SUPPLEMENTARY MATERIAL

Secondary metabolites from *Verbascum bugulifolium* Lam. and their bioactivities

Ayşe Gökmen[a], Norbert Kúsz[b], Nursenem Karaca[c], Fatih Demirci[d,e], Judit Hohmann[b], Hasan Kırmızıbekmez[a,*]

[a]Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, TR-34755, Kayışdağı, Ataşehir, İstanbul, Turkey, [b]Department of Pharmacognosy, Faculty of Pharmacy, University of Szeged, 6720 Szeged, Hungary, [c]Department of Pharmacognosy, Graduate School of Health Sciences, Anadolu University, 26470 Eskisehir, Turkey, [d]Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey, [e]Faculty of Pharmacy, Eastern Mediterranean University, 99450-Famagusta, N.Cyprus

*Corresponding author

Prof. Hasan Kırmızıbekmez

Yeditepe University, Faculty of Pharmacy,

Department of Pharmacognosy, TR-34755, Kayışdağı, Ataşehir, İstanbul, Turkey

e-mail: hasankbekmez@yahoo.com

ABSTRACT

Five iridoid glycosides catalpol (**1**), specioside (**2**), ajugol (**3**), ajugoside (**4**), 8-*O*-acetylharpagide (**5**), two phenylethanoid glycosides, verbascoside (**6**) and glucopyranosyl-(1→G_i-6)-martynoside (**7**), four flavonoids, luteolin (**8**), luteolin 7-*O*-β-glucopyranoside (**9**), luteolin 7-*O*-rutinoside (**10**), apigenin 7-*O*-rutinoside (**11**), quercetin 3-*O*-rutinoside (**12**) and β-sitosterol **(13)** were isolated from the aerial parts of *Verbascum bugulifolium* Lam. for the first time. Their structures were elucidated by NMR and MS experiments. The extracts, and the isolates were evaluated for their *in vitro* antioxidant (DPPH•, ABTS• and CUPRAC), anti-inflammatory (LOX inhibition) and antimicrobial activities. Compounds **6** and **8** showed the highest antioxidant activity in all tests, where luteolin (**8**) showed the relatively best anti-inflammatory activity compared to other samples with 54.1 ±5.0 % inhibition at 1 µg/mL. All the tested compounds showed weak antimicrobial activity against tested microorganisms. This is the first phytochemical and bioactivity study on *V. bugulifolium*.

KEYWORDS*: Verbascum bugulifolium* Lam.; Scrophulariaceae; secondary metabolites, antimicrobial, antioxidant and anti-inflammatory activities

---

Part of this work was presented at the 30th International Symposium on the Chemistry of Natural Products and the 65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 3-7 September 2017. Basel, Switzerland, 2017, Poster No: PO-98.

1. Experimental

1.1. General

NMR spectra were recorded in CD_3OD and $CDCl_3$ on a Bruker Avance DRX 500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR. The signals of the deuterated solvents were taken as references. The chemical shift values were shown in ppm in the NMR spectra. HRESIMS data were recorded on a Thermo Scientific Q-Exactive Plus Orbitrap mass spectrometer equipped with ESI ion source in positive ionization mode. Fractions were monitored by TLC analyses on silica gel 60 F_{254} precoated plates (Merck, Darmstadt, Germany), and spots were visualized under UV light (254/366 nm) or by spraying with 1% vanillin/H_2SO_4 followed by heating at 100 °C. For Medium-Pressure Liquid Chromatographic (MPLC) separations, Sepacore® Flash Systems X10/X50 (Buchi Labortechnik AG, Flawil, Switzerland) system was used with Redi sep columns (LiChroprep C_{18}: 130, 100 and 30 g; SiO_2: 12 and 24 g, Teledyne Isco). Column chromatography (CC) was performed with Polyamide (Sigma–Aldrich, St. Louis, MO, USA), and Sephadex LH-20 gel (Sigma–Aldrich, St. Louis, MO, USA).

1.2. Plant material

The aerial parts of *Verbascum bugulifolium* L. were collected from from Kayışdağı, İstanbul, Turkey (40°58'28.2"N 29°09'15.3"E) in May 2015. A voucher specimen (YEF 15003) is deposited at the Herbarium of the Faculty of Pharmacy, Yeditepe University, İstanbul, Turkey. Plant material was identified by HK. The aerial parts of plant were dried at shadow and fresh air at room temperature.

1.3. Extraction, isolation and structure elucidation

The air-dried and powdered aerial parts of *V. bugulifolium* (300 g) were extracted twice with MeOH (2 x 1.2 L) at 45 °C. The combined methanolic extracts were evaporated to dryness in vacuo (92.88 g, 30.96 %). The crude extract was dissolved in H_2O and partitioned EtOAc, *n*-BuOH and $CHCl_3$, respectively. The lyophilized EtOAc phase (590 mg) was subjected to C_{18}- medium pressure liquid chromatography (C_{18}-MPLC, 130 g) eluting with increasing amount of MeOH in H_2O (15– 80%) to afford 3 fractions, 1–3. Fraction 2 (250 mg) was likewise subjected to C_{18}-MPLC (43 g) (20–70%, MeOH) to afford fraction 2i (17.4 mg). Compound **8** (6 mg) was purified by Sephadex LH-20 CC (6 g) (MeOH) from fraction 2i.

The lyophilized *n*-BuOH phase (7.25 g) was fractionated over polyamide CC, using MeOH (0→100%) as eluent and afforded seven main fractions, A-G. An aliquant of Fr. A (2 g) was subjected to C_{18}-MPLC (150 g) eluting with increasing amount of MeOH in H_2O (0→50%) to yield fraction A_7. Purification of fraction A_7 (23.2 mg) by MPLC (SiO_2, 4 g) eluting with CH_2CI_2-MeOH mixture yielded **3** (6.9 mg). An aliquot of fr. A (2.4 g) was subjected to silica gel CC (120 g) eluting with CH_2Cl_2–MeOH–H_2O (90:10:1→61:32:7) to afford fr. A_9 (98 mg) was subjected to C_{18}-MPLC (15.5 g) (MeOH, 5→70%) to yield **4** (5.1 mg). Fr. A_{10} was further applied to C_{18}-MPLC (43 g) (MeOH, 0-60%) to obtain **7**. Fr. A_{11} was similarly subjected to C_{18}-MPLC (43 g) (MeOH, 0-60%) to purify **5**. Fr. B (798.3 mg) was applied to silica gel (80 g) CC using CH_2Cl_2–MeOH–H_2O (95:15:0 to 61:32:7) mixtures to yield a mixture of 1 (43 mg), fractions B_2 and B_5. Fraction B_5 was purified to yield **11** (2 mg) by C_{18}-MPLC (15.5 g) (MeOH, 5-30%). Fr. B_2 was subjected to silica gel (12 g) CC using CH_2Cl_2–MeOH (95:5 and 90:10) to afford fr. B_{2b} (33 mg) which was further separated by C_{18}-MPLC (15.5 g) (MeOH, 20-50%) to obtain **2** (43 mg). Fr. C (103.1 mg) and D (120.6 mg) were combined (223.7 mg) and applied to C_{18}-MPLC (43 g) (MeOH, 15-70%) to obtain **6** (5.3 mg) and fraction CD_2. Fraction CD_2 (36.9 mg) was subjected to MPLC (SiO_2, 12 g) eluting with increasing amount of CH_2CI_2-MeOH (90:0→60:40) to obtain fraction CD_{2b}.

Fraction CD_{2b} (9 mg) was further purified by Sephadex LH-20 CC (MeOH, 6 g) to give a mixture of **10** and **12** (9.8 mg). Compound **9** (7.9 mg) was purified by Sephadex LH-20 CC (MeOH, 10 g) from fraction G (147.6 mg).

The $CHCI_3$ extract (2.74 g) was fractionated over Sephadex LH-20 (100 g) using MeOH as eluent and afforded 3 fractions, 1-3. Fraction 2 was applied to silica gel (100 g) CC using CH_2Cl_2–MeOH (95:5-60:40) mixtures. Fraction 2e (433.9 mg) was further subjected to silica gel (50 g) CC using CH_2Cl_2–MeOH (97.5:2.5→80:20) mixtures. Fraction 2e2 (365.7 mg) was similarly applied to silica gel (40 g) CC using *n*-hexane-EtOAc (80:20-30:70) mixtures. Fraction 2_{e2a} (209.9 mg) was further subjected to silica gel (25 g) CC using *n*-hexane-EtOAc (95:5-65:35) mixtures. Compound **13** (6.6 mg) was purified by silica gel (CC) (25 g) using *n*-hexane-EtOAc (100:0-67.5:22.5) mixtures.

1.4. Antioxidant Activity

1.4.1. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The *in vitro* $DPPH^{\bullet}$ activities of the samples (**1-9, 11, 13**) were tested. In the scavenging activity of the extracts and isolated compounds against DPPH radical, samples were dissolved in DMSO and the concentrations were adjusted 0.5-0.001 mg/mL. Then, 100 mL of 80 µg/mL DPPH were added. The mixture was shaken and incubated at room temperature for 60 min. After incubation, the absorbance was measured at 517 nm with UV spectrophotometer. Ascorbic acid was used as the positive control. The % inhibition values were calculated, and the results were given as IC_{50} as shown in Tables S1 and S3. All tests were repeated in triplicate (Blois, 1958, Kumarasamy et al, 2002).

1.4.2. ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] radical anion scavenging assay

Samples **1-9**, **11**, **13** were tested for their $ABTS^{\bullet}$ activities. 7 mM ABTS, which was prepared with 0.1 M pH 7.4 phosphate buffer and 2.45 mM sodium persulfate ($Na_2S_2O_8$) mixed at the ratio of 1:2 in the dark at room temperature for 12 h on the shaker. The absorbance of the ABTS solution at 734 nm was 0.700±0.025 nm. The working concentration range of the samples is 0.5-0.001 mg / mL. 150 µL of the sample was added to 30 µL ABTS solution and allowed to incubate at room temperature in the dark for 30 min. Ascorbic acid was used as standards. The absorbance was measured at 734 nm and the results as IC_{50} are shown in Tables S1 and S3. All tests were repeated in triplicate (Re et al, 1999).

1.4.3. Cupric reducing antioxidant capacity (CUPRAC) assay

Samples **1-9**, **11**, **13** were tested for their CUPRAC activities. 50 µL copper(II) chloride ($CuCI_2$) solution (0.01 M), 50 µL ammonium acetate (NH_4Ac) buffer at pH=7.0 and 50 µL neocuproine solution ($7.5x10^{-3}$ M) were added to a 27.5 µL test samples (0.5-0.001 mg/mL). And this solution was shaked in the dark at the room temperature for 30 min. Their A_{450} values were measured, and results are shown as IC_{50} in Tables S1 and S3. All tests were repeated in triplicate (Dasgupta, 2014: Apak et al, 2007).

