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Phytochemical Investigation of *Rumex Abyssinicus* Root Barks and *In Vitro* Evaluation of Its Antibacterial Activities

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Abstract: Different parts of *Rumex abyssinicus* have been used as a traditional medicine by local community in Ethiopia. Root and root barks of this plant are used traditionally by people of Ilu Ababor (Ethiopia) to lower blood pressure, heal wound and treat stomach ache. This paper presents the isolation and characterization of compound from the root barks of *Rumex abyssinicus* and evaluation of its antibacterial activity. The powdered plant material was sequentially extracted using *n*-hexane, chloroform, acetone and methanol. The crude extracts and the isolated compound (**RA-3**) were evaluated against four bacterial strains: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella thyphimurium* as a potential antibacterial agent. Both the acetone and methanol crude extracts showed promising inhibitory effects against all the tested bacterial strains. The strongest inhibitory activity was observed for acetone extract against *Staphylococcus aureus* (21 mm) as compared to the standard Gentamicin (25 mm). The isolated compound showed inhibitory effect only on *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The acetone extract was subjected to column chromatographic separation and resulted in the isolation of one pure compound (**RA-3**). The structure of this compound was characterized with the help of spectroscopic methods (IR and NMR). The isolated compound was characterized as Emodin based on spectroscopic data and in comparison with literature reports.

Keywords: *Rumex abyssinicus*, Phytochemicals, Emodin, Anthraquinone.

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INTRODUCTION

Plants contain chemical compounds called natural products that almost every plant can produce. Although any biological molecule is a natural product, the term is more precisely used for secondary metabolites which are not strictly required for the survival of the organism (1). They are derived by unique biosynthetic pathways from primary metabolites and intermediates. In Ethiopia, different plant varieties are used in the traditional health care system and remain to be the main resource of treatment for a large majority (80%) of the people (2).

Rumex abyssinicus (*R. abyssinicus*), which belongs to the family polygonaceae, is a perennial herb, up to 3m tall. It is locally called Dhangaggoo in Afan Oromo (Figure 1). It is one of the medicinal plants used in Ethiopia and is a common weed of cultivated lands or disturbed grounds ranging from North Africa to Ethiopia (3). Different parts of this plant, including root and root bark are used as a traditional medicine among Ilu Ababor people (Ethiopia) to lower blood pressure, heal wound and treat stomach ache. The rhizomes are used to treat malaria, gonorrhea, hepatitis, constipation, hypertension, migraine, rheumatism, breast cancer, stomach distention, earache, liver diseases, hemorrhoids, typhus, rabies and wound (4-7). Decoction of leaf or root powder of the plant was

taken as vermifuge. The rhizomes yield a yellow and red dye which is also used to impart a red color to the feet and hands of women of this area. The rhizomes are also used to refine butter and give it a rich yellow color.

Figure 1: Aerial part of *Rumex abyssinicus*.

Getie *et al*., (8) reported that the 80% methanol extract of the rhizomes of *R. abyssinicus* demonstrated antimicrobial and *in vitro* anti-inflammatory activities. Similarly, extracts of the plant have been proved to have diuretic and analgesic (3), and antimalarial (9) activities. *Rumex* species are known to be rich in anthraquinones, particularly in the roots (10). Betulone and Oleic acid were recently isolated from acetone extracts of the roots of *Rumex abyssinicus* (11). This research reports the phytochemical investigation of the extracts from the root bark of *R. abyssinicus* and evaluation of the antibacterial activities of the crude extracts as well as the purified compound.

EXPERIMENTAL SECTION

Plant material

Botanical identification was made by Mr. Etana Tolesa (a botanist) and a specimen was deposited (voucher number Ze01) in the Herbarium of Department of Biology, Wollega University. Then after authentication, fresh root barks of *Rumex abyssinicus* were collected from Oromia region around Ilu Ababor Zone, Metu College of Teachers' Education campus. The collected plant root barks were washed with distilled water and dried under shade in laboratory at room temperature. The dried root bark was ground with manual grinder so as to enhance effective contact of solvent with plant material.

