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 (1) 247-257*
https://doi.org/10.20372/ejncs/ch.2022.16

ORIGINAL RESEARCH ARTICLE

Phytochemical Investigation and Antibacterial Activity Assessment of *Zehneria scabra* (L.f) Sond (ሀረግ ፊሃ) Leaves Extract

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Received: 14 June 2021 / Accepted: 21 December 2021 / Published online: 16 June 2022

Abstract

This study was intended to identify chemical constituents of methanol (99%) leaves extract of Zehneria scabra (L.f) Sond. The crude extract fractions were tested against Staphylococcus aureus and Escherichia coli using the agar well diffusion method at 100 mg/ml concentrations in the presence of positive control. The phytochemical was isolated and purified by using preparative thin layer chromatography (in a solvent system of 10% ethyl acetate: 90% chloroform). The structure of the isolated component was determined by using a combination of spectroscopic techniques such as IR, ^{1}H NMR, ^{13}C NMR and DEPT-135 spectra. The phytochemical screening of methanol crude extract indicated the presence of alkaloids, flavonoids, terpenoids, glycosides, and tannin. The chloroform and ethyl acetate fraction revealed more potent antibacterial activities.

Keywords: *Zehneria scabra, leaves extracts, antibacterial assay, phytochemical screening*

Introduction

Plants and humans are intimately linked on this planet (Anjaneyulu and Sudarsanam, 2013). Organic Chemistry as stands today has developed largely from the chemistry of natural products. Using modern spectroscopic techniques such as UV, IR, MS, NMR, and improved chromatographic methods, a generation of new organic substances from plants, animals and microorganisms are being discovered, of which many have biological activity (Torsell, 1997). The wild plants' genus *Zehneria* which belongs to the family of *Cucurbitaceae*, commonly known as cucurbits is most diverse in tropical and subtropical regions with hot spots in South East Asia, Madagascar, Africa, and Mexico. In general, members of *Cucurbitaceae* (*pumpkin, luffas, zucchini, watermelons, cucumbers, courgettes,* and *summer squash)* are edible and found on all continents of the world (Pandey, 2014). In the African continent, this family is represented by 24 genera and 54 species. The wild genera are *Langeneria, Luffa, Momordica* and *Zehneria* (Pandey, 2014).

Z. scabra is a climber perennial herb, locally called "Hareg Ressa" (ሀረግ ፈሣ) and it has high medicinal value according to herbal folklore practices. However, there is a lack of adequate information on the nature of bioactive principles and their therapeutic action. In Ethiopian folk medicine, this plant is used for various infectious diseases and has enormous ethnobotanical value. Tribal people used the root of *Z. scabra* to hang in front of their house believing that it will prevent the entry of disease-causing pathogens and also, used it with milk for the treatment of fever and diarrhea (Doss and Jeyachandran, 2012). The leaves of *Z. scabra* were used traditionally for the treatment of malaria, inflammation, pain, bacteria, and parasitemia management. It acts as an important medicine for livestock in various ailments and fruits are reported to cure stomachache (Tesfaye and Alamneh, 2014).

Figure 1. *The experimental plant Zehneria scabra*

Previous work (Tesfaye and Alamneh, 2014) on *Z. scabra* indicates the presence of alkaloids, glycosides, tannins, flavonoids, phenols, and terpenoids in leaves. These secondary metabolites have been used as ethnobotanical medicine to cure and protect against various diseases in humans and animals. Some species have shown antibacterial activity against *E. coli* and *Pseudomonas aeroginosa*, antimalarial activity, skin rashes, diabetes, etc. (Jeyachandran, 2014). Many plant species commonly contain monoterpenoids that are used in cosmetic, non-cosmetic, and pharmacological preparations, as well as in the food industry, and also exhibit the effect of relaxation on ileum smooth muscle (Hamdard, 2007). Due to a wide range of biological activity, terpenoids have extensive applications in the fields of pharmaceuticals, cosmetics, colorants, disinfectants, fragrances, flavorings, and agrochemicals. All terpenoids are made from the same five-carbon building blocks, isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP). IDP and DMADP in turn originate from either of two distinct pathways (Pattanaik, and Lindberg, 2015). A group of terpenoids that are attracting attention lately is pentacyclic tri-terpenoids (PCTs). PCTs are a class of C30 terpenoid compounds that occur widely in plants (Babalola and Shode, 2013). Motriterpene triterpene skeletons are tetracycles, containing three six-membered and one five-membered ring, and pentacycles, either with four six-membered and one five-membered ring or five six-membered rings. However, acyclic, mono-, di-, tri-, and hexa cyclic scaffolds have also been isolated and identified from natural sources (Sandjo and Kuete, 2013). It is well-recognized that triterpenes have long been used as flavors, pigments, polymers, fibers, glues, and waxes. In many Asian countries, herbal products containing triterpenes are widely prescribed to prevent or treat a variety of dies by traditional healers (Parmar *et al.,* 2013). Gypenoside is another compound isolated and characterized from the roots of *Z. scabra (*Anand, *et al.,* 2011). There is a scarcity of scientific research on the phytochemical investigation of *Z. scabra* leaves from Ethiopian flora. Therefore, this study was intended to isolate phytochemicals from the leaves of *Z. scabra* and examined fractions on selected pathogenic microorganisms and elucidate the structure of the compound isolated from the bioactive fractions.

