Natural Products Chemistry & Research

ISSN: 2329-6836

Zelalem and Dula, Nat Prod Chem Res 2019, 7:1
DOI: 10.4172/2329-6836.1000353

Research Article Open Access

Isolation, Characterization and Antibacterial Activities Evaluation of *Rumex abyssinicus* Rootbark Extracts

Zelalem GA[1*] **and Dula DE**[2]

[1]*Department of Material Science and Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan*

[2]*Department of Chemistry, Nekemte College of Teacher Education, Nekemte, Ethiopia*

[*]**Corresponding author:** Zelalem GA, Department of Material Science and Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan, Tel: +886966493801; E-mail: zelalemgudeta5@gmail.com

Received: December 22, 2018; **Accepted:** January 11, 2019; **Published:** January 18, 2019

Abstract

The bioactive phytochemical constituents present in the plant play a significant role in the development of medicines and drug discovery. *Rumex abyssinicus* is one of the plants that grow in Ethiopia and different parts of this plant are used as a traditional medicine. The aim of this research was isolation and characterization of bioactive compounds from *Rumex abyssinicus* rootbark. The plant material was collected from Oromia region around Ilu Ababor Zone, Metu College of teachers' education campus during February 2016. The dried and powdered plant material was subjected to sequential solvent extraction using maceration technique to prepare the crude extract which was directly used for antibacterial studies. The lists of solvents taken for the extraction were n-hexane, chloroform, acetone and methanol. Antibacterial evaluation of crude extracts of the plant rootbark were screened using *in vitro* method against four pathogenic bacteria species namely Gram-positive *Staphylococcus aureus*, Gram negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella thyphimurium*. These sequentially obtained solvent extracts were filtered and concentrated under reduced pressure. The results showed the acetone extract followed by methanol crude extracts showed inhibitory effects against all of the tested bacterial strains, but the isolated compounds showed inhibitory effects on *S. aureus* and *P. aeruginosa* bacterial strains. The acetone extract was subjected to column chromatographic separation that led to isolation of two compounds. The structures of these compounds were characterized with the help of spectroscopic methods (IR and NMR). The isolated compounds were characterized as diisobutyl phthalate (RA-2) (isolated for the first time from the plant) and Emodin (RA-3) based on spectroscopic data and in comparison, with literature reports.

Keywords: Antibacterial activity; Characterization; Extraction; Emodin; Di-isobutyl phthalate; Isolation; *Rumex abyssinicus*; Structural elucidation

Introduction

In Ethiopia medicinal plants play major supplementary roles to the limited modern health care available. The rich traditional knowledge of the people has over the centuries led to the application of plants for food, medicine and other uses. In the country traditional health care is culturally deep rooted with oral and written pharmacopoeias. Ethiopian plants have shown very effective medicinal value for some ailments of human and domestic animals. Thus, medicinal plants and knowledge of their use provide a vital contribution to human and livestock health care needs throughout the country. The major reasons why medicinal plants are demanded in Ethiopia are due to culturally linked traditions, the trust the communities have in the medicinal values of traditional medicine and relatively low cost in using them. Medicinal plants obtained from wild habitats are found in different natural ecosystems of the forests, grasslands, woodlands, wetlands, in field margins and garden fences, as weeds and in many other microhabitats from where they are harvested when the need arises [1].

Rumex abyssinicus is widely spread medicinal plant in the highlands of tropical Africa and is a common weed of cultivated lands or disturbed grounds ranging from North Africa to Ethiopia [2]. It is one of medicinal plants used by Ilu Ababor people (Southwest Oromia, Ethiopia) and its root as well as rootbark is used to lower blood pressure, heal wound and treat stomach ache. 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 present study was aimed at the evaluation of antibacterial activity, and thereby carrying out the isolation and characterization of bioactive compounds from the rootbark of *Rumex abyssinicus*.

