SUPPLEMENTARY MATERIAL

Secondary metabolites from the aerial parts of *Sideritis germanicopolitana* and their *in vitro* enzyme inhibitory activities

Hasan Kırmızıbekmez[a,*], Murat Erdoğan[a], Norbert Kúsz[b], Nursenem Karaca[c], Umur Erdem[d], Fatih Demirci[c], Judit Hohmann[b]

[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, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey, [d]Faculty of Pharmacy, Yeditepe University, TR-34755, Kayışdağı, Ataşehir, İstanbul, Turkey

*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

Three iridoid glycosides, 5-allosyloxy-aucubine (**1**), melittoside (**2**), ajugol (**3**), five phenylethanoid glycosides, verbascoside (**4**), martynoside (**5**), leucoseptoside A (**6**), lamalboside (**7**), decaffeoylverbascoside (**8**), four flavonoids, xanthomicrol (**9**), isoscutellarein 7-*O*-[6'''-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside (**10**), 4'-*O*-methylisoscutellarein 7-*O*-[6'''-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside (**11**), 3'-hydroxy-4'-*O*-methylisoscutellarein 7-*O*-[6'''-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside (**12**), and two lignan glycosides dehydrodiconiferylalcohol 4-*O*-β-D-glucopyranose (**13**) and pinoresinol 4'-*O*-β-glucopyranoside (**14**) were isolated from the aerial parts of *Sideritis germanicopolitana*. Their structures were determined on the basis of detailed NMR and HRESIMS analyses. To our knowledge, all compounds are being reported for the first time from *S. germanicopolitana,* while the isolated lignans (**13** and **14**) are new for the genus *Sideritis*. *In vitro* evaluation of AChE, BChE and LOX inhibitory effects of all the tested compounds (**1**-**14**) resulted in low to moderate activities.

KEYWORDS*: Sideritis germanicopolitana;* secondary metabolites, iridoid glycosides; phenolic compounds; AChE and BChE inhibitory activities

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. 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 *Sideritis germanicopolitana* were collected from Sarıcakaya, Eskişehir (Turkey) in July 2017. The plants were identified by one of us. Voucher specimen (YEF 17011) has been deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Turkey.

1.3. Extraction, isolation and structure elucidation

The air-dried and powdered aerial parts of *S. germanicopolitana* (200 g) were extracted twice with MeOH (2 L) at 45 °C for four h. (Scheme 1) The pooled MeOH extract was evaporated to dryness (43 g, yield: 21.5 %). The crude MeOH extract was dispersed in 150 mL of MeOH (25%) and partitioned with *n*-hexane (3 x 150 mL) to get rid of lipids and chlorophylls. Then MeOH was removed from the aqueous layer and final volume was

adjusted to 150 mL with H_2O. This extract was further partitioned against chloroform ($CHCl_3$) (3x150 mL) to obtain $CHCl_3$ (1.029 g) and the remaining H_2O (31.56 g) subextracts. The H_2O subextract was fractionated over polyamide CC (100 g) eluted with H_2O/MeOH mixtures in different ratios (0-100 % MeOH, in steps of 20% MeOH) to give seven fractions, frs. A-G. Fr. B (7.5 g) was subjected to C_{18}-MPLC (130 g) eluting with MeOH (0 to 100%) to give seven fractions, B_1-B_7 as well as compound **12** (9 mg). Fraction B_2 (54 mg) was separated by SiO_2-MPLC (12 g) eluting with CH_2Cl_2-MeOH (90:10 to 60:40) to give compounds **1** (2 mg) and **2** (2 mg) together with three fractions, B_{2a}-B_{2c}. Fraction B_{2b} (20 mg) was separated by Sephadex LH-20 CC (20 g, MeOH) to give compounds **3** (5 mg) and **8** (2 mg). Fr. B_4 (34 mg) was subjected to SiO_2-MPLC (12 g) eluted with CH_2Cl_2-MeOH mixtures (100:0 to 70:30) to give compounds **13** (4 mg) and **7** (4 mg). Fr. C (1.5 g) was subjected to C_{18}-MPLC (130 g) eluting with stepwise gradient (10-100% MeOH) to obtain fractions $C_{1\text{-}14}$. Purification of C_7 (206 mg) by SiO_2 (24 g) MPLC and eluted with CH_2Cl_2-MeOH gradient (95:5 to 70:30) yielded compound **14** (7 mg). Fraction D (1.26 g) was separated by C_{18}-MPLC (100 g) eluted with MeOH (20 to 100%) to give three fractions, D_1-D_3. Fraction D_2 (40 mg) was applied to a Sephadex LH-20 column (20 g) eluted with MeOH to give compound **4** (10 mg). Fraction D_3 (955 mg) was separated by C_{18}-MPLC (130 g) eluted with MeOH (30 to 100%) to give seven fractions, frs. D_{3a}-D_{3g}. Fraction D_{3e} (49 mg) was separated by SiO_2-MPLC (12 g) eluted with CH_2Cl_2-MeOH mixture (90:10 to 60:40) to give compound **6** (12 mg). Purification of fr. D_{3f} (95 mg) by Sephadex LH-20 (20 g, MeOH) yielded compound **5** (12 mg). Fr. F was applied to C_{18}-MPLC (130 g, 20-100% MeOH) to yield compound fr. $F_{1\text{-}13.}$ Purification of fr. F_{10} (24 mg) by Sephadex LH-20 CC (6 g, MeOH) gave compound **11** (4 mg). Fr. G (374 mg) was separated by 35 g Sephadex LH-20 CC eluted with MeOH to give fr. G_2 (185 mg) which was further applied to C_{18}-MPLC (30 g) eluting with MeOH (10-100%) to obtain 5 fraction,

G_{2a}-G_{2e}. Fr. G_{2d} (43 mg) was separated by Sephadex LH-20 CC (20 g, MeOH) to give compound **10** (8 mg). The $CHCl_3$ subextract (1 g) was loaded onto a Sephadex LH-20 (95 g, MeOH) column to give four main fractions, frs.1-4. Rechromatography of fr. 3 (111 mg) by SiO_2-MPLC (24 g) using the gradient mixture of *n*-hexane-EtOAc (90:10 →50:50) yielded compound **9** (6 mg).

