Print ISSN 2710-0200 *Full text available online at* *Ethiop J Nat Comp Sci.*
Electronic ISSN 2710-0219 *http://journal.uog.edu.et/index.php/EJNCS* *Vol. 2 (2) 327-341*
***https://doi.org/**10.20372/ejncs/Ch.2022.29*

ORIGINAL RESEARCH

Phytochemical Investigation and Determination of Anti-bacterial Activities of the Leaf Extract of Chenopodium ambrosioides L.

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Received: 19 February 2022; Accepted: 7 August 2022; Published online: 15 August 2022
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Abstract

Chenopodium ambrosioides L. is an herbaceous shrub that belongs to the family Chenopodiaceae. In Ethiopia, it has been used to treat different alignments, particularly to detoxify snake bites, as anthelmintic, analgesic and anti-inflammatory. This study aimed to investigate the phytochemical constituents, determine the anti-bacterial activities and characterize and elucidate the structure of pure isolated compounds from the leaves of C. ambrosioides. The powdered leaves were extracted through maceration using hexane, acetone and methanol as solvents. About 2% of the crude leave extract obtained was the methanol extract, which indicated the leaves of C. ambrosioides contain more polar phytochemicals. The phytochemical screening of the extracts revealed the presence of alkaloids, terpenoids, flavonoids, tannins, saponins and phenols. The crude leave extracts of hexane, acetone and methanol, each 100 mg/mL, were tested for the in-vitro antibacterial activity using disc diffusion method against three pathogens- S. aureus (gram-positive), E. coli and Ps. Aeruginosa (gram□negative). The maximum antibacterial activity was exhibited in acetone crude extract against S. aureus and E. coli with the inhibition diameter of 11 mm and 10 mm, respectively. The standard drug Erythromycin was used as a positive control and dimethyl sulfoxide was used as negative control. The acetone crude extract was subjected to further chromatographic separation using n□hexane:ethyl acetate solvent ratio in increasing order of polarity and a pure compound was isolated. The structure of the pure compound was elucidated using FTIR, 1H-NMR, 13C-NMR and DEPT-135. Spectroscopic characterization and comparison with literature reports revealed that the proposed structure of the compound is 8-ethoxy-2-oxo-2-chromene-3-carbaldeyde.

Keywords: Phytochemicals, Chenopodium ambrosioides, Anti-bacterial Activity

Introduction

According to the world health organization (WHO) report 80% of the world's populations depend on traditional medicine (TM) and a major part of traditional therapies involve the use of medical plant extracts or their active constituents (Al-Qura'n, 2005). Traditional remedies are the most important and sometimes the only source of therapeutics for more than 80% of the Ethiopian population and 90% of livestock population (WHO, 2002). Due to the cultural acceptability of traditional medicinal practitioners (TMPs) Ethiopians continue to use traditional medicine (Kassaye et al., 2007). TMPs mostly implement herbs, spiritual healing and minor surgical procedures in treating disease.

Ethiopian traditional medicine is vastly complex and diverse and varies greatly among different ethnic groups.

Chenopodium ambrosioides L. is an herbaceous shrub that belongs to the family of *Chenopodiaceae* (Pare *et al.*, 1993). Species of the family *Chenopodiaceae* are widely distributed in the East Mediterranean area, where they are often used commercially as drugs because of the presence of useful secondary metabolites where the most characteristic constituents are flavonoids, essential oils and terpenes (Sagrero-Nieves and Bartley, 1995; Pino, 2003; Song, 2011).

The genus *Chenopodium* includes varieties of weedy herbs (more than 200 species) native to much of Europe, Asia, India, and China and both North and South America. Even though *C. ambrosioides* originates in the Americas, at present it is found around the world including the warmer regions of North, Central and South America, Europe, Asia, and Africa. Various plant parts of different species of *Chenopodium* have been traditionally used in the treatment of several disorders (Gracius, 2020; Singh, 2021). *C. ambrosioides* oil is rich in monoterpenes (Chu, 2011; Li, 2020). The seeds and fruits contain a large amount of essential oil which has a main active compound in it called ascaridole (Monzote, 2007; Pandey, 2013; Wang, 2016; Fidalgo, 2019). Neeru J. reported that they have isolated and characterized two new flavonol glycosides, kaempferol 3-rhamnoside-4′-xyloside and kaempferol 3-rhamnoside-7-xyloside along with kaempferol, isorhamnetin and quercetin from the fruits of *Chenopodium ambrosioides*. (Jain *et al.*, 1990) C.*ambrosioides* has been used to treat different alignments as traditional medicinal plant in Ethiopia. Its infusion can be used as a digestive remedy to treat intestinal and stomach pains and externally as a poultice to detoxify snake bites and also used for expulsion of intestinal worms and skin parasites, used as analgesic and as anti-inflammatory (Amole and Izegbu, 2005; Amole and Yusuf, 2011).

A close literature investigation show that the phytochemical investigation of crude leave extracts *of C. ambrosioide* and its anti-bacterial activity and isolation and characterization of the bioactive component is insufficient in Ethiopia. Therefore, the main aim of this work was to isolate and elucidate the structure of possible bioactive chemicals from the leave crude extracts of *C. ambrosioide* by using chromatographic techniques.

