, leave and on root part of *C. anisata* to isolate, purify and identify bioactive principles responsible for antibacterial and antifungal activities of the plant.
- The antimicrobial activities of the plant should also be tested on other microbial species which were not addressed by this study.
- Alternative method of extraction and isolation should be done well as insecticide activity of this plant is recommended for further researchers.
- Conservation of the plant family should be recommended for further human usage and scientific study.

Reference

1. Limenew, A.; T.M. Phytochemical screening and peroxide value determination of methanolic extract of four traditional medicinal plants from Debre Tabor Town, Ethiopia. *Journal of Medicinal plants Research.***2018**, 12 , 203-208.
2. Neelan, B. K.; D. M. Natural product source of potential drugs. *Africa Journal of Basic and Applied Science*. **2014**, 6, 171-186.
3. Daniel, D. ; Sylvia, U. R. A historical overview of natural Products in Drug discovery. *Metabolites* .**2012**, 2 , 303-336.
4. Batista, O.; Simoes, M. F.; Duarte, A.; Vadeira, M. L.; Dela, M .C.; Rodriguez, B. Antimicrobia from the roots of *Plectranthu shereoensis*. *Phytochemisrty*.**1995**, 38, 167-169.
5. Ghulam, A. A.; Sumaira, A. J. Bioactive compounds from medicinal plants and their importance in Drug discovery in Pakistan .*Matrix Science Pharmacetical*.**2017**, 1,17,26.
6. Kebede, T. B. ;D. D. Ethnobotanical study of medicinal plants in Akaki district, East Shewa Zone, Oromia regional state Ethiopia. *Journal of medicinal plants studies*. **2017**, 5 , 353-360.
7. Abdul, M. G. ; Syed, M. N. ; Rukhsana, A. Phytochemical Analysis of medicinal plants occurring in local Area of Mardan. *Biochemistry and Analytical Biochemistry* .**2013**, 2, 2161-1009.
8. Daniel, D.; Sylvia, U. R. A Historical overview of natural products in Drug discovery. *Metabolites* .**2012**, 2 , 303-336.
9. Zoemerculieff, S. R. ; Seethalakshimi, S. V. ; Gantis, M. R. Phytochemical , antioxidant and antibacterial. *Potential of Elaeagnus Kologa leaf Asian pacific Jurnal of Tropical medical* .**2014**, 7, 150-155
10. Mounyr, M .S.; S. K. Methods for in vitro evaluating anti microbial activity. *Journal of pharmaceutical Analysis*. **2016**, 6, 71-79.
11. Vermani, G. S.; Herbal medicines for sexually transmitted diseases and AIDS. *Journal of ethno pharmacology* .2002, 80, 49-66.
12. Mujumdar , M. V. ; Salaskar , U. S. Antidiarrheal effect of an isolated fraction of *Jatrophacurcas* roots in mice. *Journal natural remedies.*2001, 1, 89-93.

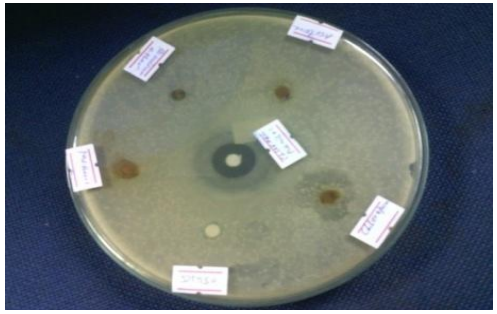
13. Roy, A. H.; R. Systematic study and medicinal uses of Rutaceae family of Rajshahi district, Bangladesh.2016, 5 , 26-32
14. Thomas, A. phylogeny of the Rutaceae and abiogeographic study of its subfamily Aurantioideae. 2010 ,15, 1372-3.
15. Shan-Shan , Y. W. ; Zhen, Z. Z. ; Ju-Qin , X. P. ; ,Zhu-Feng, S. D. essential oils from *Clausena* species in China: Santalene Sesquiterpenes Resource and Toxicity against Liposcelis bostrychophila.**2018**, 8, 1-2.
16. Ismail, A. A.; Mohamed, R. A.; Si , Mohammed, L. Z . A review of traditional uses, phytochemical and pharmacological aspects of selected members of *Clausena genus*. *Journal of Medicinal Plants Research*.**2012**, 6,107-118.
17. Mkhombo, The effects of *Clausena anisata* leaf extracts on selected diabetic related carbohydrate metabolizing enzymes.**2010**, 27-28
18. Venugopalan, A. S. Phytochemical analysis and Antibacterial Activity of the Essential Oil of *Clausena anisata* (Willd.) Hook. f. ex Benth. International Journal of Integrative Biology. **2009**, 5, 116-117.
19. Uwaifo , The mutagenic ties of seven coumarins derivatives and a furan derivative isolated from three medicinal plants. Journal Toxicol Environ Health .**1984**, 13, 521–530.
20. Winisia, M.; L. J.; Chauka , M. C. Antimicrobial and Cytotoxicity Activity of *Clausena anisata, Acokantherashemperii* and *Oleaeuropaea* growing in Tanzania. European *Journal of Medicinal Plants*.**2016**, 14 , 1-9
21. Olufemi, P. ; Ayelaagbe, O. O.; Hong, C. W. Identification, isolation and characterization of the antifeedant constituent of Clausena anisata against (Lepidoptera: Noctuidae), *Insect Science* .**2009**, 16, 247–253.
22. Adesina, S. K.; Adewunmi, C. O. Molluscicidal agents from the root of *Clausena anisata*. *Fitoterapia*, .**1985**, 56**,** 289-292.
23. Lawal, I. O.; Grierson, D. S.; Afolayan, A. J. Phytochemical and antioxidant investigations of a Clausena anisata hook, a South African medicinal plant. *Africa Journal of Traditional Complement Altern Medical*. **2015**, 12, 28-37
24. Mukandiwa, A.A.; Eloff, N. V. Isolation of seselin from *Clausena anisata* leaves and its effects on the feeding and development of Lucilia cuprina larvae may explain its use in ethno veterinary medicine.**2012**, 5, 60-57.

