

Full Length Research Paper

Flavonoids from the rhizomes of *Elephantorrhiza elephantina* and *Pentanisia prunelloides*

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This paper reports on the isolation of flavonoid compounds from two medicinal plant species that are widely used in Southern Africa, *Elephantorrhiza elephantina* and *Pentanisia prunelloides* which belong to families Fabaceae and Rubiaceae, respectively. The identification and comparative analysis of flavonoids in *E. elephantina* and *P. prunelloides* as well as the structural elucidation of flavonoids were performed using liquid-chromatography electron-spray ionization mass spectroscopy (LC-ESI-MS) and spectroscopic techniques such as Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). Among the flavonoids identified include epigallocatechin gallate (EGCg), epicatechin (EC), quercetin, epicatechin gallate (ECg), kaempferol and their polymeric derivatives. The analysis revealed that, *P. prunelloides* contains more flavonoids (0.81%) than *E. elephantina* (0.77%). 2,2-Diphenylpicrylhydrazyl (DPPH) radical antioxidant tests confirmed the presence of these compounds.

Key words: Flavonoids, Fabaceae, Rubiaceae, liquid-chromatography electron-spray ionization mass spectroscopy (LC-ESI-MS).

INTRODUCTION

Flavonoids are one of the most important groups of bioactive compounds in plants, which exist in the free aglycones and glycoside forms showing a diverse structure and a broad range of biological activities (Tapiero et al., 2002; Harborne and Williams, 2002; Hung et al., 2006; Yonathan et al., 2006). They include several classes of compounds with similar structure having a C₆-C₃-C₆ flavone skeleton which differ in the degree of

unsaturation and oxidation of their three carbon segments. Within different subclasses of these species further differentiation occurs which is based on the number and nature of substituent groups attached on the ring. Normally glycosylated flavonoids occur mostly in the O-glycosidic forms with a number of saccharides such as glucose, galactose, rhamnose, arabinose, xylose and rutinose but also found as C-glycosides. Mass spectra of

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glycosylated flavonoids have typical patterns, which depend mainly on either the number or nature of the bound saccharides and their C- or O-glycosidic linkages (Ferrerres et al., 2004). The loss of fragments with well-defined masses from the pseudomolecular ion can provide precise information about the linked saccharides. The C-C bond of C-glycosyl flavonoids is known to be resistant to fragmentation and in the C-glycosides the fragmentation of the sugar unit is the one that is usually observed (Stoick, 2002). On the other hand, fragmentation pathway of O-glycosylated flavonoids has been reported to start with the cleavage of the glycosidic bonds and elimination of sugar moieties with charge retention on the aglycone (Cuyekens and Claeys, 2004).

Studies have shown extracts from *Elephantorrhiza elephantina* contain 5.8 to 22.3% tannins (Watt et al., 1962) which explains the redness of the root. Moreover, several phenolic compounds, including flavonoids such as kaempferol, dihydroxykaempferol, ethyl β -D-galactopyranoside, quercetin-3-O- β -D-glucoside, trans-3-O-galloyl-3',5',7-pentahydroxyflavan and taxifolin-3'-O-glucoside (Polombo, 2006) as well as (+)-catechin, ethyl gallate, methyl gallate and gallic acid form part of the chemical composition of this plant (Polombo, 2006). Other compounds identified in the roots of this plant include sugars (Mthembu, 2007) and β -sitosterol (Polombo, 2006). The roots of *Pentanisia prunelloides* are reported to contain high levels of alcohol precipitable solids (0.7 to 7.0%, and these include polysaccharides and glycoproteins), amino acids (α -aminobutyric acid, valine, allo-isoleucine, serine, aspartic acid, asparagine and alanine, of which the latter is most abundant) as well as several unidentified terpenes of medium and low polarity (Ndlovu, 2007). Palmitic acid was further identified as a major non-polar compound in *P. prunelloides* by Yff et al. (2002). Recently, some non-polar fatty acids have been reported in *E. elephantina* (Msimanga et al., 2013).

In this work, the identification and structural elucidation of epigallocatechin gallate, epicatechin, epicatechin gallate and their dimeric derivatives from the rhizomes of the two plant species were reported. The techniques employed were mainly liquid-chromatography-electrospray ionisation mass spectrometry (LC-ESI-MS), nuclear magnetic resonance (NMR) and infra-red (IR). To the best of our knowledge, this is the first report of the detection and isolation of epigallocatechin gallate (EGCg) and its polymeric derivatives as well as epicatechin gallate (ECg), epicatechin gallate (EGC), kaempferol taxifolin-O-glucuronic acid from the rhizomes of *E. elephantina* and *P. prunelloides*.

MATERIALS AND METHODS

Apparatus and reagents

The standards EGCg (AR grade), ECg (AR grade), EC (AR grade),

acetonitrile (AR grade), butanol and vanillin were purchased from Sigma-Aldrich (St Louis, Mo, USA). Methanol (high performance liquid chromatography (HPLC) grade) was purchased from Merck, (Darmstadt, Germany). Ultra-pure water with resistivity > 18.4 M Ω /cm was used for all aqueous preparations. Silica gel type 60 (Merck, 230-400 mesh) was used for column chromatography. Thin layer chromatography (TLC) was carried on a 0.25 mm thick silica gel plate (Merck silica gel 60 GF₂₅₄) and elution was carried out using a solvent system comprising of butanol/acetone/water (4:1:2, v/v). Compounds were detected either under ultraviolet (UV) light (at 254 or by spraying with vanillin/sulphuric acid (1:1, v/v).

Plant and collection

Fresh plant rhizomes of *E. elephantina* and *P. prunelloides* were collected in June 2010 from seven different Southern African regions in Swaziland, South Africa and Zimbabwe and identified by Dr Anna Moteete, University of Johannesburg. Voucher specimens (SJM-0, SJM-01 to SJM-07) were deposited in JRAU Herbarium, Department of Botany and Plant Biotechnology (Kingsway Campus, University of Johannesburg). Fresh plant rhizomes were washed with water, dried marcerated and kept in the fumehood at room temperature. The dried plant materials were then ground into fine powders, extracted using appropriate solvent and water, evaporated under reduced pressure and then stored in sample bottles at room temperature.

Quantitative determination of catechins

Volumes of 20 ml acetone, 20 ml of 25% hydrochloric acid and 1 ml of 0.5% hexamethylenetetraamine were added to 25 ml extract (corresponding to 2 g of plant material) then heated under reflux at 56°C for 30 min. The extract was filtered then re-extracted twice with 20 ml acetone for 10 min. The reconstituted extracts were cooled then made up to 100 ml with acetone in a volumetric flask. This basic solution (BSS) was mixed with 50 ml of water then extracted with ethyl acetate (first with 15 ml then 3 times with 10 ml). The resultant reconstituted extract was rinsed two times with water then filtered and made up to 50 ml with ethyl acetate. This solution was labeled S1. To 10 ml of S1, 0.5 ml of 0.5% solution of sodium citrate and 2 ml of aluminum (prepared by dissolving 2 g of aluminum chloride in 100 ml of 5% acetic acid in methanol were added and then made up to 25 ml with 5% methanolic solution of acetic acid. This sample solution was labeled (SS). The same procedure was performed with the blank sample solution without aluminum chloride. After 45 min, the solutions were filtered and absorbance measured at 446.5 nm.

The content of total flavonoids was evaluated upon 4 independent analyses. The yield was calculated using the formulae below (Gayatri et al., 2014).

$$g (\%) = A \times 0.772/b \quad (1)$$

where A is the absorbance and b represents the mass of dry herbal material extracted in grams.

This procedure was carried out for the two samples; *E. elephantina* from Zvamagwiro (Zimbabwe) and *P. prunelloides* from Orange Farm South Africa). The absorbance and the respective percentage yields calculated from Equation 1 are shown in Table 1.

Thin layer chromatography of extracts

Portions of both *E. elephantina* and *P. prunelloides* were spotted on separate TLC plates and eluted with butanol/acetone/water (4:1:2).

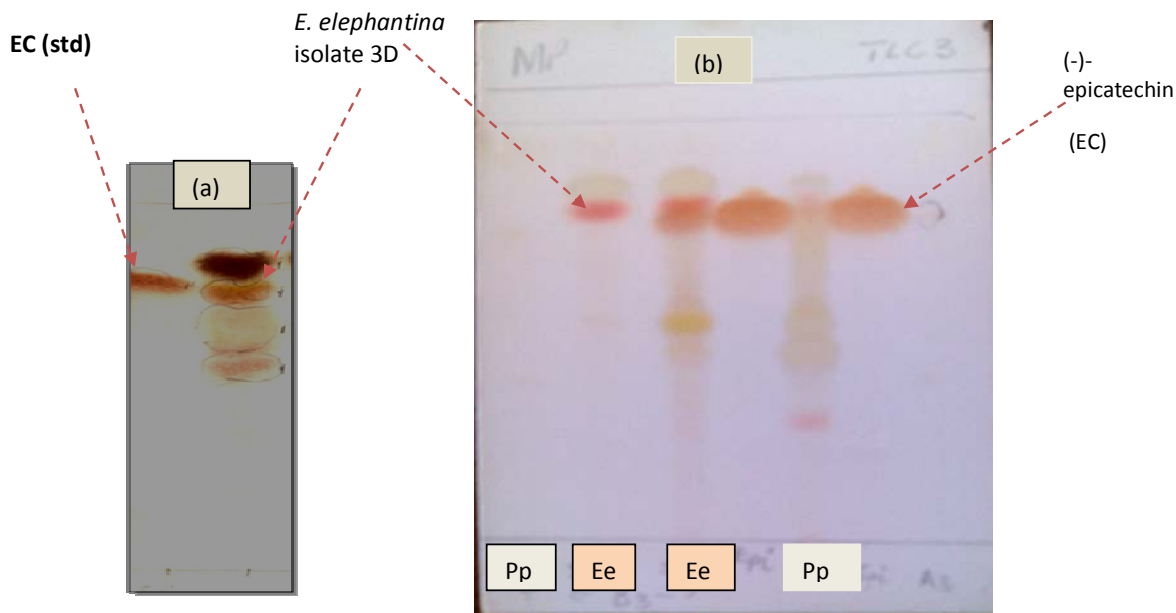


Figure 1. TLC of (-)-epicatechin isolates from *P. prunelloides* and *E. elephantina* against standard (-)-epicatechin.

The developed TLC plates were sprayed with vanillin sulphuric acid and then heated in the oven at 110°C for 5 min. Portions of both species were subjected to column chromatography using the same eluent applied for TLC. The resultant fractions of the two species were spotted on the same TLC plate against standard epicatechin for flavonoids (Figure 1). This was done for comparison purposes. For the preparation, TLC was then used to isolate the major compounds by TLC (MeOH/H₂O/Acetone/Ethyl acetate/Chloroform) 10:8:30:40:12 as eluent. Standard EGCg and epicatechin were also spotted against the isolates for comparison purposes (Figure 1).

