World Journal of Chemistry 13 (1): 01-13, 2018
ISSN 1817-3128

DOI: 10.5829/idosi.wjc.2018.01.13

Phytochemical Investigation and Isolation of Compounds From *Ajuga integrifolia* Root Extract

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Abstract: *Ajuga integrifolia* is one of the medicinal plants found in Ethiopia. Its different morphological parts are used for treatment of several human illnesses suggesting the need of identification of secondary metabolite that are responsible for the observed traditional medicinal use of the plant. The objective of study is to carry out phytochemical screening tests of crude extracts of the roots of this plant "species" and also to isolate compounds from extracts. Crude extracts were obtained from dried ground roots using solvents of different polarity (n-hexane, dichloromethane/methanol (1:1) and methanol) for extraction and employing sequential extraction approach. The phytochemical screening tests carried out on the extracts revealed the presence of alkaloids, flavonoids, phenols, glycosides, terpenoids, tannins, saponins and steroids. Chromatographic separation from dichloromethane/methanol (1:1) extract lead to isolation of two compounds (**DB6** and **DB4**). The compounds were identified to be 1-O-3,4-(dihydoxyphenyl)-ethyl-β-D-apiofuranosyl-(1→4)-α-L-rhamnopyranosyl- (1→3)-4-O-caffeoyl-β-D-glucopyranoside and (24S)-24-ethylcholesta-5,25-diene-11α-hydroxy-1-one, respectively, based on the spectral data and comparison with literature reports. This is the first report of isolation of the compounds from this species.

Key words: *Ajuga Integrifolia* · Phytochemical Investigation · Phenylpropanoid Glycosides · Terpenoids · Secondary Metabolites

INTRODUCTION

Natural products/Medicinal plants are known to be the major sources of several drugs that have been used for treatment of human diseases [1] and are also expected to be the sources of future (modern) drugs [2]. The genus *Ajuga* is a plant traditionally used for its medicinal value in many parts of the world including Ethiopia. The most important parts of the plant used in traditional medicine are leaves, stems and roots. Ethnopharmacological surveys have revealed that some 20 species of *Ajuga* plants are used in traditional medicine mostly in Africa, Asia and China [3]. *Ajuga integrifolia* is one of species in genus *Ajuga.* It is distributed from northeast Africa, through Arabia, temperate and tropical Asia to New Guinea. It has a very bitter taste, moderately to densely hairy grayish green leaves with pale violet, light blue or white flowers (Figure 1).

In Ethiopia, *Ajuga integrifolia* grows in different parts of especially in Southern Ethiopia. Some of the areas are Wolayta, Dawuro, Bench-Maji, Sidama, Hadiya and Kambata zones. The plant is known by several local names in these areas. These include "*Annamura*" (Wolaita), "*Anamuro*" (Sidama), "*Anamunure*" (Gedeo), "*Anamuro*" (Amharic), "*Annamura*" (Hadiyigna) [4-9]. The aqueous and sometimes alcohol infusion of the fresh or dried leaves or root of the plant are traditionally used for treating various types of diseases such as diabetes, malaria, pain and fevers, toothache, skin disease, hypertension, stomachache, pneumonia, liver problem, swelling of legs, diarrhea (child), evil eye, retained placenta and ascariasis [6, 7, 10] and epilepsy [11]. Though the plant is widely used traditionally to treat the aforementioned human health problems, there are no reports on its phytochemical investigations. Thus, this study was initiated to carry out phytochemical screening and isolation of compounds from roots of *Ajuga integrifolia*.

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Fig. 1: *Ajuga integrifolia* (*Anamuro*) (Photo taken by Derilo Bekere, Shonie area, Ethiopia, November, 2016).

MATERIALS AND METHODS

General Experimental Material: UV-Vis spectrum was measured with a GENESY's spectrometer (800-200 nm) in methanol at room temperature. Infrared (KBr pellet) spectrum was recorded on a Perk-Elmer BX infrared spectrometer in the range 4000-400 cm^{-1}. Nuclear Magnetic Resonance (NMR) analysis was recorded on a Bruker avance 400MHz spectrometer with tetramethylsilane as internal standard and DMSO-d_6 as solvent. Thin Layer Chromatography (TLC) was done using silica gel 60 F_{254}. Column chromatography was performed on silica gel 60 (60-120 mesh). All the spectral analyses were carried at the Department of Chemistry, Addis Ababa University, Ethiopia.

Plant Material Collection: The root of *Ajuga integrifolia* was collected from Shonie town, *Hadiya* Zone, South Nations Nationalities Peoples Region (SNNPR), Ethiopia. The area is found at a distance of 338 km south of Addis Ababa, capital of Ethiopia and at 125 km west of Hawassa University. The plant species was authenticated by a botanist Mr. Reta Regasa at The Department of Plant Science in Hawassa College of Teachers Education and the specimen was deposited at the Herbarium of Hawassa College of Teacher Education, Hawassa, Ethiopia.

Plant Material Preparation and Extraction: The collected roots of *Ajuga integrifolia* were dried under shade at room temperature for 15 days and then ground to powder to facilitate the extraction. The powder (500 g) was divided into two portions (250 g each) and was soaked in 2 l Erlenmeyer flasks containing 1.5 l n-hexane. The contents of the flasks were mixed well and put on orbital shaker and left for 24 hrs at a speed of 120 rpm. After 24 hrs, the solution was filtered using 15-cm size Whatmann filter paper and with the help of suction filtration. The filtrate was dried under reduced pressure using rotary evaporator at temperature of about 40-45 °C to get crude extract. The crude extract was dried and kept in refrigerator until used for further analyses. The residues left after n-hexane extraction was subjected to 3 l of dichloromethane: methanol (1:1) extraction following similar procedure used above. The extraction with this solvent system was done twice to increase yield. Similarly, the residue left after dichloromethane:methanol (1:1) extraction was subjected to methanol extraction employing similar procedure discussed above [12].

Phytochemical Screening Tests: Phytochemical screening tests were carried out on the n-hexane, dichloromethane: methanol (1:1) and methanol extracts using standard procedures reported in literature [13-20] in order to identify classes of secondary metabolites (alkaloids, flavonoids, phenols, glycosides, terpenoids, tannins, saponins and steroids) present in the crude extracts.

Test for Alkaloids: To 1ml of 1% HCl was added to the 1 g of crude extract of *roots of Ajuga integrifolia* of n-hexane extract was dissolved with 3 mL methanol in test tube. The mixture was heated (40 ^{O}C) for 20 min, cooled and filtered. Then 1 ml of the filtrate was tested with 0.5ml Dragendroff's reagents (solution of Potassium Bismuth iodide). Similar procedures were employed to dichloromethane: methanol (1:1) and methanol extracts. The color change upon addition of the reagent was used to confirm presence and absence of alkaloids [13-20].

