Supporting Information

***Rec. Nat. Prod*. 8:2 (2014) 93-109**

Daphne oleoides Schreber ssp. *oleoides* Exhibits Potent Wound Healing Effect: Isolation of the Active Components and Elucidation of the Activity Mechanism

Ipek Süntar[1], Esra Küpeli Akkol[1]*, Hikmet Keles[2], Erdem Yesilada[3], Satyajit Sarker[4] and Turhan Baykal[1]

[a]*Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Etiler 06330, Ankara, Turkey*

[b]*Department of Pathology, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03200, Afyonkarahisar, Turkey*

[c]*Faculty of Pharmacy, Yeditepe University, Atasehir 34755, Istanbul, Turkey*

[d] *School of Pharmacy and Biomolecular Sciences,* Liverpool John Moores University *Liverpool, Merseyside, United Kingdom*

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S1: Extraction, fractionation and isolation procedures for the bioassays

The plant material was shade dried and powdered. 1000 g of aerial parts were extracted with 85% MeOH at room temperature for 24 hours. After filtration the extract was evaporated to dryness under reduced pressure not exceeding 40°C to give "DOO-MeOH" (yield: 18.53%). Dried methanol extract was then dissolved in 400 mLof methanol/H_2O (9:1) and further extracted with *n*-hexane (20×500 mL) in a separatory funnel. *n*-Hexane subextracts were collected and evaporated to dryness to give "DOO-*n*-Hexane" (yield: 4.20%). Methanol was evaporated from the remaining extract and diluted with distilled H_2O. The extract then successively extracted with dichloromethane (20×500 mL), EtOAc (20×500 mL) and *n*-butanol saturated with water (20×500 mL). Each solvent extract was evaporated to dryness to give "DOO-CH_2Cl_2" (yield: 12.08%), "DOO-EtOAc" (yield: 6.48%) and "DOO-*n*-BuOH" subextracts (yield: 22.24%), respectively. The remaining water subextract was also evaporated to dryness "DOO-R-H_2O" (yield: 23.80%).

S2: Preparation of test samples for bioassay

For the assessment of wound healing potential, the test materials were topically applied in an ointment base onto the wounded area on the dorsal part of the experimental animals. The extracts/subextracts/fractions and isolated compounds were mixed thoroughly in a mortar with a mixture of glycol stearate: propylene glycol: liquid paraffin (3:6:1) into an ointment form. Treatments were started immediately after the production of wound by daily application of the sample ointments on the wounded area. The control group animals were topically treated with blank ointment base, while the animals in negative control group were not treated with any product. A commercial wound ointment [Madecassol®, Bayer] (0.5 g) was used topically as the reference drug.

For the anti-inflammatory test model, samples were administered orally to test animals after suspended in a mixture of distilled water and 0.5% sodium carboxymethyl cellulose (CMC). The control group animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the dosing vehicle. Indomethacin (10 mg/kg) in 0.5% CMC was used as a reference drug.

S3: Linear incision wound model

Animals, six rats in each group, were anaesthetized with 0.05 cc Xylazine (2% Alfazine®) and 0.15 cc Ketamine (10% Ketasol®). The dorsal part hairs were shaved and cleaned with 70% alcohol. 5cm length two linear incisions were created with a sterile surgical blade through the full thickness of the skin. The wounds were closed with three surgical sutures. The test ointments were topically applied on the wounds in each group of animals once daily throughout 9 days. All the sutures were removed on the last day and tensile strength of the treated skin was measured with a tensiometer (Zwick/Roell Z0.5, Germany)

S4: Circular excision wound model

The mice were anaesthetized with 0.02 cc Xylazine (2% Alfazine®) and 0.08 cc Ketamine (10% Ketasol®). The dorsal hairs of the mice were shaved. The circular wound was created on the dorsal interscapular region of each animal by excising the skin with a 5 mm biopsy punch (Nopa instruments, Germany); and then wounds were left open. Test samples, the reference drug (Madecassol®) and the vehicle ointments were applied topically once a day

till the wounds completely healed. The progressive changes in wound area were monitored by a camera (Fuji, S20 Pro, Japan) every other day. Wound areas were calculated by AutoCAD program. Wound contraction was calculated as percentage of the reduction in wounded area. A specimen sample of tissue was removed for the histopathological analyses.

S5: Histopathology

The skin specimens from each group were collected at the end of the experiment (on day 12). Samples were fixed in 10% buffered formalin, processed and blocked with paraffin and then sectioned into 5 micrometer sections and stained with hematoxylin & eosin (HE) and Van Gieson (VG) stains. The tissues were examined under light microscope (Olympus CX41 attached with Kameram® Digital Image Analyze System) and graded as mild (+), moderate (++) and severe (+++) based on the degree of epidermal or dermal re-modeling. Re-epithelization or ulcus in epidermis; fibroblast proliferation, mononuclear and/or polymorphonuclear cells, neo-vascularization and collagen depositions in dermis were also analyzed to score the epidermal or dermal re-modeling. Van Gieson stained sections were analyzed for collagen deposition. At the end of the examination, all the wound healing processes were combined and staged for wound healing phases as inflammation, proliferation, and re-modeling in all groups.

S6: Hydroxyproline estimation

Tissues were dried in hot air oven at 60-70^{o}C till consistent weight was achieved. Afterwards, samples were hydrolyzed with 6 N HCl for 3 hours at 130^{o}C. The hydrolyzed samples were adjusted to pH 7 and subjected to chloramin T oxidation. The colored adduct formed with Ehrlich reagent at 60^{o}C was read at 557 nm. Standard hydroxyproline was also run and values reported as µg/mg dry weight of tissue.

S7: Anti-inflammatory activity: acetic acid-induced increase in capillary permeability

Each test sample was administered orally to a group of 10 mice in 0.2 mL/20 g body weight. Thirty minutes after the administration, tail of each mouse was injected with 0.1 mL of 4% Evans blue in saline solution (i.v.) and waited for 10 min. Then, 0.4 mL of 0.5% (v/v) AcOH was injected i.p. Twenty minutes after injection, the mice were killed by dislocation of the neck, then abdomen of each mouse was cut open and the viscera was exposed and irrigated with distilled water, which was then poured into 10 mL volumetric flasks by filtering through glass wool. Each flask was made up to 10 mL with distilled water, 0.1 mL of 0.1N NaOH solution was added to the flask, and the absorption of the final solution was measured at 590 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA). A mixture of distilled water and 0.5% CMC was given orally to control animals, and they were treated in the same manner as described above.

S8: Determination of antioxidant activity and total phenolics

The antioxidant activity of the extracts was determined according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. In this method, the hydrogen atom or electron donation capacity of the extracts were computed from the bleaching property of the purple-colored MeOH solution of DPPH. The samples and reference were dissolved in MeOH and mixed with DPPH solution (80 µg/mL). The amount of remaining DPPH was determined

spectrophotometrically at 517 nm. Quercetin was used as reference compound. DPPH inhibitory activity was estimated by using the following formula:

Inhibition (%) = $(A_{control}-A_{sample}) \times 100 / A_{control}$

where $A_{control}$ was the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} was the absorbance of the test/reference. Experiments were run in duplicate and the results were expressed as inhibition values.

