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HPLC-HRESI-MS^n Characterization of Polyphenolic Compounds in the Stem Bark of *Chlorophora regia* A. Chev (Moraceae)

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Abstract

Isolation and identification of secondary metabolites from medicinal plants could be tedious and time consuming. Therefore, any technique that could be used to confirm the identity of medicinal plant constituents without isolating them will save time and resources. Chlorophora similar to many genera in the Moraceae family have been demonstrated to be rich sources of polyphenolic compounds with important biological activities. The current study was designed to employ HRESI-MS^n analyses to qualitatively examine isolated polyphenolic compounds from the stem bark of *Chlorophora regia*. Based on the HRESI-MS^n data obtained, the fragmentation patterns of the compounds under study will be proposed and could be used in their identification in a matrix. Five polyphenolic compounds were successfully isolated and purified using various chromatographic techniques including column chromatography, thin-layer chromatography (TLC) and *preparative* HPLC. The structures of the isolated compounds were elucidated by in-depth analyses of their 1D and 2D NMR and mass spectroscopic data. HRESI-MS^n was further used to characterize the isolated compounds. Five polyphenolic compounds including three Diels-Alder type adducts: sanggenon C, kuwanol E and chalcomoracin; two stilbene derivatives: chlorophorin and isochlorophorin were isolated from the stem bark. The tandem MS fragmentation patterns of the compounds in positive mode, were successfully proposed. The fragments obtained and proposed fragmentation patterns of the isolated compounds could be employed qualitatively in the identification of the studied polyphenolic compounds in a matrix.

Keywords: *Chlorophora regia*, polyphenolic compounds, HPLC-HRESI-MS/MS, fragmentation pattern

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1 Introduction

Chlorophora regia A. Chev. (Moraceae) is an important dioecious medicinal tree that belongs to the family Moraceae. Chlorophora similar to many genera in the family Moraceae have shown to be an abundant source of polyphenolic compounds [1], [2]. Many of the identified polyphenolic compounds isolated from these genera are biologically active [3], [4], [5], [6], [7], [8]. HRESI-MS^n analyses provide useful information about the fragmentation patterns of compounds which are usually characteristic. The data obtained could therefore be employed as a qualitative tool in establishing the identity of biologically active natural products from a matrix without the tedious approach of fractionation and isolation. Again, the fragments from the chromatogram may provide information about the biosynthetic pathways of natural products.

Our previous investigation of the chemical composition of the stem bark of *C. regia* afforded several polyphenolic compounds including three Diels-Alder type adducts: sanggenon C (**1**), kuwanol E (**2**) and chalcomoracin (**3**); two stilbene derivatives: chlorophorin (**4**) and isochlorophorin (**5**) [9]. In this study we report the use of HPLC-HRESI-MS^n to characterize five polyphenolic compounds isolated from *C. regia* and their proposed fragmentation pathways. The identities of the isolated compounds were established based on comprehensive analyses of their 1D and 2D NMR, HRESI-MS^n spectral data and correlating them to those reported in literature.

2 Materials and Methods

2.1 Plant Material

The stem bark of *Chlorophora regia* was collected from Asakraka forest 6°37'48,39''N0°41'6,87''W) in the Eastern Region of Ghana. The sample was authenticated by Mr. Cliford Asare at Department of Herbal Medicine, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. A voucher specimen (KNUST/HM/CR1/2014/R002) was deposited at the herbarium.

2.2 Extraction of plant materials

The extraction, isolation and purification of the compounds were as described previously [9]. The collected stem bark was air-dried, powdered (2 Kg) and extracted with a mixture of methanol–chloroform (80:20) by cold maceration. The crude extract obtained was concentrated using the rotary evaporator to afford a brownish residue (140 g). The concentrated residue was successively extracted with cyclohexane, dichloromethane and methanol. The methanolic fraction (110 g) was subjected to silica gel column chromatography using variable compositions of cyclohexane–ethyl acetate and ethyl acetate–methanol to obtain fourteen major fractions following HRESI–MS and TLC monitoring. The fourteen obtained fractions were subjected to various separation techniques including sephadex LH–20 column chromatography, further silica gel column chromatography and *semi-preparative* HPLC to obtain compounds **1**–**5** (Figure 1).

2.3 General Experimental Procedure

NMR spectral data were obtained on a Bruker DRX–500 spectrometer operating at 500 and 600 MHz (^{1}H) and 125 and 150 MHz (^{13}C) using deuterated methanol (CD_3OD) as solvent. Chemical shifts (δ) were quoted in parts per million (ppm) using tetramethylsilane (TMS) as internal standard. *Semi-preparative* HPLC was carried out on a Gynkotek pump equipped with a Dionex DG-1210 degasser, a Dionex Gina 50 auto-sampler, a Dionex UVD 340S detector, and a Phenomenex Gemini C18 column (10 x 250 mm, 10µm particle size) using a Chromeleon software system. Column chromatography was performed on Silica gel 60 (70-230 mesh; AppliChem, GmBH, Darmstadt, Germany) and Sephadex *LH*–20 (25-100 µm, Amersham

Biosciences). TLC was carried out on pre-coated silica gel 60 plates (0.25 mm; Merck, Darmstadt, Germany). The developed spots were visualized under UV light by spraying with H_2SO_4–EtOH (1:9, v/v). All the solvents used were of analytical grade.

