SUPPLEMENTARY MATERIAL

A new aliphatic ester of hydroxysalicylic acid from fermented *Carica papaya* L. preparation with a potential hair growth stimulating activity

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Abstract

An aliphatic ester of hydroxysalicylic acid (**6**), reported for the first time from a natural source in addition to five known compounds were isolated from the fermented *Carica papaya* L. preparation, a commercialized functional food. The known compounds were identified as 5-hydroxymethylfurfuraldehyde (**1**), *trans*-caffeic acid (**2**), butyl 4-hydroxybenzoate (butylparaben) (**3**), lycopene (**4**), benzyl isothiocyanate (**5**). Compounds **1** and **3** were reported for the first time from Papaya fruits through this study. The new compound showed a moderate antioxidant activity and a potent hair growth stimulating activity *in vitro.*

Keywords: *Carica papaya* L.; fermented preparation; anti-oxidant; hair growth

Experimental

General experimental procedures: ^{1}H and ^{13}C-NMR spectra were obtained on a Bruker DRX 600 NMR spectrometer (Bruker Daltonics Inc., MA, USA) using TMS as an internal standard for chemical shifts. Chemical shifts (δ) were expressed in ppm with reference to the TMS resonance. HR-FAB-MS were measured with a JEOL JMS 700 spectrometer (JEOL, Japan). Dimethylsulfoxide (DMSO) and organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Diaion HP-20 was purchased from Mitsubishi Chemical Co. (Tokyo, Japan). Silica gel (75–120 mesh) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Thin layer chromatography (TLC) silica gel 60 F_{254} plates were purchased from Merck Co., Darmstadt, Germany. Plates were developed in a solvent mixture of different organic solvents, and the developed chromatograms were visualized under 254 nm UV light and the spots were made visible by spraying with vanillin/H_2SO_4 reagent before warming in an oven preheated to 110 °C for 5 min. Preparative TLC (PTLC) was performed on precoated silica gel 60 GF_{254} (20 x 20 cm x 0.2 mm thick) or precoated RP-C18 F_{254} plates (5 x 7.5 cm x 0.2 mm thick) on glass plates.

Material: Fermented Papaya Preparation (FPP, SAIDO-PS501, Lot No. M20170306) was kindly provided by CARICA CELAPI Co. (Japan). The fermented preparation contains 95.1 % glucose as prepared by the company.

Extraction and isolation: Fifteen Kilograms of the fermented preparations were exhaustively extracted with methanol at room temperature. The combined extracts were then concentrated to dryness in vacuo on a rotary evaporator to get 450 g methanolic extract. The total methanolic extract was chromatographed on Diaion HP-20 stationary phase eluted with water then 100 % MeOH to yield the methanolic fraction (7.0 g). The methanolic fraction was applied onto the top of a silica gel column (500 g, 30 x 6 cm) previously packed in *n*-hexane. The column was gradiently eluted with *n*-hexane containing increasing proportions of EtOAc (50:50 → 0:100) followed by EtOAc with increasing proportions of MeOH (100:0 → 50:50). The effluents were collected in 250 mL fractions. Each fraction was concentrated to a small volume under reduced pressure at 40 °C and monitored by TLC. Fractions with the same chromatographic pattern were pooled together and evaporated to dryness. Compounds **4** (1.4 mg) and **5** (1.2 mg) were purified from subfraction 3 {eluted with

n-hexane–EtOAc (80:20)} by PTLC (on precoated silica gel plates F_{254}) using *n*-hexane–EtOAc (50:50). The same technique was used for purification of compound **3** (2 mg) from subfractions 4-5 {eluted with *n*-hexane–EtOAc (75:25)} using *n*-hexane–EtOAc (50:50). PTLC (on precoated silica gel F_{254} plates) was performed again for subfractions 13 {eluted with *n*-hexane–EtOAc (60:40)} using *n*-hexane – EtOAc (60:40) to yield compound **1** (15 mg). The same technique was applied for subfractions 24-27 {eluted with *n*-hexane–EtOAc (10:90)} using 100 % EtOAc to yield compound **2** (15 mg). PTLC (on precoated RP-C18 F_{254} plates) was performed for subfractions 34-35 {eluted with EtOAc – MeOH (80:20)} using MeOH – H_2O (50:50) to yield compound **6** (3.8 mg).