1.5. In vitro anti-inflammatory activity: 5-Lipoxygenase (5-LOX) enzyme activity inhibition assay

Samples **1-9**, **11**, **13** were tested for their *in vitro* anti-inflammatory activities. The inhibition of lipoxygenase activity was determined by a spectrophotometric method with 96 quartz plate (Baylac, 2003: Tappel, 1962: Demirci et. al., 2018). 1.94 mL 100 mM phosphate buffer (pH 8.0), 40 µL various concentration of samples and 20 µL lipoxygenase mixed and incubated for 10 min at 25 °C. 300 µL of this mixture added to each well. And then, 7.5 µL of the linoleic acid was added and mixed for 30 seconds. The absorbance change was

measured at 243 nm for 10 minutes in 10 intervals. Nordihidroguairatic acid (NDGA) was used as positive control compound. All tests were repeated in four times, results are shown as % inhibitions in Tables S1 and S3.

The percentage inhibition (%) of lipoxygenase activity was calculated as:

Inhibition (%) = (E-S) / E x100
E: the enzyme activity without sample
S: the enzyme activity with sample

1.6. Antimicrobial Activity

Microorganisms were grown using Potatoe Dextrose Agar (PDA, Merck) and RPMI medium for *Candida* sp., Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) for bacterial cultures. After 24 hours incubation at 37 °C, the bacterial density was adjusted according to McFarland No: 0.5 (approx. 10^8 CFU/mL) by using turbidimeter (CLSI, 2002; CLSI, 2006).

Test compounds **1-9**, **11** and **13** were tested for their antimicrobial activities. For this study, 96-well flat bottom microtiter plates were used. 0.62-0.00125 mg/mL concentrations of samples were prepared. The density-adjusted microorganisms (100 µL solution) was added to plates. Then, they incubated 24 h at 37 °C (CLSI,2007; Demirci et al.,2009: Karaca et. al., 2018). Chloramphenicol was used as antibacterial standard and amphotericin B was used as antifungal standard. The concentration of antimicrobials which applied were 0.001-64 µg/mL. After incubation time, 20 µL %0.01 (w/v) resazurin solutions were added to give colour to every plate and incubated 3 h at 37 °C. Results were given as minimum inhibitory concentration (MIC). Tests were repeated two times, and average values were given in Tables S2 and S4.

Table S1. The antioxidant and anti-inflammatory activities of the extracts from *V. bugulifolium*.

Extracts/References	**IC_{50} (μg/mL)**			**Inhibition % (50 µg/mL)**
	DPPH	**ABTS**	**CUPRAC**	**LOX**
MeOH	380 ±0.02	200 ±0.03	170 ±0.01	26.6 ±4.7
***n*-Hexane**	NA	450 ±0.01	50 ±0.02	25.8 ±3.3
$CHCl_3$	220 ±0.01	220 ±0.02	80 ±0.02	22.6 ±4.9
EtOAc	30 ±0.01	20 ±0.01	20 ±0.03	21.4 ±2.5
***n*-BuOH**	90 ±0.00	70 ±0.01	20 ±0.01	35.7 ±4.1
H_2O	NA	370 ±0.03	250 ±0.01	29 ±4.6
Ascorbic acid	10 ±0.00	30 ±0.01	-	-
Gallic acid	-	-	4 ±0.01	-
NDGA	-	-	-	100

NA: not active

Table S2. Antimicrobial activity results of extracts, and standards (µg/mL)

Extracts/Standards	***Bacillus cereus* NRRLB 3711**	***Pseudomonas aeruginosa* ATCC 10145**	***Staphylococcus aureus* ATCC 6538**	***Candida albicans* ATCC 90028**	***Candida parapsilosis* ATCC 22019**	***Candida krusei* ATCC 6258**
***n*-BuOH**	NA	NA	NA	NA	1000	500
MeOH	NA	NA	NA	NA	1000	500
$CHCI_3$	NA	1000	NA	1000	1000	250
***n*-Hexane**	NA	125	NA	NA	1000	500
EtOAc	NA	125	NA	NA	NA	-
Amphotericin-B	-	-	-	0.031	0.062	0.125
Chloramphenicol	0.002	0.062	0.001	-	-	-

NA: Not active (>1000 µg/mL)

Table S3. The antioxidant and anti-inflammatory activities of the compounds from *V. bugulifolium* (**1-9**, **11**,**13**).

Compounds		**IC_{50} (µg/mL)[a]**		**% Inhibition**
	DPPH	**ABTS**	**CUPRAC**	**LOX**
1	490± 0.05	NA	290±0.01	NA
2	NA	30 ±0.00	NA	3.9±1.0/(1.6 µg/mL)
3	NA	NA	NA	NA
4	NA	NA	NA	NA
5	NA	NA	NA	6.5±0.8/(1.6 µg/mL)
6	10±0.01	10±0.00	10±0.00	NA
7	50±0.00	20 ±0.00	70±0.00	5.0±1.2/(1.6 µg/mL)
8	5±0.00	10±0.00	20±0.00	54.1±5.0/(1 µg/mL)
9	10±0.01	40 ±0.00	50±0.01	4.8±1.5/(1.6 µg/mL)
11	NA	90±0.01	NA	NA
13	NA	NA	NA	6.1±2.4/(1.6 µg/mL)
Ascorbic acid	5±0.00	30 ±0.00	20±0.00	
Gallic acid			4±0.01	
NDGA IC_{50}				2.95±0.21 µg/mL

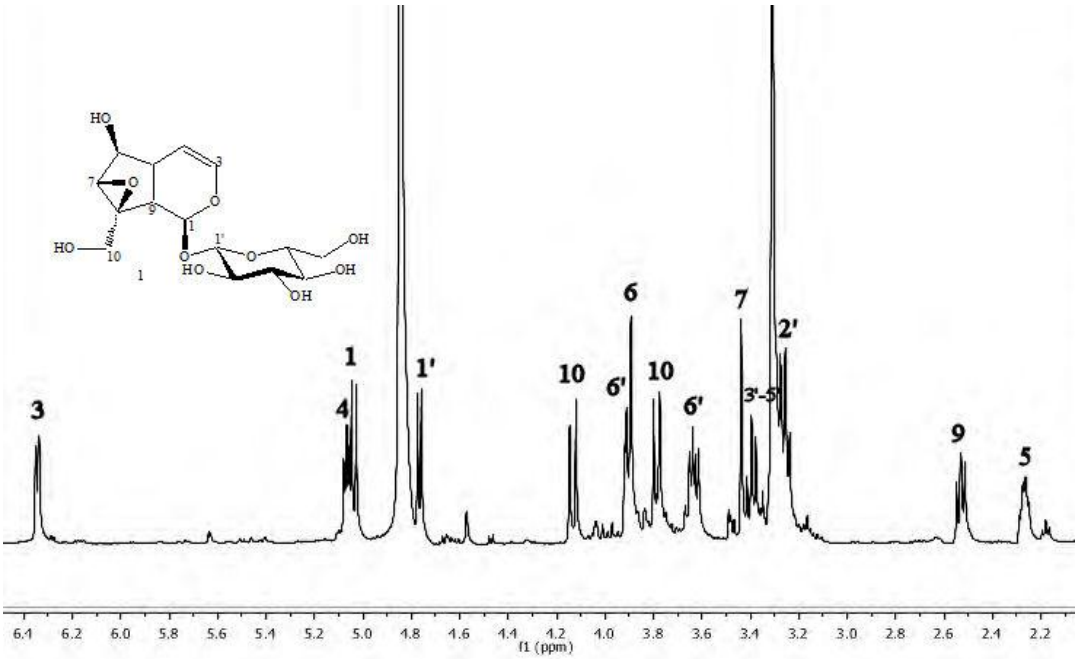
[a]Data expressed as mean ± SD (n = 4). NA: Not active

Table S4. Antimicrobial activities of isolated compounds and standards (μg/mL)

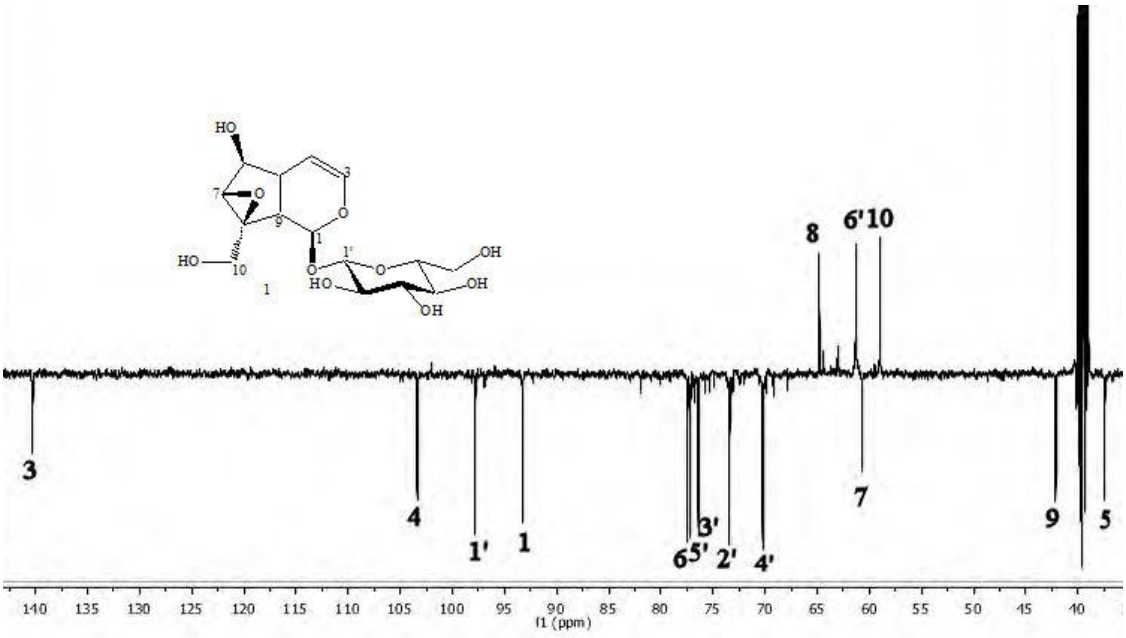
Compounds	***B. cereus* NRRLB 3711**	***P. aeruginosa* ATCC 10145**	***S. aureus* ATCC 6538**	***C. albicans* ATCC 90028**	***C. parapsilosis* ATCC 22019**	***C. krusei* ATCC 6258**
1	16	NA	8	NA	310	NA
2	31	620	31	NA	NA	NA
3	62	NA	16	NA	NA	310
4	16	620	8	NA	620	NA
5	31	NA	31	NA	620	NA
6	NA	NA	NA	NA	NA	620
7	2	NA	2	NA	620	NA
8	16	NA	8	NA	NA	NA
9	NA	NA	NA	NA	80	20
11	8	NA	4	NA	20	NA
13	16	NA	16	NA	NA	NA
Amphotericin-B	-	-	-	0.031	0.062	0.125
Chloramphenicol	0.002	0.062	0.001	-	-	-

NA: Not active (>620 μg/mL)