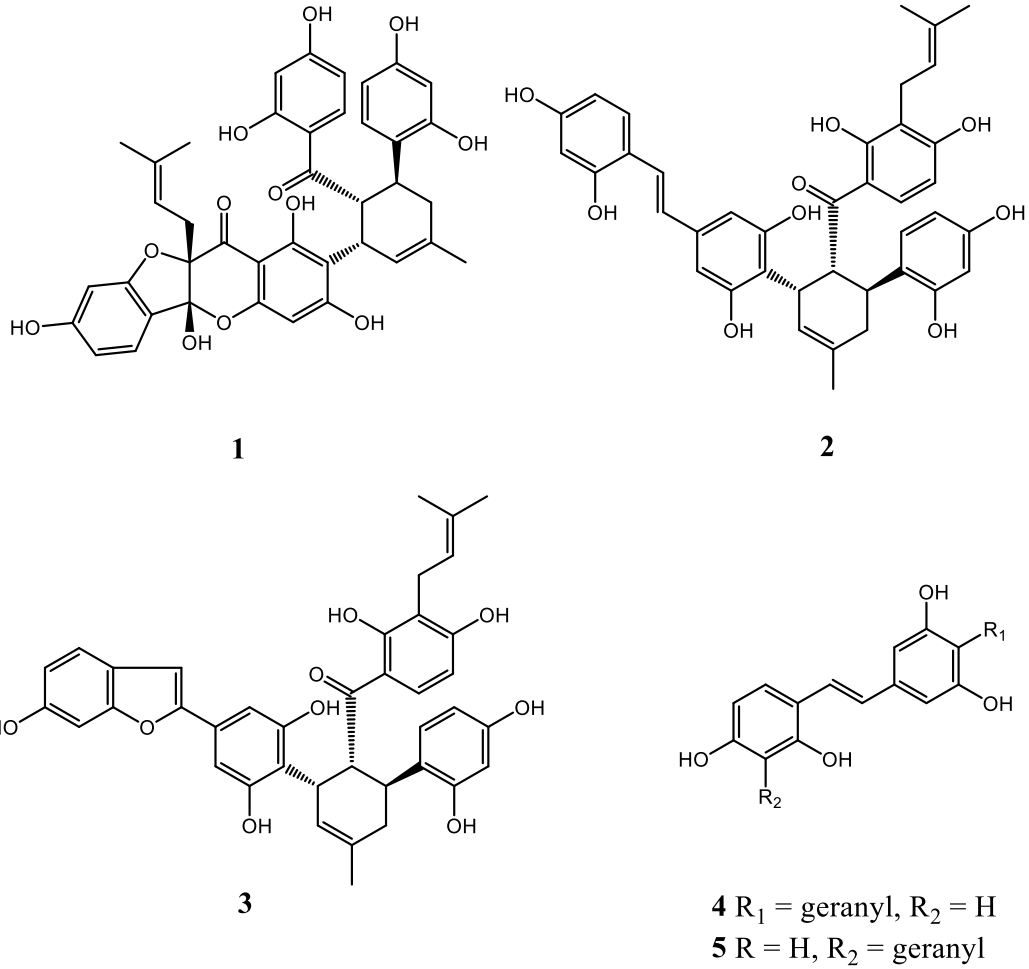

Figure 1. Chemical structures of compounds **1-5**

2.4 HPLC-HRMS^n

The HPLC–HRMS^n experiments were conducted on an LTQ–Orbitrap spectrometer (Thermo Fisher, USA) equipped with a HESI-II source. The spectrometer comprised an Agilent 1200 HPLC system (Santa Clara, USA) including pump, PDA detector, column oven (30°C), and auto-sampler (injection volume 5 µL for Fullscan, 7 µL for MS^n). MS^2 experiments were measured by CID (collision-induced decay, 35 eV) mode. HPLC analyses were carried out on a Luna C18 (2) column (50×3 mm, 3 µm particle size) from Phenomenex (Torrance, USA) using a mobile phase system of water (+ 0.1 % formic acid) (A) and methanol (B) gradient (flow rate 350 µL/min). The gradient parameters were set as follows: linear gradient from 95 % A to 100 % B over 14 min, 100 % B isocratic for 4 min, the system returned to initial conditions within 0.5 min of 95 % A and was equilibrated for 4.5 min.

3 Results and Discussion

Compound **1** was obtained as yellow amorphous solid with molecular formula of $C_{40}H_{36}O_{12}$, HRESI–MS (**S1**), showed a pseudo–molecular ion at *m/z* 709.2283 $[M + H]^+$ (calcd. for 709.2279). The HRESI–MS/MS study (**S2**) of **1** produced fragments at *m/z* 641.1646 [M + H–