Materials and Methods

Plant Material Collection

Fresh leaves of *Z. scabra* were collected from Azezo (Gondar, Amhara National Regional State), located 10 km away from Gondar city, on the way to Addis Ababa. The collected plant materials were identified and authenticated by a botanist from the Department of Biology, University of Gondar. The leaves were thoroughly washed and dried at room temperature in the laboratory for about 10 days and then chopped into smaller sizes to enhance drying. The specimen of dried leaves was deposited at the herbarium of the University of Gondar as a reference.

Extraction and Isolation

The leaves of *Z. scabra* were dried and powdered using an electrical grinder (Model: IKA-WERKE GMBH AND CO.KG). Two hundred and sixty grams of coarsely powdered leaves were weighed and soaked in a 6000 ml flask containing 1000 ml of 99% methanol for 72 hrs. at room temperature, shaking gently three times a day. The extract was filtered with Whatman filter paper No.1. The marc left was further extracted with the same solvent for 72 hrs. in a similar manner and continued for another 144 hrs. to obtain a better yield. After filtration, the extracted solutions were combined and concentrated using a rotary evaporator (Model: RE200) at a temperature of 40-45^{0}C which produces blue-green crude extract (29 g, 11.15%). Then, the concentrated filtrates were kept in a refrigerator until required for further experiments. The methanol crude extract was subjected to partition or separation by using a separating funnel with petroleum ether, chloroform, ethyl acetate, and methanol and each fraction was monitored by TLC with increasing polarity of ethyl acetate and chloroform in n-hexane. All solvent fractions were collected in a different flask and concentrated by rotary evaporator under reduced pressure and all crude fractions were kept in the refrigerator until required for further experiments and antibacterial activity study. From the four fractions, the chloroform fraction in the eluent system chloroform: ethyl acetate (9:1) gives three separate spots in TLC. By using chloroform: ethyl acetate (9:1) eluent system, the chloroform fraction was subjected to preparative thin layer chromatography and gives separate-colored compounds. However, all compounds were not visible with necked eyes and some of them are visible under a UV lamp at 254 nm and 365 nm. The six different colored compounds were separated and each one of them was collected in a 50 ml flask. Then, each collected sample was dissolved in acetone and filtered with Whatman filter paper No.1.

The yellowish-colored compound gives a single spot in TLC with chloroform: ethyl acetate (9:1) eluent system. It was re-purified with preparative thin layer chromatography with chloroform: ethyl acetate (9:1). After re-purifying in the preparative thin layer visible yellowish component was isolated. This component was collected in a 50 ml flask and the colored compounds were dissolved with acetone and filtered with Whatman filter paper No. 1. The filtered yellowish-colored compound was kept in a refrigerator. After 4 days the acetone dissolved yellowish-colored compound was concentrated by rotary evaporator under reduced pressure. Only yellowish oil-colored film was left in the flask and washed with a small amount of acetone, and using a clean capillary tube, the yellowish -colored compound was transferred into sample vials for spectroscopic analysis.