The structures of the isolates were identified as 5-allosyloxy-aucubine (**1**), melittoside (**2**) (Serrilli et al. 2006), ajugol (**3**) (Elusiyan et al. 2011), verbascoside (**4**), martynoside (**5**) (Ersöz et al. 2002), leucoseptoside A (**6**), lamalboside (**7**) (Budzianowski and Skrzypczak 1995), decaffeoylverbascoside (**8**) (Ersöz et al. 2002), xanthomicrol (**9**) (Jahaniani et al. 2005), isoscutellarein 7-*O*-[6'''-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside (**10**), 4'-*O*-methylisoscutellarein 7-*O*-[6'''-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside (**11**), 3'-hydroxy-4'-*O*-methylisoscutellarein 7-*O*-[6'''-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside (**12**) (Kırmızıbekmez et al. 2012) dehydrodiconiferylalcohol 4-*O*-β-D-glucopyranose (**13**) (Changzeng and Zhongjian 1997) and pinoresinol 4'-*O*-β-glucopyranoside (**14**) (Mai et al. 2017) by comparing their spectroscopic data with those published previously.

1.4. LOX inhibition assays

The inhibitory activities of the isolates (**1**-**14**) on lipoxygenase (1.13.11.12, Type I-B, 7.9 Unit/mg) enzyme activity was performed spectrophotometrically on a 96-well quartz plate according to previous work (Tappel 1962; Baylac and Racine 2003) 1.94 mL of potassium phosphate buffer (100 mM; pH: 8.80), 40 µL test substances and 20 µL lipoxygenase enzyme were incubated for 10 min. at 25°C. 300 µL of this mixture was added to each well. The reaction was then initiated by the addition of 7.5 µL linoleic acid solution, the change of absorbance at 243 nm was followed for 10 min. The experiments were carried out in 4

replicates, and the results are given in Table S1 as percent inhibition (%). Nordihydroguaiaretic acid (NDGA) was used as a positive control.

% Inhibition = (E − S) / E x 100

E: the absorbance of the enzyme without sample.

S: the absorbance of the enzyme with the test sample.

1.5. AChE and BuChE inhibitory assays

AChE and BuChE inhibitor activity were measured by using the method of Ellman et al. and Dohi et al. with minor modifications (Ellman et al. 1961; Dohi et al. 2009) Before the starting of test procedure, all solutions are carried out to 25°C. For this reason, 140 µL phosphate buffer (pH: 8.0), 20 µL calibrated concentration of samples, and 20 µL enzyme solutions (prepared in %1 gelatin AChE/BChE) were prepared at 96 micro-well plate. The first test solution was prepared by including 70 µL phosphate buffer, 20 µL enzyme solution and 20 µL of DTNB and the second solution were prepared by including 70 µL phosphate buffer and 10 µL ATC/BTC solution. Initially, first test solution and inhibitor compound solutions with different concentrations were added to wells. Then, mixture was incubated at 25°C for 15 minutes. After incubation, second solution added to be 80 µL to each plate. After the adding second solution, 30 seconds of fast mixing process carried out. Then, the first absorbance was measured at 412 nm. Microplates left 5 min. to maintain reaction and at the end of this time, second absorbance was measured. Taking the differences between two absorbance, inhibition ratio was calculated according to below formula.

% Inhibiton=([(A(C)-A(B))-(A(I)-A(B))])/((A(C)-A(B))) x 100

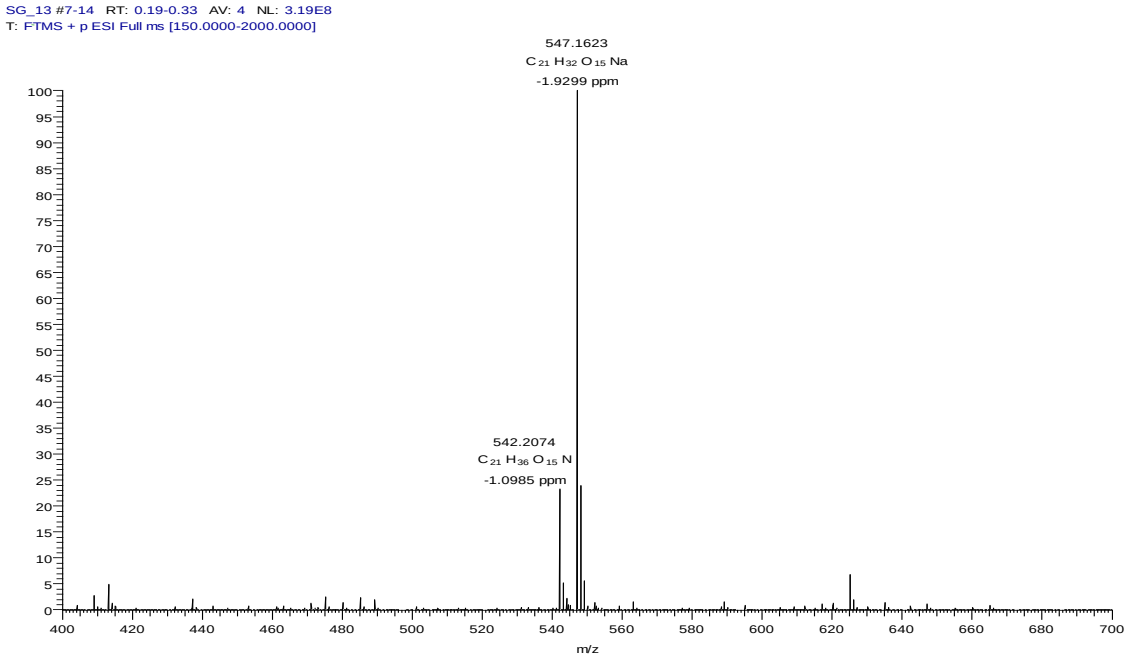
A(B): Absorbance reading differences belonging to Blank wells
A(C): Absorbance reading differences belonging to Control wells
A(I): Absorbance reading differences belonging to Inhibitor substances

Galantamine, tacrine and donepezil were used as standard substances. Experiments were repeated in quadruplicate and the results are given as % inhibition in Table S1.