$C_5H_8]^+$, *m/z* 623.1542 [M + H–C_5H_8-$H_2O]^+$, *m/z* 339.1225 [M + H–$C_{20}H_{18}O_7]^+$, *m/z* 491.1332 [M + H–$C_{13}H_{14}O_3]^+$, *m/z* 573.2114 [M + H–$C_7H_4O_3]^+$. The ^{1}H NMR spectrum (**Table 1** and **S3**) revealed signals typical of Diels–Alder type adducts. The spectrum showed three sets of ABX type aromatic protons at δ_H 6.79 (1H, d, *J* = 8.5 Hz, H–33), δ_H 6.21 (1H, d, *J* = 1.5 Hz, H–30), δ_H 6.11 (1H, dd, *J* = 8.5, 2.0 Hz, H–32); δ_H 8.01 (1H, d, *J* = 9.0 Hz, H–27), δ_H 6.23 (1H, d, *J* = 2.5 Hz, H–26), δ_H 6.05 (1H, d, *J* = 2.5 Hz, H–24); δ_H 7.14 (1H, d, *J* = 8.5 Hz, H–6'), δ_H 6.35 (1H, dd, *J* = 8.5, 2.0 Hz, H–5') and δ_H 6.24 (1H, d, *J* = 1.5 Hz, H–3'). Furthermore, protons of an isoprenyl group were observed at δ_H 1.51 (3H, s, H–12), 1.48 (3H, s, H–13), δ_H 2.94 (1H, dd, *J* = 14.5, 9.0 Hz, H–9a), δ_H 2.62 (1H, dd, *J* = 14.5, 6.0 Hz, H–9b) and δ_H 5.12 (1H, t, *J* = 6.5 Hz, H–10). The spectrum showed proton signals of a trisubstituted methylcyclohexene ring at δ_H 1.75 (3H, s, H–17), δ_H 2.32 (1H, dd, *J* = 17.5, 5.0 Hz, H–18a), δ_H 2.21 (1H, dd, *J* = 18.5, 4.5 Hz, H–18b), δ_H 4.10 (1H, br s, H–14), δ_H 3.80 (1H, dd, *J* = 13.5, 6.5 Hz, H–19), δ_H 4.42 (1H, t, *J* = 6.5 Hz, H–20) and an aromatic proton at δ_H 5.57 (1H, s, H–8). The ^{13}C NMR data (**Table 1** and **S4**) and HMBC correlations (**S5**) indicated the methylcyclohexene ring was connected to δ_C 109.5 (C–6) through δ_C 34.3 (C–14) with further substitution at δ_C 49.1 (C–20) and δ_C 36.1 (C–19). Detailed analysis of the 1D and 2D NMR data and comparing to literature confirmed compound **1** to be sanggenon C [10]. The proposed HRESI-MS^n fragmentation pattern is as shown in Figure 2.

Table 1: NMR spectral data for Compound 1 in CD_3OD

Position	δ_H[a] multi. (*J* in Hz)	δ_C[b]
2		92.4
3		103.7
4		189.1
4a		100.9
5		161.7
6		109.5
7		168.4
8	5.57, s	96.3
8a		162.5
9	a. 2.94, dd (14.5, 9.0)	32.7
	b. 2.62, dd (14.5, 6.0)	
10	5.12, t (6.5)	119.1
11		137.5
12	1.51, s	18.4
13	1.48, s	26.3
14	4.10, br s	34.3
15	5.38, br s	123.4
16		134.9
17	1.75, s	24.1
18	2.32, dd (17.5, 5.0)	35.8
	2.21, dd (18.5, 4.5)	
19	3.80, dd (13.5, 6.5)	36.1
20	4.42, t (6.5)	49.1
21		209.2
22		115.2
23		166.3
24	6.05, d (2.5)	103.7
25		166.7
26	6.23, d (2.5)	108.9
27	8.01, d (9.0)	135.1
28		124.0
29		157.2
30	6.21, d (1.5)	104.2
31		157.7
32	6.11, dd (8.5, 2.0)	107.8
33	6.79, d (8.5)	130.0
1'		121.9
2'		161.8
3'	6.24, d (1.5)	99.9
4'		161.8
5'	6.35, dd (8.5, 2.0)	110.1
6'		125.9

