

Full Length Research Paper

Assessment of the DPPH and α -glucosidase inhibitory potential of gambier and qualitative identification of major bioactive compound

F. B. Apea-Bah^{1*}, M. Hanafi², R. T. Dewi², S. Fajriah², A. Darwaman², N. Artanti², P. Lotulung²,
P. Ngadymang² and B. Minarti²

¹Biotechnology and Nuclear Agriculture Research Institute, Ghana Atomic Energy Commission, P. O. Box LG 80, Legon, Ghana.

²Research Centre for Chemistry, Indonesian Institute of Sciences, Kawasan Puspiptek, Serpong, Tangerang, Indonesia.

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Gambir (*Uncaria gambir*) and other plants belonging to the genus *Uncaria* have been used in traditional medicine in southeastern Asia, Africa and South America and they have been studied widely over the past century. Gambier, the dried leaf extract from gambier is known to have antioxidant properties and some studies have attributed it to the presence of tannins and condensed tannins. The objective of this study was to investigate the potential of commercial gambier on the Indonesian market as a scavenger of reactive free radicals, evaluate its ability to inhibit α -glucosidase and determine the bioactive compound responsible for these activities. An ethanolic extract of commercial gambier was extracted with ethyl acetate. The ethanol and ethyl acetate extracts as well as the aqueous extract after ethyl acetate extraction and residue from ethanol extraction were tested for free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH). They were also tested for α -glucosidase inhibitory activity. The extracts were then studied using reverse phase HPLC, LCMS and NMR to identify the bioactive compound. It was observed that all the extracts had high activity for DPPH inhibition but moderate activity for inhibiting α -glucosidase *in vitro*. Apart from the aqueous extract, 92% DPPH inhibition by the extracts was achievable at 30 μ g/ml. The ethanol and ethyl acetate extracts had significantly higher ($p < 0.01$) DPPH inhibitory activity than the aqueous extract. IC_{50} of the organic extracts and residue ranged between 13.8 to 16.2 μ g/ml for DPPH inhibition while that of the aqueous extract was 27.4 μ g/ml. With regards to α -glucosidase inhibition, however, IC_{50} range of 15.2 and 49.5 μ g/ml was recorded. Catechin was identified as the major bioactive compound present.

Key words: *Uncaria gambir*, gambier, DPPH, alpha-glucosidase, HPLC, LCMS, NMR

INTRODUCTION

Gambir (*Uncaria gambir*) is a vine belonging to the family *Rubiaceae* and genus *Uncaria* (Heitzman et al., 2005). Species of the genus *Uncaria* have many traditional medicinal uses including treatments for wounds and ulcers, fevers, headaches, gastrointestinal illnesses and bacterial and fungal infections (Aquino et al., 1991; Cerri et al., 1988; Chang et al., 1989; Lemaire et al., 1999; Rizzi et al., 1993; Wurm et al., 1998). In Malaysia, Singapore, Borneo and Sumatra where *U. gambir* is common or indigenous, aqueous extracts of the leaves are

processed and used for medicinal purposes (Ahmed et al., 1978; Das and Griffiths, 1967; Phillipson et al., 1978). The aqueous extracts of leaves and young twigs of *U. gambir* are used traditionally for the treatment of diarrhea and dysentery. They may also be used as gargle for treatment of sore throat (Taniguchi et al., 2007). Dried hooks of some *Uncaria* species have also been integral components in traditional oriental medicines, where they are used as spasmolytics, analgesics and sedatives for symptoms associated with nervous disorders and in the treatment of hypertension (Heitzman et al., 2005).

Gambier, the dried leaf extract from *U. gambir* is believed to have antioxidant properties which are attributed to the presence of tannins and condensed tannins

*Corresponding author. E-mail: franklinapeabah@yahoo.com.

(Desmarchelier et al., 1997; Wirth and Wagner, 1997). Gambir was formerly cultivated extensively in Malaysia and Indonesia as a commercial source of tanning materials (Das and Griffiths, 1967) and catechin is the most abundant polyphenolic constituent in the plant (Taniguchi et al., 2007; Das and Griffiths, 1967). Other polyphenols extracted and identified in gambir include gambirins which are chalcane-flavan dimers, (+)-epicatechin and dimeric proanthocyanidins (Taniguchi et al., 2007). The presence of catechin in green tea and fermented tea has been associated with health protective and cancer preventive properties in animal models due to their antioxidant activity (Sang et al., 2002). Of particular importance in cancer prevention is the ability of catechin to scavenge reactive oxygen species that play a role in carcinogenesis (Sang et al., 2002).

Diabetes mellitus is a serious chronic metabolic disorder characterized by high blood glucose levels (Corry and Tuck, 2000). Worldwide, the number of patients is rapidly growing with increasing obesity (King et al., 1998) and ways to control postprandial hyperglycemia involve medication with dietary restriction and body exercise (Goke and Herrmann-Rinke, 1998). Among the several therapeutic approaches is retarding glucose absorption using inhibitors of carbohydrate-hydrolyzing enzymes such as α -amylase and α -glucosidase (Holman et al., 1999; Saito et al., 1998; Toeller, 1994). Although Acarbose (Schmidt et al., 1977), miglitol (Standl et al., 1999), voglibose (Saito et al., 1998) and nojirimycin (Hansawasdic et al., 2000) are known to be potent inhibitors of α -glucosidase, further screening for α -glucosidase inhibitors from natural sources such as plants and microorganisms will help in reducing overdependence on synthetic drugs. Pine bark extract which is rich in polyphenols such as catechin, quercetin, dihydroquercetin, taxifolin and phenolic acids (Markham and Porter, 1973; Packer et al., 1999), is reportedly effective in suppressing postprandial hyperglycemia in diabetics (Kim et al., 2005). Even though gambir is also rich in polyphenols, it is not used traditionally in treating diabetes mellitus. There is the need to study its potential as an inhibitor of α -glucosidase. In the present study, we investigated the free radical scavenging and α -glucosidase inhibitory potential of commercial gambir sold in a local Indonesian market in Tangerang. The major compound responsible for the bioactivity was qualitatively identified using reversed-phase HPLC, LCMS and NMR.

MATERIALS AND METHODS

Materials

Distilled water, methanol, deuterated methanol (CD_3OD), ethanol, ethyl acetate, hexane, chloroform, sulphuric acid in methanol, 2,2-diphenyl-1-picrylhydrazyl, catechin standard (Sigma-Aldrich Chemical Co.), α -glucosidase (Sigma-Aldrich Chemical Co.), p-nitrophenyl- α -D-glucopyranoside (Sigma-Aldrich Chemical Co.), Na_2CO_3 (E. Merck), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (E. Merck), KH_2PO_4 (E. Merck), bovine serum albumin (E. Merck), dimethyl sulphoxide (E. Merck),

phosphate buffer pH 7.0, analytical balance (Mettler Toledo AT400), rotary evaporator (Buchi Rotavapor R-124), column chromatograph, thin layer chromatograph plates, forced convection oven (Memmert 854 Schwabach, Germany), UV-Visible spectrophotometer (Hitachi U-2000, Japan), HPLC (Shimadzu, Japan), liquid chromatograph-mass spectrometer (LC: Hitachi L 6200; Mariner Biospectrometry, Electrospray Ionisation System), micropipettes, glassware, water bath, stopwatch and other supplies.

Sample preparation

A dried leaf extract of gambir was purchased from a local market in Serpong, a suburb of Tangerang on the Java Island of Indonesia. 10 g of the gambier (dried leaf extract of gambir) was dispersed in 100 ml of 95% aqueous ethanol and stirred. It was then filtered through Whatmann No.1 filter paper into a conical flask. The residue from the ethanol filtration was dried for further analysis while a portion of the ethanol filtrate was evaporated on a rotary evaporator (50°C, 90 -100 rpm) to obtain the ethanol extract. The other portion of the ethanol filtrate was further extracted with ethyl acetate, resulting in ethereal and aqueous layers which were separated using a separating funnel. The ethereal and aqueous layers were concentrated using rotary evaporator (45°C, 90 - 100 rpm for ethereal layer and 53°C, 90 - 100 rpm for aqueous fraction) to obtain the ethyl acetate extract and the aqueous extract (after ethyl acetate extraction). The samples were dried in an oven at temperature of about 45°C overnight (24 h).

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined using the method of Hatano et al. (1988). 4 mg of each sample was weighed and added to 4 ml of methanol. 4 concentrations, 10, 50, 100 and 200 $\mu\text{g}/\text{ml}$, were prepared from each sample solution using methanol as solvent and 500 μl DPPH was added giving a total volume of 2.5 ml per sample concentration. Standards were also prepared at similar concentrations using standard catechin while the blank was prepared using methanol and DPPH without any sample. They were then incubated in a water bath at 37°C for 30 min after which absorbance of samples and standards were read against the blank at 517 nm. The percent inhibition was estimated as % inhibition = $[(C - S)/C] \times 100\%$, where S is the sample absorbance and C is absorbance of the blank. Scatter diagrams were plotted and linear regression estimated using the equation $y = ax + b$, where y is % inhibition and x is concentration ($\mu\text{g}/\text{ml}$). IC_{50} was calculated as the concentration that caused 50% inhibition of DPPH.

α -Glucosidase inhibitory activity

The α -glucosidase inhibitory activity was determined using the method described by Sutedia (2005). The enzyme solution was prepared by dissolving 0.5 mg α -glucosidase in 10 ml phosphate buffer (pH 7.0) containing 20 mg bovine serum albumin. It was diluted further to 1:10 with phosphate buffer just before use. Sample solutions were prepared by dissolving 4 mg sample extract in 400 μl DMSO. 3 concentrations; 6.25, 12.5 and 25 $\mu\text{g}/\text{ml}$ were prepared and 5 μl each of the sample solutions or DMSO (sample blank) was then added to 250 μl of 20 mM p-nitrophenyl- α -D-glucopyranoside and 495 μl of 100 mM phosphate buffer (pH 7.0). It was pre-incubated at 37°C for 5 min and the reaction started by addition of 250 μl of the enzyme solution, after which it was incubated at 37°C for exactly 15 min. 250 μl of phosphate buffer was added instead of enzyme for blank. The reaction was then stopped by addition of 1000 μl of 200 mM Na_2CO_3 solution and the amount of p-nitrophenol released was measured by reading the

absorbance of sample against sample blank (containing DMSO with no sample) at 400 nm using UV-visible spectrophotometer. Standard solutions of same concentrations were also prepared using 'koji', an extract from *Aspergillus terreus*. Scatter diagrams were plotted and linear regression estimated. IC_{50} was calculated as the concentration that caused 50% inhibition of enzyme activity.

HPLC analysis

The samples were analyzed using reverse phase HPLC to ascertain presence of catechin. Wavelength for maximum absorption of catechin was determined using the UV-visible spectrophotometer. The standards and samples were then analyzed using HPLC at 210 nm. Water and methanol (30:70) was used as the eluent and was set to a flow rate of 1 ml/min and elution time of 20 min.

Identification and structure elucidation of active component

A sample of the ethyl acetate extract was purified using column chromatography with hexane, ethyl acetate and methanol in different proportions as eluent. The purified extract and other samples were analyzed using liquid chromatograph-mass spectrometer (LC: Hitachi L 6200; Mariner Biospectrometry; Electrospray Ionisation System) to determine the molecular weights (m/z values) of the major compounds. 20 μ l of the samples were injected and run at a flow rate of 1 ml/min using acetonitrile: water (70:30) as eluent and C18 column (Supelco: 150 mm x 2 mm x 5 μ m). Nuclear magnetic resonance spectroscopy (JEOL) with radio frequency of 500 MHz was then used to elucidate the structure of the active compound in the purified sample which was prepared using deuterated methanol (CD_3OD) as solvent. One and two dimensional NMR spectra were developed and analyzed.

Data analysis

Two way analysis of variance (Montgomery, 1991) was used to determine the effects of concentration and type of extraction on the DPPH and α -glucosidase inhibitory activities. Where significant differences existed ($p < 0.05$), least significant difference (LSD) was used for mean comparison. Statgraphics centurion XV (Statgraphics, 2005) statistical software was used for all statistical analysis.

RESULTS AND DISCUSSION

DPPH Radical scavenging activity

Figures 1 to 4 show the linear regression curves of concentration of catechin standard and gambier extracts plotted against percent inhibition of DPPH. The regression coefficient (R^2) of the logarithmic trend line obtained for the extracts and standard was between 0.79 - 0.80, implying that 80% of the variability in DPPH inhibition can be attributed to the concentration of the extracts. Analysis of variance (ANOVA) showed concentration to significantly ($p < 0.01$) affect percent inhibition. From the mean comparison using least significant difference, it was observed that inhibition of DPPH was significantly low at 10 μ g/ml concentration (Table 1) compared to all the other concentrations. The extracts from gambier were not significantly different ($p > 0.05$) from the catechin standard with regards to DPPH inhibition. The IC_{50} for the

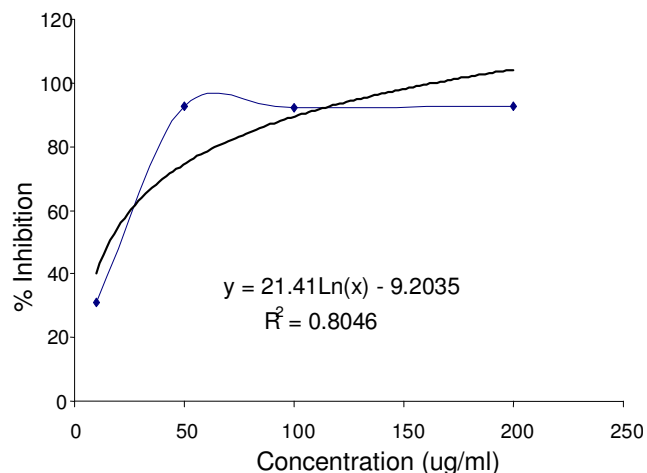


Figure 1. Percent inhibition of DPPH against catechin concentration.

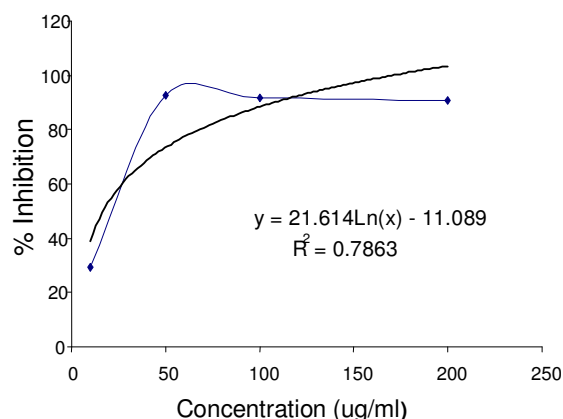


Figure 2. Percent inhibition of DPPH against concentration of ethanol extract

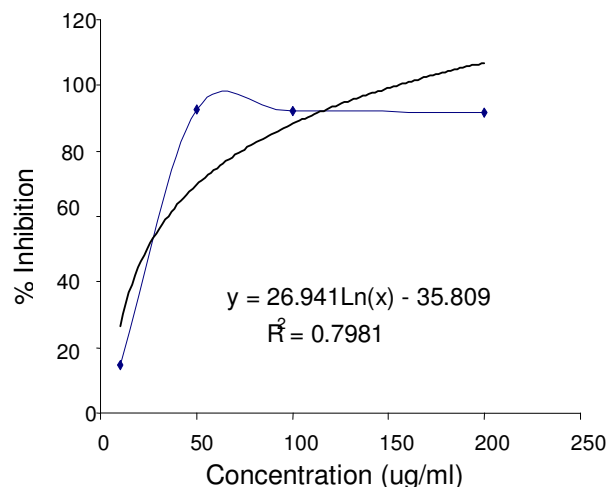


Figure 3. Percent inhibition of DPPH against concentration of Residue after Ethanol extraction.