Column chromatography and spectroscopy performance of extracts

The dried residue (10.0 g) was suspended in a minimum amount of methanol, adsorbed on silica gel (8 g), dried, and subjected to column chromatography (CC), using a 38 × 4.5 cm glass column filled with Merck silica gel 60 F₂₅₄ in chloroform to a level about 8 cm from the top. The adsorbed extract was then charged on the column. Fractionation was done by successive applications of chloroform, chloroform/methanol (2:1), and methanol. One of the fractions was non-polar (NP), another was of medium polarity (MP) and the last was a polar (HP) fraction. Three major fractions were collected and the solvent was evaporated in the fume hood overnight. The fraction with medium polarity and the polar were used to isolate two of the major compounds.

FT-IR spectroscopic analysis of extracts

A little powder of plant specimen was mixed with KBr salt (1:1, w/w) using a mortar and pestle, and compressed into thin pellets. Infrared spectra were recorded between 4000 and 500 cm⁻¹ as KBr pellets on the TL 8000 Balanced Flow FT-IR EGA (Perkin Elmer Spectrum series) instrument at the University of Johannesburg, Department of Applied Chemistry, Doornfontein Campus. Infrared

absorption spectra were recorded and are shown in Figures 2 and 3.

LC-ESI-MS spectroscopy

The LC-MS instrument used for the analysis was Waters E2695, 3100 mass selective detector equipped with the electron spray ionization source (ESI) and a single quadrupole analyzer. The quadrupole temperature of the MS was set at 120°C, while the dissolution temperature was 350°C, the capillary voltage was at 3500 V and the spectra were acquired in a full scan mode with polarity switching (+ve and -ve mode). The temperature of the drying gas, nitrogen (N₂) was 300°C and the flow rate of the nebulising gas, nitrogen (N₂) was 450 L/h. The eluent was system comprised of acidified aqueous solvent (FA), acetonitrile and methanol. Acidification provides better retention and separation in the C18-RP columns. The Vensil HPLC Column was used (Vensil × BP C₁₈ (2) 5 μm100Å, 4.6×250 mm, Cat. No V×952505 S/N V95105258/00036. The solvent system [A] ACN-H₂O-FA (3:1:1%) and [B] MeOH:H₂O (3:17) were run isocratically at a flow rate of 0.3 μl/min for run period of 20 min.

RESULTS

TLC for the fractions of *E. elephantina* and *P. prunelloides*

The results obtained by thin layer chromatography indicated brownish spots of the flavonoid compounds after spraying with vanillin/sulphuric acid (Figure 1). Apart from the colour of the alleged (-)-epicatechin, the R_f value (about 0.77) of the isolates corresponded with standard (-)-epicatechin suggesting the presence of epicatechin in both *E. elephantina* and *P. prunelloides*.

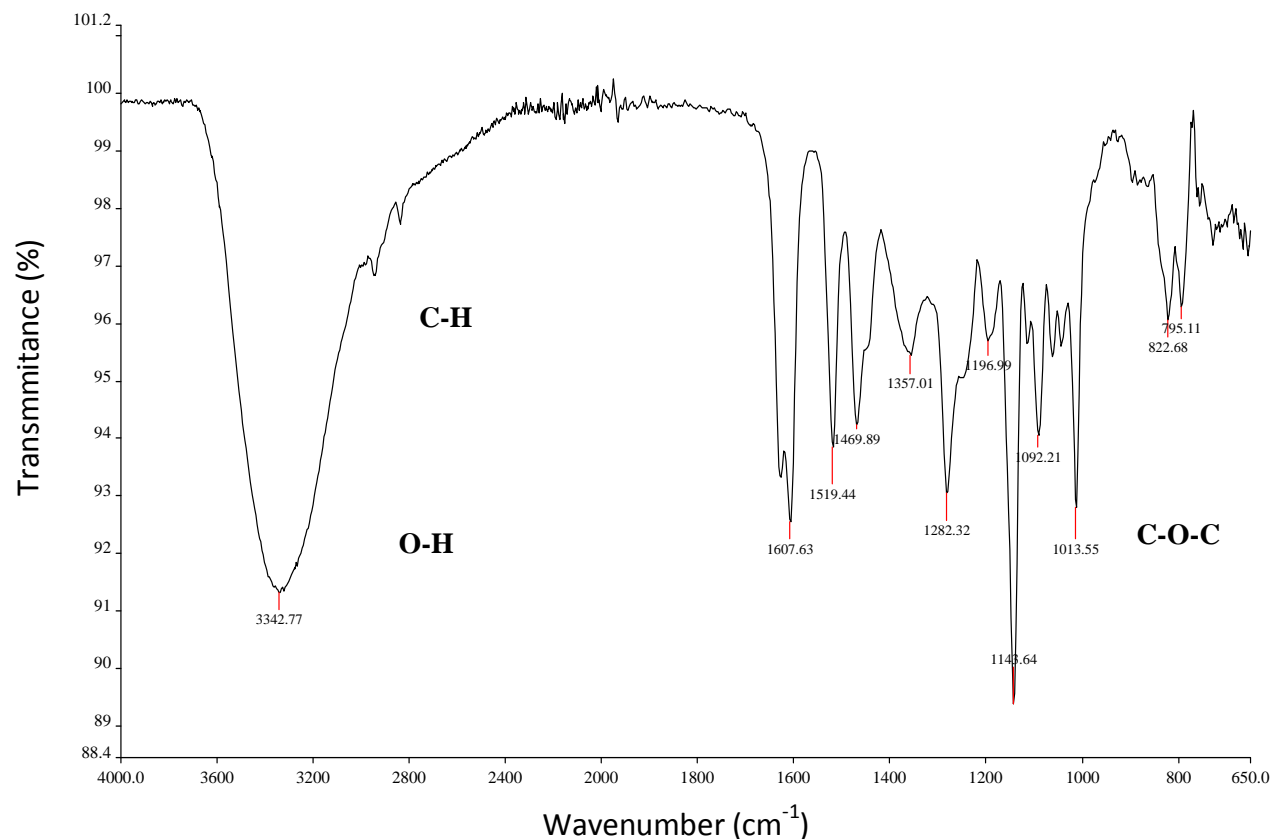


Figure 2. FT-IR spectra of (a) (-)-epicatechin standard.

(Figure 1). The intensity of the colour for the alleged epicatechin fractions was observed to be more pronounced for *E. elephantina* suggesting a greater concentration of this compound in *E. elephantina* compared to *P. prunelloides*. This observation was in agreement with the findings from electrochemical determination of (-)-epicatechin for the two species (Arotiba et al., 2013). CC afforded fractions of flavonoids suspended in methanol for *E. elephantina* (0.77% yield) and *P. prunelloides* (0.81% yield) as shown in Table 1. The samples from CC confirmed the presence of flavonoids that were conspicuous by their yellow colour.

FT-IR spectra of (-)-epicatechin standard and (-)-epicatechin from *E. elephantina*

The phytochemical analyses of *E. elephantina* and *P. prunelloides* gave positive results for flavonoids which was also confirmed by the IR spectra of the standard (-)-epicatechin which was almost identical to that of the isolates obtained from preparatory thin layer chromatography as depicted in the IR spectra (Figures 2 and 3). (-)-Epicatechin showed characteristic infrared absorption bands (Figure 2) of the hydroxyl group (-OH)

ranging from 3332 (extract) to 3343 cm^{-1} (standard epicatechin), C-H ranging from 2900 (standard epicatechin) to 3000 cm^{-1} (extract), C=C ranging from 1607.6 (standard epicatechin) to 1608.1 cm^{-1} (extract), C-O-C ranging from 1013.6-1092.2 (standard epicatechin) to 1012 cm^{-1} -1090.8 cm^{-1} (extract). In all cases, the fingerprint of the isolated sample was almost similar to that of the standard clearly confirming the identity of the isolated compound to be (-)-epicatechin.

Quantitative composition of catechins in *E. elephantina* and *P. prunelloides*

Generally, the two species exhibited comparable yields of flavonoids and this also confirms their high antioxidant capacities as demonstrated by the 2,2-diphenylpicrylhydrazyl (DPPH) radical antioxidant tests as well as the highly positive qualitative tests reported (Mpofu et al., 2014). NMR spectra for (-)-epicatechin isolated from *P. prunelloides*.

Table 2 shows that there were 15 carbon atoms which were bonded to nine hydrogens as suggested by the ^1H and ^{13}C NMR together with the heteronuclear single quantum coherence (HSQC) and distortionless

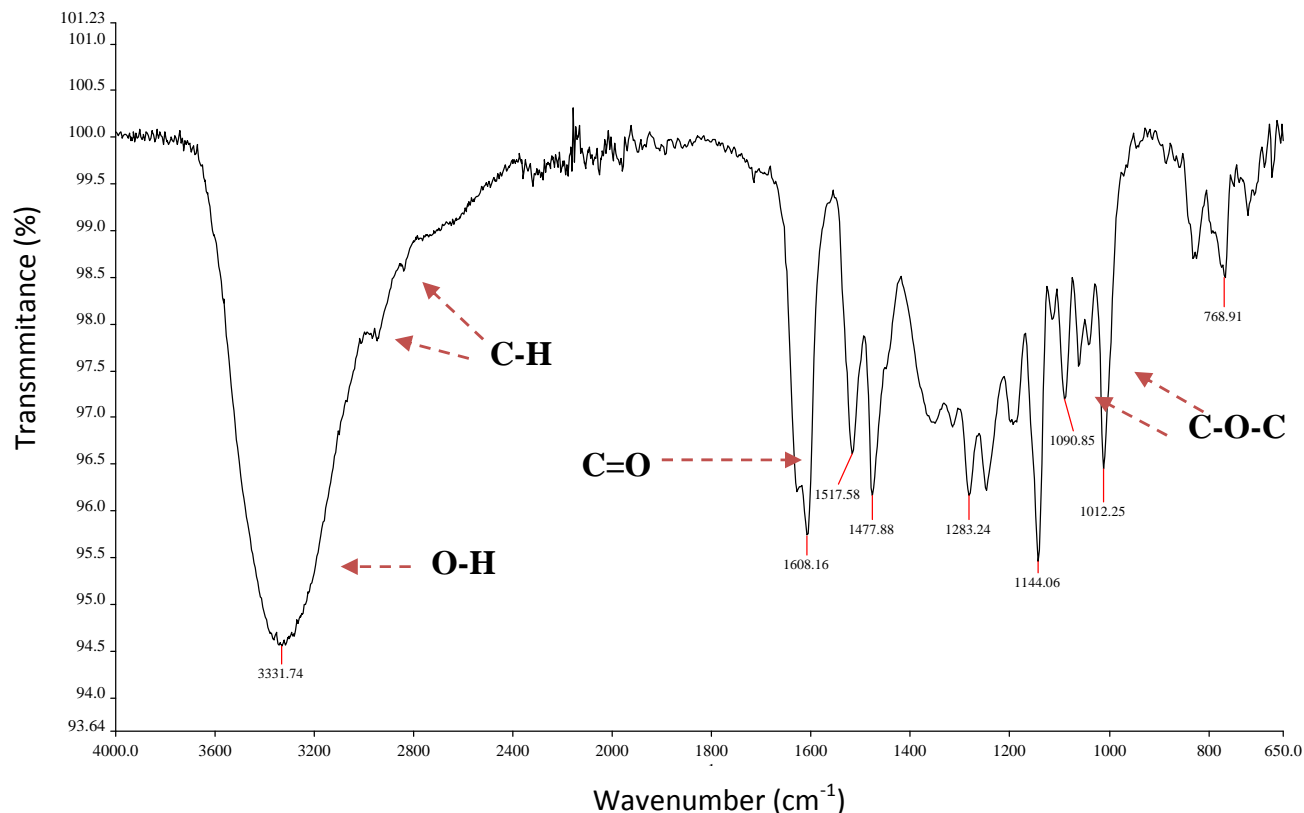


Figure 3. (-)-epicatechin isolated from *E. elephantina*.

