SUPPLEMENTARY MATERIAL

Gymnocarpos decandrus Forssk: A new promising drug for diabetes management

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Abstract:

Gymnocarpos decandrus Forssk. is a well-known grazing wild plant. This study targets scientific validation of its claimed antidiabetic activity and exploring its bioactive metabolites. Chromatographic purification of *G. decandrus* ethanol extract (GDEE) allowed isolation of vitexin (**C1),** protocatechuic acid (**C2)** and quercetin (**C3)**. HPLC-PDA-MS/MS enabled identification of nineteen metabolites; 13 flavonoids, 5 saponins, and 1 phenolic acid in *G. decandrus* and four in the genus *Gymnocarpos* for the first time. The antidiabetic potential was evaluated *via* testing the *Coxsackie B4* virus and *α*–glucosidase inhibitory potentials. C3 exhibited its potent antiviral activity through blocking of the virus attachment (96.28%, SI 4.41) and virus inactivation before adsorption (91.47%, SI 4.78). GDEE and C1-C3 showed dose dependent *α*–glucosidase inhibitory activity with IC_{50} of 733.9, 293.3, 118.1 and 69.1 µg/mL respectively. Our study represents the sole complete map for *G. decandrus* secondary metabolites and presents it as promising drug for diabetes management.

Key words: *Gymnocarpos decandrus*, Caryophyllaceae, *Coxsackie B4* virus, *α*–Glucosidase enzyme, flavonoids, HPLC- PDA-MS/MS.

Experimental:

Plant material

Flowering aerial parts of *G. decandrus* Forssk. were collected during the flowering stage from the western Mediterranean coastal region (Alexandria- Mersa Matrooh road 80- 140 km.) during April, 2018. The plant was identified by Prof. Dr. Azza El Hadidy, Professor of Taxonomy and Flora- Herbarium, Faculty of Science, Cairo University. Voucher specimen of the plant under investigation was deposited with code no. (7.12.15.1) at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

General

General UV lamp (Spectroline Model CM-10, New York, USA) was used for location of fluorescent spots. UV spectra were determined in methanol and after addition of different reagents on a Hewlett Packard 8452A diode array spectrophotometer in the region of 200-500 nm. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (75 MHz) were measured on Varian MercuryVX-300 NMR spectrophotometer. NMR spectra were recorded in CD3OD, DMSO-d6 and chemical shifts were given in δ (ppm) relative to TMS as internal standard. For column chromatography (Scazzocchio *et al.*, 2011), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), silica gel 60 (Fluka, 70-230 mesh ASTM, Germany) were used. Thin-layer chromatography (TLC) was performed on silica gel GF254 precoated plates (Fluka, Germany). TLC plates were visualized under visible and UV light before and after exposure to ammonia vapor. *Para*-anisaldehyde sulfuric acid and aluminum chloride spray reagents were used. Standards for co-TLC: several flavonoids and phenolic acids were obtained from Fluka, Sigma, Germany.

Extraction and isolation of the major phenolics

Air dried flowering aerial parts of *G. decandrus* Forssk. (1.5 kg) was powdered then extracted by maceration in ethanol (70 %) and filtered off. The combined *G.*

decandrus ethanol extract (Sharma *et al.*, 2016) was concentrated under reduced pressure at temperature not exceeding 50 ºC till dryness. The dry extract (80 g) was subjected to fractionation by solvents of increasing polarities, concentrated and dried, to yield 12.2 g, 2.1 g, 4.95 g and 22 g of *n*-hexane, methylene chloride, ethyl acetate and remaining aqueous fractions respectively.

The ethyl acetate fraction was fractionated on polyamide column and eluted with gradient elution of water and ethanol. Fractions of (500 ml each) were collected, concentrated and monitored by TLC. Similar fractions were combined together yielding two major sub-fractions A and B.

Sub-fraction A which was eluted using ethanol 20% and re-fractionated on sephadex LH-20 using distilled water as eluent. Further purification on preparative (TLC) using ethyl acetate: methanol: water 30:5:4 resulted in the isolation of single pure compound **C1** (35 mg), after purification on silica column (50 X 1.5 cm), using methylene chloride.

Sub-fraction B which was eluted using ethanol 40-50 % was then applied on sephadex LH-20 using saturated *n*-butanol as eluent yielding two fractions. (F_{B1} 0.12 g & F_{B2} 0.3 g) containing two compounds **C2** and **C3**, respectively with several minor spots. F_{B1} was subjected to preparative paper chromatography (PC) using system *n*- butanol: acetic acid: water (4:1:5). Further purification on sephadex LH-20 using isocratic elution with water yielded pure compound **C2** (19 mg). Compound **C3** (27 mg) was isolated from F_{B2} after applying double run descending preparative PC using acetic acid: water (15: 85).

HPLC-PDA-MS/MS metabolites profiling:

A Thermo Finnigan HPLC system using a Discovery HS F5 bonded phase column (Discovery HS F5, 15cm x 4.6mm ID, 5µm particles, 4.6 × 150 mm, 3 µm, Sigma-Aldrich, Germany) was used. Water and acetonitrile (ACN) (Sigma-Aldrich GmbH, Germany) (0.1 % formic acid each) were used as a mobile phase. At 0 min,

ACN was 5% and then increased to 30% in 60 min at 1 mL/min with a 1:1 split before the ESI source. Autosampler survey or Thermo Quest was utilized to inject GDEE and the system was controlled by Xcalibur software (Xcalibur TM 2.0.7, Thermo Fischer Scientific, Waltham, Ma, USA). Thermo Finnigan LCQ-Duo ion trap mass spectrometer (Thermo Electron Corporation, Waltham, Ma, USA) with an ESI source (Thermo Quest Corporation, Austin, Tx, USA) was used. The MS was operated in the negative mode so that the phenolics and flavonoids could be readily ionized (Okba, El Gedaily, and Ashour 2017; Ashour et al. 2019). The ions were detected in a full scan mode and mass range of 50–2000 *m/z*.

Biological evaluation

Cytotoxicity

The MTT colorimetric assay was performed as described (Elaissi et al. 2012; Takeuchi, Baba, and Shigeta 1991). Cytotoxicity assessment is based on the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), by mitochondrial dehydrogenase of viable cells, to give a blue formazan product measured at 540 nm. Every day the cells were examined using a phase-contrast microscope to determine the minimum concentration that caused alterations in cell morphology which is the maximum non-toxic concentration (MNTC). Optical density (OD) was measured at 540 nm using a Perkin-Elmer ELISA reader (HTS 7000 plus) (Goodger and Woodrow 2011). The cytotoxicity % was calculated as [(A - B)/A] x 100, where A & B were the optical density of untreated and treated cells, respectively.

The sample concentration that caused 50% reduction in the cell viability (CC_{50}) when compared to untreated controls was determined graphically. Standard graph was plotted using different concentrations of tested samples on X axis and cell viability on Y axis.

Cell viability (%) = Mean OD/Control OD x 100%.

Antiviral activity

Cell culture and virus.

The Vero cell line (*Cercopithecus aethiops* kidney epithelial cells) was maintained as described by (Okba, El Gedaily, and Ashour 2017). *Coxsackie* (*CoxB4*) virus was provided by Dr. Mohammed Ali, Laboratory of virology, Science Way for scientific researches and consultations, Faculty of medicine, Al-Azhar University, Egypt.

Antiviral Protocols

In order to study the mechanism of the antiviral activity of the tested samples; the virus and the Vero cell cultures were treated with the MNTC of the tested samples and their serial dilution according to the following three protocols (Gong 2013; Ocazionez et al. 2010; Gescher et al. 2011):

protocol A (virus pretreatment):

To test the virucidal activity of the tested samples this protocol was applied. Virus was exposed to the tested samples for one hr at 37°C. Then 100 µL of the mixture were added to the cells cultured fluently in 96-well flat-bottom microtiter plate.

protocol B (cell pretreatment):

This protocol was used to test the virus entry into the host cells by blocking attachment to the cell surface through incubation of the tested samples on Vero cells for one hour before the virus was added.

protocol C (post infection treatment):

This protocol was designed to test the effect of the tested samples on virus replication; incubation of the virus on Vero cells for one hour then the tested samples were added.

