Supporting Information

Salvia officinalis for Hot Flushes: Towards Determination of Mechanism of Activity and Active Principles

Sinikka Rahte[1], Richard Evans[2, 3], Philippe J. Eugster[4], Laurence Marcourt[4], Jean-Luc Wolfender[4], Andreas Kortenkamp[2, 3], Deniz Tasdemir[1, 5]

Affiliation

[1] Centre for Pharmacognosy and Phytotherapy, School of Pharmacy, University of London, London, United Kingdom

[2] Centre for Toxicology, School of Pharmacy, University of London, London, United Kingdom

[3] Current address: Institute for the Environment, Brunel University, Uxbridge, United Kingdom

[4] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland

[5] Current address: School of Chemistry, National University of Ireland, Galway, Ireland

Correspondence

Prof. Dr. Deniz Tasdemir – School of Chemistry, National University of Ireland, Galway, University Road, Galway, Ireland. Email: deniz.tasdemir@nuigalway.ie. Phone: +353 91 49 24 50. Fax: +353 91 49 55 76

ERLUX assay

T47D-Kbluc cells were routinely grown in growth media (GM) consisting of RPMI (L-glutamine free), which contained glucose (2.5 g/L; Sigma Aldrich), HEPES (10 mM), sodium pyruvate (1 mM; Sigma Aldrich), insulin (0.2 U/mL), 10% foetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). Briefly, T47D-KBluc cells were transferred into a low-estrogen condition by using pre-assay media (PAM; identical to GM, but 10% carcol-dextran stripped FBS, no antibiotics) one week prior to testing. 10 000 cells/well in PAM were seeded into white, sterile, 96-well plates with lids (Greiner BIO-ONE Ltd.). Over the next 24 h the cells were allowed to attach. PAM was then removed from the plate and the samples, positive controls (E2, genistein), and solvent control (0.5% DMSO) were applied in dosing media (DM; phenol-red free RPMI, 5% carcol-dextran stripped FBS, no antibiotics). Eight replicates of solvent controls plus an antiestrogen, fulvestrant (1 μM), and eight replicates of the positive control plus fulvestrant (1 μM), were used to monitor the background estrogenicity in each assay. The performance of the assay was considered to be satisfactory when the antiestrogen sufficiently blocked the estrogenic effect of the positive control.

Cytotoxicity assessment in the ERLUX assay

40 000 cells/well were seeded and incubated as described for the ERLUX assay and 24 h after the application of the treatment, the DM was gently removed and replaced by 100 μL DM and an additional 20 μL of MTT solution (5 mg/mL in HBSS) per well. Cells were incubated for 4 h and lysed afterwards with 150 μL of a mixture consisting of 250 mL dimethylformamide, 250 mL deionised H_2O, 100 g sodium dodecyl sulphate, 10 mL glacial acetic acid, and 5 mL hydrochloric acid (2 M). The solubilisation solution was adjusted to pH 4.7 [1]. The optical density of the

formazan was quantified at 570 nm (Labsystem Multiscan). 40 000 cells/well was chosen for the cytotoxicity testing, as the original number of 10 000 cells/well was too low to observe mitochondrial activity by the formation of purple coloured formazan. The results of the cytotoxicity assay were normalised by subtraction of the background absorbance of the DM (100 μL), the MTT solution (20 μL), and 40 000 cells (blank wells). The normalised raw data were then divided by the average of on-plate solvent controls (n = 8) and multiplied by 100, to express the results as % of viable cells compared to solvent controls.

Modification of the SSRI assay

The cells (100 000 cells/well) were seeded into poly-D-lysine (50 mg/mL) coated 24-well plates (Nunc, Thermo Scientific) and allowed to attach for 48 h at 37 °C (5% CO_2, humidified incubator). The medium was removed and wells were washed with 200 μL wash-Krebs-Ringer-Hepes-buffer [wash-KRH, 120 mM NaCl, 4.7 mM KCl, 2.2 mM Ca_2Cl, 10 mM Hepes, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, endotoxin-free water (cell culture grade)]. Test samples were diluted with assay KRH identical to wash-KRH except for the addition of D-glucose (10 mM), ascorbic acid (100 μM), and paragyline (100 μM). The highest DMSO concentration in the wells was 1%. The test sample in the assay-KRH solution was applied (500 μL/well) and cells were incubated for 10 min at 37 °C, before 5-HT solution (20.5 pmoles 5-HT and 0.1 pmoles [^{3}H]-5-HT) was added to each well. Plates were incubated for a further 10 min and washed with ice-cold wash-KRH (3×) before the cells were solubilised in 0.1% NaOH.