Materials/Instruments

Pestle and mortar was used to powder the dried plant material. Rotary evaporator (RE 52-F) was employed to remove solvents from extracts. Thin-layer chromatography (TLC) analysis was performed on alumina plates precoated with silica gel (Merck 60 F_{254}). Spots were determined using UV lamp under UV radiation (λ_{max} = 254 and λ_{max} = 365 nm). Infrared (IR) spectra (KBr) were obtained from Perkin-Elmer BX infrared spectrophotometer (400-4000 cm^{-1}). One dimensional nuclear magnetic resonance (NMR) spectra were recorded on Bruker 400 MHz Avance spectrometer at Addis Ababa University, Ethiopia using DMSO-d_6 as a solvent.

Chemicals and Reagents

Solvents used for extraction were *n*-hexane, chloroform, acetone and methanol. Petroleum ether and ethyl acetate were utilized for elution. Column was packed with silica gel (100-120 mm mesh size). Chromatograms were visualized on TLC by spraying with 10% H_2SO_4 and heating on hot plate. Dimethyl sulfoxide (as a solvent), Mueller Hinton agar, nutrient broth and standard antibiotic drug gentamicin were used as a culture media during antibacterial test. All the chemicals and reagents of analytical grade were used.

Extraction and Isolation

Air dried and powdered root barks of *Rumex abyssinicus* (670 g) were sequentially extracted with *n*-hexane, chloroform, acetone and methanol by cold maceration method. The powdered material was socked with *n*-hexane (3350 mL) for 72 hours with occasional shaking and filtered first using cotton plug followed by Whatmann No.1 filter paper and concentrated by means of rotary evaporator. The marc was air dried and then socked with similar volume of chloroform for 72 hours. The extract was again filtered and concentrated. The same steps were repeated for acetone and methanol.

The crude acetone extract of *R. abyssinicus* root barks was subjected to chromatographic separation using petroleum ether/ethyl acetate solvent combinations. Crude extract (2.5 g) was adsorbed

onto 7 g of silica gel. The dry adsorbed sample was applied onto the top of column that was packed with 80 g silica gel (100-120 mesh size) slurry dissolved in petroleum ether. The column was eluted with petroleum ether and ethyl acetate mixture in different ratio with increasing polarity (Table 1).

Table 1: Column purification for acetone extract of *Rumex abyssinicus root* bark.

Fractions	Solvent ratio	Solvent used (mL)
F1-F17	Pet. ether 98:2 ethyl acetate	340
F18-F34	Pet. ether 95:5 ethyl acetate	340
F35-F51	Pet. ether 90:10 ethyl acetate	340
F52-F68	Pet. ether 85:15 ethyl acetate	340
F69-F85	Pet. ether 80:20 ethyl acetate	340
F86-F102	Pet. ether 75:25 ethyl acetate	340
F103-F119	Pet. ether 70:30 ethyl acetate	340
F120-F136	Pet. ether 65:35 ethyl acetate	340
F137-F152	Pet. ether 60:40 ethyl acetate	320

A total of 152 fractions each with 20 mL were collected. Fractions F120-F136 were combined and purified on silica gel column chromatography eluting with petroleum ether: ethyl acetate (65:35) and a total of 52 (f1-f52) fractions were collected each with 20 mL. Fractions f23-f52 were combined, solvent removed and resulted in an orange crystal solid (**RA-3**; 25mg) with R_f value of 0.57 (petroleum ether/ethyl acetate; 65:35).

Antibacterial assay

Test organisms

Four pathogenic bacterial strains, one Gram-positive (*Staphylococcus aureus* (ATCC25923)) and three Gram-negative (*Pseudomonas aeruginosa* (ATCC27853), *Escherichia coli* (ATCC25922) and *Salmonella typhimurium* (ATCC13311)) were obtained from Department of Biology, Wollega University and used to test the antibacterial activities of the root bark extracts of *R. abyssinicus* using agar well diffusion method (12).