[a]Recorded at 500 MHz; [b]Recorded at 125 MHz

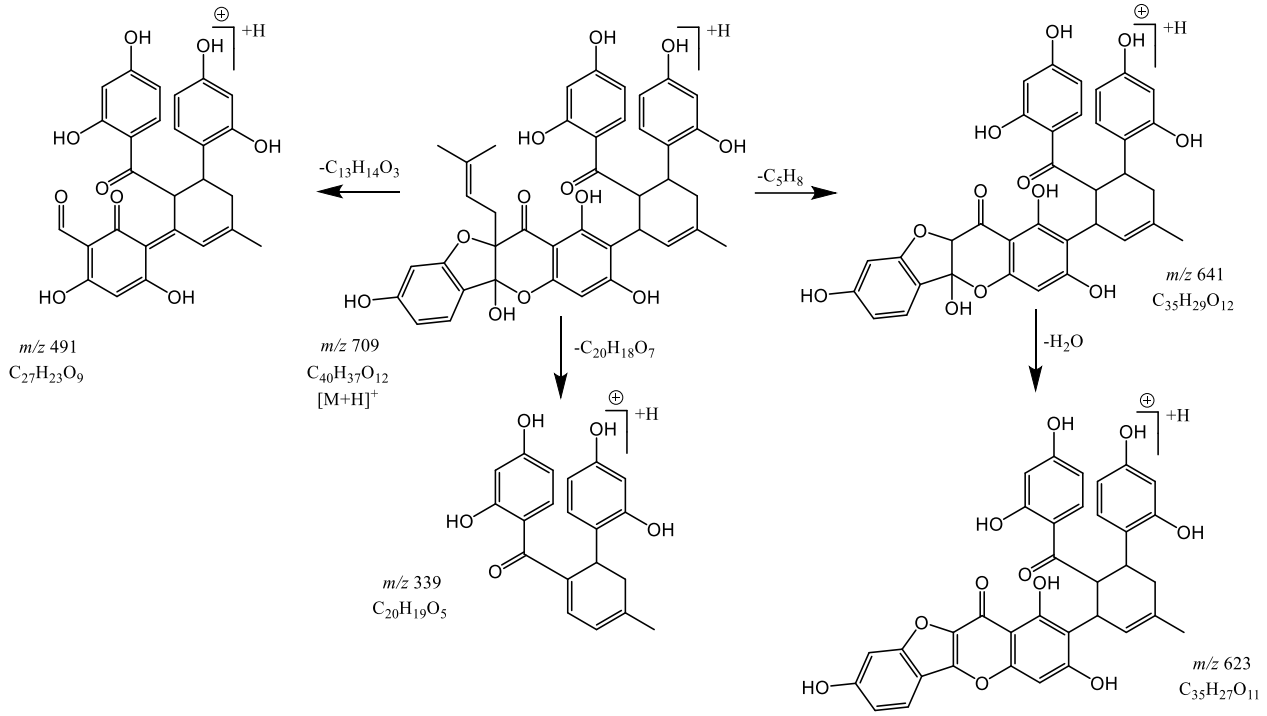

Figure 2 Proposed collision–induced mass spectral fragmentation pattern of compound 1 in positive ESI mode.

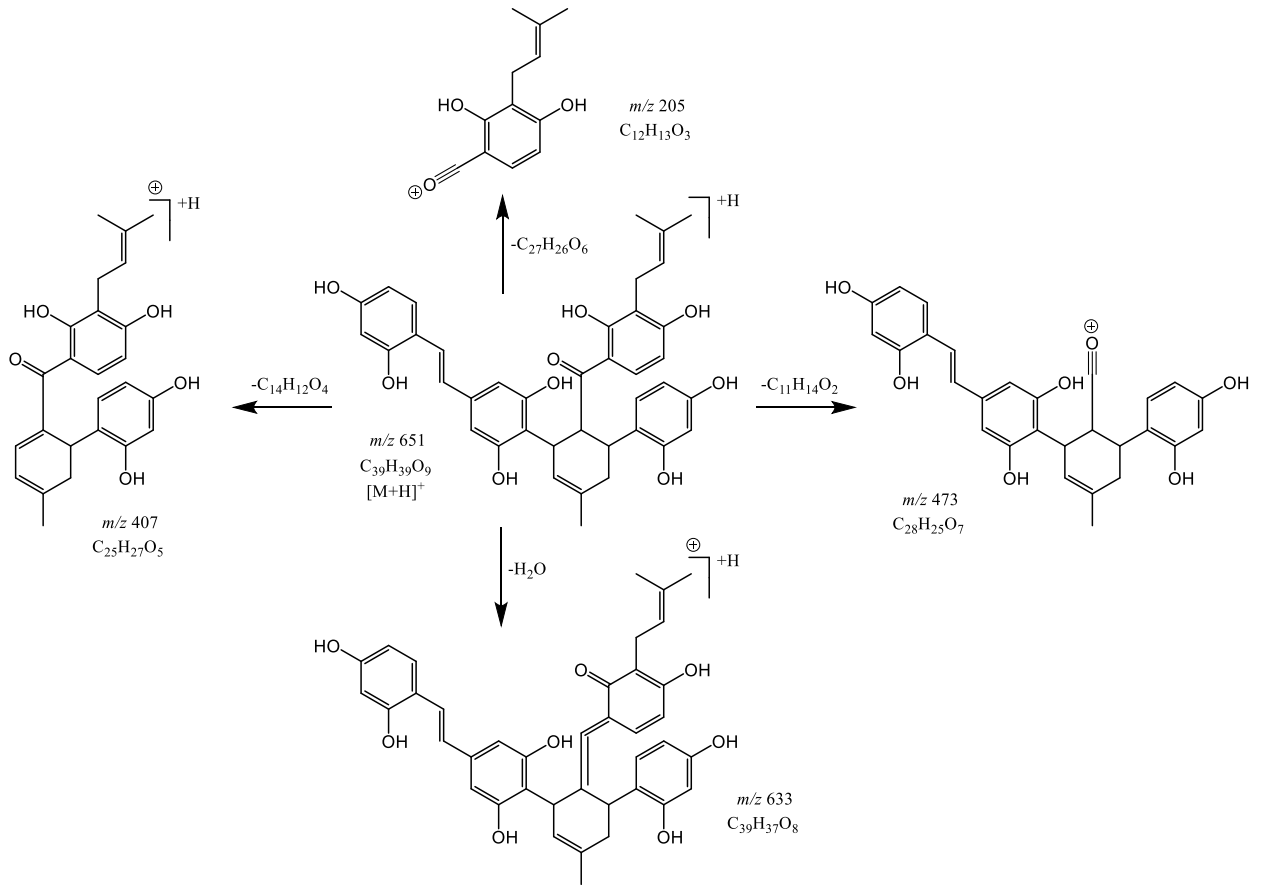

Figure 3. Proposed collision–induced mass spectral fragmentation pattern of compound 2 in positive ESI mode.