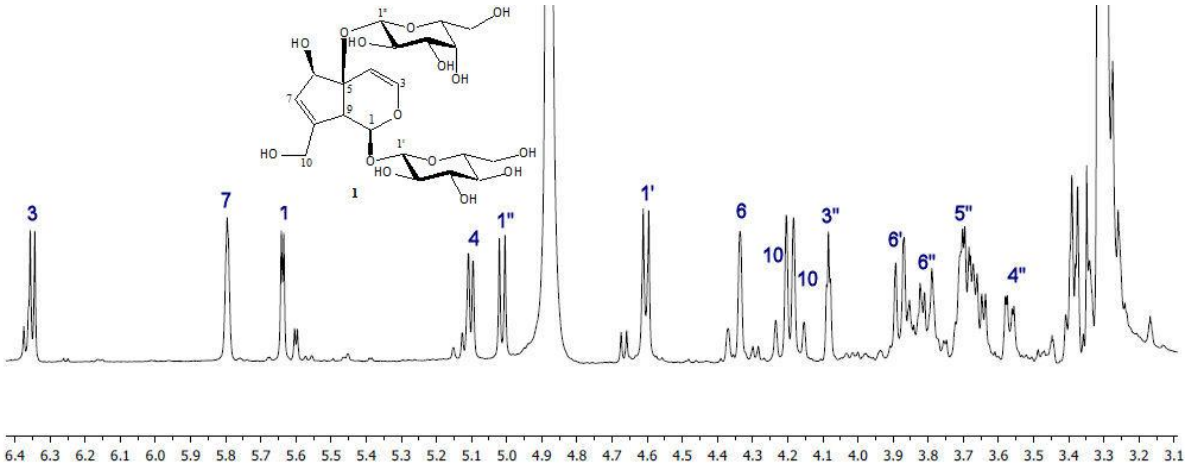
Table S1. LOX, AChE and BChE inhibitory activities of the isolates (**1**-**14**).

Compounds	**AChE Inhibition (%)[a] (250 µg/mL)**	**BChE Inhibition (%)[a] (250 µg/mL)**	**LOX Inhibition (%)[a] (5 µg/mL)**
1	20.36±0.70	14.22±0.61	12,20±4,55
2	24.63±0.44	12.75±0.40	NA
3	21.29±0.48	10.51±0.32	13,32±1,93
4	25.18±0.62	13.11±0.51	NA
5	20.75±0.50	12.08±0.33	NA
6	21.30±0.71	11.50±0.48	4,87±3,60
7	21.37±0.69	16.92±0.48	8,69±2,57
8	18.35±0.53	11.05±0.25	5,05±2,31
9	19.47±0.66	11.36±0.36	4,77±0,64
10	18.47±0.41	12.91±0.39	NA
11	27.90±0.68	16.32±0.29	9,41±4,63
12	22.79±0.78	19.41±0.63	NA
13	24.32±0.57	14.28±0.41	6,02±4,46
14	20.17±0.44	11.02±0.35	8,87±3,86
Donepezil[b] (250 µg/mL)	98.25±0.93	79.41±0.83	
Tacrine[b] (250 µg/mL)	97.69±1.02	98.58±0.99	
Galantamine[b] (250 µg/mL)	84.26±0.86	63.95±1.06	
NDGA[b] (12 µg/mL)			98,14±0,28

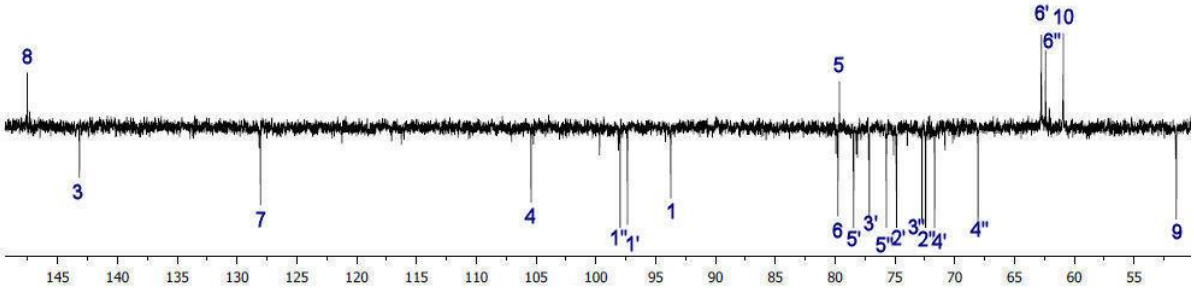
[a]Data expressed as mean ± SD (n = 4). [b]Positive controls. NA: Not active