Evaluation of infected and non-infected cells viability was done using the absorbance values of formazan used in the MTT assay, as described previously. The 50% inhibition concentration (IC_{50}) and the selectivity index ($SI = CC_{50}/IC_{50}$) were calculated. Each experiment was repeated six times.

α ***-Glucosidase inhibitory activity***

α-Glucosidase (*Saccharomyces cerevisiae*) and 3, 5, di-nitro salicylic acid (DNS) were purchased from Sigma-Aldrich. *p*-nitro-phenyl-α-D-glucopyranoside (*p*-NPG), sodium carbonate, sodium dihydrogen phosphate and di-sodium hydrogen phosphate were purchased from Hi-Media.

The inhibitory activity of GDEE and the isolated compounds was carried out according to (Shai et al. 2011) with minor modification . Reaction mixture containing 50 µl phosphate buffer (100 mM, pH=6.8), 10 µl *α*-glucosidase and 20 µl of varying concentrations of each sample (1000 to 7.81 *µ*g/mL) was preincubated for 15 min at 37 °C in a 96-Well plate. Then, 20 µl P-NPG (5 mM) was added as a substrate and incubated further at 37 °C for 20 min. 50 µl Na_2CO_3 (0.1 M) were added to stop the reaction. The absorbance of the released *p*-nitrophenol was measured using Multiplate Reader at 405 nm. Acarbose at various concentrations (1000 to 7.81 *µ*g/mL) was included as a standard. Without test substance was set up in parallel as a control and each experiment was performed in six replicates. The results were expressed as inhibition percentage which was calculated as following:

Inhibitory activity (%) = $(1 - As/Ac) \times 100$

As: absorbance in the presence of tested sample

Ac: absorbance of control.

Statistical analysis

The data were analyzed using analysis of variance (Krasteva et al.). Determination of the significance of differences between means was done at $p < 0.05$ using Duncan's multiple range tests.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest statement:

The authors wish to declare that no conflicts of interest associated with this publication and there has been no financial support for this work.

Table (S1): ^{1}H-NMR and ^{13}C-NMR spectroscopic data for compounds C1-C3

No.	C1		C2		C3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1				118.58		
2		**167.58**	7.33 (1H, d, J= 3.0)	115.11		147.91
3	6.63 (1H, s)	104.85		148.45		136.52
4		**181.29**		154.91		176.58
5		**160.29**	6.61 (1H, d, *J* = 8.6)	115.12		161.37
6	6.32 (1H, s)	99.11	6.73 (1H, dd, J=3.0, 8.6)	119.79	6.19 (1H, d, *J* =1.6)	98.51
7		**164.32**		165.34		166.01
8		107.42			6.39 (1H, d, *J* =1.6)	94.57
9		**157.33**				156.78
10		107.02				104.01
1'		123.66				123.15
2'	7.94 (2H, d, *J* = 8.6)	129.61			7.64 (1H, d, *J* =1.5)	116.23
3'	6.91 (2H, d, *J* = 8.6)	118.64				145.72
4'		**161.58**				148.11
5'	6.91 (2H, d, *J* = 8.6)	118.64			6.89 (1H, d, *J* =8.5)	116.57
6'	7.94 (2H, d, *J* = 8.6)	129.61			7.75 (1H, dd, *J* =1.5, 8.5)	122.41
1''		75.43				
2''		71.07				
3''		79.32				
4''		71.50				
5''		81.54				
6''		61.44				

Table (S2): HPLC-MS/MS detected secondary metabolites in *G. decandrus* aerial parts

Pk. no.	R_t (min)	$[M-H]^-$	Fragment ions	Tentatively identified compounds	Class
1	2.44	289.02	271, 247, 245, 205, 179	(epi)catechin	fla-3-ol
2	2.78	783.34	741, 723, 633, 591, **315**	isorhamnetin acetyl-*O*-dipentosyl-*O*-hexoside	fl-*O*-gly.
3	3.06	637.26	595, 577, 463, **301**	quercetin -acetyl-*O*-pentosyl - *O*- hexoside.	fl-*O*-gly.
4	3.41	609.40	591, 477, **315**	isorhamnetin-*O*- pentosyl-*O*- hexoside	fl-*O*-gly.
5	3.53	1221.53	1203, 1089, 957, 811, 679, **503**	tetrahydroxyoleanen-*O*-dipentosyl-*O*-rhamnosyl- *O*-pentosyl- *O*-hexuronide	tr. sap.
6	4.00	1235.65	1219, 1089, 957, 679, **503**	tetrahydroxyoleanen *O*- rhamnosyl- *O*-pentosyl-*O*- rhamnosyl- *O*- pentosyl- *O*- hexuronide	tr. sap.
7	4.12	651.23	609, 591, 477, **315**	isorhamnetin -acetyl-*O*-pentosyl -*O*-hexoside	fl-*O*-gly.
8	4.99	735.29	693, 651, 609, 591, 477, **315**	isorhamnetin -triacetyl- *O*-pentosyl-*O*-hexoside	fl-*O*-gly.
9	5.17	825.27	783, 765,741, 723, 693, 675, 609, **315**	isorhamnetin-diacetyl-di-*O*-pentosyl-*O*-hexoside	fl-*O*-gly.
10	5.31	1263.50	1131, 721,679, **503**	tetrahydroxyoleanen acetyl-tri-*O*-pentosyl-*O*-rhamnosyl- *O*-hexuronide	tr. sap.
11	5.49	187.20	187, 169, 125	hydroxygallic acid	ph. acid
12	5.70	1219.60	1201, 941, 663, **487**	trihydroxyoleanen di-*O*- pentosyl- di- *O*-rhamnosyl- *O*- hexuronide	tr. sap.
13	5.79	679.19	637, 619, 595, 463, **301**	quercetin diacetyl-*O*-pentosyl-*O*-hexoside	fl-*O*-gly.
14	6.07	1219.42	1087, 955, 809, 663, **487**	trihydroxyoleanen di-*O*-pentosyl, di-*O*-rhamnosyl-*O*- hexuronide	tr. sap.
15	7.24	693.26	633, 609, 591, 477, **315**, 271	isorhamnetin diacetyl -*O*-pentosyl, *O*-hexoside	fl-*O*-gly.
16	7.37	431.15	413, 341, 311, 285, 269	(iso) vitexin	fl-*C*-gly.
17	10.14	721.17	702, 679, 637, 631, 595, 463, **301**	quercetin triacetyl *O*-pentosyl-*O*-hexoside	fl-*O*-gly.
18	19.73	299.17	299, 284, 271, 255, 243	gliricidin/ methylorobol	iso.agly.
19	25.82	283.02	283, 268, 255, 239, 227, 220	Calycosin	iso.agly.

fla-3-ol: flavan-3-ol; fl-*C*-gly.: flavone-*C*-glycoside; fl-*O*-gly.: flavonol-*O*-glycoside; iso. agly.: isoflavonoid aglycone; ph. acid.: phenolic acid; Pk.: peak; tr. sap.: triterpenoidal saponin

Table (S3): Cytotoxicity effect of *G. decandrus* Forssk. and its isolated compounds on Vero cells