Total phenolic contents of the methanolic extract and its subextracts were performed employing the methods involving Folin-Ciocalteu reagent and gallic acid as a standard. Test solution (100 µl) containing 1 mg of extract/or subextract was transferred in a volumetric flask, distilled water and Folin-Ciocalteu reagent were added and flask was shaken thoroughly. Na_2CO_3 solution (4 mL) was added and the mixture was allowed to stand for 2 h with intermittent shaking at room temperature. Then the absorbance of the test solution was measured at 765 nm. The same procedure was repeated for various concentrations of gallic acid solutions (0.05 mg/mL; 0.1 mg/mL; 0.15 mg/mL; 0.25 mg/mL and 0.5 mg/mL) and standard curve was obtained.

S9: Determination of hyaluronidase inhibitory activity

The inhibition of hyaluronidase enzyme was assessed by the measuring the amount of *N*-acetylglucosamine released from sodium hyaluronate. 50 µl of bovine hyaluronidase (7900 units/mL) was dissolved in 0.1M acetate buffer (pH 3.6). Then this solution was mixed with 50 µl of different concentrations of the extracts dissolved in 5% DMSO. For the control group 50 µl of 5% DMSO was added instead of the extracts. After 20 min incubation at 37^{o}C, 50 µl of calcium chloride (12.5 mM) was added to the mixture and again incubated for 20 min at 37^{o}C. 250 µl sodium hyaluronate (1.2 mg/mL) was added and incubated for 40 min at 37^{o}C. After incubation the mixture was treated with 50 µl of 0.4 M NaOH and 100 µl of 0.2 M sodium borate and then incubated for 3 min inside the boiling water bath. 1.5 mL of *p*-Dimethylaminobenzaldehyde solution was added to the reaction mixture after cooling to room temperature and was further incubated at 37^{o}C for 20 min to develop a color. The absorbance of this colored solution was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA).

S10: Determination of collagenase inhibitory activity

The samples were dissolved in DMSO. *Clostridium histolyticum* (ChC) was dissolved in 50 mM Tricine buffer (with 0.4M NaCl and 0.01M $CaCl_2$, pH 7.5). Then, 2 mM N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) solution was prepared in the same buffer. 25 µl buffer, 25 µL test sample and 25 µL enzyme were added to each well and incubated for 15 minutes. 50 µL substrate was added to the mixture to immediately measure the decrease of the optical density (OD) at 340 nm using a spectrometer.

The ChC inhibitory activity of each sample was calculated according to the following formula:

ChC inhibition activity (%)= $OD_{Control} - OD_{Sample} \times 100 / OD_{Control}$

where $OD_{control}$ and OD_{sample} represent the optical densities in the absence and presence of sample, respectively.

S11: Determination of elastase inhibitory activity

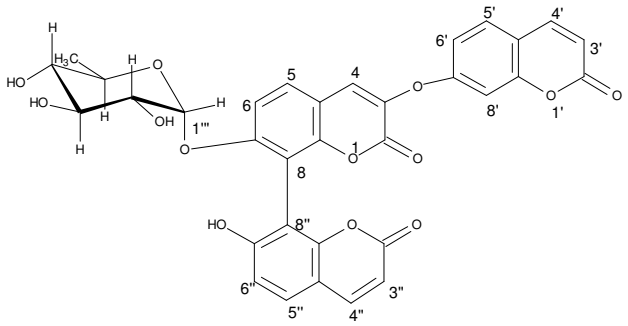
The sample solution and human neutrophil elastase enzyme (HNE) (17 mU/mL) were mixed in 0.1M Tris-HCl buffer (pH 7.5), then incubated at 25°C for 5 minutes. N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MAAPVN) was added to the mixture and incubated at 37°C for 1 hour. Afterwards, the reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/mL) and the optical density due to the formation of *p*-nitroaniline was immediately measured at 405 nm. The HNE inhibitory activities were calculated as described in the ChC inhibitory activity.

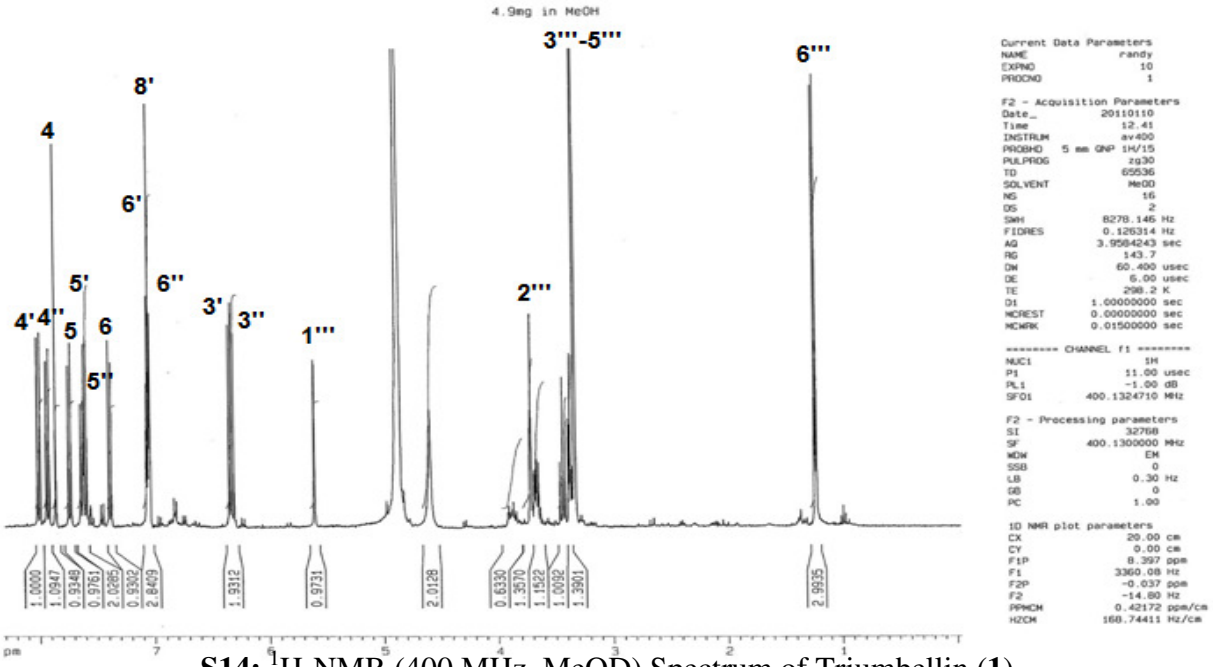
S12: Statistical analysis of the data

The data on percentage anti-inflammatory and wound healing was statistically analyzed using one-way analysis of variance (ANOVA). The values of $p \leq 0.05$ were considered statistically significant. Students-Newman-Keuls posthoc was used from the active extract and fractions. Histopathologic data were considered to be nonparametric; therefore, no statistical tests were performed.