S1: HRESIMS of **1**



S2: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **1**



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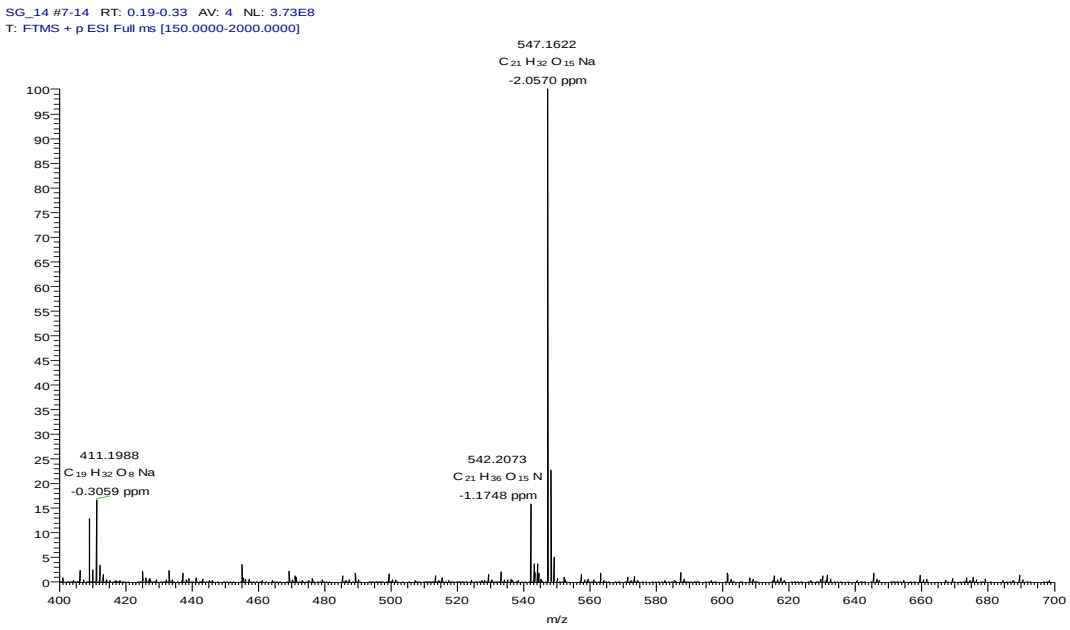
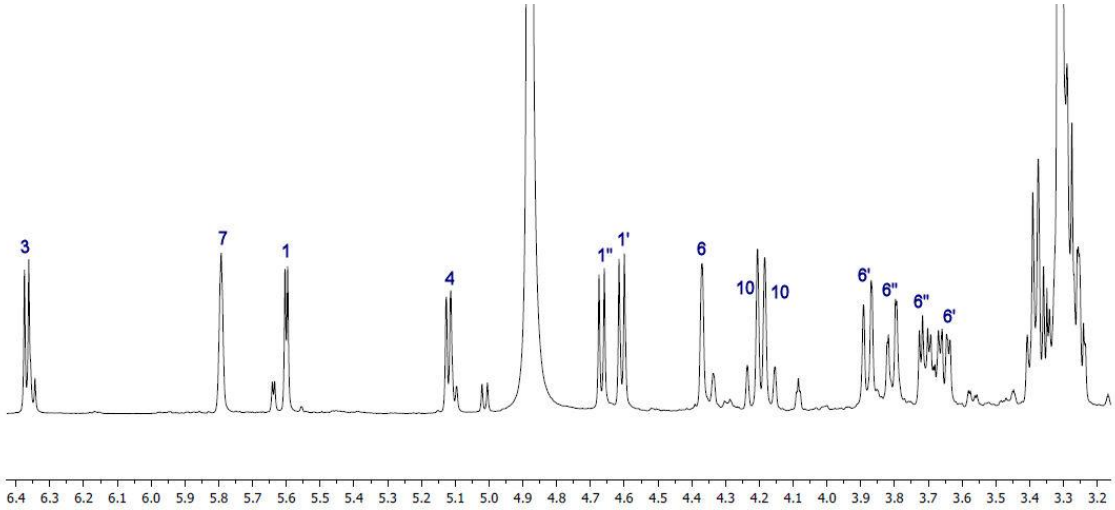
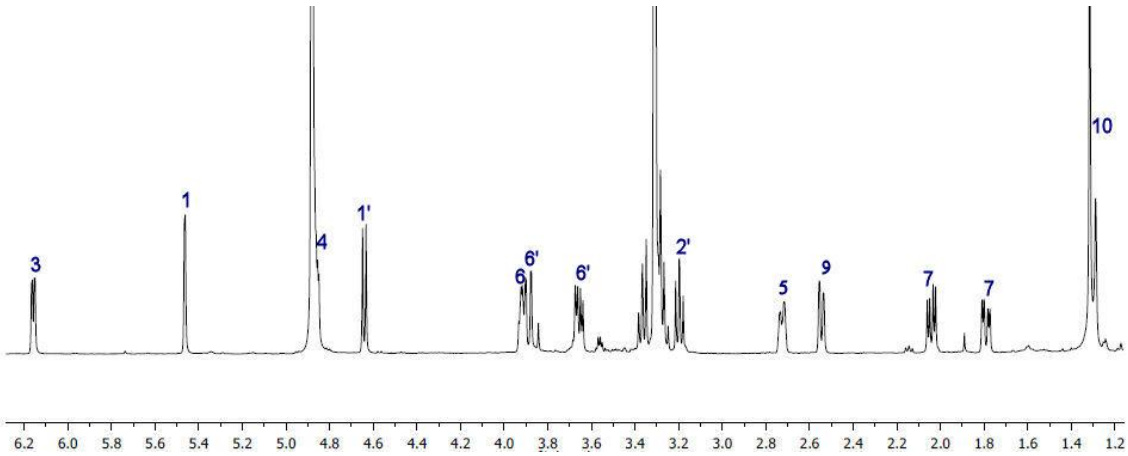