Table 1. Concentration of Catechin and extracts from Gambier and their IC₅₀ for DPPH inhibition.

Concentration (µg/ml)	% Inhibition				
	Catechin standard	Ethyl acetate extract	Ethanol extract	Residue from ethanol extraction	*Mean
200	92.41	91.39	90.67	91.61	91.52 ^a
100	92.26	92.33	91.75	92.19	92.13 ^a
50	92.48	92.62	92.48	92.48	92.51 ^a
10	31.14	21.59	29.11	14.72	24.14 ^b
IC ₅₀ (µg/ml)	15.88	20.70	16.88	24.17	

*Means in a column with same superscript are not significantly different from each other (p<0.01)

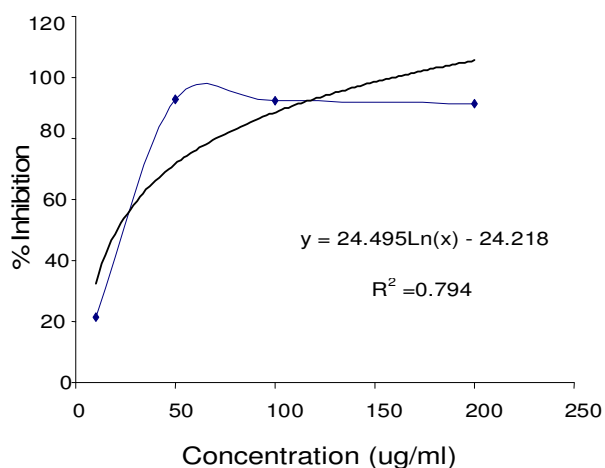


Figure 4. Percent inhibition of DPPH against concentration of ethyl acetate extract.

catechin standard was 15.9 µg/ml while it was in the range of 16.9 to 24.2 µg/ml for the extracts (Table 1). Since IC₅₀ was lowest for ethanol extract and highest for residue, it implies that the ethanol extract had the highest free radical scavenging activity at concentrations between 10 and 200 µg/ml, while that of the residue was least. This suggests that the compounds present in gambier that are responsible for scavenging DPPH free radicals are very soluble in ethanol. It is worth noting from the linear regression curves (Figures 1 to 4) that increasing sample concentration above 50 µg/ml did not cause a corresponding increase in percent inhibition of DPPH. There was therefore the need to study concentrations between 10 and 50 µg/ml.

It was observed from studies using lower concentrations of standard catechin and gambier extracts that maximum DPPH inhibition was measured at 30 µg/ml (Table 2), except for the aqueous extract obtained after ethyl acetate extraction of gambier, which showed percent DPPH inhibition to increase correspondingly with concentration until 50 µg/ml. At the lower concentrations, a coefficient of regression (R^2) of 0.87 for catechin and 0.94 - 0.97 for gambier extracts were obtained (Figures 5

to 10) from the logarithmic trend line. The IC₅₀ of the ethyl acetate extract was least and closest to that of catechin standard while that of the aqueous extract was highest (Table 2) indicating that the active compound is very soluble both in ethanol and ethyl acetate.

From the ANOVA, both sample type or extract and concentration significantly affected ($p < 0.01$) percent DPPH inhibition. Concentrations of 30 to 50 µg/ml were not significantly different from each other but resulted in significantly higher DPPH inhibition than 5 to 20 µg/ml (Table 2). The lower concentrations of 5, 10 and 20 µg/ml were all significantly different from each other. This means therefore that for optimum free radical scavenging using organic extracts of commercial gambier, a concentration of 30 µg/ml is adequate.

With regards to the samples, however, ethanol and ethyl acetate extracts were statistically not different ($P < 0.01$) from the catechin standard. They were also not significantly different from the residue from ethanol extraction. They all, however, showed significantly higher potential for DPPH inhibition than the aqueous extract (Table 2). This suggests that the major bioactive compound has lower solubility in water than in ethanol and ethyl acetate.

α-Glucosidase inhibitory activity

Figures 11 to 16 show the linear regression curves of concentration of koji extract and gambier extracts plotted against percent inhibition of α-glucosidase. The regression coefficient (R^2) of the trend lines obtained for the extracts and standard was between 0.94 - 0.99, implying that 94 to 99% of the variability in α-glucosidase inhibition can be attributed to the concentration of the extracts. Since koji, an extract from *Aspergillus terreus*, is known to have high activity for α-glucosidase inhibition, the gambier extracts were compared to it for α-glucosidase inhibitory activity. Three concentrations, 6.25, 12.5 and 25 µg/ml, were studied. For all extracts, percent α-glucosidase inhibition increased with increasing concentration. Koji extract showed a mean of 74% inhibition while ethanol, ethyl acetate and the aqueous extraction caused

Table 2. Percent DPPH inhibition by catechin and Gambier Extracts at lower concentrations.

Concentration ($\mu\text{g/ml}$)	% Inhibition					
	Catechin Standard	Ethanol Extract	Ethyl acetate extract	Residue after ethanol extraction	Aqueous extract	Mean
50	91.89	91.77	92.63	92.24	92.44	92.20 ^h
40	92.48	91.50	92.44	92.63	73.28	88.47 ^h
30	92.99	91.93	92.56	92.36	67.80	87.53 ^h
20	92.60	68.07	70.66	54.24	33.01	63.71 ⁱ
10	35.71	27.68	33.32	17.22	14.16	25.62 ^j
5	14.32	5.90	6.72	4.45	0.61	6.40 ^k
Mean	70.00 ^m	62.81 ^{mn}	64.72 ^{mn}	58.86 ⁿ	46.88 ^p	
IC ₅₀ ($\mu\text{g/ml}$)	11.62	14.54	13.78	16.20	27.35	

*Means with same superscript are not significantly different from each other ($p < 0.01$).

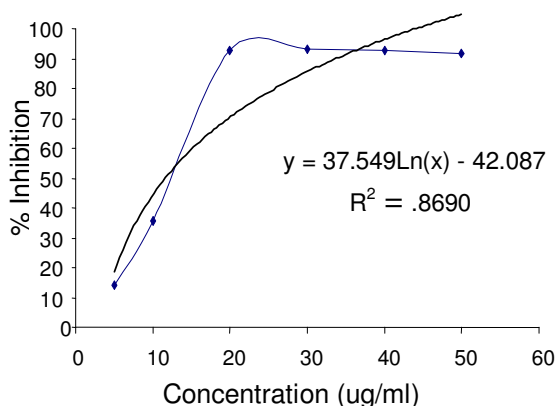


Figure 5. Linear regression curve of percent DPPH inhibition at lower concentration of catechin standard.

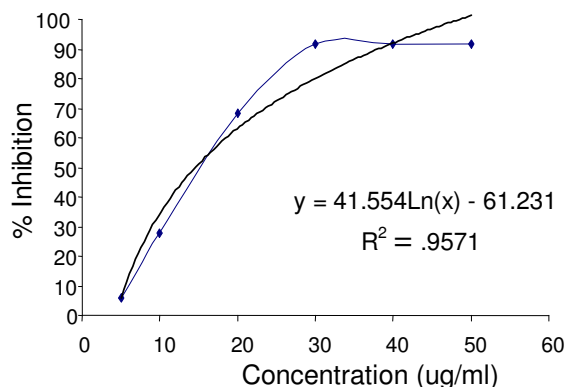


Figure 7. Linear regression curve of percent DPPH inhibition at lower concentration of ethanol extract.

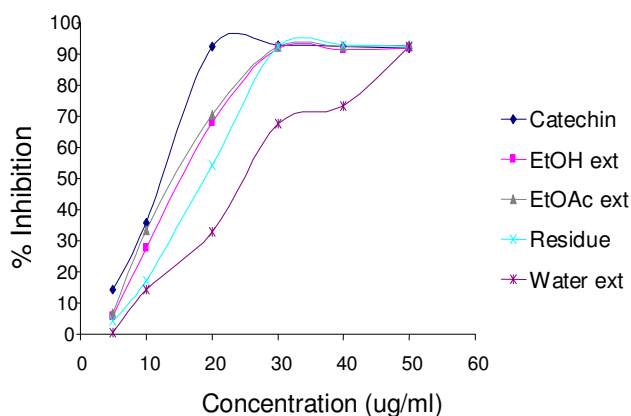


Figure 6. Linear Regression curve of percent DPPH inhibition lower concentrations of catechin standard and gambier extracts.

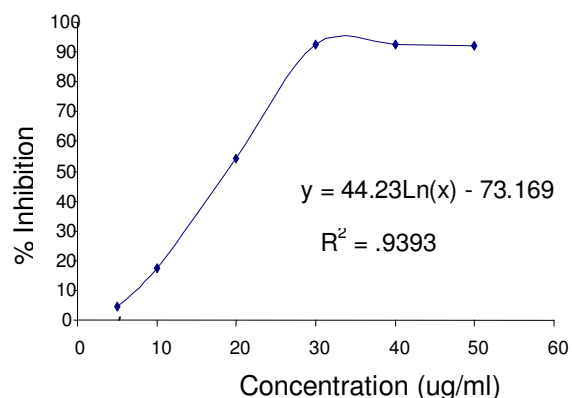


Figure 8. Linear Regression Curve of Percent DPPH inhibition at lower concentration of residue from ethanol extraction.

extracts caused below 50% inhibition of α -glucosidase activity (Table 3). This suggests that the extracts have

moderate α -glucosidase inhibitory activity *in vitro*. 2 way ANOVA showed both the extracts and their concentrations

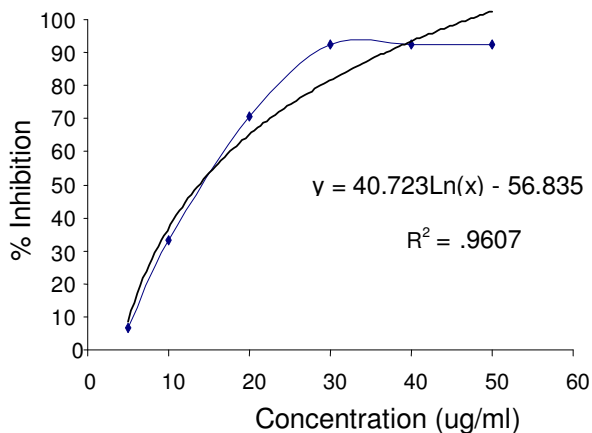


Figure 9. Linear regression curve of percent DPPH inhibition at lower concentration of ethyl acetate extract.

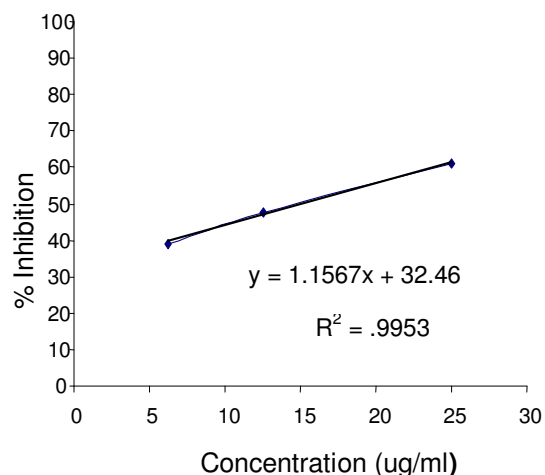


Figure 12. Linear regression curve of percent α -glucosidase inhibition at lower concentration of ethanol extract.

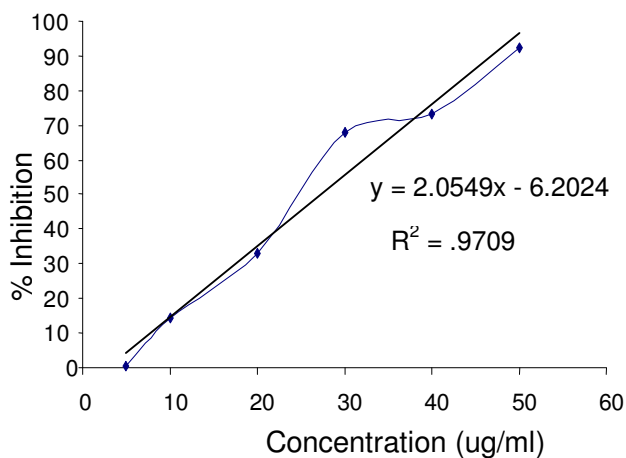


Figure 10. Linear regression curve of percent DPPH inhibition at lower concentration of water extract.

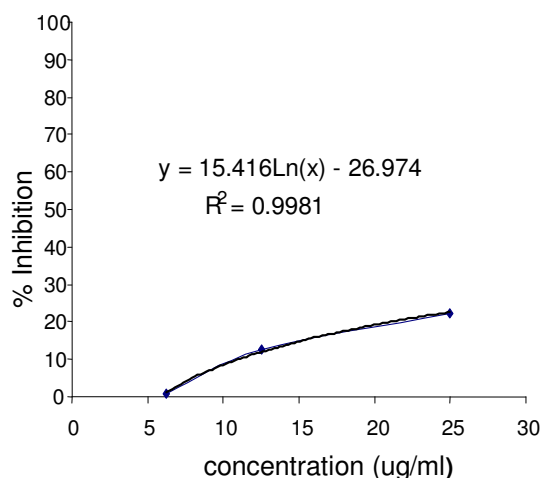


Figure 13. Linear Regression curve of percent α -glucosidase inhibition at lower concentration of residue of ethanol extract

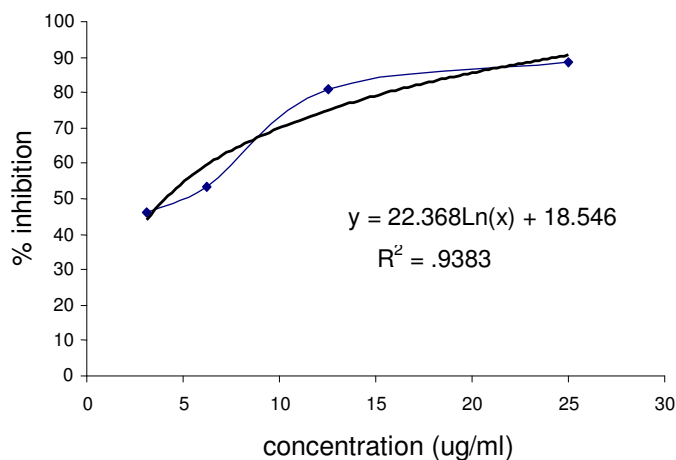


Figure 11. Linear regression curve of percent α -glucosidase inhibition at lower concentration of 'koji' extract from *Aspergillus terreus* extract.

to significantly ($p < 0.01$) affect percent inhibition. Inhibition of α -glucosidase was significantly highest for koji and lowest for residue after ethanol extraction. The other solvent extracts did not differ significantly from each other. Percent inhibition was also significantly highest at 25 $\mu\text{g/ml}$ concentration compared to the lower concentrations of 6.25 and 12.5 $\mu\text{g/ml}$, which did not significantly differ from each other (Table 3).