	Conc. (µg/mL)	O.D* ± S.E		Toxicity %	CC_{50} µg/mL
Vero		0.25	0.0056	0	
GDEE	10000	0.01	0.0010	95.56	441.38
	5000	0.01	0.0009	94.49	
	2500	0.02	0.0009	90.19	
	1250	0.04	0.0048	83.33	
	625	0.07	0.0081	70.16	
	313	0.15	0.0023	40.99	
	156.25	0.21	0.0009	13.58	
	78.12**	0.25	0.0023	0.27	
C1	1000	0.01	0.0015	95.83	22.04
	500	0.01	0.0006	94.35	
	250	0.01	0.0009	94.22	
	125	0.02	0.0012	92.74	
	62.5	0.03	0.0053	89.52	
	31.25	0.07	0.0058	70.83	
	15.63	0.14	0.0041	45.43	
	7.81**	0.24	0.0015	2.55	
C2	1000	0.01	0.0007	95.7	23.54
	500	0.01	0.0012	95.97	
	250	0.01	0.0012	94.49	
	125	0.02	0.0012	93.15	
	62.5	0.03	0.0028	88.58	
	31.25	0.08	0.0015	67.88	
	15.63	0.16	0.0030	36.96	
	7.81**	0.24	0.0044	2.55	
C3	1000	0.01	0.0006	96.37	23.86
	500	0.01	0.0018	95.3	
	250	0.01	0.0009	94.22	
	125	0.02	0.0034	91.8	
	62.5	0.03	0.0028	87.23	
	31.25	0.09	0.0012	65.05	
	15.63	0.15	0.0066	41.4	
	7.81**	0.25	0.0021	0	

* Mean of six determinations; **: maximum nontoxic concentration (MNTC); C1: vitexin; C2**:** protocatechuic acid; C3: quercetin; CC_{50}, concentration that kill 50% of Vero cells, conc., concentrations; S.E., standard error; O.D., optical density; GDEE: *G. decandrus* ethanol extract.

Table (S4): Inhibitory effect of GDEE and C1-3 on *α*-glucosidase enzyme.

Conc.		Acarbose	GDEE	C1	C2	C3
1000		90.10±0.58	53.24±1.5	59.31±1.5	67.32±1.2	72.65±1.5
500		86.34±1.2	47.15±1.3	53.01±2.1	60.48±2.5	68.28±0.92
250		71.34±1.5	29.18±2.1	49.37±0.58	54.18±0.58	61.08±1.5
125	µg/mL	63.42±2.1	12.34±0.58	41.32±0.92	51.32±1.6	56.85±1.6
62.5		60.14±0.72	0	37.25±0.63	39.24±0.58	49.18±0.63
31.25		50.31±1.5	0	29.32±2.1	25.73±1.5	31.25±0.58
15.63		43.28±1.2	0	17.16±1.3	10.34±0.92	18.68±1.5
7.81		32.15±0.58	0	6.28±1.6	0	8.32±2.1
IC_{50}	µg/mL	30.57	733.90	293.30	118.17	69.18
	mM			0.678	0.767	0.229

C1: vitexin; C2**:** protocatechuic acid; C3: quercetin

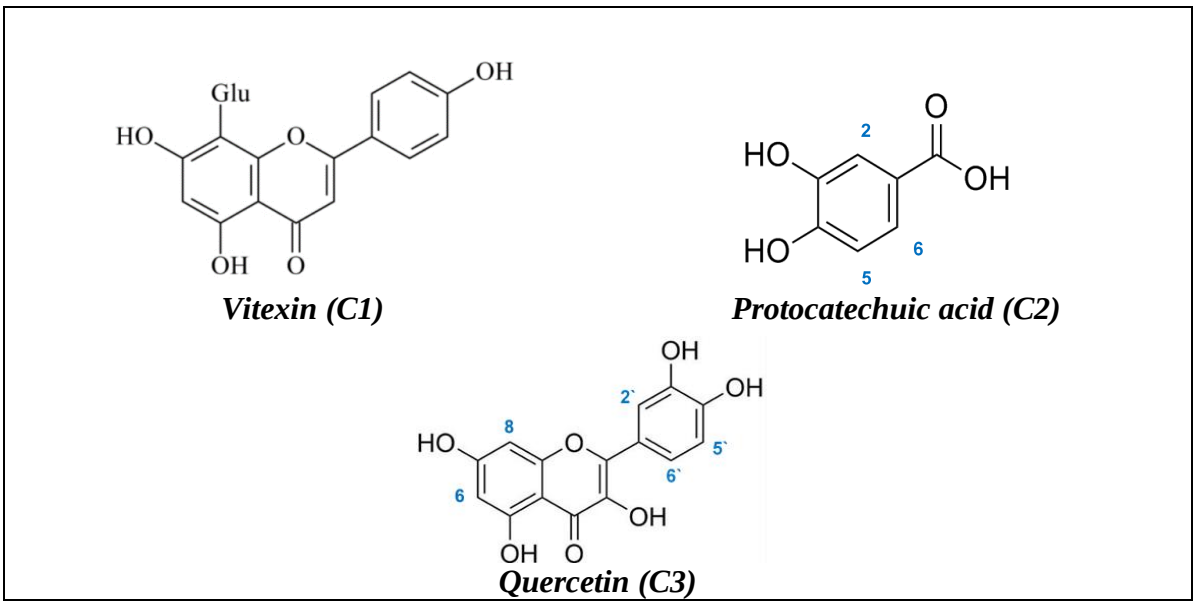

Fig. (S1): Structures of *G. decandrus* Forssk. aerial parts isolated compounds

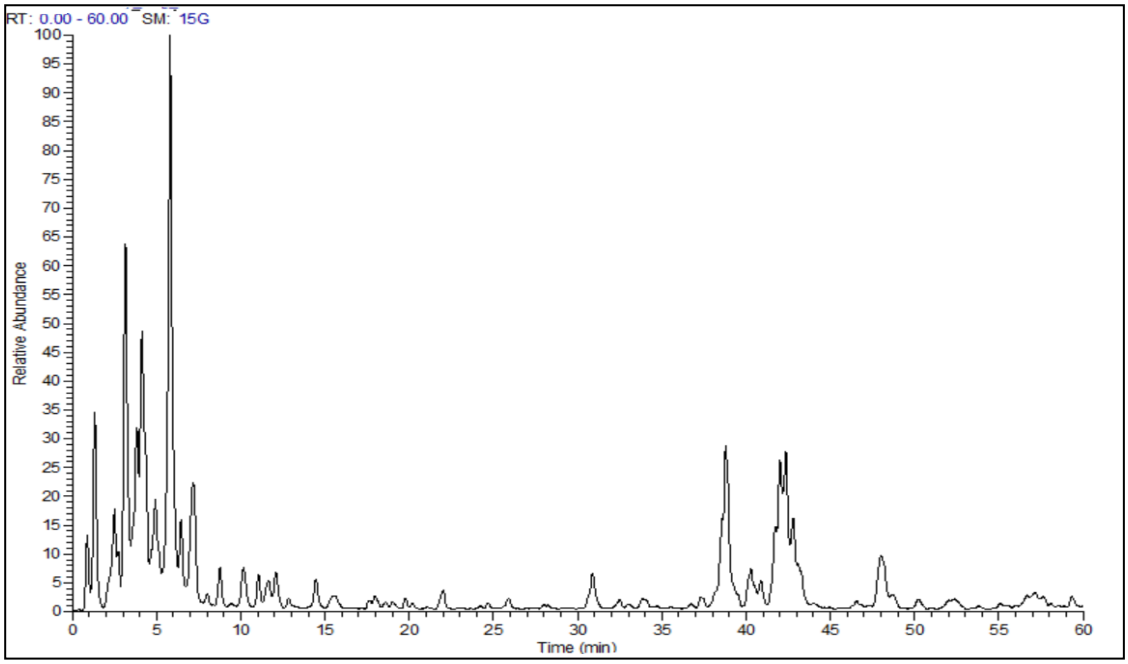

Fig. (S2): LC base peak chromatogram of ethanol extract of *G. decandrus* Forssk. aerial parts