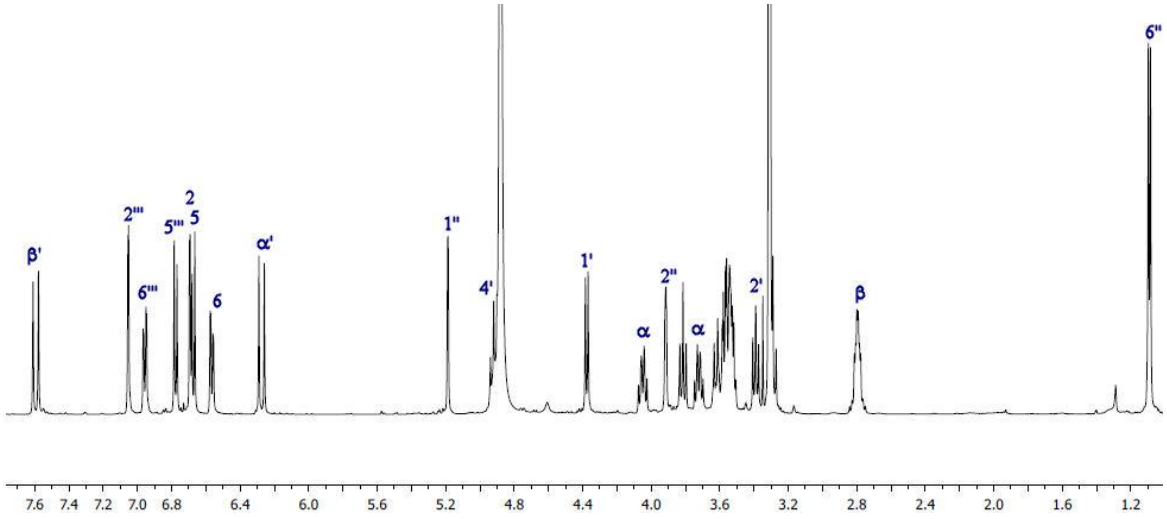
S4: HRESIMS of **2**



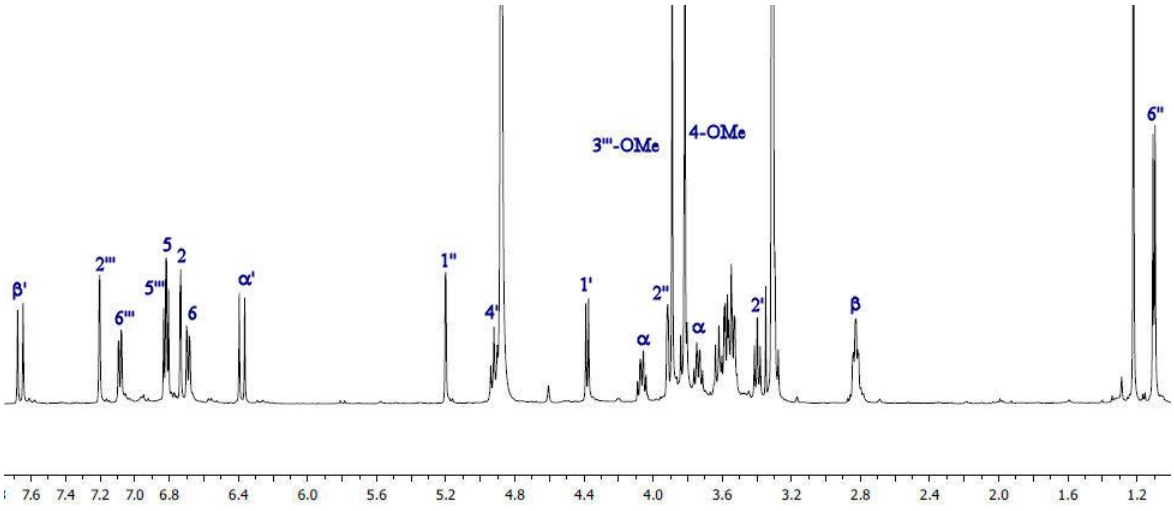
S5: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **2**



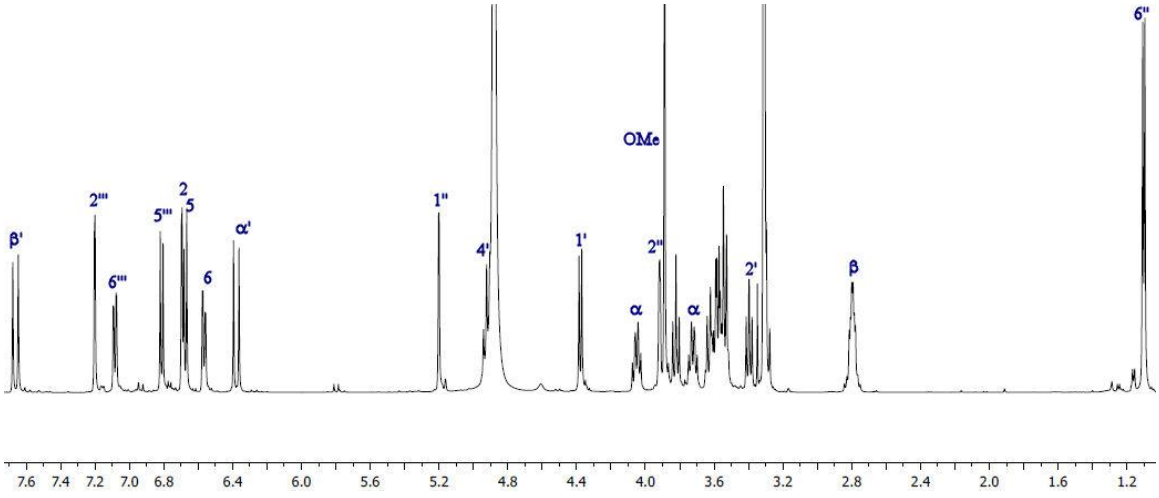
S6: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **3**



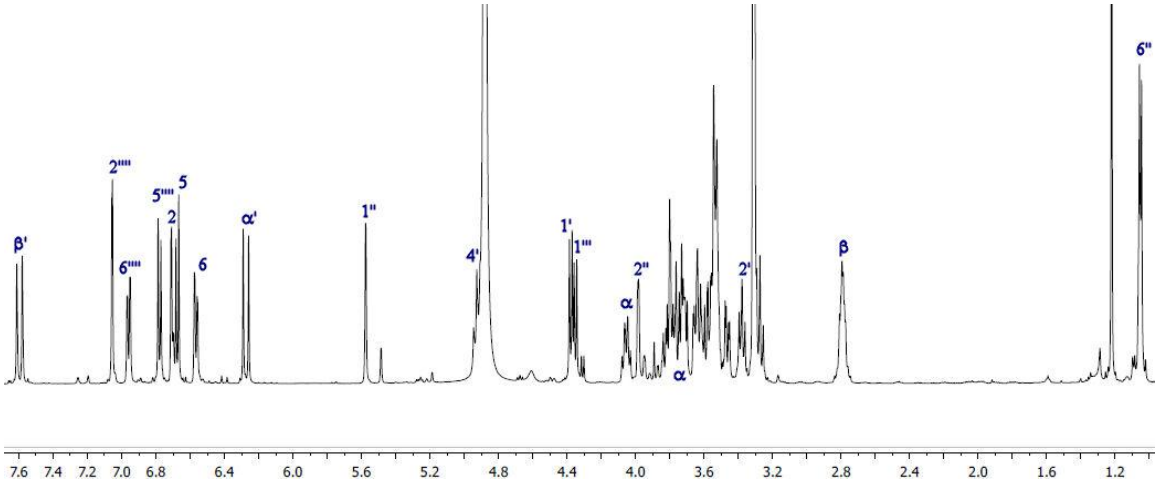
S7: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **4**



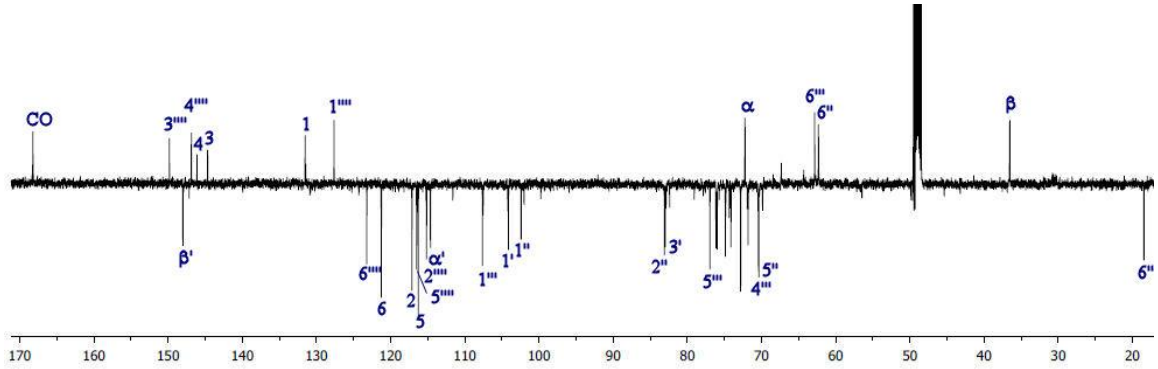
S8: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **5**