enhancement by polarization transfer (DEPT). One of the carbons was a methylene ($-\text{CH}_2$) group, seven were methyne ($-\text{CH}$) groups while the other eight were quaternary carbons. Of the seven quaternary carbons signals, none could be assigned to be carbonyls ($\text{C}=\text{O}$). These types of carbons are usually seen above 200 ppm, but carbons from esters and carboxylic acids are usually seen upfield at approximately 170 ppm. It is possible that the carbonyl signals were just weak under these experimental conditions. It is true that $\text{C}=\text{O}$ carbons show weak peaks but they are usually there. Their complete absence can be due to conjugation with another double bond or bonding electron rich atoms such as nitrogen in amides for example. Other types of carbons that are found in this region are those with a double bond and hydroxyl groups attached to them. They were then assigned as shown in Figure 4 using correlation spectroscopy (COSY) and heteronuclear multiple-bond correlation spectroscopy (HMBC). The location of the quaternary carbons was confirmed by their chemical shifts which were consistent with those reported in literature.

The proposed structure of compound B is therefore shown in Figure 4 where the quaternary carbons were assigned to be carbons number 1, 2, 3, 5, 1'', 3'' and 4''

of which some had hydroxyl groups (3,5,3'',4''), with a bridging ether between C1 and C3' and the last a normal aromatic bond (2)

The COSY experiment showed a few correlations as almost half of the carbon atoms did not have any hydrogen atoms. A few links were made from these results where the hydrogens of the middle C3 moiety showed correlation with one another. The connectivities in the aromatic rings were confirmed using mainly HMBC. C-2 showed correlation with C-1' confirming the linkage of the first aromatic ring. The second one was observed in the correlation of carbon 3' with carbons 2'' and 6''. The IR spectrum showed the presence of OH groups (bonded) at 3488 cm^{-1} , confirming the presence of hydroxyl groups in this molecule. There were no $\text{C}=\text{O}$ bands observed in the IR spectrum (usually seen at about 1610 cm^{-1}) thus confirming the absence of this type of bonds in the NMR. The structure obtained from the NMR data has a theoretical molecular weight of 290 g/mole. This was confirmed by the LC-ESI-MS results which also suggested that the compound could dimerise (link two of its molecules) to give double its mass (a fragment found at $m/z = 578.8$) (Figures 5 and 6). This type of compound is a flavanol which has the basic structure as shown in Figure 7. Such compounds have

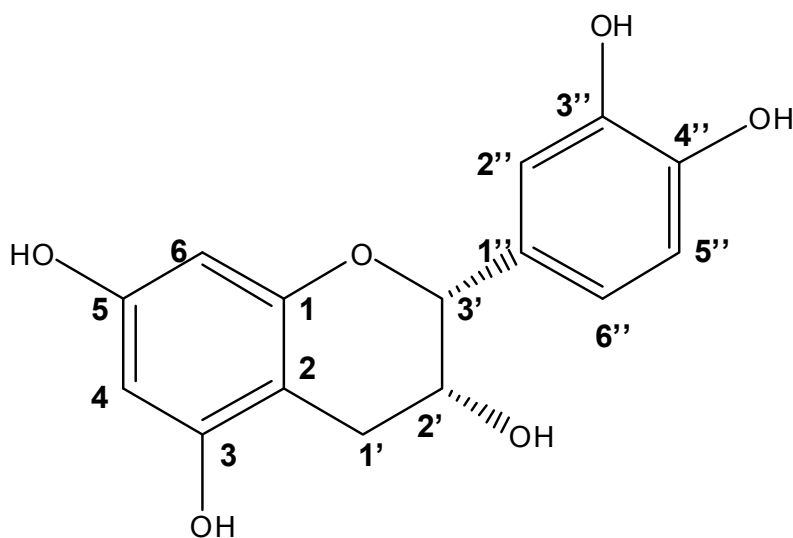


Figure 4. The structure of Compound B [(-)-epicatechin isolate].

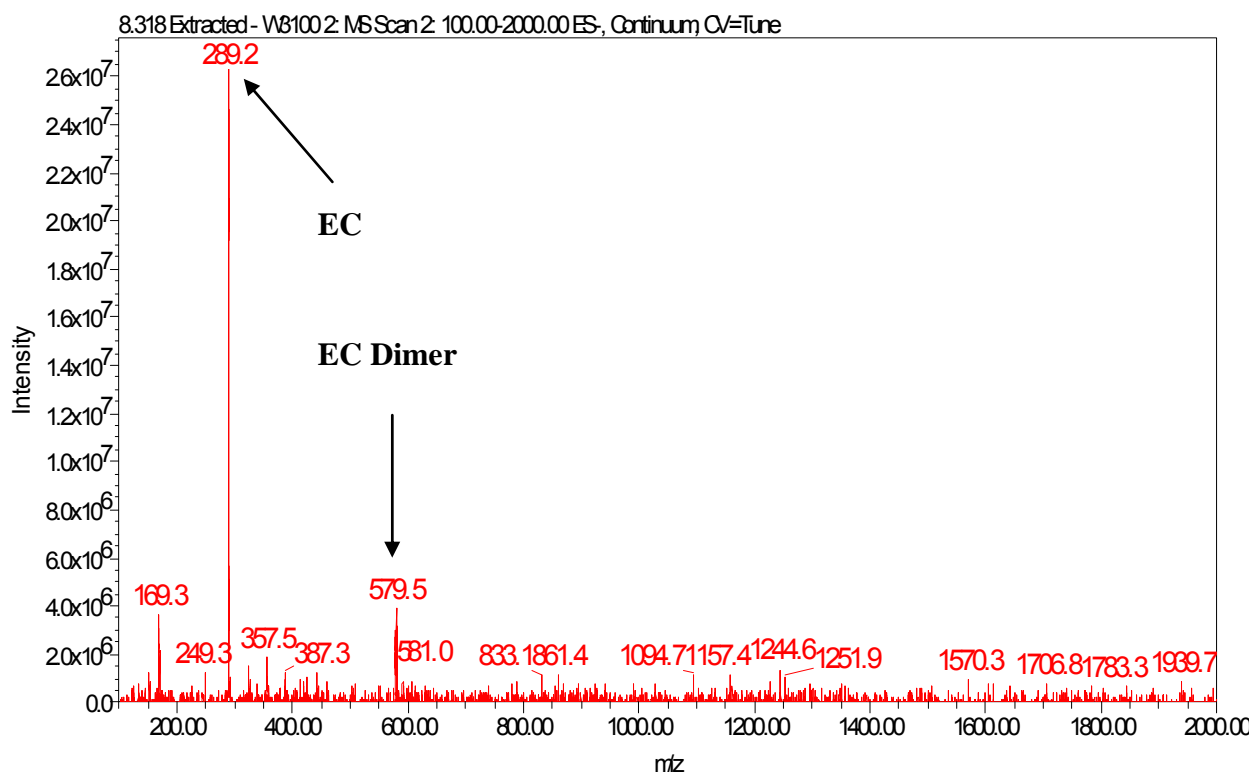


Figure 5. LC-ESI-MS spectra and chromatogram of isolated epicatechin.

C6-C3-C6 units where the C6 moieties are aromatic rings (Baskarajan and Chezhiyan, 2002). After comparing this data and the structure with what is available in literature, it was concluded that the name of compound B is (-)-epicatechin. (-)-Epicatechin is a major polyphenolic component of green tea and was reported to have insulin

mimetic action with protective effects on erythrocytes in a manner similar to insulin and it would be beneficial in supporting the management of secondary complications of diabetes. It should also be noted that epicatechin has been isolated in the Rubiaceae family earlier. The bark of *Cinchona pubescens* (belonging to the Rubiaceae family)

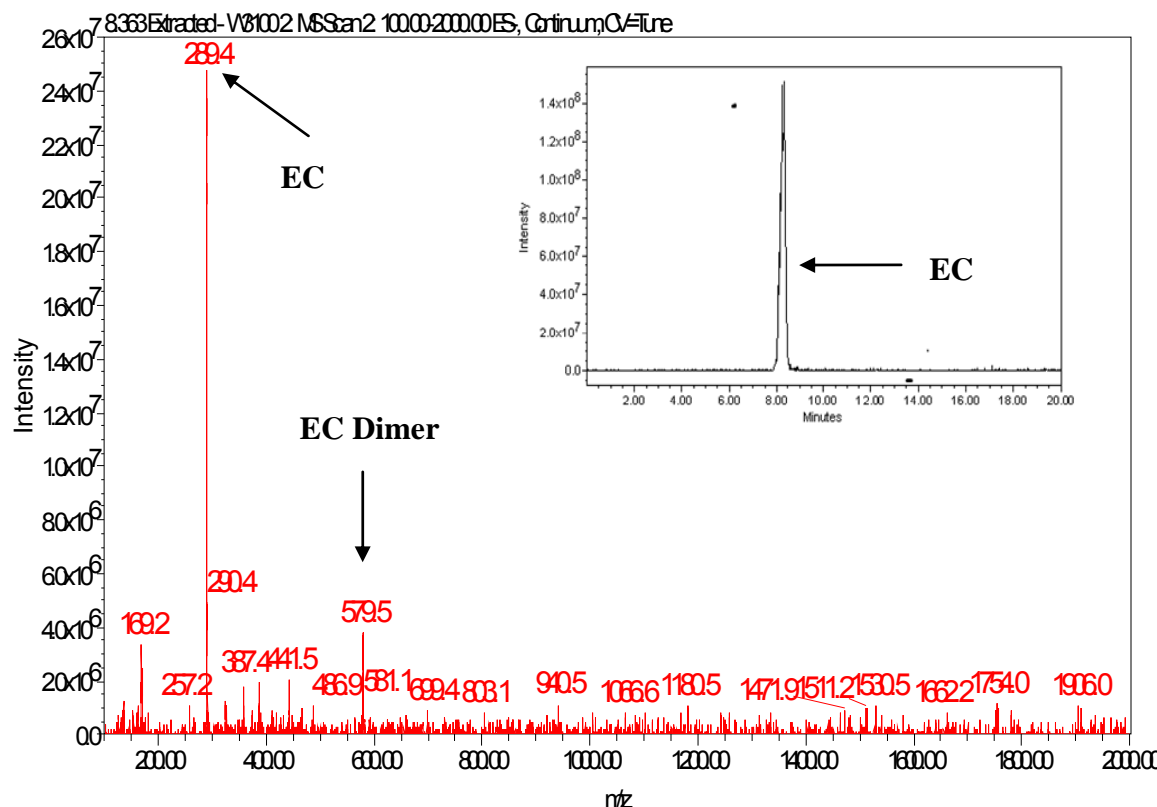


Figure 6. LC-ESI-MS spectra and Chromatogram of standard (-)-epicatechin.

is known to contain catechins (Van Wyk and Wink, 2004). This information can be of chemotaxonomic importance to the Rubiaceae family. It also offers antioxidant protection against lipid peroxidation and inhibits activity of radical oxygen species.