Materials and Methods

Chemicals

Chemicals that were used during this study include petroleum ether, hexane, chloroform, acetone, ethyl acetate and methanol. Silica gel (100-120 mm mesh size), TLC plates, vanillin, Dimethyl sulfoxide (DMSO), Mueller Hinton agar, nutrient broth and standard antibiotic gentamicine. All the chemicals and reagents used were of analytical grade.

Apparatus and instruments

Apparatuses like rotary evaporator (RE 52-F), round bottom flask (50 mL, 100 mL, and 250 mL), volumetric flask, measuring cylinder, pestle and mortar, filter papers, cotton, weighing balances, water bath, oven for drying purpose, reflux condenser and glass columns for column chromatography were used for the study. Spectral recording were done using Bruker 400 MHz advance NMR spectrometer and Deutrated DMSO as solvent. Infrared (IR) spectra (KBr) were obtained

from Perkin-Elmer BX infrared spectrometer (400-4000 cm^{-1}). All spectroscopic analysis were carried out at the Department of Chemistry, Addis Ababa University.

Collection and preparation of plant material

Fresh rootbark of *Rumex abyssinicus* were collected from Oromia region around Ilu Ababor Zone, Metu College of Teachers' Education campus during February 2016. The collected plant rootbark were washed with distilled water and shade dried in laboratory at room temperature. The dried part was grounded with manual grinder so as to enhance effective contact of solvent with plant material and make more homogenizing of the sample, increasing the surface area, and facilitating the penetration of solvents into cells of the plant powder. 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.

Extraction of *Rumex abyssinicus* rootbark

Air dried powdered rootbark of *Rumex abyssinicus* was sequentially extracted with n-hexane, chloroform, acetone and methanol. The successive extraction with solvents of increasing polarity ensures that wide polarity range of compounds were extracted. Six hundred seventy grams (670 g) of fine powdered material was socked with hexane (3.35 L) for 72 hours (with manual shaking) and extracted with hexane. The combined extracts were filtered and concentrated by means of a rotary evaporator. The marc obtained from filtration of the hexane extract was then socked with chloroform twice for 72 hours and extracted with the same solvent and the combined extracts were filtered and concentrated. The marc was then socked with acetone twice for 72 hours and extracted and the combined extracts were filtered and concentrated. The methanol extract was also prepared in the same way as the chloroform and acetone extract (Scheme 1). The mixtures were filtered first by cotton plugged followed by Whatman No.1 filter paper and residual solvent in each gradient extract was removed using Rotary evaporator under reduced pressure. Fractions were then being placed in an oven at not more than 40°C for about 48 hours to remove any residual solvent. The resulting semidried mass of each fraction were stored at 4°C until used for experiments [3].

Antimicrobial assay of the crude rootbark extracts: Under the antimicrobial assay, of course, evaluation of antibacterial activities were done for both crude rootbark extracts and isolated compounds in which both cases were explained in result and discussion sections.

Test strains: Microorganisms used for determination of antibacterial activities of the crude plant extracts were: gram positive (*Staphylococcus aureus*), gram negative (*Escherichia coli*), gram negative (*Pseudomonas aeruginosa*) and gram negative (*Salmonella thyphimurium*).

Preparation of test samples: The test solution 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.

Preparation of fresh inoculums: 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 hours at 37°C. A cell suspension of each organism was freshly prepared by transferring isolated colonies selected from a 24 hours agar plate in to a broth and the suspension turbidity adjusted to a 0.5 McFarland turbidity standard (1×10^8 CFU/mL) in sterile saline solution [4].

