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Research Paper

Phenolic constituents from *Platycerium bifurcatum* and their antioxidatant properties

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ABSTRACT

Semi-preparative HPLC separation of the ethyl acetate fraction of the leaf extract of *Platycerium bifurcatum* (Cav.) C. Chr. lead to the isolation of *a* polyphenolic compound- quercetin 3-O-β-D-glucopyranoside (**1)** and a carboxylic acid-chlorogenic acid (**2**).The structures of the compounds were elucidated by 1D (^{1}H, ^{13}C), 2D NMR (COSY, HSQC, and HMBC); mass spectroscopy (HPLC/ESI-MS) and by comparism with the reported data. The antioxidant activities of the isolated compounds (**1–2**) were evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. These secondary metabolites were isolated for the first time from *Platycerium bifurcatum.*

Keywords: *P. bifurcatum*; Polyphenolic; Structure elucidation; Antioxidant activities.

INTRODUCTION

Platycerium bifurcatum (Cav.) or "staghorn fern" is an epiphyte, growing naturally on branches and trunks of trees in the tropical, subtropical jungles and rain forest (Bode and Oyedapo, 2011; Hemipman and Roos, 1982). *Platycerium bifurcatum* is a lower plant since it does not have roots and produce spores to reproduce, rather than flowers. It belongs to the family *Polypodiaceae* and is propagated from its spores. Apart from its ornamental uses, it has been reported to have wide medicinal uses. In Nigeria, young leaves of *Platycerium bifurcatum* are prescribed as a common anti-ulcer remedy (Pemberton, 2003). The leaf extract of *Platycerium bifurcatum* is

reported to have diverse uses such as preventing miscarriages in women when taken two months after conception (Flora and Ubah, 2006), treating oedema, coughs and hypertension (Mensah, et al., 2006). Antibacterial potency of methanol extract of *P. bifurcatum* using agar dilution method has been reported (Ojo, et al., 2007). The isolation and characterization of polysaccharides from *P. bifurcatum* has also been reported (Omeje, et al., 2007). Currently, there is great interest in finding antioxidants from natural sources to minimize oxidative damage to cells. Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative health conditions such as Parkinson's and Alzheimer's diseases. Oxidative damage is caused by free radicals and reactive oxygen species, mostly generated endogenously (Aniya, 2002). Free radicals are atoms or groups of atoms that have at least one unpaired electron, which make them highly unstable and reactive. Living organisms accumulate free radicals through both normal metabolic processes and exogenous sources. Although free radicals have beneficial effect during energy production and as antibacterial, excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death (Asres, et al., 2006; Pham-Huy, et al., 2008). Plant polyphenols provide a protection against these diseases, since they have high antioxidant properties. Here, we isolated two pure secondary metabolites from the air-dried leaves of *Platycerium bifurcatum* by the method of Agbo, et al. (2013). The anti-oxidative potentials of these isolated compounds were assessed by using DPPH radical scavenging method previously described by Tsevegsuren, et al. (2007). This is the first report of polyphenols from the leaves of *Platycerium bifurcatum* and its antioxidant activities. *P. bifurcatum* is widely distributed in Nigeria and this has given an insight into its ethno medicinal uses.

MATERIALS AND METHODS

Instruments and materials: The optical rotation was recorded on a Perkin-Elmer 241 MC polarimeter. 1D and 2D NMR spectra were recorded using Bruker ARX 500 NMR spectrometer. LC-MS measurements were performed on a Thermofinnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system. Analytical HPLC analysis was performed with a HPLC system (Dionex, Munich, Germany). Semi-preparative HPLC was performed on a MERCK HITACHI system equipped with a UV Detector L-7400 and a Pump L-7100 connected to a Kipp & Zonen Flatbed Recorder. Vacuum liquid chromatography (VLC) was performed on silica gel (230–400 mesh, Merck) using a glass column (i.d. 3×30cm). Gel permeation column chromatography (CC) was performed on Sephadex LH-20 (Merck, Germany) using a glass column (i.d. 3×110cm). TLC was performed on TLC plates pre-coated with silica gel 60 F_{254} (0.20 mm thickness, Merck, Darmstadt, Germany) using various solvent combinations as the mobile phase.