Preliminary Phytochemical Screening

Phytochemical screening was carried out on the crude extract using standard procedures to identify the constituents such as alkaloids, flavonoids, phenols, terpenoids, tannins, saponins, carbohydrates, and glycosides

Test for Alkaloids

Mayer's reagent: To 2N HCl was added into 2 ml of the crude extract in the test tube. The mixture was heated for 20 min, cooled, and filtered. Then 1 ml of filtrate was tested with Mayer's reagents. Formation of cream precipitate for Mayer's indicated presence of alkaloids (Thamacin *et al.*, 2014).

Test for Flavonoids

Shinoda test: Pieces of magnesium ribbon and concentrated HCl was mixed with crude plant extract and after a few minutes pink colored scarlet appeared which indicated the presence of flavonoids.

Alkaline reagent test: 2 ml of 2% NaOH solution was mixed with plant crude extract, the intensive yellow color was formed, which turned into colorless when 2 drops of diluted acid were added to the solution, this result indicated the presence of flavonoids (Jaradet *et al.,* 2015).

Test for Terpenoids

Salkowski's test: 2 ml of the crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes. The development of a grayish color indicated the presence of terpenoids (Bargah, 2015).

Test for Glycosides

Liebermann's test: 2 ml of acetic acid and 2 ml of chloroform were mixed with 2ml of crude extract. The mixture was then cooled and concentrated H_2SO_4 was added. The green color indicated the entity of the aglycone steroidal part of glycosides (Kebede *et al.*, 2013).

Salkowski's test: about 2 ml of concentrated H_2SO_4 was added to the 2ml of crude extract. A reddish-brown color was produced indicating the entity of the steroidal aglycone part of the glycoside. 2ml of crude extract solution, an equal quantity of Fehling's solution was added and the solution was heated for a few minutes. A brick red precipitate indicated the presence of glycosides (Thamacin *et al.*, 2014).

Test for Phenols

2ml of 2% solution of $FeCl_3$ was mixed with the crude extract. The Black or blue-green color indicated the presence of phenols (Jaradet *et al.,* 2015).

Test for Carbohydrates

500 mg of powdered sample was taken and dissolved into 5 ml of distilled water and then filtered. The filtrate was added with a few drops of Molisch's reagent, followed by the addition of 1 ml of concentrated H_2SO_4 by the side of the test tube. After two minutes, 5 ml of distilled water was added. Red or dull violet color formation at the interphase of the two layers was taken as a positive test (Rajaraman *et al.*, 2016).

Test for Tannins

About 2 ml of the crude extract was stirred with 2ml of distilled water and a few drops of ferric chloride ($FeCl_3$) solution were added to the solution. The formation of green precipitate indicated the presence of tannins (Bargah, 2015).

Test for Saponins

Foam test: 10 g of powdered sample was boiled in 10 ml of distilled water and then filtered. 3 ml of distilled water was added to the filtrate and shaken vigorously for about 5 min. The formation of foam after shaking was taken as a confirmation of the presence of saponins (Rajaraman *et al.*, 2016).

Experimental Procedure

A UV-Vis spectrum was measured using a GENESYS spectrometer (200 – 800 nm) in methanol at room temperature. The infrared spectrum was recorded using KBr pellets on Perk-Elmer BX Infrared Spectrometer in the range 4000-400 cm^{-1}. Nuclear Magnetic Resonance (NMR) analysis was recorded on a Bruker Avance 400 MHz spectrometer with deuterated CD_3COCD_3 using tetramethylsilane (TMS) as the internal standard. Structural elucidations were conducted based on 1D NMR Spectra (^1H NMR, ^{13}C-NMR, and DEPT-135). Analytical thin layer chromatography (TLC) was carried out with pre-coated 0.2mm silica gel 60 F_{254} on aluminum foil and compounds on TLC were detected under a UV lamp at 254 and 365 nm. The crude extract was fractionated by a different solvent system and the bioactive component was isolated and purified by using preparative thin layer chromatography in a chosen solvent system.