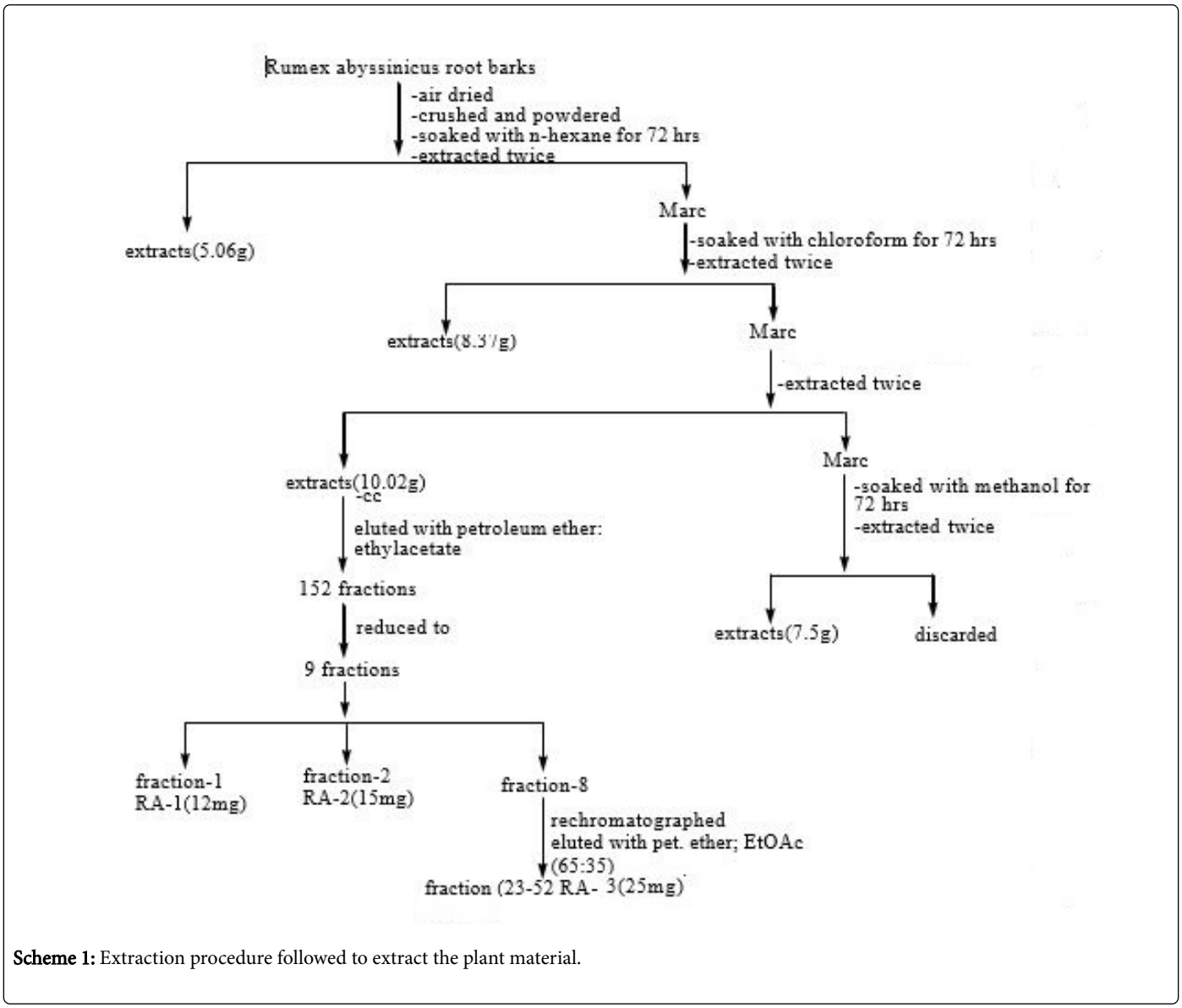
Disc diffusion method: About 100 µL of bacterial suspensions obtained above was spread over the 90 mm Petri dishes containing Mueller-Hinton agar using a sterile cotton swab. Then six mm diameter sterile discs were bore red by cork borer on the surface of the inoculated Agar in Petri dishes, and 20 µL each test solutions were applied onto the discs. After addition of test solutions on the discs, the extract was allowed to diffuse for 5-10 minutes and then the plates were kept in incubator at 37°C until 24 hours. The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs in millimeter with ruler [5]. The disk diffusion assay was used as a preliminary test to select the most efficient extracts.