The findings of this study will have significant contribution in supporting and promoting the usage of this plant in disease control and their conservation by the community. Knowledge of the compounds contained in this plant is expected to be useful in the development of new antimicrobial drugs and to introduce the leaf of *C. ambrosioides* as a local raw material for use in the traditional medicine production. The information will help to bridge the gap between modern and traditional medicine.

Materials and Method

Collection of Plant Materials and Preparations

The leaves of *C.ambrosiode* were collected from Mida Kegn and Dire Weni districts of West Shewa Zone, Oromia Region, Ethiopia. The samples were collected in June, 2012. The botanical identification of the plant was done by Botanist Dr. Fikadu Gurmessa at the Department of Biology, Wollega University, Ethiopia. After collection, the plant material was washed repetitively with tap water and air dried in the shade to make it easy for grinding and made ready for extraction. Air dried leaves of *C.ambrosioide* were grounded by an analytical mill and packed in a polyethylene bags to avoid entrance of air and any other contaminants from the surrounding.

Equipment and Chemicals

Rotary Evaporator (Heidolph Laborata 4000) was used for solvent evaporation from extracts. TLC analyses was carried out on Aluminum precoated Silica gel plates (Merck, Kiesel gel 60 F254, 0.25mm). Compounds on TLC were detected using UV light (254 nm and 365 nm). The FT-IR samples were prepared using spectral grade KBr and made into pellets and measured on Perkin-Elmer IR spectrophotometer in the range between 4000 cm^{-1} and 400 cm^{-1}. Spectra of 1D ^{1}H-NMR, ^{13}C -NMR and DEPT-135 were recorded using a

Bruker advance spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C) at room temperature using $CDCl_3$ as solvent and TMS as the internal standard. The chemical shifts were reported in δ (ppm) units relative to TMS signal. Milling machine and Whatmann number 1 filter paper (110x100 circles) were used for powdering sample and filtration, respectively. The chemicals and regents used were analytical grade methanol, acetone , hexane DMSO, ethyl acetate, chloroform, hydrochloric acid, sulfuric acid, glacial acetic acid, acetic anhydride, ferric chloride ($FeCl_3$), iodine, Benedict reagent, Mayer's reagent, Muller Hinton Agar, erythromycin, distilled water and bases like dilute ammonia were used.

Extraction of Plant Material

A 500 g of the powder of *C.ambrosioides* was weighted using digital balance and was macerated in a 2.5 L conical flask using 2 L of n-hexane. Then, the mixture was shaken well and stored for 72 h with occasional shaking. The mixture was filtered using Whatmann filter paper. Then, the filtrate was concentrated using rotary evaporator at 40 ^{o}C and 5gm of dark green hexane crude extractwas obtained. Then, n-hexane extract leave marc was macerated with 2 L of acetone for three days with occasional shaking and then the acetone extract was concentrated using rotary evaporator at 40°C and 8 g of dark green acetone crude extract was obtained. Finally, the leave marc was further macerated with 2 L of methanol for three days again with occasional shaking, the methanol crude extract was concentrated using a rotary evaporator at 50°C and afforded 10 g of black methanol crude extract.

From these three crude extracts acetones crude extract showed a better TLC profile as compared to other solvent extracts and showed positive preliminary anti-bacterial activity test .Thus, it was subjected to column chromatography with increasing solvent polarity.

Isolation and Purification of the Crude Extract

The crude extract of acetone showed positive preliminary anti-bacterial activity test and better TLC profile as compared to other solvent extracts. Therefore, it was selected for column chromatography. Thus, 4 g of acetone crude extract was subjected to column chromatography by using n-hexane ðyl acetate solvent system. The following ratios of solvent combinations were sequentially used in the elution process; hexane: ethyl acetate (10:0, 9:1, 8:2,7:3,6:4,1:1,4:6 ,3:7 & 2:8) as shown in Table1.

Table 1. Column chromatographic separation of acetone crude extract.

No.	Solvents	Fraction	Code of Fraction	Solvent ratios
1	Hexane: Ethyl acetate	F1	C1-C5	10:0
2	Hexane: Ethyl acetate	F2	C6-C10	9:1
3	Hexane: Ethyl acetate	F3	C11-C15	8:2
4	Hexane: Ethyl acetate	F4	C16-C20	7:3
5	Hexane: Ethyl acetate	F5	C21-C24	6:4
6	Hexane: Ethyl acetate	F6	C25-C30	1:1
7	Hexane: Ethyl acetate	F7	C31-C35	4:6
8	Hexane: Ethyl acetate	F8	C36-C40	3:7
9	Hexane: Ethyl acetate	F9	C41-C45	2:8

A total of 45 fractions were collected and the TLC profile of each fraction was checked using n-hexane: ethyl acetate (6:4) eluent system. Fractions having the same Rf value are combined together and nine fractions were obtained. Fraction F5 having code C21-C24 showed only one spots on TLC by using the mobile phase hexane (60%) and ethyl acetate (40%), finally the solvent was concentrated by using rotary evaporate and it was afforded 54 mg of dark green crystal material which was easily soluble in chloroform. The Rf of isolated crystal was 0.72 (3.3/3.58) (hexane: ethyl acetate 6:4) mobile phase and this was submitted to spectroscopic analysis. The TLC chromatogram of the isolated compound from the crude extract of acetone is shown in Figure 1.