Plant Material: *Platycerium bifurcatum* leaves were collected from Orba in Enugu State, Nigeria in January, 2012. The leaves were identified by Mr. A. O. Ozioko of the Bio resources Conservation and Development Program (BDCP), Nsukka. A voucher specimen (PB 2076) was deposited at the herbarium of the Institute.

Extraction, Purification and Isolation: The air dried powdered leaves (500g) were macerated five times with 500ml of methanol and extracted at room temperature for 48h with agitation. The filtrate was evaporated in vacuum (40°C) to obtain the crude methanol extract (20g), which was suspended in 400ml of 10% methanol (MeOH) in water and the resulting mixture successively partitioned against *n*-hexane, ethyl acetate (EtOAc) and butanol (*n*-BuOH) to obtain *n*-hexane (HF, 1.24g), EtOAc (EF, 9.83g), *n*-BuOH (BF, 5. 87g) and water (WF, 0.92g) fractions, respectively. The EtOAc fraction (7.20g) was purified by vacuum liquid chromatography using silica gel (230–400 mesh, 3.0×30 cm, 500g) as the stationary phase and eluted with a gradient of *n*-hexane in EtOAc (10:0, 8:2, 6:4, 4:6, 2:8, 0:10, each 500ml) and of dichloromethane (DCM) in methanol (9:1, 7:3, 9.5:5, 3:7,1:9, each 1000ml) to afford 11 sub-fractions (EF1-EF11). Fraction EF7 (233.7mg) was further fractionated on Sephadex LH-20 (DCM/MeOH, 1:1) to afford nine sub-fractions (EF7A–EF7I). Fraction EF7B (41.3mg) was separated by semi-preparative HPLC using MeOH–H_2O as mobile phase to give compound **1** (8.6 mg, t_R = 20.2min). Similarly, fraction EF11 (162.3mg) was further fractionated on Sephadex LH-20 (100% MeOH) to afford four sub-fractions (EF11A–EF11D). Fraction EF11C (43.1mg) was separated by semi-preparative HPLC using MeOH–H_2O as mobile phase to give compound **2** (10.0 mg, t_R = 13.2min). Structure elucidation of the isolated compounds was carried out by using ^1H,^{13}C-NMR, ^1H,^1H-Correlation spectroscopy (COSY), ^1H,^{13}C- Heteronuclear Multiple Quantum Coherence (HMQC) experiment, ^1H,^{13}C-Heteronuclear Multiple Bond Correlation Spectroscopy (HMBC), mass spectroscopy (HPLC/ESI-MS) and UV spectroscopy.

DPPH radical scavenging activity: The radical scavenging activity of the compounds was determined by the DPPH assay (Tsevegsuren, et al., 2007). The compounds were dissolved in methanol to give a concentration of 1mg/1000µl stock solution. Ten microliter (10µl) of the test samples was added to 490µl DPPH solution (4.5mg/100ml) in an ependorf vial. The mixture was incubated for 3 min and the color change (from deep violet to light yellow) of the DPPH free radical was measured by recording the absorbance using a UV/Visible spectrophotometer (Perkin Elmer, Lambda 25) at 517nm. Prior to the measurement, the difference in absorption between a DPPH blank solution and the positive control (propyl gallate, 76µM) was determined. This difference was then taken as 100% antioxidative activity. The percent antioxidative activity was determined from the difference in absorption between the samples at 76 µM and the DPPH blank as follows:

$$aA(\%) = \frac{AB - A_p}{AB - A_{pos}} \times 100$$

- aA=% antioxidative activity compared to the positive control, AB=absorption of the DPPH blank solution, Ap = absorption of the sample, and Apos = absorption of the positive control (propylgallate).
- Measurements were done in triplicates, and the IC_{50} values were determined by linear regression.