Isolation and characterization of compounds: The crude acetone extract of *Rumex abyssinicus* rootbark was subjected to chromatographic separation using petroleum ether/ethyl acetate solvent combinations. Solvent system for elution of the column was determined after carrying out the TLC analyses in various combinations of petroleum ether, chloroform and ethyl acetate with different polarity. Among the solvent combinations tried petroleum ether/ethyl acetate combination showed better separation of the visible spots. From the crude 10.02 g of acetone extract, 2.5 g was adsorbed onto 10 g of silica gel. Then the dry sample was applied into the column that was packed with 100 g silica gel (100-120 mesh) slurry dissolved in petroleum ether. The column was eluted with petroleum ether and ethyl acetate mixture in different combination with increasing polarity (in the ratio 98:2, 95:5, 90:10, 85:15, 80:20, 75:25 up to 60:40%) which is tabulated in Table 1 below.

S. No	Fractions	Solvent ratio	Volume of solvent used (mL)
1	1-17	Petroleum ether 98: 2 ethyl acetate	340
2	18-34	Petroleum ether 95:5 ethyl acetate	340
3	35-51	Petroleum ether 90:10 ethyl acetate	340
4	52-68	Petroleum ether 85:15 ethyl acetate	340
5	69-85	Petroleum ether 80:20 ethyl acetate	340
6	86-102	Petroleum ether 75:25 ethyl acetate	340

7	103-119	Petroleum ether 70:30 ethyl acetate	340
8	120-136	Petroleum ether 65:35 ethyl acetate	340
9	137-152	Petroleum ether 60:40 ethyl acetate	340

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



Scheme 1: Extraction procedure followed to extract the plant material.

A total of 152 fractions each with 20 mL were collected in small beakers and TLC analysis was done. According to TLC profiles, these fractions were reduced to 9 fractions. This was done by comparison of observed spots on TLC and their R_f values, i.e., fractions with similar R_f values were mixed. The developed spots on TLC plates were visualized by spray of vanillin. Among reduced 9 fractions, fraction 1 eluted with petroleum ether: ethyl acetate (98:2) resulted single spot on TLC and showed an R_f value of 0.46 (98:2 petroleum ether: ethyl acetate). The product was yellow-orange crystalline solid and designated as Compound RA-1 (12 mg). Fraction 2 eluted with petroleum ether: ethyl acetate (95:5) showed single spot on TLC and showed an R_f value of 0.35 (98:2 petroleum ether: ethyl acetate). The product was yellowish crystalline solid and designated as compound RA-2 (15 mg). Fraction-8 was rechromatographed on silica gel eluting with petroleum ether: ethyl acetate (65:35) and a total of 52 fractions were collected each with 20 mL.

Fractions 23-52 were combined and the product was yellowish- red crystal solid (25 mg) and its R_f value was determined as 0.57 in petroleum ether: ethyl acetate (65:35). The compound was labeled as

RA-3. The isolated compounds were then characterized by the spectral techniques namely, IR, ^{1}HNMR and ^{13}C NMR.

Results and Discussion

Fractional extraction of rootbark extracts of *Rumex abyssinicus* plant species

The rootbark part of the plant species was successively extracted with n-hexane, chloroform, acetone and methanol by using maceration. These extracts after removal of the solvents were used for the both antibacterial assay and isolation. The yield from different solvent extracts is presented in Table 2 below.

Extraction solvents	Mass extracted (g)	% Yield
n-hexane	5.06	0.755
Chloroform	8.37	1.249
Acetone	10.02	1.5
Methanol	7.5	1.119

Table 2: Percentage yields of *Rumex abyssinicus* rootbark extracts.