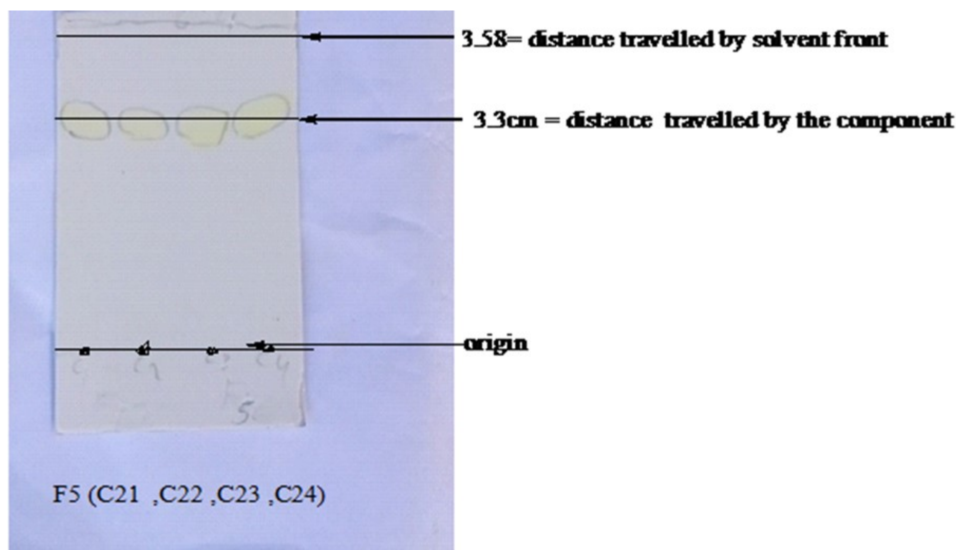


Figure 1. TLC Chromatogram of Fraction F5.

Phytochemical Screening Tests

The phytochemical analysis of the powdered leaf of *C. ambrosioides* was performed using the standard methods (Harborne, 1984; Price, 1985; Richardson and Harborne, 1990; Paterson, 1999; Hussain *et al.*, 2011).Thus, phytochemical analysis was carried out on the crude extracts of hexane,acetone and methanol to determine the phytochemical constituents *of C. ambrosioides*.

Test for flavonoids
About 5 mL of dilute sodium hydroxide solution was added to the portion of the plant extract followed by the addition of 2 mL concentration H_2SO_4. A yellow coloration was observed which confirms the presence of flavonoids.

Test for Tannins
About 5 mL of the extract was dissolved in 5 mL of chloroform and 2 mL of acetic anhydride was added to the mixture. Finally, 2 mL of concentrated Sulphuric acid (H_2SO_4) was added carefully to the solution along the wall sides of the vessel. A green coloration was observed which confirms presence of tannins.

Test for Alkaloids
About 0.5 g of the extract was dissolved in 5 mL of NH_4Cl and filtered. Filtrates were then treated with Mayer's reagent. Red coloration was observed which confirms the presence of alkaloid in acetone and methanol extracts.

Test for Phenols
5 mL of hexane, acetone & methanol crude extracts were treated with 4 drops of ferric chloride solution. Bluish black color was

observed which indicates the presence of phenols in the crude extracts.

Test for Terpenoids

About 5 mL of extracts were mixed in 2 mL of chloroform and 3 mL concentrated sulphuric acid (H_2SO_4). A reddish brown color was observed which indicates the presence of terpenoids in the crude extracts.

Test for Saponins

0.5 g plant leave crude extract was vigorously shaken with water in a test tube andthen heated to boil. Frothing was observed that indicates the presence of saponin in crudeextracts.

Antibacterial Activity

Methanol, acetone and hexane crude extracts of plant material were tested for antibacterial assay by using the disk diffusion method. Microorganisms used for evaluation of antibacterial activities of the crude extracts were Gram-positive bacteria (*S. aureus (ATCC20911*), and gram-negative bacteria (*E. coli (ATCC 25922)) and Ps. aeruginosa (ATCC 24853)*.

The bacteria cultures were inoculated into the Mueller Hinton Agar (MHA) and Incubated at 37 ^{o}C. All the bacteria were obtained from Biotechnology laboratory of Wollega University, Ethiopia, and Erythromycin was used as standard drug against bacteria.

Sample solutions preparation for antibacterial activity test

To test antibacterial activities the crude extracts of each 100 mg was separately dissolved in 1ml of dimethyl sulfoxide (DMSO, used as a negative control for antibacterial tests) for each of the three bacteria.