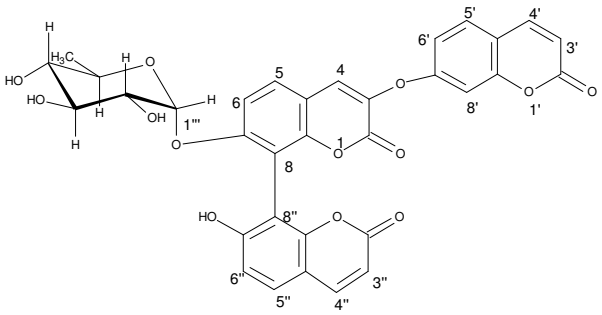
S13: NMR and MASS data for Triumbellin (**1**)

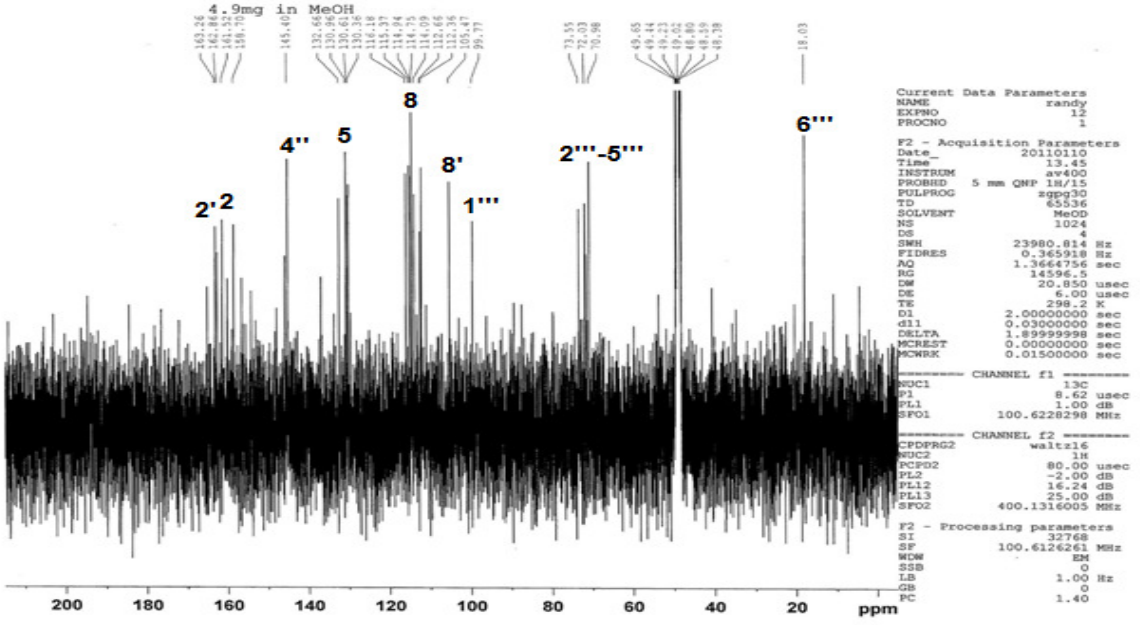
Triumbellin **(1,** 23.8 mg): Amorphous powder, FABMS *m/z* (+ve ion mode) 1274 $[2M + NH_4]^+$, 646 $[M + NH_4]^+$; ^{1}H NMR (400 MHz, MeOD): 8.05 d (*J* =9.5 Hz, H-4'), 7.95 d (*J* =9.5 Hz, H-4''), 7.85 s (H-4), 7.70 d (*J* =9.2 Hz, H-5'), 7.70 d (*J* = 8.8 Hz, H-5), 7.60 d (*J* =8.6 Hz, H-5''), 7.35 d (*J* =8.8 Hz, H-6), 7.05 d (*J* =9.2 Hz, H-6'), 7.05 d (*J* =8.6 Hz, H-6''), 7.05 s (H-8'), 6.30 d (*J* =9.5 Hz, H-3'), 6.30 d (*J* =9.5, H-3''), 5.56 d (*J* =1.8 Hz, H-1'''), 3.53 m (H-2'''), 3.35 m (H-3''', H-4''' and H-5'''), and 1.20 d (*J* =3.0 Hz, H-6'''); ^{13}C-NMR (100 MHz, MeOD): 163.3 (C-2'), 162.9 (C-2), 161.5 (C-7), 161.5 (C-2''), 158.7 (C-7'), 158.1 (C-7''), 156.8 (C-9'), 154.9 (C-9''), 151.9 (C-9), 144.7 (C-4'), 145.4 (C-4''), 137.8 (C-3), 132.6 (C-5'), 131.0 (C-4), 130.6 (C-5), 130.4 (C-5''), 116.2 (C-10'), 115.3 (C-3'), 114.9 (C-6'), 114.8 (C-8), 114.1 (C-10''), 112.8 (C-6''), 112.7 (C-6), 112.4 (C-3''), 111.5 (C-9), 107.3 (C-8''), 105.5 (C-8'), 99.8 (C-1'''), 73.6 (C-4'''), 72.0 (C-2'''), 71.0 (C-3'''), 71.0 (C-5''') and 18.0 (C-6''').



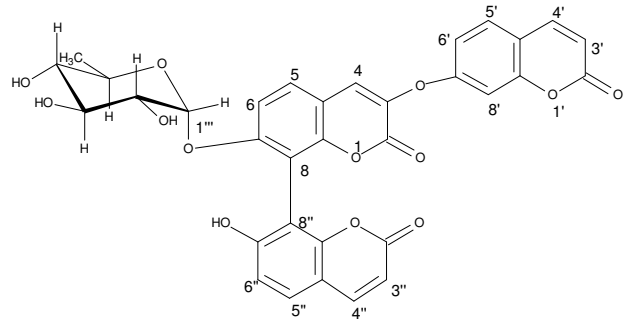


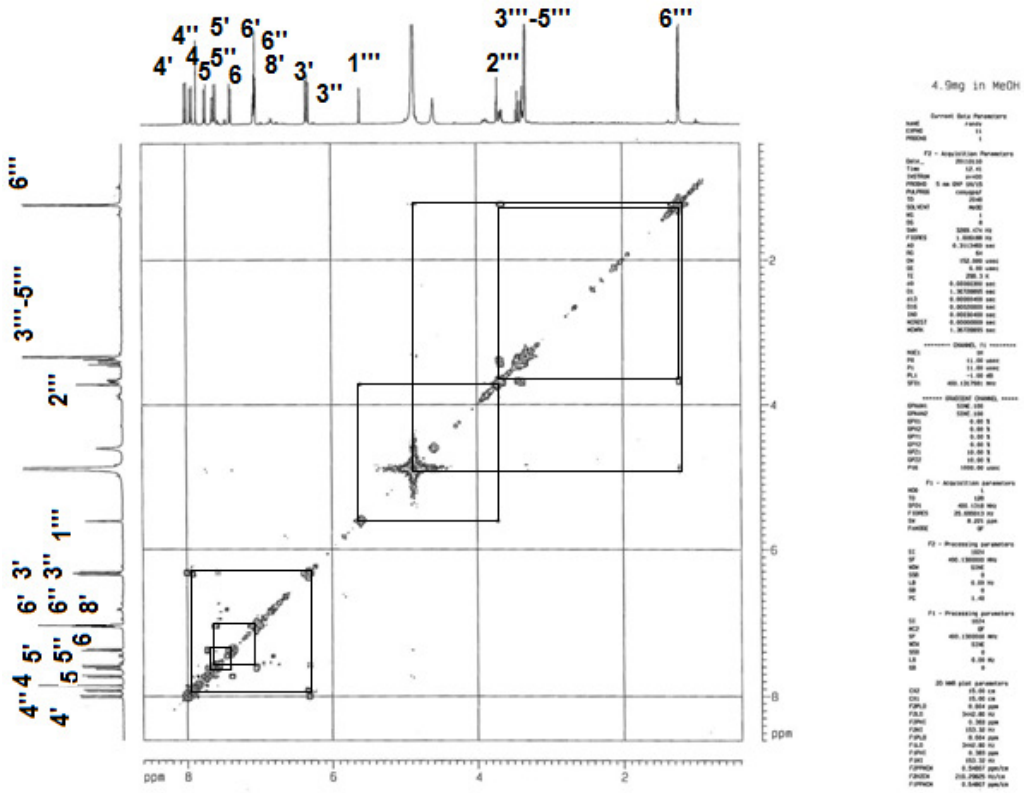
S14: ^{1}H-NMR (400 MHz, MeOD) Spectrum of Triumbellin (**1**)