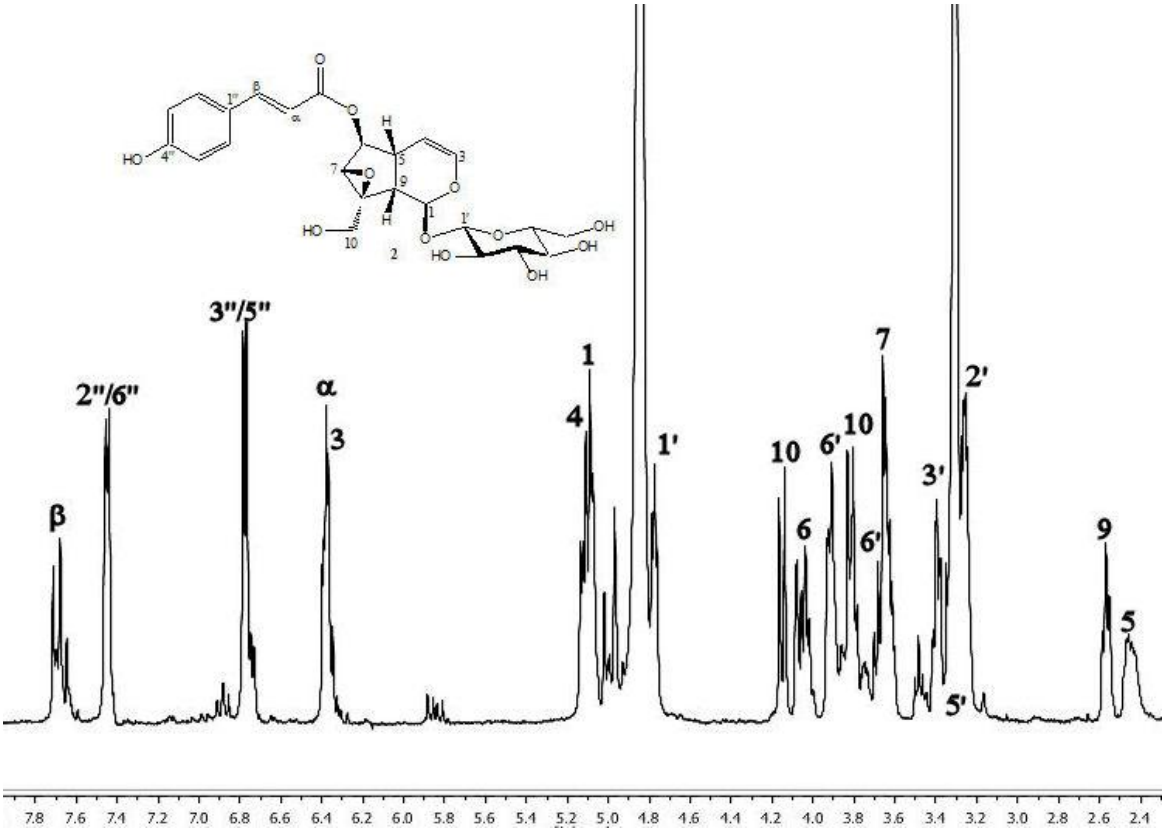
Spectra	**Page**
S1: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **1**	**12**
S2: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **1**	**12**
S3: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **2**	**13**
S4: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **2**	**13**
S5: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **3**	**14**
S6: HR-ESI-MS of **4**	**14**
S7: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **4**	**15**
S8: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **4**	**15**
S9: HR-ESI-MS of **5**	**16**
S10: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **5**	**16**
S11: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **5**	**17**
S12: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **6**	**17**
S13: HR-ESI-MS of **7**	**18**
S14: ^{1}H-NMR (500 MHz, DMSO-*d6*) Spectrum of **7**	**18**
S15: APT-^{13}C-NMR (125 MHz, DMSO-*d6*) Spectrum of **7**	**19**
S16: HMBC Spectrum of **7**	**19**
S17: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **8**	**20**
S18: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **9**	**20**
S19: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **9**	**21**
S20: HR-ESI-MS of **10** and **12**	**21**
S21: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **10** and **12**	**22**
S22: APT-^{13}C-NMR (125 MHz, CD_3OD) of **10** and **12**	**22**
S23: HMBC Spectrum of **10** and **12**	**23**
S24: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **11**	**23**
S25: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **13**	**24**



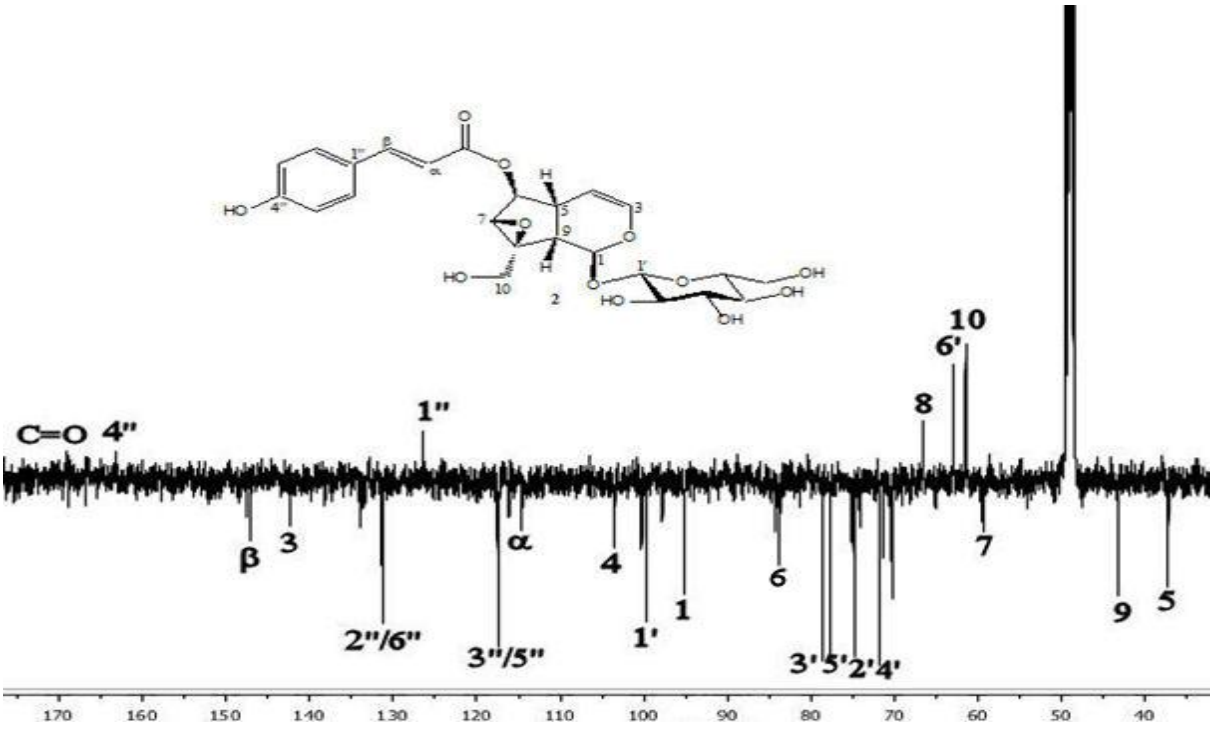
S1: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **1**



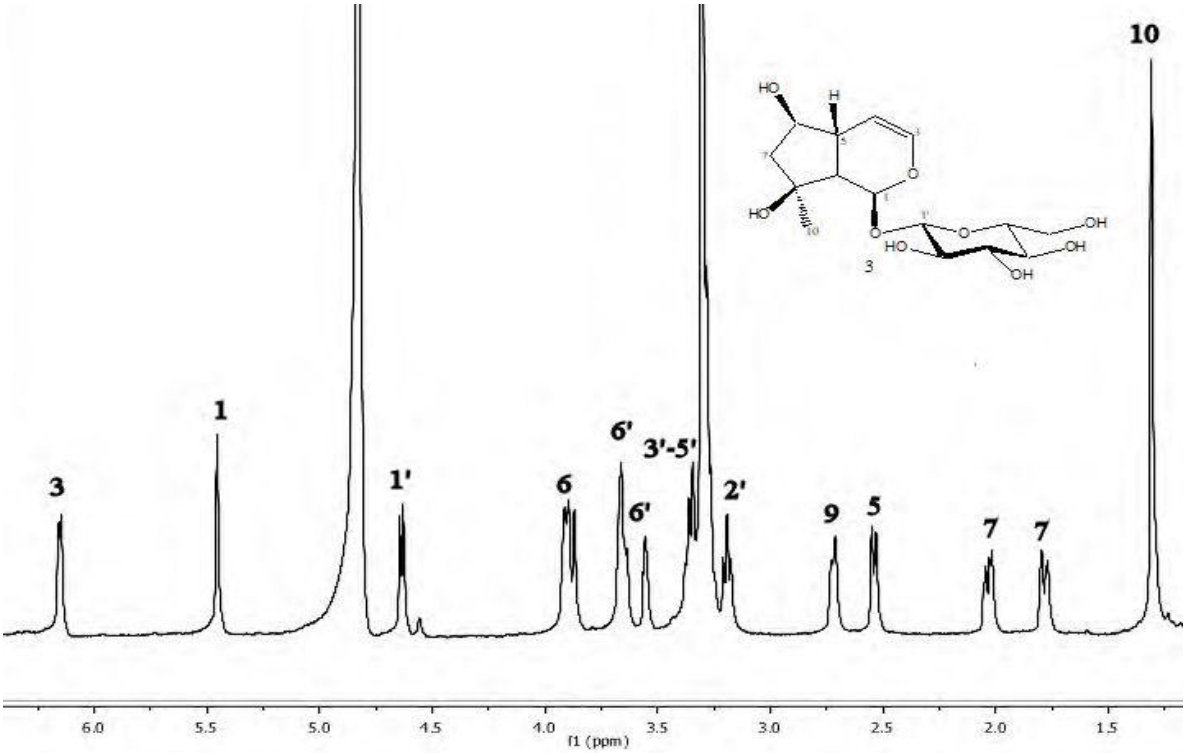
S2: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **1**



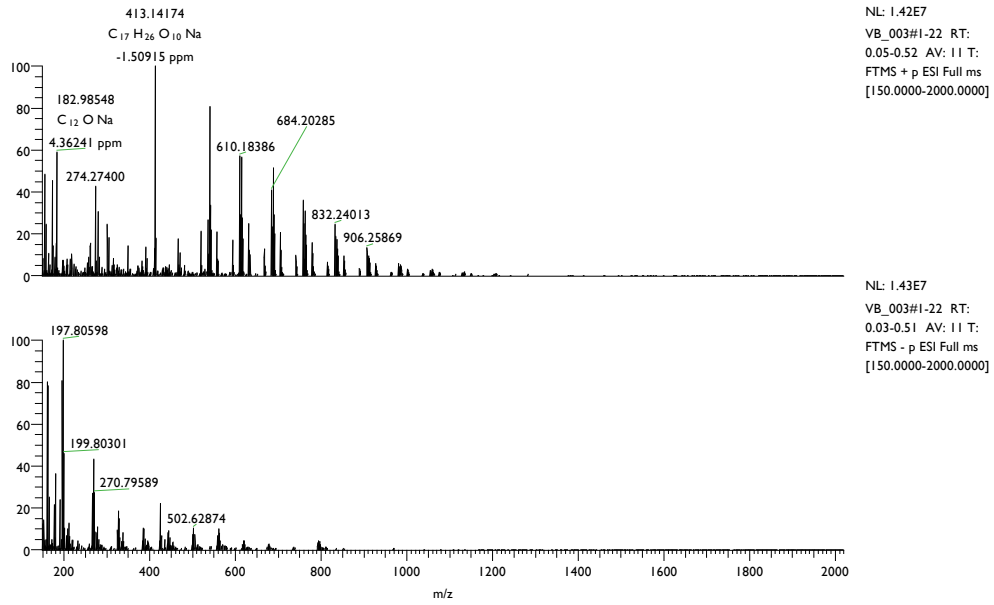
S3: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **2**