RESULTS

Semi-preparative HPLC of the EtOAc fraction of the leaves of *Platycerium bifurcatum* resulted in the isolation a polyphenolic glycoside and a carboxylic acid. These known compounds were identified as quercetin 3-O-β-D-glucoside and 5'-O-caffeoyl qunic acid (chlorogenic acid) by spectroscopic (1D and 2D NMR, and mass spectra) comparison with the reported values. The antioxidative properties of the isolated compounds showed that the compounds (IC_{50} = 31.27±0.12 and 47.21±0.11) were more active compared to the positive control with IC_{50} values of 68.46±0.16.

DISCUSSION

Compound **1** was obtained as a pink amorphous powder, $[\alpha]_D^{20}$= -10.9 (*c* 0.100, methanol). The UV spectrum showed maximum absorption bands of a flavonoid system at λ_{max} 360.0, 256.0, and 220.0 nm. The HPLC-MS (positive ion mode) at m/z 465 $[M+H]^+$ was compatible with the molecular formula $C_{12}H_{20}O_{12}$. The ^{1}H NMR (Table 1) showed aromatic protons with signals at [δ_H 6.20 (1H, d, *J*=2.0 Hz), δ_H 6.39 (1H, d, *J*=2.1 Hz)] consistent with the meta-coupled protons H-6 and H-8 of ring-A and an ABX system at [δ_H 7.71 (1H, d, *J*=2.2 Hz, H-2'), δ_H 7.59 (1H, dd, *J*=8.5, 2.3 Hz, H-6'), δ_H 6.87 (1H, d, *J*=8.5 Hz, H-5')] corresponding to the catechol protons on ring-B (Ebada, et al., 2008). The spectroscopic data indicated that this compound was a flavonoid glycoside. From the mass spectrum data, fragments at *m/z* 303 $[M-162 + H]^+$ (loss of glucose), together with the occurrence of an anomeric proton at δ_H 5.25 d (*J* =7.6 Hz) was indicative of a glucose unit in the molecule (Figure 2). The ^{1}H NMR data also revealed that the aglycone part was glucose by comparison of spectroscopic data with those of the reported values (Islam, et al., 2012). The rest of the protons in the sugar moiety resonated between 3.42 and 3.71 ppm. The anomeric proton coupling constant of compound **1** (H-1", J=7.6 Hz) define the stereochemistry of glycosidic linkage as β (Mina, et al., 2013; Guo et al., 2012).The ^{1}H NMR data recorded on MeOH-d_4 were in good agreement with published values (Jan, 2003; Li, et al., 2011). Thus, compound **1** was elucidated as quercetin 3-O-β-D-glucoside.

Compound **2** was obtained as a yellow amorphous powder, $[\alpha]_D^{20}$= -21.4 (*c* 0.100, methanol). The UV spectrum showed absorption maxima λ_{max} 325.7, 239.9 and 217.0 nm. The molecular formula $C_{16}H_{18}O_9$ was deduced from the ESI-MS with ion peaks at *m/z* 355.1 $[M+H]^+$ (Figure 3). Thus the molecular mass of compound **2** was found to be 354 g/mol with nine double-bond equivalent. The ^{1}H and ^{13}C NMR assignments were based on the DEPT and 2D-NMR (COSY, HMQC and HMBC) experiments. The ^{1}H NMR spectrum of **2** in MeOH-d_4 exhibited signals in aliphatic and aromatic range. In aromatic range, the ^{1}H-NMR spectrum (Table 2) revealed an ABX system at [δ_H 7.03 (1H, d, *J*=2.0 Hz, H-2'), 6.91 (1H, dd, *J*= 8.2, 2.1 Hz, H-6'), 6.73 (1H, d, *J*=8.0 Hz, H-5')] characteristic for 1, 3, 4-trisubstituted benzene. The ^{1}H NMR spectrum also showed a set of *trans*-olefinic protons at [δ_H (7.57 and 6.25 (each d, *J* = 15.8 Hz, H-7' and H-8')], indicating the presence of an (*E*)-caffeic acid moiety (Satake, et al., 2007; Sri, et al., 2007). The presence of a quinic acid moiety was further confirmed by the signals from two methylene protons at [δ_H 2.10 (d, *J* = 7.8 Hz), 2.16 (dd, *J* = 14.7,3.0 Hz) and 2.00 (d, *J* =11.8 Hz), 1.96 (d, *J* = 6.3 Hz)] for H-2