Isopropyl 5-β-D-glucopyranosyloxy-2-hydroxybenzoate (6): white amorphous powder, ^{1}H NMR (600 MHz, CD_3OD): δ_H 6.78 (d, *J*= 9.0 Hz, H-3), δ_H 6.96 (dd, *J*= 9.0, 3.0 Hz, H-4), δ_H 7.22 (d, *J*= 3.0 Hz, H-6), δ_H 5.26 (q, *J*= 6.6 Hz, H-1'), δ_H 1.38 (d, *J*= 6.0 Hz, H-2'). ^{13}C NMR (150 MHz, CD_3OD): δ_C 113.8 (C-1), δ_C 156.2 (C-2), δ_C 119.0 (C-3), δ_C 124.9 (C-4), δ_C 150.7 (C-5), δ_C 115.4 (C-6), δ_C 170.9 (C-7), δ_C 70.5 (C-1'), δ_C 22.1 (C-2').

Anti-oxidant (ORAC) assay: In this assay, compound **6** was estimated by oxygen radical absorbance capacity (ORAC) assay. The assay was conducted as described previously (Garrett et al. 2010; Roy et al. 2010). The compound was dissolved in 75 mmol/L phosphate buffer (pH 7.4) and diluted with the buffer at a moderate concentration. This concentration of the buffer should be appropriate for the different concentrations of the trolox solution used for making the standard curve. The standard curve was made with 50, 25, 12.5, and 6.25 μmol/L trolox solution. Mixtures of fluorescein with the compound solution or trolox were incubated at 37 ºC for 10 min. Then, AAPH (2.2'-azobis(2-amidinopropane) dihydrochloride) was added and the absorbance at excitation and emission wavelengths of 485 and 515 nm respectively were measured with a fluorescence spectrophotometer for 90 min each 30 sec. The results were expressed as mg of trolox equivalents (TE) on the basis of the compound (mg TE/mg compound) (Figure S12). All chemicals used for this assay were of analytical grade and were purchased from Wako Chemical, Osaka, Japan.

HFDPC (Human Hair Follicle Dermal Papilla Cells) assay: HFDPC were cultured in follicle dermal papilla cell medium (FDPC) (PromoCell, Heiderberg, Germany)

containing 10% FBS (GE Healthcare life sciences Hyclone lab., Utah, USA) and 1% streptomycin/penicillin (Wako, Osaka, Japan). HFDPC cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. For the cell viability, the experiment was conducted as follows. HFDPC were cultured on a 96 well plate (2 x 10^4 cells/well) for 24 h, and then the medium was replaced with FDPC medium containing 0.5% FBS with the tested compound (final concentrations: 10 μg/mL) followed by 72 h incubation. And then, the cells were replaced with FDPC medium with 10% FBS and MTT [3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide)] (5 mg/mL) for 4 h followed by replacement with 100 μL of acidified isopropanol (containing 0.04 N HCl) which was then incubated overnight in the dark at room temperature. After keeping in dark for 4 h, the absorbance was measured at 570 nm using a Microplate Reader (Shimadzu, Japan). The results of compound **6** are shown in (Figure S13).