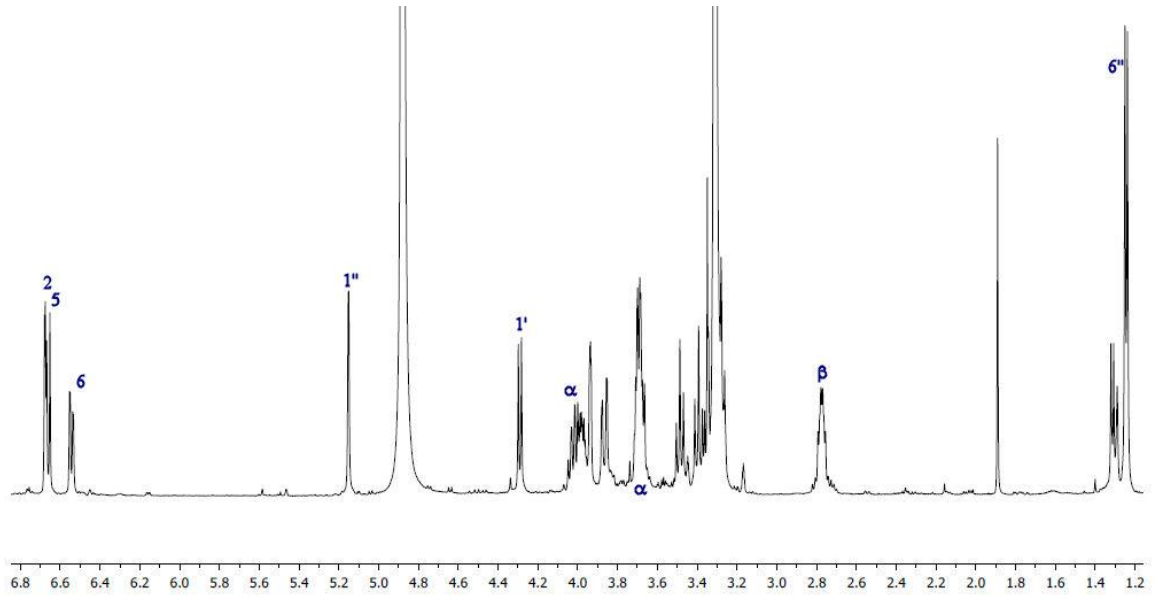
S9: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **6**



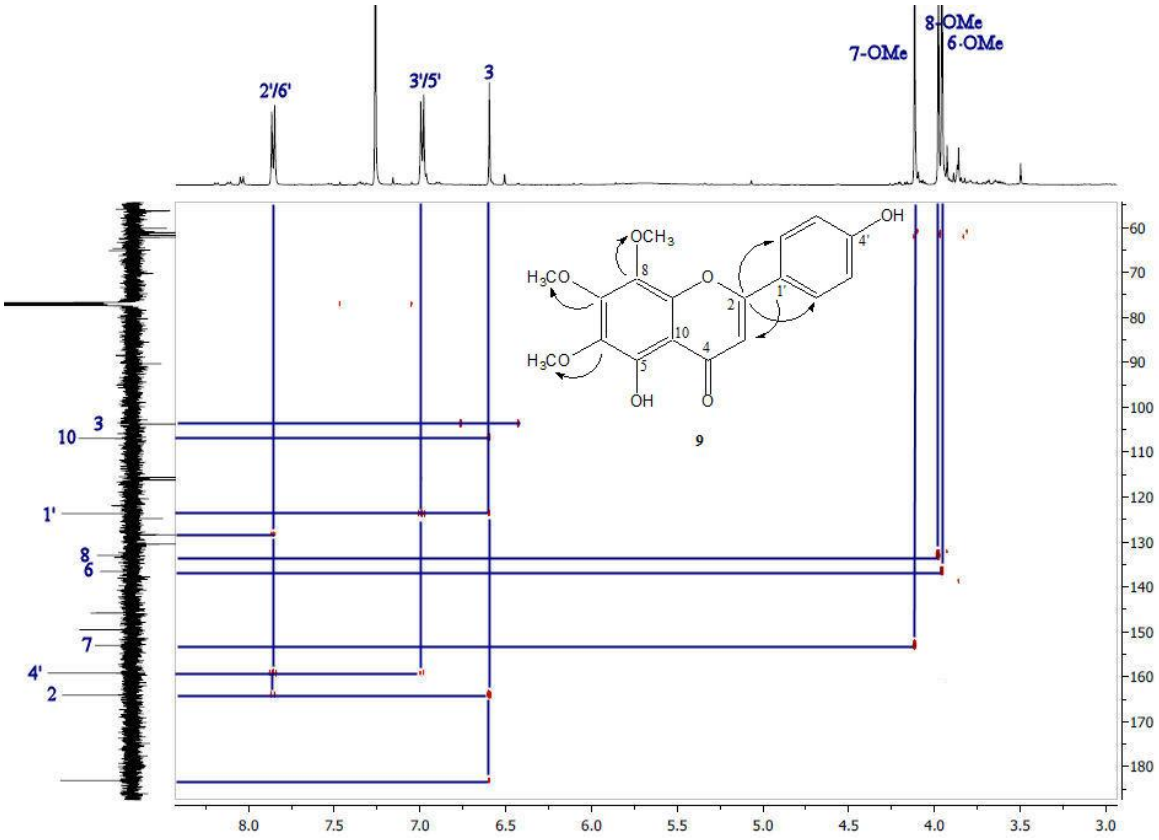
S10: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **7**