Test for Flavonoids: Flavonoids were tested using Alkaline Reagent Test (sodium hydroxide solution). 0.3 g crude extracts of n-hexane extract was dissolved with 3 ml methanol in test tubes. Then few drops of sodium hydroxide solution were added into the test tube containing the solution. Similar procedures were applied to dichloromethane: methanol (1:1) and methanol extracts. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicated the presence of flavonoids [13-20].

Test for Phenols

Ferric chloride Test: 0.2 g crude extract of n-hexane extract was taken in a test tube containing few ml of water and was mixed 2 ml of ferric chloride solution. Similar procedures were applied to dichloromethane methanol (1:1) and methanol extracts. Formation of green/blue color was taken as an indicator of presence of phenols [13-20].

Test for Glycosides

Keller-killani Test: 0.5 g crude extracts of n-hexane was taken and subject to the following test. 1 ml of glacial acetic acid containing traces of ferric chloride and 1 ml of conc. sulphuric acid were added to the extract. Similar procedures were applied to dichloromethane: methanol (1:1) and methanol extracts. Formation of reddish brown color at the junction of two layers and bluish green in the upper layer indicated the presence of glycosides [13-20].

Test for Terpenoids

Salkowski Test: Small amount (0.5 g) of crude extracts of n-hexane extract was taken in test a tube and mixed with 2 mL of chloroform. 3 ml of concentrated H_2SO_4 was added to the mixture. Similar procedures were applied to dichloromethane: methanol (1:1) and methanol extracts. Observation of a reddish brown coloration at the interface was taken as indicator for the presence of terpenoides [13-20].

Test for Tannins: Small amount (0.5 g) of crude extracts of n-hexane was taken in a test tube and boiled with 20 ml distilled water. The mixture was then filtered and few drops of 0.1% ferric chloride ($FeCl_3$) were added to it. Finally, the mixture was shaken to get it mixed well. It was then allowed to stand for some time. Similar procedures were employed to dichloromethane:methanol (1:1) and methanol extracts. Observation of brownish green/blue-black coloration was taken as indicator of presence of tannins [13-20].

Test for Saponins: Froth test: Small amount (0.2 g) of crude extracts of n-hexane was boiled in 2 ml of distilled water on a water bath and followed by filtration. A fraction of filtrate (1 mL) was mixed with 2 ml of distilled water in a test tube and was shaken vigorously to form a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously. Similar procedures were employed to dichloromethane: methanol (1:1) and methanol extracts. The formation of an emulsion was taken as confirmation for the presence of saponins [13-20].

Tests for Steroids: 2 mL of acetic anhydride was added into a test tube containing 0.5 g of n-hexane extract. Then 2 ml of sulphuric acid was added slowly into the test tube containing the above mixture. Similar procedures were employed to dichloromethane methanol (1:1) and methanol extracts. Formation of violet/blue-green solution was used as indicator for the presence of steroids [13-20].

Chromatographic Isolation of Compounds from Dichloromethane Methanol (1:1) Crude extracts: A 17 g of the dried crude extract was adsorbed onto 17 g of silica gel and loaded to column chromatography packed with 170 g silica gel as the stationary phase. It was then eluted with chloroform at the beginning and gradually increasing the polarity by adding ethanol to chloroform. The different fractions (each 50 ml) were collected and monitored using TLC. Fraction 25 was subjected for further fractionation using small glass column and chloroform: ethanol (60:40) solvent system. This led to isolation of compound 55 mg pure compound (DB6).

A portion of dichloromethane/methanol (1:1) extract (5 g) was also subjected to solvent extraction using chloroform repeatedly before subjecting it to further column chromatographic separation. The extract was then adsorbed onto silica gel (4 g) for further purification using n-hexane: ethyl acetate solvent systems and isocratic separation technique. Combing and concentration of fractions 28-32 afford a yellow solid compound DB4.

RESULTS AND DISCUSSION

Solvent Extraction and Yield of Extracts: As discussed above, the extraction of the plant material (root) was carried out using different solvents (of different polarity) and in sequential extraction approach. It started with n-hexane (least polar) and followed by dichloromethane: methanol (1:1) (intermediate polarity) and methanol

Table 1: The masses and percent yields of solvent extraction

S.No	Solvent used for extraction	Mass of extract (g)	% yield of extracts
1	n-hexane	2.3	0.46
2	Dichloromethane:methanol(1:1)	26.5	5.3
3	Methanol	13.3	2.66

Table 2: The phytochemical screening test results of extracts of roots of *Ajuga integrifolia*

Classes of secondary metabolites	Reagent used	n-hexane extract	Dichloromethane:methanol (1:1) extract	Methanol extract
		Result	Result	Result
Alkaloids	Dragendorff's reagents	+	+	+
Flavonoids	Alkaline reagent test (NaOH)	+	+	+
Phenol	$FeCl_3$ solution	+	+	+
Glycosides	Treated with a mixture of acetic acid, $FeCl_3$ an Conc. H_2SO_4	+	+	+
Terpenoids	Chloroform and conc. H_2SO_4	+	+	+
Tannins	$FeCl_3$ solution	+	+	+
Saponins	Warming in water bath	-	+	+
Steroids	Warming in water bath	+	+	+

Key: + (presence) - (absence)

(the most polar). The amount of extracts collected were 2.3 g, 26.5 g and 13.3 g for n-hexane, dichloromethane: methanol (1:1) and methanol, respectively (Table 1). The percent yields of the extracts were calculated using the formula given in Equation 1.

$$\frac{\text{Mass of the extract}}{\text{Mass of the plant material used for extraction}} \times 100\% \quad \text{(Eq. 1)}$$

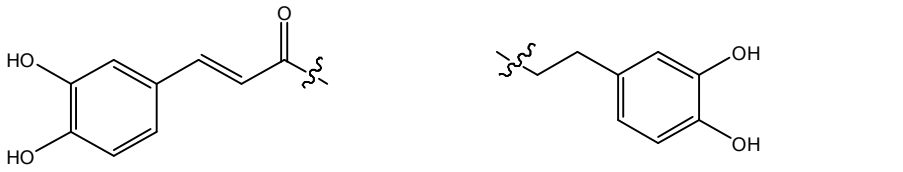
As shown above (Table 1), the n-hexane (2.3 g) and the methanol (13.3 g) crude extracts were not enough to carry out chromatographic isolation of compounds. They were used only for phytochemical screening tests. On the other hand, the dichloromethane: methanol (1:1) extract (26.5 g) was enough for phytochemical tests as well as column chromatographic isolation. Thus, it was subjected to chromatographic separation to isolate compounds (Section 4.3). The data also showed that dichloromethane: methanol (1:1) is optimal solvent for extraction as compared to the other two solvent systems.