LC-ESI-MS for catechins from *E. elephantina* and *P. prunelloides*

The retention time of the isolate and standard were almost identical 8.318 and 8.363 (Figures 5 and 6), respectively. The LC-ESI-MS spectra and chromatogram of the isolated and standard (-)-epicatechin samples are shown in Figures 5 and 6, respectively. The pseudomolecular mass of 289.4 in each case is consistent with values reported in literature. The mass ion at m/z 289.4 is $[M]^-$ because the LC-ESI-MS instrument was operated in the negative mode. The m/z at 579.5 for both standard and isolate suggested dimerisation of (-)-epicatechin (Figure 7). This data was consistent with the proposed fragmentation pattern and structure as shown in Figure 8.

Figure 8 shows another isolate with pseudomolecular ion with m/z 607 which on fragmentation gives a species with m/z = 303 which is proposed to be quercetin. On

dimerisation, the proposed quercetin gives its dimer as shown in Figure 9. The m/z at 911.4 suggests the trimer of quercetin ($303.4 \times 3 = 910.2$). The presence of this compound is confirming results from literature where its glycoside was reported for *E. elephantina* (Mthembu, 2007). Hydrolysis of the glycoside may result in the formation of free quercetin.

LC-ESI-MS spectra and chromatogram of isolated EGCg

The LC-ESI-MS spectra for both standard EGCg and the isolate from *E. elephantina* were recorded (Figures 10 and 13). The isolate from *E. elephantina* was assumed to exist as a glycoside bonded to sugar moieties which on fragmentation gave the pseudomolecule at m/z 457 which is consistent with literature. Figure 11 confirmed EGCg as the aglycone (m/z 459) but bonded differently as depicted by its early elution (R_t = 8.338). The presence of epicatechin is exhibited by the adduct with m/z 288.9 formed as a result of the hydrolysis of EGCg while the fragment with m/z 139 is diagnostic of the fragmentation of catechins. The fragmentation pattern (Figure 12) suggested the presence of arabinose, m/z = 132.9, glucuronic

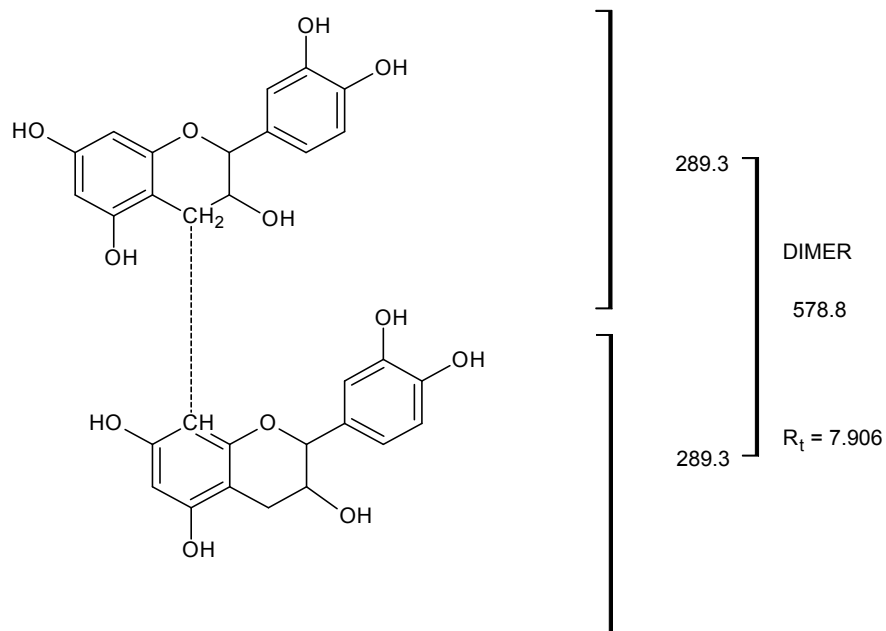


Figure 7. Fragmentation pattern of standard and isolated (-)-epicatechin.

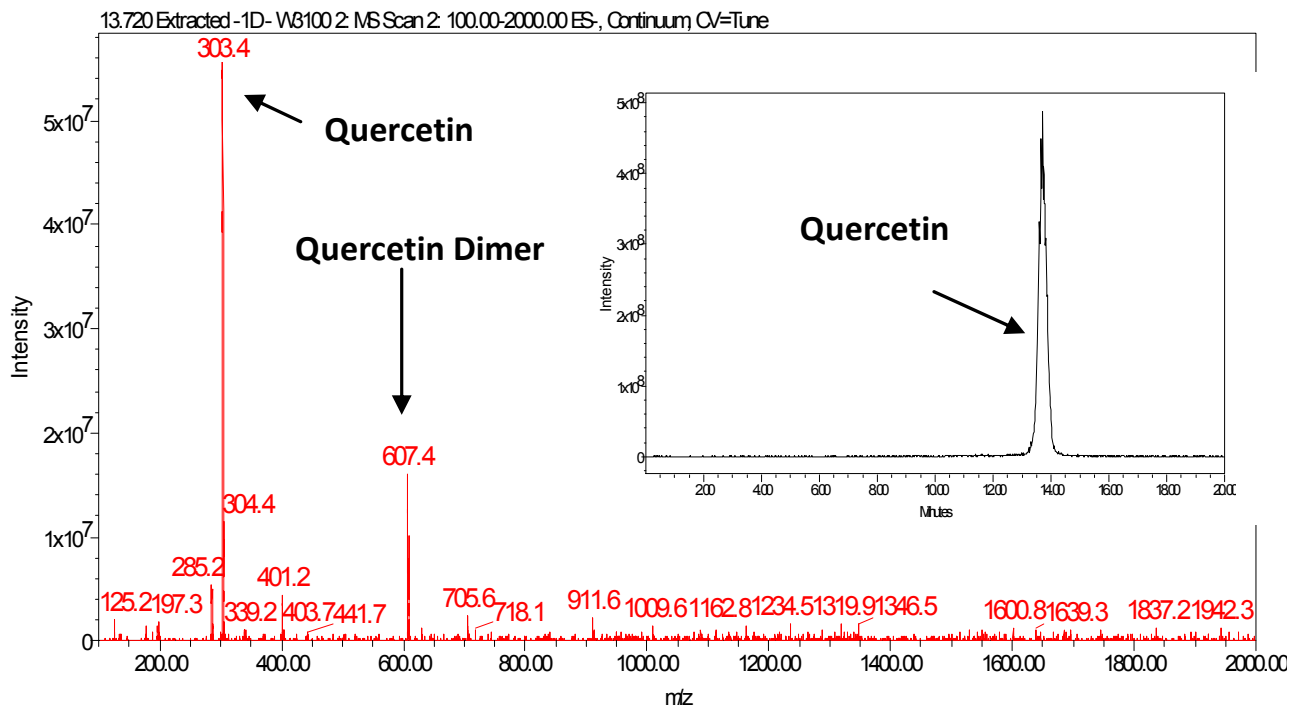


Figure 8. LC-ESI-MS spectra and chromatogram of isolated Quercetin.

acid, m/z 176/195, and a hexose sugar, m/z 162.3. The LC-ESI-MS spectra and fragmentation pattern of standard EGCg also assumed to be arising from m/z 457

for the monomer EGCg and m/z 916 for the dimer of EGCg. Based on these results, this is the first time EGCg reported in *E. elephantina*.

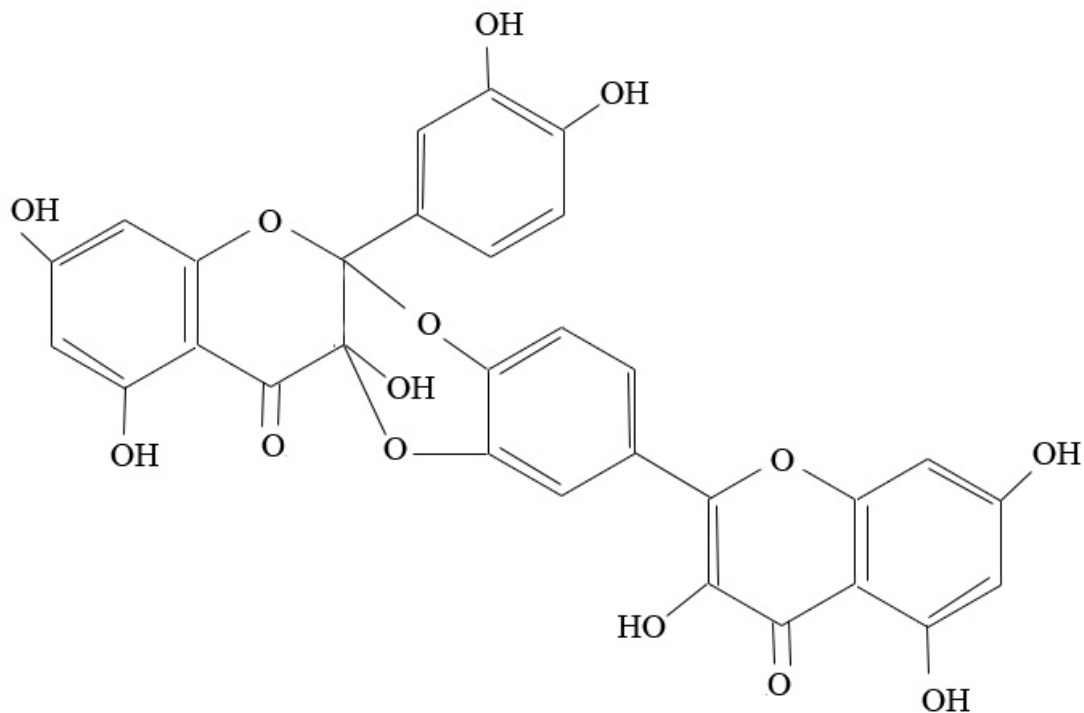


Figure 9. The dimer of Quercetin.