Increasing the concentrations of the extracts up to 50 $\mu\text{g/ml}$ did not improve their α -glucosidase inhibitory activity (Figure 17). The IC_{50} for koji extract was 4.08 $\mu\text{g/ml}$ while it was in the range of 15.16 $\mu\text{g/ml}$ to 49.48 $\mu\text{g/ml}$ for the extracts (Table 3). Since IC_{50} was lowest for

Table 3. Concentration of Koji and Extracts from Gambier and their IC₅₀ for α-glucosidase inhibition.

Concentration (µg/ml)	Koji extract	Ethanol extract	Ethyl acetate extract	Residue after Ethanol extraction	Aqueous extract	*Mean
25.0	88.71	61.09	45.77	22.38	66.73	56.94 ^c
12.5	80.85	47.78	43.15	12.50	38.51	44.56 ^d
6.25	53.43	39.11	40.73	1.01	35.08	33.87 ^d
*Mean	74.33 ^e	49.33 ^g	43.21 ^g	11.96 ^f	46.77 ^g	
IC ₅₀ (µg/ml)	4.08	15.16	40.65	49.48	16.41	

*Means with same superscripts are not significantly different from each other (p>0.01). *Koji is an extract from *Aspergillus terreus*.

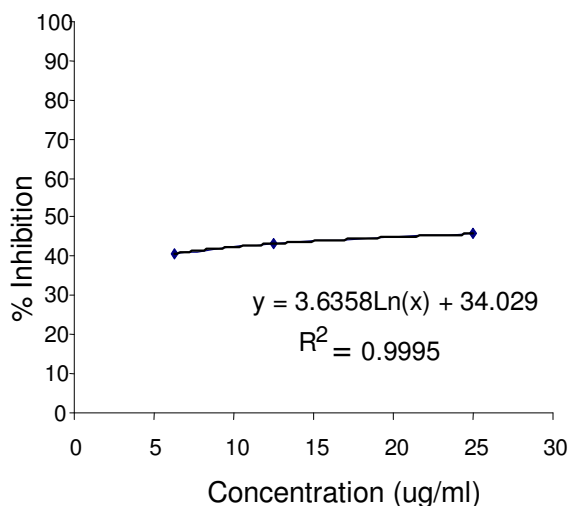


Figure 14. Linear regression curve of percent α-glucosidase inhibition at lower concentration of ethyl acetate extract

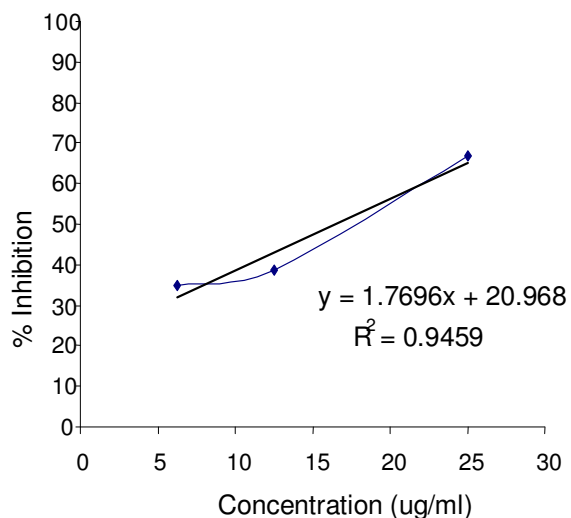


Figure 15. Linear regression curve of percent α-glucosidase inhibition at lower concentration of water extract.

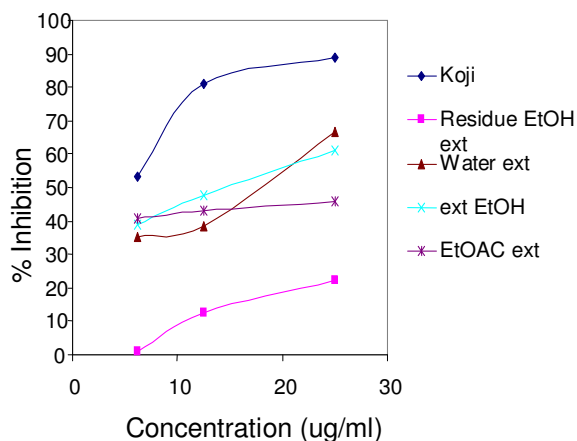


Figure 16. Linear regression curve of percent α-glucosidase inhibition at lower concentrations of gambier extracts.

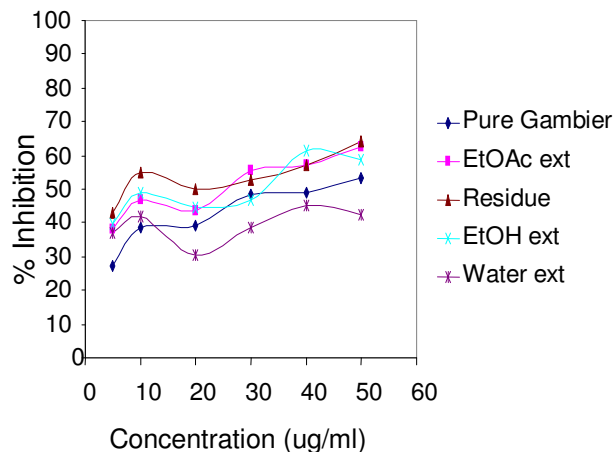


Figure 17. Linear regression curve of percent α-glucosidase inhibition at higher concentrations of gambier extracts.

ethanol extract and highest for residue from ethanol extract, it implies that the ethanol extract had the highest α-glucosidase inhibitory activity *in vitro* while that of the residue from ethanol extraction was least.

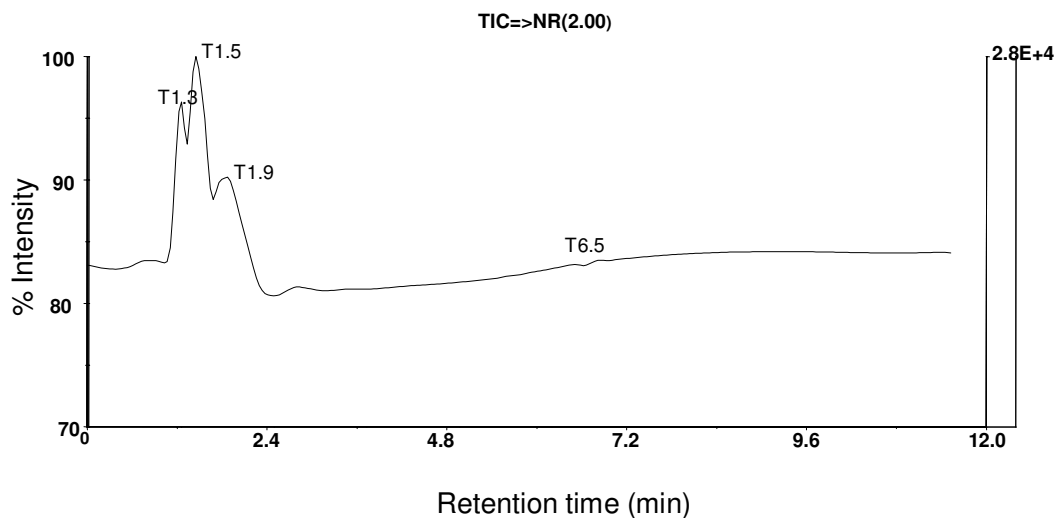


Figure 18. LCMS spectrum for ethanol extract showing three peaks at T1.3, T1.5, T1.9.

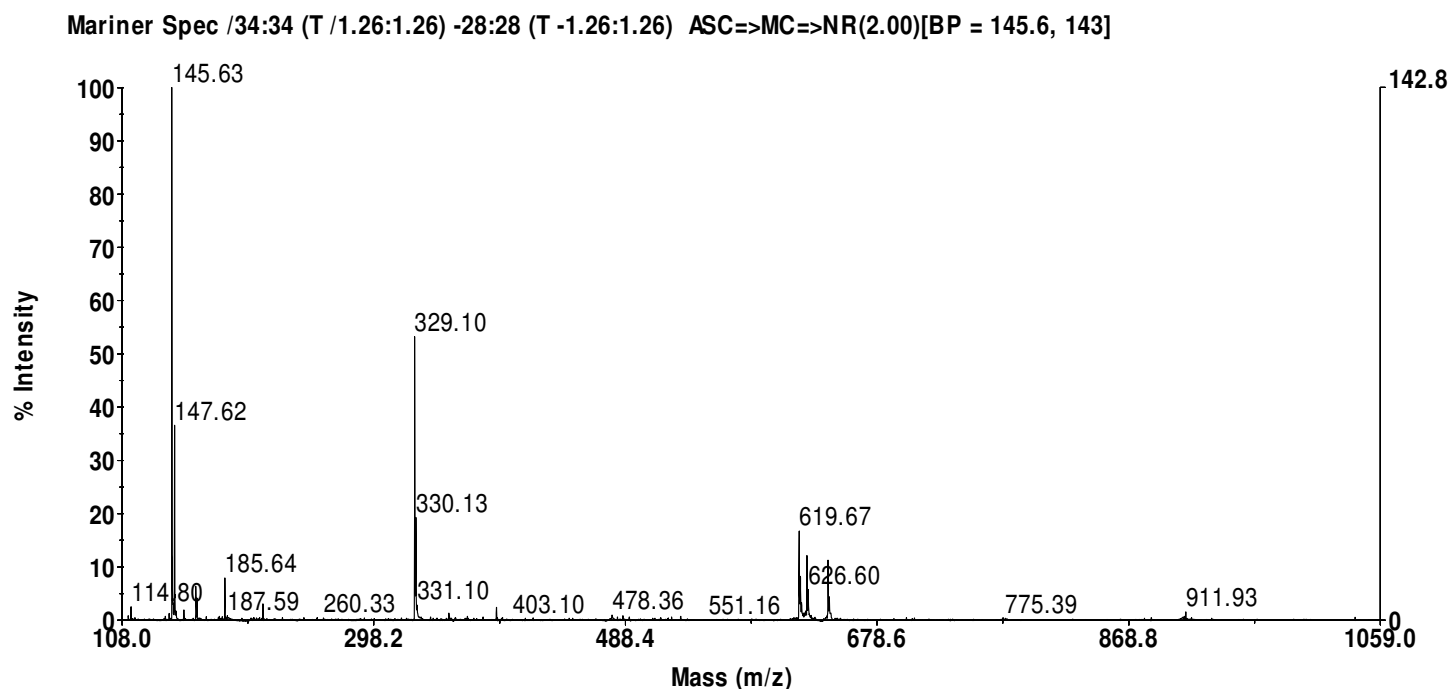


Figure 19. m/z spectral lines of compounds present in peak T1.3 for ethanol extract.

HPLC analysis

Preliminary work on the wavelength for maximum absorption using the UV-visible spectrophotometer resulted in 5 ppm standard catechin giving best results at 209.4 nm. All HPLC analyses were therefore done at 210 nm wavelength. Catechin standard gave maximum peak at retention time range of 3.258 to 3.271 min (Table 4) for the various concentrations studied.

At sample concentrations of 500 and 1000 $\mu\text{g/ml}$, for all the extracts, retention times were similar (Table 5) to that of standard catechin indicating that the bioactive compound could be catechin.

LCMS analysis

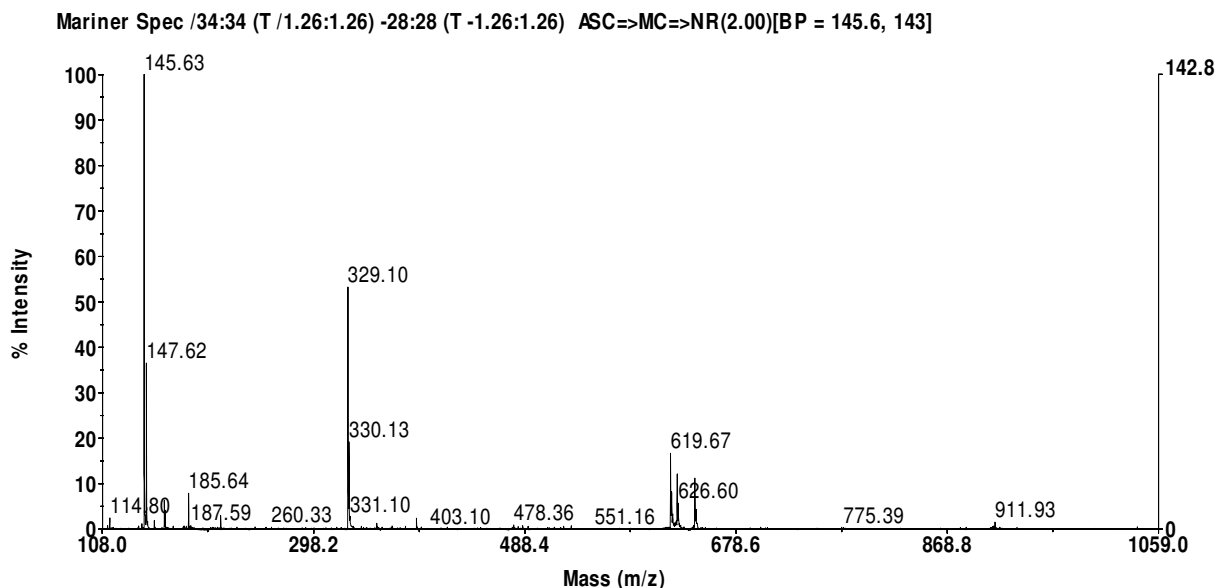
The extracts upon analysis using liquid chromatograph-

Table 4. Retention time of standard catechin at 210 nm using HPLC.

Retention time (min)	Concentration ($\mu\text{g/ml}$)	Area
3.266	5	1650628
3.263	10	3495729
3.264	25	7766748
3.271	50	15226023
3.266	100	28116464
3.263	500	70398534
3.258	1000	83927104

Table 5. HPLC Retention time of Samples at higher concentration at 210nm

Sample	Concentration ($\mu\text{g/ml}$)	Retention time	Area
Ethyl Acetate	500	3.299	60923005
	1000	3.275	70239090
Ethanol	500	3.274	62442591
	1000	3.266	75125886
Water	500	3.256	65592871
	1000	3.227	92219895
Residue	500	3.277	79735339
	1000	3.265	92755082

**Figure 19.** m/z spectral lines of compounds present in peak T1.3 for ethanol extract.

mass spectrometer (LCMS) showed 2 or 3 board peaks (Figures 18 to 37) each comprising different compounds and hence different m/z ratios. Notable among the m/z values was 291.23 with its major peak, which is indicative of catechin. NMR was used to then elucidate and confirm the structure of the major bioactive compound.