25. Lillian, J. N. ;Eloff, S.; V. N. Acetone extract of *Clausena anisata* may be a potential control agent for flies encountered in cutaneous myiasis. **2016**, 83,1-7
26. Arsia, ,N .B.; Deepa N. S. Study of invitro antioxidant, ant diabetic and antibacterial activity of *Clausena anisata* leaf extracts. *International journal of research in pharmacology and pharmacotherapeutics*.**2014**, 3 ,112-125.
27. Ntam. M. D.; Paul , J.S., Leon, L. B.; Melvin, M. G. The effect of an aqueous leaf extract of *Clausena anisata* on blood pressure, urine output, angiotensi II levels and cardiac parameters in spontaneously hypertensive rat. *Journal of Medicinal plants research*.**2016**, 10 ,425-434
28. Yakoob, A. T.; Tajuddin, N.B.; Mathew, S.; Hussian, M. M. QadrilI. GC-Ms Analysis of Ethanolic Stem Extract of *Clausena anisata Pharmacognosy Journa*l .**2016**, 8, 576-578
29. Chiramet, S. L.; Kongkiat, S. G.; Pyne, T. R .Carbazole alkaloids and coumarins from the roots of *Clausena guillauminii , Phytochemisy letters*.**2014** , 9, 113-116.
30. Charaborty,C, C. S.; Podder, K.; Chowdhury, P. B. Carbazole alkaloid with antimicrobial activity from *Clausena heptaphylla. Phytochemistry*. **1995**,38,787-789.
31. Wisanu, S. L.; Thuwadee, S. C. Carbazole alkaloids from *Clausena lansium* roots *Phytochemistry Letters*.**2012**, 5, 26-28.
32. Jules, K.; E ,Theophile, R. W. Chemical constituents from stem bark and roots of *Clausena anisata, Molecules*. **2012,**17,13673-13686.
33. Dandena , M .E. Antibacterial Activity of Coumarins and Carbazole Alkaloid from Roots of *Clausena anisata.*2019, 8, 4-5
34. Cao, N.; Chen, Y.; Ma, X.; Zeng, K.; Zhao, M.; Tu, P.; Li, J.; Jiang, Y. Bioactive carbazole and quinoline alkaloids from *Clausena dunniana*. *Phytochemistry.* **2018**, 151, 1–8
35. Hidayati, S. M. ; Yun, Mohd, G. C. L. Cytotoxicity constituents of *Clausena excavate*. *Africa Journal of Biotechnology*. **2011**, 10, 16337-16341.
36. Faiza, M. D.; Abdullah, M. A. 7-Methoxy-8-Prenylated Coumarins from *Murraya Koenigii* Ructacea family. **2016**, 15 , 155-159.
37. Javed, M. A. Coumarins from the root of *Clausena pentaphylla*. *Jornal of pharmaceutical sience*.**2008**, 33, 67-70.
38. Hangliu, F. C. ;Jing, L. ; Nai, D. M.. Bioactive furanocoumarins from stem of *Clausena anisata. Phytochemistry*.**2014**, 17, 141-147.

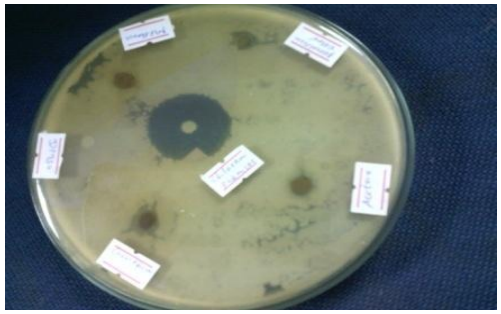
39. Mounyr, M. S.; K .I. Methods for invitro evaluating antimicrobial activity. *Journal of Pharmaceutical Analysis*.**2016**, 6, 71-79.
40. Machesney, Clark , S. R. . Antimicrobial diterpenes of Croton sonderianus, hardwickic and 3, 4-secotrachylobanoic acids. Journal of Natural Products.1991, 54, 1625-1633.

Appendices

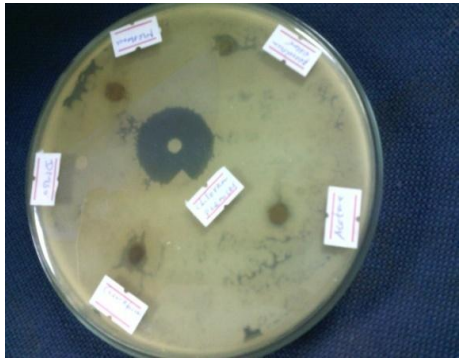
Appendix 1. Bioassays Tests of Crude Extracts Zone of Growth Inhibition in mm antibacterial