Modification of the AChEI assay

Briefly, 25 μL Tris buffer (50 mM, pH 8) was added to all test wells (duplicate columns of 6 wells/sample). 25 μL of test solution were added to the first wells of the columns and by removing 25 μL from those wells and adding them to the next column; a 1:2 serial dilution was

performed. Subsequently, 50 μL of Tris buffer, 25 μL acetylthiocholiniodide solution (15 mM in Millipore water), and 125 μL Ellman's reagent (3 mM in 50 mM Tris–HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2 \times 6H_2O$) were added to each well. The plates were mixed on the plate shaker for 2 min in the dark, before the absorbance (background reading, spontaneous hydrolysis of substrate) was read at 405 nm on a plate reader (Labsystem Multiskan Multisoft). Subsequently, 25 μL of AChE solution (0.22 U/mL in 50 mM Tris–HCl, pH8, containing 0.1% bovine serum albumin) was added to each well. Plates were again mixed in the dark for 2 min on the plate shaker and read at 405 nm to determine the development of the resulting, yellow anion 5,5′-thio-2-nitro-benzoic acid. Solutions of acetylthiocholiniodide, DTNB and AChE were freshly prepared.

References

1 *Hansen MB, Nielsen SE, Berg K.* Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 1989; 2: 203-210

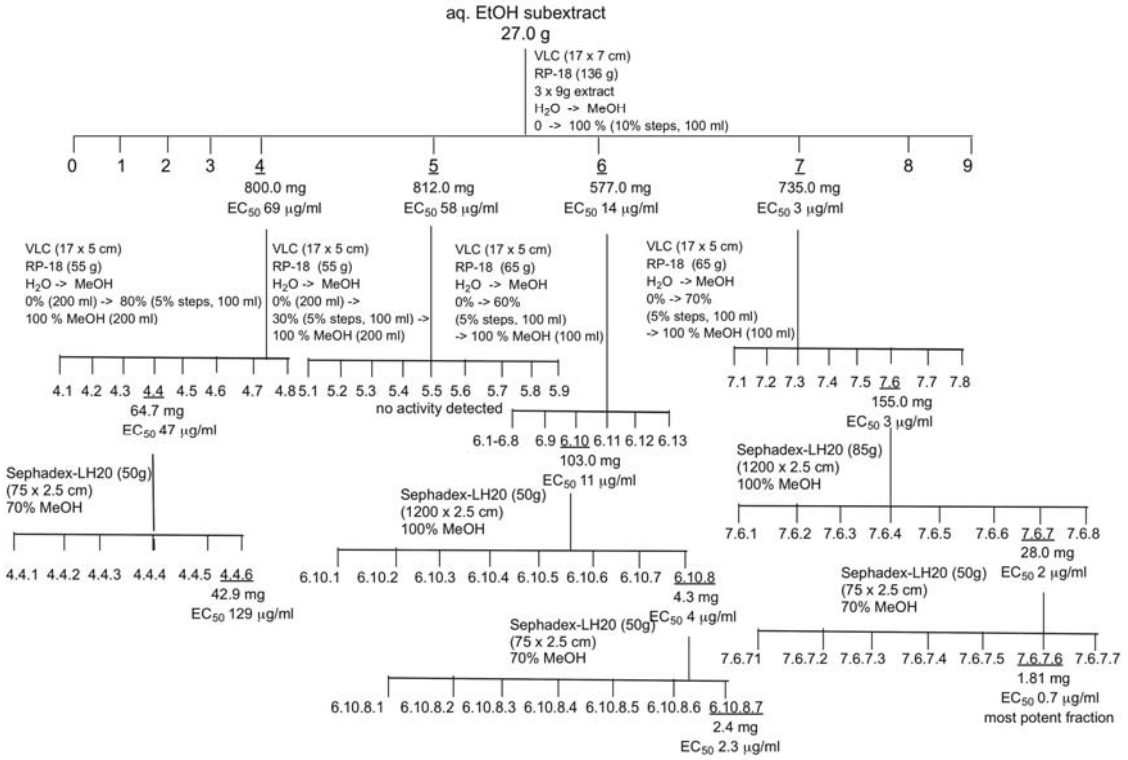


Fig. 1S Estrogenicity-guided isolation scheme of the aq. EtOH SE. Chromatographic methods are indicated on the left of each fractionation step. Underlined fractions were chosen for further fractionation based on estrogenic potential (EC_{50} values).