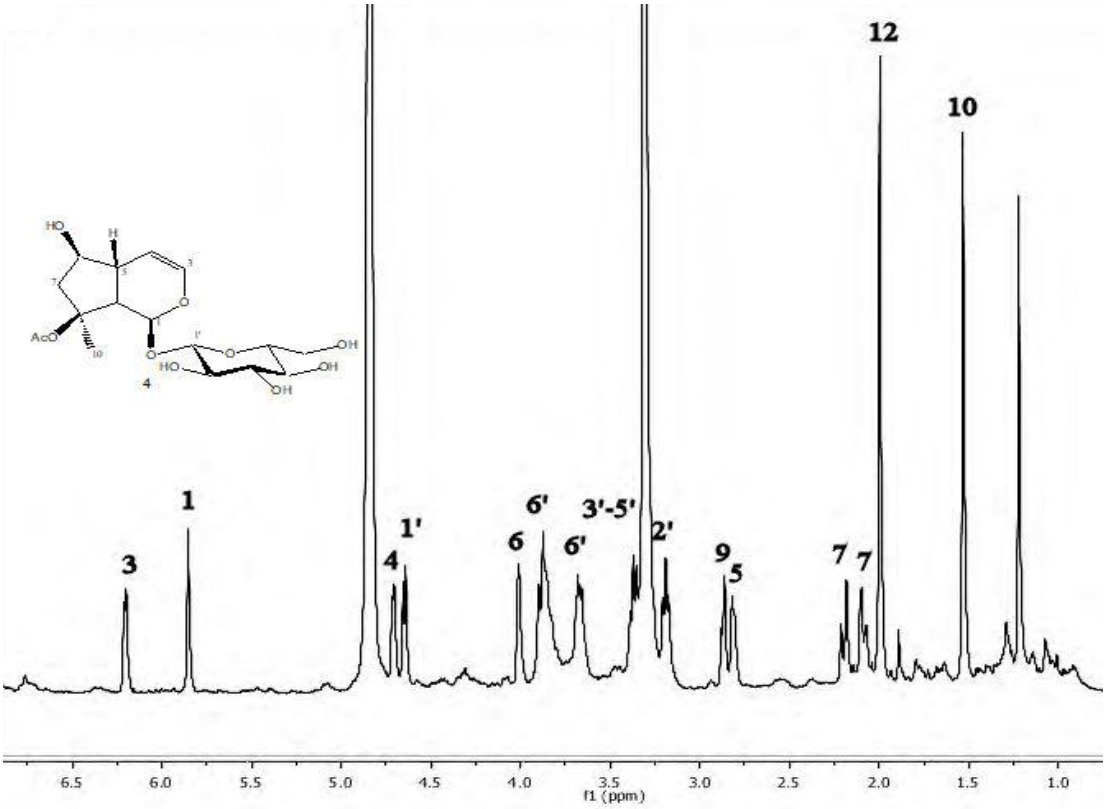
S4: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **2**



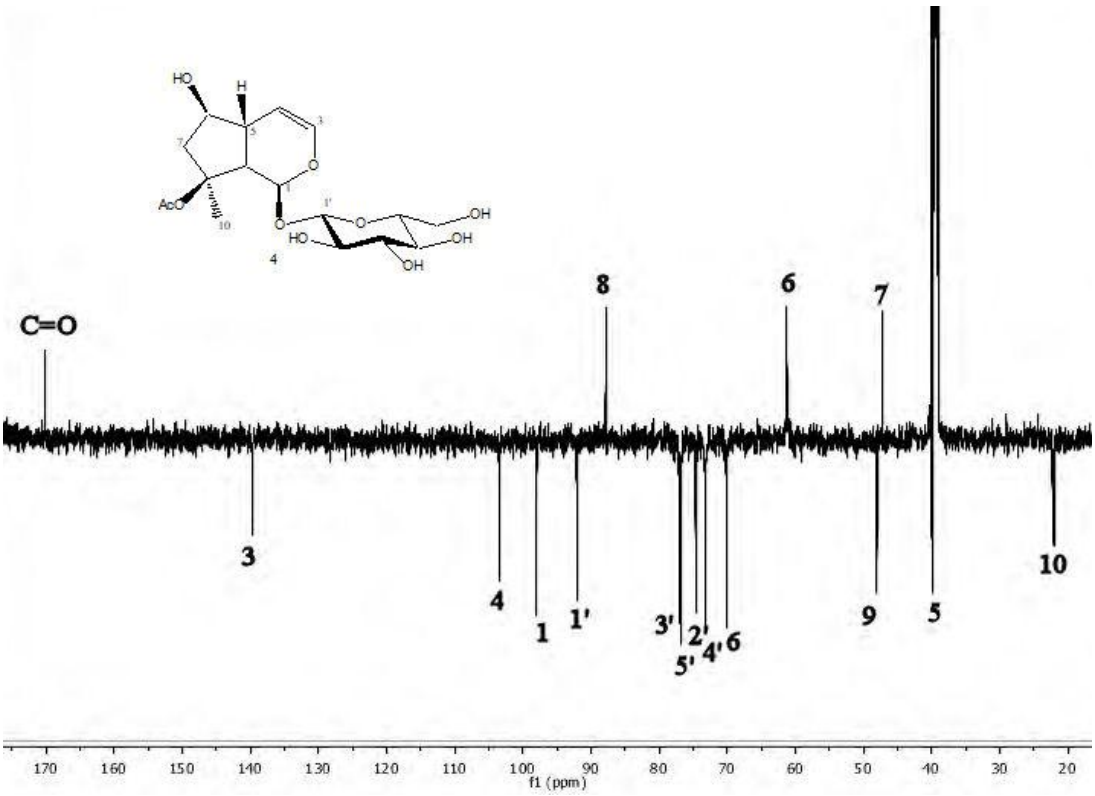
S5: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **3**



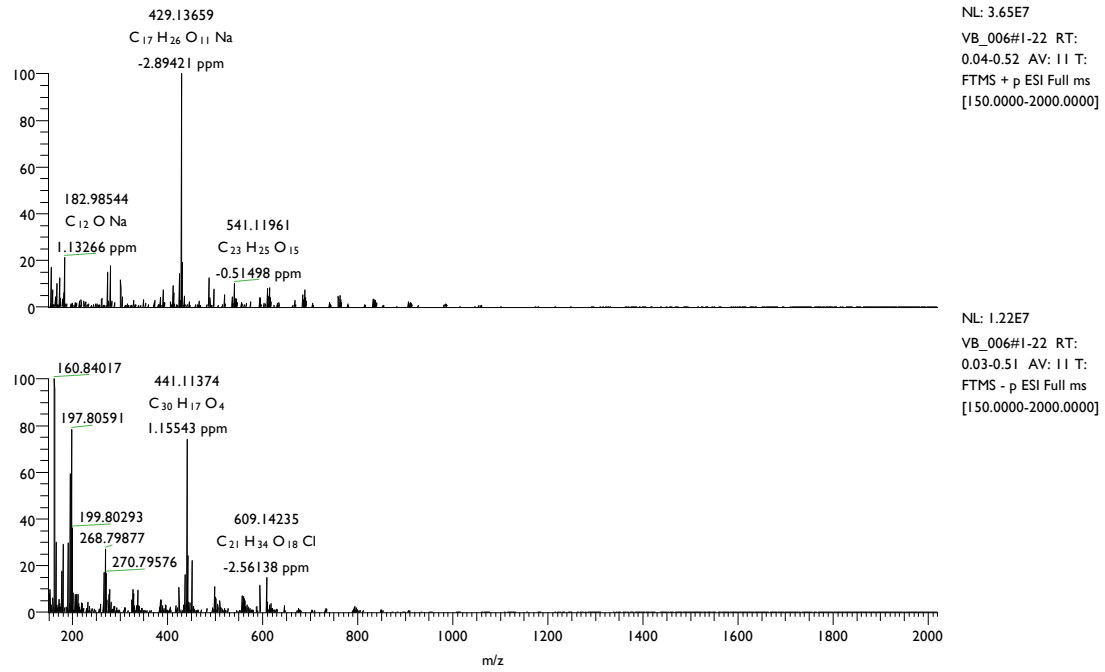
S6: HR-ESI-MS of **4**



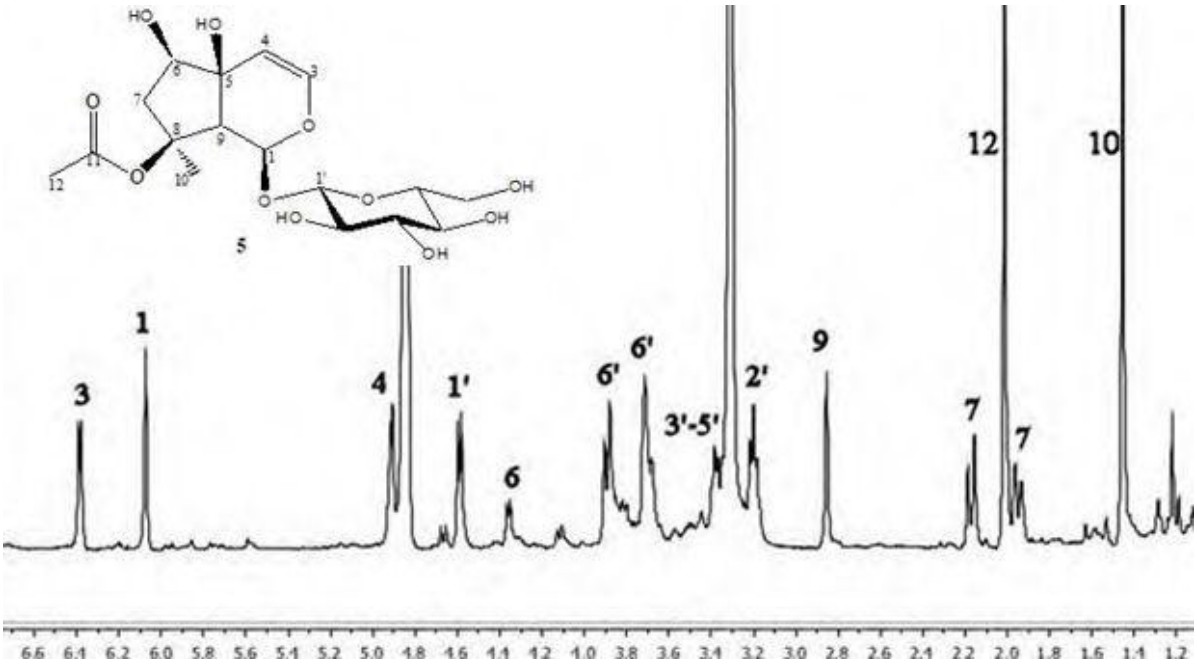
S7: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **4**