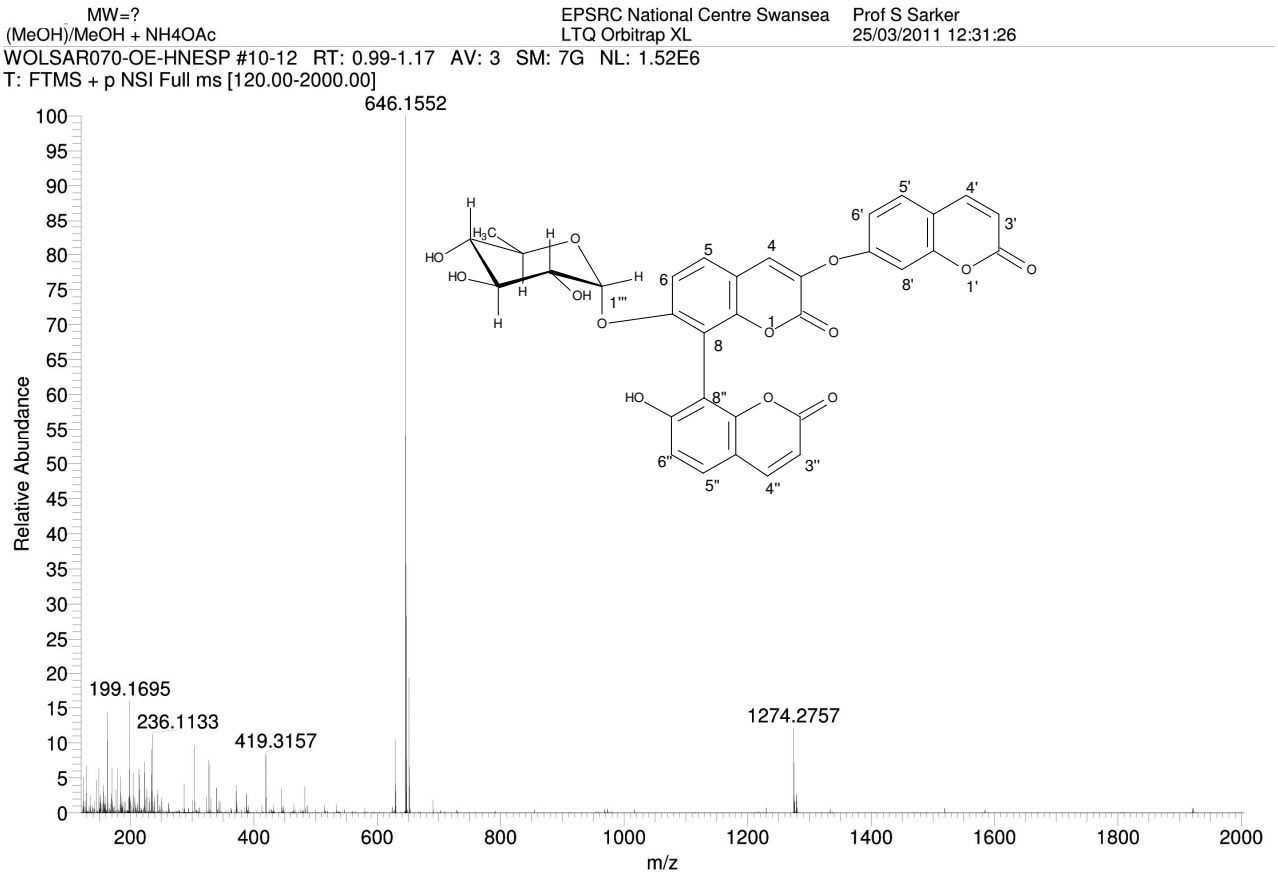


S15: ^{13}C-NMR (100 MHz, MeOD) Spectrum of Triumbellin (**1**)





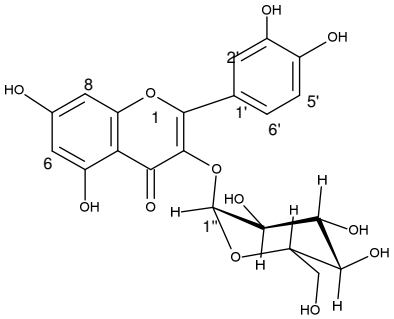
S16: HOMO-COSY ^{1}H-NMR (400 MHz, MeOD) Spectrum of Triumbellin (**1**)

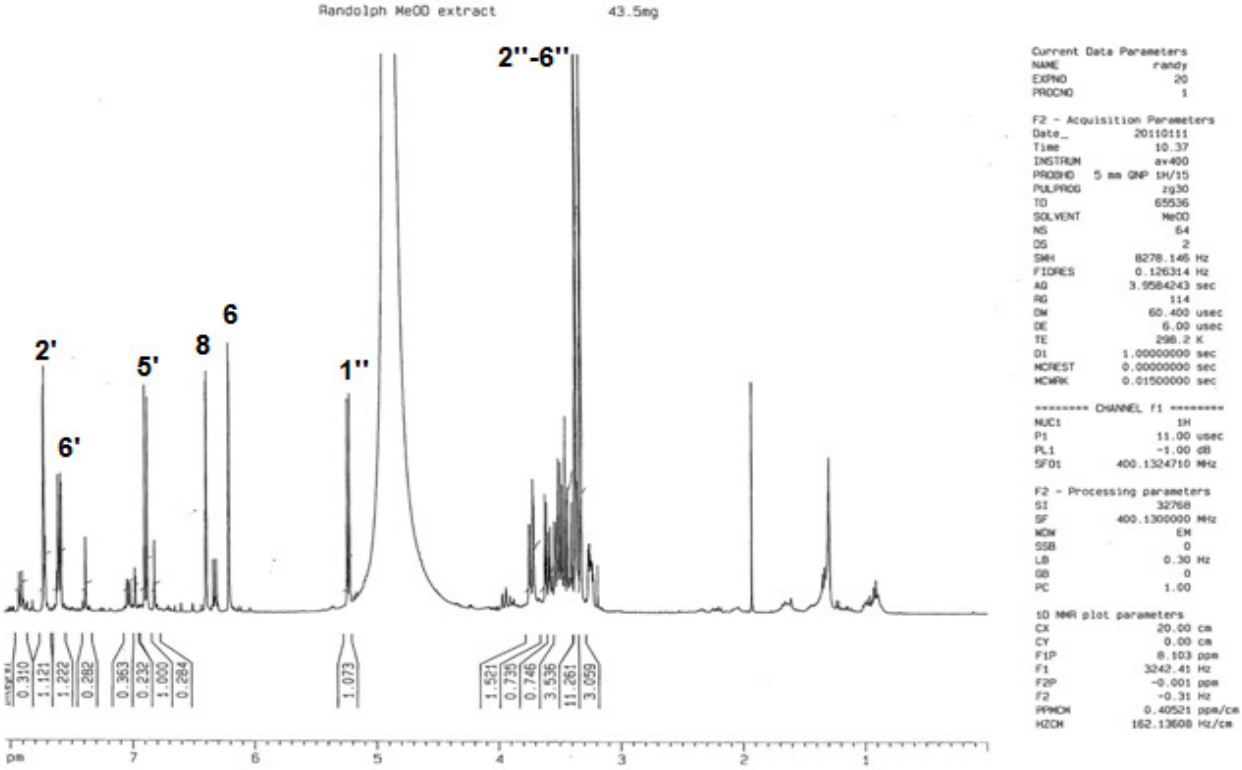


S17: FTMS Spectrum of Triumbellin (**1**)