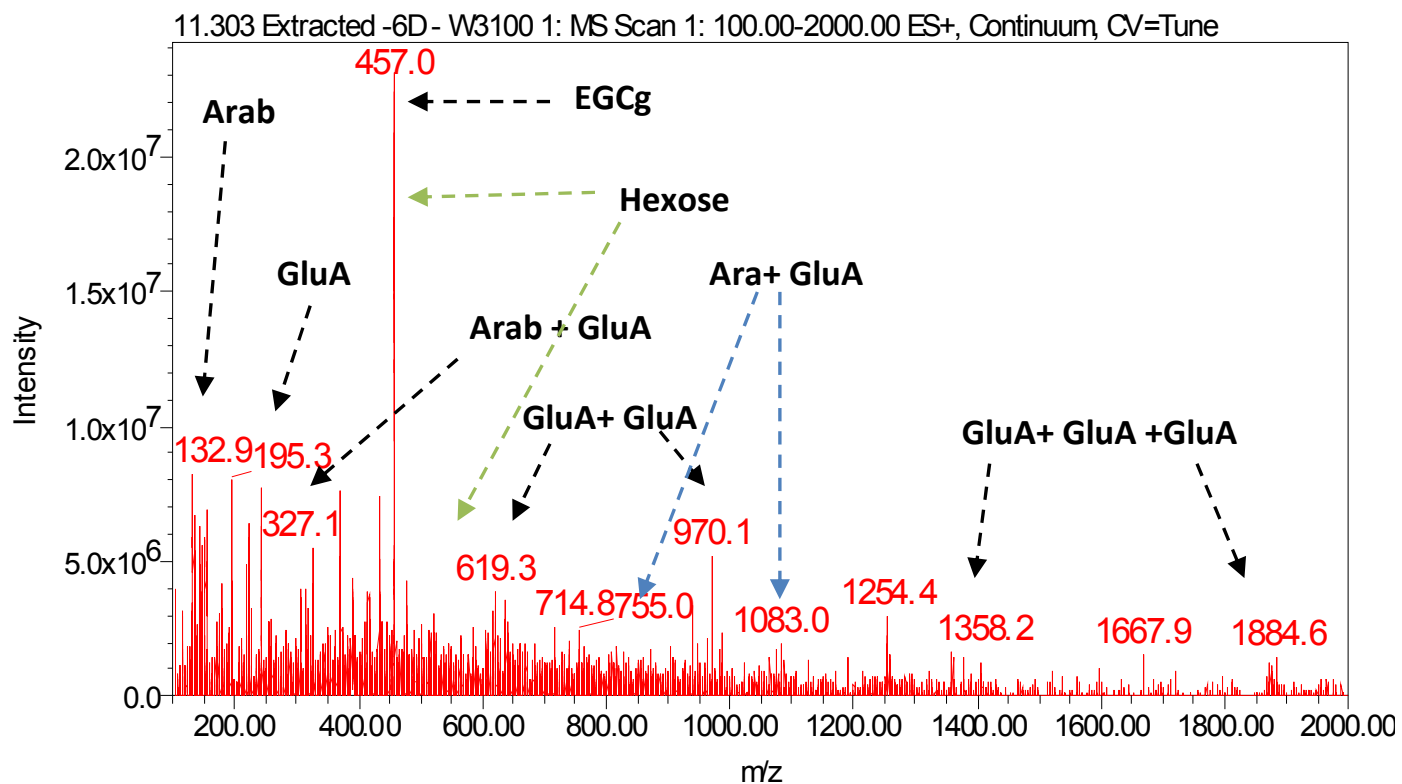


Figure 10. LC-ESI-MS spectra of EGCg glycoside isolated from *E. elephantina*.

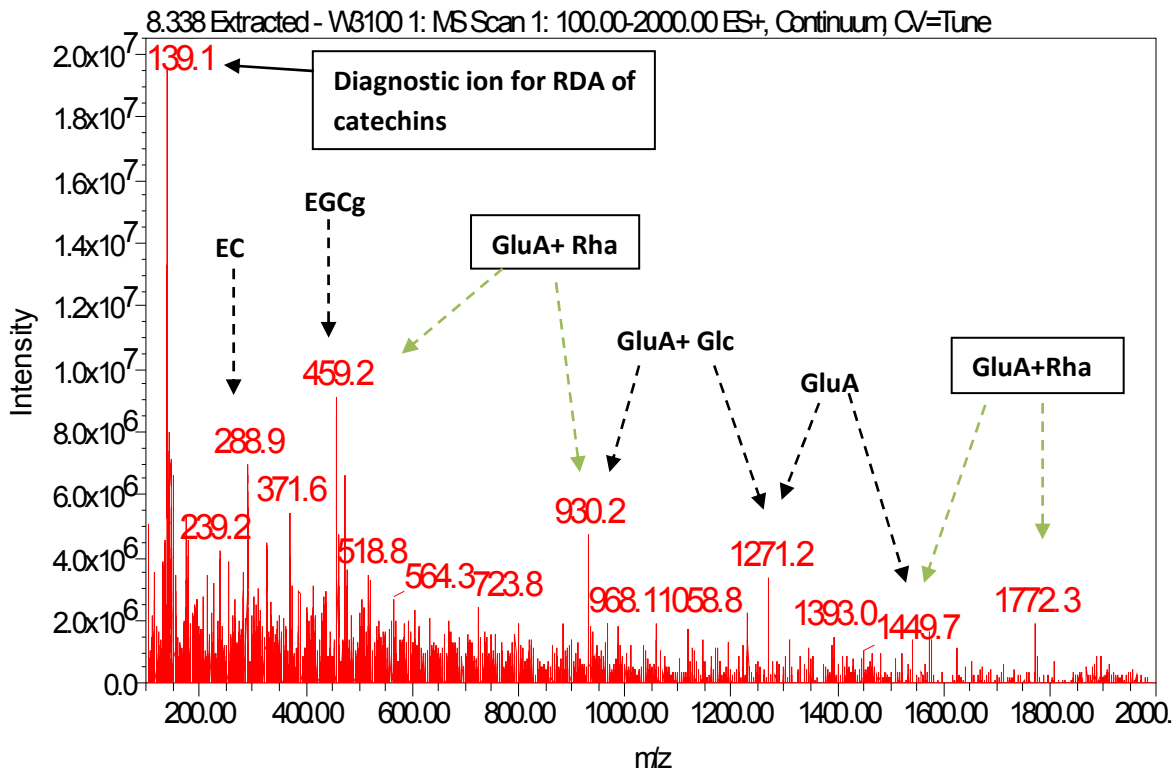


Figure 11. LC-ESI-MS spectra of EGCg glycoside isolated from *P. prunelloides*.

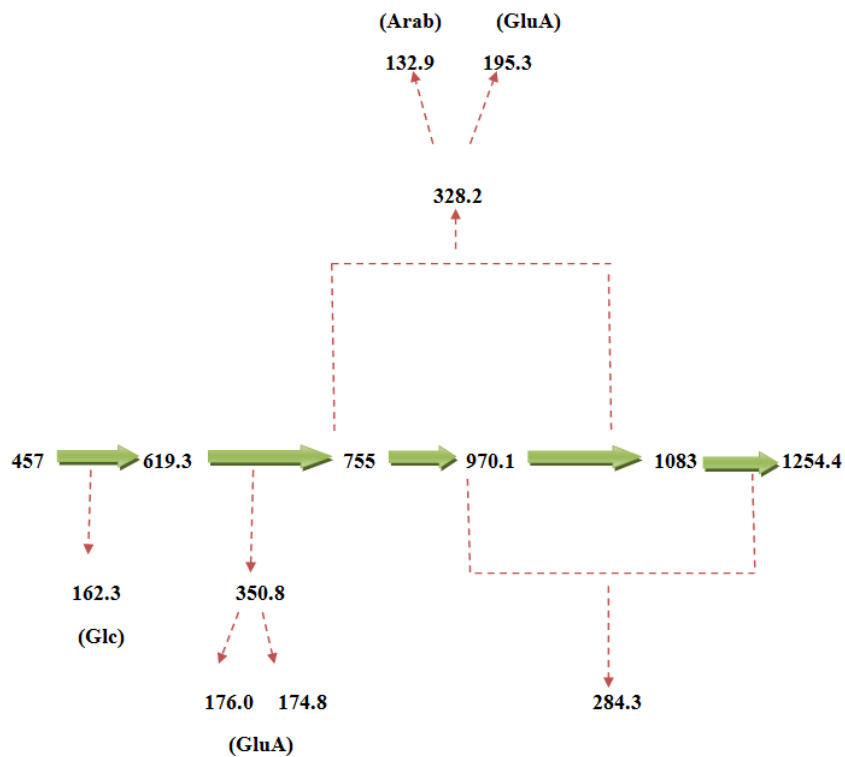


Figure 12. The proposed fragmentation pattern of EGCg isolated from *E. elephantine*.

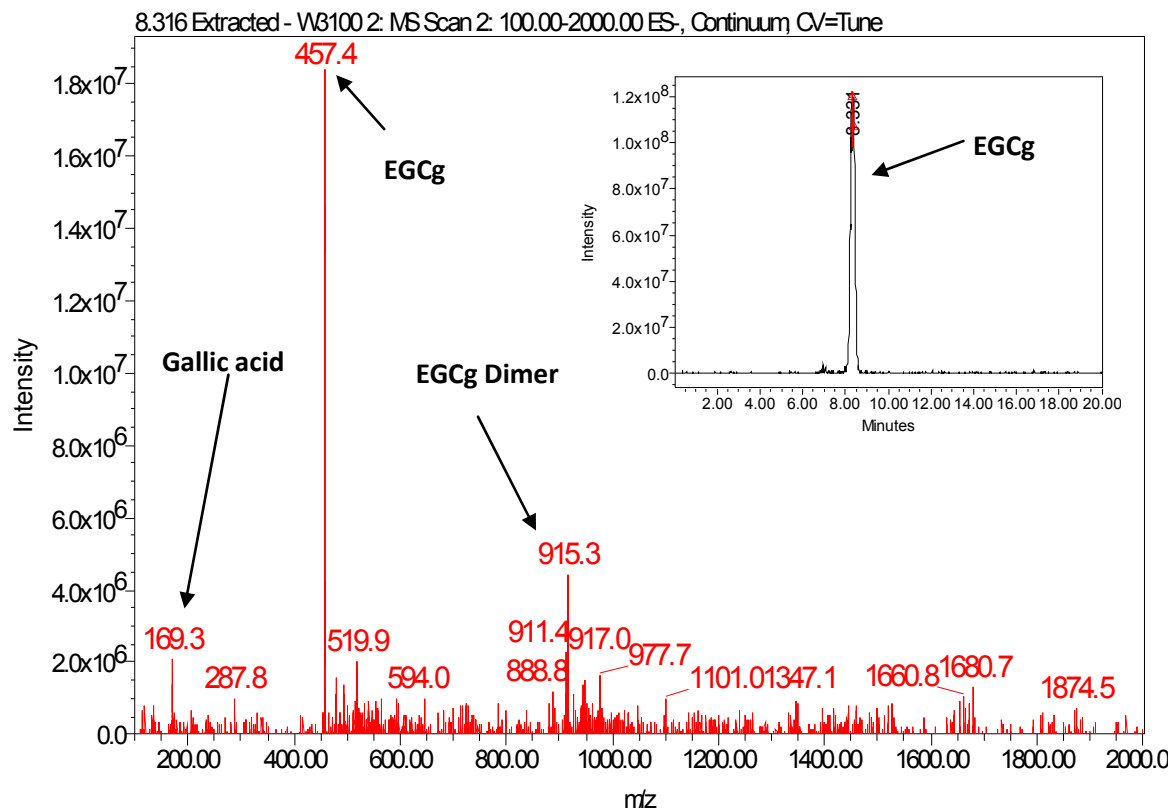


Figure 13. LC-ESI-MS spectra and chromatogram of standard EGCg.