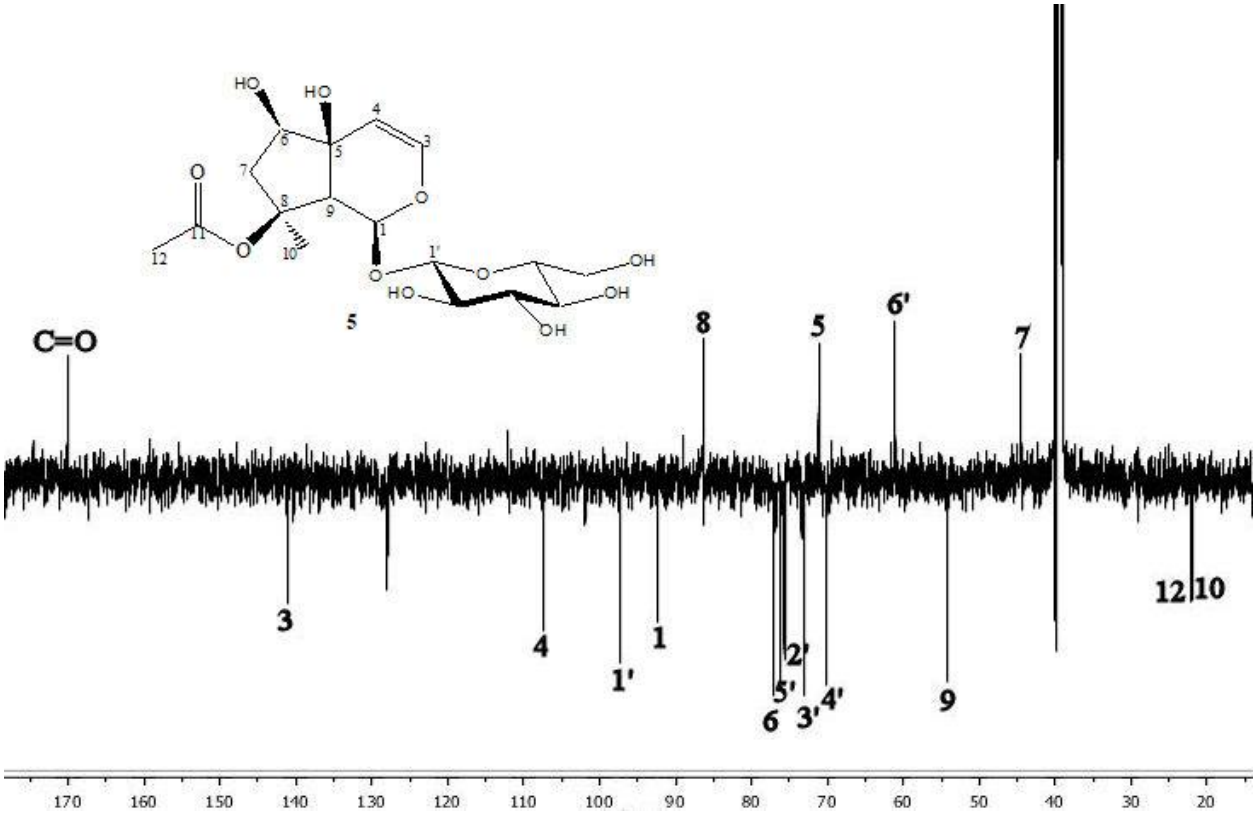
S8: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **4**



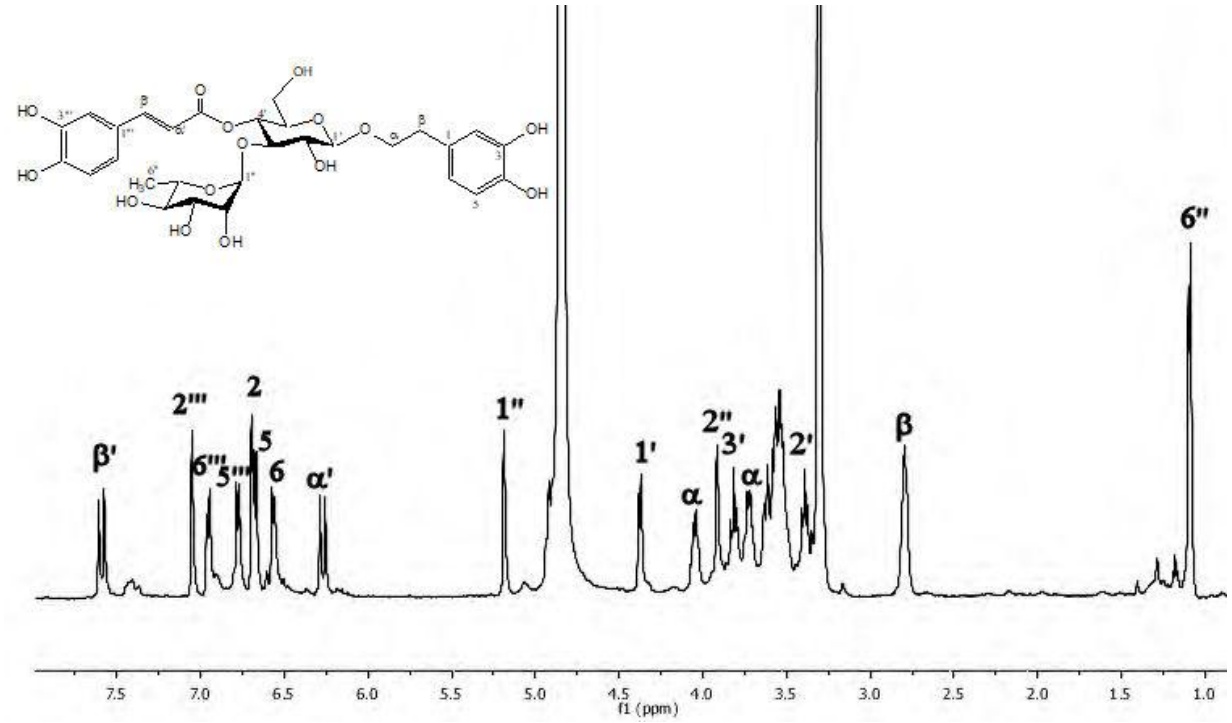
S9: HR-ESI-MS of **5**



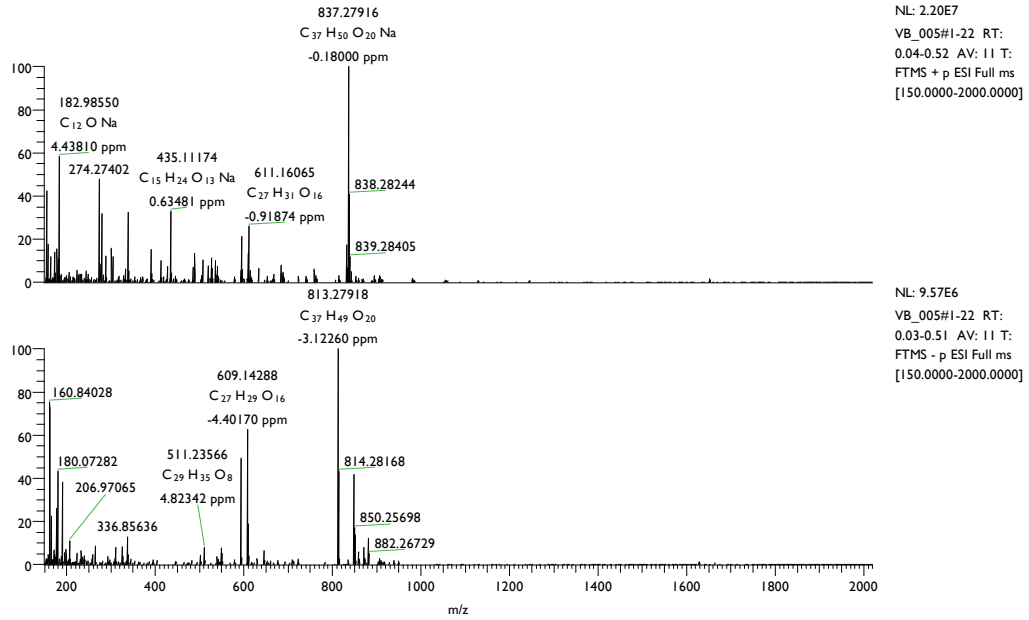
S10: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **5**



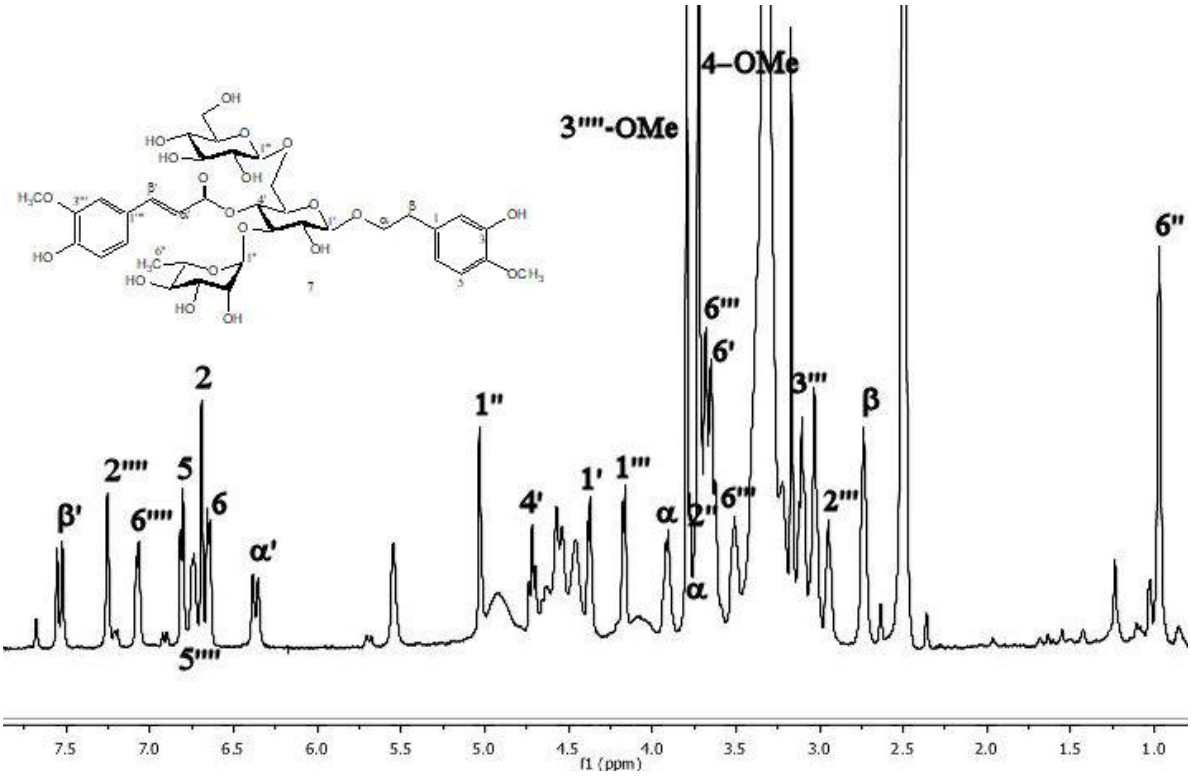
S11: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **5**