Table 2. NMR spectral data for Compounds 2 and 3 in CD_3OD

Position	2		3
	δ_H[a] multi. (*J* in Hz)	δ_C[b]	δ_H[a] multi. (*J* in Hz)
1		118.3	
2		158.1	
3	6.29*, d (3.5)	103.8*	6.76, br s
4		159.4	7.24, d (8.5)
5	6.22**, d (2.5)	108.3**	6.64, dd (8.0, 2.0)
6	7.21, d (9.0)	128.6	
7			6.79, br s
α	7.12, d (16.5)	124.5	
β	6.66, d (16.5)	126.7	
1'		140.0	
2'	6.33, s	107.0	6.64, s
3'		157.9	
4'		116.0	
5'		157.9	
6'	6.33, s	107.0	6.64, s
1''		134.2	
2''	5.65, br s	125.6	5.66, s
3''	3.96, br s	33.9	4.00, br s
4''	4.47, t (5.0)	48.9	4.50, br s
5''	3.62, m	37.4	3.65, m
6''	a. 2.39, d (18.0)	33.2	a. 2.40, d (18.0)
	b. 2.09, d (18.0)		b. 2.12, d (17.5)
7''	1.85, s	24.1	1.86, s
8''		210.6	
9''		114.1	
10''		164.9	
11''		116.5	
12''		164.1	
13''	6.21**, d (2.0)	108.6**	6.29, d (2.5)
14''	8.28, d (9.0)	132.9	8.28, d (9.0)
15''		123.1	
16''		157.5	
17''	6.27*, d (3.0)	103.9*	6.27, d (3.5)
18''		157.3	
19''	6.16, dd (8.5, 2.5)	107.5	6.17, dd (8.5, 2.5)
20''	6.86, d (8.5)	129.2	6.87, d (8.5)
21''	3.18, d (7.0)	22.7	3.17, d (7.0)
22''	5.11, t, (7.0)	123.9	5.10, t, (7.0)
23''		132.1	
24''	1.67, s	18.2	1.66, s
25''	1.56, s	26.2	1.55, s

[a]Recorded at 500 MHz; [b]Recorded at 125 MHz; *Overlapping signals

Compound **2** was isolated as yellow amorphous solid and the molecular formula was assigned as $C_{39}H_{38}O_9$ based on the ion *m/z* 651.2592 $[M + H]^+$ (calcd. for 651.2590) in the HRESI–MS spectrum (**S6**). The HRESI–MS/MS, in positive mode, (**S7**) gave fragment ions at *m/z* 407.1853 $[M + H–C_{14}H_{12}O_4]^+$, *m/z* 271.1329 $[M + H–C_{22}H_{20}O_6]^+$, *m/z* 205.0861 $[M + H–C_{27}H_{26}O_6]^+$, *m/z* 473.1599 $[M + H–C_{11}H_{14}O_2]^+$, *m/z* 633.2476 $[M + H–H_2O]^+$. Similar to compound **1**, the ^1H NMR spectrum (**Table 2** and **S8**) of **2** showed signals typical of Diels–Alder type adducts. Two sets of ABX type aromatic protons were observed at δ_H 7.21 (1H, d, *J* = 9.0 Hz, H–6), δ_H 6.29 (1H, d, *J* = 3.5 Hz, H–3), δ_H 6.22 (1H, d, *J* = 2.5 Hz, H–5); δ_H 6.86 (1H, d, *J* = 8.5 Hz, H–20''), δ_H 6.27 (1H, d, *J* = 3.0 Hz, H–17'') and δ_H 6.16 (1H, dd, *J* = 8.5, 2.5 Hz, H–19''). Also observed were four aromatic protons at δ_H 8.28 (1H, d, *J* = 9.0 Hz, H–14''), δ_H 6.21 (1H, d, *J* = 2.0 Hz, H–13''), δ_H 6.33 (2H, s, H–2'/6') and olefinic protons at δ_H 7.12 (1H, d, *J* = 16.5 Hz, H–α), δ_H 6.66 (1H, d, *J* = 16.5 Hz, H–β). In addition, the spectrum indicated the presence of an isoprenyl moiety with proton signals at δ_H 5.11 (1H, t, *J* = 7.0 Hz, H–22''), δ_H 3.18 (2H, d, *J* = 7.0 Hz, H–21''), δ_H 1.67 (1H, s, H–24'') and δ_H 1.56 (1H, s, H–25''). Proton signals of the trisubstituted methylcyclohexene ring were observed at δ_H 5.65 (1H, br s, H–2''), δ_H 4.47 (1H, t, *J* = 5.0 Hz, H–4''), δ_H 3.96 (1H, br s, H–3''), δ_H 3.62 (1H, m, H–5''), δ_H 2.39 (1H, d, *J* = 18.0 Hz, H–6''a), δ_H 2.09 (1H, d, *J* = 18.0 Hz, H–

6''b) and δ_H 1.85 (1H, s, H–7''). The ^{13}C NMR spectrum (**Table 2** and **S9**) showed signals confirming **2** was a Diels–Alder type adduct. Based on the analyses of the 1D, 2D and comparing the data to reported literature, the structure of **2** was elucidated as kuwanol E [11]. The proposed HRESI-MS^n fragmentation pattern is as shown in Figure 3.

Compound **3** was yellow amorphous powder with a molecular formula of $C_{39}H_{36}O_9$ determined by HRESI–MS spectrum (**S11**) showing a pseudo-molecular ion at *m/z* 649.2437 $[M + H]^+$ (calcd. for 649.2439). The ^{1}H NMR spectrum (**Table 2** and **S12**) were similar to that of compound **2** except for the absence of the olefinic proton signal at δ_H 6.66 and the broad singlet appearance at δ_H 6.84 (1H, br s, H–3) indicating H–3 was not involved in scalar coupling. This observation together with the double bond equivalent (DBE) of twenty–two in **3** suggested the difference in structure between **2** and **3** was an additional ring system. The HRESI–MS/MS spectrum (**S13**) of **3** showed fragment losses similar to that of **2**. Detailed analysis of the ^{1}H NMR, HRESI–MS^n, comparing to reported literature confirmed compound **3** to be the Diels–Alder type adduct chalcomoracin [12]. The proposed HRESI-MS^n fragmentation pattern is as shown in Figure 4.