Phytochemical Screening Tests: It is known that phytochemical constituent's secondary metabolites are responsible for most of observed biological activities (antibacterial, antifungal, antiviral and pesticide) of medicinal plants [21-27]. In this research work it was found that phytochemical screening tests of n-hexane, dichloromethane:methanol (1:1) and methanol extracts of the roots of *Ajuga integrifolia* revealed presence of alkaloid, flavonoid, phenols, glycoside, terpenoides, tannins and steroids in the three solvent systems but negative test for saponins n-hexane extract and positive in rest solvent system (Table 2). The presence of these secondary metabolites justifies various medicinal uses of the plant mentioned in the introductory section [6, 7, 10, 11, 28].

Structural Elucidation of Isolated Compounds

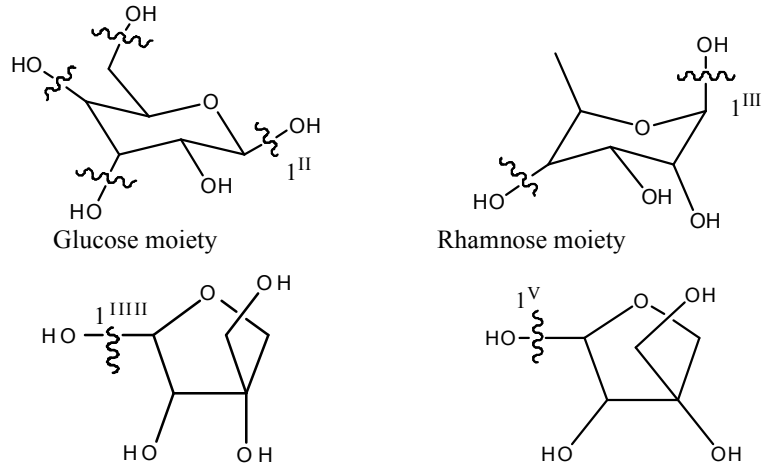
Characterization of Compound DB6: Compound DB6 was obtained as a golden brown amorphous powder (55 mg) and with R_f value of 0.60 in chloroform: ethanol (60:40%) solvent system. The UV-Vis spectrum (Appendix 1) indicated absorbance peaks at λ_{max} 290 and 330 nm that could be attributed to π-π* transition of C=C double bond conjugated with C=O group and transitions of lone pair of electrons (n-π* transition), respectively. The IR spectrum (Appendix 2) indicated vibrations at 3363 cm^{-1} (indicates hydroxyl group stretching), 2921 cm^{-1} (indicates C-H stretching vibrations), 1599 cm^{-1} and 1700 cm^{-1} (due to α, β-unsaturated ester) and medium band at 1516 cm^{-1} indicates the presence of benzene ring).

The ^{1}H-NMR spectrum (Appendix 3) this compound revealed the presence six aromatic protons o different types. These are protons that belong to aromatic ABX coupling system resonating at δ6.7 (1H, *d*), δ6.6 (1H, *d*) and δ6.5 (1H, *dd*) for the 3,4-dihydroxy-β-phenylethoxyl moiety and δ6.9 (1H), δ 6.7(1H, *d*) and δ6.90 (1H, *dd*) for the caffeoyl moiety. The other types are vinyl protons of AB-type (trans-olefinic) that were observed at δ7.5 (1H*d*, H-8I) and δ6.2 (2H*d*, H-7I) (Fig. 2).



The caffeoyl moiety The 3,4-dihydroxy-β- phenylethoxyl moiety

Fig. 2: The caffeoyl and the 3, 4-dihydroxy-β-phenylethoxyl moieties



Glucose moiety Rhamnose moiety

Apiose moiety-I Apiose moiety-II

Fig. 3: The glucose, rhamnose-I, apiose-I and Apiose -II moieties

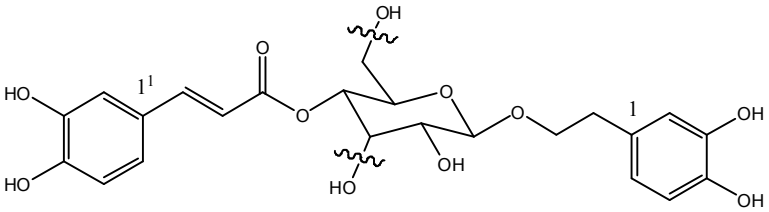


Fig. 4: Partial structure of compound-1 made from caffeol, glucose and aglycone moieties

The proton peaks observed at δ4.3 (1H, *d*), δ5.3 (1H, *nwr*), δ4.9 (1H, *d*) and δ5.2 (1H, *d*) could be attributed to anomeric protons of sugar moieties; more specifically to β-configuration of an anomeric proton [24]. The peak at δ0.8 integrated for three protons is attributed to a methyl group (H-6^{III}) of rhamnose (Table 3). The anomeric proton peaks at δ5.3 and δ0.8 (*d*) could also be attributed to α–L-configuration of the rhamnopyranoside moiety. The peak at δ3.7 could be attributed to H-3^{II} of glucose moiety (Table 3). The observed NMR data was consistent with literature report [29] indicated that the configuration of glucose moiety to be β-configuration whereas that of a rhamnose to be α-configuration. The configurations of both the apiose moieties in the structure to be *β*-configuration (Fig. 3).

The ^{13}C-NMR spectrum (Appendix 4) also confirmed the presence of four anomeric carbons that resonate at δ102.45 (β-glucose), 100.49 (δ-rhamnose), 109.04 (β-apiose) and 106.31 (β-apiose). The peaks are attributed to anomeric sugar carbons of glucose (C-1^{II}), rhamnose (C-1^{III}) and apiose I and II (C-1^{IIII} and C-1^{IIIII}) (Table 3), respectively. The peaks at δ129.01, δ125.95, δ 144.25, δ148.95 and δ116.75 and δ 115.95 belong to C-1, C-3 and C-5 of both aromatic nuclei, respectively. The signals at δ113.99 and δ146.02 indicate the presence of ester carbonyl carbon (δ166.18) of α and β conjugated system. The signals observed at δ144.92, δ148.95, δ143.99 and δ145.43 are attributed to oxygenated sp^2 quaternary carbons of a phenyl group at C-3, C-4, C-3^{I} and C-4^{I} (Table 3)[29], respectively (Fig. 4 and 5).