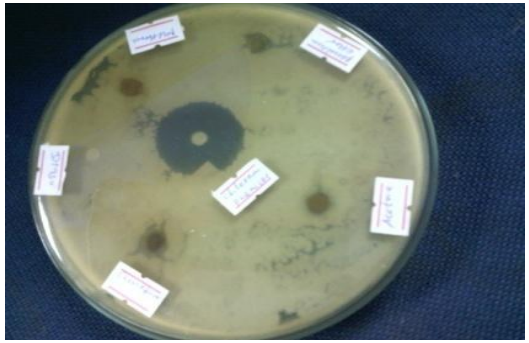
Bacillus Subtilis (+)



Staphylococcus aureus(+)

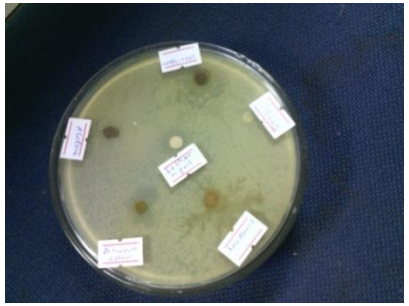


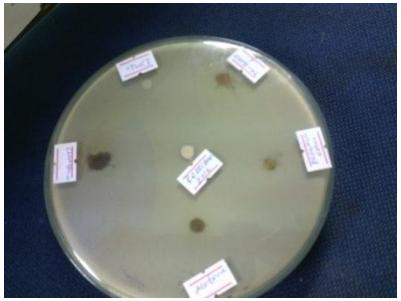
Escherichia coli(-)



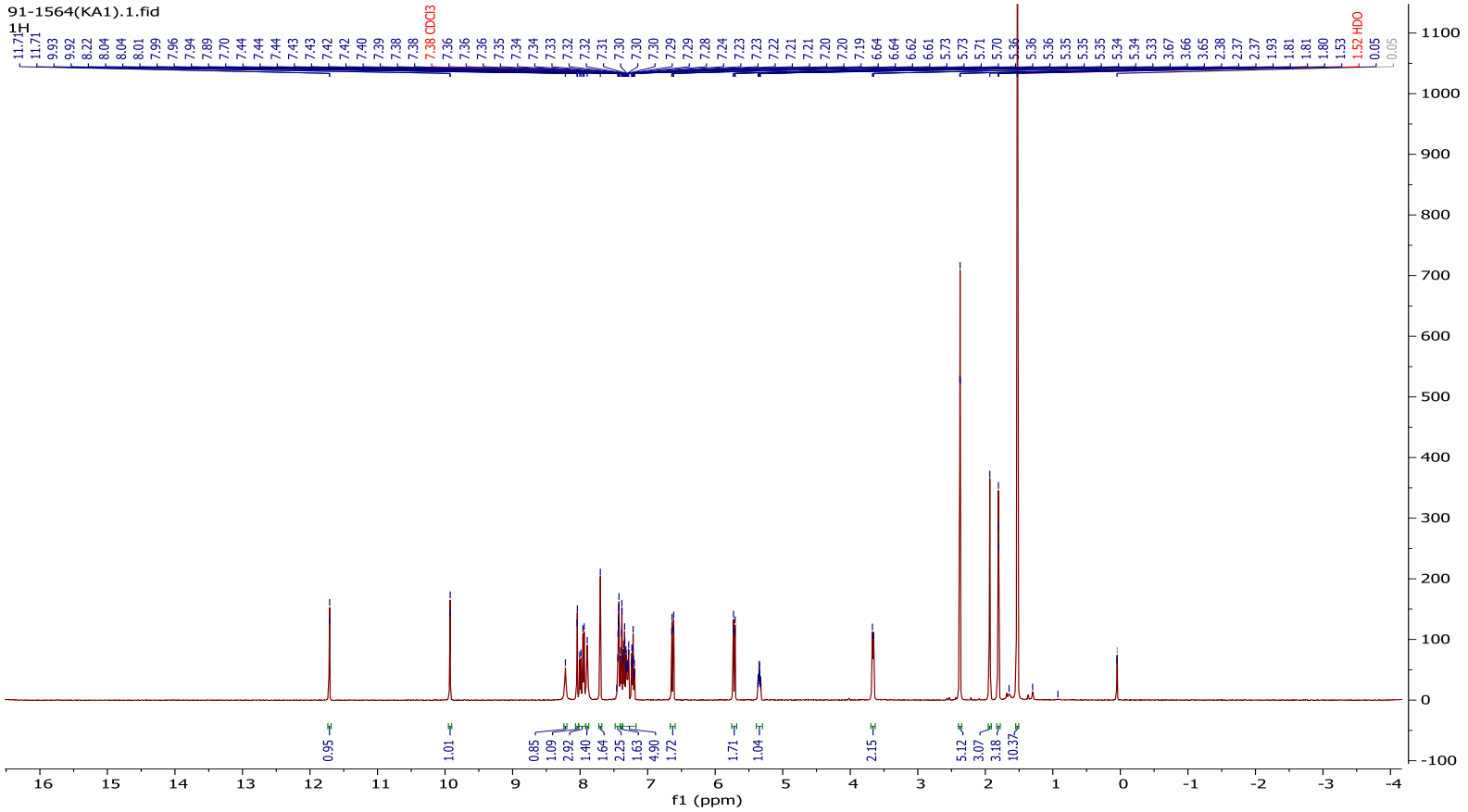
Pseudomonas aeruginosa, (-)

Appendix 2. Bioassays Tests of Crude Extracts Zone of Growth Inhibition in mm anti fungal