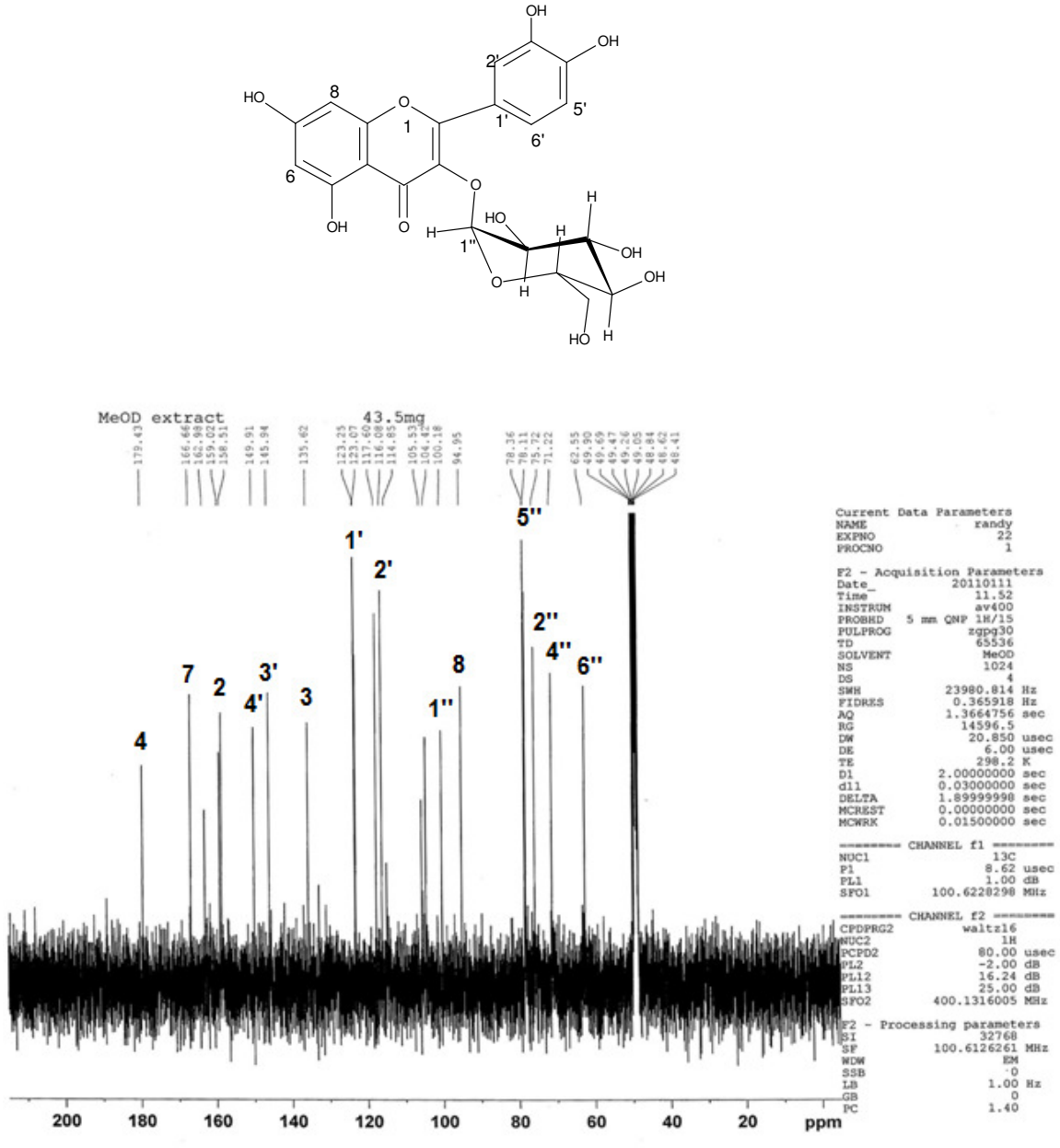
S18: NMR and MASS data for Quercetin-3-*O*-glucoside (**2**)

Quercetin-3-O-glucoside (**2**, 177.2 mg), Yellow amorphous powder, FABMS *m/z* (+ve ion mode) 951 $[2M + Na]^+$, 465 $[M + H]^+$; ^{1}H NMR (400 MHz, MeOD): 7.70 d (*J* =2.0 Hz, H-2'), 7.57 dd (*J* = 8.0, 2.0 Hz, H-6'), 6.90 d (*J* = 8.0 Hz, H-5'), 6.40 d (*J* =2.0 Hz, H-8), 6.20 d (*J* =2.0 Hz, H-6), 5.10 d (*J* =7.7 Hz, H-1'') and 3.30-3.80 m (H-2'', H-3'', H-4'', H-5'' and H-6''); ^{13}C-NMR (100 MHz, MeOD): 179.4 (C-4), 166.7 (C-7), 163.0 (C-5), 159.0 (C-9), 158.5 (C-2), 149.9 (C-4'), 145.9 (C-3'), 135.6 (C-3), 123.3 (C-1'), 123.1 (C-6'), 117.6 (C-5'), 116.1 (C-2'), 105.5 (C-10), 104.4 (C-6), 100.2 (C-1''), 95.0 (C-8), 78.3 (C-5''), 78.1 (C-3''), 75.7 (C-2''), 71.2 (C-4'') and 62.5 (C-6'').