Procedure for Antibacterial Activity

Clinically isolated Gram-positive bacterial species (*Staphylococcus aureus)*, and Gram-negative bacterial species (*Escherichia coli)*, obtained from the College of Medicine and Health Science, University of Gondar were used for this study. They were maintained on Mueller Hinton Agar at 4 ^0C before use. The methanol crude extract of *Zehneria scabra* leaves fraction (petroleum ether fraction, chloroform fraction, ethyl acetate, and methanol fraction) were screened for *in-vitro* antibacterial activity in comparison with standard antibiotic vancomycin (100 mg/ml)

by using disc diffusion method. For antibacterial screening 100 mg/ml of each extract was used. This was done by dissolving 1.0 g of each crude extract in 2 ml of acetone. Each of the solid extracts was reconstituted in acetone solvent to obtain a stock solution of 100 mg/ml. The Mueller Hinton Agar medium was prepared by dissolving 11.4 g in 300 ml of distilled water and heated on a hot plate, and the Petri dishes and dispensed at 20 ml per plate in 12 x 12 Petri dishes. The Petri dishes were incubated overnight before use to ensure sterility. After that, microorganisms were inoculated by using a sterile cotton swab rolled in the suspension to streak the medium. A sterile cork borer of 5 mm diameter was used to make wells on the medium and added 100 mg/ml of the methanol crude extract fractions, labeled and incubated at 37 ^{0}C for 24 hrs. Antibacterial activity was determined by measuring the diameter of zones of inhibition (mm) produced after incubation.

Result and Discussion

Phytochemical Screening

The result of the phytochemical analysis of the methanol crude extract is presented in Table 1. This phytochemical study revealed the presence of alkaloids, flavonoids, glycosides, terpenoids, phenols and tannins.

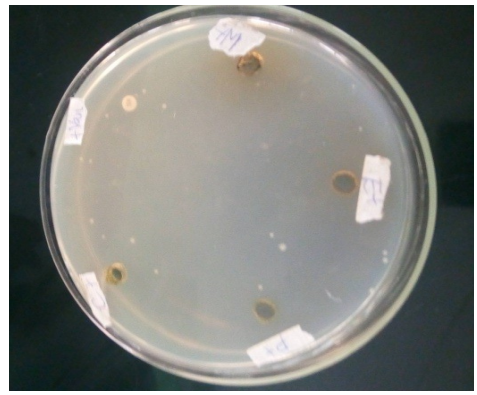
Table 1: Phytochemical screening test of methanol crude extract of *Z. scabra* leaves

Phytochemical	Test Method	Test Result
Alkaloids	Mayer'stest	+
Flavonoids	Shinoda test	+
Terpenoids	Salkowski test	+
Glycosides	Libermann's test	+
Phenols	Ferricchloride test	+
Carbohydrates	Molisch's test	-
Tannins	Ferric chloride test	+
Saponins	Foam test	-

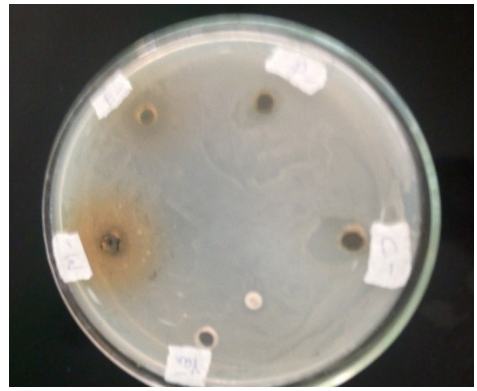
Note: + indicates present and - indicates absent

Antibacterial Activity

Antibacterial study results revealed that the fraction of methanol crude extract (100 mg/ml) of various solvent systems (petroleum ether, chloroform, ethyl acetate, and methanol fraction) inhibited both gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacterial species. Maximum inhibition zone was observed in chloroform (100 mg/ml) and ethyl acetate (100 mg/ml) solvent fraction against gram-negative bacteria (*E. coli*) at 100 mg/ml concentration. This result was corroborated with an earlier report of another study, chloroform fraction and ethyl acetate fraction of methanol crude extract of *Z. scabra* leaves against *S. aureus* and *E. coli*.



A. Gram negative (*E. coli*)



B. Gram positive (*S. aureus*)

Figure 2: Growth Inhibition Zone of *E. coli* (A) and *S. aureus* (B) (M indicator: methanol, E, ethyl acetate, C, chloroform and P, petroleum ether fraction).