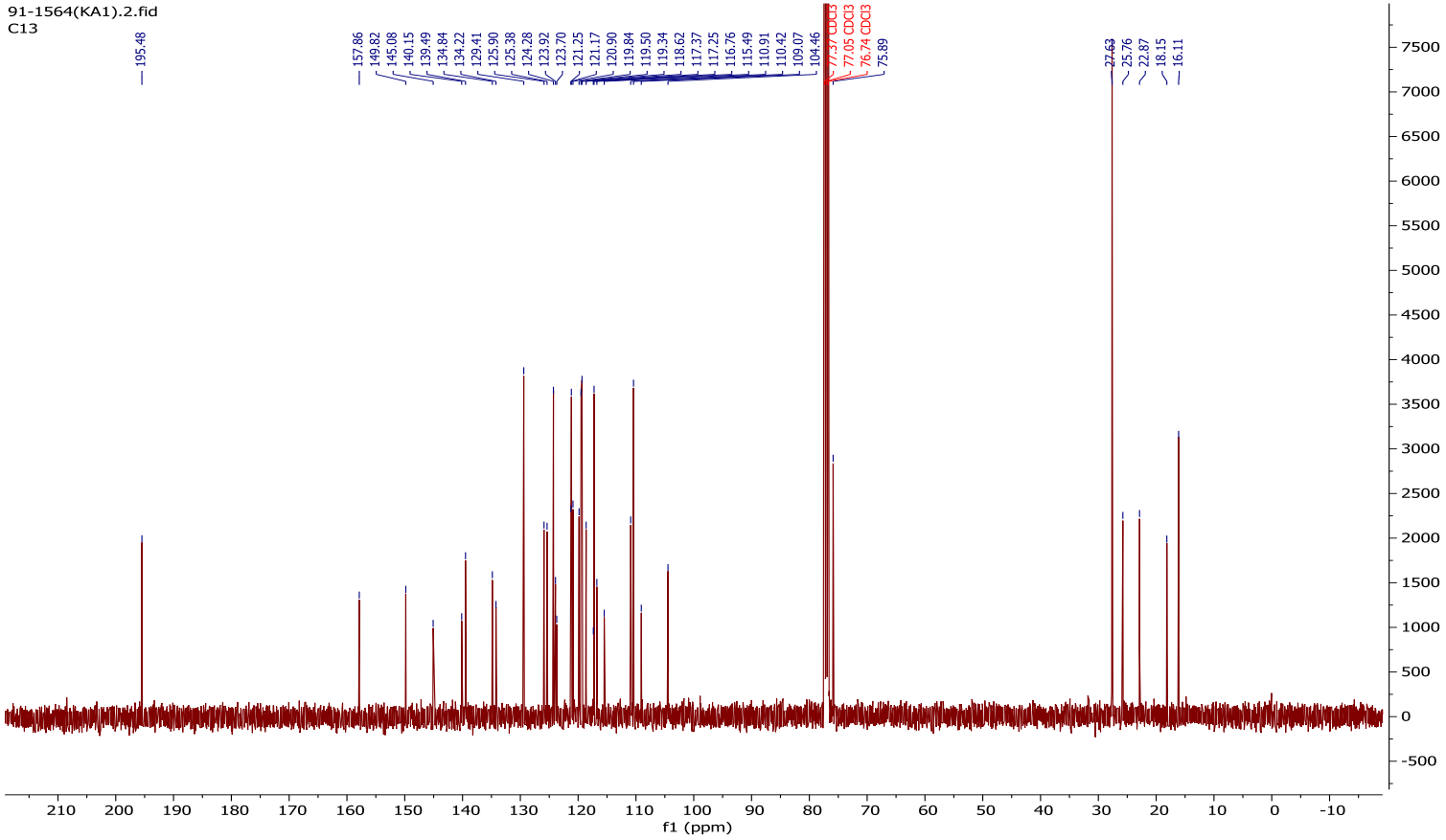




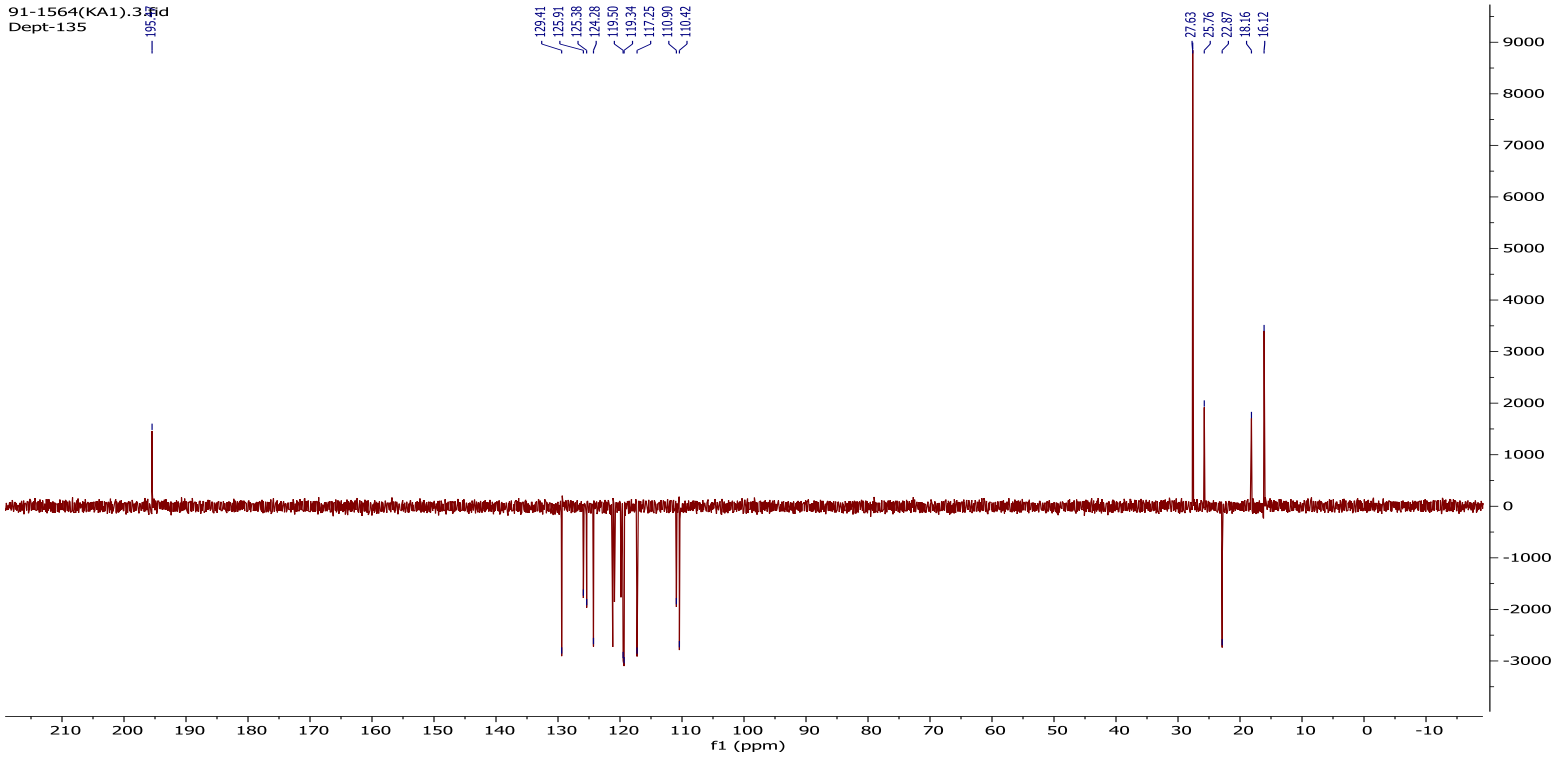
Appendix 3.