NMR analysis

Plates 1 to 5 show the ^1H , ^{13}C , DEPT, HMQC and HMBC spectra generated from the NMR analysis at 500MHz. From the ^1H NMR spectra (Plate 1), 2 double doublet peaks each with one proton (^1H) are observed at chemi-

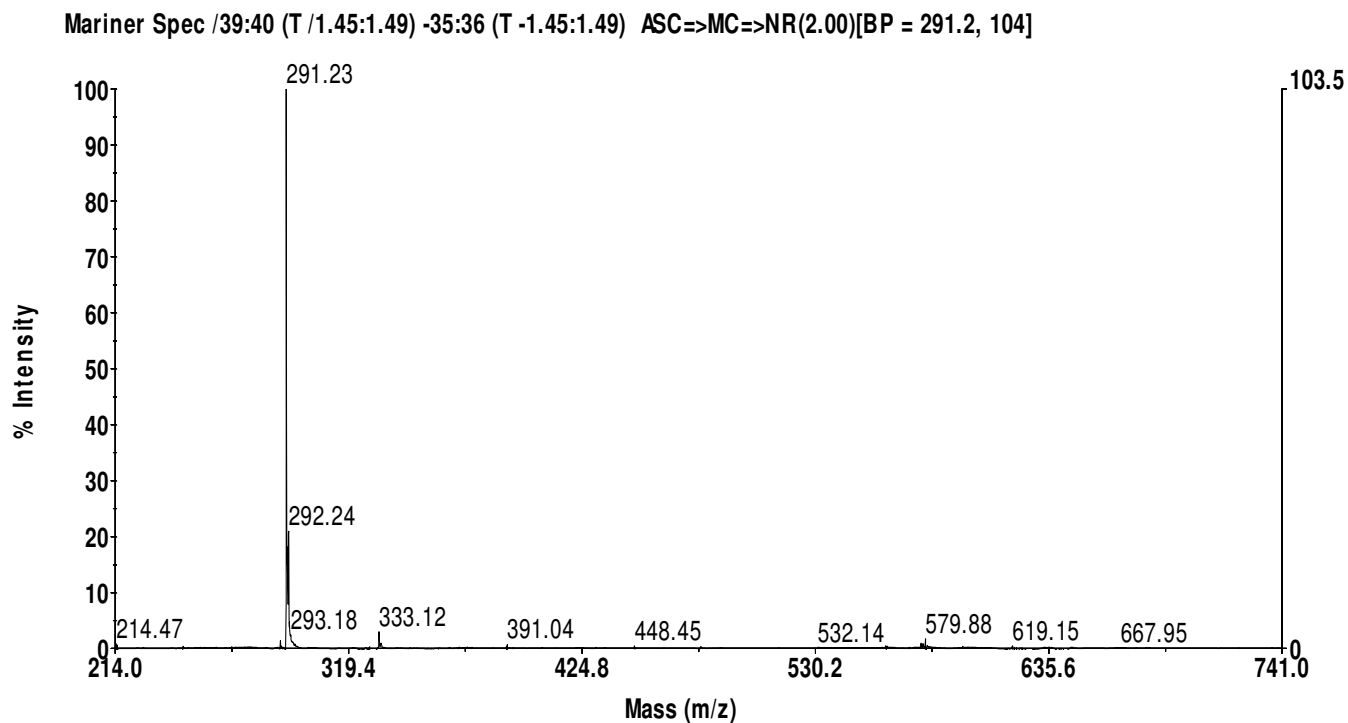


Figure 20. m/z spectral lines present in peak T1.5 for ethanol extract

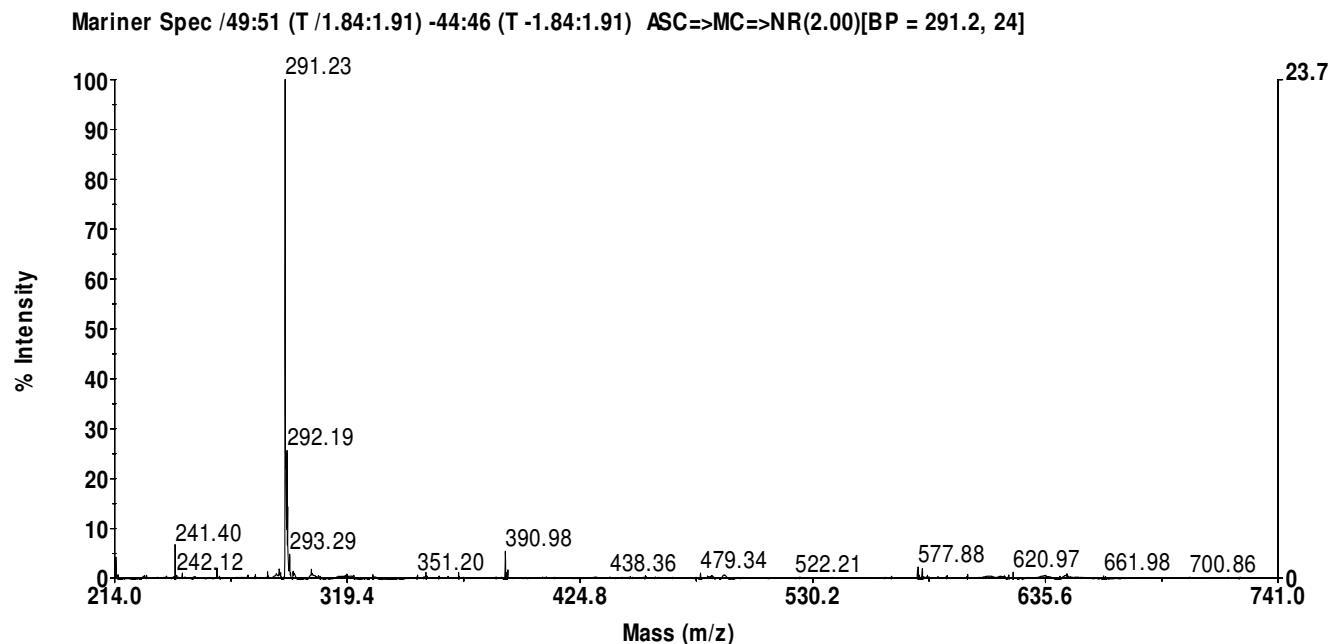


Figure 21. m/z spectral lines present in peak T1.9 for ethanol extract.

cal shifts (δ), 2.48 - 2.53 ppm (J-coupling, 7.95Hz) and 2.82 - 2.87 ppm (J-coupling 5.50 Hz). These peaks represent an aliphatic group having a single near ^1H and another longer range ^1H coupling per each double doublet.

The ^1H s are in axial and equatorial positions at the bonded site (Plate 1). At δ 3.98 - 4.0ppm, a quartet peak (J-coupling, 7.95Hz, 2.45 Hz) is observed indicating 3 neighboring ^1H -atoms. At δ 4.45 - 4.58 ppm, there is a

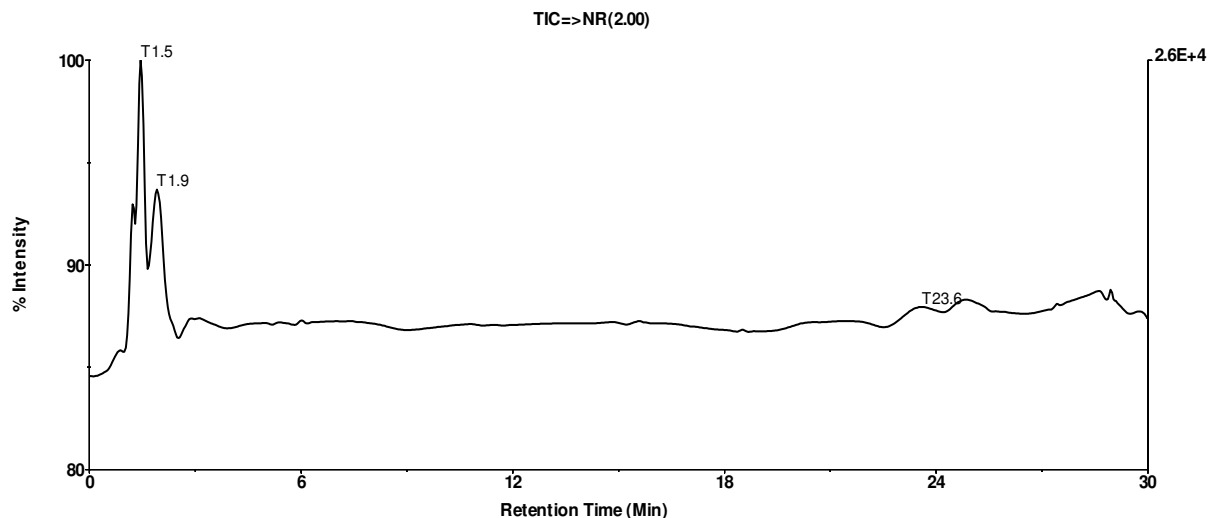


Figure 22. LCMS spectrum for ethyl acetate extract showing three peaks at T1.2, T1.5, T1.9.

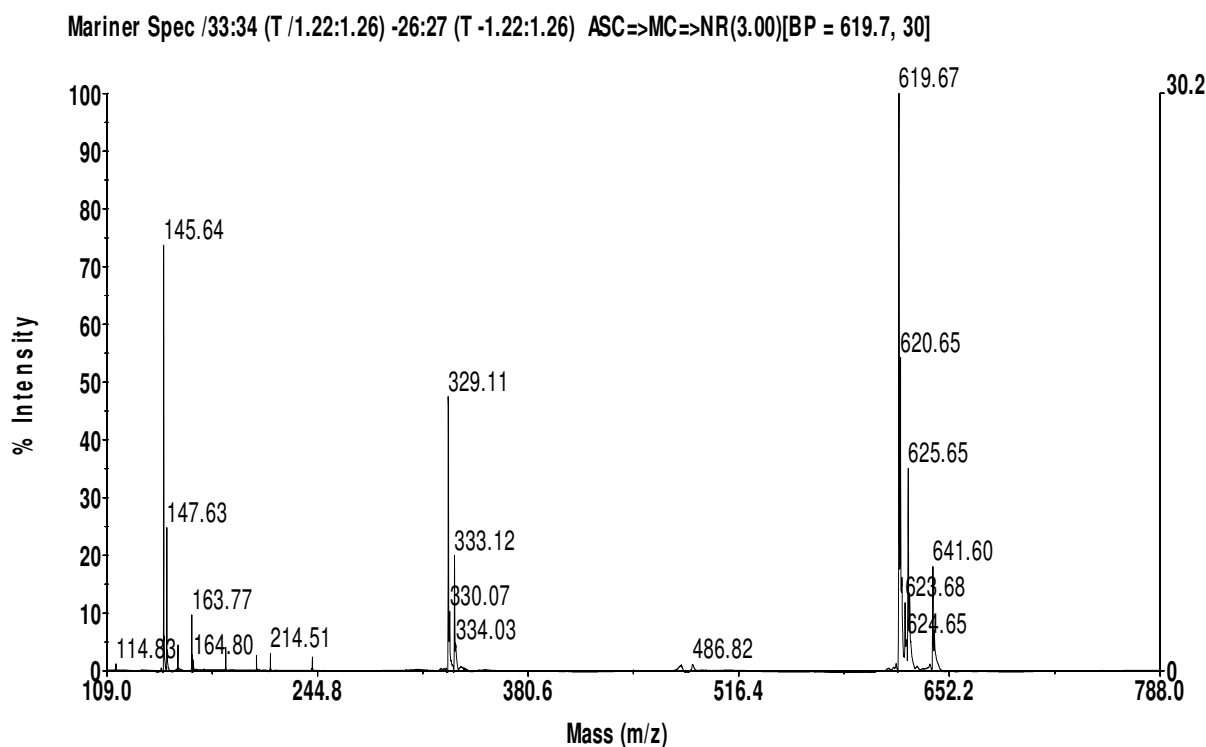


Figure 23. m/z spectral lines present in peak T1.2 for ethyl acetate extract.

doublet peak (J-coupling, 7.95 Hz) with one ^1H indicating only one neighboring ^1H and there is likelihood of a neighboring O-atom at this δ value. Both the quartet and doublet having J-coupling of 7.95 Hz are in long range coupling with the double doublet at δ 2.48 - 2.53 ppm. The deuterated methanol (CD_3OD) produced its peak at δ 3.31 ppm and another peak for water produced at δ 4.67 - 4.95 ppm (Plate 1). At 5.86 and 5.94 ppm, there are 2

doublets, each with one ^1H , having meta coupling (J-coupling 2.45 Hz) with each other in an aromatic ring. At δ 6.71 - 6.73 ppm, there is a double doublet (J-coupling, 8.8 Hz, 1.85 Hz) representing 2 neighboring ^1H s in meta and ortho positions in an aromatic ring. The neighboring ortho ^1H -atom produced a doublet at 6.76 - 6.78 ppm (J-coupling, 8.55 Hz) while the neighboring meta ^1H (J-coupling, 1.85 Hz) produced a doublet at 6.84 ppm (Plate 2).

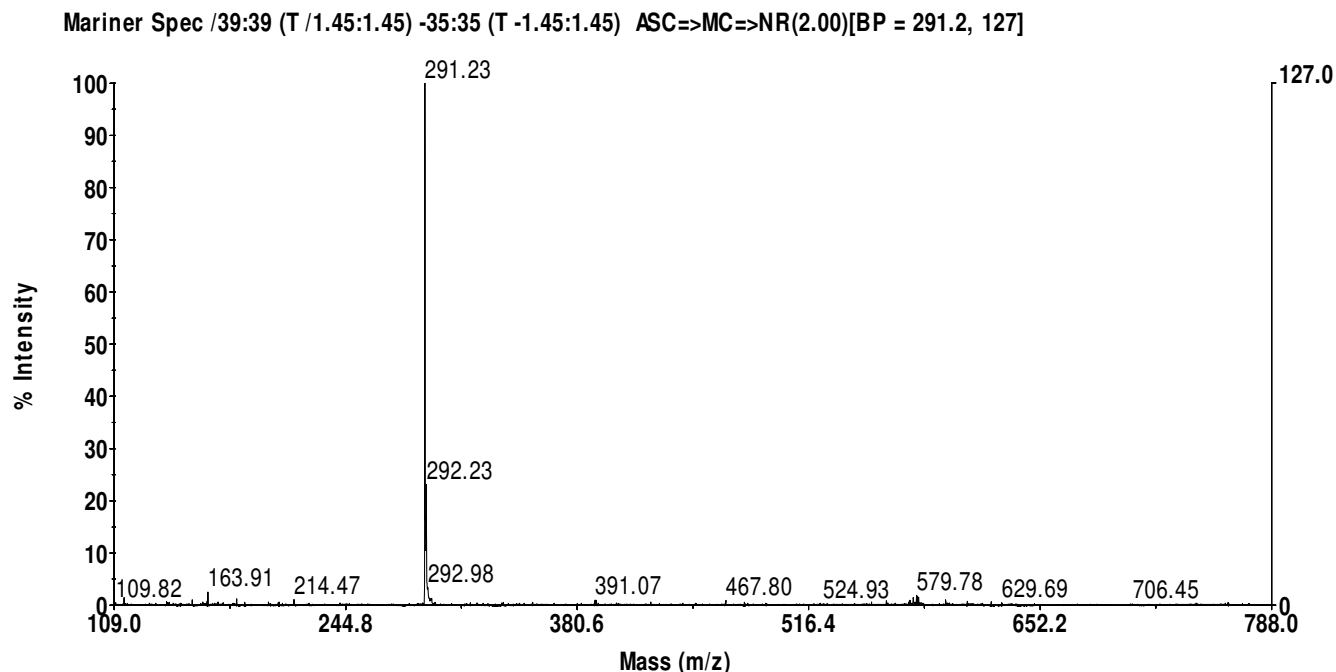


Figure 24. m/z spectral lines present in peak T1.5 for ethyl acetate extract.