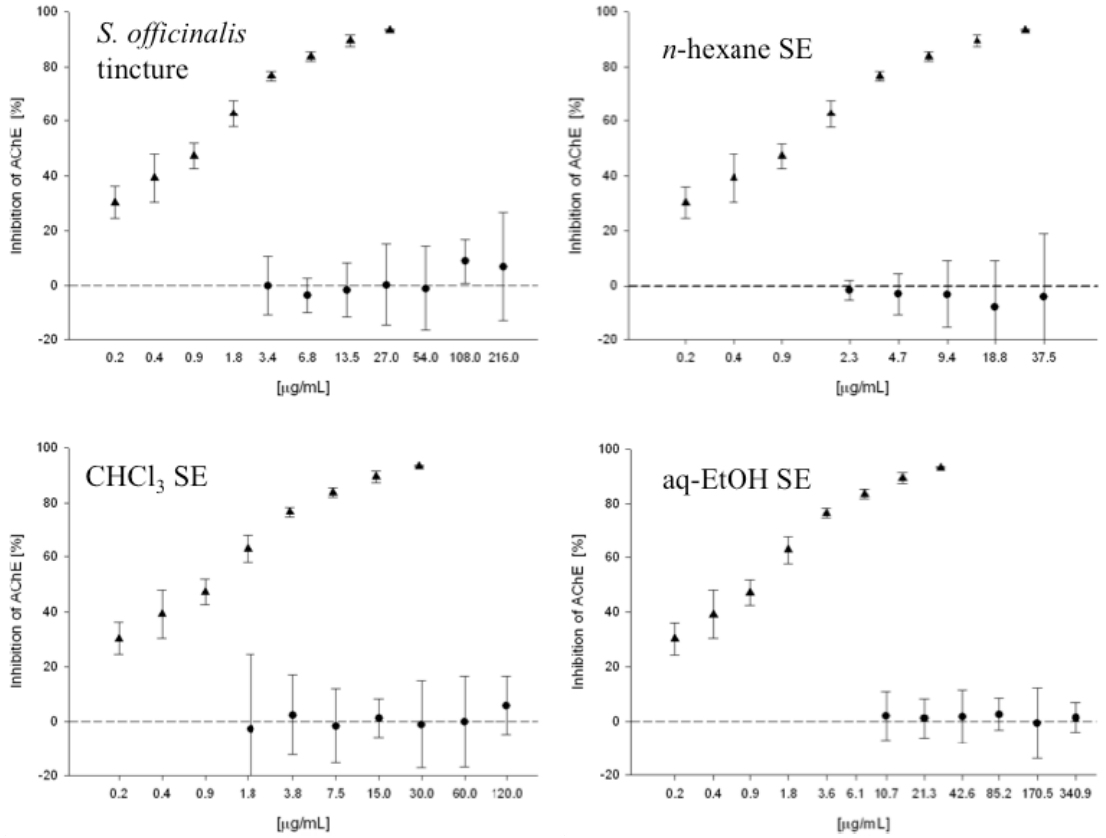


Fig. 2S AChE inhibition by the crude *S. officinalis* tincture, the *n*-hexane, the $CHCl_3$ and the aq-EtOH SEs and the positive control galanthamine (triangle). Data are from at least three independent experiments, tested in duplicate. Error bars represent 95% confidence intervals. *P < 0.05, significant inhibition (> 0).

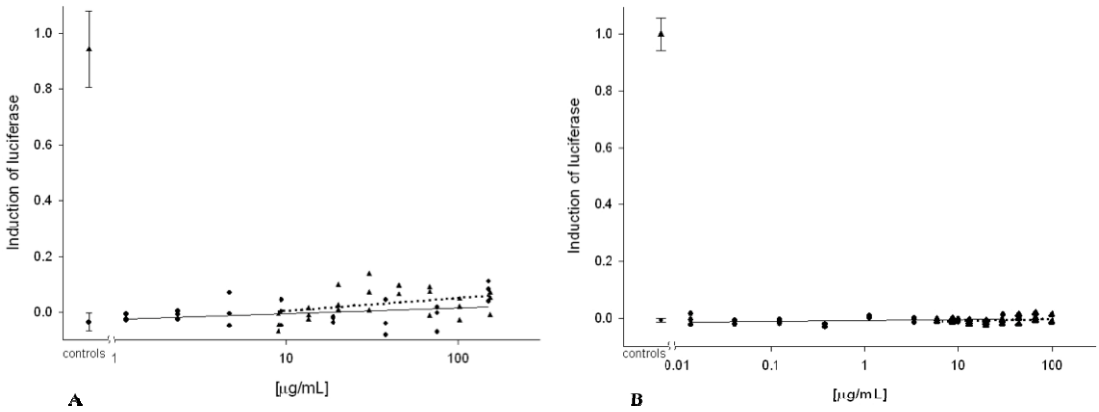
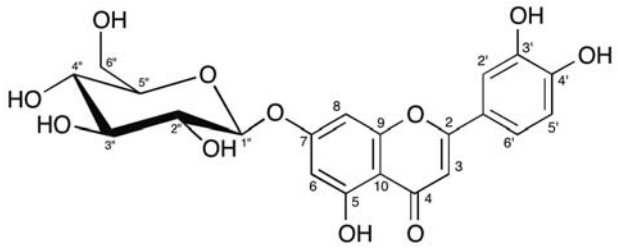
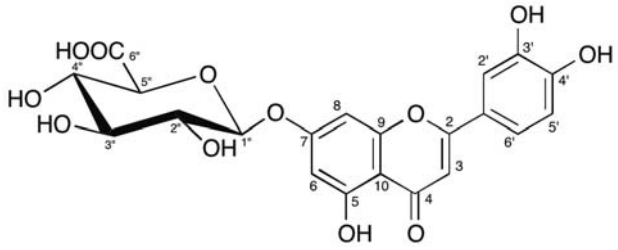