Evaluation of antibacterial activities of crude extracts from *Rumex abyssinicus* rootbark

The inhibitory activity of the four different extracts of *Rumex abyssinicus* against some human pathogenic bacteria was presented in Table 3. Among the four different extracts tested, acetone extract showed good antibacterial activity followed by methanol extract, whereas no significant antibacterial activity was observed in n-hexane and chloroform extracts (Appendix 1). While the standard test gentamicine showed the maximum antibacterial activity compared to the four extracts, the negative control DMSO did not inhibit any of the bacteria tested. The acetone extract exhibited antibacterial activity with zone of inhibition ranging from 17 mm to 21 mm at 100 mg/mL concentration depending upon bacterial species. The most susceptible organism in the present investigation was *Staphylococcus aureus* followed by *Pseudomonas aeruginosa* and *Salmonella thyphimurium*, whereas *Escherichia coli* were found to be most resistant bacteria against all the extracts tested (Table 3). The effectiveness of the extracts varies with the kind of bacteria used in the study. These differences in the susceptibility of the test organisms to the different extracts might be due to the variation in the rate at which active ingredients penetrate their cell wall and cell membrane structures.

The results suggest that the acetone extract has broad spectrum against bacterial pathogens. Therefore, the crude acetone extract of *Rumex abyssinicus* rootbark was selected as the best candidate for chromatographic isolation of compounds.

Structural elucidation of the isolated compounds

The structures of compounds (RA-2 and RA-3) those isolated from acetone extract of *Rumex abyssinicus* rootbark were discussed below. But the structure of RA-1 was not analyzed due to small (insufficient) quantity of the sample for NMR spectroscopic techniques. The compounds were characterized using spectroscopic techniques (IR and NMR) and the structural elucidation was done by comparing the observed spectra with the reported data of these compounds in the literature.

Bacteria strain	conc. mg/mL	Diameter of zone in mm				
		HE	CE	AE	ME	Ge (10 µg)
Escherichia coli	100	na	na	17	16	25
Staphylococcus aureus	100	na	na	21	20	25
Salmonella thyphimurium	100	na	na	19	13	21
Pseudomonas aeruginosa	100	na	na	19	19	21
Note: na=not active; HE-hexane extract; CE-chloroform extract; AE-acetone extract; ME-methanol extract; Ge-Gentamicin						

Table 3: Antibacterial activity of different solvent extracts of *Rumex abyssinicus* rootbark against bacterial strains.

The structural elucidation of RA-2

The IR spectrum of RA-2 (Appendix 2) showed the absorption band at 2924 cm^{-1} corresponding to the stretching of C-H of alkane. The band at 1670 cm^{-1} most probably belongs to the carbonyl frequency and the band at 1250 cm^{-1} showed the C-O stretching carbonyl frequency of ester. The broad band around 3400 cm^{-1} indicates the presence of hydroxide group. This may be due to the moisture absorbed by the sample or by KBr.

^{1}H NMR spectrum of compound RA-2 (Appendix 3) showed a peak at δ 2.5 observed due to solvent (DMSO-d6); the peak at δ 0.95 (H-1) indicates protons of methyl groups. Signal at δ 4.00 (H-3) indicates protons that were attached with oxygenated carbon, the peak at δ 7.74 (H-6) double doublets and δ 7.69 (H-7) triple triplets might be proton on aromatic ring, and a peak at δ 1.90 (H-2) septets indicates the signal of methine carbon flanked between methylene and methyl carbons. The ^{1}H NMR data of RA-2 is shown in Table 4 below.

Position of H	Observed data of ^{1}H-NMR (δ in ppm)
H-1	0.95 d J=6.8 Hz
H-2	1.95 m
H-3	4.00 d
H-6	7.74 dd
H-7	7.69 tt

Table 4: ^{1}H-NMR data of RA-2.