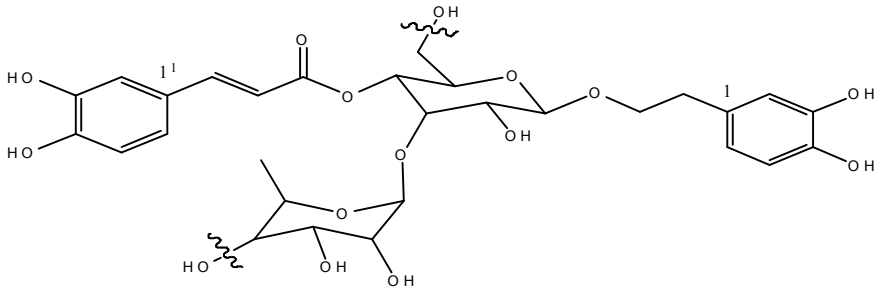


Fig. 5: Partial structure of compound-1 made from caffeoyl, glucose, rhamnose and aglycone moieties.

Table 3: ^{1}H-NMR and ^{13}C-NMR (DMSO-d_6, 400MHz) data of compound DB6 and data reported for 1-O-3,4-(dihydoxyphenyl)-ethyl-β-D-apiofuranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)-4-O-caffeoyl-β-D-glucopyranoside [29]

Position	Observed ^{1}H-NMR data of compound DB6	Observed ^{13}C-NMR data of compound DB6	Reported ^{1}H-NMR and ^{13}C-NMR data [29]	
			δH (ppm)	δC (ppm)
Aglycone				
1	-	129.01	-	129.7
2	6.7(1H,dd)	116.25	6.66 (*d*, *J*=2.0)	116.2
3	-	144.92	-	146.9
4	-	143.99	-	144.7
5	6.6(1H,d)	116.76	6.63 (*d*, *J*=8.1)	116. 8
6	6.5(1H,d)	120.01	6.52 (*dd*, *J*=2,8.0)	120.0
α	2.7(2H,t)	35.46	2.75 (*t*, *J*=7.3)	35.5
β	3.9(2H,t)	71.63	3.96/3.7 (*m*)	72.3
Glucose				
1^{II}	4.3(1H,d)	102.45	4.38(*J*=7.8)	104.5
2^{II}	3.4(1H,dd)	73.47	3.41 (*dd*, *J*=7.96/9.3)	73.3
3^{II}	3.7(1H,t)	81.36	3.83 (*t*, *J*=9.1)	80.5
4^{II}	4.9(1H,t)	69.49	4.93 (*t*, *J*=9.5)	69.2
5^{II}	3.58(m)	72.74	3.59 (*m*)	72.1
6^{II}	3.6(dd)	61.47	3.54/3.66 (*dd*, *J*=12.0/5.5)	62.3
Rhamnose				
1^{III}	5.3 nwr	100.49	5.28 (*d*, *J*=1.5)	102.7
2^{III}	3.8(1H,s)	70.72	3.9 (*s*)	70.8
3^{III}	3.6(1H,dd)	70.18	3.67 (*dd*, 9.5/3.3)	70.0
4^{III}	3.4(nwr)	76.62	3.41 (*t*, *J*=9.3)	76.4
5^{III}	3.58(1H,m)	68.15	3.55 (*m*)	67.6
6^{III}	0.8 (1H,d)	18.50	1.05 (*d*, *J*=6.0)	18.6
Apiose I				
1^{IIII}	4.9(1H,d)	109.04	5.17 (d, J=2.2)	111.5
2^{IIII}	3.91H,d)	74.73	3.9 (*d*, *J*=1.8)	74.7
3^{IIII}	-	74.85	-	74.8
4^{IIII}	3.95(2H,d)	70.55	3.97 (*d*, 9.9),3.70 (*d*, 9.9)	70.8
5^{IIII}	3.58(2H,s)	63.83	3.55 (*s*)	63.6
Apiose II				
1^{IIIII}	5.2(1H,d&nwr)	106.31	5.22 (*d*, *J*=2.7)	109.6
2^{IIIII}	3.6(1H,d)	73.47	3.65	73.9
3^{IIIII}	-	80.43	-	79.3
4^{IIIII}	3.4(2H,d)	71.00	3.39/3.27 (*d*, *J*=11.6)	71.0
5^{IIIII}	3.3(2H,d)	61.47	3.27 (*d*, 11.6)	62.5
Acyl moiety				
1^{I}	-	125.95	-	125.9
2^{I}	6.9(1H,nwr)	115.13	7.01 (*d*, *J*=2.0)	115.2
3^{I}	-	148.95	-	148.0
4^{I}	-	145.43	-	146.2
5^{I}	6.7(1H,d)	115.92	6.73 (*d*, *J*=8.1)	115.9
6^{I}	6.9(1H,dd)	121.93	6.90 (*dd*, *J*=2.8/8.1)	122.0
α	6.2(1H,d)	113.99	6.26 (*d*, *J*=15.9)	113.8
β	7.5(1H,d)	146.02	7.59 (*d*, *J*=15.9)	149.0
C=O	-	166.18	-	166.3