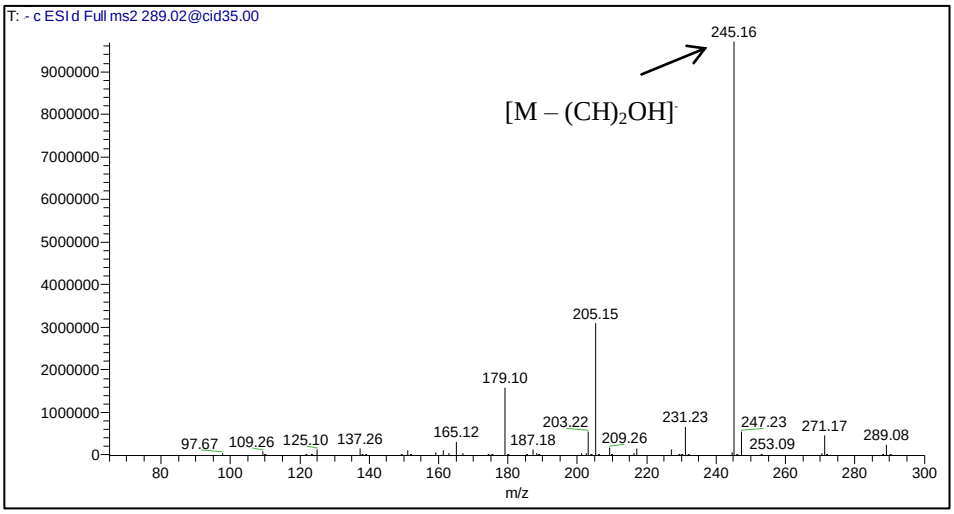

Fig. (S3): MS/MS chromatogram of peak 1

Pk.1 was the only identified flavan-3-ol. Fragment ions at *m/z* 245, 205, and 179 characteristic for catechin were observed (Sobeh et al. 2018).

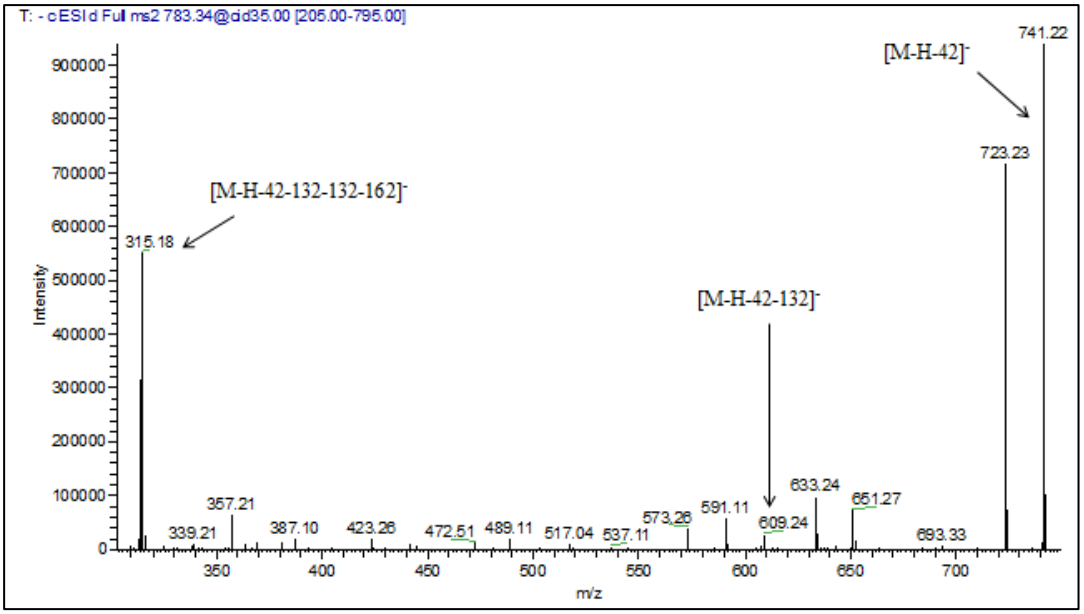

Fig. (S4): MS/MS chromatogram of peak 2

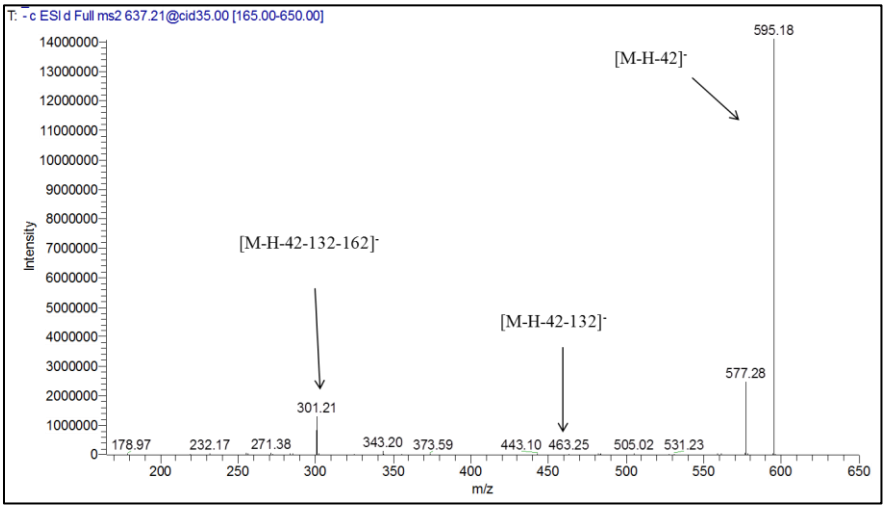

Fig. (S5): MS/MS chromatogram of peak 3

Pk. 3 showed $[M-H]^-$ at *m/z* 637.26 yielded upon fragmentation daughter ions at *m/z* 595 $[M-H-42]^-$, 463 $[M-H-132]^-$, and 301 $[M-H-162]^-$ due to loss of acetyl, pentose, and hexose moieties respectively. It was tentatively identified as quercetin acetyl-*O*-pentosyl-*O*-hexoside. Pks. 13 and 17 with $[M-H]^-$ at *m/z* 679.19 and 721.17 showed molecular weight higher than pk. 3 by 42 and 42x2 Da which in turn means extra acetyl groups. The fragmentation pattern of these two pks. 13 and 17 showed fragments at *m/z* 679 and 637 due to loss of 2 acetyl moieties (2x42 amu) in addition to same fragments observed in pk. 3 (at *m/z* 595, 463 and 301) which confirmed their identification as acetyl derivatives of pk. 3 (Cuyckens and Claeys 2004; Bechlem et al. 2017)

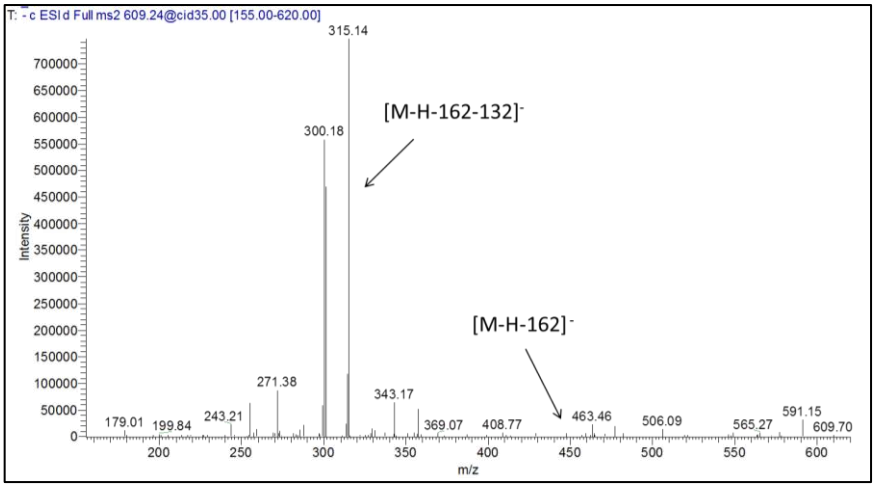

Fig. (S6): MS/MS chromatogram of peak 4

Pk. 4 was characterized by $[M-H]^-$ at *m/z* 609.40 and upon fragmentation produced daughter ion at *m/z* 477 $[M-H-132]^-$ due to loss of pentose moiety, as well as *m/z* 315 $[M-H-132-162]^-$ due to loss of pentose and hexose moieties. It was tentatively identified as isorhamnetin-*O*-pentosyl-*O*-hexoside (Bechlem et al. 2017). Pks. 2 and 9 $[M-H]^-$ at *m/z* 783.34, and 825.27 respectively showed molecular weight exceeds that of pk.4 by 132+42 and 132+(42x2) which in turns indicated acetylated derivatives of isorhamnetin-*O*-dipentosyl-*O*-hexoside (Bechlem et al. 2017).