[1]H-NMR Spectrum of compound **1**



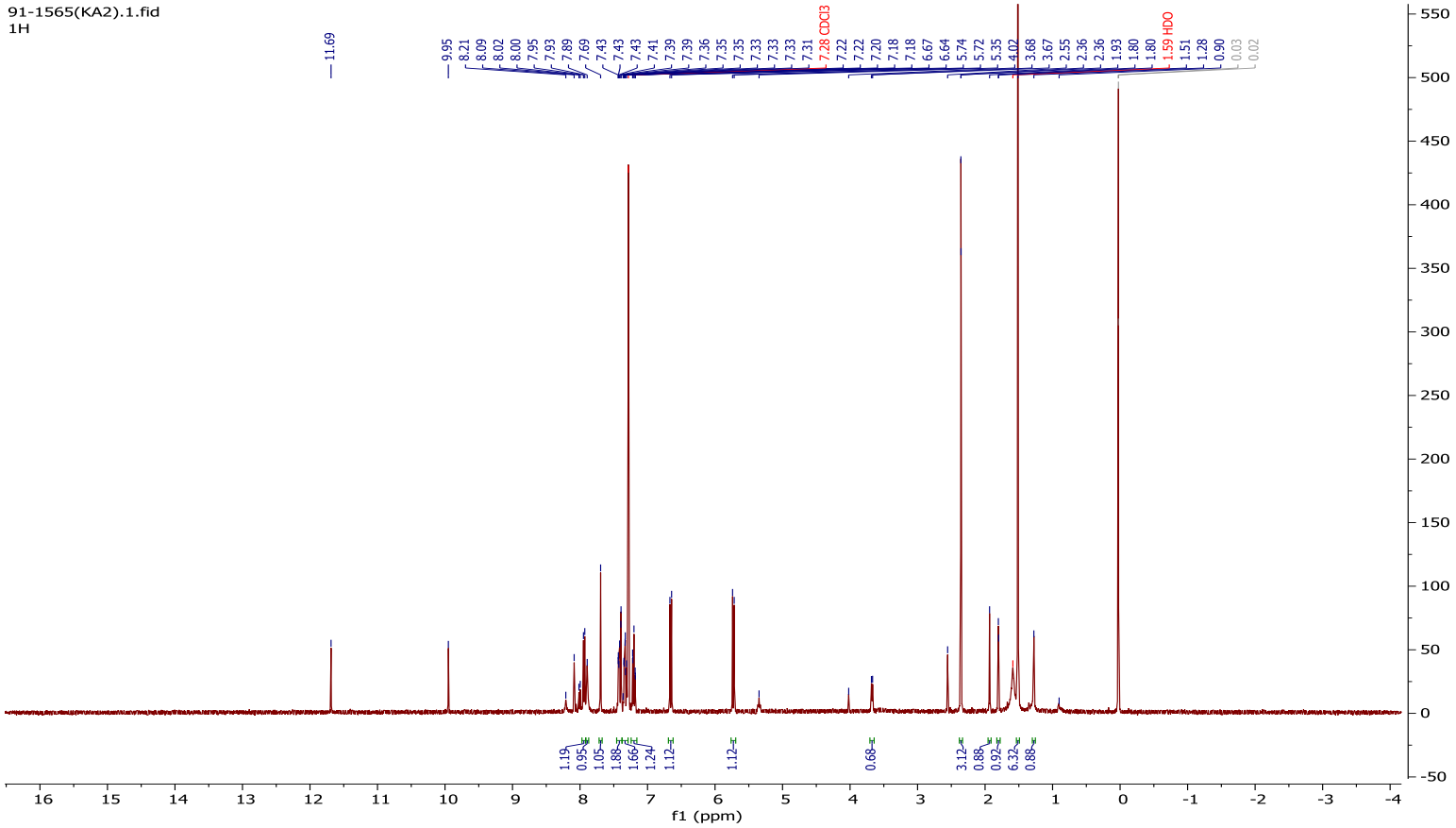
Appendix 4.[13]C-NMR Spectrum of compound **1**