Spectral data of compound 6

Figure S1. HR-FAB-MS of compound **6**

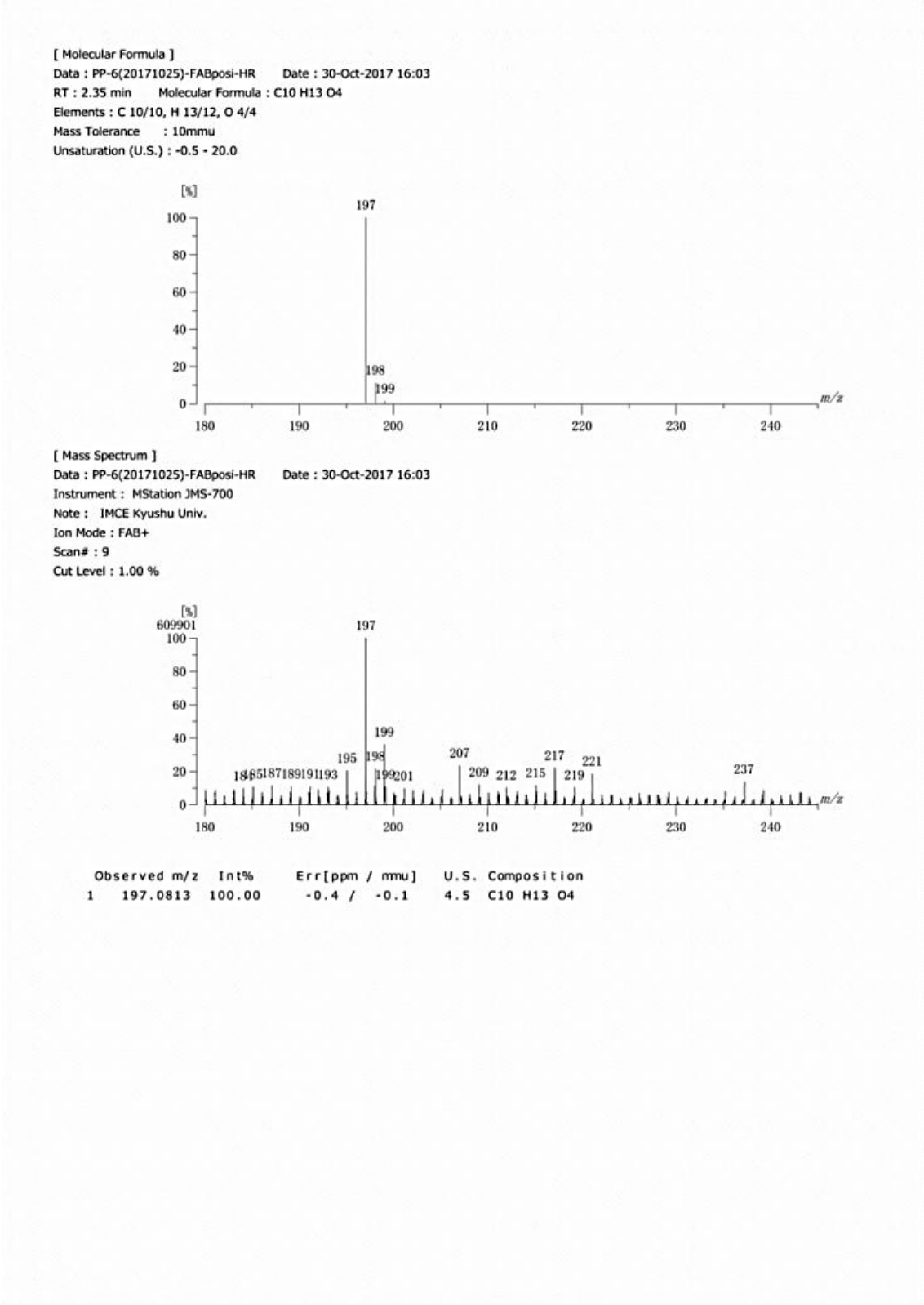

Figure S2. ^{1}H-NMR spectrum of compound **6**

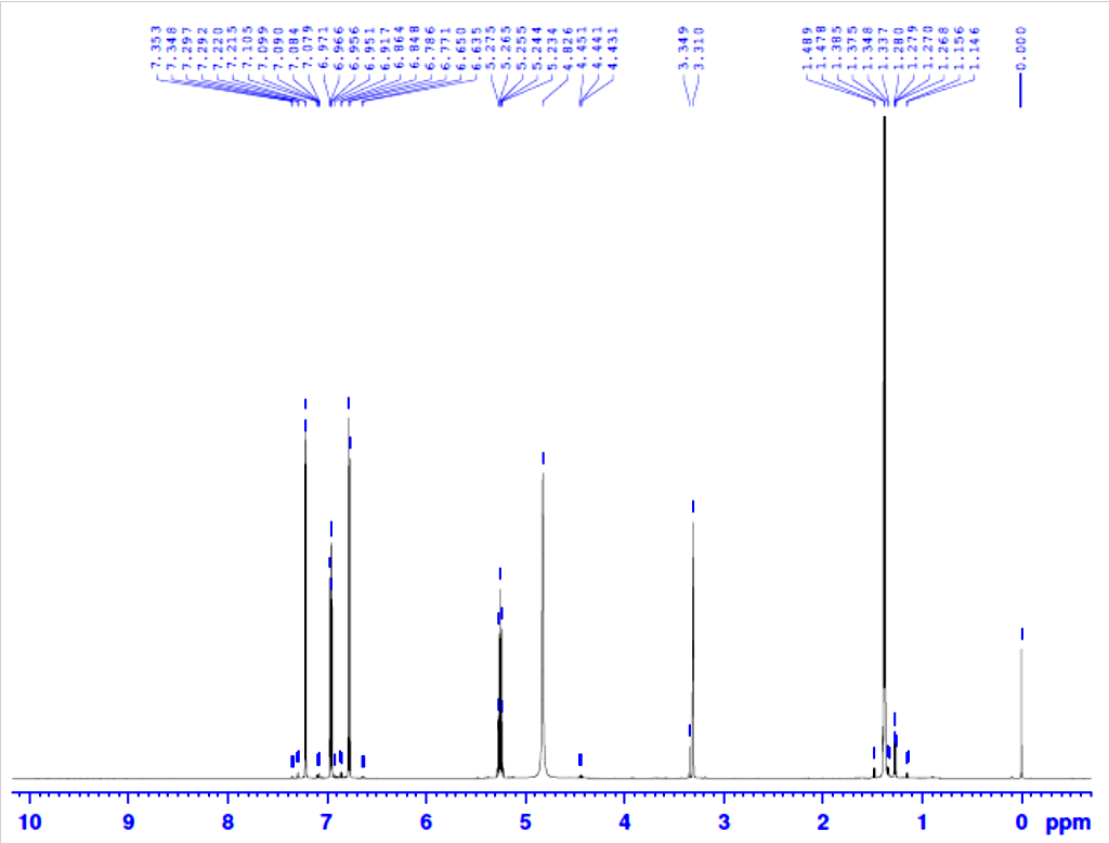