Other compounds isolated from *E. elephantina*

LC-ESI-MS spectra and chromatograms (Figure 14) exhibited the presence of the dimer of epicatechin and epigallocatechin gallate. This observation is consistent with the proposed fragmentation pattern where m/z 289.2 was deemed to be epicatechin, m/z 169.0 galloyl fragment and 441.4 for the epicatechin gallate after the hydroxyl ion fragment has been eliminated from epigallocatechin gallate (Figure 14).

LC-ESI-MS spectra (Figure 15) suggested the presence of glycosylated catechin. The proposed fragmentation patterns are shown in Figure 15a to e. In Figure 15a, cleavage of the gallate would result in the adduct m/z 465.5 and the galloyl fragment m/z 153 and the fragmentation of the hexose sugar results in the formation of the adduct with m/z 465.5 again (Figure 16). The retro-Diels-Alder (RDA) cleavage of the C ring (Figure 16) also results in the formation of the same fragment (465); hence, justifying the prominence and intensity of the spectra for the adduct with m/z 465.5 (Figure 15). The pseudomolecule at m/z 1083.5 was proposed to be a polymer of pseudomolecule m/z 617.4 (Figure 16) and pseudomolecule with m/z 466.1 bound by the interflavonic bond as shown in Figure 16. The

pseudomolecule at m/z 931.3 was proposed to be a dimer of pseudomolecule 465 ($465.5 \times 2 = 931$). While the precise structure of this compound could not be unambiguously elucidated, this information acts as a starting point for further studies. This LC-ESI-MS spectra confirmed the presence of a mixture of flavonoids in fractions of *E. elephantina* (Figure 15).

The LC-ESI-MS spectra for another catechin at $R_t = 10.660$ (not shown) exhibited pseudomolecules at m/z 745, 442, 303 and daughter ions at m/z 169 and 153. The proposed fragmentation pattern is as shown in Figure 17. The presence of dimers of quercetin and epicatechin gallate was proposed which confirms that catechins exist as complex mixture of closely related compounds that have very close R_t values (Figures 14 to 17). The same observation was made for various flavonoids that were identified in both *E. elephantina* and *P. prunelloides* (Table 3).

Schemes 1 to 2 and 3 to 4 represent the proposed fragmentation patterns for *P. prunelloides* and *E. elephantina*, respectively as derived from the LC-ESI-MS in the negative mode spectra data (not included). The fragmentation pattern of the observed pseudomolecular ions and fragments were characteristic for the sugar moieties (Schemes 1 to 4).

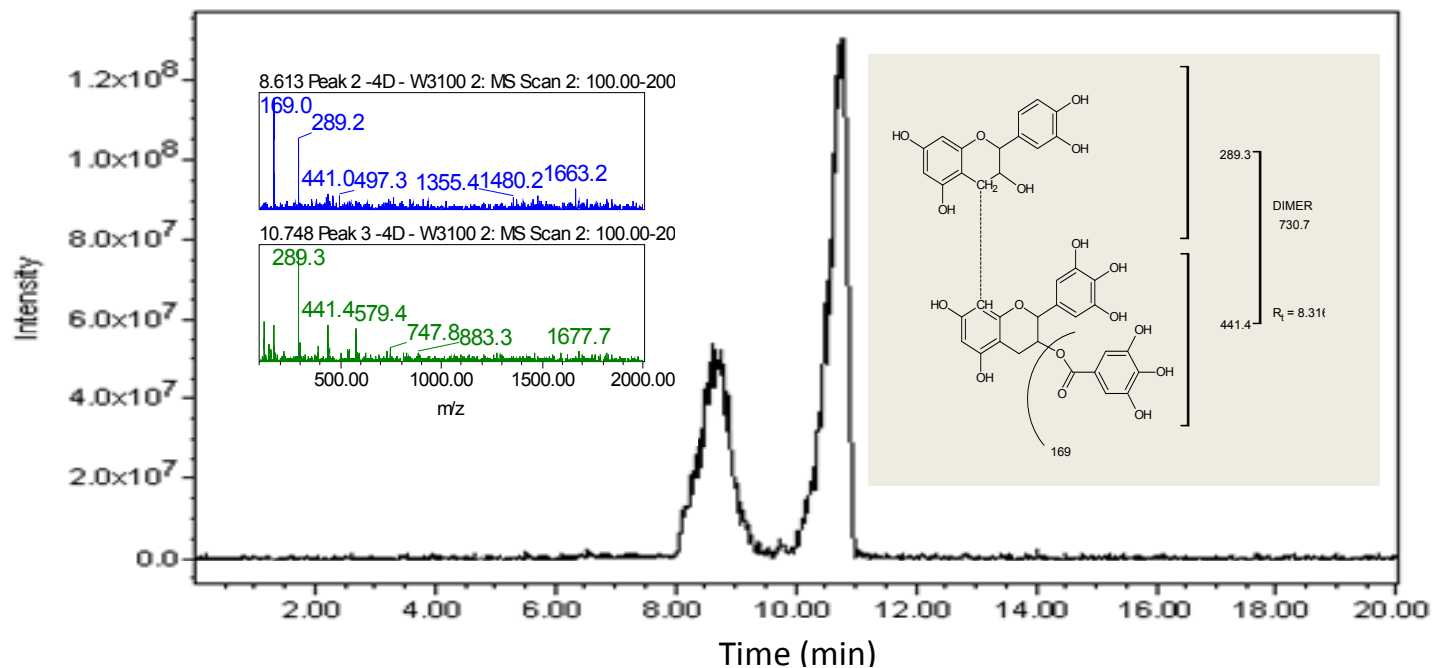


Figure 14. LC-SEI-MS spectra, chromatogram and proposed fragmentation pattern of *E. elephantina* isolate 4D.

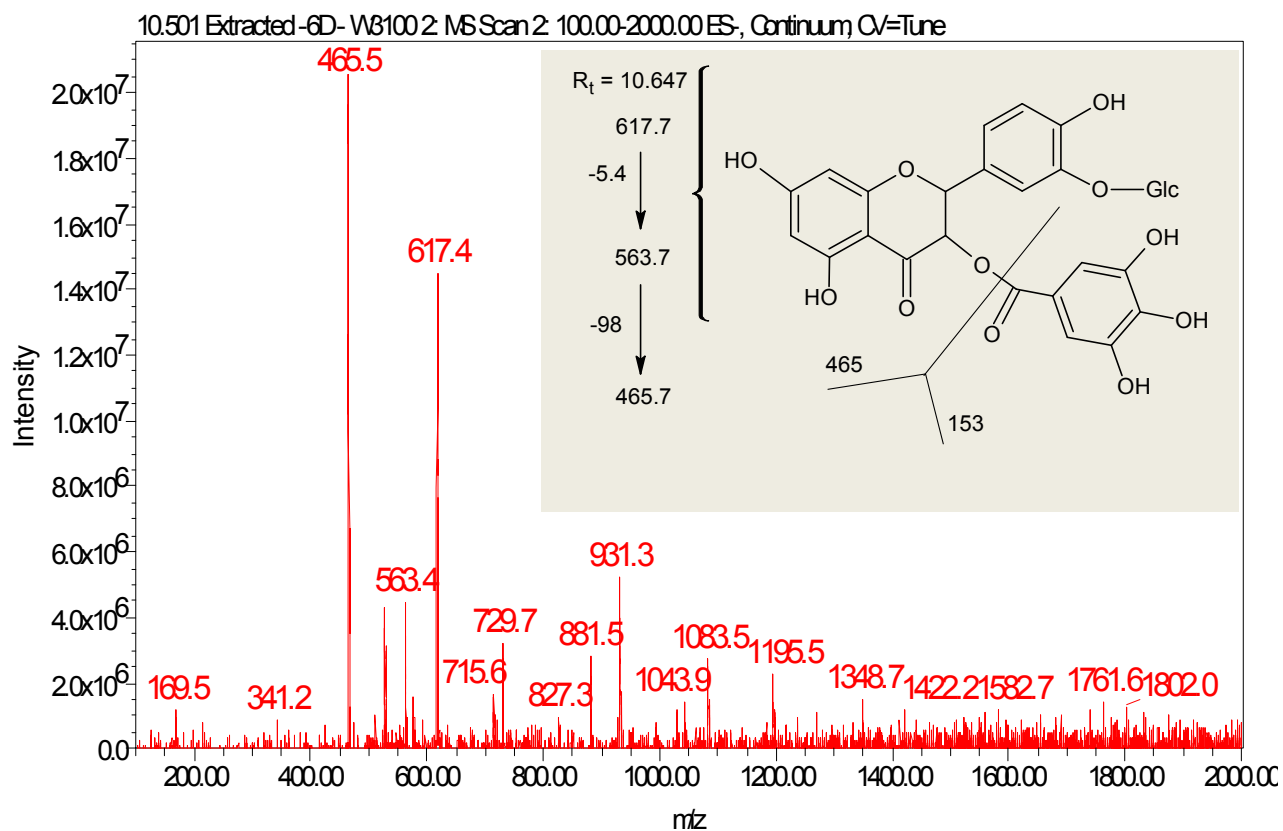


Figure 15. LC-ESI-MS spectra and proposed fragmentation pattern of compound 4D at $R_t = 10.501$.