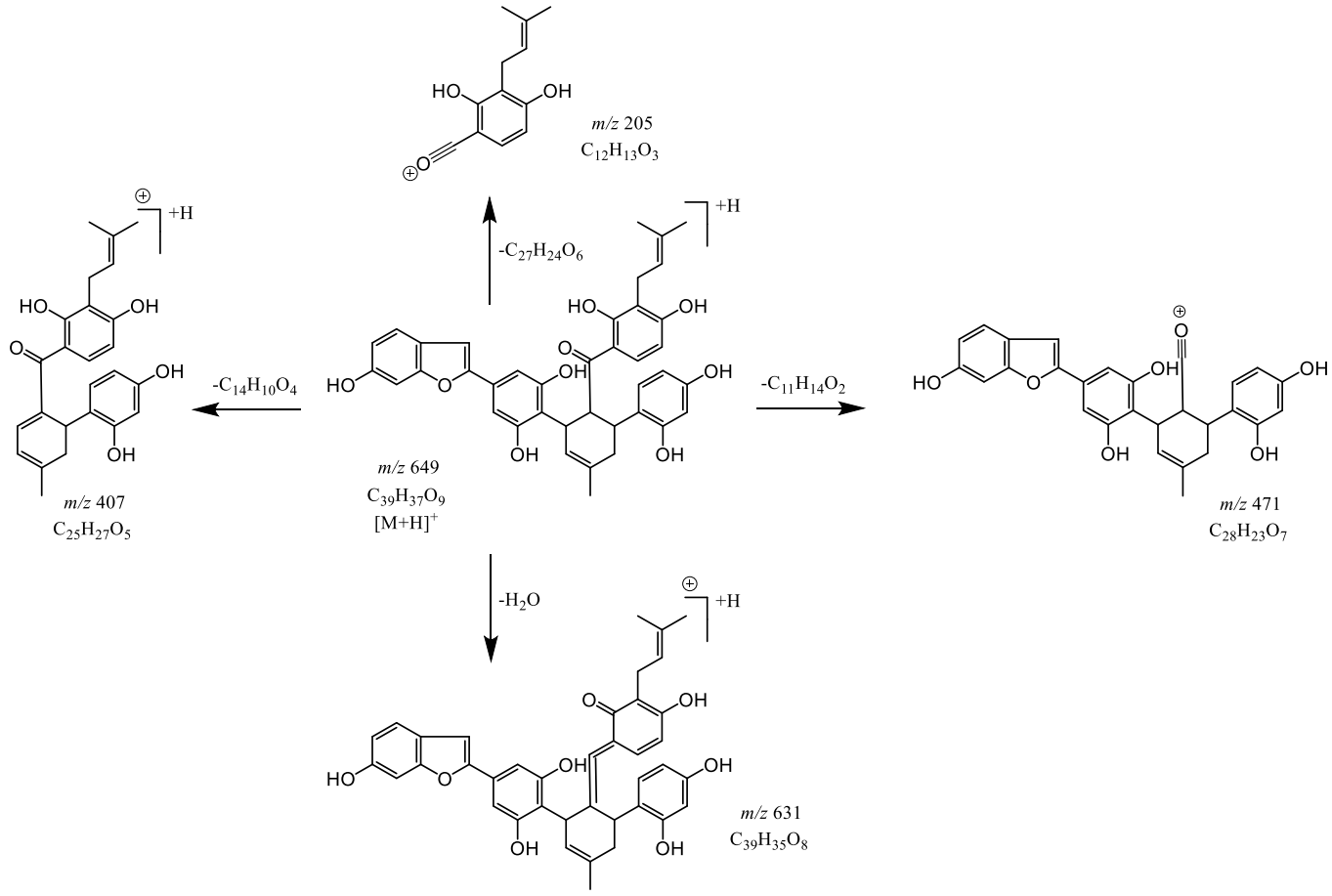

Figure 4. Proposed collision–induced mass spectral fragmentation pattern of compound 3 in positive ESI mode.

Table 3. NMR spectral data for Compounds 4 and 5 in CD_3OD

Position	4		5	
	δ_H[a] multi. (*J* in Hz)	δ_C[b]	δ_H[a] multi. (*J* in Hz)	δ_C[b]
1		138.8		142.3
2	6.39, br s	105.9	6.38, d (2.4)	106.1
3		157.5		159.8
4		115.9	6.06, t (2.4)	102.6
5		157.5		159.8
6	6.39, br s	105.9	6.38, d (2.4)	106.1
1'		118.4		119.4
2'		157.4		154.8
3'	6.21*, m	103.8		117.8
4'		159.3		157.3
5'	6.23*, m	108.6	6.31, d (8.4)	109.4
6'	7.23, d (9.0)	128.5	7.13, d (8.4)	125.1
7'	7.14, d (16.8)	124.0	7.25, d (16.2)	125.3
8'	6.69, d (16.8)	127.0	6.67, d (16.2)	127.3
1''	3.28, d (7.2)	23.5	3.29, d (7.2)	23.7
2''	5.17, tm	125.0	5.15, tm	124.6
3''		135.1		136.1
4''	1.88, m	41.3	1.90, m	41.2
5''	1.99, m	28.1	1.99, m	28.0
6''	5.00, tm	125.9	4.99, tm	125.7
7''		132.3		132.4
8''	1.55, br s	26.2	1.55, br s	26.2
9''	1.49, br s	18.0	1.49, br s	18.0
10''	1.69, br s	16.6	1.71, br s	16.6