and H-6 respectively, with three methine protons at [δ_H 3.68 (dd, *J*= 9.9,3.2 Hz, H-3), 4.12 (d, *J* = 3.1Hz, H-4), and 5.38,m (H-5)] together with the corresponding carbon resonances at δ_C 39.48, 41.14, 75.54, 73.54, 72.88 for C-2, C-6, C-4, C-3, and C-5 respectively.In the COSY spectrum, H-2 was found to correlate with H-3, which further coupled to H_2-2 and H-4. The latter proton showed correlation to H-5 which in turn correlated to H_2-6 thus revealing a typical coupling pattern attributable to a quinic acid residue. The *trans*-olefinic protons were seen to correlate with the ester C=O (δ_C 168.75) as was observed in the HMBC spectrum (Pauli et al., 1999). The ^{13}C NMR spectrum for **2** (Table 2) displayed 16 carbon signals differentiated as two CH_2 at δ_C 41.14 and 39.48, eight CH (including two olefins at δ_C 147.71 and 114.88, three oxygenated at δ_C 75.54, 73.54, and 72.88, and three sp^3 carbons at δ_C 123.61, 117.05, and 114.96), and six C [(including two oxygenated at δ_C 148.11, and 147.71, one keto carbonyl at δ_C 169.85, one ester carbonyl at δ_C 168.75, one quaternary carbon at δ_C 78.18, well as one further quaternary carbon whose carbon signal was not detected (C-1')] corresponding to $C_{16}H_{18}O_9$. The HMQC spectroscopic data were used to assign protons attached to their corresponding carbons (Table 2). The complete structure was established by the HMBC spectrum, which shows correlation between protons and carbons for two bonds. On the basis of these spectroscopic data, the structure of **2** was identified as 5'-O-caffeoyl qunic acid (chlorogenic acid) (Saracoğlu, et al., 2002).

The antioxidative potential of the isolated compounds of *Platycerium bifurcatum* was investigated by DPPH free radical scavenging method. The isolated compounds showed stronger antioxidative potential than Propyl gallate (positive control) (Table 3). Some structure-activity relationships were observed with the anti-oxidative properties of compound **1**.These include the 3-glucosylation of the quercetin nucleus (Williamson, et al., 1996); the presence of an *ortho*-dihydroxyl group in the ring-B of the quercetin nucleus together with the 2,3-double bond in conjugation with the 4-oxo functional groups. These functional groups help in the delocalisation of electrons on the quercetin nucleus (Procházkova, et al., 2011). The anti-oxidative properties of chlorogenic acid has been reported by Li et al., (2011) with an IC_{50} of 0.1135mg/ml which compares favourably with our own value of IC_{50} = 0.1175mg/ml (49.21μM).

Isolated compounds

(-)- Quercetin 3-O-β-D-glucopyranoside (1): Pink amorphous powder, $C_{12}H_{20}O_{12}$, LC-MS: *m/z* 464 $[M]^+$, 465 $[M+H]^+$, 950 $[2M + Na]^+$ (Figure 2); UV λ_{max} (MeOH) nm: 360.0, 256.0, 220.0; $[\alpha]_D^{20}$= -10.9 (*c* 0.100, MeOH); ^{1}H-NMR (CD_3OD, 500 MHz): Table 1.

Chlorogenic acid (2): Yellow amorphous powder, $C_{16}H_{18}O_9$, LC-MS: *m/z* 354 $[M]^+$, 355 $[M+H]^+$, 163 $[caffeoyl\text{-}OH]^+$, 164 $[caffeoyl\text{-}O]^+$ (Figure 3); UV λ_{max} (MeOH): 325.7, 239.9, 217.0; $[\alpha]_D^{20}$= -21.4 (*c* 0.10, MeOH); ^{1}H-NMR (CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 125.76 MHz): Table 2.