Preparation of test samples

The test solutions were prepared by dissolving known weight of crude extract by serial dilution methods (400, 200, 100 and 50 mg) in 1 mL of Dimethyl Sulfoxide (DMSO) to achieve final stock concentration of 400, 200, 100 and 50 mg/mL, respectively. Stock bacterial cultures were maintained at 4 °C on slants of nutrient agar. Active cultures for experiments were prepared by transferring a loop full of bacterial cells from the stock cultures to test tubes of Mueller-Hinton broth (MHB) that was incubated without agitation for 24 hrs at 37 °C. A cell suspension of each organism was freshly prepared by transferring isolated colonies selected from a 24 hrs agar plate in to a broth and the suspension turbidity adjusted to a 0.5 McFarland turbidity standard ($1x10^8$ CFU/mL) in sterile saline solution (13). About 100 µL of bacterial suspensions obtained above was spread over the 90 mm Petri dishes containing Mueller-Hinton agar using a sterile cotton swab. Then 20 µL each test solutions were applied onto 6 mm diameter sterile discs, the extract was allowed to diffuse for about 10 minutes and then the plates were kept in incubator at 37 °C for 24 hrs. The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs in millimeter with ruler (14). Control wells containing neat solvent (DMSO) and gentamicin were run parallel in the same plate as negative and positive controls, respectively.

RESULTS AND DISCUSSION

Extraction yield

The crude extraction yields obtained for *n*-hexane, chloroform, acetone and methanol were 5.06g (0.75%), 8.37g (1.25%), 10.02g (1.52%) and 7.5g (1.16%), respectively (Table 2).

Table 2: Percentage yields of *Rumex abyssinicus* root barks extract.

Extraction solvents	Mass extracted (g)	% Yield
n-hexane	5.06	0.75
Chloroform	8.37	1.25
Acetone	10.02	1.52
Methanol	7.50	1.16

Structural elucidation of RA-3

The IR spectrum of **RA-3** (**S-1**) showed absorption band at 3424 cm^{-1} corresponding to the stretching vibration peak of the hydroxyl (OH) group. The band at 2936 cm^{-1} indicates the C-H stretching of methyl and the bands at 1680 cm^{-1} and 1650 cm^{-1} correspond to the unchelated and chelated carbonyl carbons absorption, respectively.

The ^{1}H-NMR (DMSO-d_6, 400 MHz) of **RA-3** (**S-2**) exhibited two hydroxyl protons at δ 11.87 (OH-1) and δ 11.95 (OH-8), two *meta*-coupled doublets at δ 6.49 (H-7) and δ 6.98 (H-5), two broad singlet signals at δ 7.01(H-2) and δ 7.31 (H-4) and one methyl proton group at δ 2.33. The result is also comparable with the ^{1}H-NMR spectral data of Emodin from literature (15) as shown in Table 3 below.

Table 3: ^{1}H-NMR (DMSO-d_6, 400 MHz) data of **RA-3** with reported data of Emodin (15).

Nature/position of Proton	^{1}H-NMR data of **RA-3**(δ_H, ppm)	Reported data for Emodin (2:1 $CDCl_3$ + MeOH-d_4) (δ_H, ppm)
OH-1	11.87 s	-
H-2	7.01 br. s	7.06 s
CH_3-3a	2.33 s	2.44 s
H-4	7.31 br. s	7.56 br. s
H-5	6.98 d, J = 2.4 Hz	7.21 d, J = 2.5 Hz
OH-6	-	-
H-7	6.49 d, J = 2.4 Hz	6.58 d, J =2.2 Hz
OH-8	11.95 s	-

The ^{13}C-NMR (DMSO-d_6, 100 MHz) (Table 4, **S-3**) spectrum of **RA-3** showed one methyl carbon signal at δ 21.9 and three oxygenated carbons at δ 161.9 (C-1), δ 165.8 (C-6) and δ 164.7 (C-8). The ^{13}C-NMR also showed two carbonyl carbons at δ 189.9 (C-9) and δ 181.4 (C-10) and one methyl substituted carbon at δ 148.5 (C-3). According to the DEPT-135 spectra (**S-4**) the peaks at δ 135.3 (C-11), δ 109.1 (C-12), δ 113.4 (C-13) and δ 133.0 (C-14) belong to the quaternary carbons and the peaks at δ 124.5 (C-2), δ 121.1 (C-4), δ 109.1 (C-5) and δ 108.0 (C-7) are characteristics of one proton carbons. The chemical shift of carbon at δ 165.8 (C-6) indicates the presence of hydroxyl group on the benzene ring at this position.