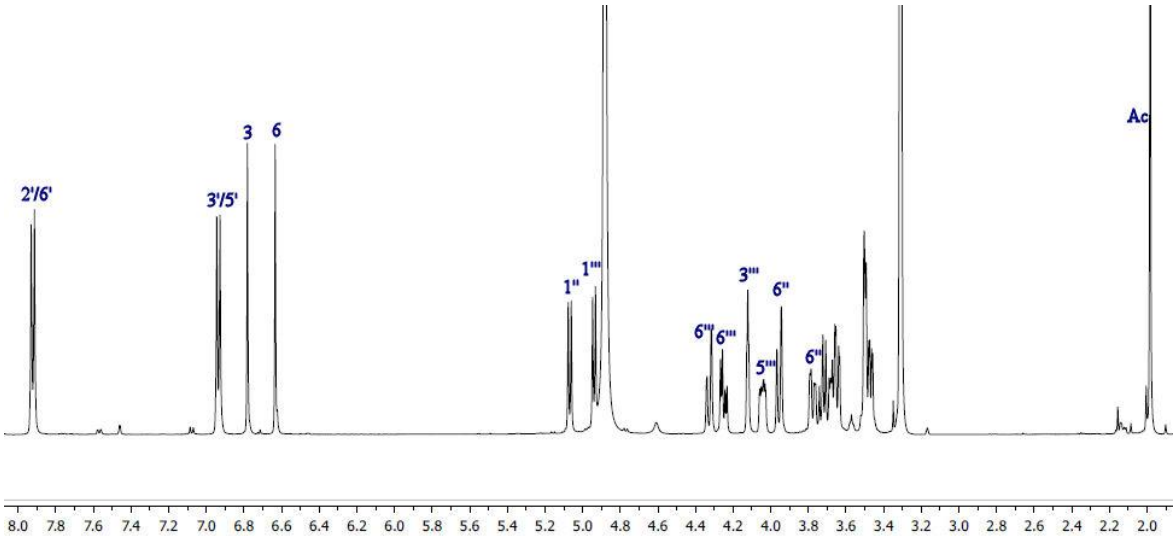
S11: APT-^{13}C-NMR (125 MHz, CD_3OD) Spectrum of **7**



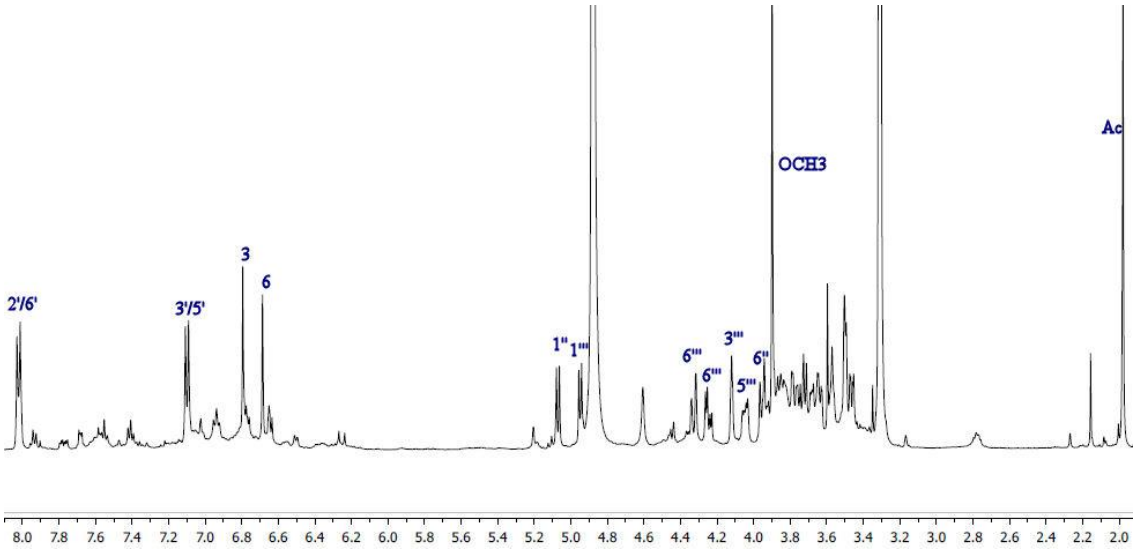
S12: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **8**



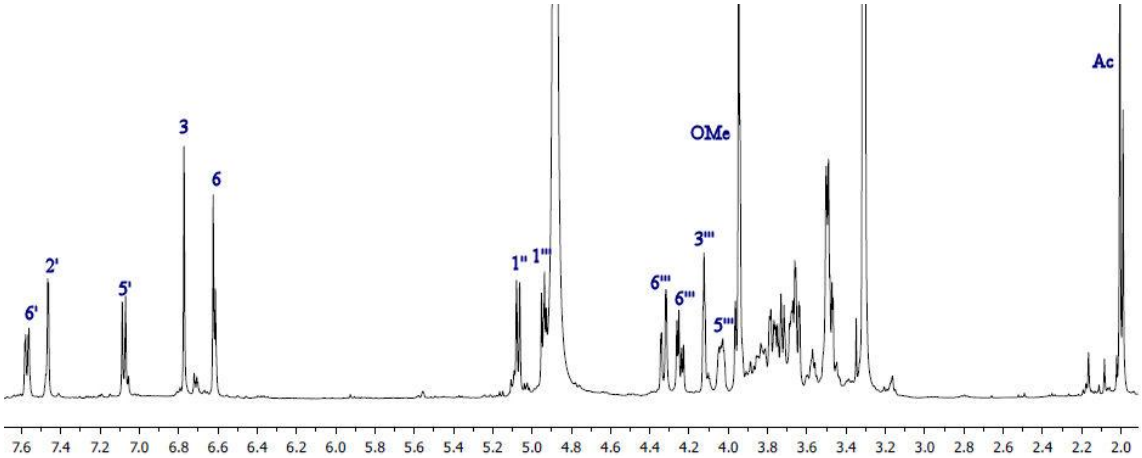
S13: HMBC Spectrum of **9**



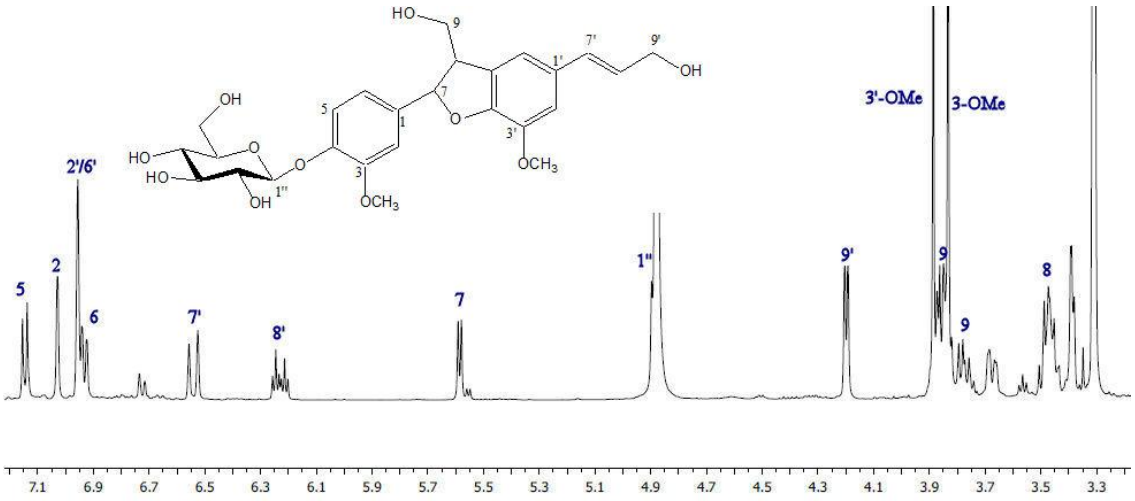
S14: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **10**