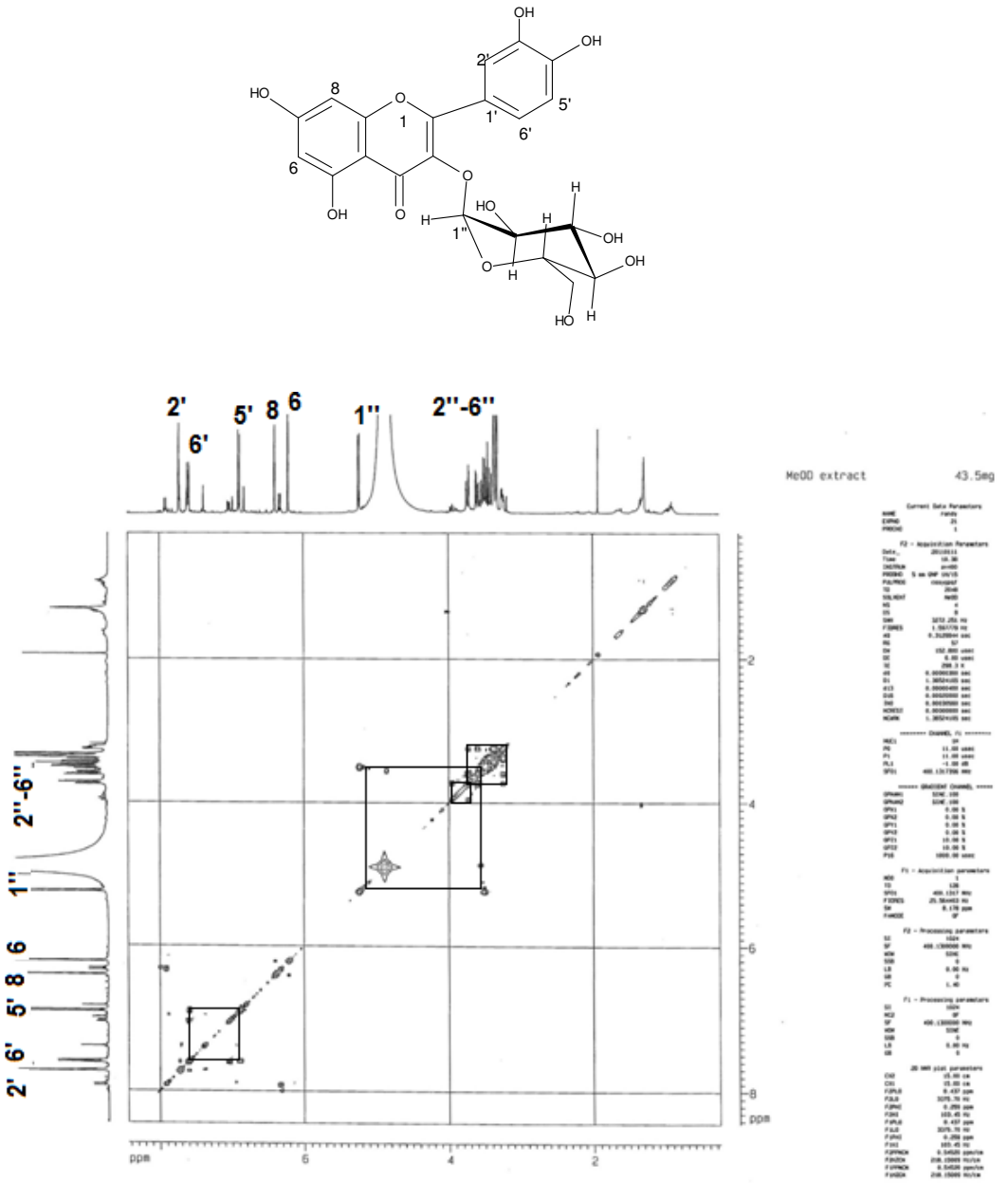




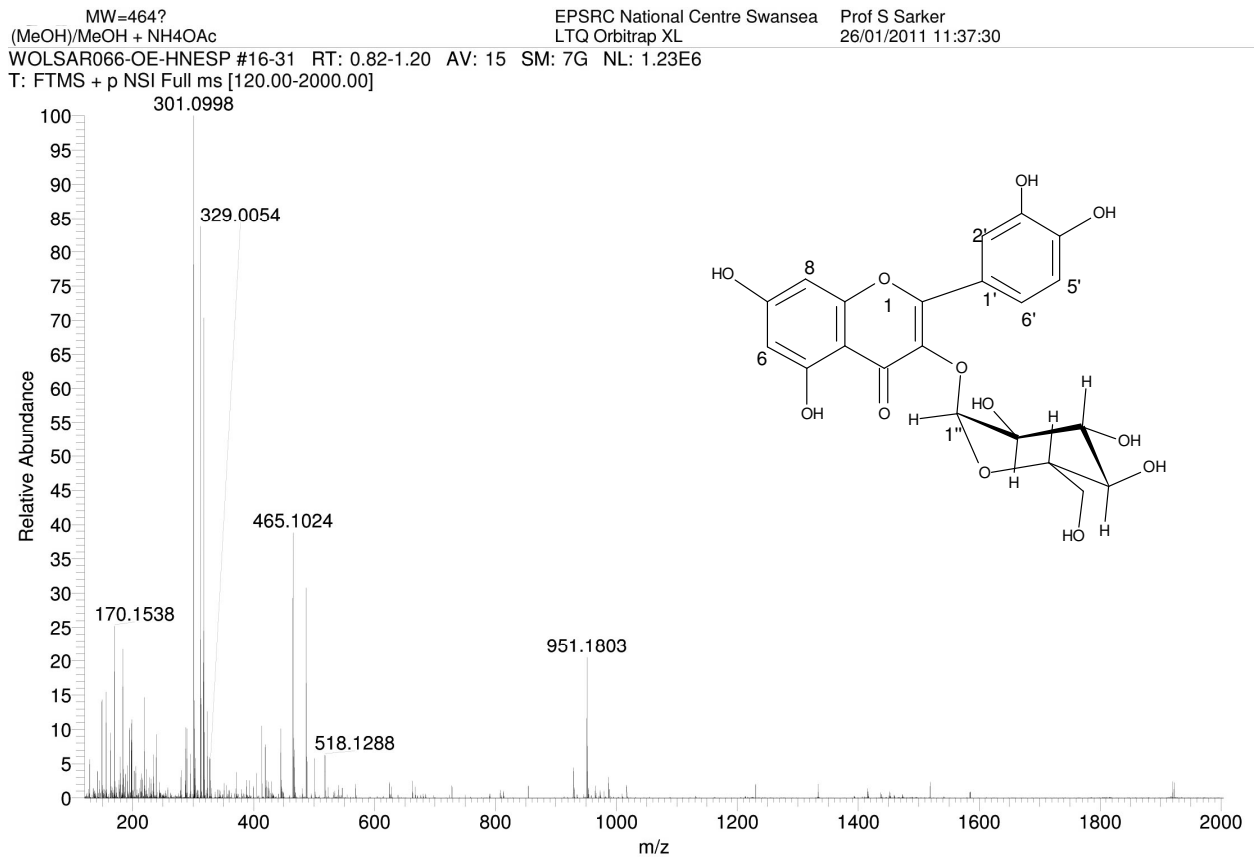
S19: ^{1}H-NMR (400 MHz, MeOD) Spectrum of Quercetin-3-*O*-glucoside (**2**)



S20: ^{13}C-NMR (100 MHz, MeOD) Spectrum of Quercetin-3-*O*-glucoside (**2**)



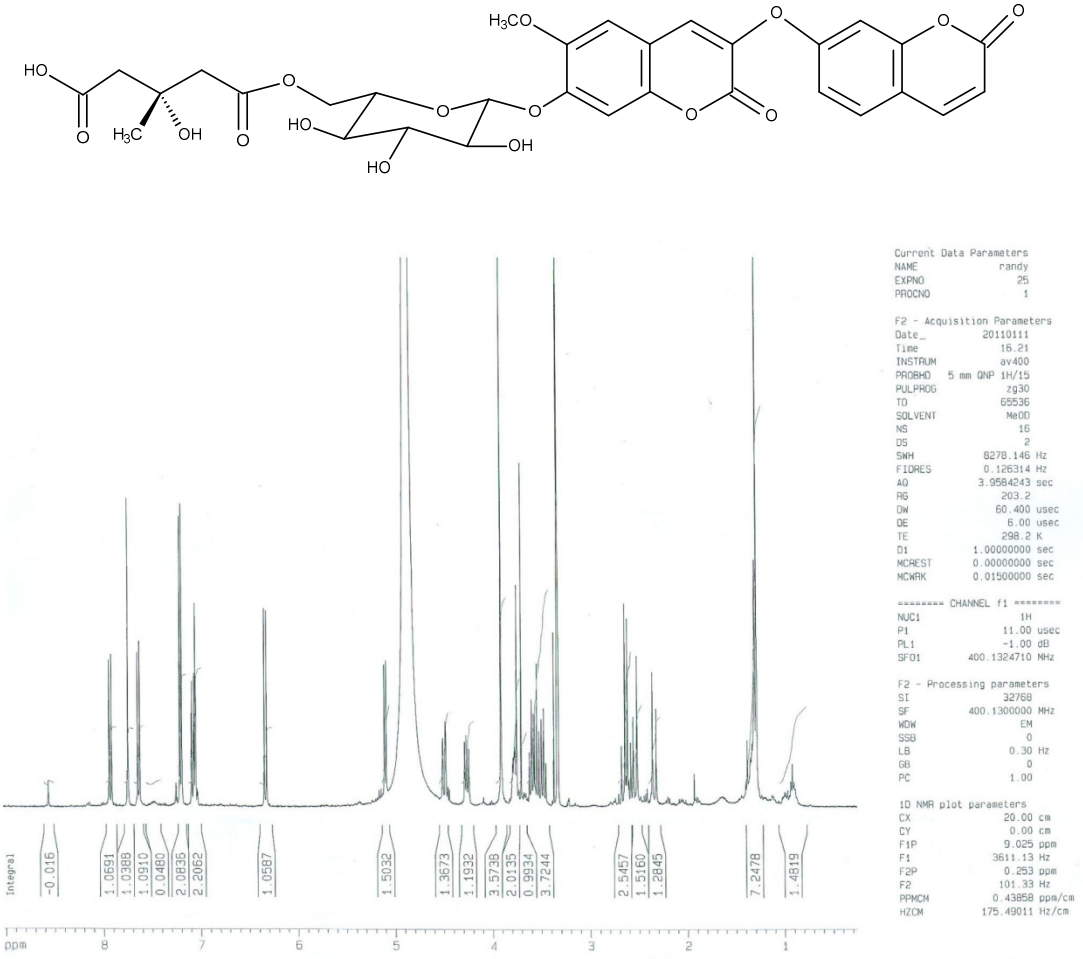
S21: HOMO-COSY ^{1}H-NMR (400 MHz, MeOD) Spectrum of Quercetin-3-*O*-glucoside (**2**)