Testing for Antibacterial Activity

In vitro antibacterial activity of crude extract from leaf of *C.ambrosioides* was checked by using agar well diffusion and the bacteria strains S. *aureus* (gram positive), *E. coli* and Ps. *aeruginosa* (gram negative). Muller Hinton Agar media (MHA) was prepared by suspending 38 gram in 1000 mL distilled water. To prepare six petri dishes Mueller Hinton Agar well diffusion based on the above rule it needs 19 gram suspended Mueller Hinton agar dissolved in 500 mL of distilled water. Muller Hinton Agar culture media were used for the growth of organisms. The culture media were boiled in distilled water to dissolve the media completely and sterilized by autoclave at 121°C for 15 minutes and then poured or dispensed into sterilized (clean) Petri dishes and allows it to cool at -40°C in the refrigerator.After the culture media was solidified, organisms *S. aureus* (gram positive), *E. coli* and *Ps. aeruginosa* (gram negative) bacteria were uniformly seeded with it. Three well-isolated colonies of the same morphological type were selected from an agar plate culture (petri dishes) and the top of each colony was touched with a loop, and the growth was transferred through a sterilized loop into a test tube having nutrient broth to grow overnight in the incubator at 37°C until it achieves the turbidity of the 0.5 McFarland standards. The turbidity of the actively growing broth culture was adjusted with sterile saline solution to obtain turbidity optically comparable to that of the 0.5 McFarland standards which were resulted in a suspension for different strains. About 100 μL of bacterial suspensions which were obtained from Biotechnology laboratory of Wollega University was spread over the petri dishes containing Mueller-Hinton agar using a sterile cotton swab. Then, six mm diameter Whatman No 1 filter paper disc was impregnated on the surface of the inoculated agar in Petri dishes with the help of micropipette containing 5 μL each test solution (methanol, acetone and hexane crude extract solutions and DMSO) were applied onto the discs. A positive control Erythromycine was assayed simultaneously. After the addition of test solutions on the discs, the extract was allowed to diffuse for 30 minutes, and then the plates were kept in an incubator at 37°C until 24 hours. After overnight incubation, the antibacterial activity was evaluated by measuring the zone of growth inhibition (ZI) surrounding the discs in millimeter with a ruler (Kambizi and Afolayan, 2001; Al-Asmari *et al.*, 2015).

RESULTS AND DISCUSSION

In this section, phytochemical analysis and antibacterial activity test on hexane, acetone& methanol crude extracts and structural elucidation of the compound isolated from the acetone crude extract of the leave of *C. ambrosioides* will be discussed.

Yield of Crude Extracts

500 g of dry powdered leaf of *C. ambrosioides* was successively extracted by using hexane, acetone and methanol organic solvents. The yield of crude extracts that were obtained (Table 2) indicates that the crude extract of the leave of *C. ambrosioides* contain more polar phytochemicals than non-polar constituents.

Table 2. Yield of crude extract of *C. ambrosioides*.

Solvent used for Extraction	Amount of Crude extract Obtained (gm)	Percentage yield	Color
Hexane	5 g	1%	dark green
Acetone	8 g	1.6%	dark green
Methanol	10 g	2%	Black

Phytochemical Screening Test Results

The phytochemical screening of hexane, acetone & methanol crude extracts of *C. ambrosioides* revealed the presence of Flavonoids, Tannins, Alkaloids, Phenols, Terpenes and Saponins as shown in Table 3. This could be responsible for the versatile medicinal properties of plant under study.

Table 3. Phytochemical screening result of leave of *Chenopodium ambrosioides*.

Crude Extract	Chemical constituents					
	Flavonoids	Tannins	Alkaloids	Phenols	Terpenes	Saponins
Methanol	+	+	+	+	+	-
Acetone	+	+	+	+	+	+
Hexane	-	-	-	-	+	+

*Where (+) = indicate the presence of chemical and (-) = indicate the absence of chemical

Antibacterial Activity Results

The anti-bacterial activities of the methanol, acetone and hexane crude extracts of *C. ambrosioides* were performed against one gram positive bacterium (*S. aureus*) and two gram negative bacteria *(E. Coli & Ps. aeruginosa*).

The acetone crude extract (100 mg/mL) showed the maximum zone of inhibition of 11 mm, 10 mm and 9 mm against the gram positive bacteria (*S. aureus*) and gram negative bacteria *(E. Coli & Ps. aeruginosa*), respectively (Table 4). The methanol and hexane crude extracts (100 mg/mL) shows weaker potency against the three clinical pathogens as compared to acetone extract.

Table 4**.** The anti-bacterial activity of *C. ambrosioides* leaves crude extract.

Extracts		Zone of inhibition (in mm)		
		S. aureus	***E. Coli***	***Ps. aeruginosa***
Methanol extract	100 mg/mL	9 mm	8 mm	8 mm
Acetone extract	100 mg/mL	11 mm	10 mm	9 mm
Hexane extract	100 mg/mL	8 mm	7 mm	7 mm
Erythromycin	10 µg /disc	12 mm	20 mm	11 mm
DMSO 10 mL		0	0	0

In anti-bacterial study of C. *ambrosioides* leave crude extract, it was found out that the maximum zone of inhibition was obtained against the gram positive bacteria *S. aureus* with inhibition zone of 11 mm.

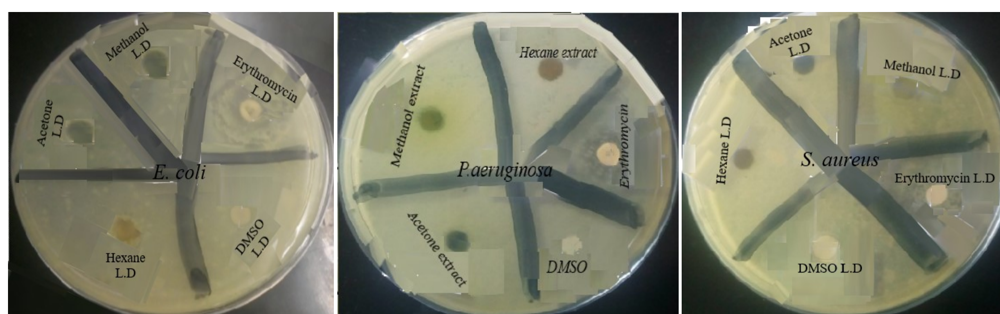


Figure 4. Acetone crude extract inhibition zone against *E. coil*, *Ps. aeruginosa* and *S. aureus* from left to right.