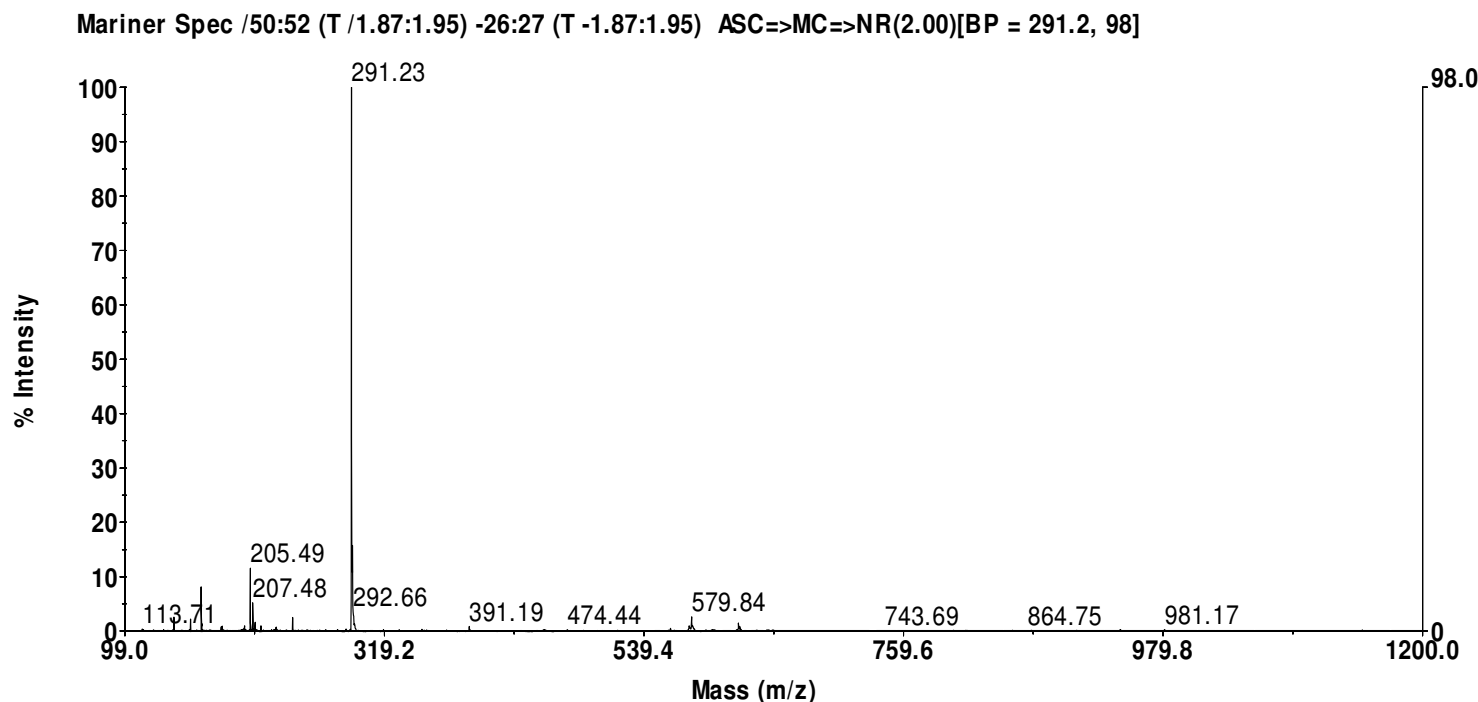


Figure 25. m/z spectral lines present in peak T1.9 for ethyl acetate extract

Comparing the ^{13}C and DEPT spectra (Plate 3) reveal CH_2 as inverted DEPT peak at 28.56 ppm and quaternary C-atoms, being present in ^{13}C spectra but absent in DEPT spectra, at 100.94, 132.19, 146.25 ppm, 146.28,

156.91, 157.57 and 157.78 ppm. δ values above 100 ppm in the ^{13}C spectra indicated aromatic C-atoms while δ values of about 150 ppm indicated aromatic C-atoms bonded to O-atom. It is therefore likely that C-atoms at

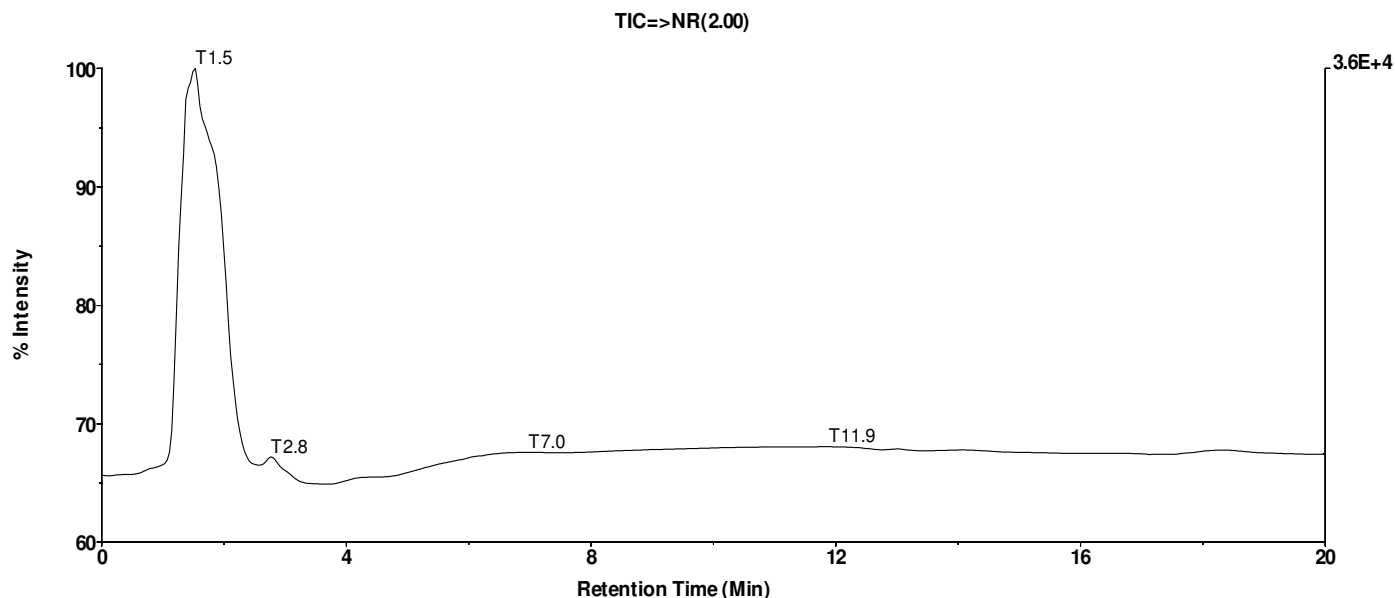


Figure 26. LCMS spectrum for residue from ethanol extraction showing broad peak at T1.5 and small peak at T2.8

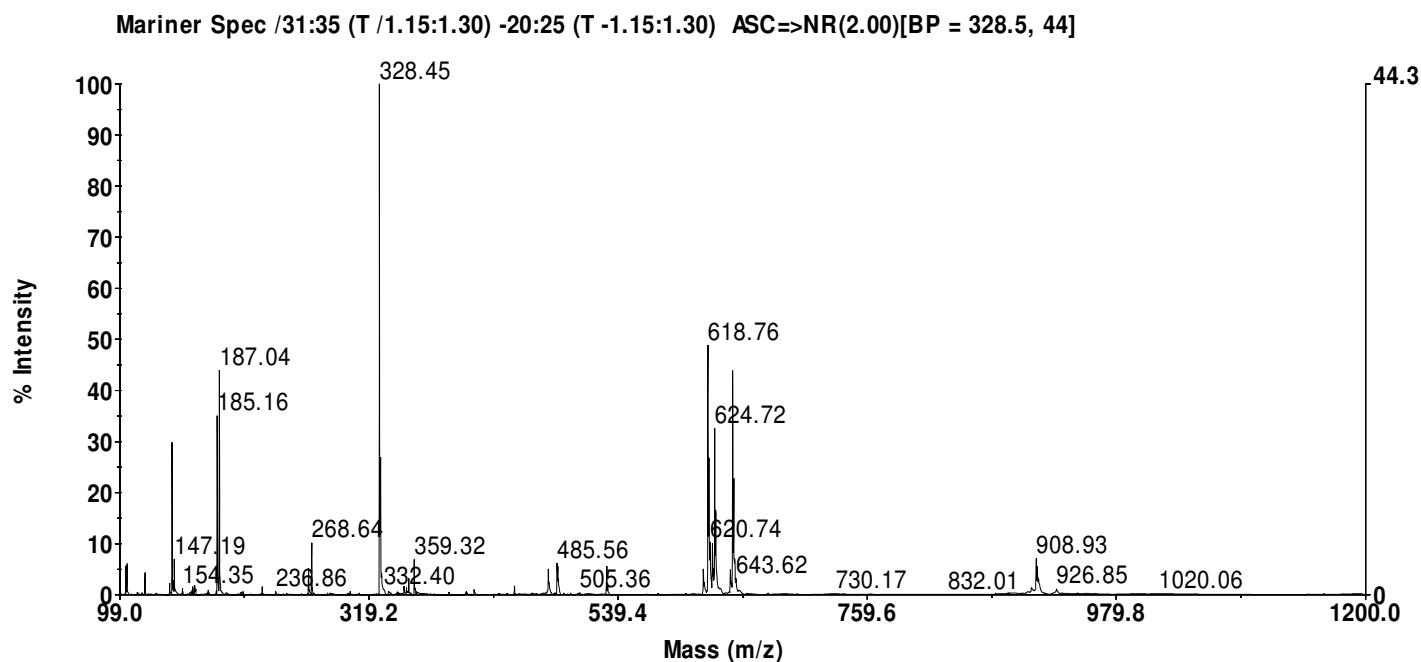


Figure 27. m/z spectral lines present in peak T1.3 for residue from ethanol extraction.

values from 146.25 to 157.78 ppm are bonded to an O-atom.

From the HMQC spectra (Plate 4), it was observed that the two ^1H -atoms with double doublet peaks at δ 2.48 – 2.53 ppm (Plate 1) were bonded to the C-atom at 28.56 ppm while the quartet ^1H at 3.98 - 4.0 ppm was bonded to the C-atom at 68.81 ppm. The ^1H with doublet peak at

4.45 - 4.58 ppm was bonded to the C-atom at 82.86 ppm while the ^1H -atoms with 2 doublets at 5.86 and 5.94 ppm, were bonded to C-atoms at 95.60 and 96.41 ppm. The double doublet ^1H at 6.71 - 6.73 ppm was bonded to the C at 120.16 ppm while the doublet ^1H at 6.76 - 6.78 ppm was bonded to the C at 116.23 ppm. The doublet ^1H at 6.84 ppm was bonded to the C-atom at 115.34 ppm.

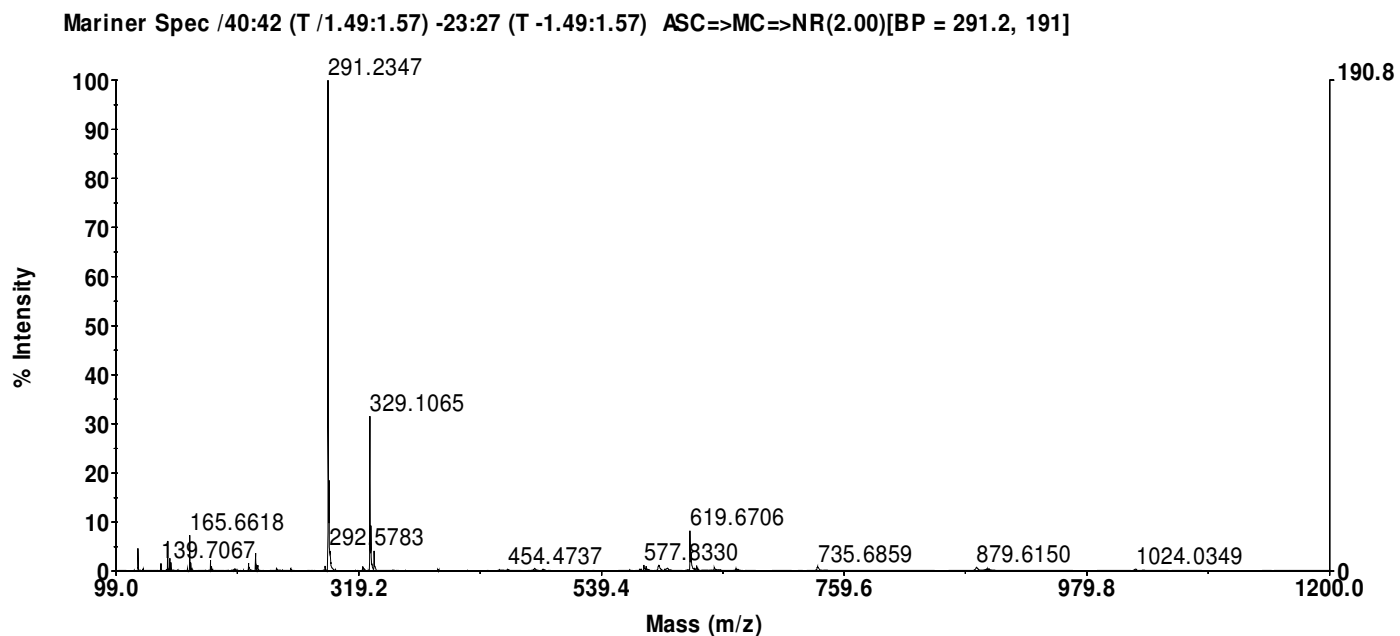


Figure 28. m/z spectral lines present in peak T1.5 for residue from ethanol extraction.

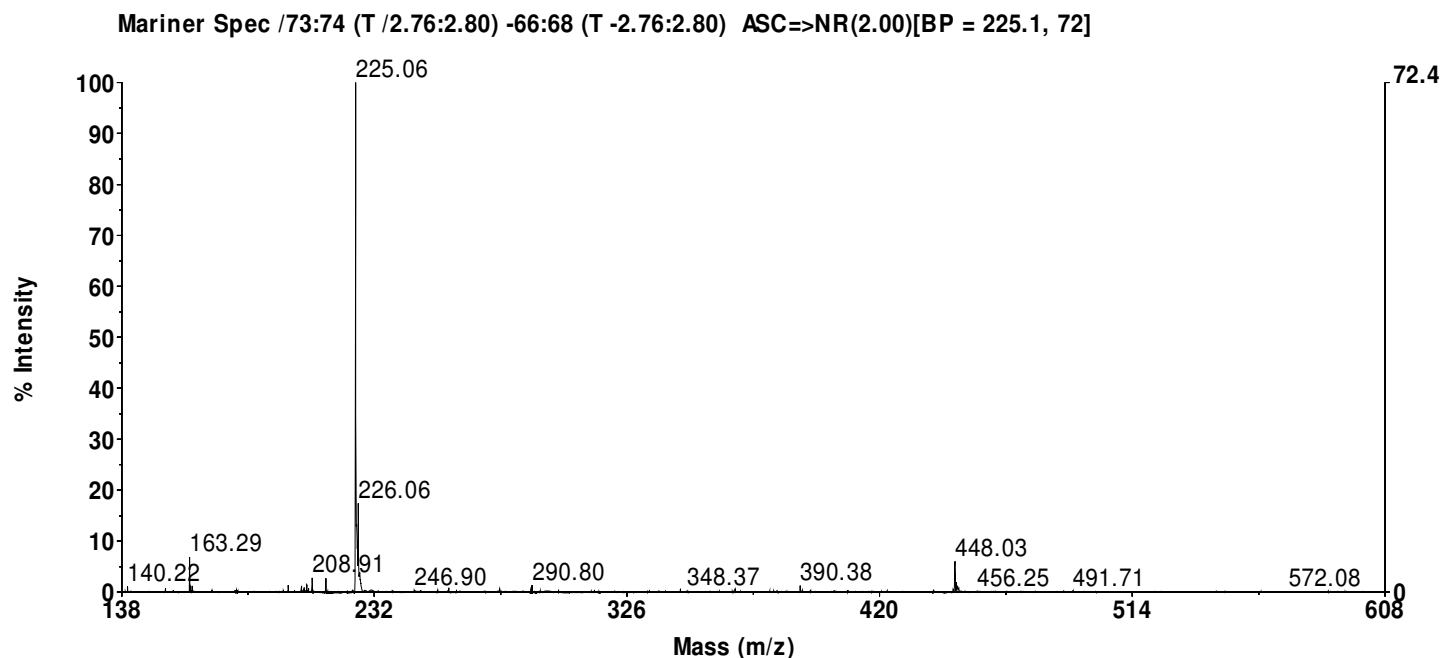


Figure 29. m/z spectral lines present in peak T2.8 for residue from ethanol extraction.