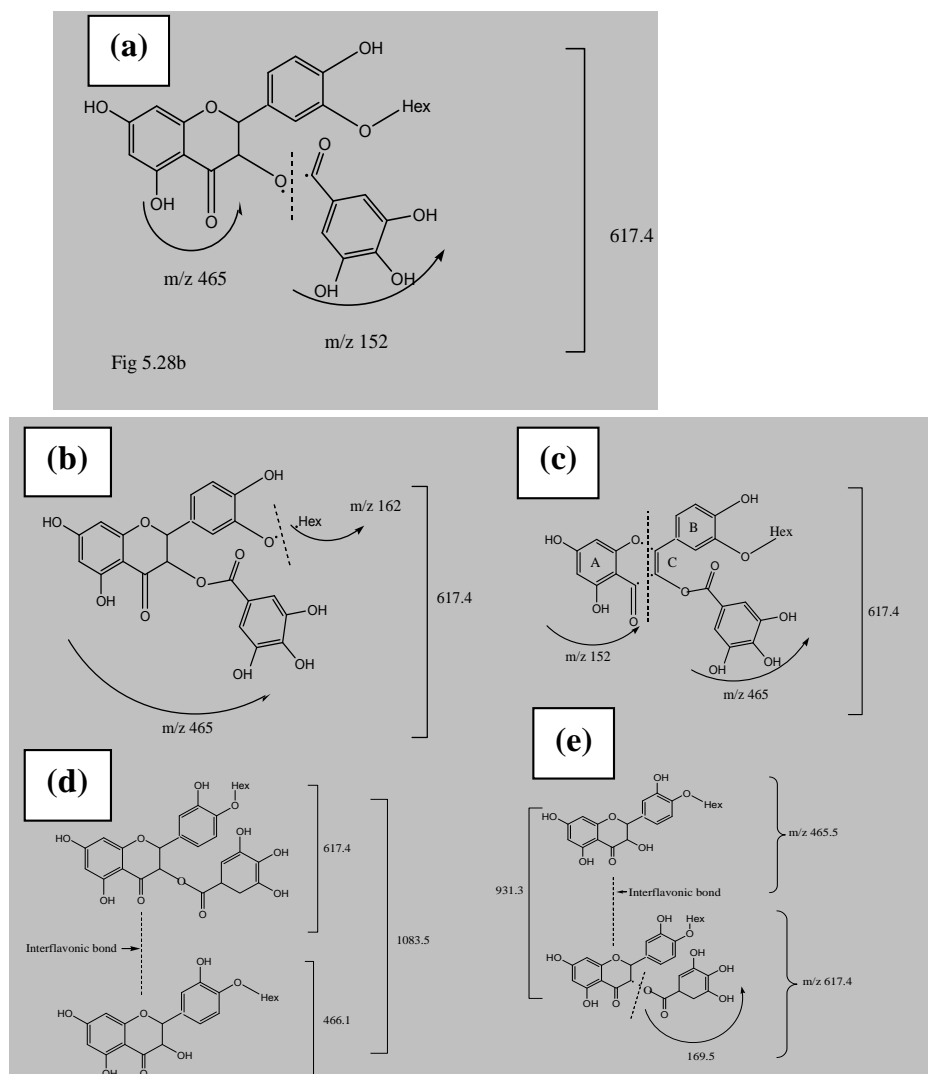


Figure 16. Fragmentation patterns. (a) Cleavage of the gallate of pseudomolecule with m/z 617.4, (b) Cleavage of the hexose sugar from the pseudomolecule m/z 617.4, (c) retro-Diels-Alder cleavage of the C-ring of pseudomolecule m/z 617.4, (d) Fragmentation of the interflavonic bond of pseudomolecule m/z 1083.5 and (e) Cleavage of the gallate fragment and interflavonic bond to form monomers with m/z 465.5.

In Schemes 1 and 2, the fragment m/z 1368 was generated by the loss of a disaccharide (m/z 342.3). Subsequent loss of three disaccharide units generated the pseudomolecular mass ion m/z 439.2 which is easily assignable to the aglycone. In Schemes 3 and 4, the pseudomolecular ion m/z 1465.5 undergoes a partial rearrangement along a fragmentation pathway which is also peculiar to flavonoid glycosides. This leads to the loss of the “internal” sugar of the disaccharide, that is, glucuronic acid m/z 196.4, as exhibited in Schemes 3 and 4. This fragmentation is concomitant with the expected pathway, that is, loss of the glucuronic moiety. The appearance of intense polysaccharide ion fragments, that

is, 1366 for *P. prunelloides* and 1882 and 1710 for *E. elephantina* suggests the presence of O-glycosidic bonds as these are prone to cleavage due to soft ionisation under the ESI-MS spectrometry apart from hydrolysis as the eluent was acidified with 1% acetic acid (Fabre et al., 2001).

The spectra and chromatogram at R_t 9.508 was observed to be common to both *E. elephantina* and *P. prunelloides* (Figure 18). The fragmentation pattern of the observed pseudomolecular ions and fragments were proposed to be characteristic for the sugar moieties (Figure 18). The fragmentation pattern exhibited the presence of glucuronic acid (m/z =177/195) three hexose

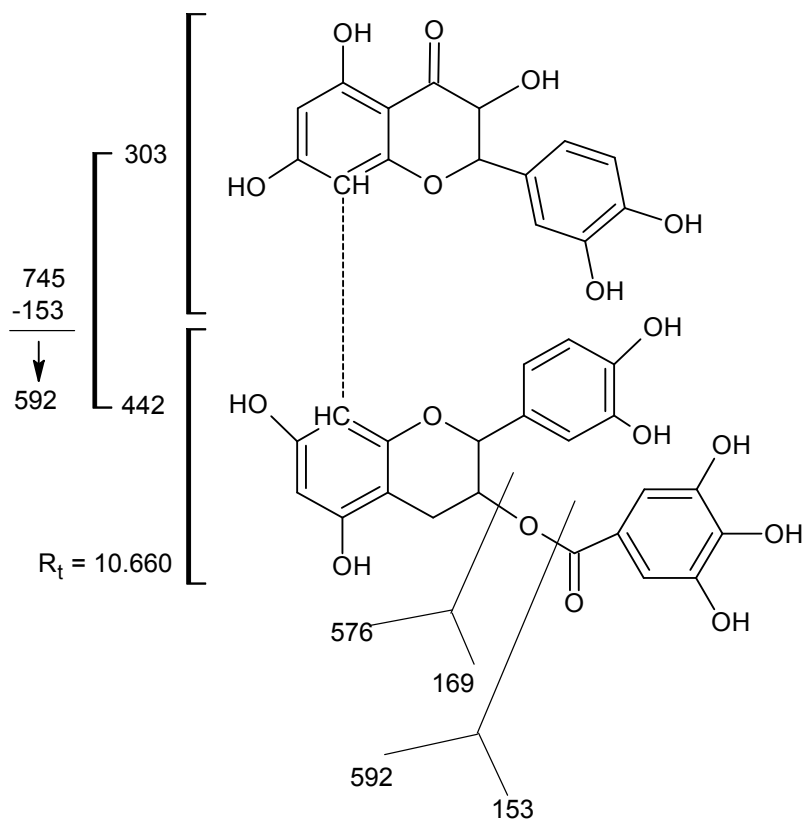


Figure 17. The fragmentation pattern of compound 4D at $R_t = 10.660$.

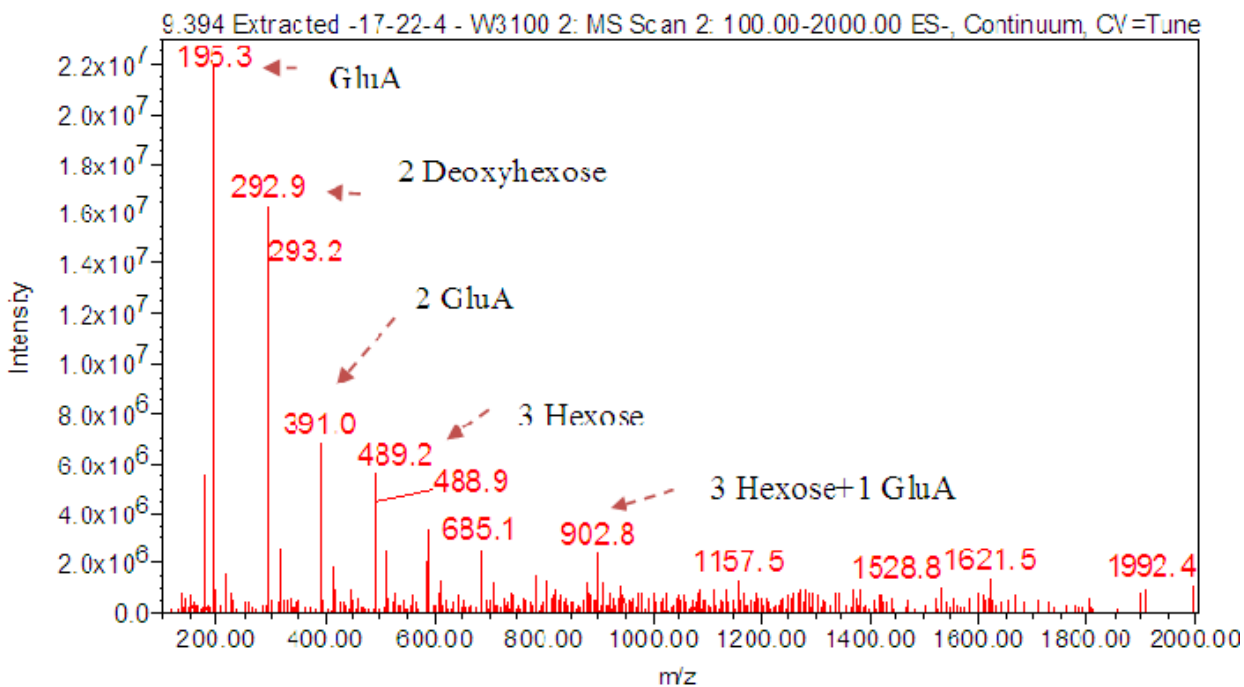


Figure 18. LC-ESI-MS spectra of sugar moieties from *P. prunelloides*.

Table 1. Absorbance and percentage yields of flavonoids for *E. elephantina* and *P. prunelloides*.

Sample	Absorbance	g% yield	Average yield (%)
<i>E. elephantina</i>	0.3975	0.153	0.77
	0.3912	0.151	
	0.4030	0.156	
	0.4101	0.158	
<i>P. prunelloides</i>	0.3224	0.124★	0.81
	0.3955	0.153	
	0.4255	0.164	
	0.4438	0.171	

★Value discarded due to its high deviation from other values.

Table 2. ^1H (400 MHz) and ^{13}C NMR (100 MHz) and 2D NMR [^1H (300 Hz) and ^{13}C NMR (75 MHz)] for Compound B (-)-epicatechin isolate.