Fig. 3S ERLUX experiments in the presence of an ER-antagonist fulvestrant. **A** shows data from experiments with crude *Salvia officinalis* extract (circles) and aq-EtOH SE (triangles) and **B** shows $CHCl_3$ SE (triangle) and *n*- hexane SE (circles) in combination with fulvestrant (1.0 µM, antiestrogen), each experiment was done in triplicate. Filled circles represent individual data points. Data were fitted using a linear regression model. On-plate controls, positive controls (1.0 nM estradiol, triangle, n = 8), and E2 in combination with fulvestrant (in 1.0 nM/1.0 µM, circles, n = 8) are shown next to the y-axis (mean ± 95% CI).



Luteolin-7-*O*-glucoside



Luteolin-7-*O*-glucuronide

Fig. 4S Chemical structures of luteolin-7-*O*-glucoside and luteolin-7-*O*-glucuronide.

Position	δ (^{13}C)	δ (^{1}H)
2	164.5 *s*	-
3	102.9 *d*	6.7 (s)
4	181.8 *s*	-
5	161.0 *s*	-
6	99.5 *d*	6.41 (d, *J* = 2.0)
7	163.0 *s*	-
8	94.5 *d*	6.76 (d, *J* = 2.0)
9	157.0 *s*	-
10	105.2 *s*	-
1'	121.0 *s*	-
2'	113.4 *d*	7.46 (d, *J* = 1.7)
3'	146.0 *s*	-
4'	150.3 *s*	-
5'	116.1 *d*	6.86 (d, *J* = 8.4)
6'	119.0 *d*	7.37 (dd, *J* = 8.4, 2.0)
1"	99.6 *d*	5.10 (d, *J* = 7.4)
2"	73.0 *d*	3.13-3.37 (m)
3"	76.4 *d*	3.13-3.37 (m)
4"	71.9 *d*	3.13-3.37 (m)
5"	73.8 *d*	3.67 (d, *J* = 9.9)
6"	172.0 *s*	-

Fig. 5S ^{1}H- and ^{13}C-NMR data for luteolin-7-*O*-glucuronide (DMSO-d_6, 500 MHz/125 MHz). Chemical shifts are reported in ppm, *J* values in Hz.