Table 2: Inhibition zone of all solvent fractions of methanol crude extract

Bacteria species	Zone of inhibition (mm) of 100 mg/ml of fractions				
	MeOH	EtOAc	$CHCl_3$	Pet. ether	Vancomycin
S. aureus	10	15	14	8	8
E. coli	15	20	21	17.5	10

Antibacterial activity assay of solvent partitions of the methanol crude extract indicated higher growth inhibition against the tested bacteria even better than the reference drug. The yellowish chloroform solvent fraction demonstrating higher inhibitory activity was subjected to repeated preparative thin layer chromatography (PTLC) until a yellowish component was obtained with chloroform: ethyl acetate (9:1) solvent system (Rf 0.39). The yellowish oil (25 mg) reconstituted in acetone was filtered and concentrated for spectroscopic analysis.

Figure 3. IR spectra of the yellowish component

The IR spectrum exhibited absorptions at 3455 cm-1 indicating the presence of hydroxyl group (-OH), 3090 cm-1 indicating the presence of =C-H stretching, 2946 cm-1 and 2840 cm-1 indicating the presence of –C-H stretching vibrations, 1690 cm-1 indicating the presence of carbonyl group and 1420 cm-1 due to the presence of C-C vibrations, 1000 cm-1 indicating the presence of C-O vibrations (Fig. 3, Table 3).

Table 3: IR spectra of the yellowish component

No.	IR Absorption region (cm^{-1})	Groups
1	≈ 3455	-OH
2	≈ 3090	=C-H
3	≈ 2946 and 2840	-C-H
4	≈ 1690	C=O
5	≈ 1420	C-C
6	≈ 1000	C-O

Inferences from ^{1}H NMR Spectrum

The ^{1}H NMR spectrum measured in deuterated acetone (Acetone-d6, Table 7) revealed the existence of aliphatic protons, hydroxyl protons, olefin protons (sp2 hybridized carbon), and aldehyde protons. The two hydroxyl protons resonating at δ3.84 and δ1.30 which are fixed at the position of C -1' and C-2' respectively, the aliphatic protons resonating at [δ2.16 (H, s), δ2.90 (2H, s) and δ1.21 (3H, s)]. The olefin protons (sp2 hybridized carbon proton) resonated at δ6.20 (H, s) and the aldehyde proton resonated at δ8.04 (H, s). The ^{13}C NMR spectrum for yellowish compounds showed 10 carbon signals of which two olefin or sp2 hybridized carbon atoms resonating at δ127.48 and δ137.57 which are assigned to positions C-2 and C-3 respectively. The ^{13}C NMR also indicated the presence of two quaternary non-protonated carbon atoms at δ33.76 and δ68.83 consistent with DEPT Spectra and assigned as C-5 and C-2' respectively. Furthermore, the ^{13}C NMR spectrum also revealed the presence of three sp3 hybridized (aliphatic) carbons, resonating at [δ26.83, δ31.00, and δ54.62] and three carbonyl groups resonating at δ205.49, δ205.09 and δ208.82 consistent with DEPT spectra and assigned as C-1, C-4, and C-6 respectively. The DEPT spectrum reveals the presence of one CH_3 resonating the peak at δ26.83 (CH and CH_3 give positive peaks in the DEPT spectrum whereas CH_2 gives negative peaks in the DEPT spectrum) and there is no peak for quaternary carbons in the DEPT spectrum. The one CH_2 groups (aliphatic carbon) are resonating at δ54.62 and also, and one CH resonated at δ31.00. The aldehyde proton resonates at δ205.49 in DEPT Spectrum. The complete ^{1}H NMR, ^{13}C NMR, and DEPT Spectra of the yellowish compound in acetone-d6, data are reported in Table 4.