Figure S3. ^{1}H-NMR spectrum of compound **6** (Expansion 6.6 – 7.4 ppm)

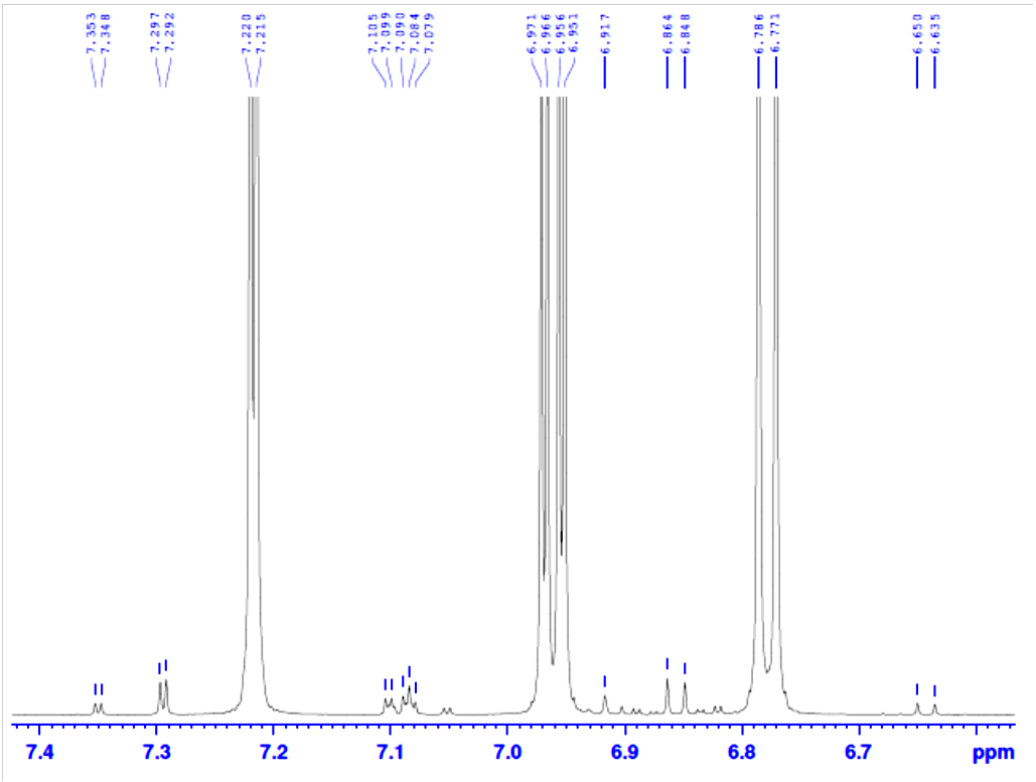

Figure S4. ^{1}H-NMR spectrum of compound **6** (Expansion 3.3 – 5.3 ppm)