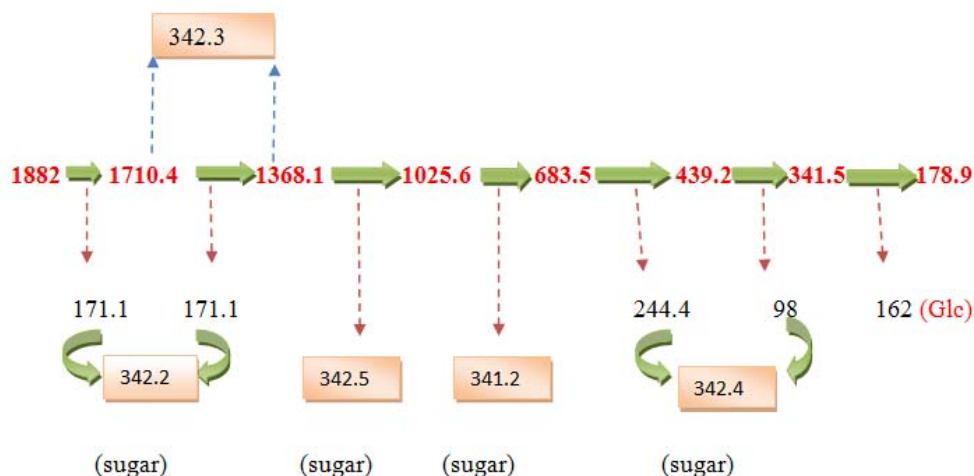
C assignments	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)	COSY	HMBC
1	158.0	—	—	3'
2	100.1	—	—	4, 6, 1'
3	157.6	—	—	4, 6, 1'
4	96.4	5.93 dd (2.40)	—	6
5	157.3	—	—	4, 6
6	95.9	5.93 dd (2.40)	—	4
1'	29.2	2.79 dq	2', 3'	3'
2'	67.4	4.17 s	1', 3'	1'
3'	79.8	4.81 s	1', 2'	1', 2'', 6''
1''	132.2	—	—	3', 2'', 5''
2''	115.3	6.78 m	3'	3', 5''
3''	145.9	—	—	5''
4''	145.7	—	—	2'', 6''
5''	115.9	6.78 m	3', 6''	3', 2'';
6''	119.4	6.96 d (1.2)	3', 5''	3', 2''

Table 3. Chromatographic data (peak number and retention time) and m/z values of flavonoids and their glycosides isolated from *E. elephantina* and *P. prunelloides*.

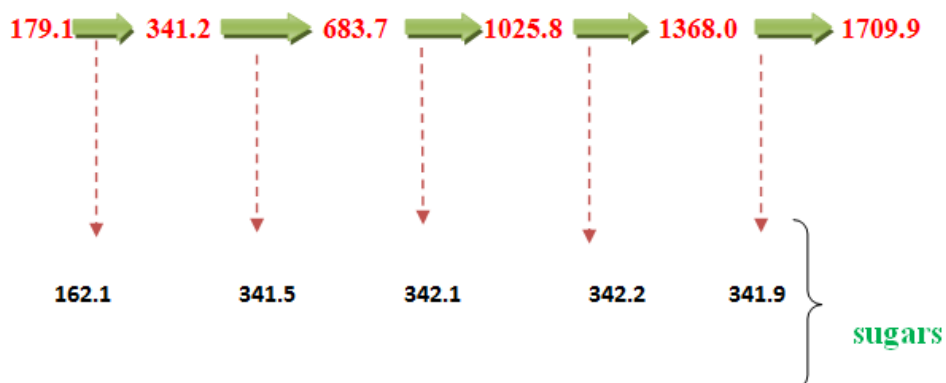
Peak	R _t (Min)	MH ⁺	Flavonoids/Glycosides
2 (Ee)	9.866	596.8	Taxifolin/Epicatechin dimer [596.8-305.5 = 291.3; 663.6-305.5 = 358.1 (2 × 179.5)]
3 (Ee)	13.065	482.2	Sodiated EGCg glycoside (482.2-23 = 459.2); 819-482.2 = 336.8 (2 × 163.4) di-hexose
3 (Ee)	8.502	457.7	EGCg glycoside 748.2-457.7 = 290.5; 748.2-169 = 579.2; 903.4-748.2 = 155.
3 (Pp)	7.426	287.3	Luteolin/EC dimer
1 (Pp)	5.915	305.1	Taxifolin/EGC
5 (Pp)	8.853	577	Kaemperol/Luteolin (577.9-287.3 = 290.6)
4 (Pp)	7.698	315.2	Dihydroxybenzoyl-O-hexose

(m/z) = 486), a tetramer of two hexose and two pentose sugars (m/z = 587), a dimer of two rhamnose, (m/z = 293), a dimer of two glucuronic acids [m/z = 2 × 195 = 390.8), a tetramer of two rhamnose (293) and two glucuronic acid (390.9), that is, m/z 684.6.

The corresponding LC-ESI-MS spectra and chromatogram of *E. elephantina* at Rt 9.508 was recorded (not included) and the fragmentation pattern showed similar fragment ions as for *P. prunelloides* explained earlier, but the major difference being a longer



Scheme 1. Fragmentation pattern of oligosaccharides isolated from *P. prunelloides*.



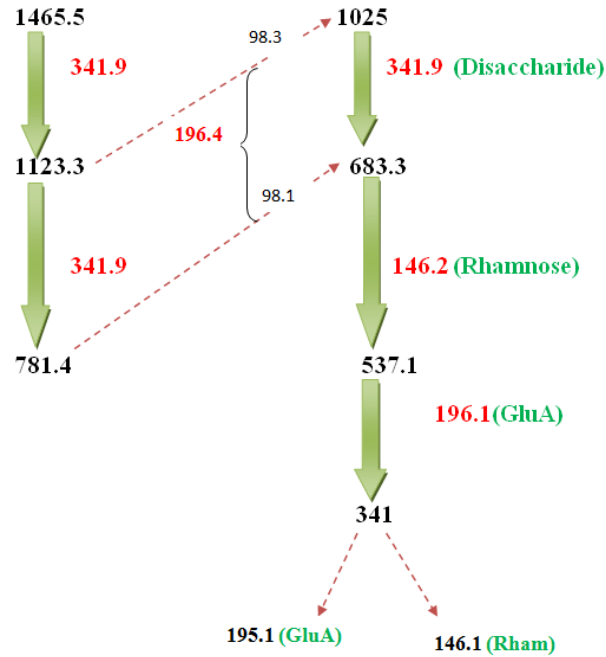
Scheme 2. Fragmentation pattern of oligosaccharides isolated from *P. prunelloides*.

sugar chain for *P. prunelloides*. The corresponding proposed fragmentation pattern suggested the linkages of disaccharides with m/z 342 and the presence of glucuronic acid m/z 179.1 and rhamnose for *E. elephantina*. The proposed fragmentation pattern further suggested about five dimers with m/z 342 each, which implied about ten monomers either a hexose and GluA (163 + 179) or GluA and rhamnose, that is, m/z (195 + 146), respectively (Schemes 1 to 4). The presence of the sugar chains in these compounds could also account for the presence of flavonoid glycosides observed in these two medicinal plants.

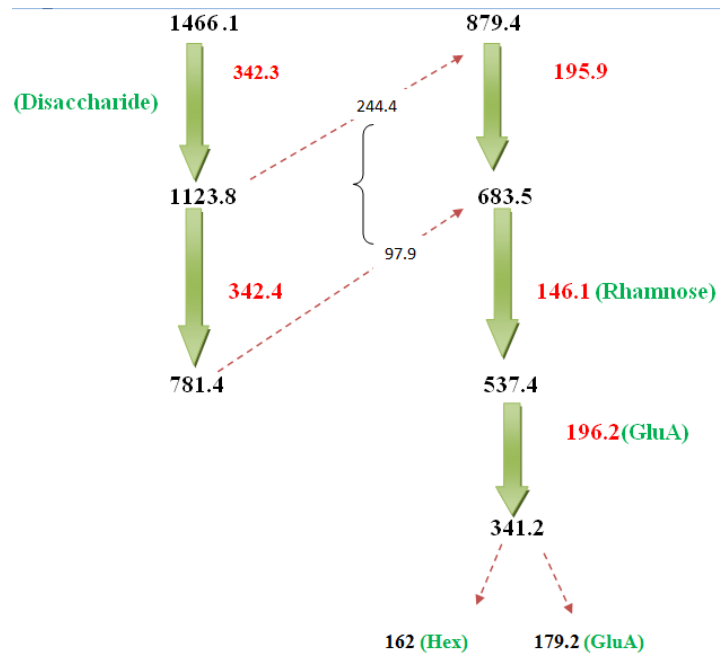
DISCUSSION

As alluded earlier, flavonoids are structurally diverse and generally part of a complex mixture of compounds isolated from plant extracts. Plants that contain flavonoids

are currently advocated for preventing and treating many diseases provided that they are of adequate quality. It is therefore essential for research to monitor the compositions of flavonoids in the complex matrix of natural product. The present study has unveiled the presence of flavonoids as some of the major secondary metabolites in both *E. elephantina* (0.77%) and *P. prunelloides* (0.81%). While some of these phytochemicals have not been fully characterized, their mere presence in large quantities supports the use of these two plant species by traditional healers. The high flavonoid content in both plant species may offer some explanation for the use of these plants as anti-inflammatory and antimicrobial agents. Palmitic acid is the only compound reportedly isolated from *P. prunelloides* in the literature (Yff, 2002), thus it is the first time that (-)-epicatechin is isolated from this plant. Epicatechin and 3,5,7,2'-tetrahydroxy flavones-3- α - β -D-glucopyranoside have been implicated for antimicrobial activity against



Scheme 3. Fragmentation pattern of oligosaccharide isolated from *E. elephantina*.



Scheme 4. Fragmentation pattern of oligosaccharide isolated from *E. elephantina*.

Escherichia coli, *Bacillus cereus*, *Staphylococcus aureus* and *Shrigella flexnen* at minimum inhibition concentration (MIC) values ranging from 12.5 to 100 mg/ml (Krause et

al., 2001; Ann and Geboes, 2002). The antimicrobial potency of these compounds against these pathogens is noteworthy, because the earlier referred bacteria have

been implicated as causal agents of diarrhoea (Ann and Geboes, 2002; McGaw, 2000) for which the two plants are administered by traditional healers. Epicatechin has also been reported (Esquenazi, 2000), to possess appreciable activity against acyclovir-resistant herpes simplex virus 1 (HSV-ACVr) and *S. aureus*. Flavonoids are reported to have antidiarrhoeal activity by inhibiting intestinal motility and hydroelectrolytic secretion (Venkatesan et al., 2006). While flavonoids are known to inhibit intestinal motility and hydroelectrolytic secretion, tannins are reported to denature proteins in the intestinal mucosa by forming tannates which make intestinal mucosa more resistant to chemical alteration and consequently reduce secretion (Havagiray et al., 2004). Therefore, the anti-diarrhoeal activities of *E. elephantina* and *P. prunelloides* may be attributed to the presence of tannins, flavonoids and saponins in the aqueous and methanol extracts. The presence of such compounds supports the use of *P. prunelloides* and *E. elephantina* decoctions as antioxidants as well as the application of these two medicinal for the remedy of various ailments for which they prescribed by traditional healers.

Conclusion

This paper reports the presence of EGCg in *E. elephantina* for the first time. The therapeutic value of this compound is extensively documented (Yang and Wang, 1993; Lin et al., 1996; Ho et al., 1992; Katiyar, 1993). Hence, its presence in *E. elephantina* as well as its glycosides may account for the alleged efficacy of this medicinal plant. The comparative analysis of samples of the two plants revealed that *P. prunelloides* contains a greater proportion of flavonoids (0.81%) than *E. elephantina* (0.77%). Some of the flavonoids identified and characterized by spectroscopic techniques include quercetin, epicatechin, epigallocatechin gallet, kaempferol and their polymeric derivatives.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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