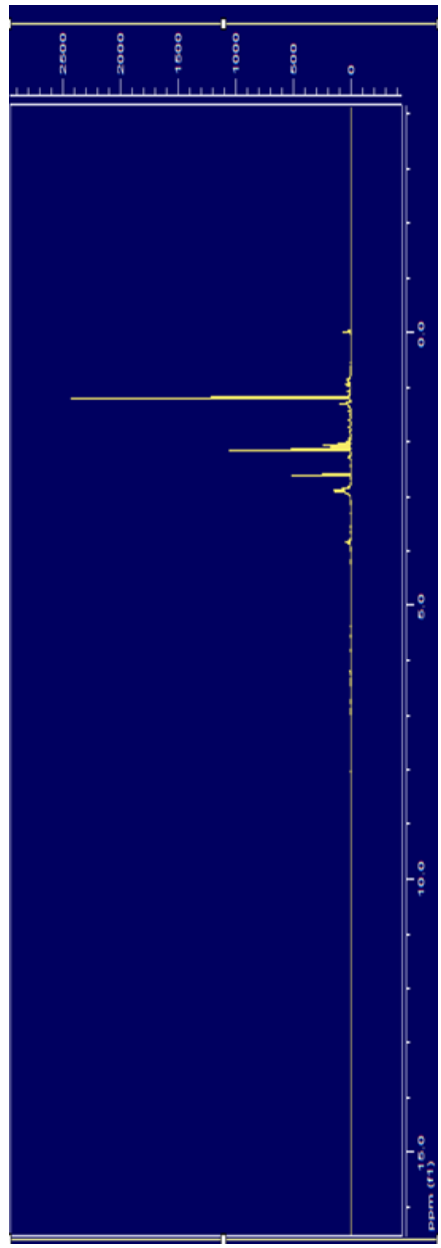
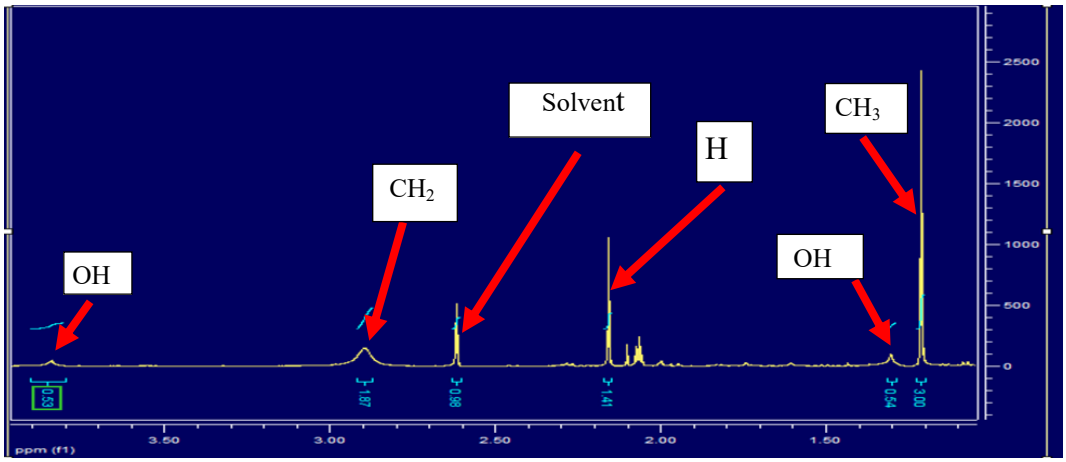
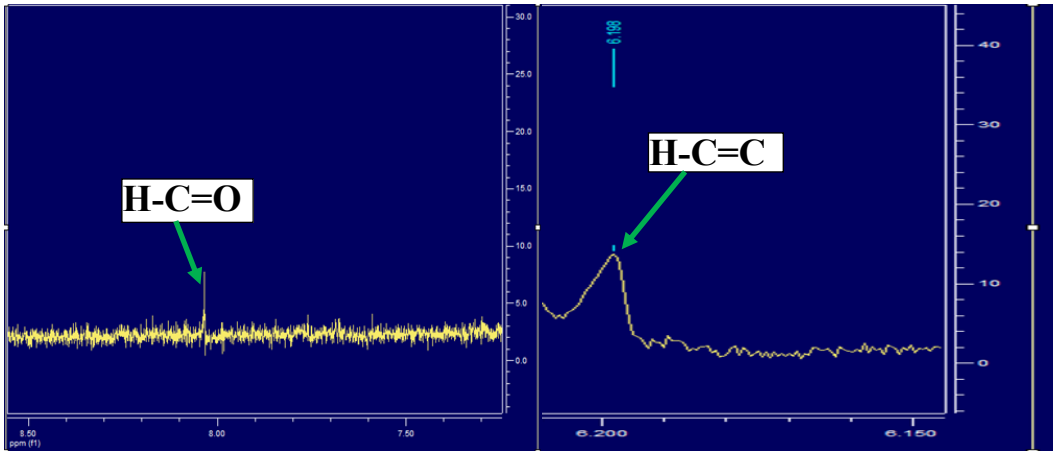


Figure 4. ^{1}H NMR Spectra of the yellowish compound.