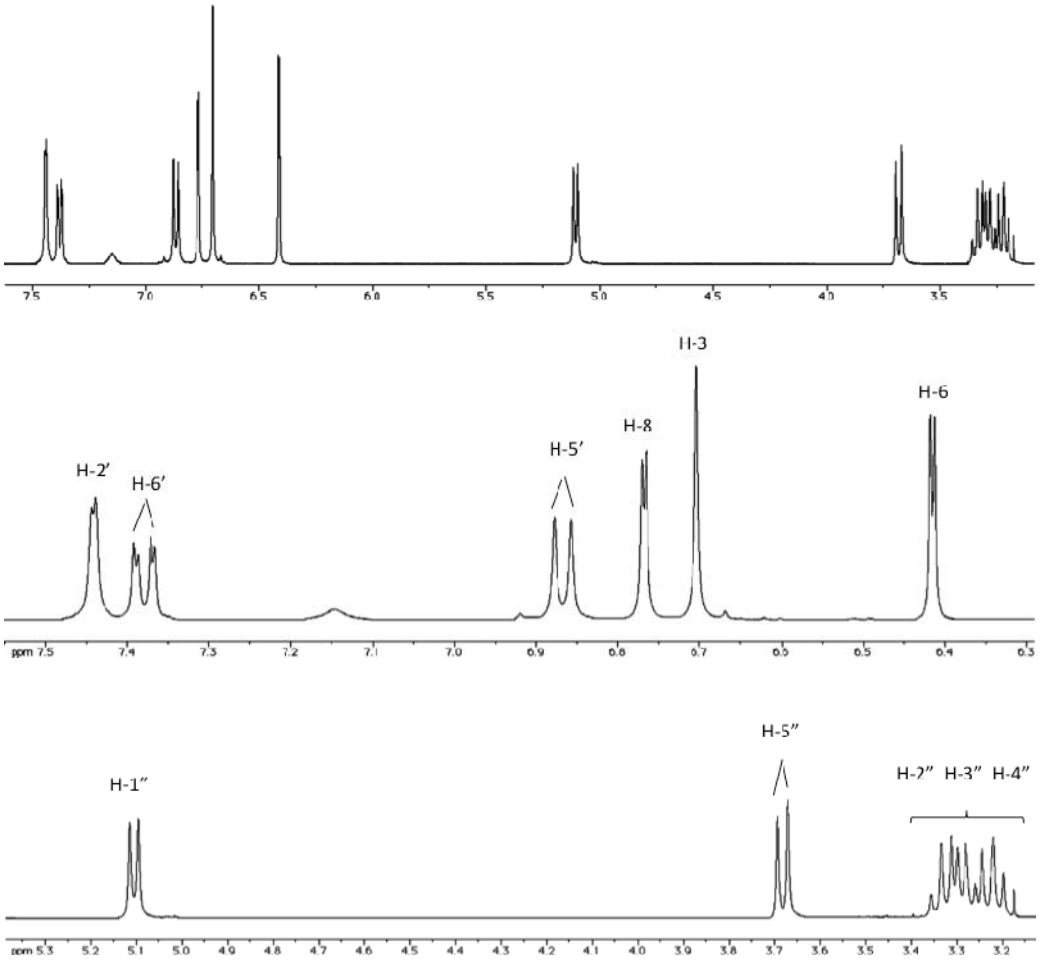


Fig. 6S ^{1}H-NMR spectrum of luteolin-7-*O*-glucuronide (DMSO-d_6, 500 MHz).

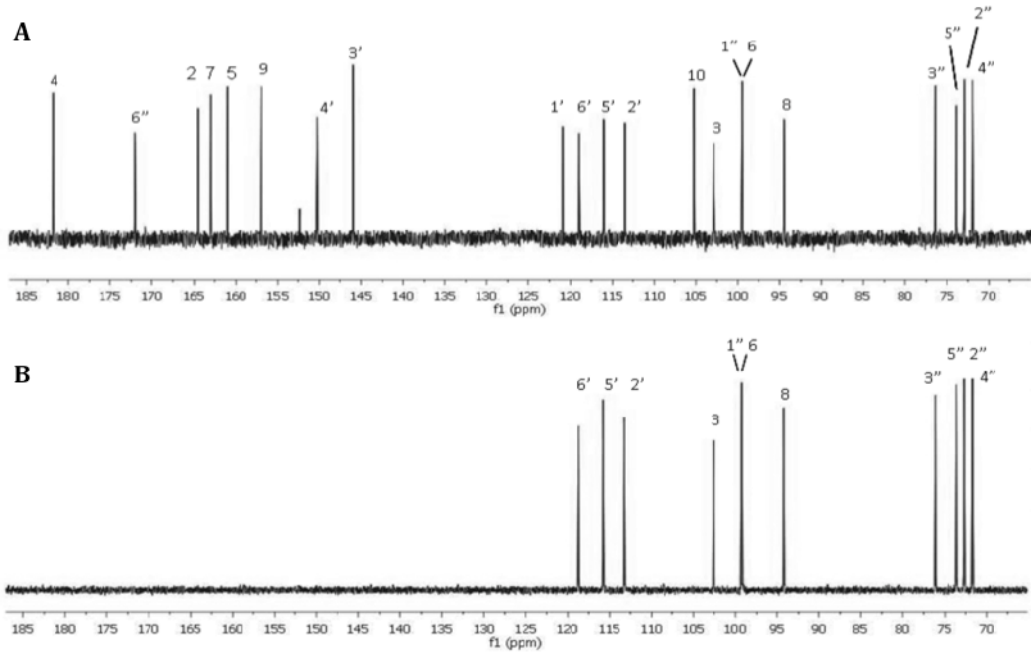


Fig. 7S ^{13}C-NMR (**A**) and DEPT-135 (**B**) spectra of luteolin-7-*O*-glucuronide (DMSO-d_6, 125 MHz).