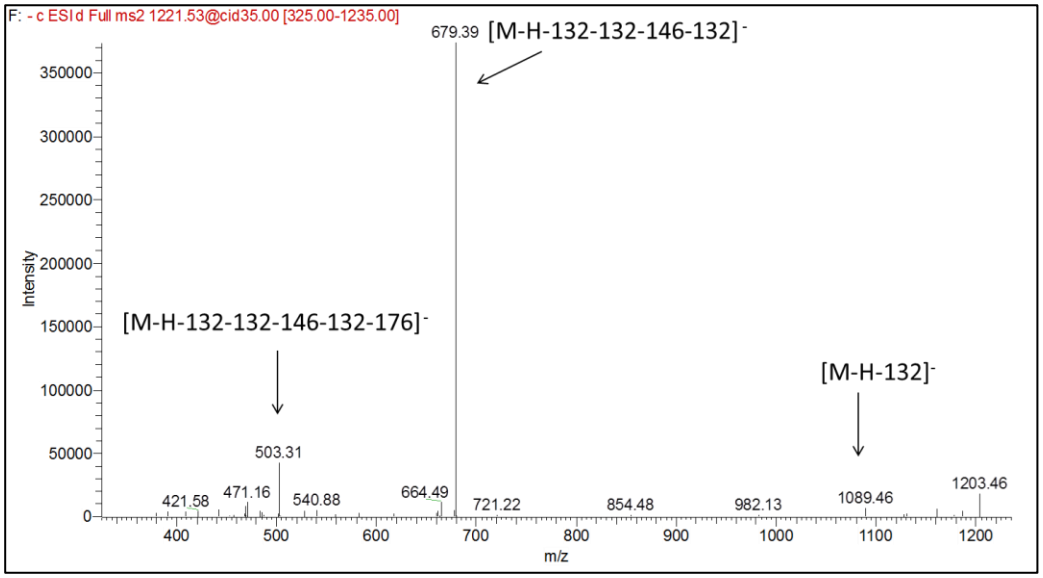

Fig. (S7): MS/MS chromatogram of peak 5

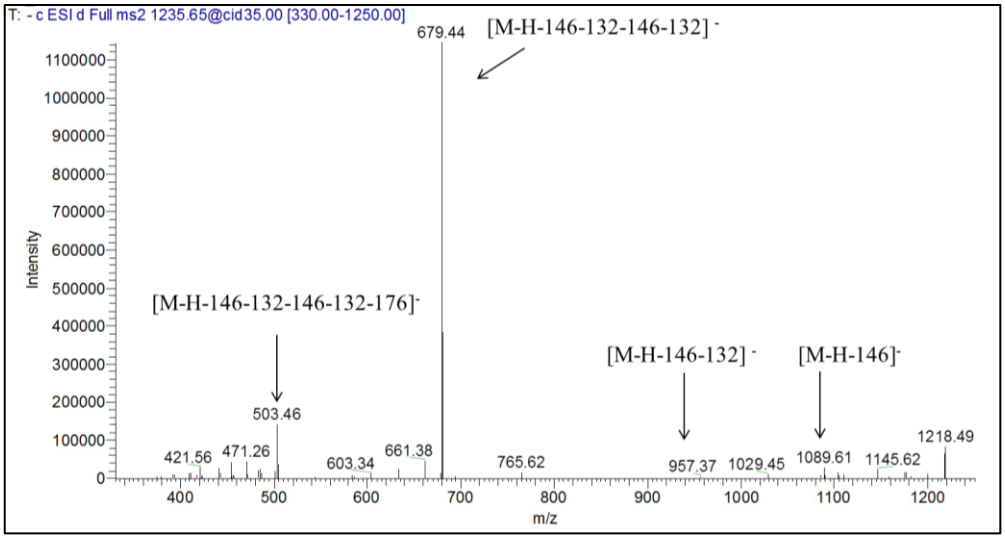

Fig. (S8): MS/MS chromatogram of peak 6

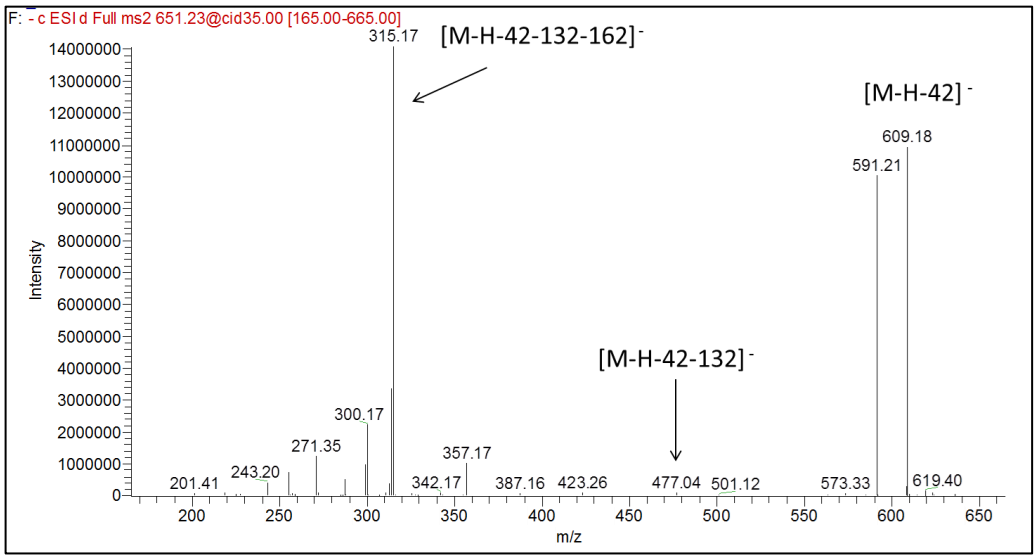

Fig. (S9): MS/MS chromatogram of peak 7

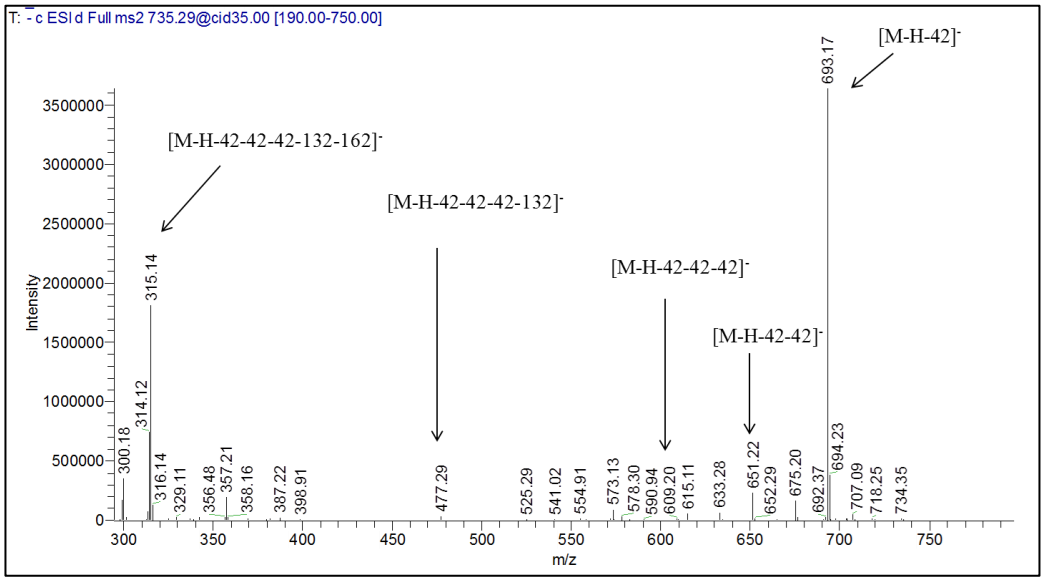

Fig. (S10): MS/MS chromatogram of peak 8

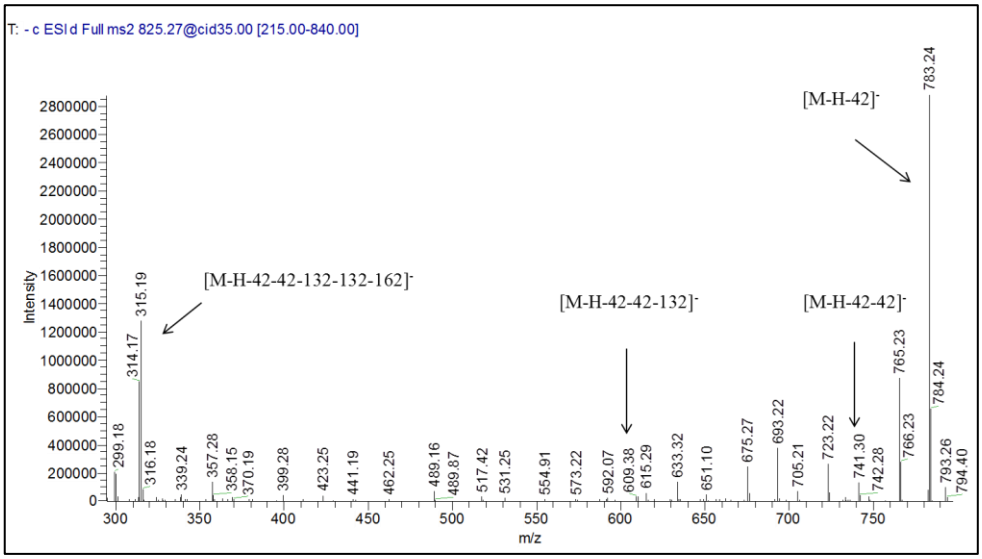

Fig. (S11): MS/MS chromatogram of peak 9

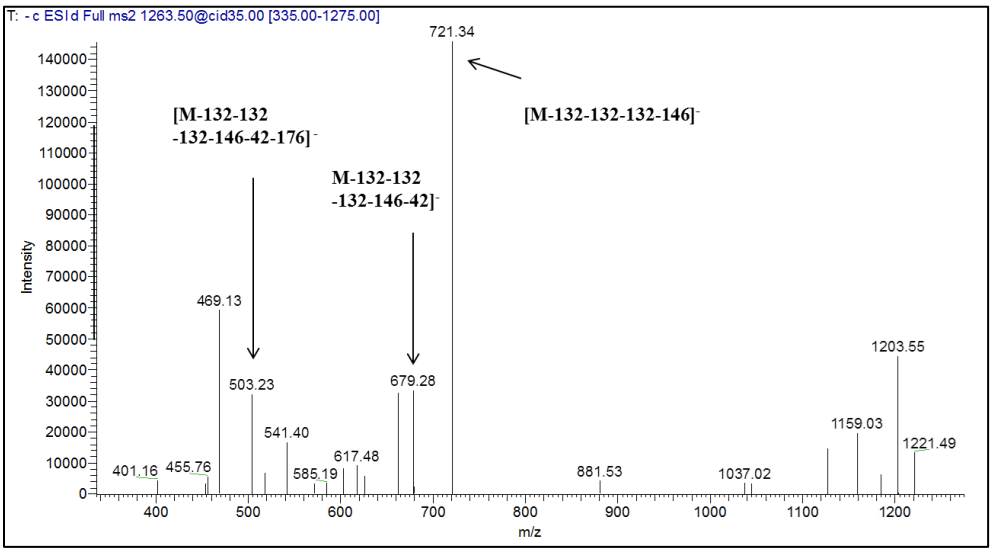