The interpreted ^{1}H NMR Spectra are given in the following spectrum





Inferences from ^{13}C NMR Spectrum

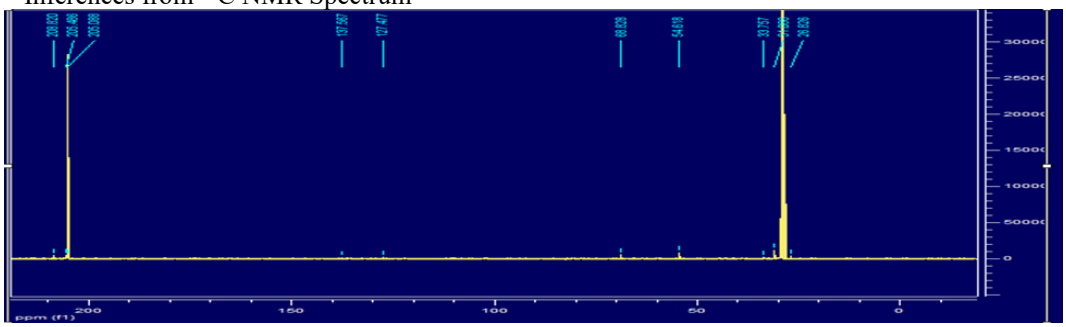
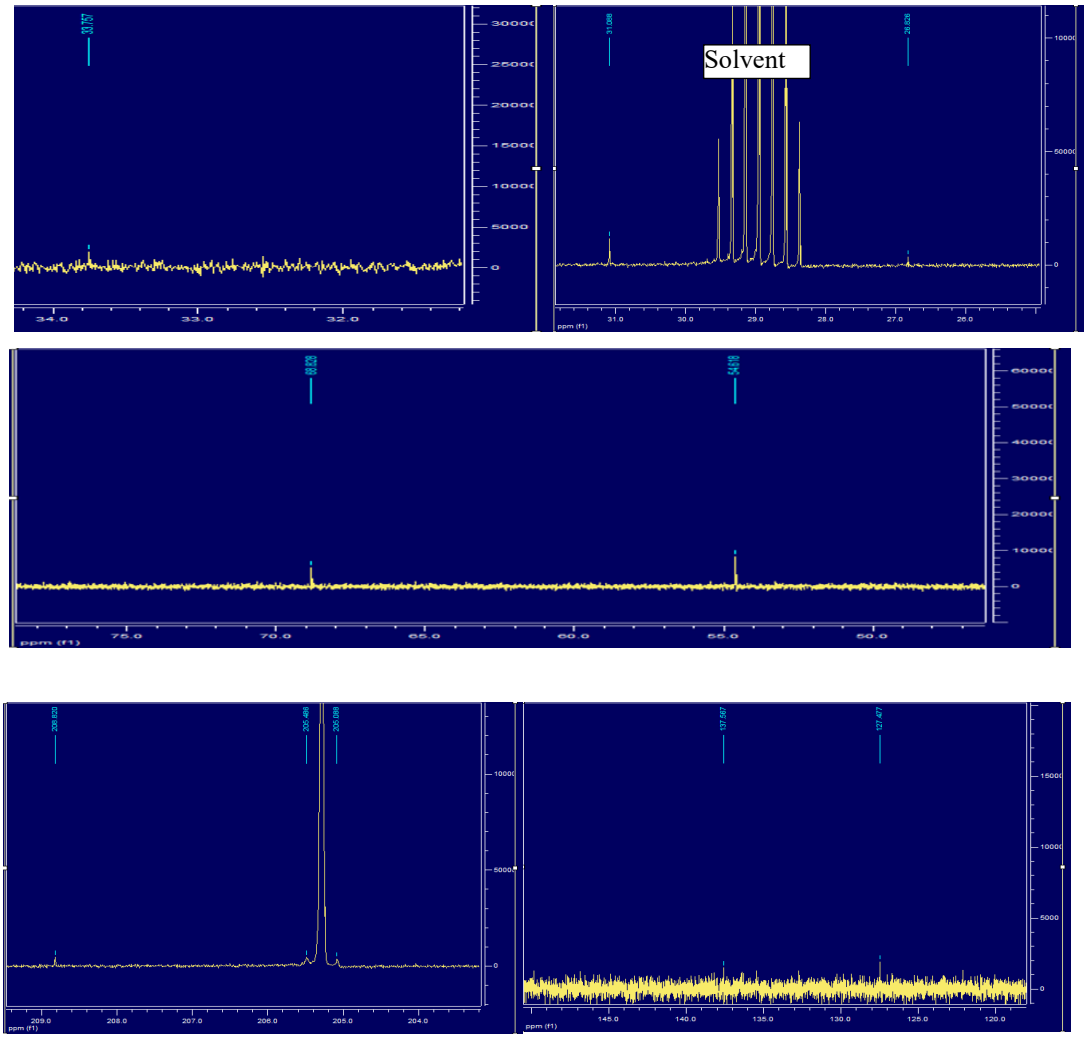