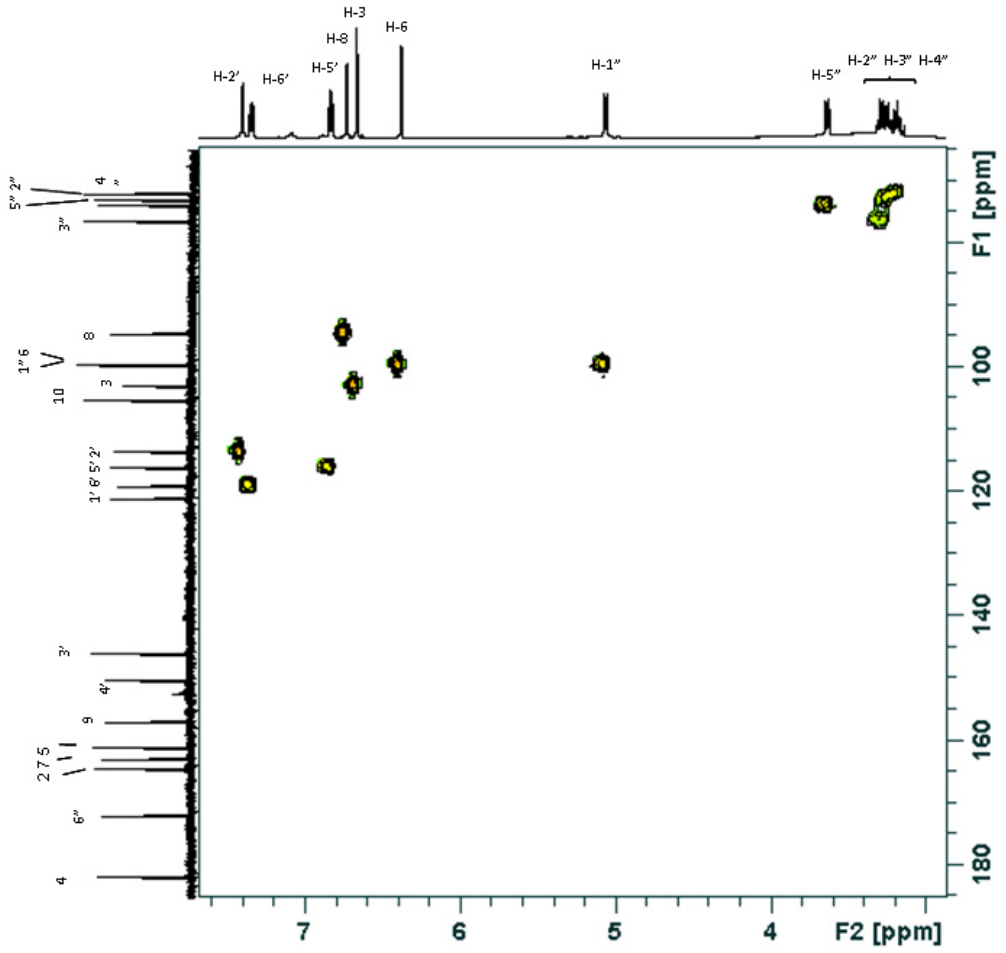


Fig. 8S The HMQC spectrum of luteolin-7-*O*-glucuronide (DMSO-d_6, 500 MHz).