Characterization of Compound CAL-2

Dry powdered leave of *C. ambrosioides* plant (500 g) was successively extracted by using hexane, acetone & methanol organic solvents. The acetone crude extract showed a better TLC profile as compared to other crude extracts. Thus, 4 gm of acetone crude extract was subjected to column chromatography by using n-hexane and ethyl acetate eluent. The following ratios of solvent combinations were sequentially used in the elution process; hexane: ethyl acetate (10:0, 9:1, 8:2,7:3,6:4,1:1,4:6 ,3:7 & 2:8) and finally provides 54 mg of pure compound named CAL -2 was isolated. The characterization of compound CAL-2 was done using spectroscopic techniques such as FT-IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectra.

FT-IR spectrum of Compound CAL-2

The FTIR spectrum of compound CAL-2 (Supplementary Figure SI 1) showed the bands 3015 cm^{-1}, 2912 cm^{-1}, 2882 cm^{-1}, 2851 and 2744, 1723 cm^{-1}, 1623 cm^{-1} which are assignable to the stretching of aliphatic =C-H, CH_2, CH_3, C-H(CHO), C=O and C=C bending, respectively. The band that raises at 1465 cm^{-1}, 1372 cm^{-1}, and 1173 cm^{-1} can be assigned to CH_2 bending, CH_3 bending and C-O stretching vibrations, respectively. The FTIR spectrum of compound CAL-2 is shown in Table 5**.**

Table 5. FTIR spectrum of compound CAL-2.

Signal	Observed Value for the compound CAL-2 (cm^{-1})	Range from IR Tables
CH stretching	2912 and 2882	3000-2850
=CH stretching	3015	3100-3000
C-H(CHO) stretching	2851 and 2744	2860-2800 and 2760-2700
C=C stretching	1623	1680-1620
C=O(aldehyde) stretching	1723	1740-1720
CH_2 bending of alkane	1465	1470-1350
CH_3 bending of alkane	1372	1470-1350
C-O stretching	1173	1260-1000

Figure 5. ^{1}H-NMR spectrum of compound CAL-2.

^{1}H-NMR Spectrum of Compound CAL-2

The chemical shifts (PPM) of the ^{1}H-NMR (400 MHZ, in $CDCl_3$) spectrum of CAL-2 (Supplementary Figure SI 2) are indicated in Figure 5 and Table 6 below. The downfield singlet signal which is integrable to one proton a d 10.51 (1H,s) is a characteristic of aldehydic proton.

A singlet signal which appeared downfield at d 8.59 is assignable for H4. The three doublet signals each integrable to one proton at d 7.85 (1H, d, J = 8.4 Hz), d 7.74 (1H, t, J = 7.2Hz) and d 7.43 (1H, t, J = 8.4 Hz) are assignable to aromatic protons H5, H6 and H7, respectively.

The quartet signal at d 4.69 (2H, q, J= 8 Hz) is a characteristic methylene protons adjacent with methyl group. The triplet signal at d 1.53 with the J value of 8 Hz is a characteristic of methyl protons adjacent with methylene group.

Table 6. The ^{1}H-NMR of Chemical Shift Values of CAL-2 in PPM.

Proton	^{1}H-NMR d (PPM), J (Hz) of CAL-2 (in $CDCl_3$
CHO	10.51 (1H, s, CHO)
C_2	-
C_3	-
C_4H	8.59 (1H, s, H4)
C_5H	7.85 (1H, d, H5, J=8.4 Hz)
C_6H	7.74 (1H, t, H6, J=8 Hz)
C_7H	7.43 (1H, t, H7, J=8 Hz)
C8	-
C9	-
C10	-
$-OCH_2-$	4.69 (2H, q, CH_2, J=8Hz)
$-CH_2CH_3$	1.53 (3H, t, CH_3, J=8Hz)

^{13}C-NMR and DEPT-135 spectra of compound CAL-2

The ^{13}C-NMR and DEPT-135 NMR spectra of compound CAL-2 (Supplementary Figure SI 3 and 4) indicate the presence of 12 carbon atoms. Out of these carbon atoms one is carbadehyde (-CHO), one is methyl ($-CH_3$), four are methine (CH), one is methylene (CH_2) and five are quaternary carbons with one overlapping. The observed ^{13}C-NMR and DEPT-135 chemical shifts are assigned as follows in Table 7 and 8.

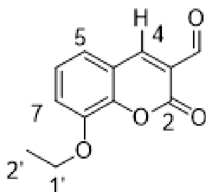
Table 7. Protons Decoupled ^{13}C-NMR spectra of compound CAL-2.

Position of Carbon	^{13}C-NMR d (PPM) of compound CAL-2 (in $CDCl_3$)
CHO	189.57
-	-
2	161.05
3	124.28
4	124.90
5	127.25
6	139.68
7	132.48
8	149.09
9’	139.64
10	129.76
1’	62.40
2.	14.51

Table 8. DEPT-135 NMR spectrum of compound CAL-2.