Based on the above spectroscopic data and comparison of this data with the literature values, the compound **RA-3** is a hydroxyl anthraquinone known as Emodin (Figure 2). Emodin forms the basis of a purgative anthraquinone derivative and from ancient times has been widely used as a laxative compound (16-17). It is believed that the presence of hydroxyl groups in position 1 and 8 of the aromatic ring system is essential for the purgative action of this compound (18).

Table 4: Comparison of the observed ^{13}C-NMR and DEPT-135 spectroscopic data (DMSO-d_6) of **RA-3** and Emodin (2:1 $CDCl_3$ + MeOH-d_4) from literature (15).

Position of carbon	^{13}C NMR data of **RA-3** (δc in ppm)	DEPT-135 data of **RA-3** (δc in ppm)	^{13}C-NMR of Emodin (δc in ppm)	DEPT-135 of Emodin (δc in ppm)	Appearance
C-1	161.9	-	161.6	-	Quaternary
C-2	124.5	124.5	123.6	123.6	CH
C-3	148.5	-	147.5	-	Quaternary
C-4	121.1	121.1	120.3	120.3	CH
C-5	109.1	109.1	108.7	108.7	CH
C-6	165.8	-	165.6	-	Quaternary
C-7	108.0	108.0	107.6	107.6	CH
C-8	164.7	-	164.7	-	Quaternary
C-9	189.9	-	189.8	-	Quaternary
C-10	181.4	-	181.9	-	Quaternary
C-11	135.3	-	134.8	-	Quaternary
C-12	109.1	-	109.0	-	Quaternary
C-13	113.4	-	113.1	-	Quaternary
C-14	133.0	-	132.7	-	Quaternary
3a-CH_3	21.9	21.9	20.9	20.9	CH3

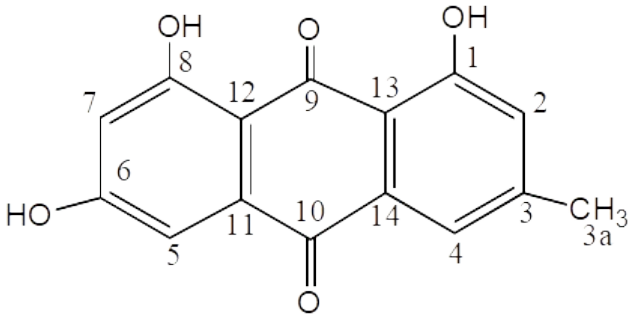

Figure 2: Proposed structure of **RA-3**.

Spectral data

The compound **RA-3** is an orange crystal, R_f 0.57 in petroleum ether: EtOAc (65:35), IRv_{max} (KBr) cm^{-1}: 2936, 1680, 1724, ^{1}H-NMR (DMSO-d_6) at δ 2.33 (3H, s, CH_3), 6.49 (1H, d, *J* = 2.4 Hz, H-7), 6.98 (1H, d, *J* = 2.4 Hz, H-5), 7.01 (1H, br s, H-2), 7.31 (1H, br s, H-4), 11.87 (OH, s, OH-1), 11.95 (OH, s, OH-8); ^{13}C-NMR (DMSO-d_6) δ: 21.9 (CH_3), 108.0 (C-7), 109.1 (C-5, C-12), 113.4 (C-13), 121.1 (C-4), 124.5 (C-2), 133.0 (C-14), 135.3 (C-11), 148.5 (C-3), 161.9 (C-1), 164.7 (C-8), 165.8 (C-6), 181.4 (C-10), 189.9 (C-9).