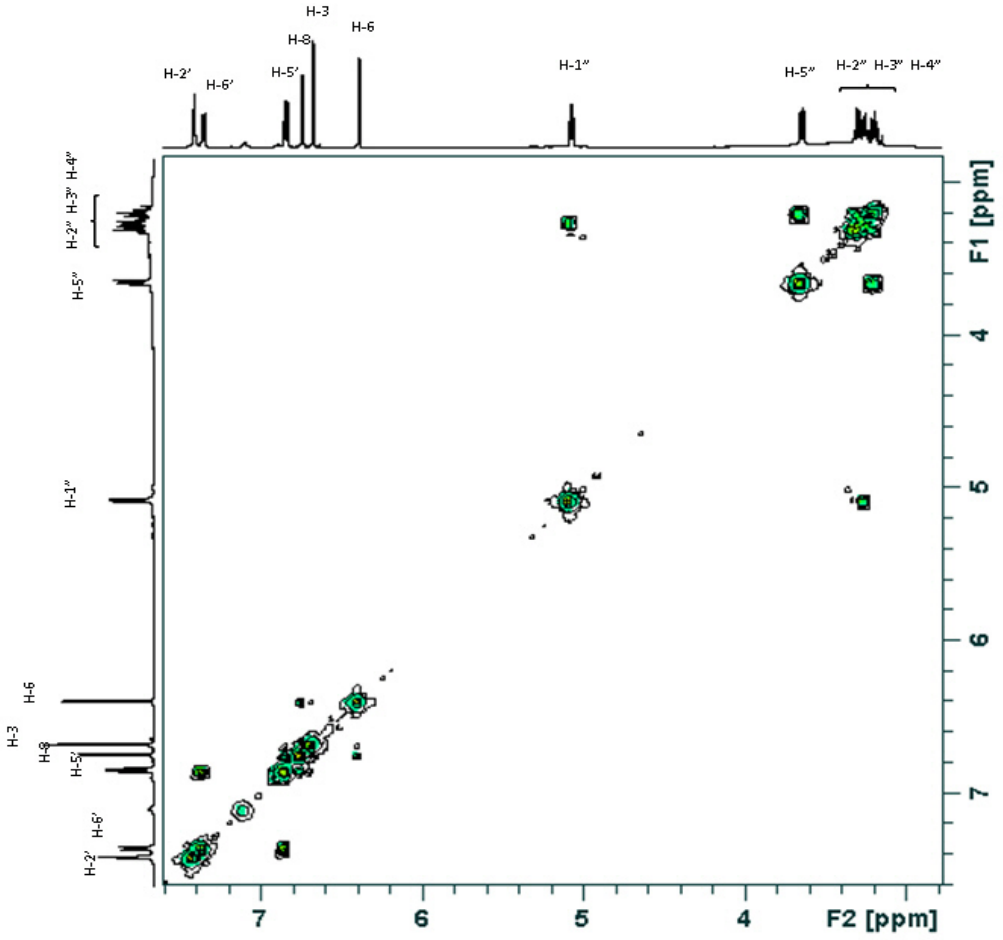


Fig. 9S The COSY spectrum of luteolin-7-*O*-glucuronide (DMSO-d_6, 500 MHz).

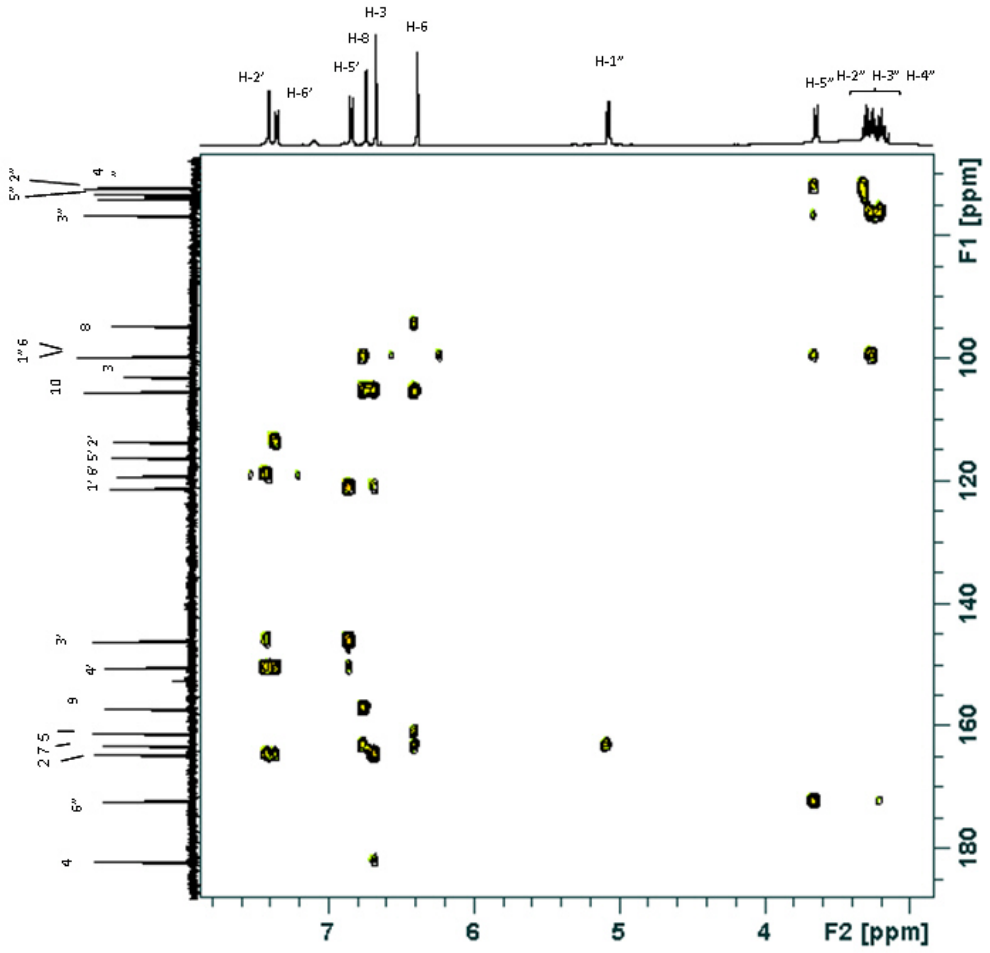


Fig. 10S The HMBC spectrum of luteolin-7-*O*-glucuronide (DMSO-d_6, 500 MHz).