Position of Carbon	DEPT-135 d (PPM) of compound CAL-2 (in $CDCl_3$)	Nature of Carbon
CHO	189.57	C=O
2	161.05	Quaternary
3	124.28	Quaternary
4	124.90	CH
5	127.25	CH
6	139.68	CH
7	132.48	CH
8	149.09	Quaternary
9’	139.64	Quaternary
10	129.76	Quaternary
1’	62.40	CH_2
2.	14.51	CH_3

The spectroscopic data suggest that the most probable structure of the compound CAL-2 is 8-ethoxy-2-oxo-2-chromene-3-carbaldeyde.



8-ethoxy-2-oxo-2*H*-chromene-3-carbaldehyde

Figure 6. Proposed structure of compound CAL-2.

CONCLUSION

When the hexane, acetone and methanol crude extracts were subjected to bacterial pathogens, acetone crude extract was more active against gram positive (*S. aureus*) bacteria with the diameter of zone of inhibition of 11mm. Whereas hexane & methanol crude extracts are less active against gram positive (*S. aureus*) and gram negative (*E. Coli and Ps. aeruginosa*) bacteria whose diameter zone of inhibition were lower than that of acetone crude extract on agar well diffusion method. The phytochemical investigation of the crude extract of the leave of *C. ambrosioides* reveals the presence of alkaloids, flavonoids, tannins, phenols, terpenes and saponin. Repeated successive solvent extraction followed by chromatographic separation afforded the isolation of one new compound from the acetone crude extract and characterized using spectroscopic techniques such as IR, ^{1}HNMR, ^{13}C-NMR and DEPT-135.The elucidated structure of the newly isolated compound is 8-ethoxy-2-oxo-2-chromene-3-carbaldeyde.

ACKNOWLEDGEMENTS

The author would like to acknowledge Dr. Fikadu Gurmessa, Depatment of Biology, Wollega University, Ethiopia, for the botanical identification of the plant under study and Dr. Zelalem Abdissa Department of Chemistry, Wollega University, Ethiopia, for laboratory support.

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ANNEX

Supplementary Material (Figure SI 1-4)

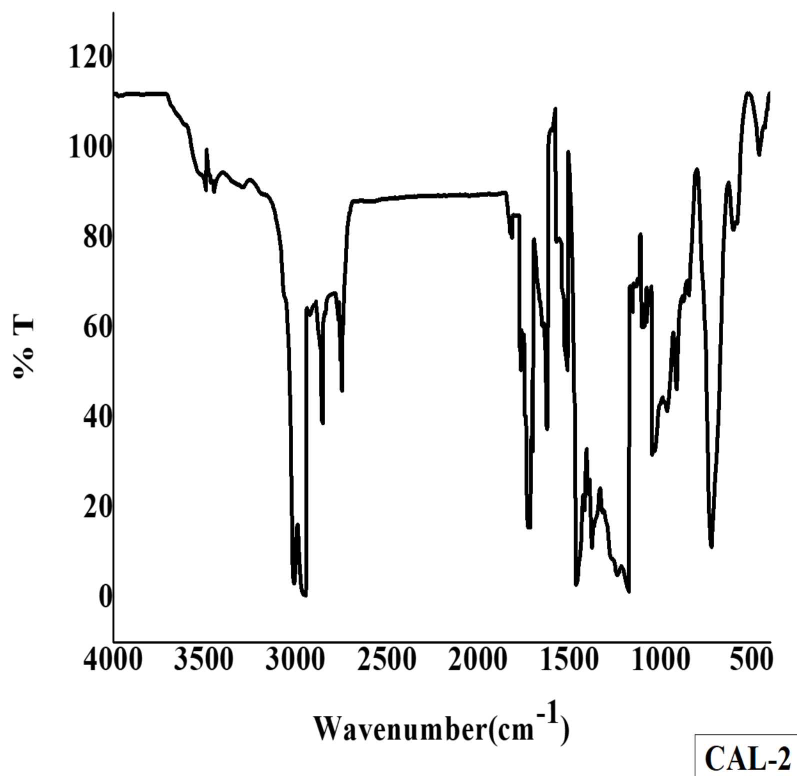

Figure SI 1. FTIR spectrum of Compound CAL-2.