The ^{13}C NMR (Appendix 4) spectrum showed one methyl carbon signals at δ 19.30, one carbonyl carbons at δ 167.41 (C-4), one oxygenated carbon at δ 71.60 (C-3) and one methyne carbon at δ 27.65 (C-2).

The DEPT-135 spectrum (Appendix 5) of RA-2 indicates the presence of one methyl proton at δ 19.30 and two aromatic C-H signals at δ 132.01 (C-6) and δ 129.13 (C-7). Additionally, two quaternary carbon signals are shown at δ 167.41 (C-4) and δ 132.14 (C-5) (Table 5).

Position of carbon	^{13}C NMR data	DEPT-135 data	Multiplicity
C-1	19.30	19.30	CH_3
C-2	27.65	27.65	CH
C-3	71.60	71.60	CH_2
C-4	167.41	-	Quaternary
C-5	132.14	-	Quaternary
C-6	129.13	129.13	CH
C-7	132.01	132.01	CH

Table 5: The observed ^{13}C NMR and DEPT-135 spectroscopic data of (DMSO-d6) RA-2.

The HSQC spectrum (Appendix 6) of RA-2 correlates the chemical shift of proton with the directly bonded carbon atom. The HSQC spectrum showed connectivity between the following pairs: δH 0.95 and δC 19.30 (C-1); δH 1.90 and δC 27.65 (C-2); δH 4.00 and δC 71.60 (C-3); δH 7.74 and δC 129.13 (C-6); δH 7.69 and δC 132.01 (C-7) (Table 6).

The HMBC (Appendix 7) was also used to propose the structure of a compound by correlation of proton with carbon that is two or three bond away (Table 7).

As can be deduced from all spectroscopic data above, the possible structure of RA-2 is given in Figure 1 and is named as diisobutyl phthalate.

Position of Carbon	^{13}C NMR data of RA-2 (δ in ppm)	HSQC Spectra data of RA-2
C-1	19.3	C-1↔H-1
C-2	27.65	C-2↔H-2
C-3	71.6	C-3↔H-3
C-6	129.13	C-6↔H-6
C-7	132.01	C-7↔H-7

Table 6: ^{13}C NMR (DEPT-135) and HSQC spectroscopic data of RA-2.

Position of carbon	^{13}C NMR data of RA-2 (δ in ppm)	HMBC spectra data of RA-2
C-1	19.30	H-1→C-1, C-2 and C-3
C-2	27.65	H-2→C-1 and C-3
C-3	71.60	H-3→C-1.C-2 and C-4
C-4	167.41	-
C-5	132.14	-
C-6	129.13	H-6→C-7 and C-4
C-7	132.01	H-7→ C-6

Table 7: Observed correlation in HMBC spectroscopic data of RA-2.

The structural elucidation of RA-3

The IR spectrum of RA-3 (Appendix 8) showed that the 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 and the bands at 1680 cm^{-1} and 1650 cm^{-1} correspond to the un-chelated and chelated carbonyl carbons absorption, respectively.

The ^{1}H-NMR of this compound (DMSO-d6, Appedix-8) exhibited two hydroxyl protons at δ 11.87 (OH-1) and δ 11.95 (OH-8), two meta coupled doublet 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 group at δ 2.33. The result is also comparable with the ^{1}H-NMR spectra data of Emodin from literature as shown in Table 8 below.

The ^{13}C NMR (Appendix 9) spectrum showed one methyl carbon signal at δ 21.92 and three oxygenated carbons at δ 161.78 (C-1), δ 165.95 (C-6) and δ 164.83 (C-8). The ^{13}C NMR also shows two carbonyl carbon at δ 189.91 (C-9) and δ 181.50 (C-10). One methyl substituted carbon at δ 148.55 (C-3). According to the DEPT 135 (Appendix 10) spectra the peaks at δ 135.26 (C-11), δ 109.16 (C-12), δ 113.53 (C-13) and δ 132.96 (C-14) belong to the quaternary carbons and the peaks at δ 124.41 (C-2), δ 120.78 (C-4), δ 109.18 (C-5) and δ 108.24 (C-7) characteristics of one proton carbons. The chemical shift of carbon at δ 165.95 (C-6) indicate the presence of hydroxyl group on the benzene ring at this position (Table 9).