Fig. (S12): MS/MS chromatogram of peak 10

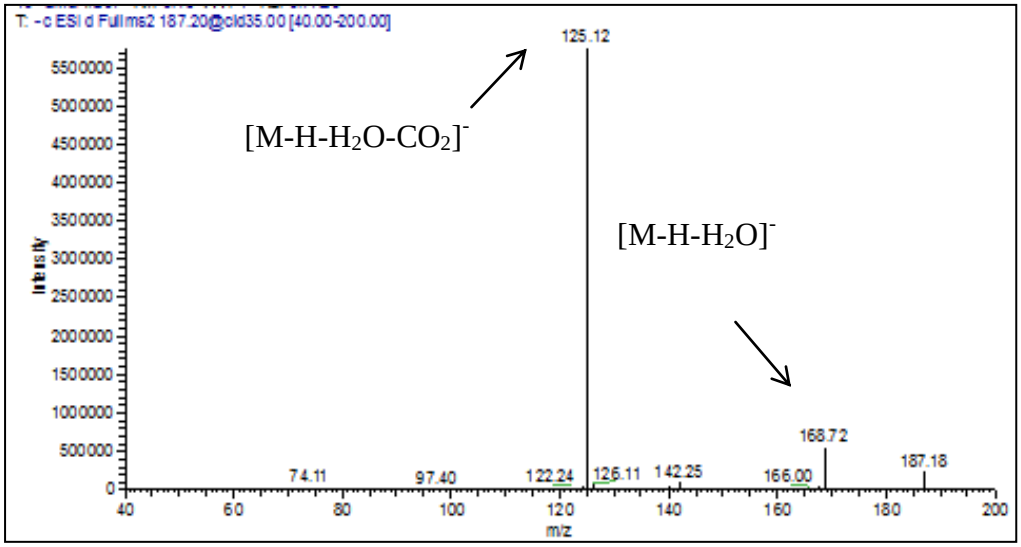

Fig. (S13): MS/MS chromatogram of peak 11

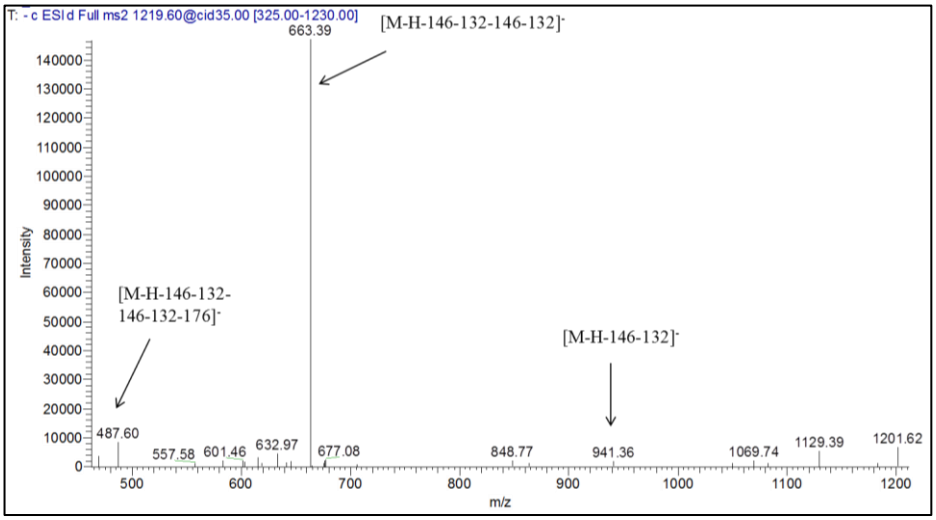

Fig. (S14): MS/MS chromatogram of peak 12

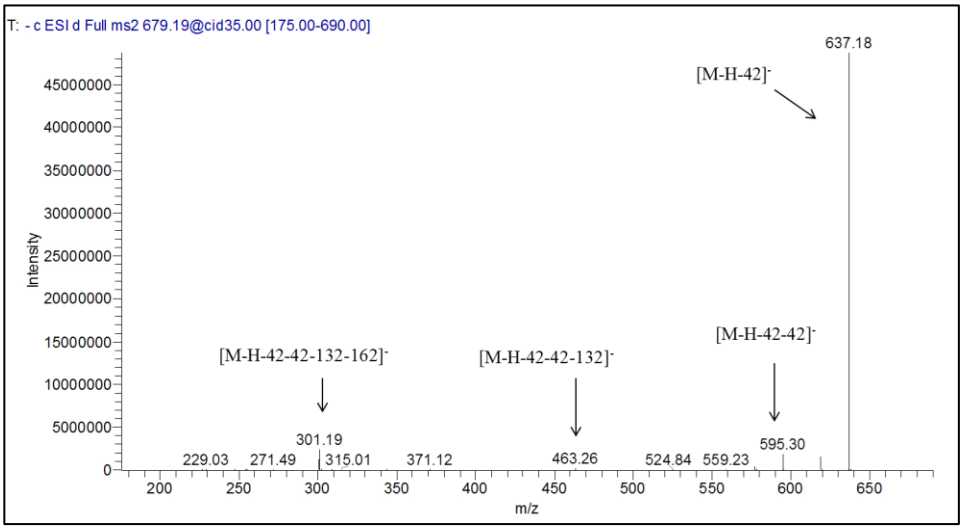

Fig. (S15): MS/MS chromatogram of peak 13

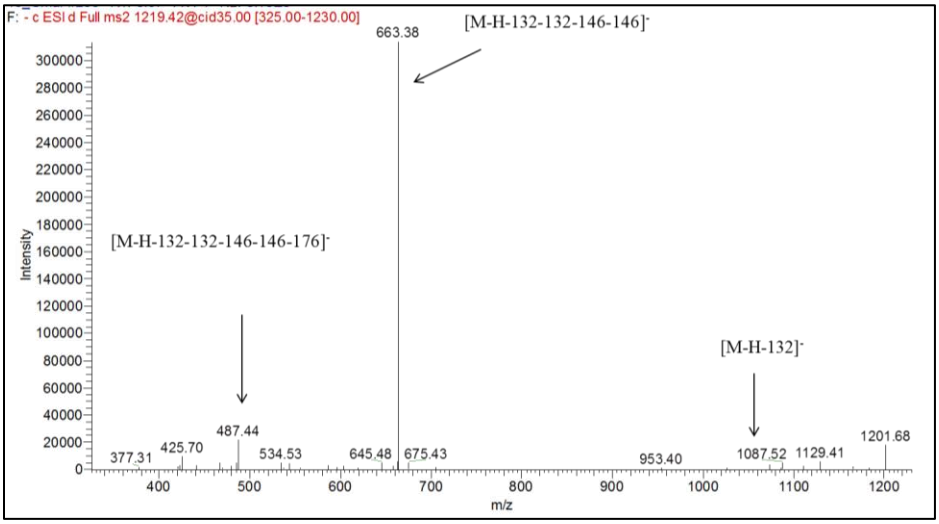

Fig. (S16): MS/MS chromatogram of Peak 14

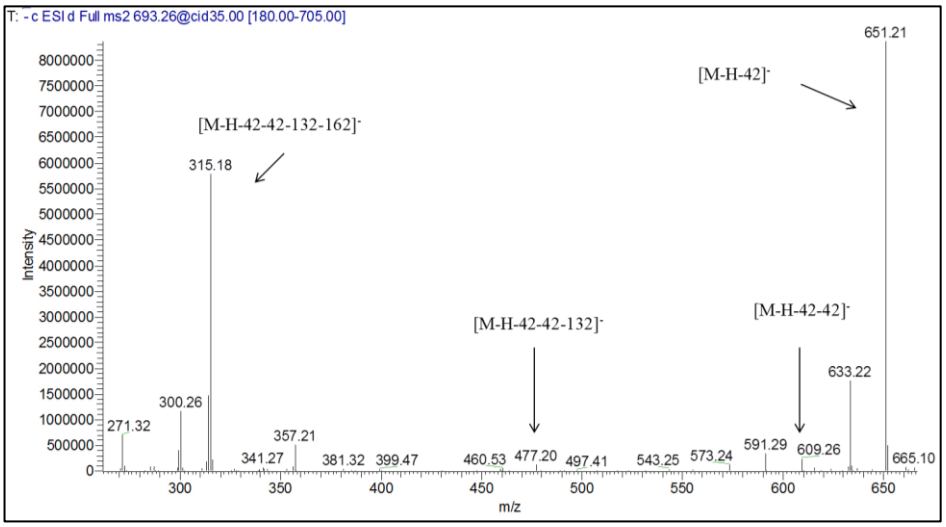

Fig. (S17): MS/MS chromatogram of peak 15