Statistical analysis: The results were expressed as Mean±SEM of three measurements. Analysis of variance was performed using one-way ANOVA.

Significant differences between means were determined by student's *t-test*, and *P* values<0.05 were regarded as significant.

CONCLUSION

Thus isolated compounds had good antioxidative activity in the DPPH assay. The structures of the isolated polyphenols were obtained by comparism with reported values. These secondary metabolites were isolated for the first time from *Platycerium bifurcatum.*

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Table -1: ^{1}H-NMR Data of Quercetin 3-O-β-D-glucoside (1).

Position	δ_H (ppm)	J (Hz)	COSY
6	6.20,d	2.0	
8	6.39,d	2.1	
2'	7.71,d	2.2	
5'	7.59,m		6'
6'	6.87,dd	8.5,2.3	5'
1"	5.25,d	7.6	2"
Sugar protons	3.71-3.42,m		

Table -2: ^{1}H-NMR and ^{13}C-NMR Data of Chlorogenic acid (2).

Position	δ_C (ppm)	δ_H (ppm)	J (Hz)	COSY	HMBC (H→C)
1	78.18				
2	39.48	2.10,d	7.8	3	3
		2.16, dd	14.7,3.0	3	4
3	73.54	4.12,d	3.1	2, 4	
4	75.54	3.68, dd	9.9,3.2	3, 5	
5	72.88	5.38, m		4, 6	
6	41.14	2.00, d	11.8	5	
		1.96,d	6.3	5	
7	169.85				
1'	nd*				
2'	114.96	7.03,d	2.0	6'	
3'	147.71**				
4'	148.11				
5'	117.05	6.73,d	8.2	6'	
6'	123.61	6.91, dd	8.2,2.1	2', 5'	
7'	147.71**	7.57, d	15.8	8'	9'
8'	114.88	6.25, d	15.8	7'	9'
9'	168.75				

- *Not detected. **Interchangeable peaks.

Table- 3: Median Inhibitory Concentration (IC_{50}) of Compounds 1-2[a].

Compounds	IC_{50} (µM)
Quercetin 3-O-β-D-glucoside (**1**)	31.27±0.12
Chlorogenic acid (**2**)	47.21±0.11
Propyl gallate	68.46±0.16