[a]Recorded at 600 MHz; [b]Recorded at 150 MHz; *Overlapping signals

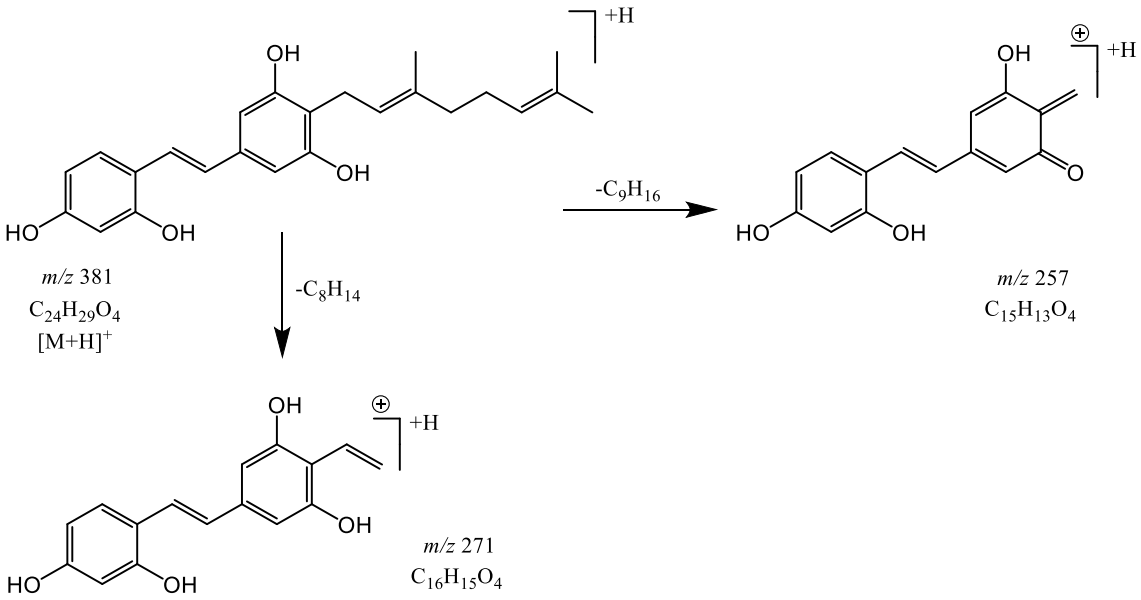

Figure 5. Proposed collision–induced mass spectral fragmentation pattern of compound 4 in positive ESI mode.

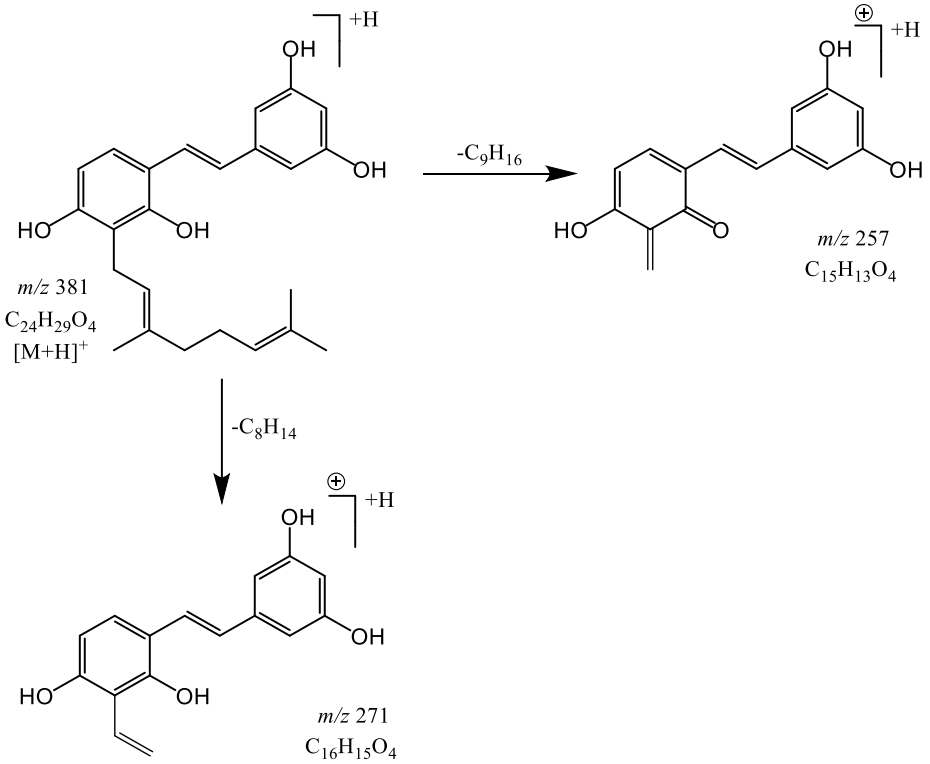

Figure 6. Proposed collision–induced mass spectral fragmentation pattern of compound 5 in positive ESI mode.