Figure 5. ^{13}C NMR Spectra of yellowish compound

The interpreted ^{13}C NMR Spectra are shown below



Inferences from DEPT Spectrum

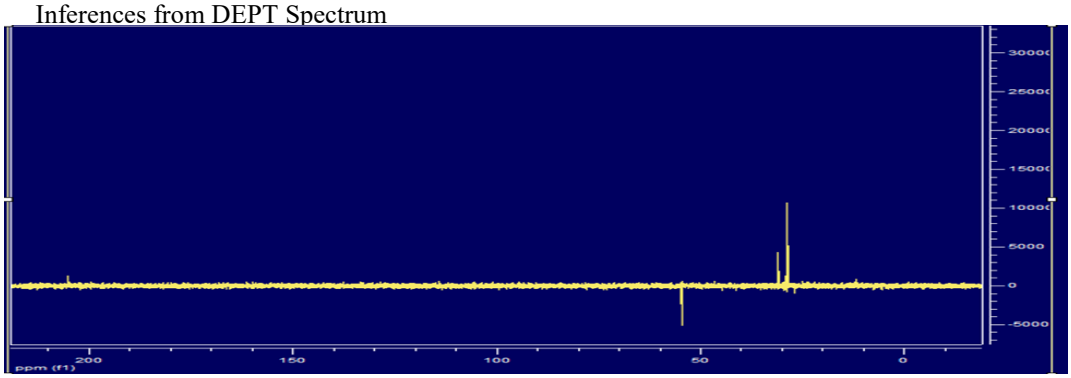


Figure 6. DEPT Spectra of yellowish compound

Table 4: Complete 1D NMR spectra of the yellowish compound in acetone-d_6

C/ H atom	Multi	δC (ppm)	δH (ppm)
C-1	CH	205.49	8.04 (s)
C-2	C	137.57	-
C-3	CH	127.48	6.20 (s)
C-4	CO	205.09	-
C-5	C	33.76	-
C-6	CO	208.82	-
C-7	CH	31.00	2.16
C-1'	CH_2	54.62	2.90 (s)
C-2'	C	68.83	-
C-3'	CH_3	26.83	1.21 (s)
1'-OH	-OH	1'-OH	1.303 (s)
2'-OH	-OH	2'-OH	3.84 (s)

From the extensive interpretation of IR, ^{1}H NMR, and ^{13}C NMR and DEPT spectrum, the possible structure of the yellowish compound is presented in Fig. 7.

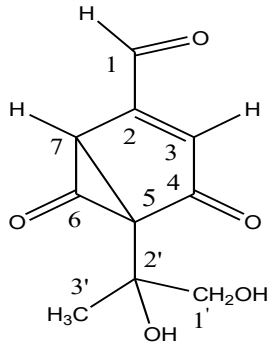


Figure 7. Possible structure of the isolated and characterized yellowish compound 5-(1',2'-dihydroxy propane-2'-yl)-4-6-dioxobicyclo [3.1.0] hex-2-ene-2-carbaldehyde

In conclusion, phytochemical constituents of leaves extract of *Zehneria scabra* from Ethiopian origin were reported for the first time. Chromatographic separation of the chloroform: ethyl acetate (9:1) yielded a mono terpenoid compound. Monoterpenoids are one of the classes of terpenoids and are common in many plant species. To make this compound applicable for clinical use, extensive studies are needed to understand its bioavailability, metabolic pathways, and toxicity to humans.

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