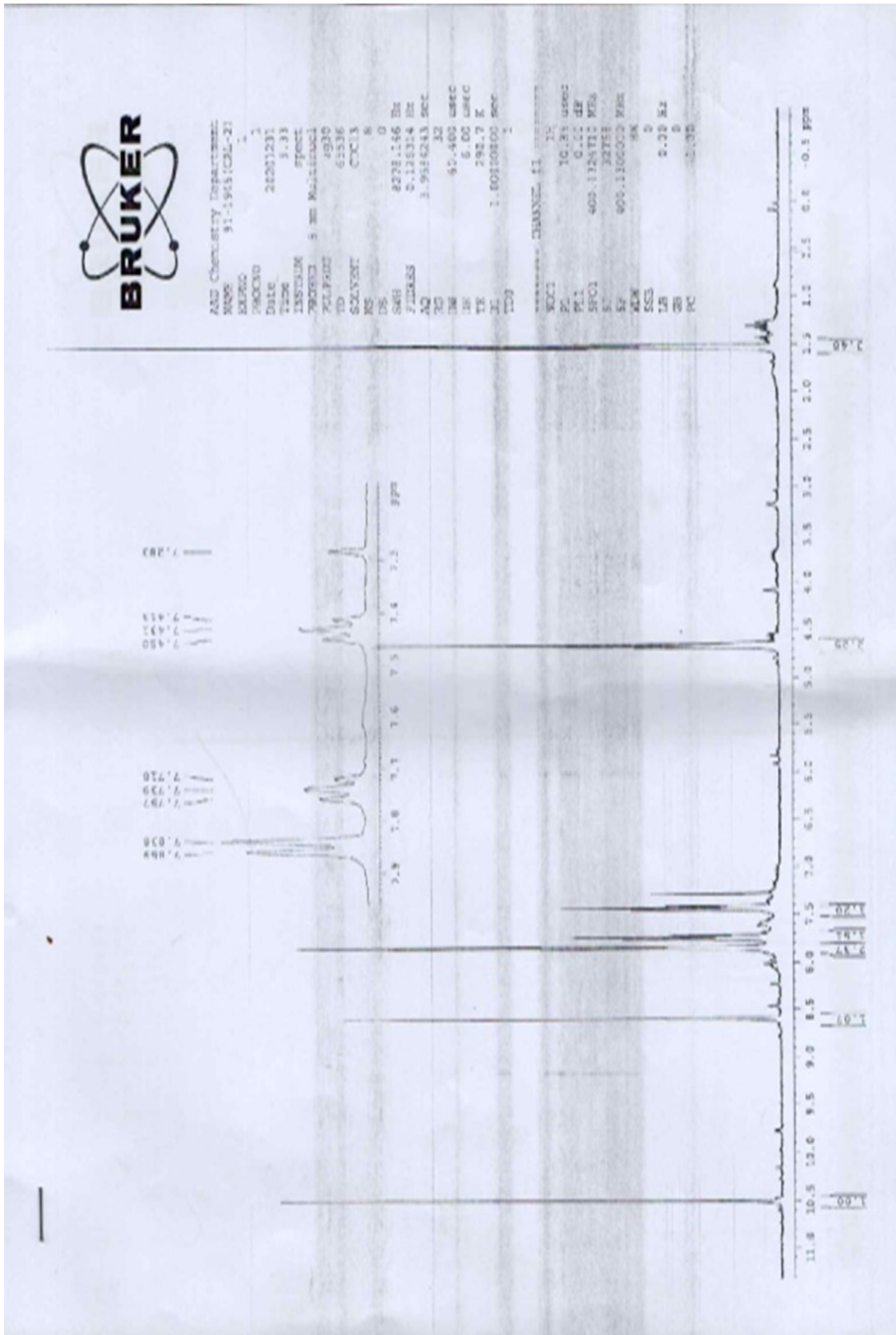

Figure SI 2. ^{1}H-NMR spectrum of compound CAL-2

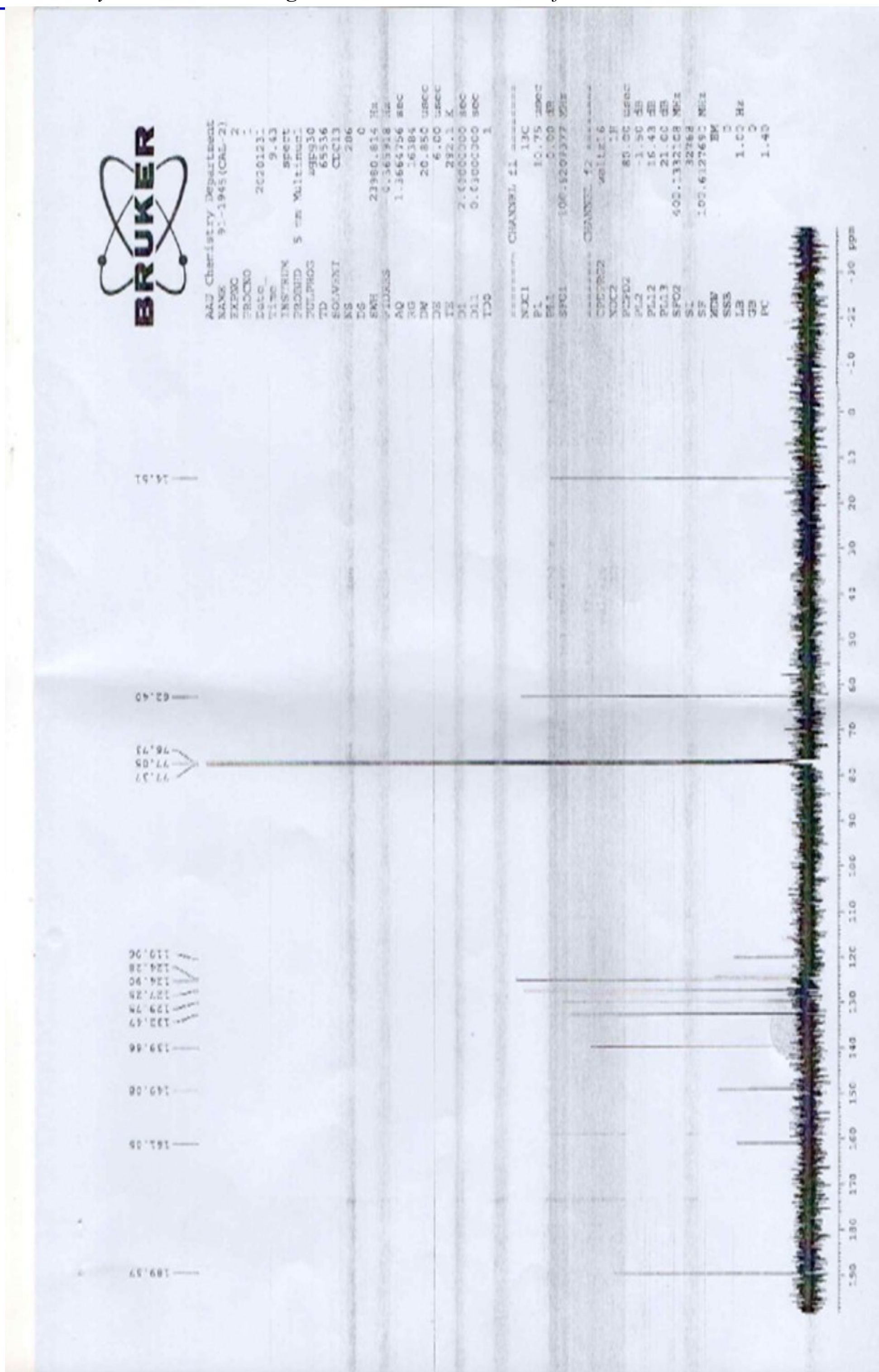