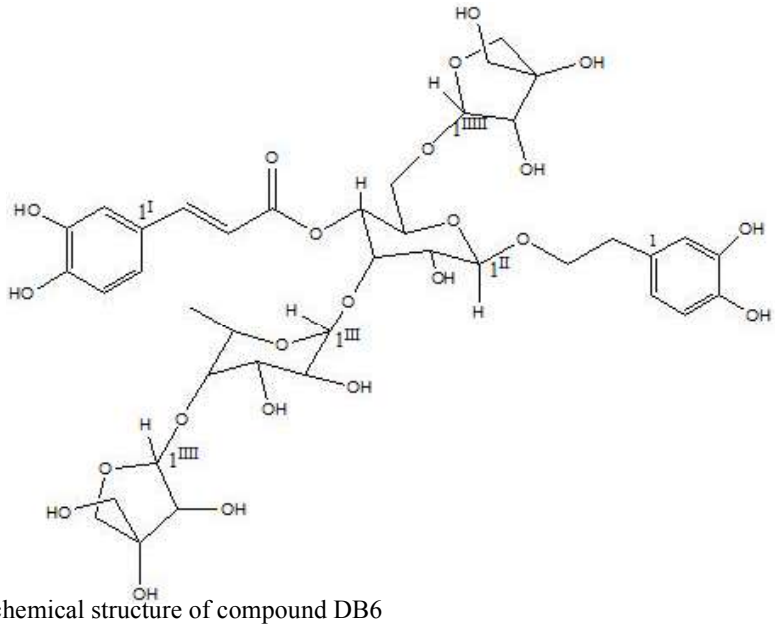


Fig. 6: The proposed chemical structure of compound DB6

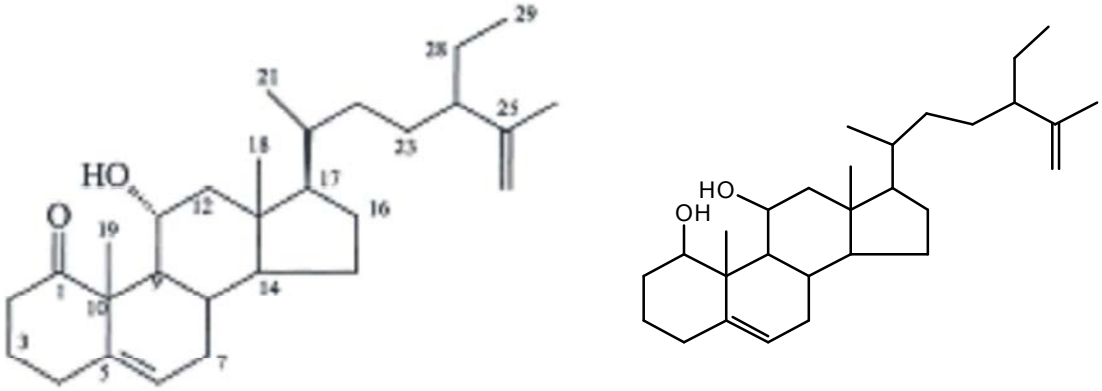
The observed spectral data and literature reports suggested that the structure of compound DB6 to be identical to the structure of a new phenylprophanoid glycoside with four sugar units reported in literature [29]. This compound was also reported previously from *Phlomis samia* and *Clerodendrum myricoides* the trivial name was given as 1-*O*-3,4-(dihydoxyphenyl)-ethyl-β-D-apiofuranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside (Figure 6) [29, 30]. This is the first report of isolation of this compound from the genus *Ajuga*.

Characterization of Compound DB4: Compound DB4 was obtained as a yellowish amorphous powder (22.2 mg) with R_f value of 0.56 in ethyl acetate: n-hexane (10:90%) solvent system. The IR spectrum (Appendix 5) revealed absorption bands at 3427, 2921, 2850 and 1628 cm^{-1} suggesting the presence of hydroxyl group, C-H stretching of methyl, C-H stretching of methylene and C=C unsaturated moieties, respectively.

Its ^{1}H-NMR spectrum of compound DB4 (Appendix 6, Table 6) exhibited five methyl signals at δ0.70 (s), 0.80 (t), 0.95 (d), 1.17 (s) and 1.57 (s). Olefinic proton signals at δ4.68 and 4.72 and also an olefinic proton signal at δ5.38 were observed. The latter signal is characteristic for olefinic proton in steroid compounds whereas the former pair could be attributed to olefinic methylene protons. The observation of a proton signal at δ4.02 as multiplet indicated the presence of sp^3 oxygenated methane. This was also supported by the presence of carbon peak at δ71.81 in the ^{13}C-NMR spectrum (Appendix 7).

The ^{13}C-NMR spectrum revealed a total of 29 carbon signals that could be attributed to five methyl, eleven methylene, nine methine and four quaternary carbon atoms (Appendix 7). The olefinic methylene signal was observed at δ111.32 pointing downward in DEPT-135 spectrum (Appendix 8). This suggested that this olefinic carbon is a terminal carbon (δ147.59) that bonded to a quaternary carbon. The other olefinic methine carbon signal observed at δ121.71 is internal carbons of which one of them is a quaternary carbon (δ140.77). The presence of an sp^3 oxygenated methine is also observed at δ74.63. Based on the above spectral data and comparison with literature report [31], a very closely related compound DB4 was found to be similar to (24S)-24-ethylcholesta-5, 25-diene-11α-hydroxy-1-one) with the only difference in carbonyl group peak appearing at δ210.6 that belongs to a ketone in this compound but a secondary alcohol (OH group) is located at C-1 in our case (compound DB4). This implies that the compound isolated in the present work is the reduced form of an already reported compound ((24S)-24-ethylcholesta-5,25-diene-11α-hydroxy-1-one) (Figure 7a) as it can be seen from the NMR data (Table 4). Thus, based on the above NMR features and spectral data of compound 2 the structure of the compound was deduced to be (24S)-24-ethylcholesta-5, 25-dien-1,11-diol [31] (Fig. 7).

This work was the first attempt to phytochemically analyze the roots of *Ajuga integrifolia* from Ethiopian flora. The phytochemical screening of the roots extracts of n-hexane, dichloromethane: methanol (1:1) and methanol revealed that the presence of alkaloids,



(a) (b)

Fig. 7: The chemical structure of ((24S)-24-ethylcholesta-5,25-diene-11α-hydroxy-1-one)(a) and proposed structure of compound DB4 (b).

Table 4: The ^{1}H-NMR and ^{13}C-NMR (DMSO-d_6, 400MHz) data of compound DB4

Position	^{1}H-NMR data of compound DB4	^{13}C-NMR data of compound DB4	Reported ^{1}H and ^{13}C-NMR data of (24S)-24-ethylcholesta-5,25-diene-11α-hydroxy-1-one [31]	
			^{1}H-NMR	^{13}C-NMR
1	-	74.63	-	210.6
2		35.77	-	40.2
3		24.28	-	24.3
4		33.68	-	33.6
5		140.77	-	139.4
6	5.4(1H,nwr)	121.12	5.6br	122.3
7	3.8(1H,m)	37.26	3.84m	32.4
8		30.26	-	30
9		50.14	-	56.8
10		36.51	-	38.8
11	4.02(2H, m)	71.83	4.04(m)	68.9
12		42.32	-	49.6
13		39.81	-	44.2
14		56.78	-	56.5
15		26.52	-	24.5
16		28.16	-	28.6
17		56.07	-	56.8
18	0.70(3H,s)	12.04	0.68 s	12.7
19	1.17(3H,s)	21.09	1.16 s	21.4
20		35.54	-	34.8
21	0.95(3H,d)	18.65	0.92 δ (7.0)	18.8
22		31.67	-	33.6
23		29.42	-	29.5
24		49.53	-	49.1
25		147.59	-	148.6
26a	4.68(1H,d)	111.35	4.63 dq (2.6 and 0.6)	109.4
26b	4.72(1H,nwr)		4.72 brd (2.6)	
27	1.57(3H,s)	19.38	1.56 s	18.1
28	0.8(3H,t)	29.92	0.79 t (7.5)	29.4
29	0.8(3H,t)	11.84	0.79 t (7.5)	12.1

flavonoids, phenols, glycosides, terpenoids and tannins. The chromatographic separation of the dichloromethane: methanol (1:1) gave phenylpropanoid glycosides and (24S)-24-ethylcholesta-5, 25-diene-11α-hydroxy-1- diol. This is the first report on the presence of such kind of phenylpropanoid derivatives from the genus *Ajuga* that is indigenous to Ethiopian flora. The identification of the above pharmacologically important secondary metabolites from root extract sheds more light on the medicinal uses of the plant used in the study. Further intensive investigations are recommended for its therapeutic benefits of this herb (*Ajuga integrifolia*).