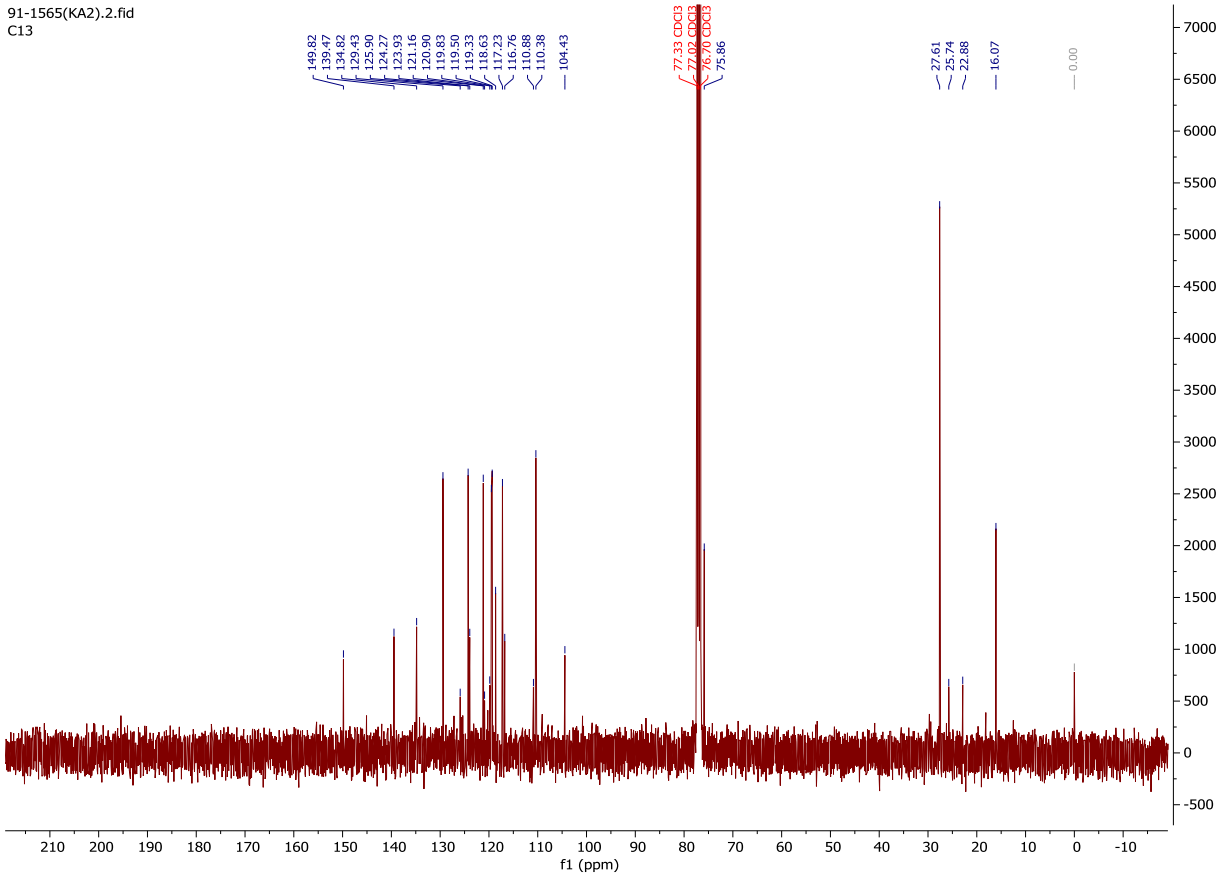
Appendix 5 . Dept-135 Spectrum of compound **1** in $CDCl_3$