Figure SI 3. ^{13}C-NMR spectrum of Compound CAL-2.

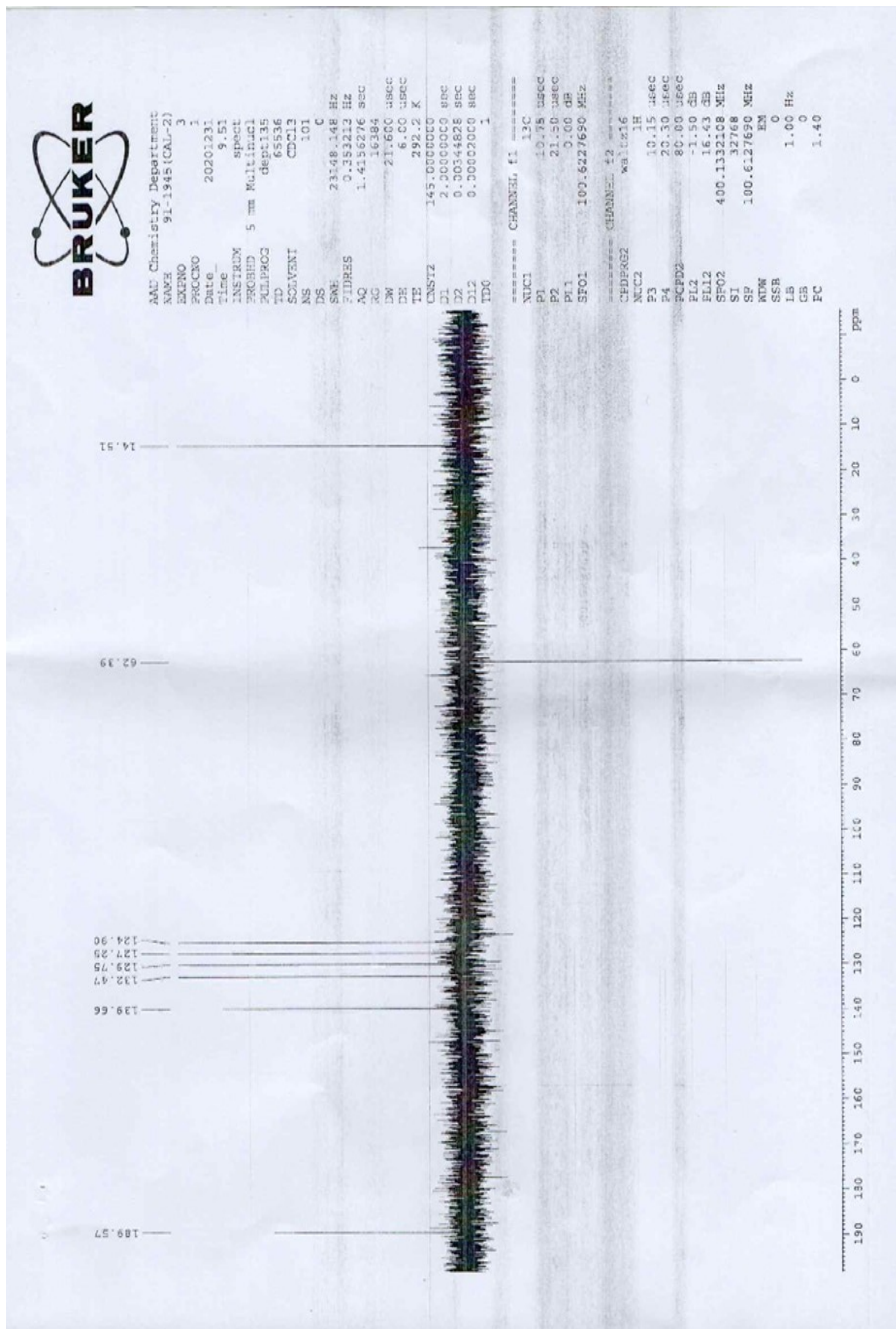

Figure SI 4. DPT-135 spectrum of CAL-2 compound.