ACKNOWLEDGMENT

The authors duly acknowledge Department of Chemistry, Hawassa University for material support.

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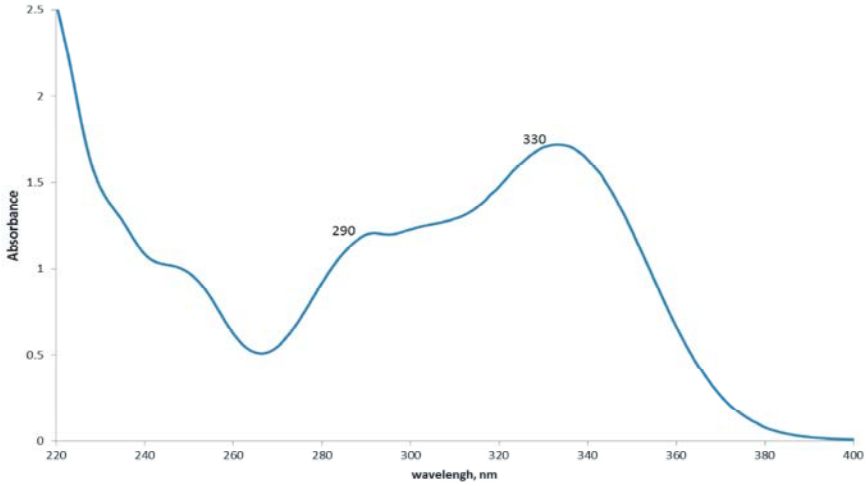
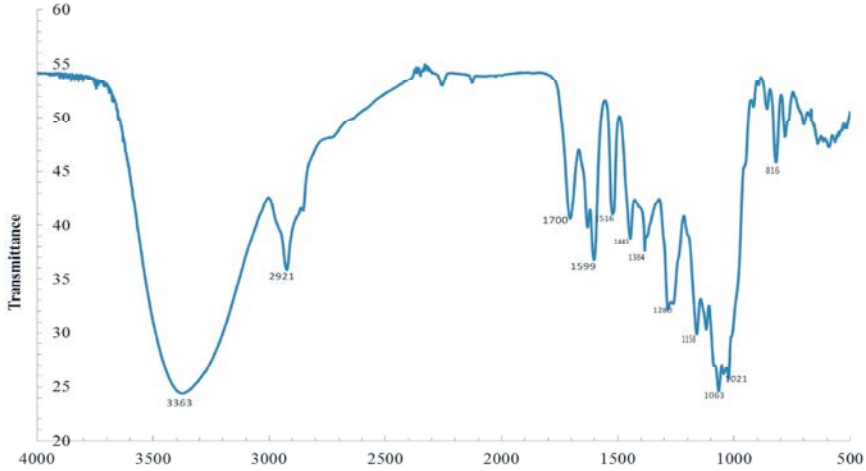
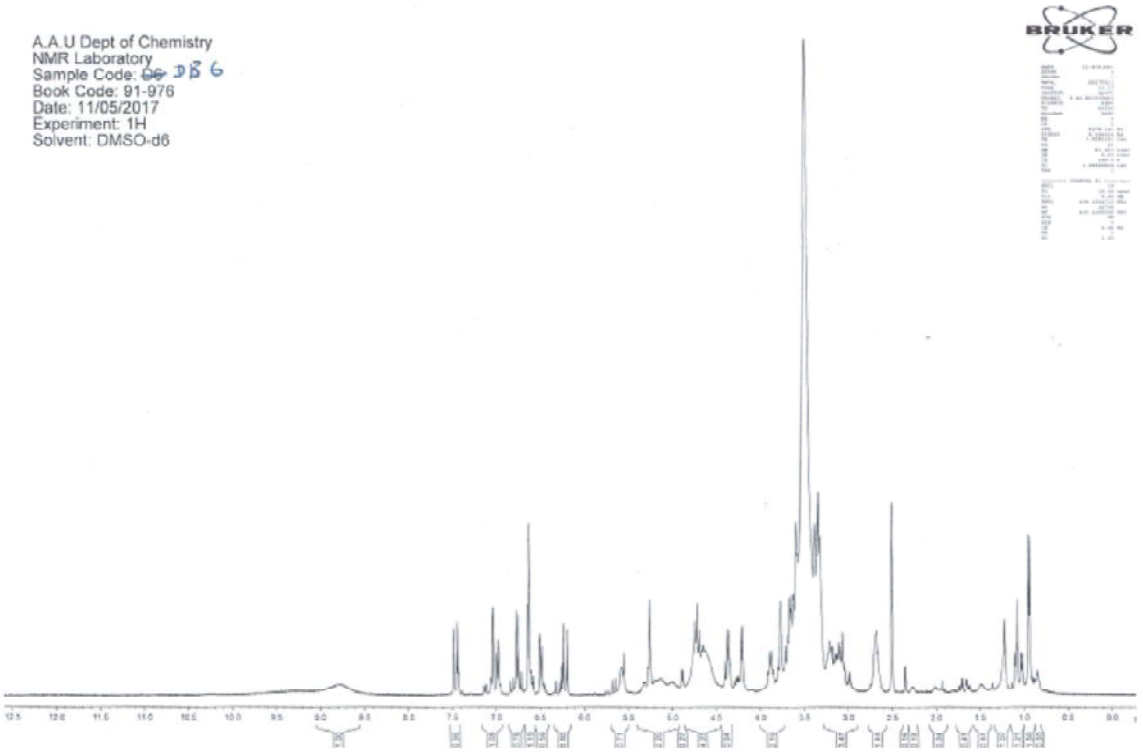