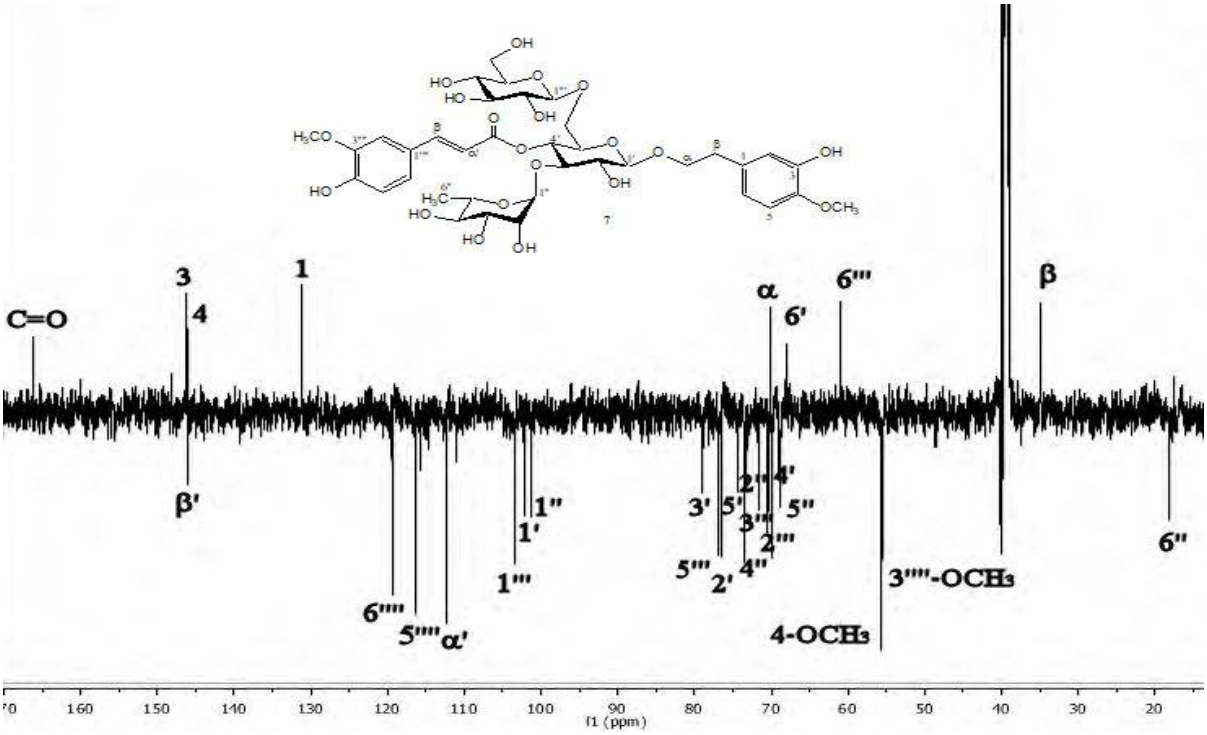
S12: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **6**



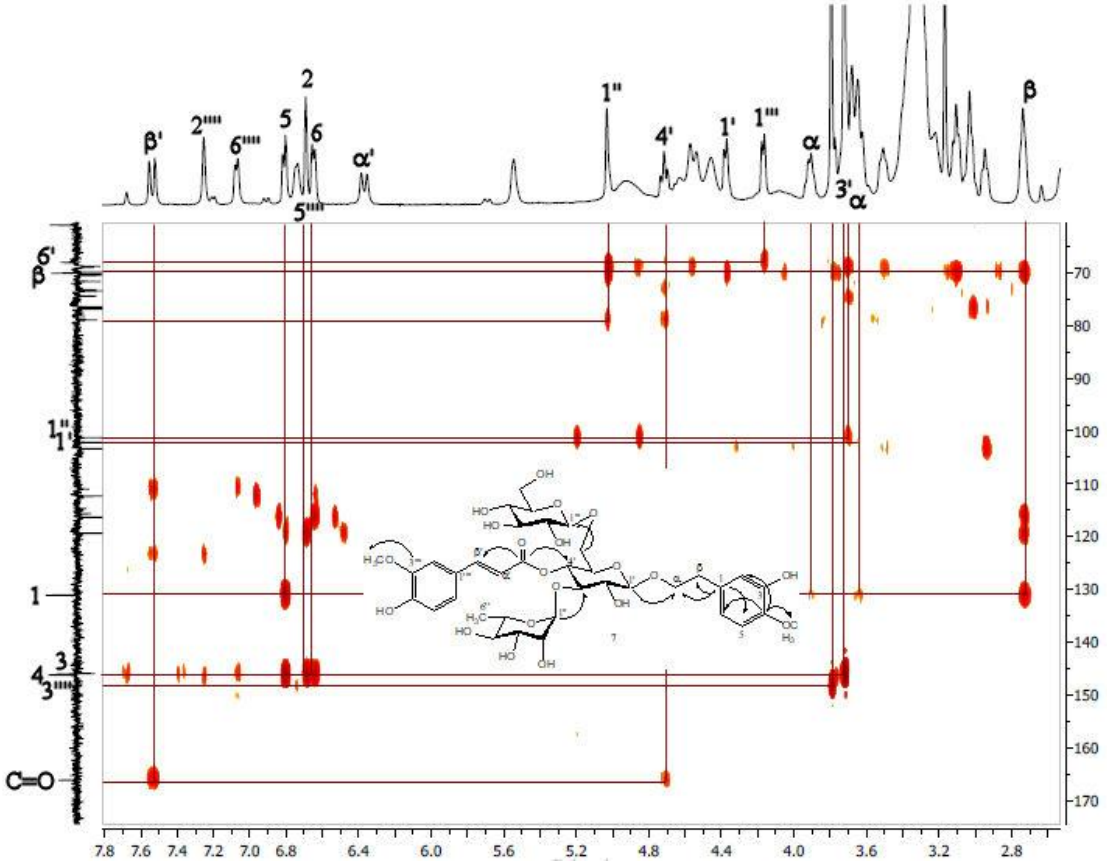
S13: HR-ESI-MS of 7



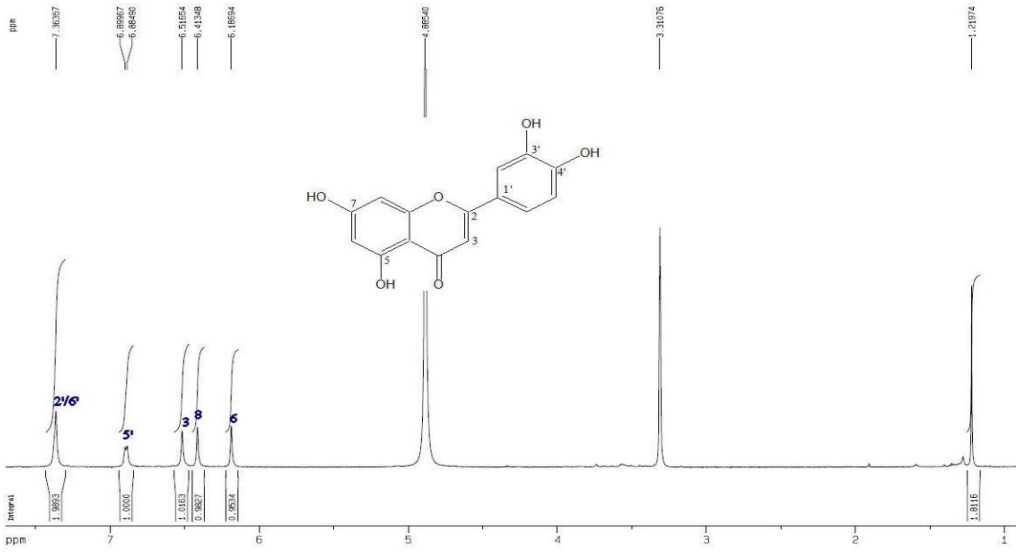
S14: ^{1}H-NMR (500 MHz, DMSO-*d6*) Spectrum of **7**



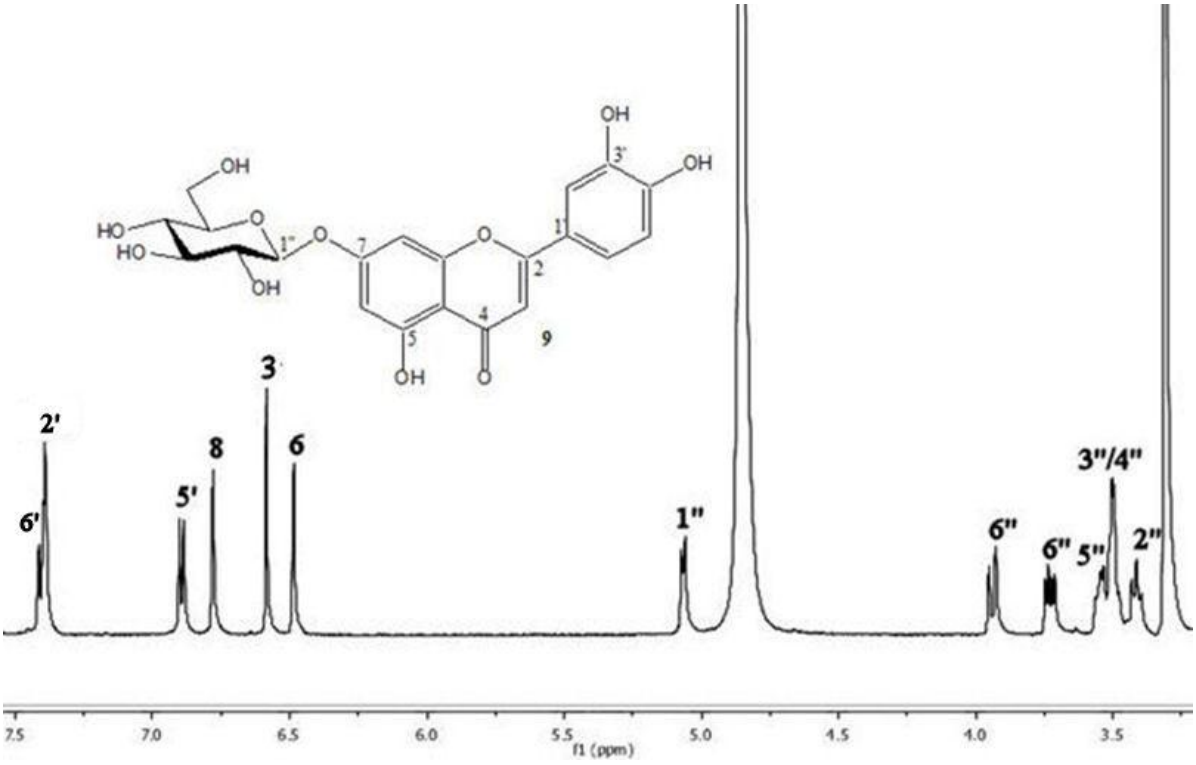
S15: APT-^{13}C-NMR (125 MHz, DMSO-*d6*) Spectrum of **7**