The HMBC spectra (Plate 5) show the long range coupling between the ^1H -atoms and the C-atoms. The ^1H -atom at 4.57 ppm was coupled to the C-atoms at 28.56, 68.81, 115.34, 116.23, 132.20 and 156.91 ppm. The ^1H -atoms at 5.86 and 5.94 ppm were coupled to C-atoms at 100.94, 95.60, 96.41 and 157 ppm while the double

doublet ^1H -atom at 6.72 ppm was coupled to 115.34, 116.23 and 146 ppm. Its ortho-coupled neighbor at 6.77 ppm had similar C-atom coupling in addition to the C-atom at 132.20 ppm while the meta-coupled neighbor at 6.84 ppm had similar C-atom coupling in addition to the C-atom at 120.16 ppm.

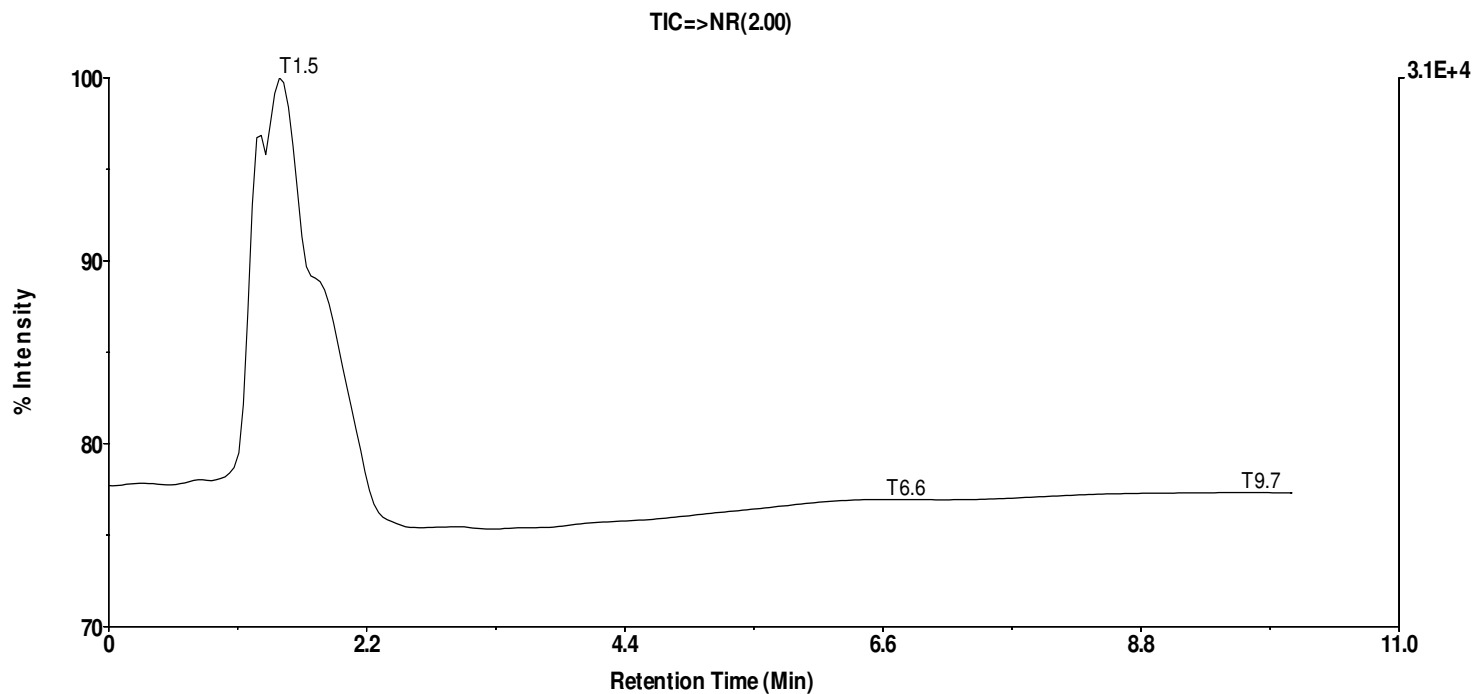


Figure 30. LCMS spectrum for aqueous extract showing broad peak at T1.5.

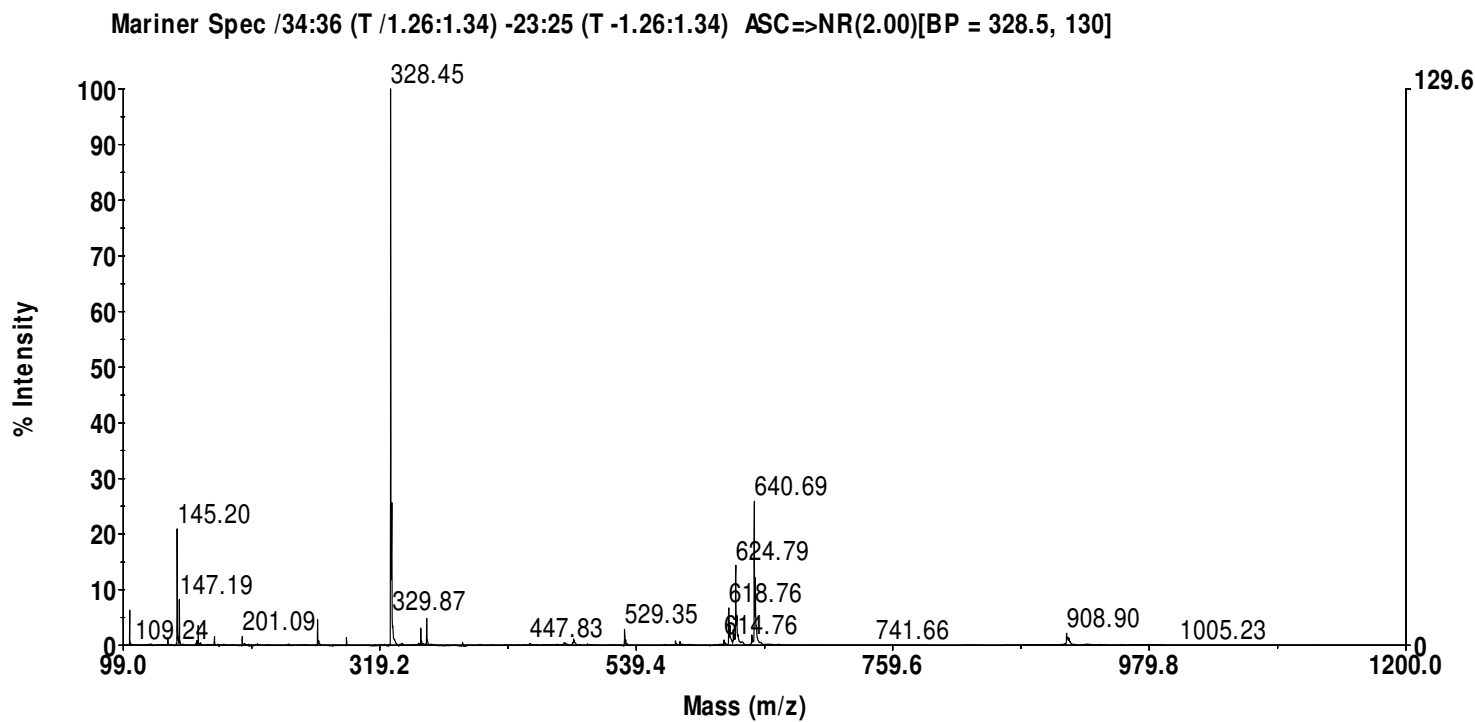


Figure 31. m/z spectral lines present in peak T1.3 for aqueous extract.

From the NMR analyses, the major bioactive compound in the purified sample was identified as (+)-catechin (Figure 38).

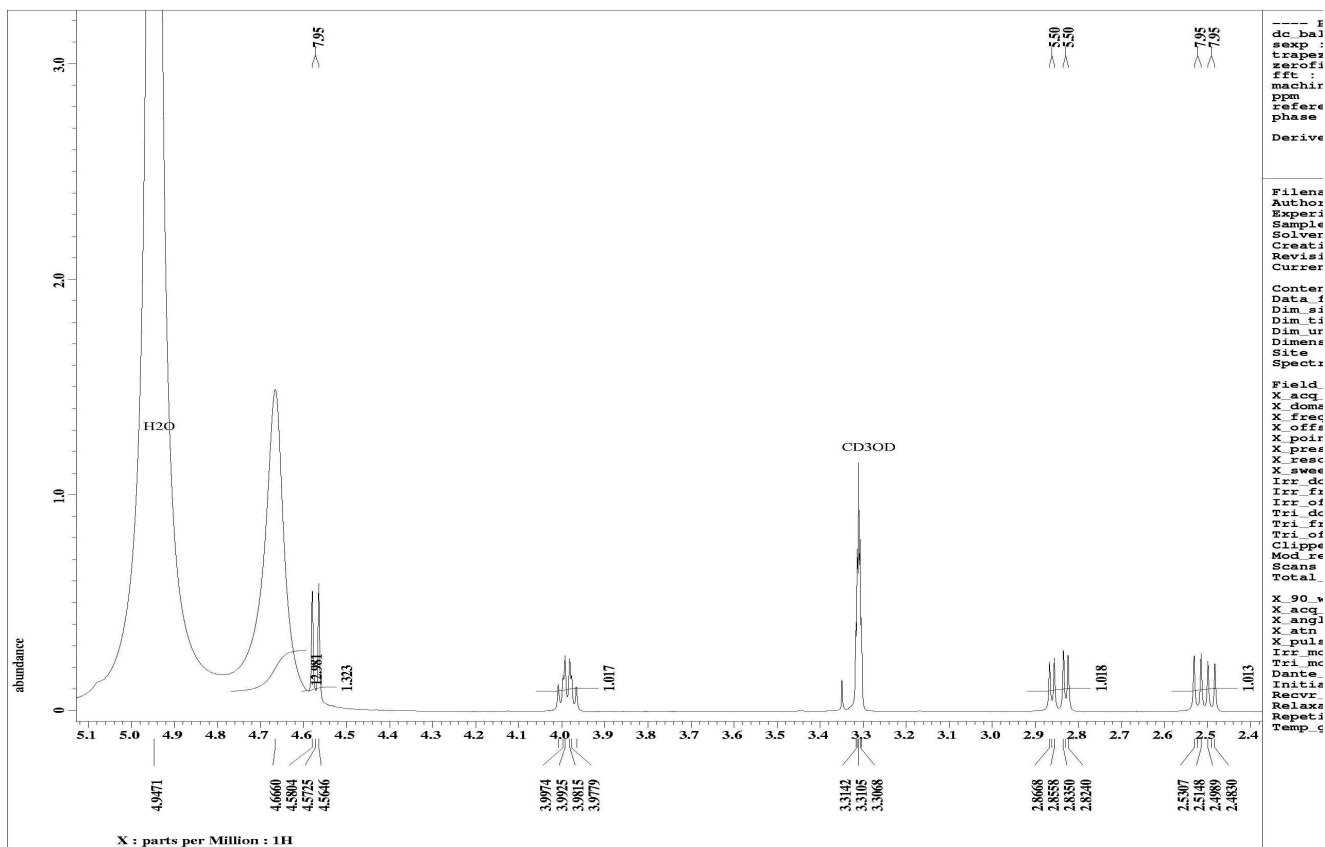


Plate 1. ¹H NMR of Purified Ethyl acetate extract of Gambier using CD₃OD at 500 MHz showing chemical shifts of 2.4ppm to 5.1 ppm.

Mariner Spec /38:40 (T /1.42:1.49) -23:25 (T -1.42:1.49) ASC=>MC=>NR(2.00)[BP = 329.1, 91]

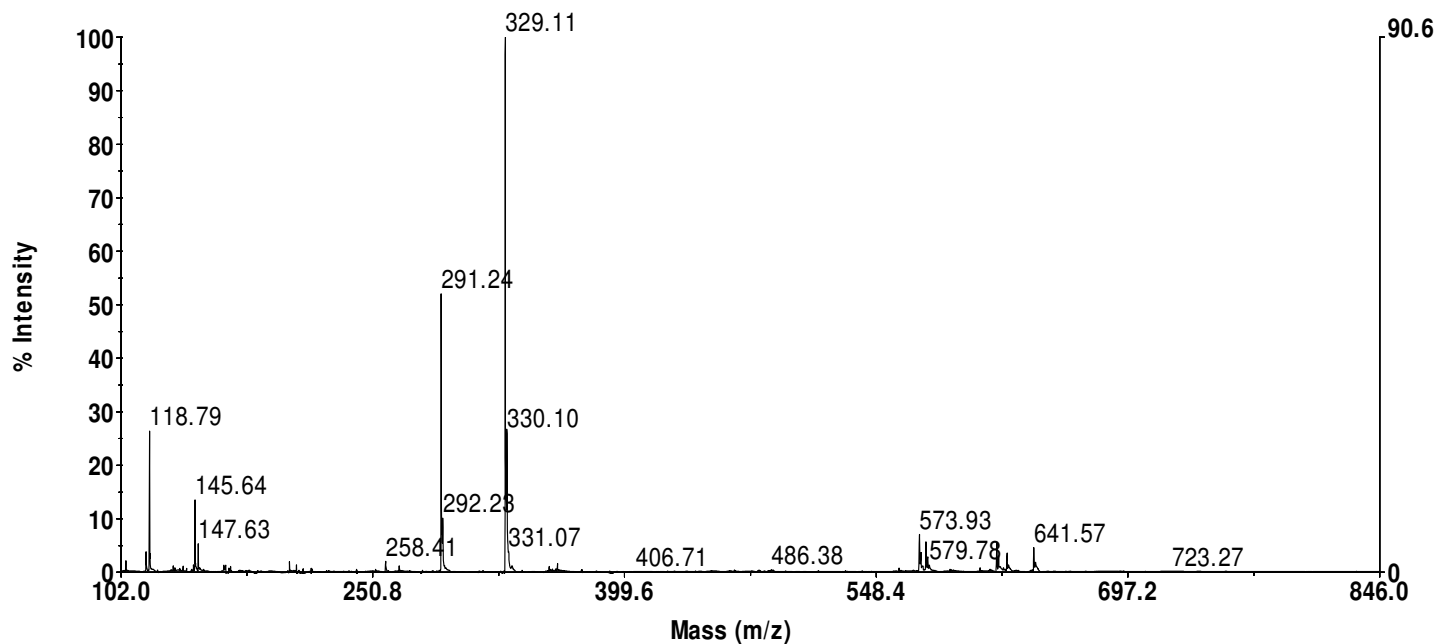


Figure 32. m/z spectral lines present in peak T1.5 for aqueous extract.