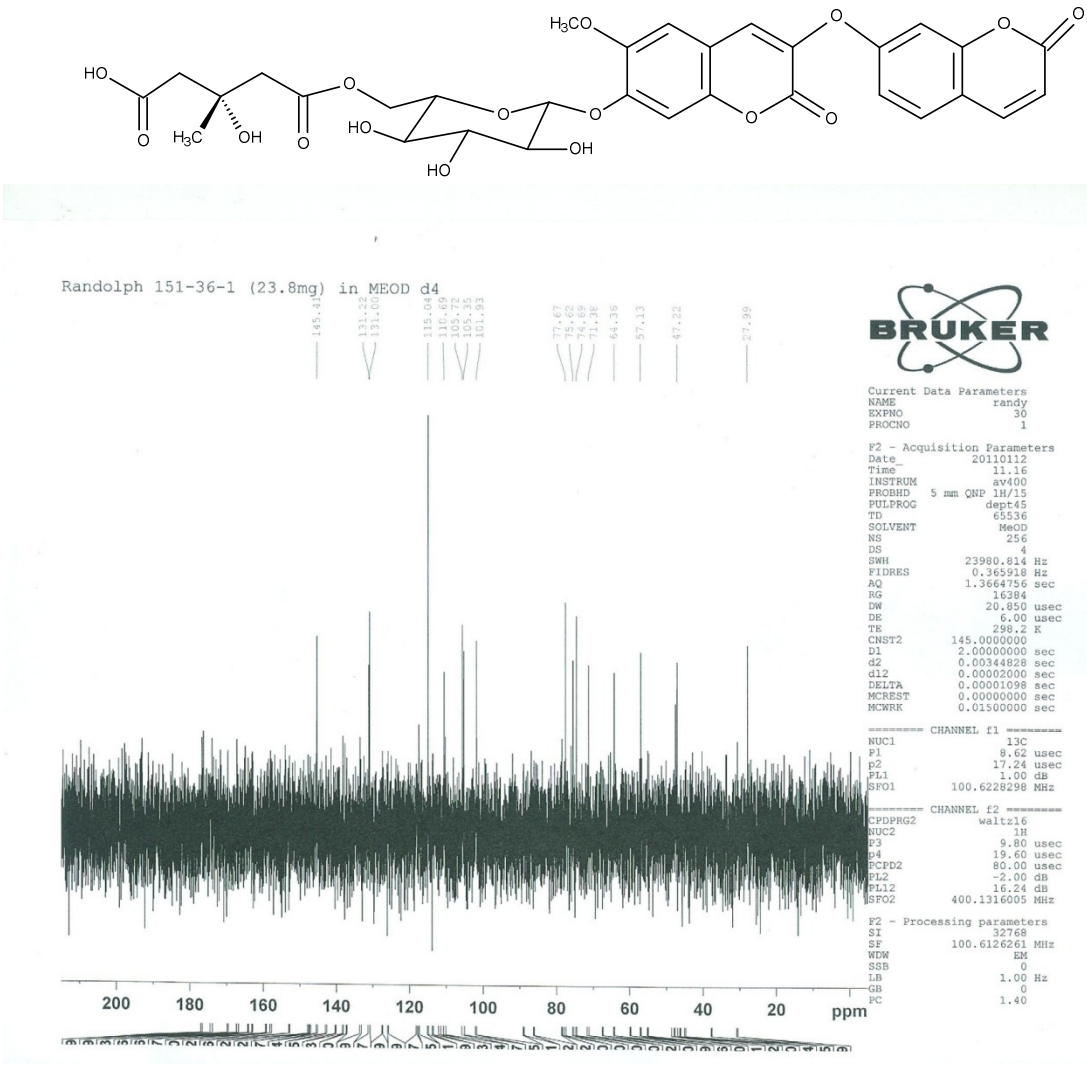
S22: FTMS Spectrum of Quercetin-3-*O*-glucoside (**2**)

S23: NMR and MASS data for Rutarensin (**3**)