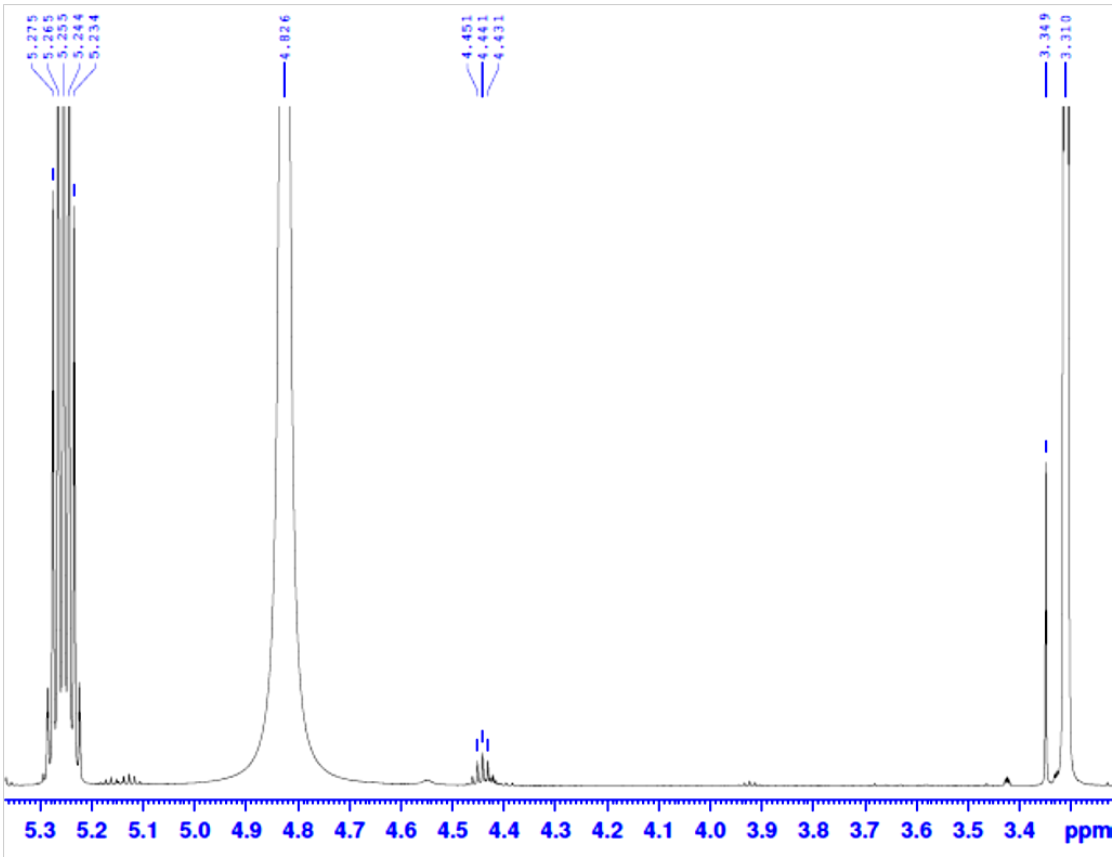

Figure S5. ^{13}C-NMR spectrum of compound **6**

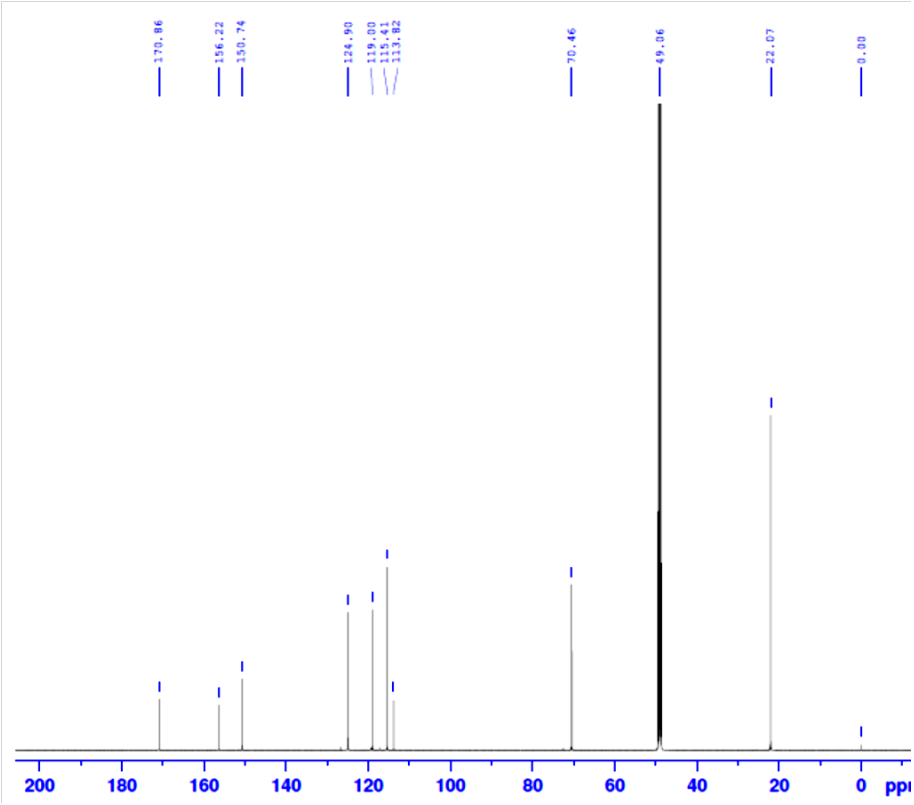