Antibacterial activity

The *in vitro* antibacterial activity test result of the root barks extract of *R. abyssinicus* and the isolated the compound **RA-3** was shown in Table 5 (**S-5** and **S-6**). The result indicated that the acetone crude extract showed relatively better antibacterial activity followed by methanol extract as compared to the reference drug gentamicin. However, the activity of the pure compound **RA-3** was observed to be lower than the crude extracts. This showed that either the more active ingredient was not isolated or the activity might result from the joint effect of the constituents. *E. coli* and *S. typhimurium* were found to be resistant against **RA-3.** This and the anti-inflammatory effect of *R. abyssinicus* could justify its traditional use for the treatment of several skin diseases (8).

Table 5: Inhibition zone of the crude extracts and **RA-3** (Conc. 100 mg/mL).

Extract/**RA-3**/control	Zone of inhibition (mm)			
	E. coli	*S. aureus*	*S. typhimurium*	*P. aeruginosa*
n-Hexane extract	NA	NA	NA	NA
Chloroform extract	NA	NA	NA	NA
Acetone extract	17	21	19	19
Methanol extract	16	20	13	19
RA-3	NA	14	NA	12
Gentamicin	25	25	21	21
DMSO	NA	NA	NA	NA

NA – not active

CONCLUSION

Herbal remedies have been used for centuries but more recently, the compounds that are active have been identified, extracted and purified. The increase in drug resistance bacteria also urges chemists and other scientists to find more and more bioactive chemicals from medicinal plants. Several species of Rumex have important medicinal properties and they have been the subject of several pharmacological investigations. In the work presented herein one compound namely Emodin was isolated from the crude acetone extract of root barks of *R. abyssinicus*. Acetone crude extract demonstrated better bioactivity against the tested bacterial strains. However; the isolated compound **RA-3** showed moderate activity only on two of the tested pathogens at the tested concentration.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGMENTS

The authors are grateful to Wollega University.

Supplementary Materials

The IR spectrum, ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 spectra generated for identifying **RA-3** (Emodin) are annexed as supporting material **S-1** to **S-4**. Zone of bacterial growth inhibition of the crude extracts and isolated compound are attached as supporting material **S-5** and **S-6**, respectively. **S-1**: IR spectrum of **RA-3**. **S-2**: 1H-NMR spectrum of **RA-3**. **S-3**: 13C-NMR spectrum of **RA-3**. **S-4**: DEPT-135

spectrum of **RA-3**. **S-5**: Zone of bacterial growth inhibition of crude extracts of root of *R. abyssinicus*. **S-6**: Zones of bacterial growth inhibition of the isolated compound **RA-3**.

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Supplementary Information

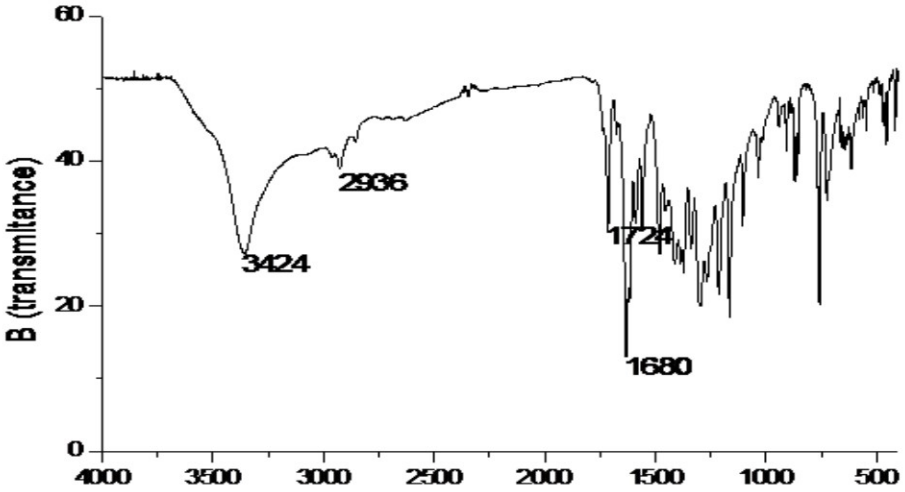

Figure S-1: IR spectrum of RA-3.