Compound **4** was obtained as a yellow amorphous solid. The molecular formula was assigned as $C_{24}H_{28}O_4$ based on the quasi–molecular ion peak at *m/z* 381.2061 $[M + H]^+$ (calcd. for 381.2060) in the HRESI–MS spectrum (**S14**). The fragmentation profile in the HRESI–MS/MS spectrum (**S15**) showed an abundant fragment at *m/z* 257.0808 $[M + H–C_9H_{16}]^+$ and further fragments at *m/z* 271.0963 $[M + H–C_8H_{14}]^+$ and *m/z* 239.0702 $[M + H–C_9H_{16}–H_2O]^+$. Analysis of the ^{1}H NMR spectrum (**Table 3** and **S16**) revealed the presence of a symmetrically substituted aromatic ring at δ_H 6.39 (2H, br s, H–2/6), an ABX pattern aromatic protons at δ_H 7.23 (1H, d, *J* = 9.0 Hz, H–6'), δ_H 6.23 (1H, m, H–5'), δ_H 6.21 (1H, m, H–3') and a *trans*–stilbene at δ_H 7.14 (1H, d, *J* = 16.8 Hz, H–7'), δ_H 6.69 (1H, d, *J* = 16.8 Hz, H–8'). Proton signals typical of a geranyl moiety were observed at δ_H 5.17 (1H, m, H–2''), δ_H 5.00 (1H, tm, H–6''), δ_H 1.99 (2H, m, H–5''), δ_H 3.28 (2H, d, *J* = 7.2 Hz, H–1''), δ_H 1.88 (2H, m, H–4''), δ_H 1.70 (3H, d, *J* = 12.6 Hz, H–10''), δ_H 1.55 (3H, br s, H–8'') and δ_H 1.49 (3H, br s, H–9''). The ^{13}C NMR spectrum (**Table 3** and **S17**) together with HMBC correlations (**S18**) from H–1'' to δ_C 157.5 (C–3/5) and δ_C 115.9 (C–4) confirmed the geranyl is connected to the symmetrically substituted aromatic ring at C–4. Based on the NMR data and comparing to reported data, the structure of **4** was elucidated as chlorophorin [13]. The proposed HRESI-MS^n fragmentation pattern is as shown in Figure 5.

Compound **5** showed the same molecular formula, $C_{24}H_{28}O_4$, as that of **4** in the HRESI–MS spectrum (**S19**). This indicated compounds **4** and **5** were isomers. The HRESI–MS/MS (**S20**) spectrum was closely related to that of **4** with the main fragment at *m/z* 257.0808 $[M + H–C_9H_{16}]^+$ and additional fragments at *m/z* 271.0963 $[M + H–C_8H_{14}]^+$ and *m/z* 239.0702 $[M + H–C_9H_{16}–H_2O]^+$. The ^{1}H NMR spectrum (**Table 3** and **S21**) displayed signals and chemical shifts similar to the ^{1}H NMR spectrum of **4**. HMBC connectivities (**S23**) from δ_H 3.36 (2H, d, *J* = 7.2 Hz, H–1'') to δ_C 155.8 (C–4'), δ_C 153.3 (C–2') and δ_C 116.3 confirmed the geranyl moiety was connected to C–3'. Compound **5** was thus elucidated as isochlorophorin [14]. The proposed HRESI-MS^n fragmentation pattern is as shown in Figure 6.

4 Conclusions

The fragments obtained and proposed fragmentation patterns of the isolated

compounds could be employed qualitatively in the identification of the studied polyphenolic compounds in a matrix thus avoiding the arduous task of isolation before identification.

5 Supplementary Data

Supporting information article can be accessed online. Figure S1 Positive HRESI–MS spectrum of compound **1**, Figure S2 Positive HRESI–MS/MS spectrum of compound **1**, Figure S3 ^{1}H NMR spectrum (CD_3OD, 500 MHz) of compound **1,** Figure S4 ^{13}C NMR spectrum (CD_3OD, 125 MHz) of compound **1**, Figure S5 HMBC NMR spectrum (CD_3OD) of compound **1**, Figure S6 Positive HRESI–MS spectrum of compound **2**, Figure S7 Positive HRESI–MS/MS spectrum of compound **2**, Figure S8 ^{1}H NMR spectrum (CD_3OD, 500 MHz) of compound **2**, Figure S9 HMBC NMR spectrum (CD_3OD) of compound **2**, Figure S10 HMBC NMR spectrum (CD_3OD) of compound **2**, Figure S11 Positive HRESI–MS spectrum of compound **3**, Figure S12 ^{1}H NMR spectrum (CD_3OD, 500 MHz) of compound **3**, Figure S13 Positive HRESI–MS/MS spectrum of compound **3**, Figure S14 Positive HRESI–MS spectrum of compound **4**, Figure S15 Positive HRESI–MS/MS spectrum of compound **4**, Figure S16 ^{1}H NMR spectrum (CD_3OD, 600 MHz) of compound **4**, Figure S17 ^{1}H NMR spectrum (CD_3OD, 150 MHz) of compound **4**, Figure S18 HMBC NMR spectrum (CD_3OD) of compound **4**, Figure S19 Positive HRESI–MS spectrum of compound **5**, Figure S20 Positive HRESI–MS/MS spectrum of compound **5**, Figure S21 ^{1}H NMR spectrum (CD_3OD, 600 MHz) of compound **5**, Figure S22 ^{13}C NMR spectrum (CD_3OD, 150 MHz) of compound **5**, Figure S23 HMBC NMR spectrum (CD_3OD) of compound **5**.

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7 Conflicts of Interest

The authors declare no conflict of interest.

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