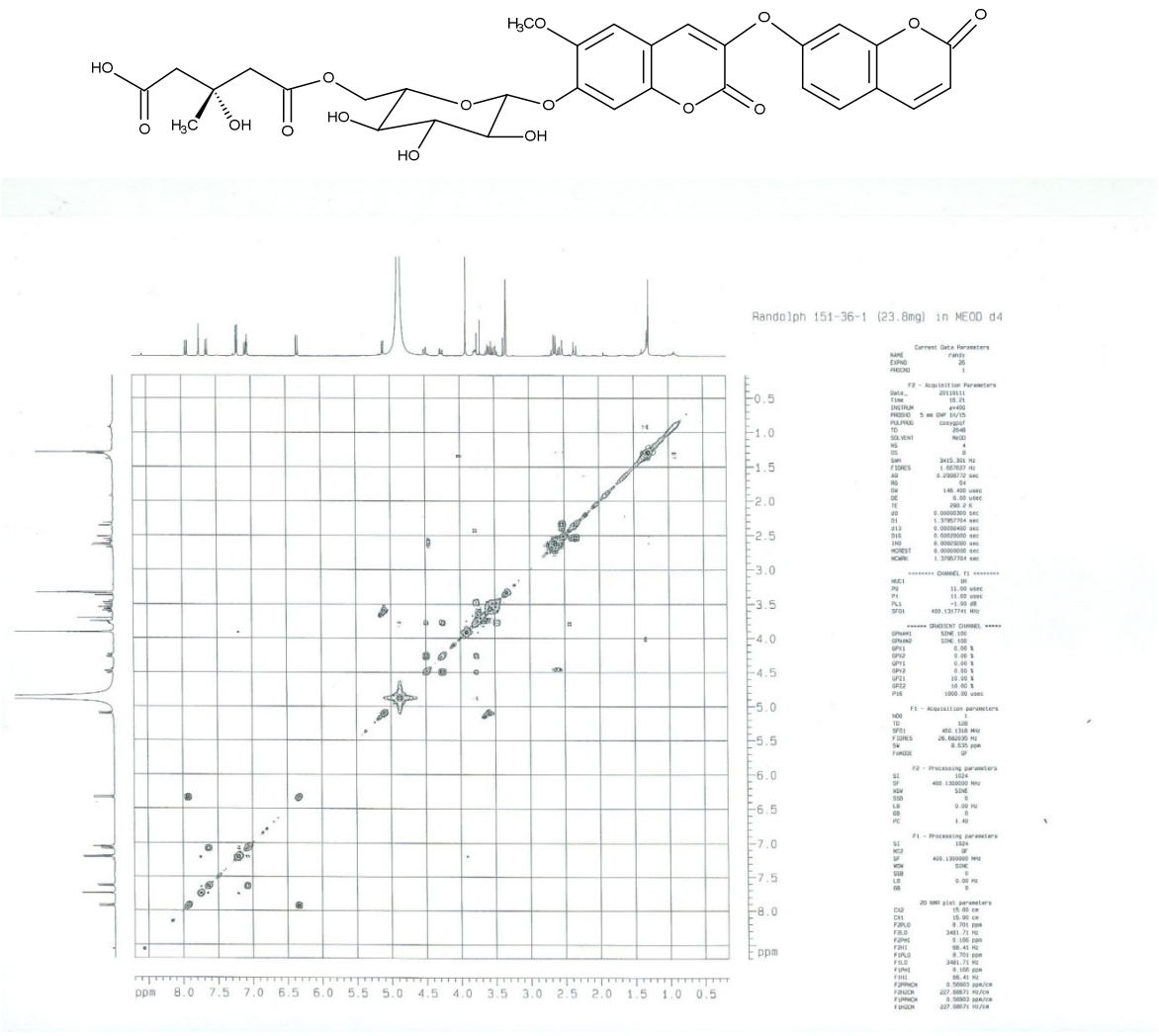
Rutarensin (**3,** 28.3 mg): Amorphous powder, FABMS *m/z* (+ve ion mode) 676 $[M + NH_4]^+$, 659 $[M + H]^+$, (-ve ion mode) 657 $[M - H]^-$; ^{1}H NMR (600 MHz, CD_3OD): 7.95 d (*J* = 9.6 Hz, H-4'),7.75 s (H-4), 7.65 d (*J* = 8.8 Hz, H-5'), 7.26 s (H-8), 7.23 s (H-5), 7.08 dd (*J* = 1.9, 8.8 Hz, H-6'), 7.06 d (*J* = 1.9 Hz. H-8'), 6.35 d (*J* = 9.6 Hz, H-3'), 5.15 d (*J* = 7.8 Hz, H-1''), 4.50 d (*J* = 12.0 Hz, H-6''a), 4.26 dd (*J* = 6.7, 12.0 Hz, H-6''b), 3.95 s (6-OMe), 3.40 – 3.85 m (H-2'', H-3'', H-4'' and H-5''), 2.65 bd (H-2'''), 2.55 bd (H-4''') and 1.30 s (3'''-Me); ^{13}C NMR (150 MHz, CD_3OD): 179.0 (C-5'''), 171.4 (C-1'''), 161.3 (C-2'), 159.9 (C-7'), 157.6 (C-2), 155.3 (C-9'), 149.3 (C-7), 147.3 (C-9), 146.9 (C-6), 144.0 (C-4'), 137.8 (C-3), 129.7 (C-4), 129.6 (C-5'), 114.9 (C-10'), 113.6 (C-3' and C-6'), 113.0 (C-10), 109.4 (C-5), 104.3 (C-8'), 104.0 (C-8), 100.5 (C-1''), 76.3 (C-3''), 74.2 (C-2''), 73.0 (C-5''), 70.0 (C-4''), 69.5 (C-3'''), 62.9 (C-6''), 55.8 (6-OMe), 46.2 (C-2'''), 45.8 (C-4''') and 26.6 (3'''-Me).



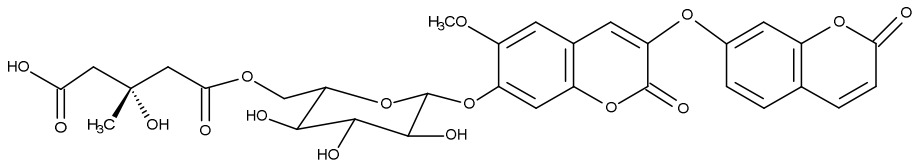
S24: ^{1}H-NMR (400 MHz, MeOD) Spectrum of Rutarensin (**3**)

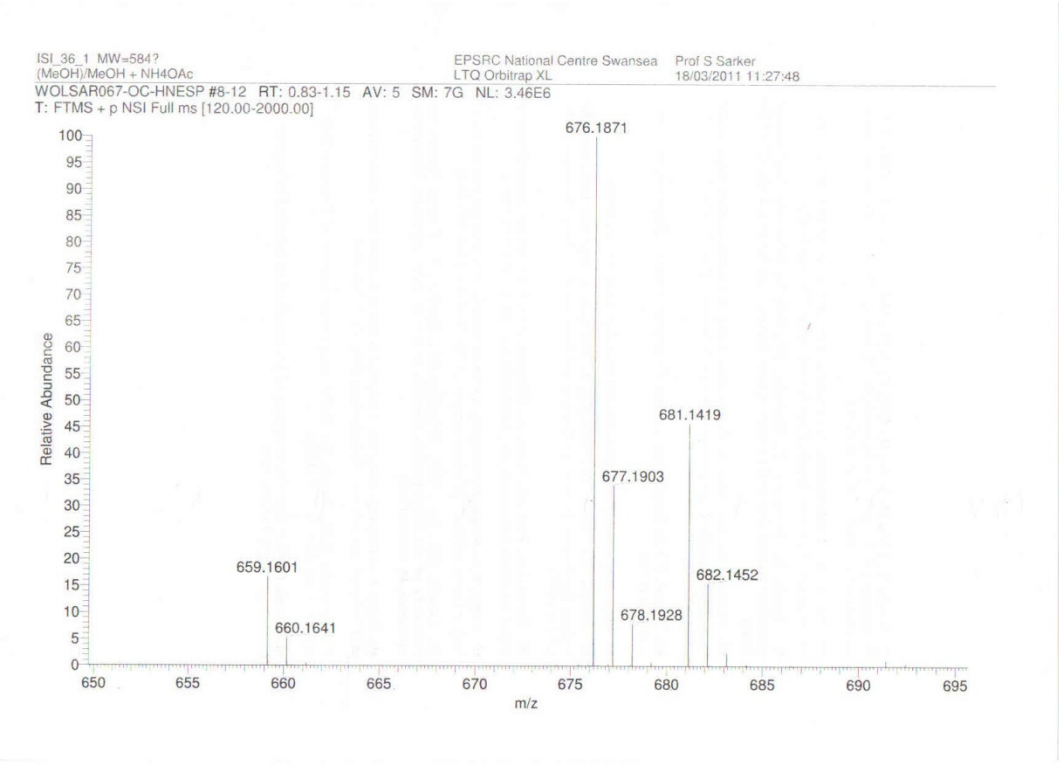


S25: ^{13}C-NMR (100 MHz, MeOD) Spectrum of Rutarensin (**3**)



S26: HOMO-COSY ^{1}H-NMR (400 MHz, MeOD) Spectrum of Rutarensin (**3**)





S27: FTMS Spectrum of Rutarensin (**3**)