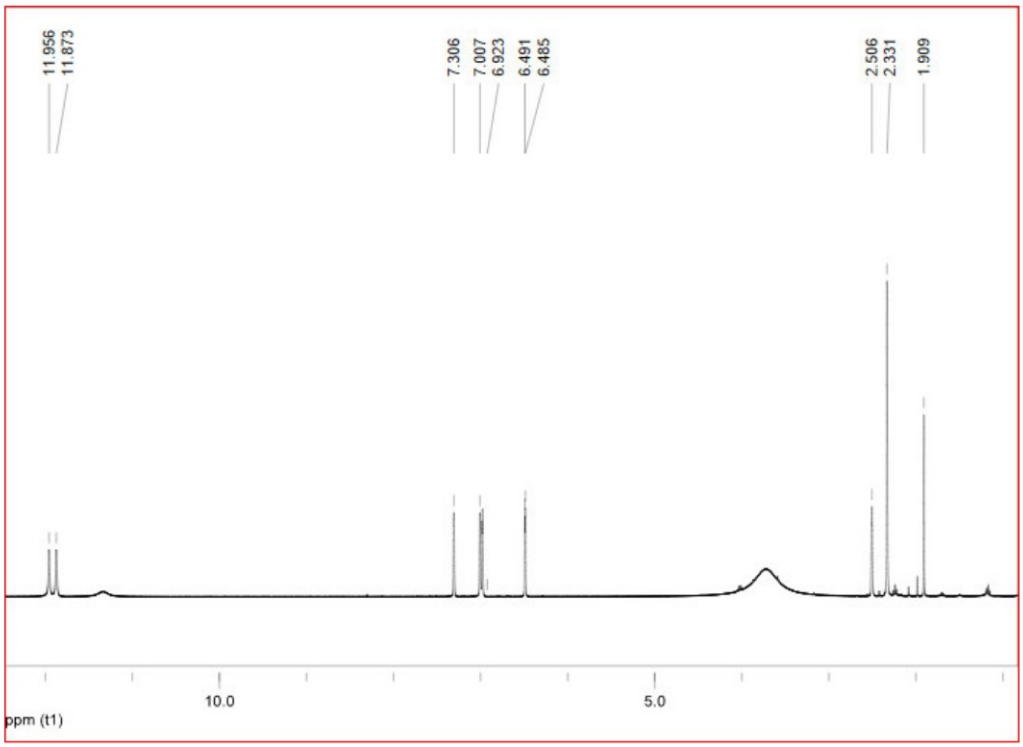

Figure S-2: ^{1}H NMR (DMSO-d_6) spectrum of RA-3.