H	^{1}H-NMR data of RA-3 (δ, ppm)	Reported data of Emodin *(δ, ppm)
OH-1	11.87 s	12.01 s
H-2	7.01 br.s	7.18br.s
CH3-3a	2.33 s	2.40 s
H-4	7.31 br.s	7.50 br.s
H-5	6.98 d J=2.4 Hz	7.20 d, J=1.6Hz
H-6	-	-
H-7	6.49 d J=2.4 Hz	6.60 d, J=1.6Hz
OH-8	11.95 s	12.15 s
*Data from Zinaye and Fiseha, 2008 AAU electronic library.		

Table 8: ^{1}H NMR data of RA-3 with reported data of Emodin.

Position of Carbon	Observed ^{13}C NMR data (δ in ppm)	Observed DEPT-135 data (δ in ppm)	Reported data of Emodin *^{13}C NMR	Reported data of Emodin *DEPT-135	Nature of Carbon
C-1	161.8	-	161.9	-	Quaternary
C-2	124.4	124.4	124.6	124.6	CH
C-3	148.5	-	148.8	-	Quaternary
C-4	120.8	120.8	120.9	120.9	CH
C-5	109.2	109.2	109.3	109.3	CH
C-6	165.9	-	166.1	-	Quaternary
C-7	108.2	108.2	108.4	108.4	CH
C-8	164.8	-	164.9	-	Quaternary
C-9	189.9	-	190.1	-	Quaternary
C-10	181.5	-	181.9	-	Quaternary
C-11	135.3	-	135.6	-	Quaternary
C-12	109.3	-	109.4	-	Quaternary
C-13	113.5	-	113.8	-	Quaternary
C-14	132.9	-	133.3	-	Quaternary
3a-CH	21.9	21.9	21.9	21.9	CH_3
*Data from Zinaye and Fiseha, 2008 AAU electronic library					

Table 9: Comparison of the observed ^{13}C NMR and DEPT-135 spectroscopic data (DMSO-d6) of RA-3 and Emodin.

From the observed and reported literature data of ^{1}H NMR, ^{13}C NMR and DEPT-135 RA-3 is a hydroxyl anthraquinone known as Emodin.

Emodin forms the basis of a purgative anthraquinone derivative and from ancient times has been widely used as a laxative compound [6]. 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 [7] (Figure 1).