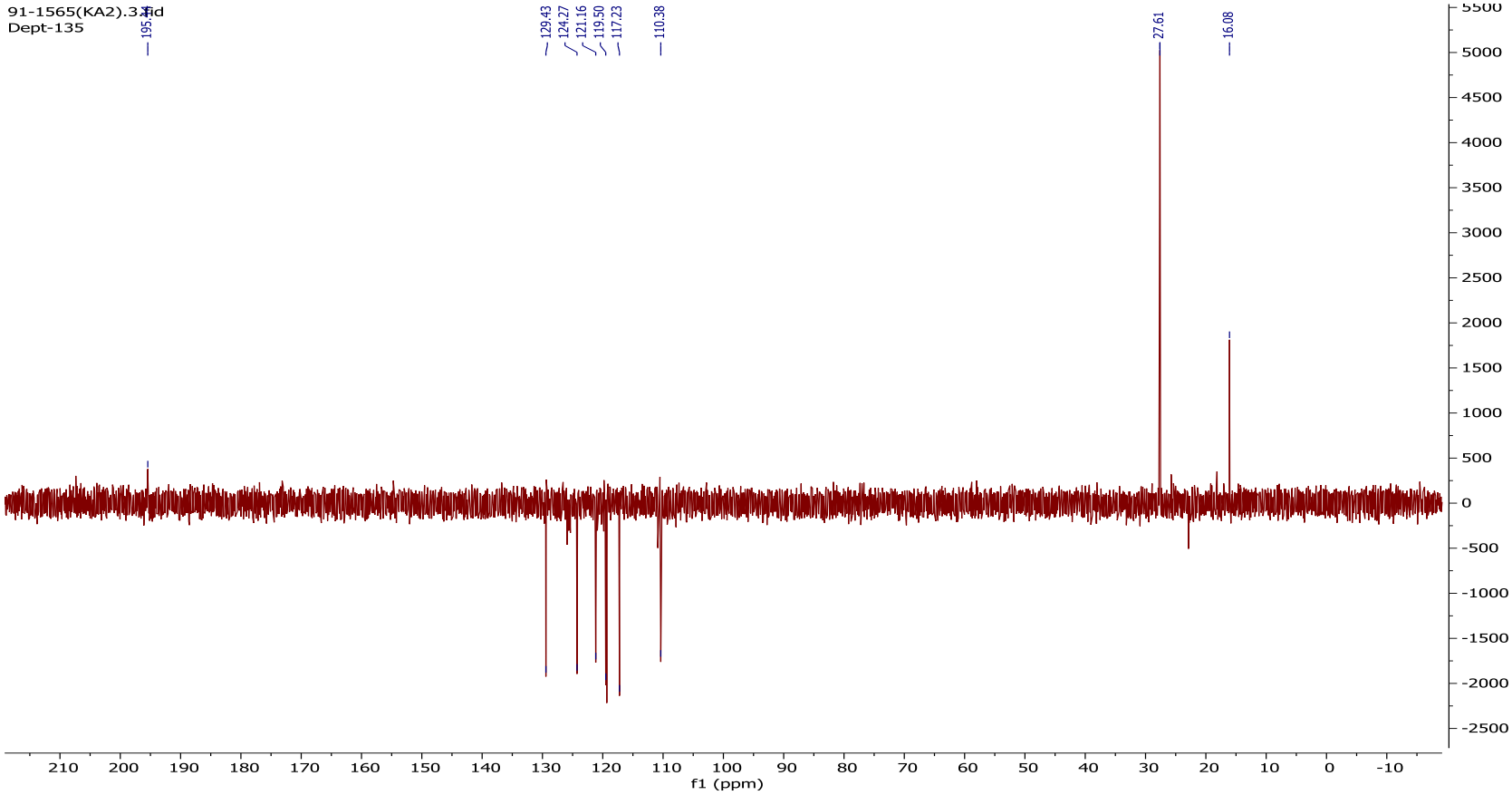
Appendix 6. ^{1}HNMR Spectrum of compound **2**



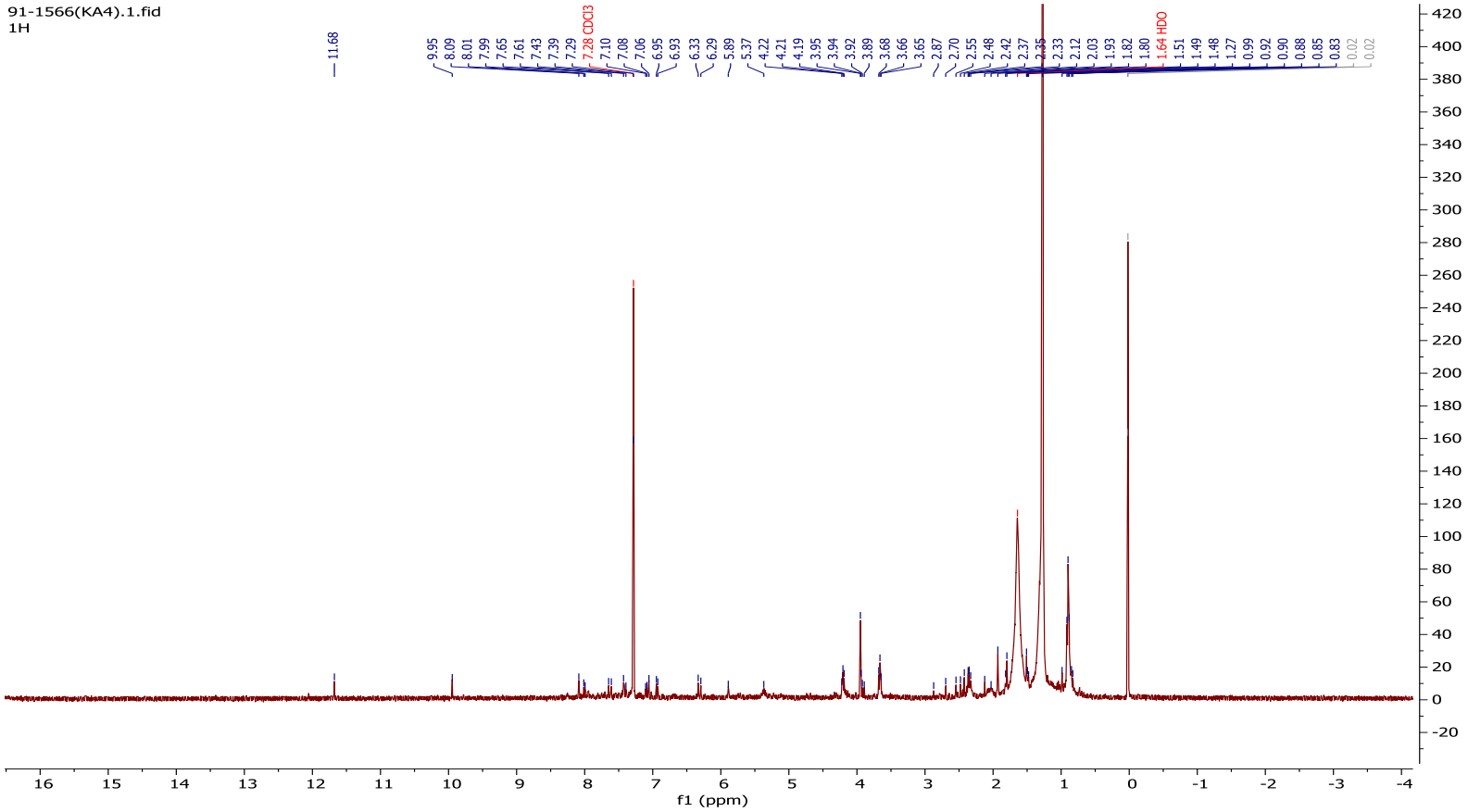
Appendix 7. ^{13}C-NMR Spectrum of compound **2**