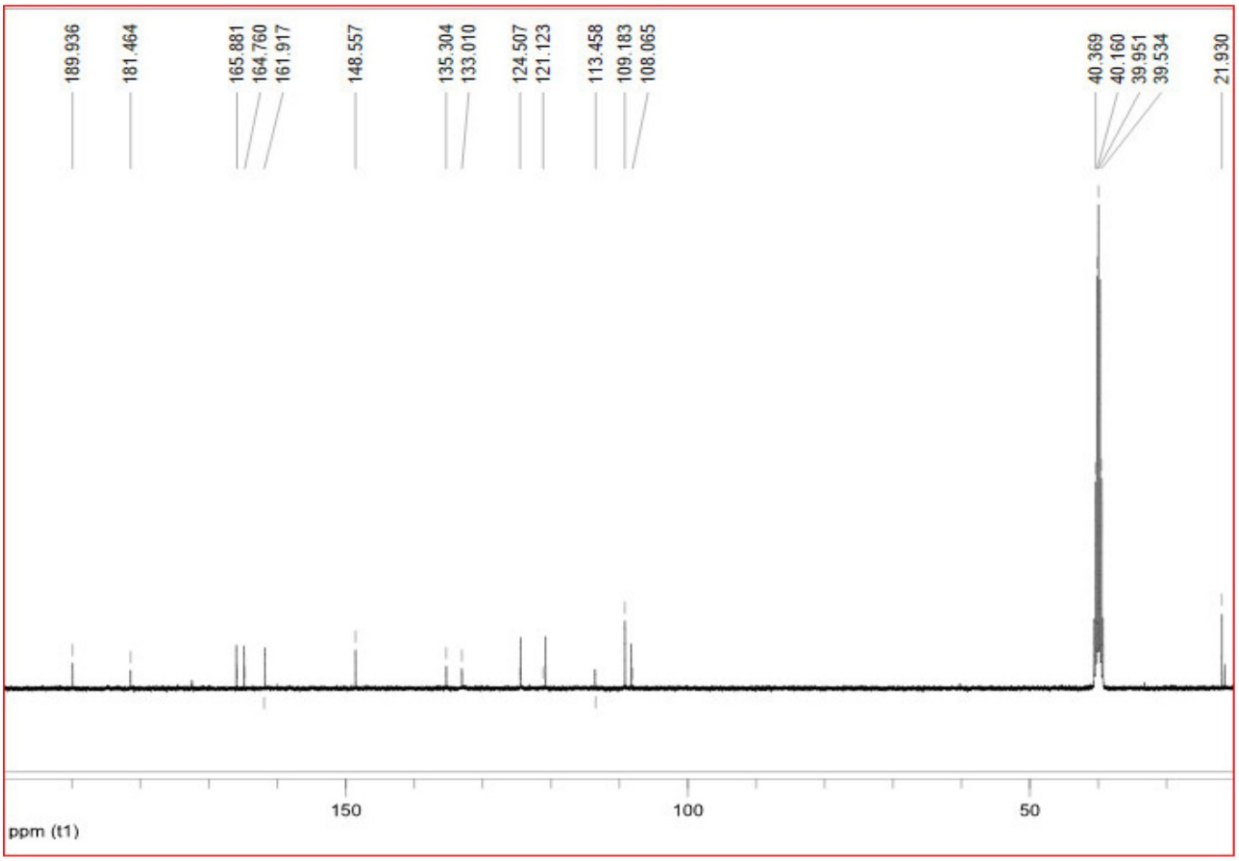

Figure S-3: ^{13}C NMR (DMSO-d_6) spectrum of RA-3.