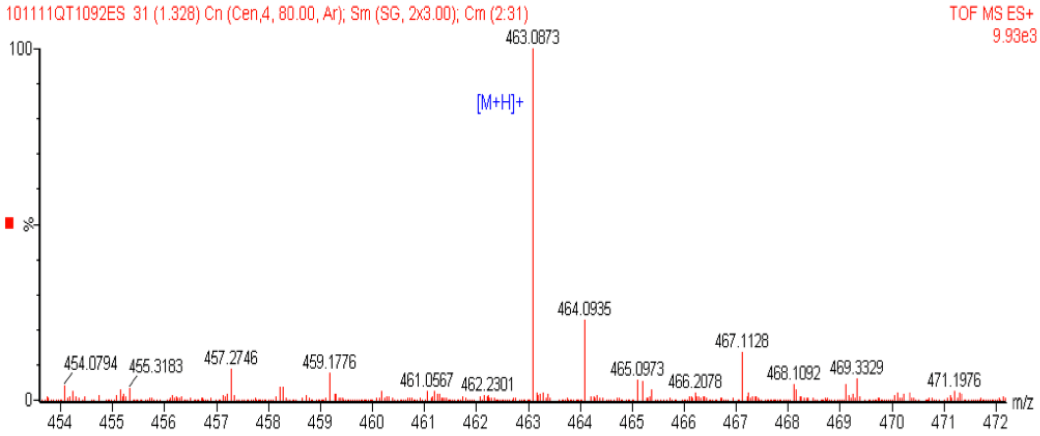


Fig. 11S The (+)-HR-ESI-MS spectrum of luteolin-7-*O*-glucuronide.

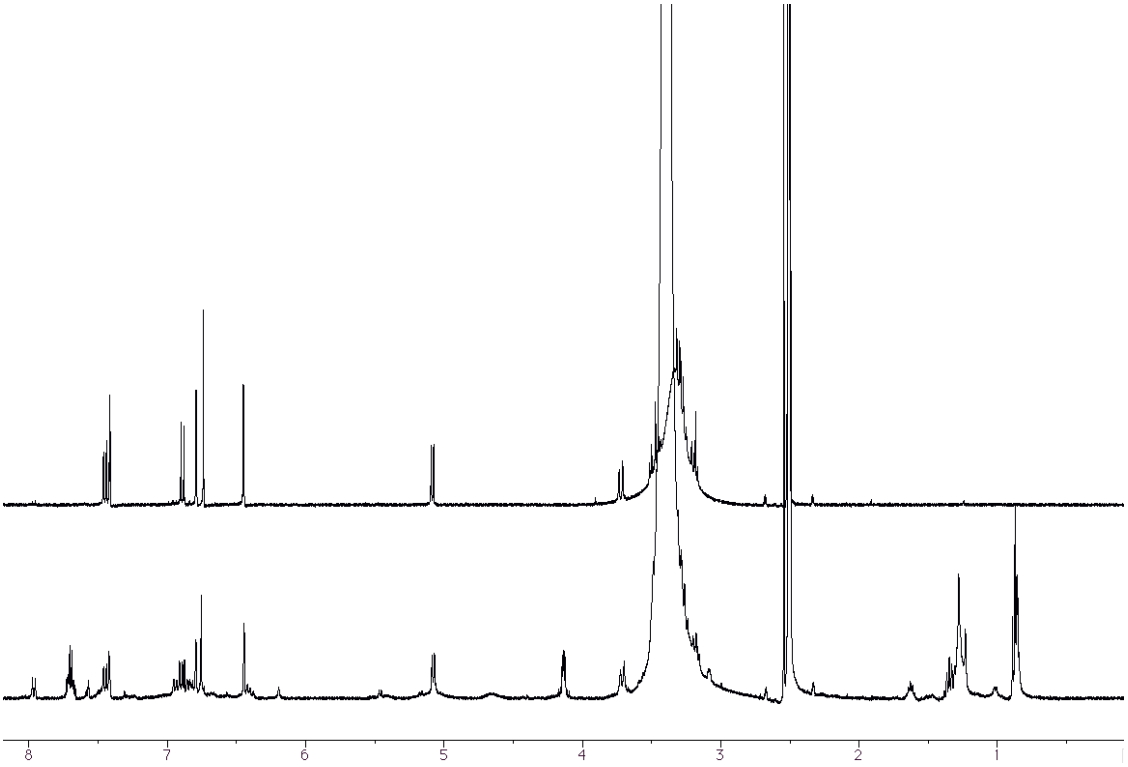


Fig. 12S ^{1}H-NMR spectrum of fraction 7.6.7.6 (bottom) and luteolin-7-*O*-glucoside (DMSO-d_6, 500 MHz).