- Values expressed are Mean ± SEM; *$P < 0.05$; n= 3

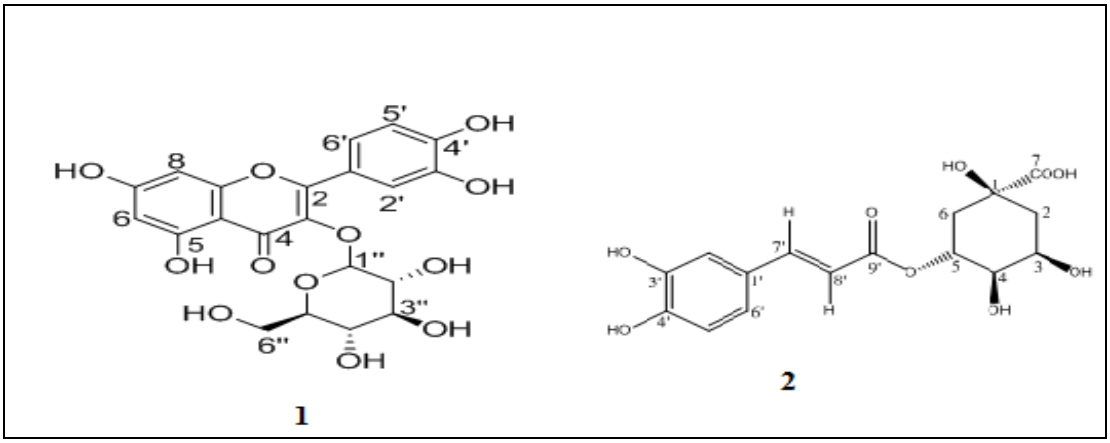
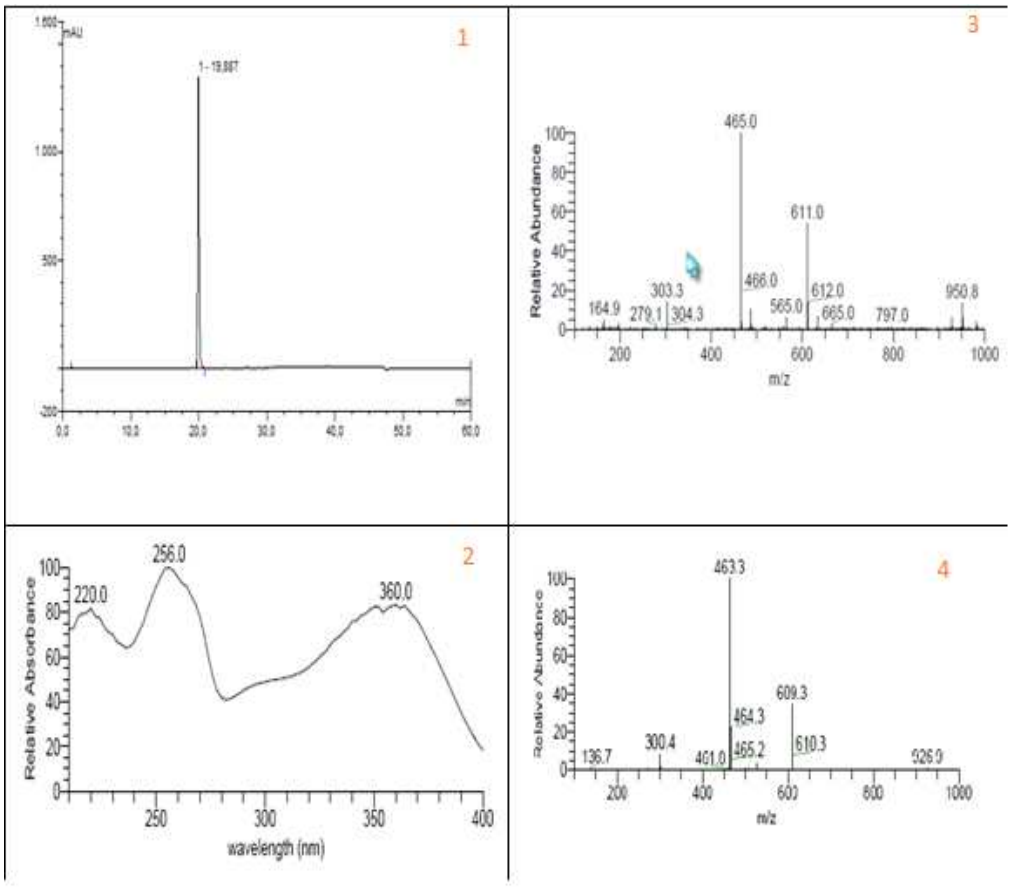


Figure-1: Structures of the isolated Compounds (1-2).