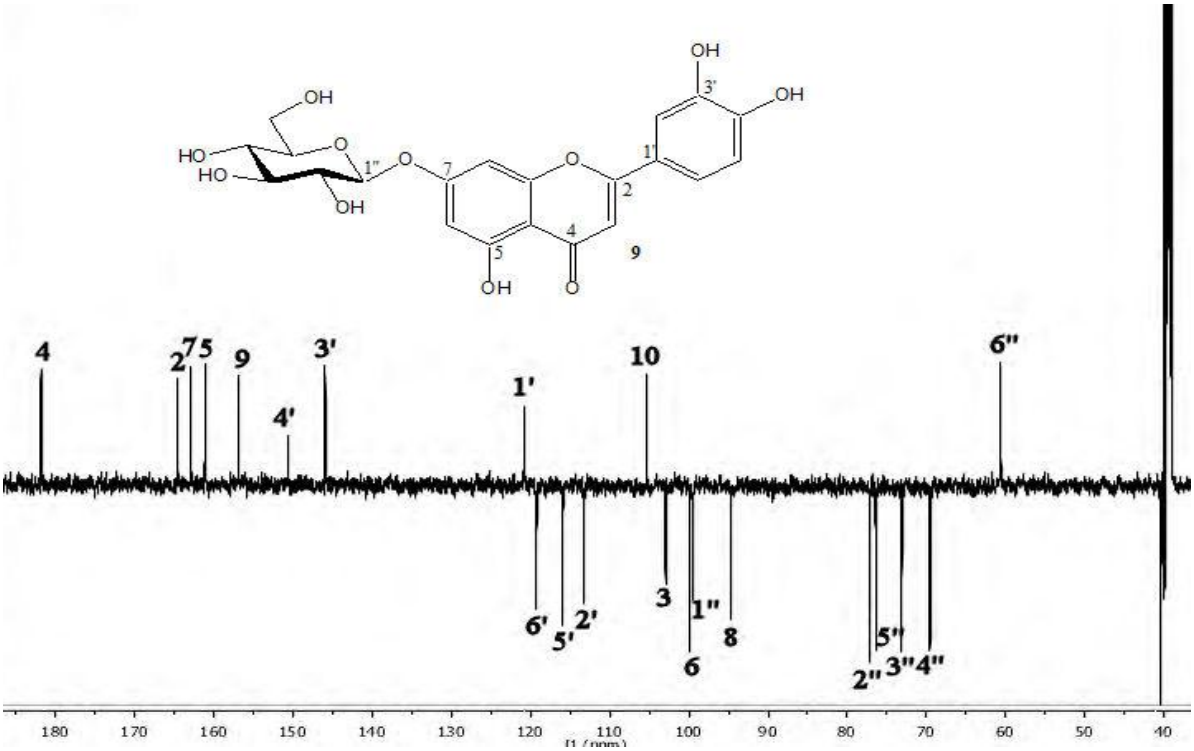
S16: HMBC Spectrum of **7**



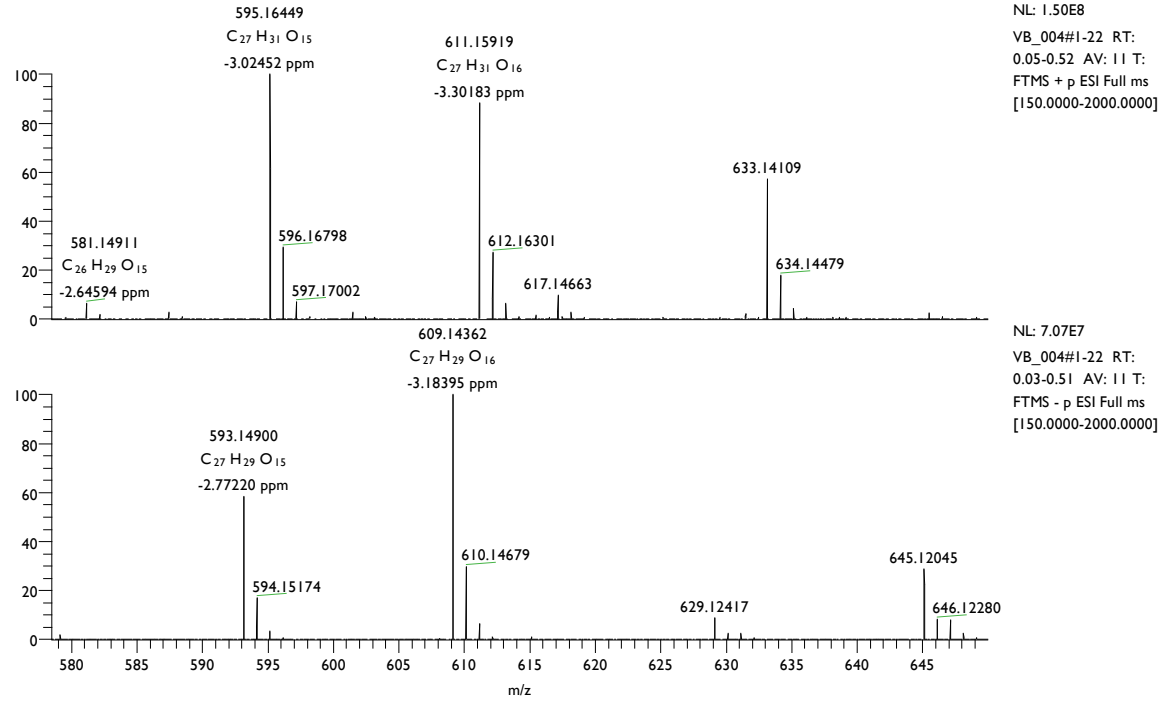
S17: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **8**



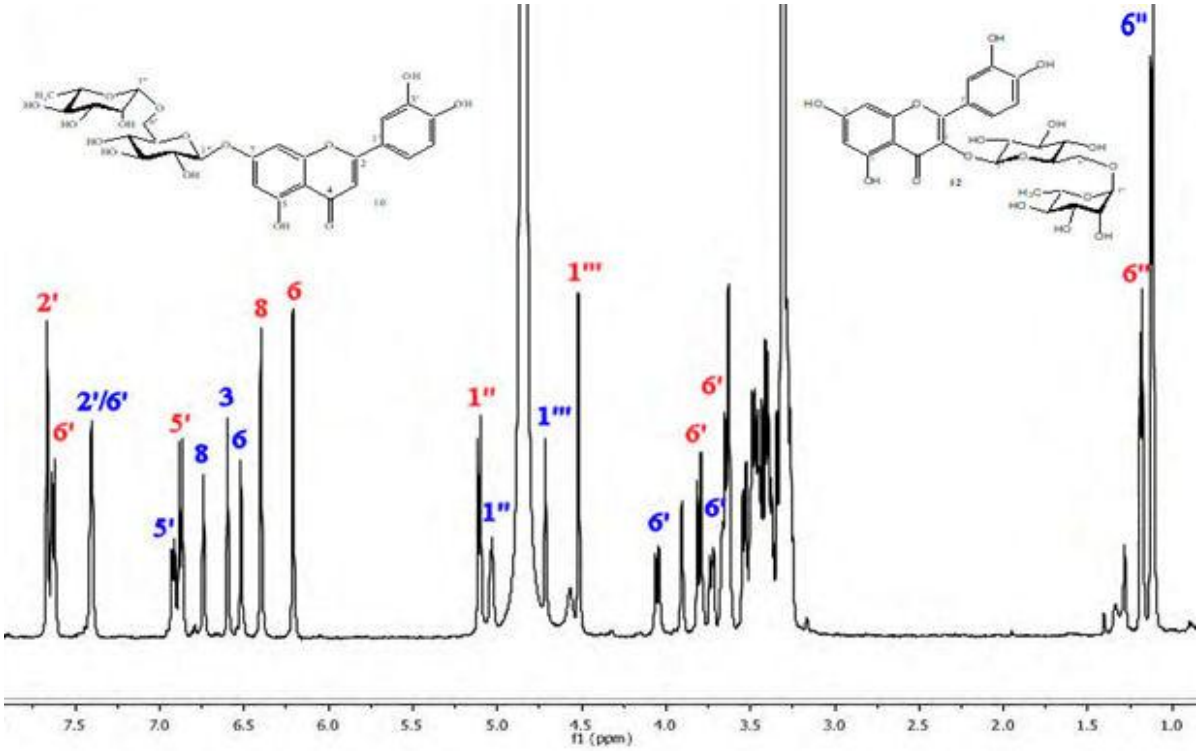
S18: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **9**



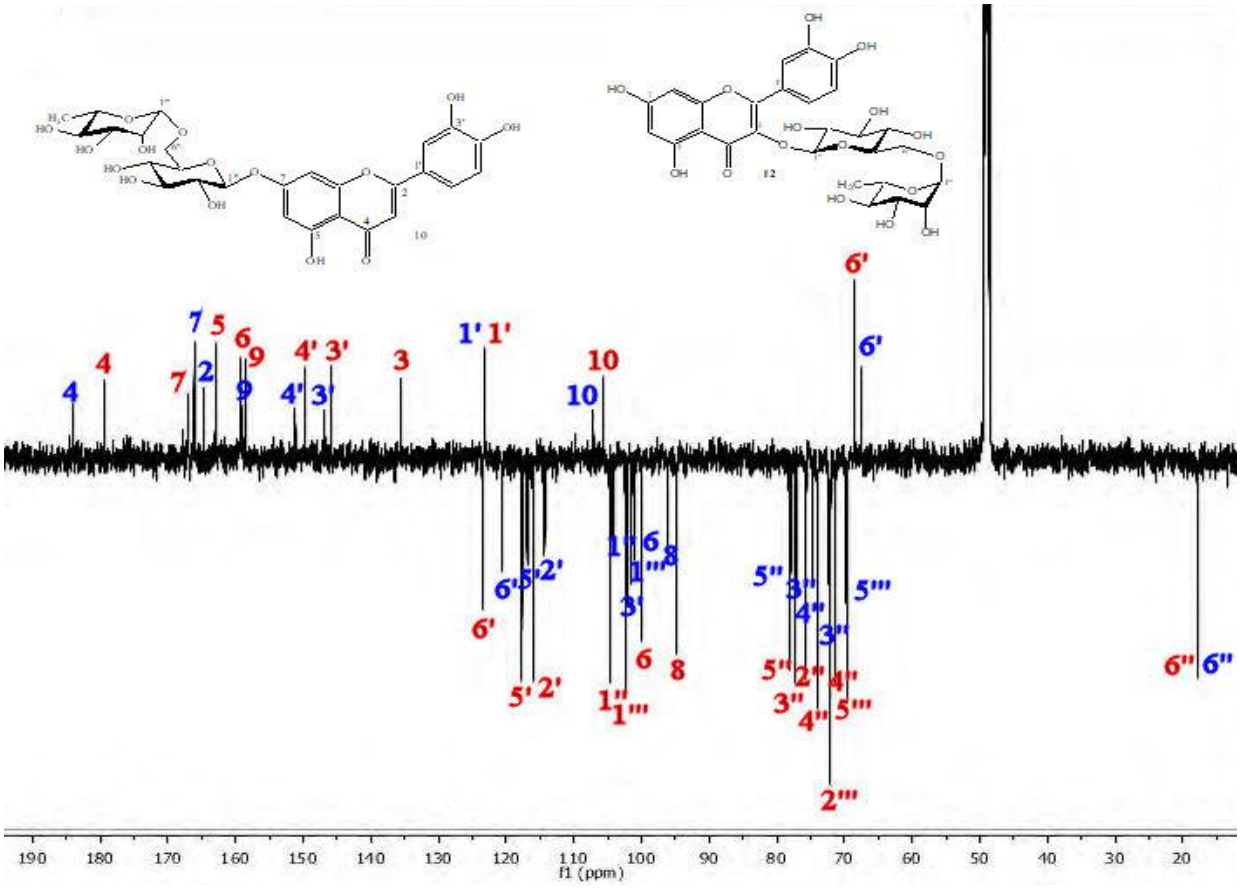
S19: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **9**