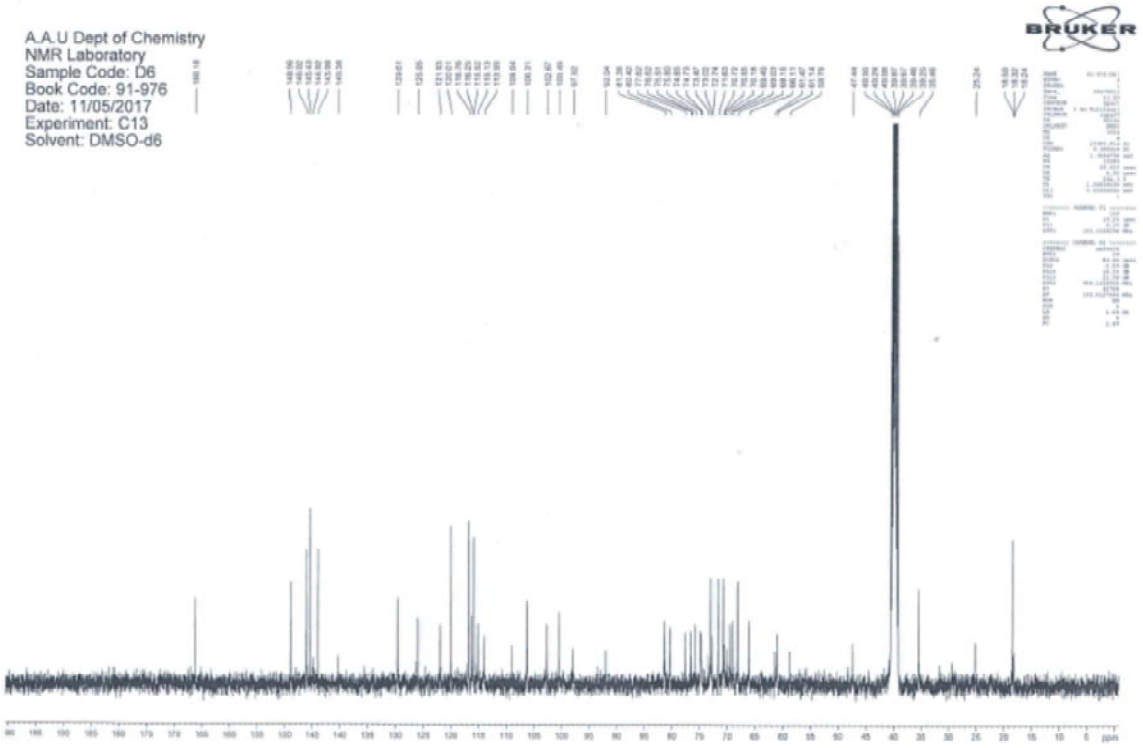
Appendix 6: 1H-NMR spectrum of compound DB4



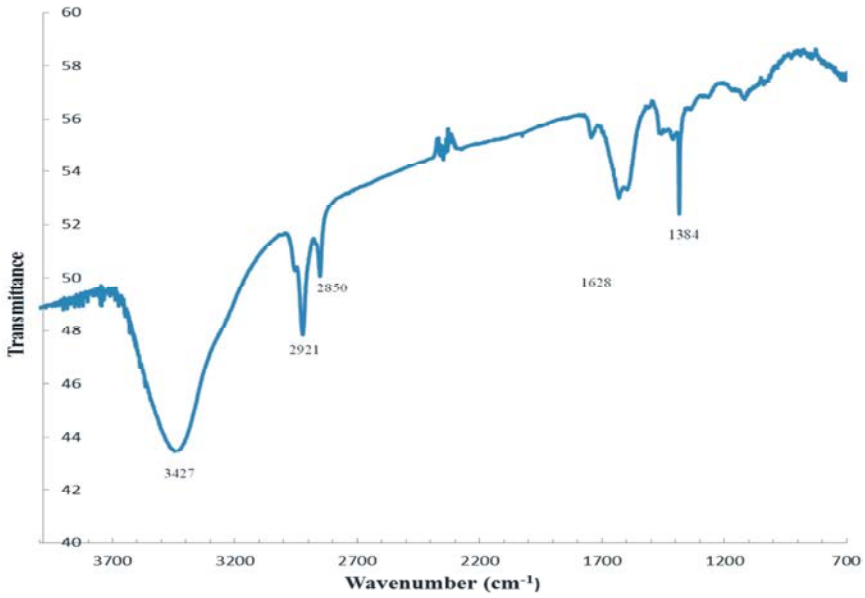
Appendix 2: FT-IR spectrum of compound **DB6**



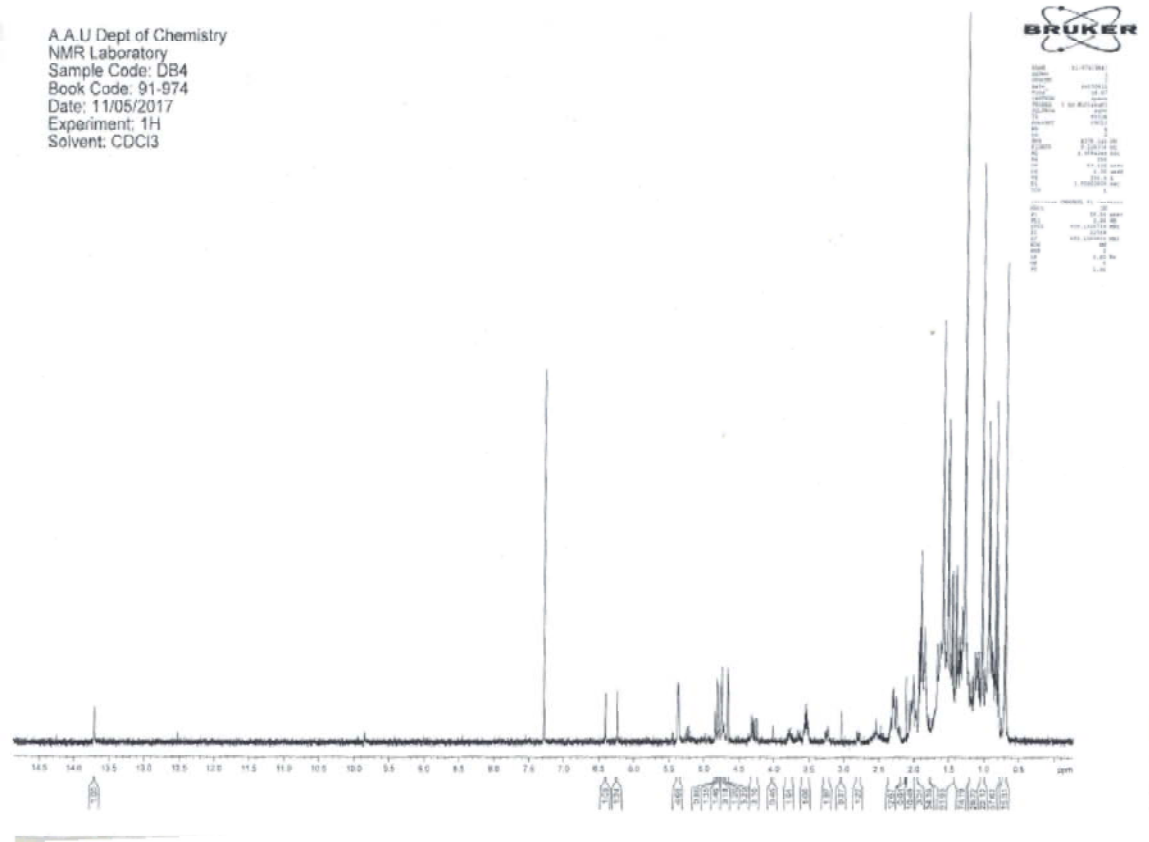
Appendix 3: 1H-NMR spectrum of compound **DB6**