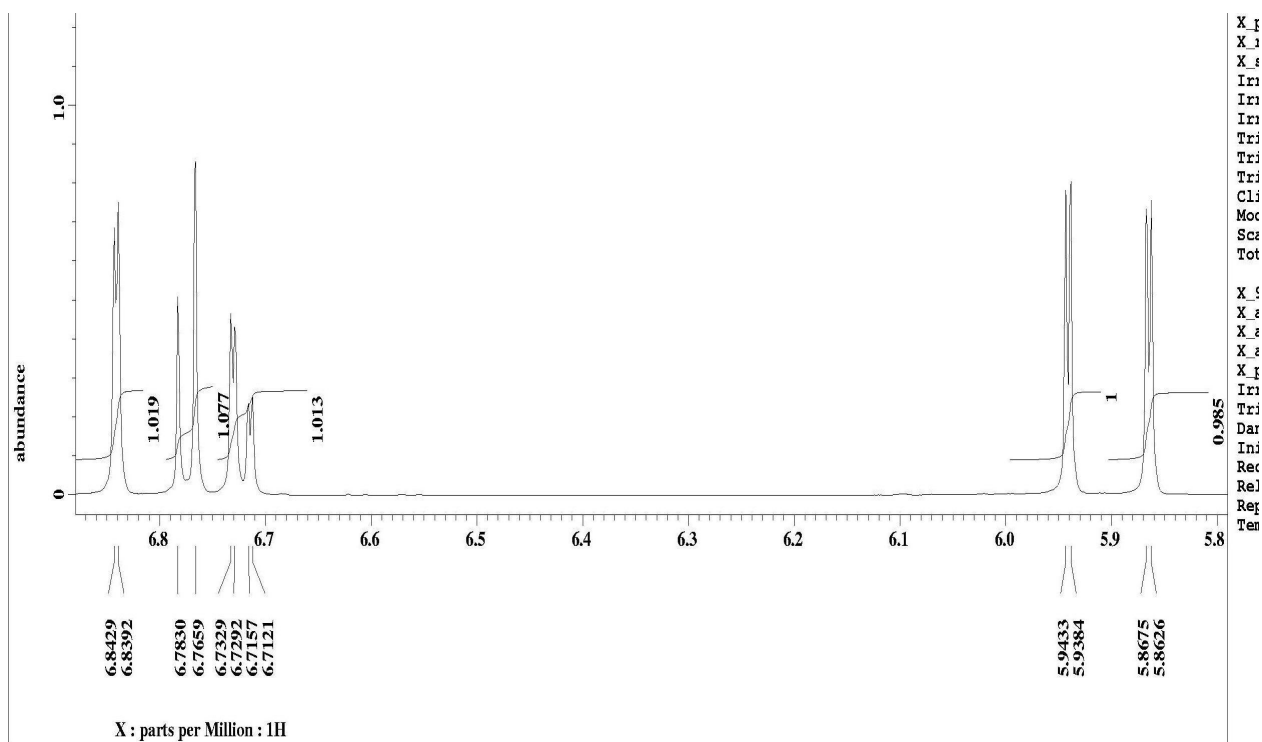


Plate 2. ^1H NMR of purified ethyl acetate extract of Gambier using CD_3OD at 500 MHz showing chemical shifts of 5.8ppm to 7.0 ppm.

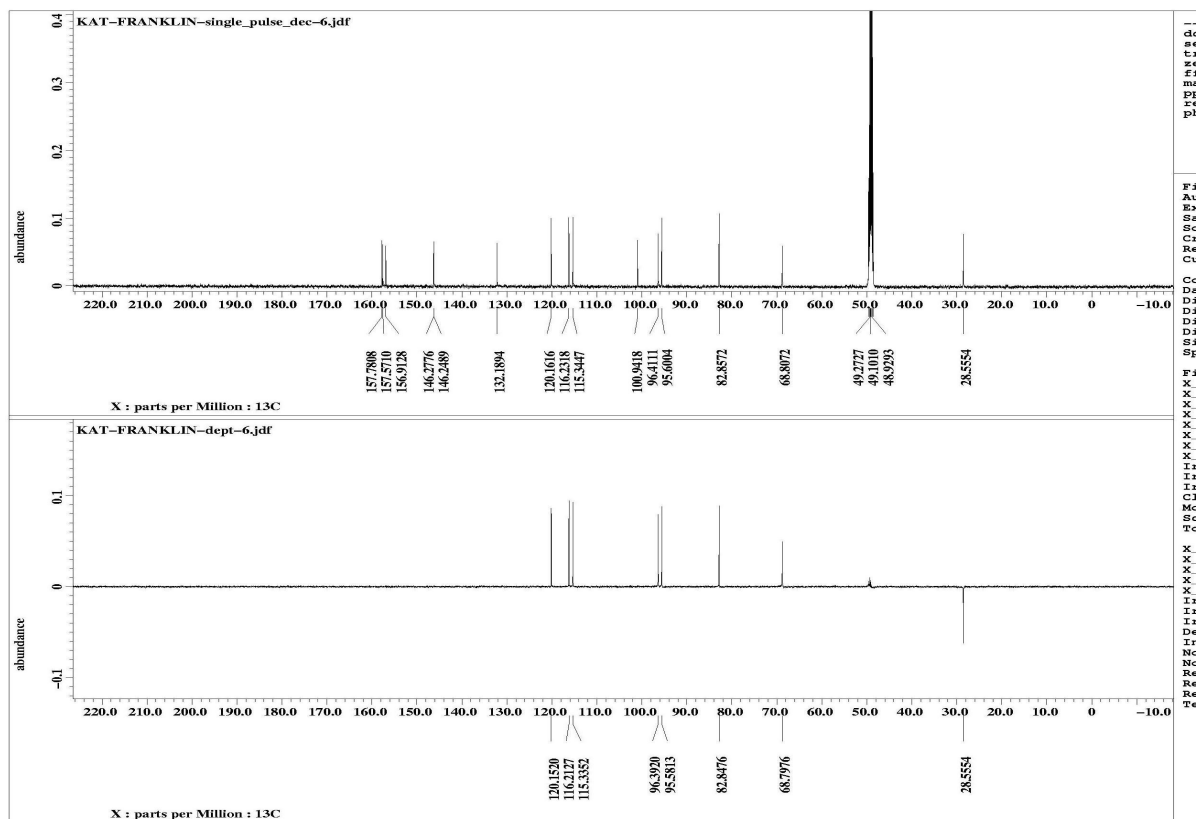


Plate 3. ^{13}C NMR and dept of purified ethyl acetate extract of gambier using CD_3OD at 500 MHz.

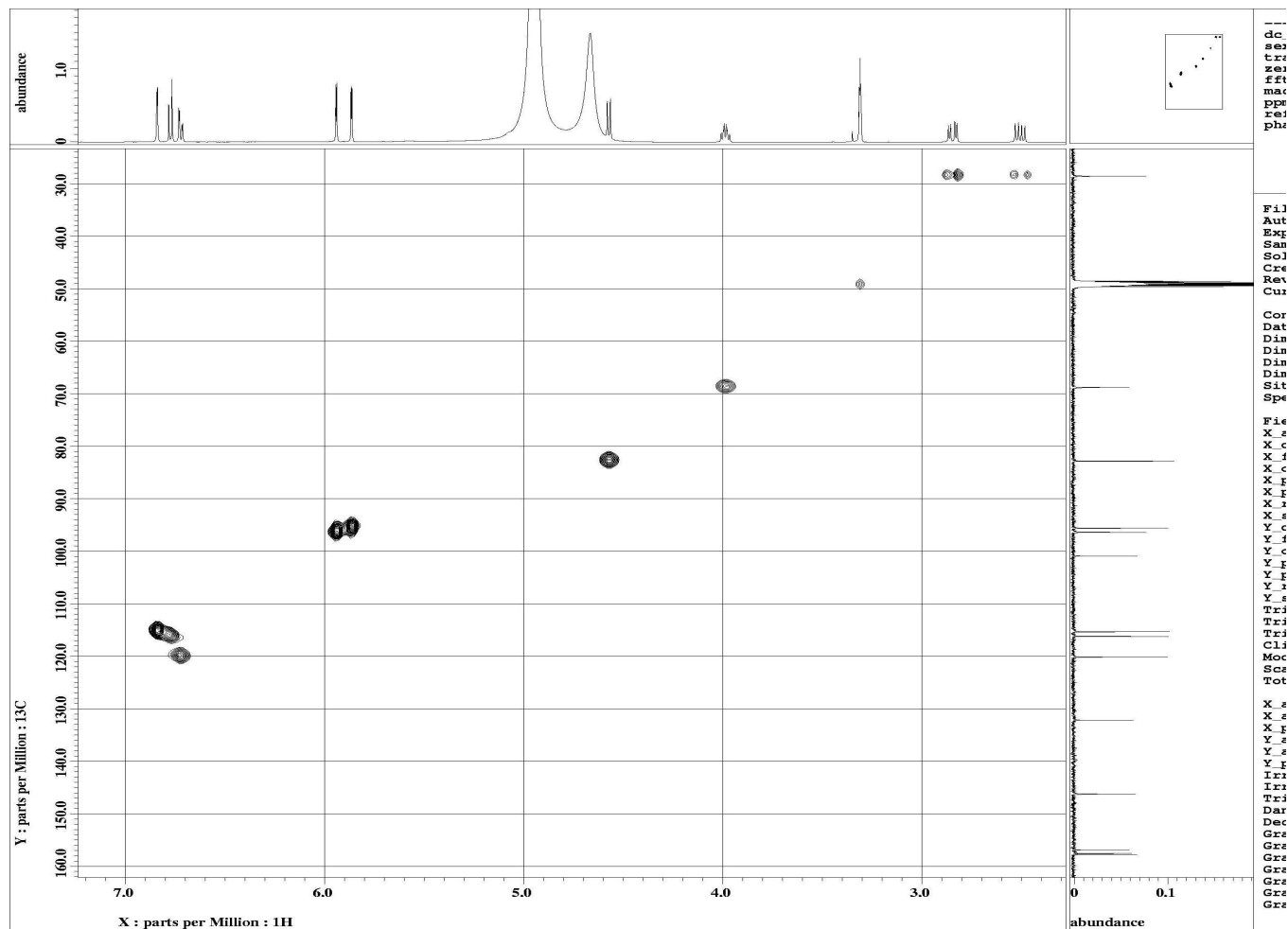


Plate 4. HMQC NMR of purified ethyl acetate extract of Gambier using CD_3OD at 500 MHz.

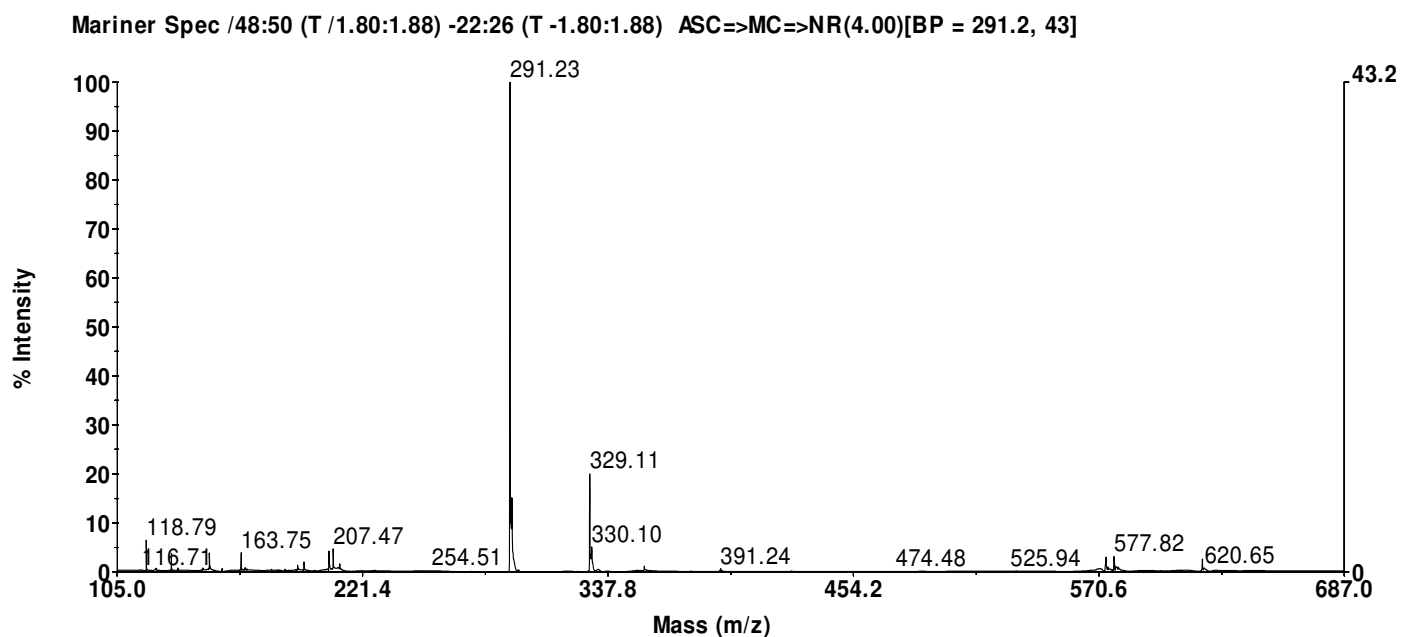


Figure 33. m/z spectral lines present in peak T1.9 for aqueous extract.