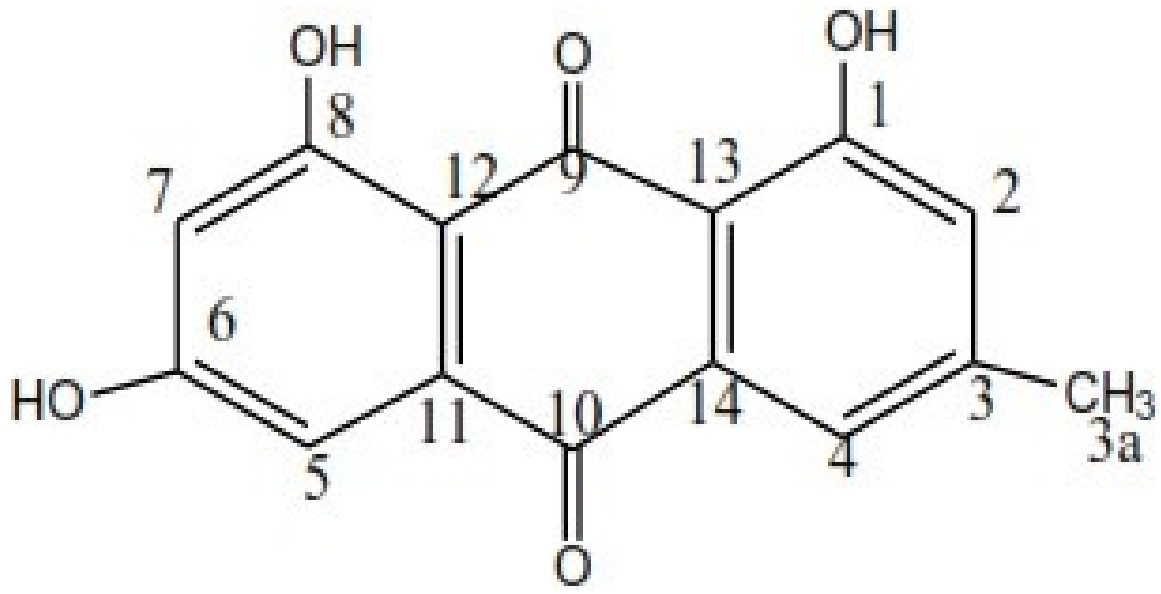


Figure 1: The structure of Emodin.

Evaluation of anti-bacterial activities of the isolated compounds

The anti-microbial activities of two isolated compounds (RA-2 and RA-3) were carried using four human pathogenic bacteria species namely *Pseudomonas aeruginosa*, *Salmonella thyphimurium*, *Staphylococcus aureus* and *Escherichia coli* by disc diffusion method. The growth inhibition zones (in mm) of the compounds were given in Table 10. The result indicated that RA-2 and RA-3 showed a better antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacterial species. On comparison basis, the antibacterial activity of RA-3 showed more than RA-2 against the two of the bacterial strains (Table 10). Thus, these results prove the antimicrobial potential of Emodin isolated from the *Rumex abyssinicus* rootbark and provide additional support for the use of this plant as traditional medicine. Both RA-2 and RA-3 showed no activity against *Escherichia coli* and *Salmonella thyphimurium* bacterial strains [8,9]. Similar to the crude extracts, the antibacterial activities of the isolated compounds were lower than that of the reference drug (gentamicine) against all bacterial species used in the experiment. However, as evidenced from the activity of the acetone crude extract compared to individual isolated compounds there are still more active and unidentified secondary metabolites. The most susceptible organism was *Staphylococcus aureus* followed by *Pseudomonas aeruginosa* whereas *Escherichia coli* and *Salmonella thyphimurium* were found to be most resistant bacteria against all the isolated compounds (Appendix 11 and 12).

Bacteria strains	Conc.	Diameter of zone in mm			
	mg/mL	RA-2	RA-3	Ge (10 µg)	DMSO
Escherichia coli	100	na	na	24	na
Staphylococcus aureus	100	11	14	25	na
Salmonella thyphimurium	100	na	na	21	na
Pseudomonas aeruginosa	100	10	12	21	na
Note: na-not active; Ge-Gentamicin.					

Table 10: Anti-bacterial activity of isolated compounds by disc diffusion methods.

Conclusion

From the results of this study two compounds (RA-2 and RA-3) were isolated from the crude acetone extract rootbark of *Rumex abyssinicus*. The compounds were identified as diisobutyl phthalate and Emodin based on spectroscopic (IR and NMR) data as well as literature reports. The isolation of RA-2 (diisobutyl phthalate) is reported for the first time from *Rumex abyssinicus* plant. The observed antibacterial activities of the crude extract and isolated compound RA-3 could justify the traditional use of the plant for the treatment of bacterial infection.

Recommendations

As evidenced from the activity of the crude extract compared to individual isolated compounds there are still more active and unidentified secondary metabolites. Further work is needed to isolate more bioactive compounds from the rootbark of *Rumex abyssinicus*. In addition, further test is recommended on large number of bacterial strains to conclude as the potential candidate in the development of antibacterial drugs.

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