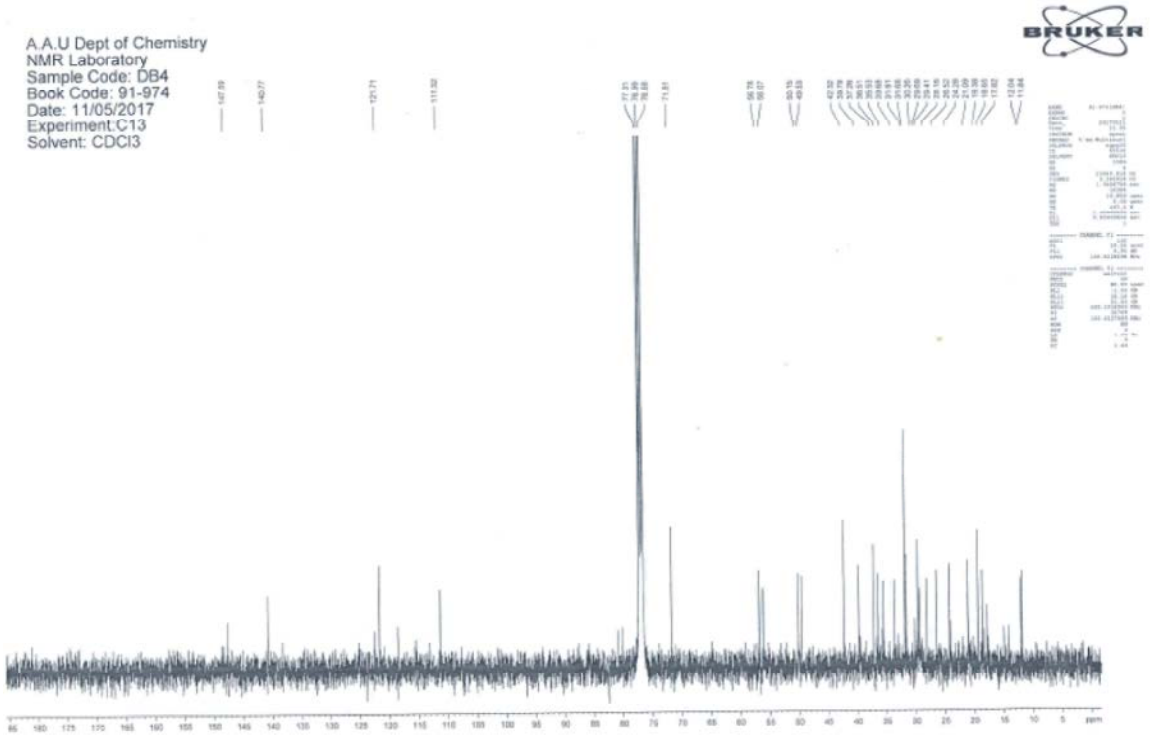
Appendix 4: ^{13}C-NMR spectrum of compound **DB6**



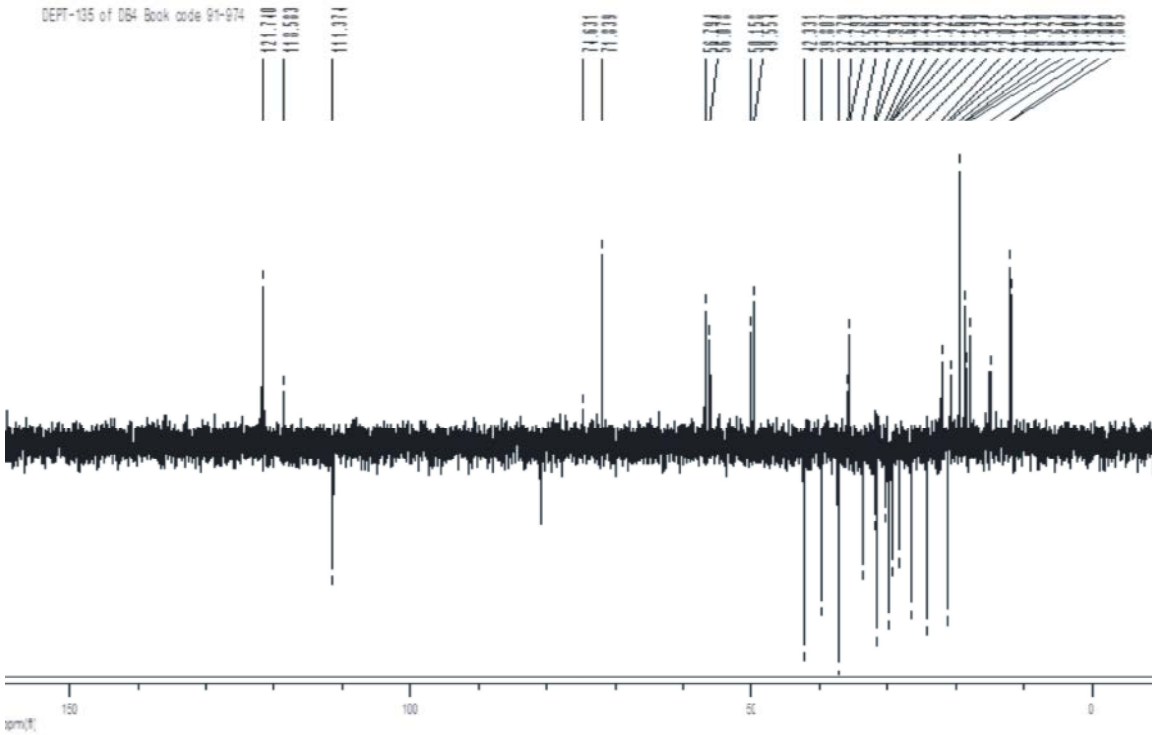
Appendix 5: IR spectrum of compound **DB4**



Appendix 6: ^{1}H-NMR spectrum of compound **DB4**



Appendix 7: ^{13}C-NMR spectrum of compound **DB4**



Appendix 8: DEPT-135 spectrum of compound **DB4**