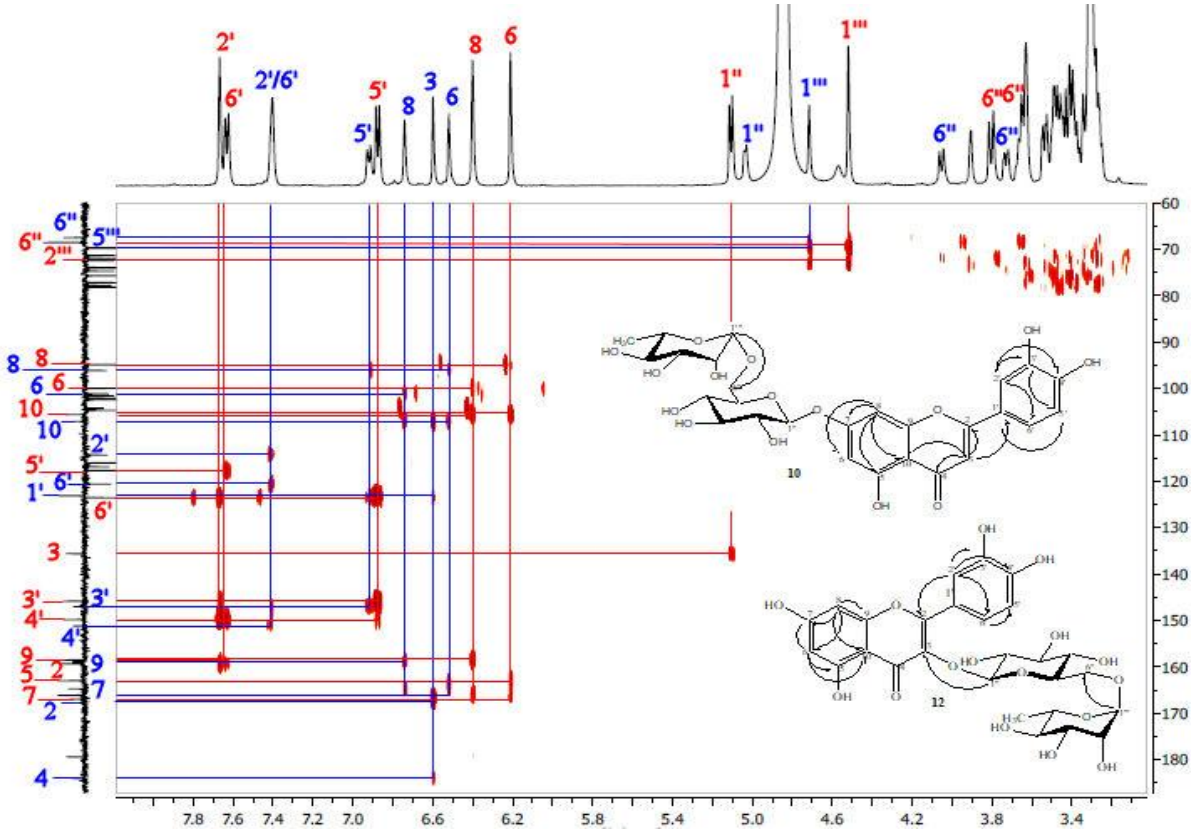
S20: HR-ESI-MS of **10** and **12**



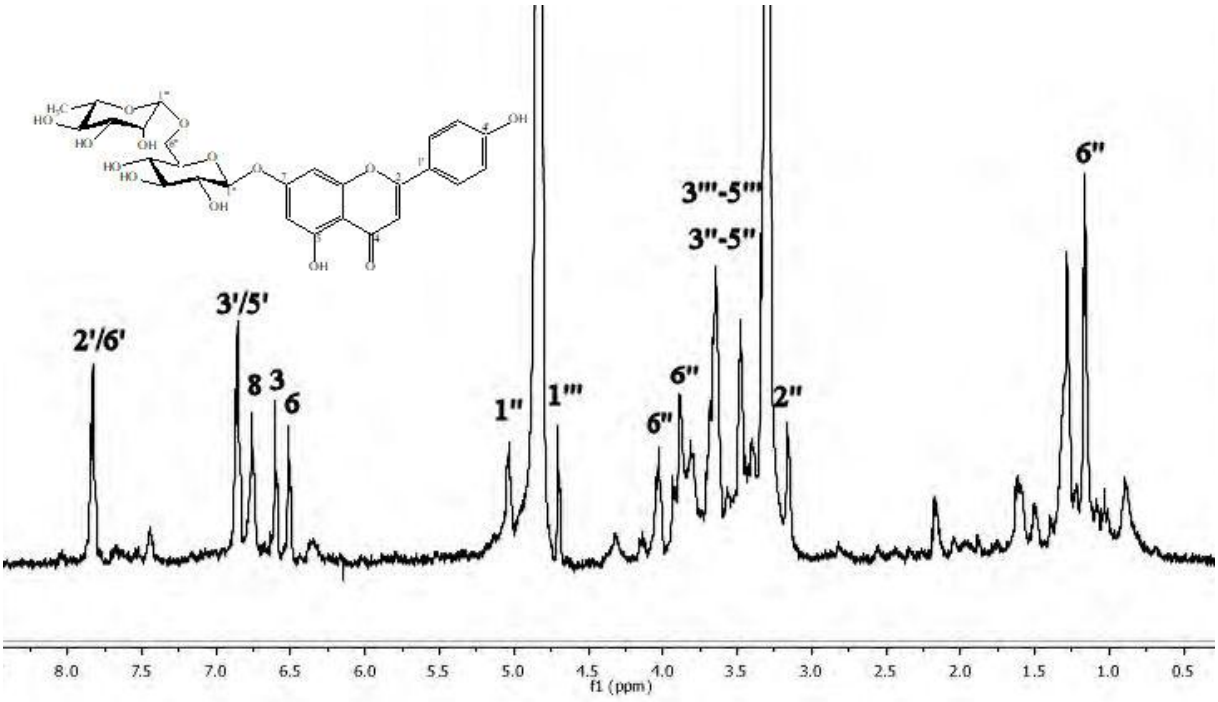
S21: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **10** and **12**



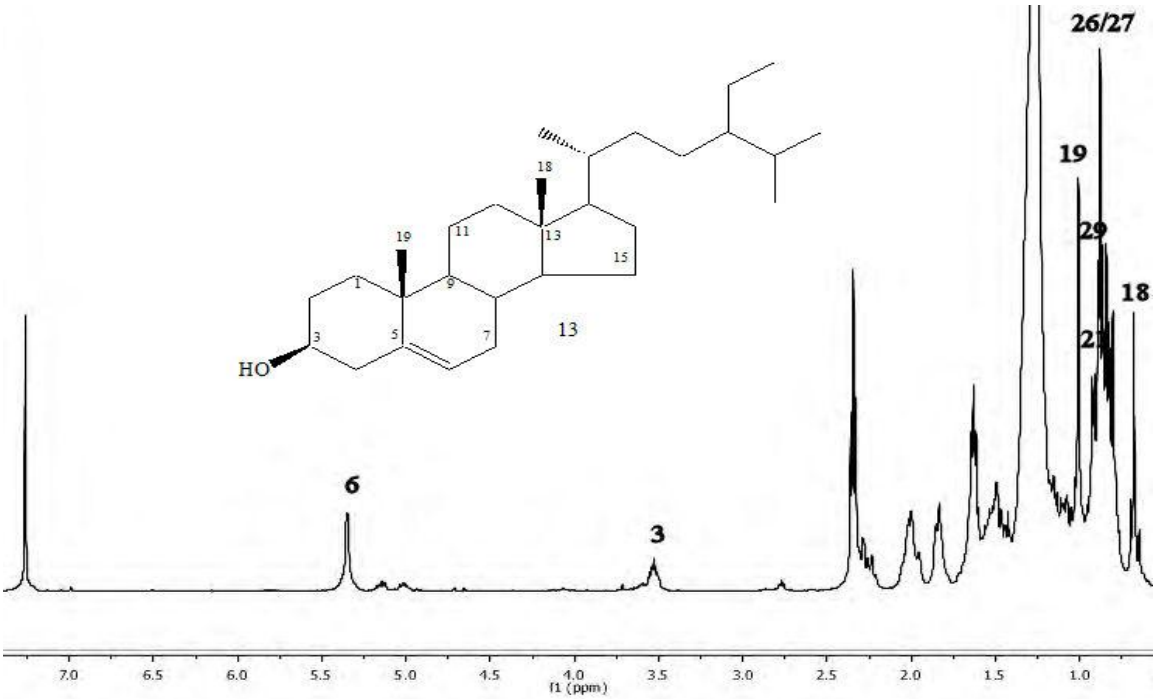
S22: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **10** and **12**



S23: HMBC Spectrum of **10** and **12**



S24: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **11**



S25: ^{1}H-NMR (500 MHz, $CDCl_3$) Spectrum of **13**

References

Apak R, Güçlü K, Demirata B, Özyürek M, Bektaioğlu K, Berker I, Özyurt, D. 2007. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC Assay. Molecules. 12:1496-1547.

Baylac S, Racine P. 2003. Inhibition of 5- Lipoxygenese by essential oils and other natural fragrant extracts. Int. J. Aromather. 13:138-142.

Blois MS. 1958. Antioxidant determination by the use of a stable free radical. Nature, 181:1199-1200.

CLSI, Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard, M27-A2, Clinical and Laboratory Standards Institute 2002;22(15).

CLSI, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, M7-A7, Clinical and Laboratory Standards Institute 2006;26(2).

CLSI, Performance standards for antimicrobial susceptibility testing; Seventeenth Informational Supplement, M100-S17 (2007). Clinical and Laboratory Standards Institute 27(1).

Dasgupta A, Klein K. 2014. Methods for measuring oxidative stress in the laboratory, Antioxidant in Food, Vitamins and Supplements, Chapter 2, 19-40.

Demirci F, Karaca N, Tekin M, Demirci B. 2018. Anti-inflammatory and antibacterial evaluation of *Thymus sipyleus* Boiss. subsp. *sipyleus* var. *sipyleus* essential oil against rhinosinusitis pathogens. Microb. Pathog. 122: 117-121.

Demirci B, Toyota M, Demirci F, Dadandi MY, Baser K.H.C. 2009. Anticandidal pimaradiene diterpene from *Phlomis* essential oils. C. R. Chim. 12: 612-621.

Karaca N, Demirci B, Demirci F. 2018. Evaluation of *Lavandula stoechas* L. subsp. *stoechas* L., *Mentha spicata* L. subsp. *spicata* L. essential oils and their main components against sinusitis pathogens. Zeitschrift für Naturforschung C, 73(9-10): 353-360.

Kumarasamy Y, Ferrusson ME, Nahar L, Sarker SD. 2002. Bioactivity of moschamindole form *Centaurea moschata*. Pharm Biol. 40: 307-310.

Re R, Pellegrini N, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad Biol Med. 26:1231-1237.

Tappel A L. 1962. Methods in Enzymology, Academic Press: New York. 5: 539.