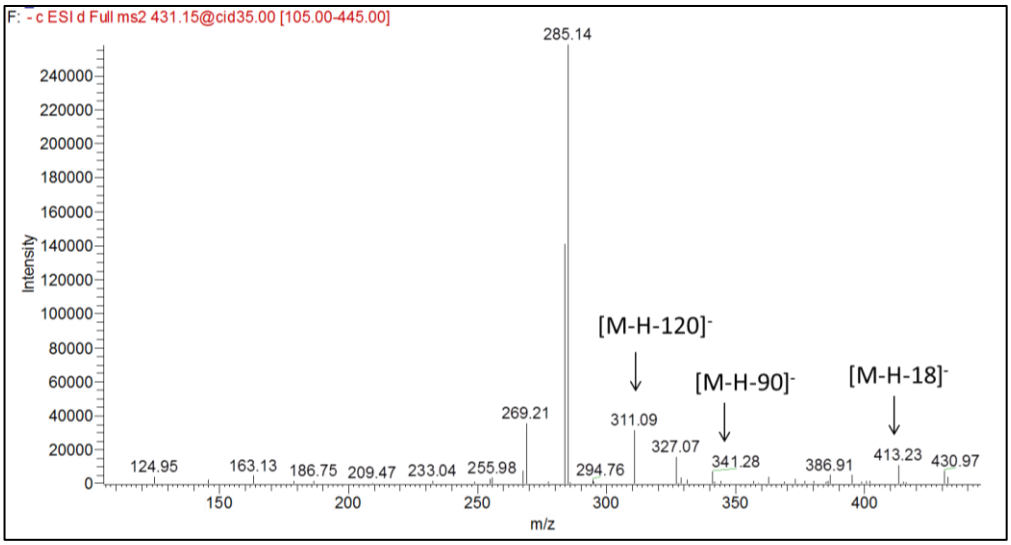

Fig. (S18): MS/MS chromatogram of peak 16

Pk. 16, the only detected flavone glycoside, $[M-H]^-$ at *m/z* 431.15 showed fragment ions at *m/z* 413 $[M-H-18]^-$, 341 $[M-H-90]^-$, 311 $[M-H-120]^-$. This loss of 90 and 120 Da indicating *C*-glycoside, as well as, fragment at *m/z* 269 Da corresponding to apigenin aglycone (Ibrahim et al. 2015; Ferreres et al. 2007). It was tentatively identified as (iso) vitexin.