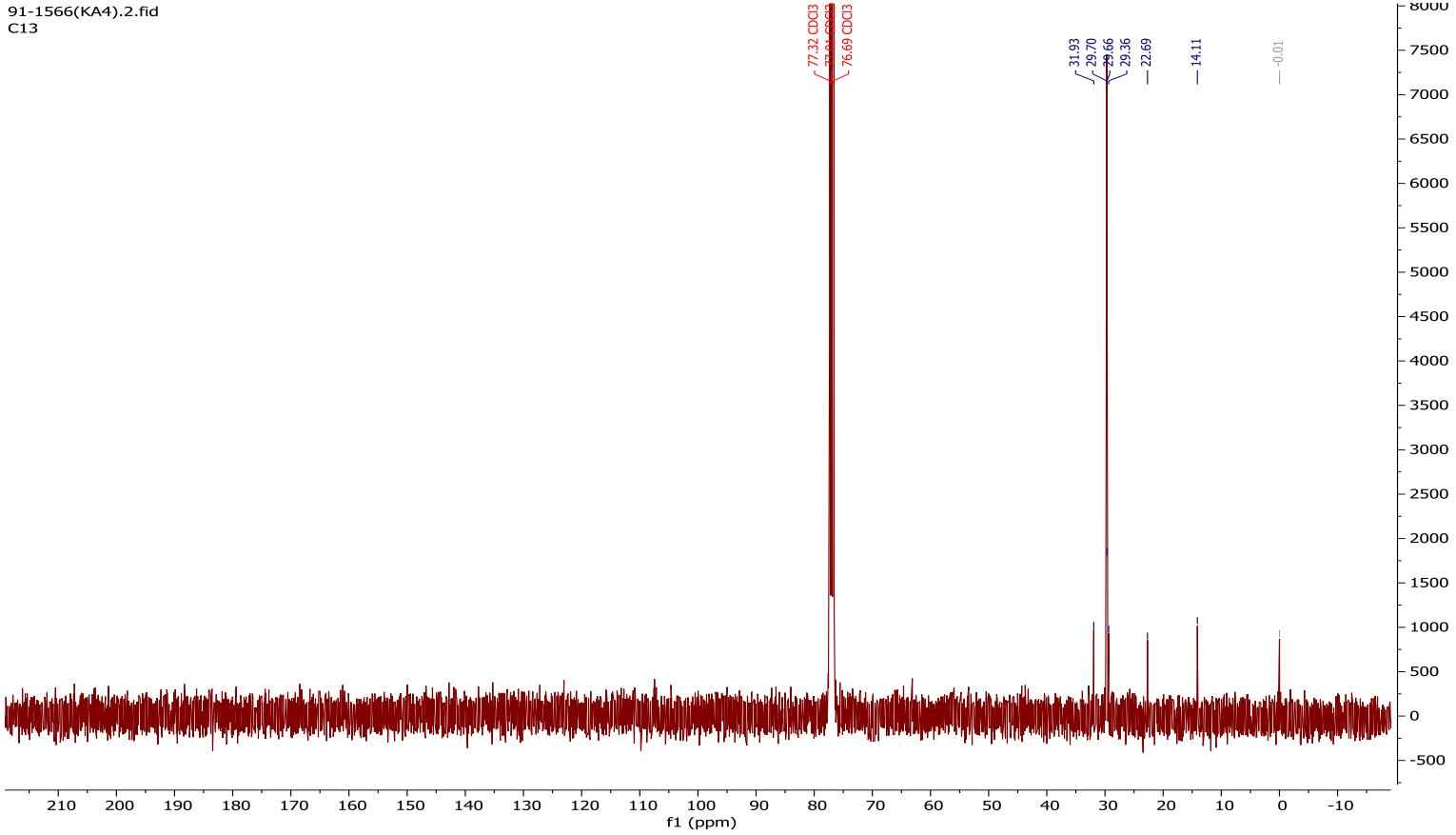
Appendix 8. Dept-135 spectrum of compound **2**



Appendix 9. ^{1}H-NR Spectrum of compound **4**



Appendix 10.[13] C-NMR of compound **4**



Appendix11.Dept-135 of compound **4**