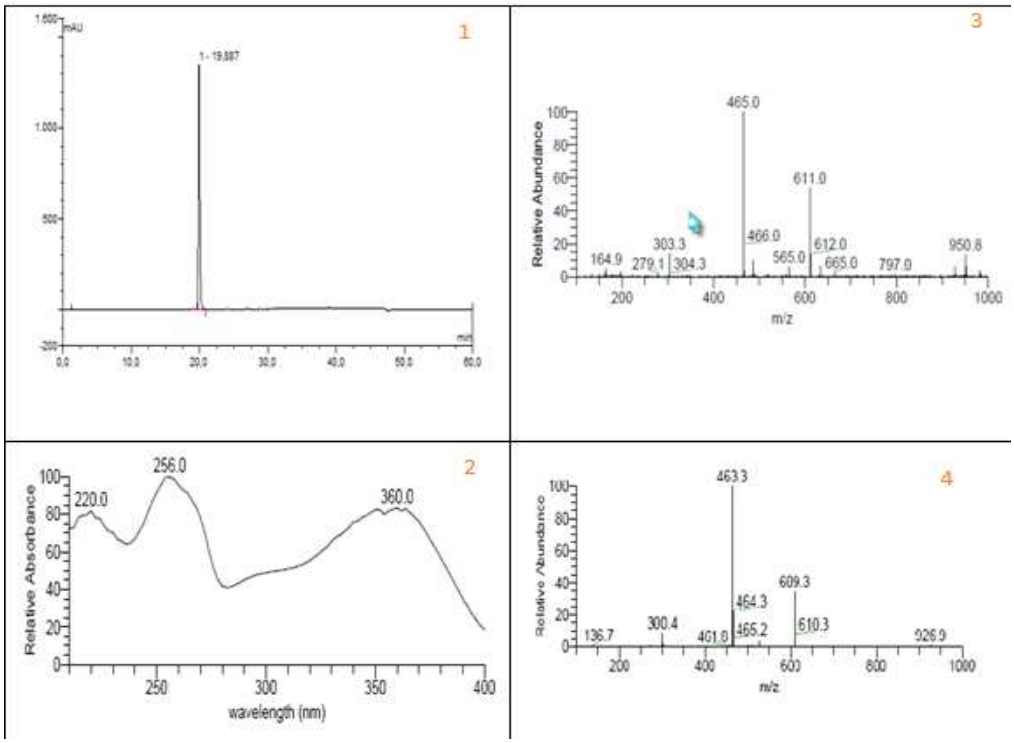


Figure -2: HPLC Analysis of Compound 1.

- 1 = HPLC Chromatogram of Compound **1**; 2 = UV Spectrum of Compound **1**
- 3 = Positive ion (3) and Negative ion (4) Modes HPLC–MS spectra of Compound **1**

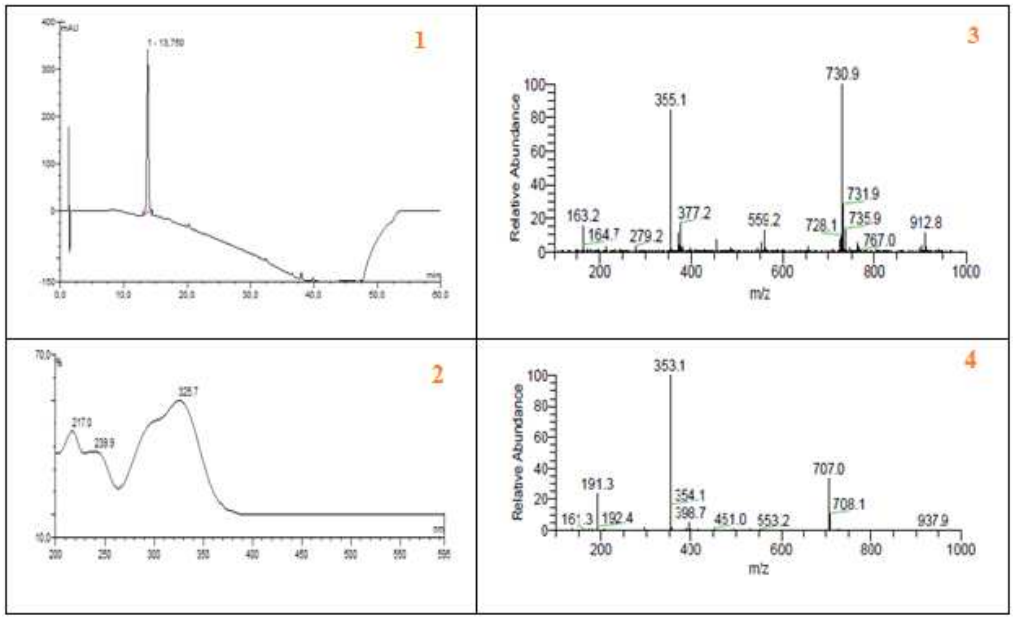


Figure-3: HPLC Analysis of Compound 2.

- 1 = HPLC Chromatogram of Compound **2**; 2 = UV Spectrum of Compound **2**
- Positive ion (3) and Negative ion (4) Modes HPLC–MS spectra of Compound **2**