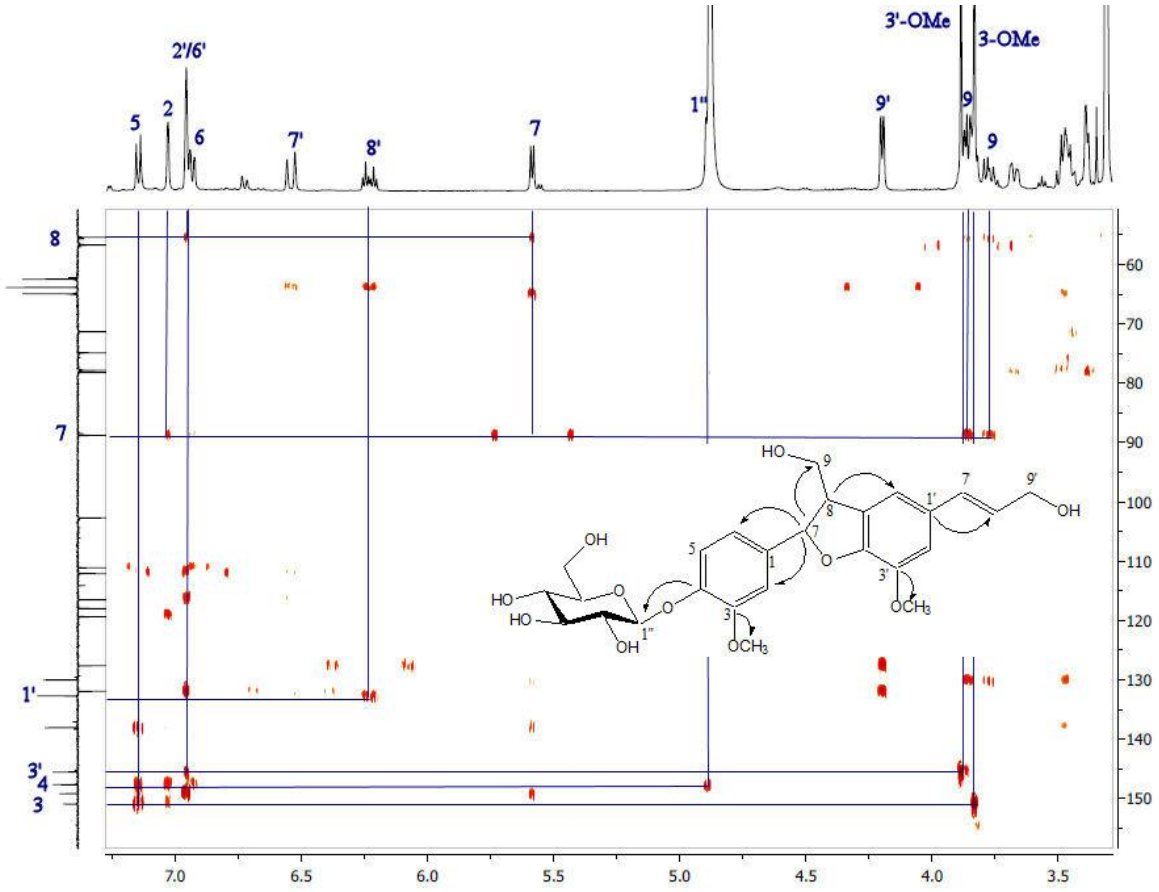
S15: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **11**



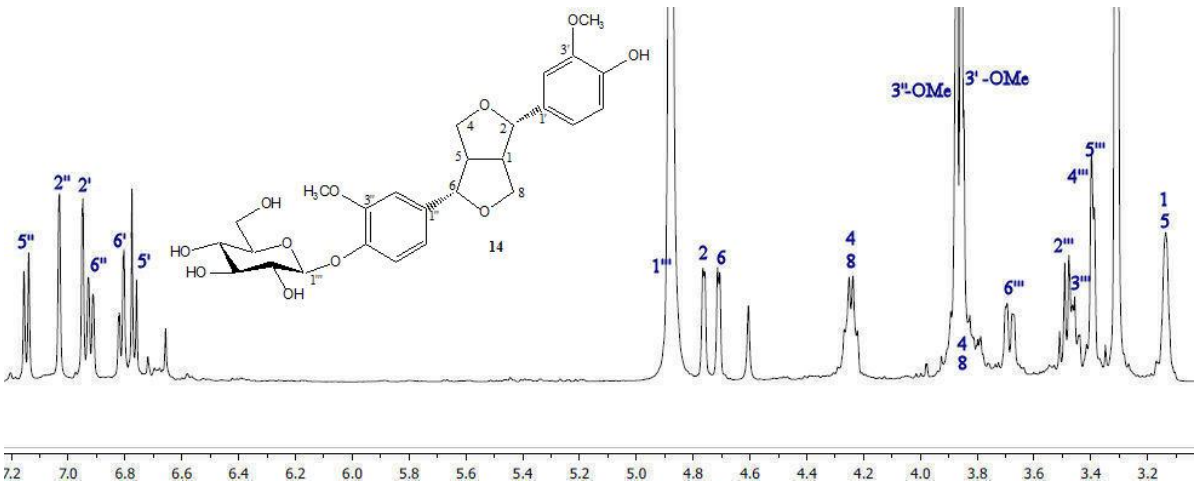
S16: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **12**



S17: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **13**



S18: HMBC Spectrum of **13**



S19: ^{1}H-NMR (500 MHz, CD_3OD) Spectrum of **14**

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