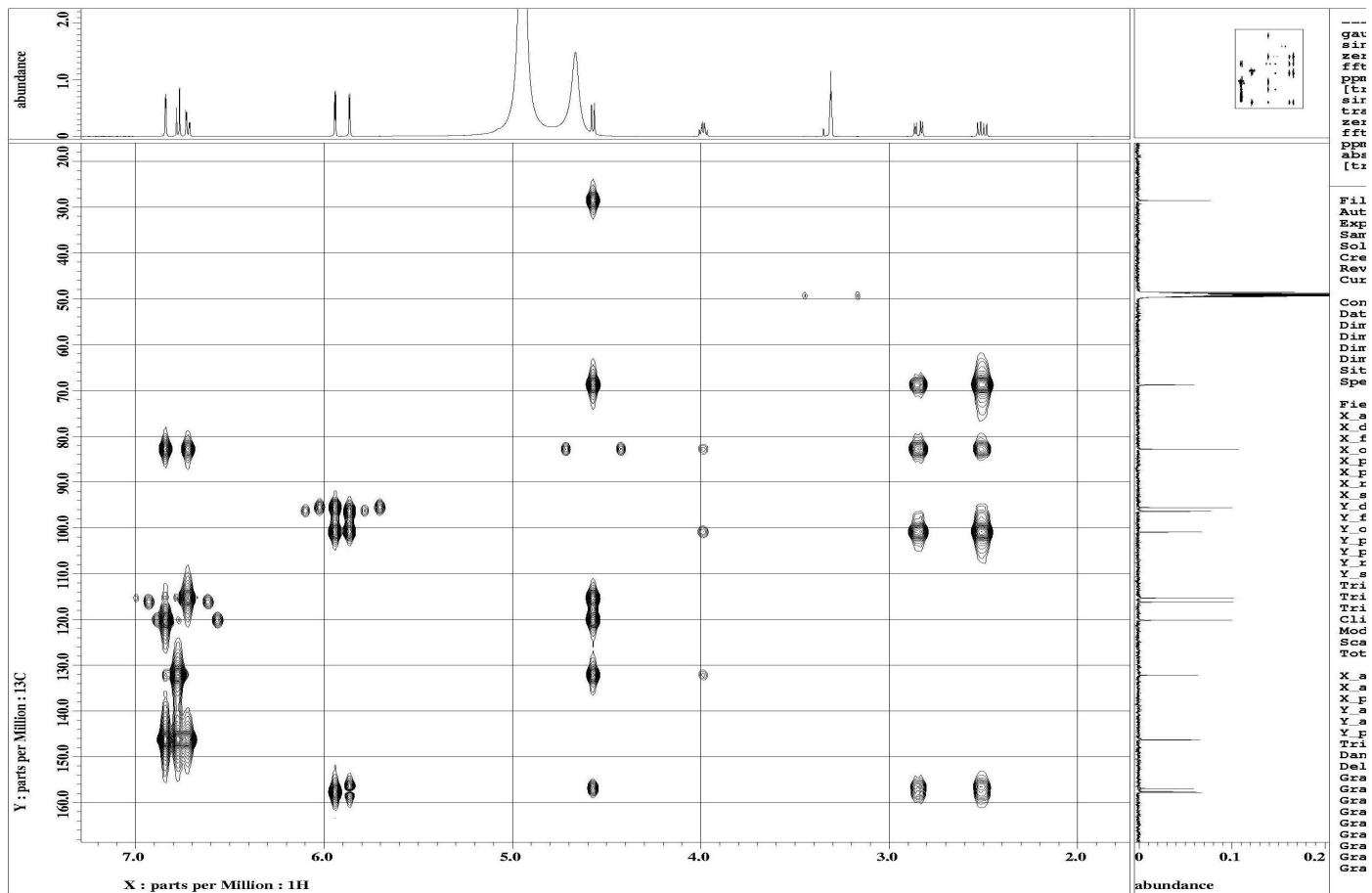


Plate 5. HMBC NMR of purified ethyl acetate extract of gambier using CD3OD at 500 MHz.

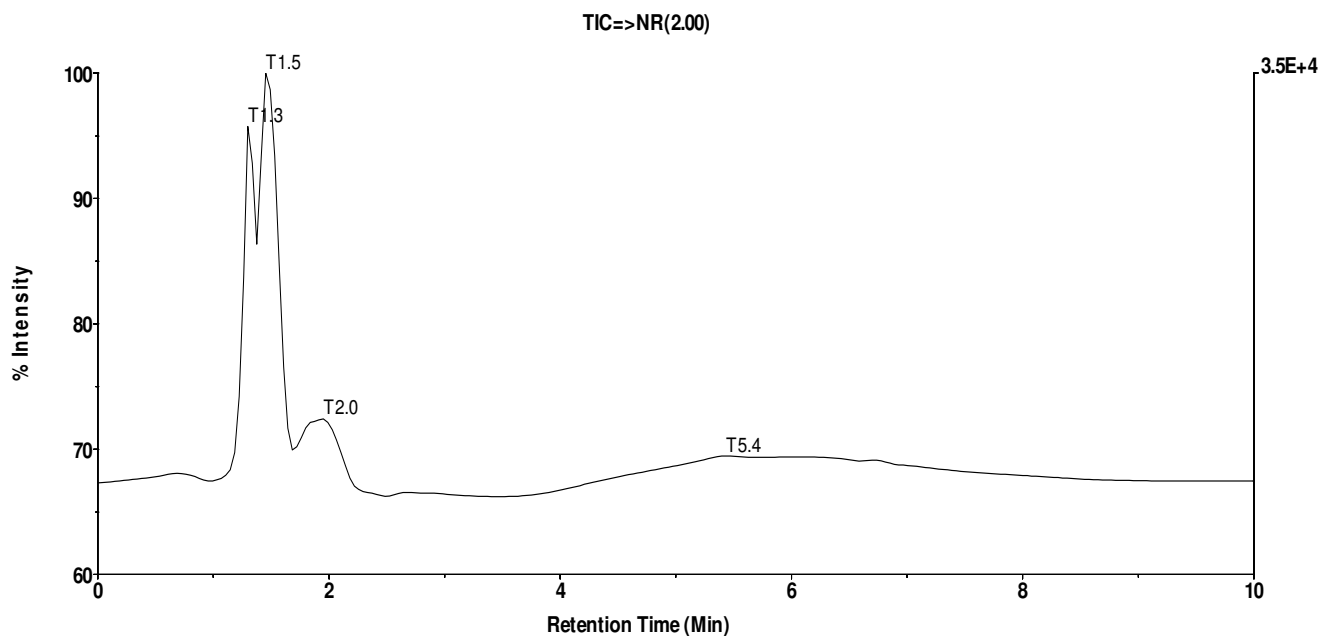


Figure 34. LCMS spectrum for purified extract from ethyl acetate fraction showing three peaks at T1.3, T1.5 and T2.0.

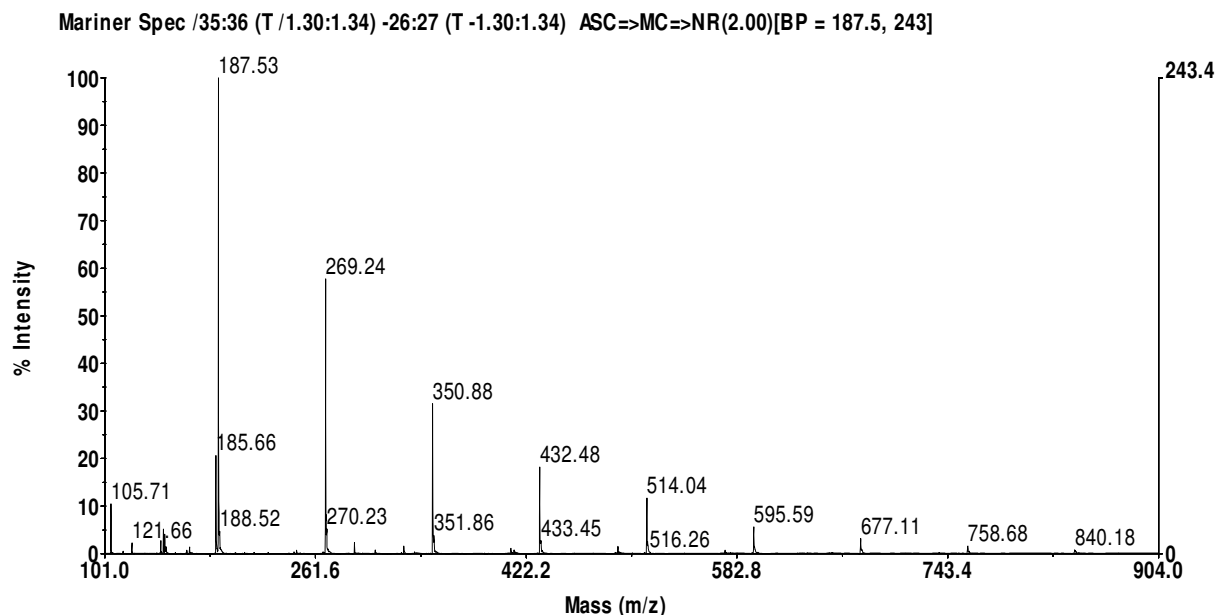


Figure 35. m/z spectral lines present in peak T1.3 for purified extract from ethyl acetate fraction.

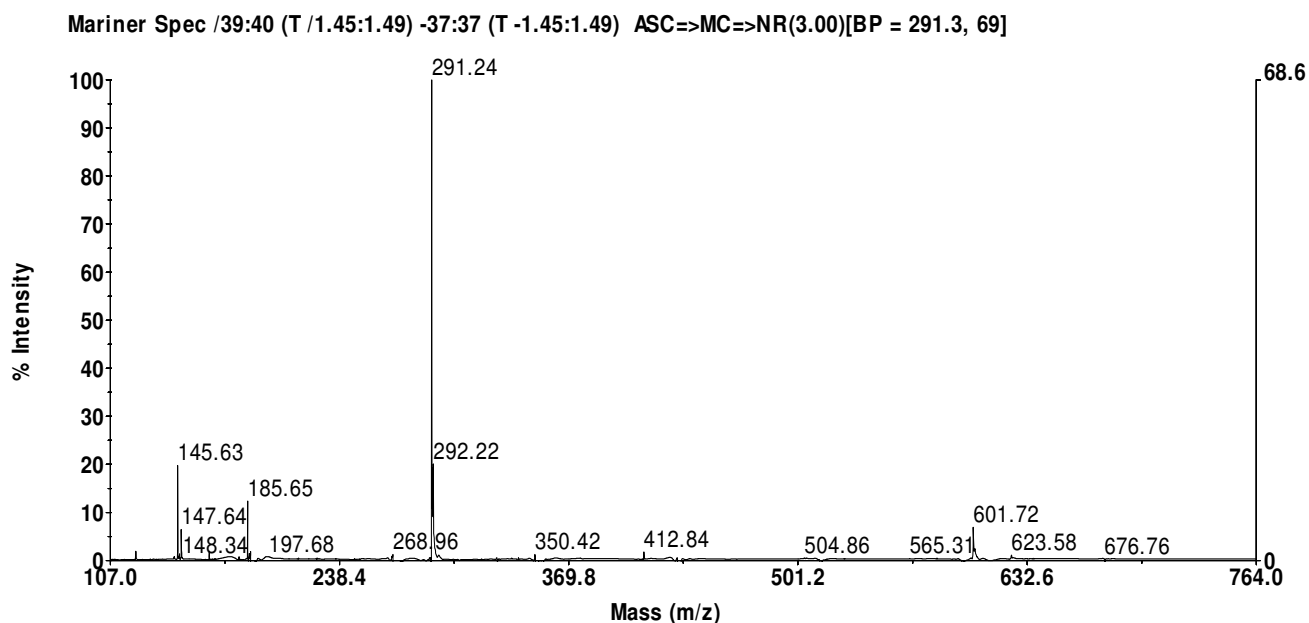


Figure 36. m/z spectral lines present in peak T1.5 for the purified extract.

Conclusion

From the study it is concluded that ethanolic and ethyl acetate extracts of commercial gambier, as well as their residues on extraction have high ability to scavenge reactive free radicals such as is produced by DPPH and hence, have high antioxidant activity *in vitro*. For optimal

in vitro free radical scavenging using organic extracts of commercial gambier, 30 µg/ml is adequate. The study has shown that the ethanolic extract of gambier and aqueous residue after ethyl acetate extraction, have moderate *in vitro* α-glucosidase inhibitory activity. Catechin was identified and confirmed as the major bioactive compound in gambier.

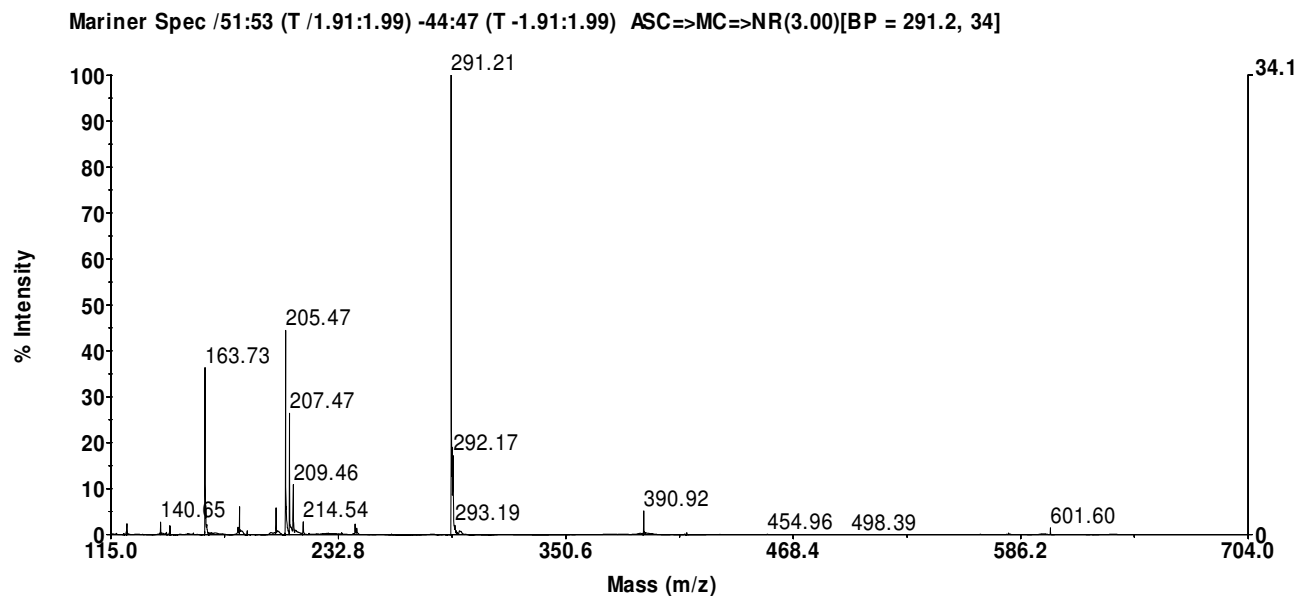


Figure 37. m/z spectral lines present in peak T2.0 for the purified extract.

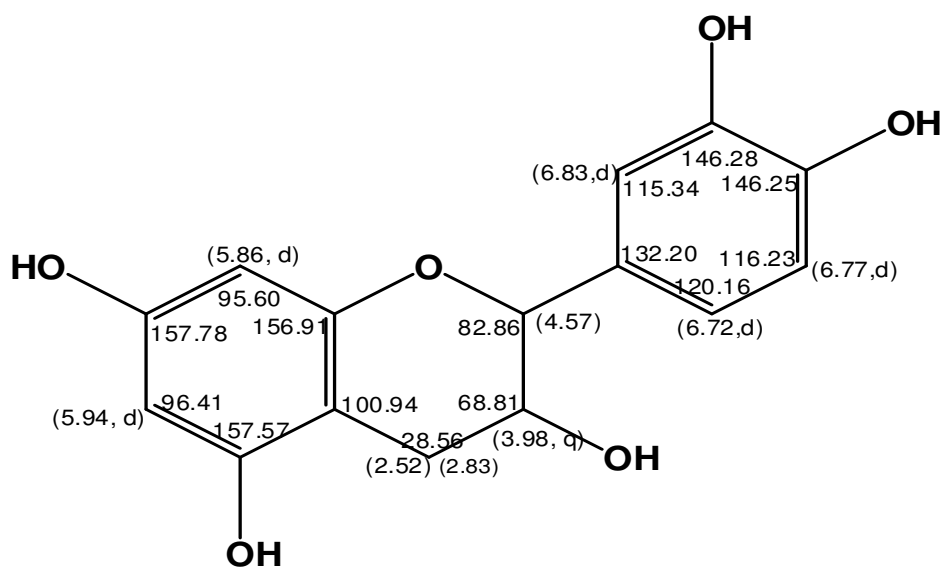


Figure 38. Structure of catechin [2-(3,4-dihydroxyphenyl)chroma-3,5,7-triol] showing ^1H and ^{13}C chemical shifts as elucidated using NMR, 500MHz.
Exact mass: 290.08, Molecular weight: 290.27. m/z: 290.08(100.0%), 3291.08(16.5%), 292.09(1.3%), 292.08(1.2%)

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