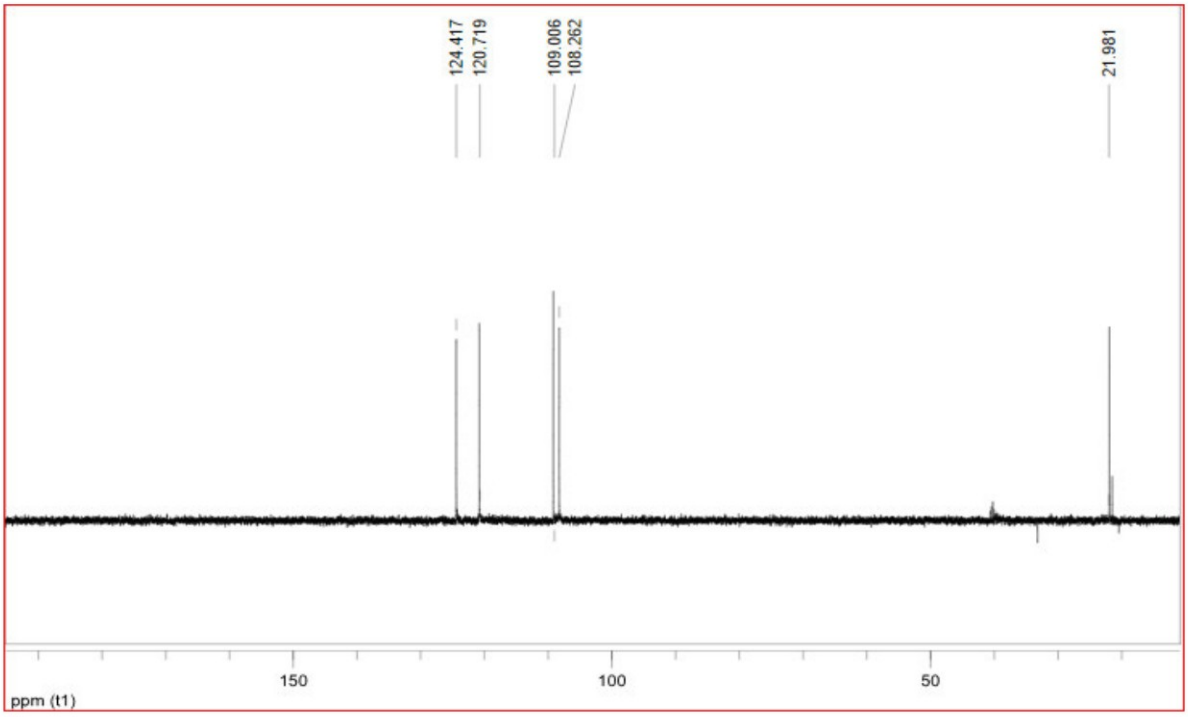

Figure S-4: DEPT-135(DMSO-d6) spectrum of RA-3.

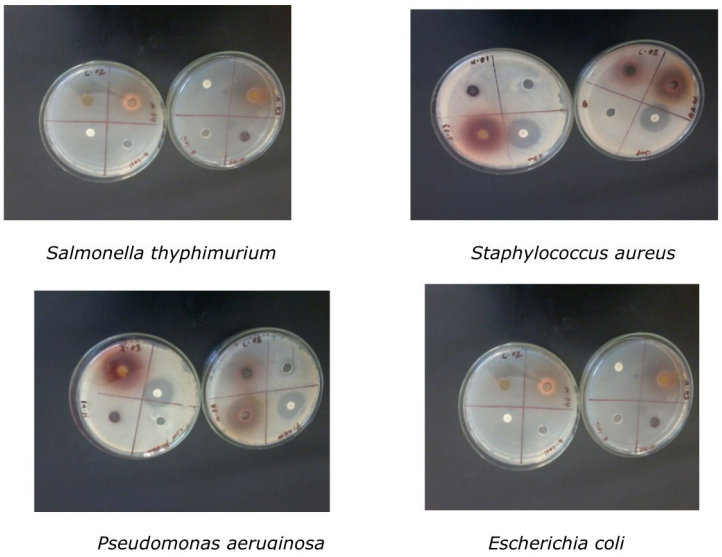

Figure S-5: Antibacterial activity of crude extracts of the root barks of *R. abyssinicus*.

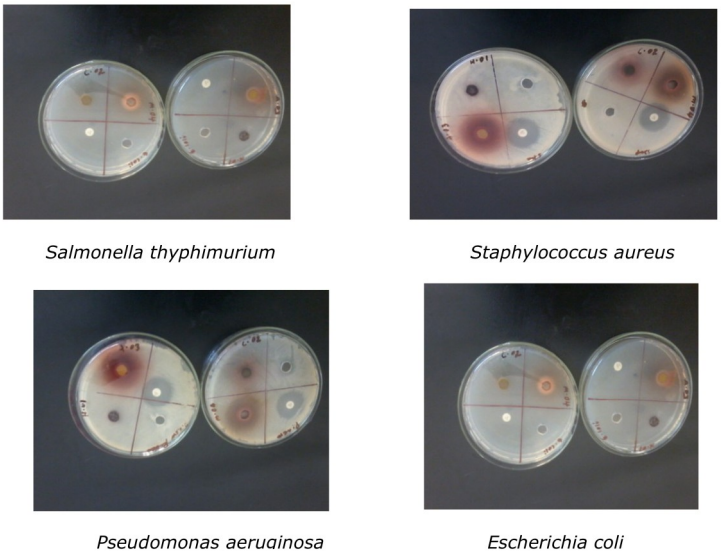

Figure S-6: Zone of bacterial growth inhibition of RA-3.