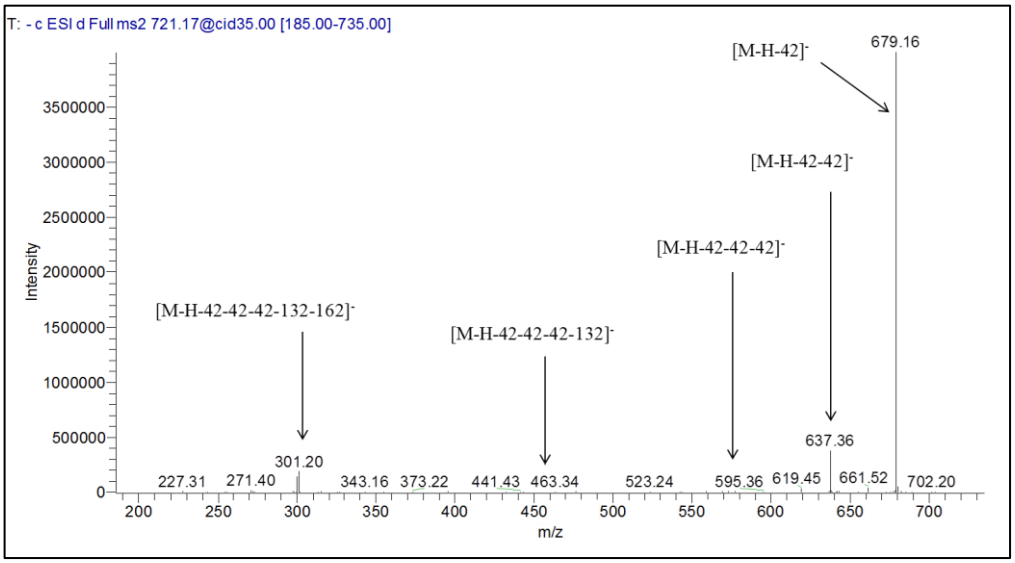

Fig. (S19): MS/MS chromatogram of peak 17

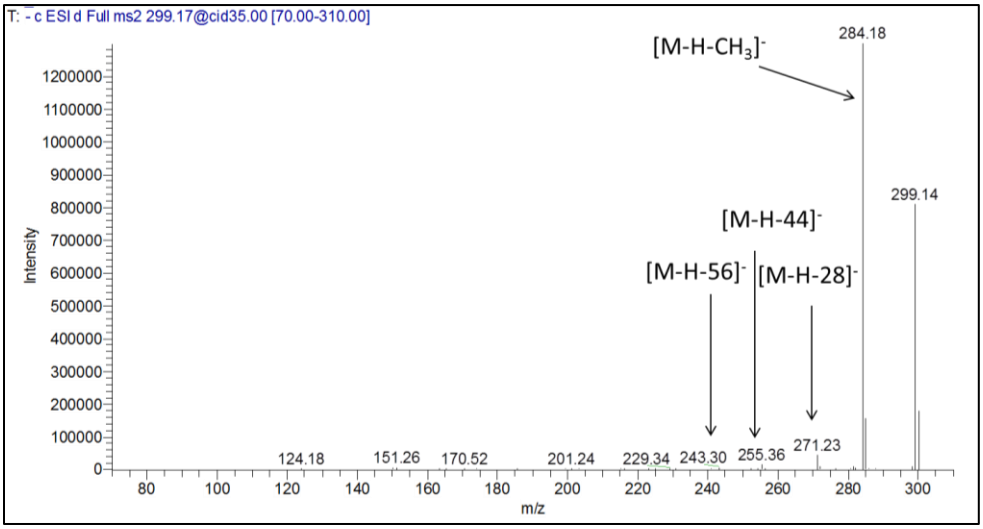

Fig. (S20): MS/MS chromatogram of peak 18

Isoflavones gave a series of regular neutral losses of 28 Da, 44 Da, 56 Da, which could be attributed to CO, CO_2 and 2CO respectively (Ye et al. 2012). MS/MS spectra of pks. 18 and 19 showed fragments corresponding to $[M-H-CO]^-$, $[M-H-CO_2]^-$, and $[M-H-2CO]^-$ at *m/z* 271, 255, 243 and 255, 239, 227 respectively. Additionally, their MS/MS spectra showed base peaks at *m/z* 299 and 268 $[M-H-CH_3]^-$, suggesting the presence of a methoxy group. Pks. 18 and 19 were tentatively identified as gliricidin/methylorobol and calycosin respectively (Ye et al. 2012).

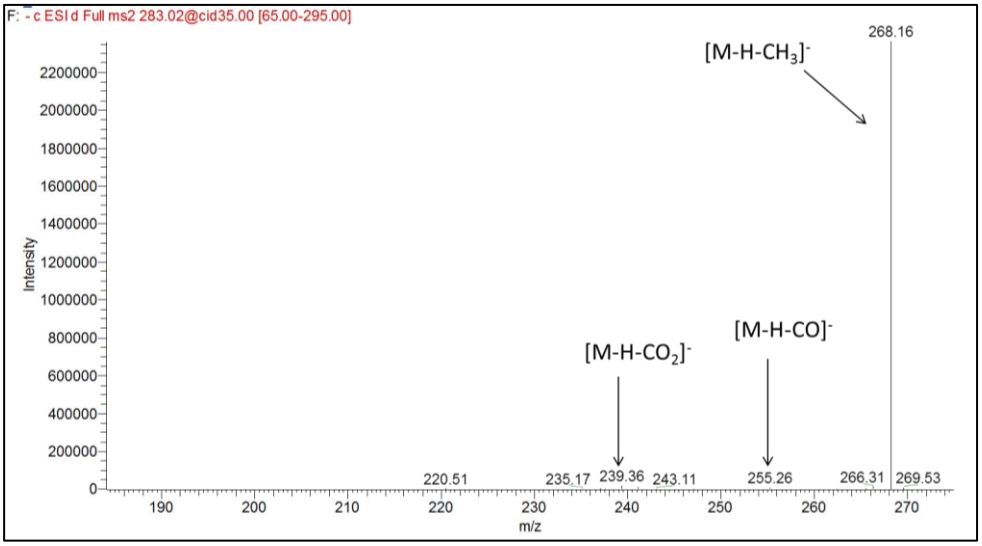

Fig. (S21): MS/MS chromatogram of peak 19

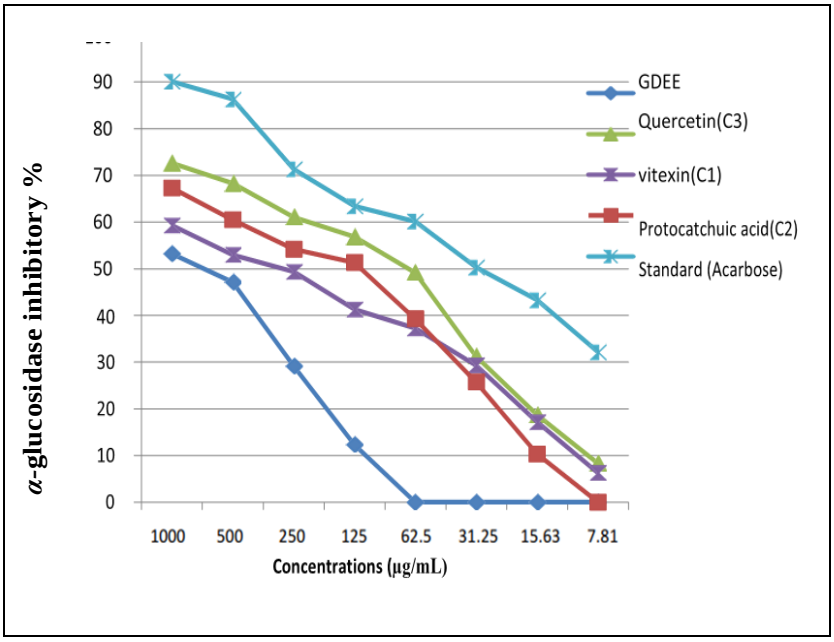

Fig. (S22): Inhibitory effect of GDEE and the isolated compounds on *α*-glucosidase enzyme

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