Figure S6. HMBC spectrum of compound **6**

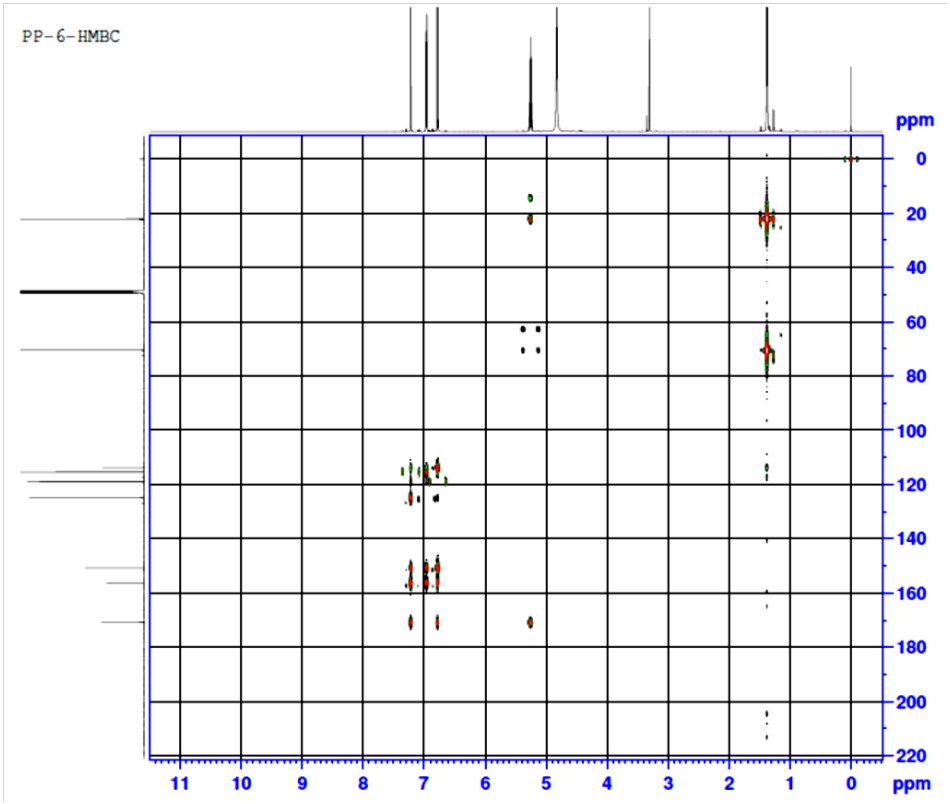

Figure S7. HMBC spectrum of compound **6** (Expansion)

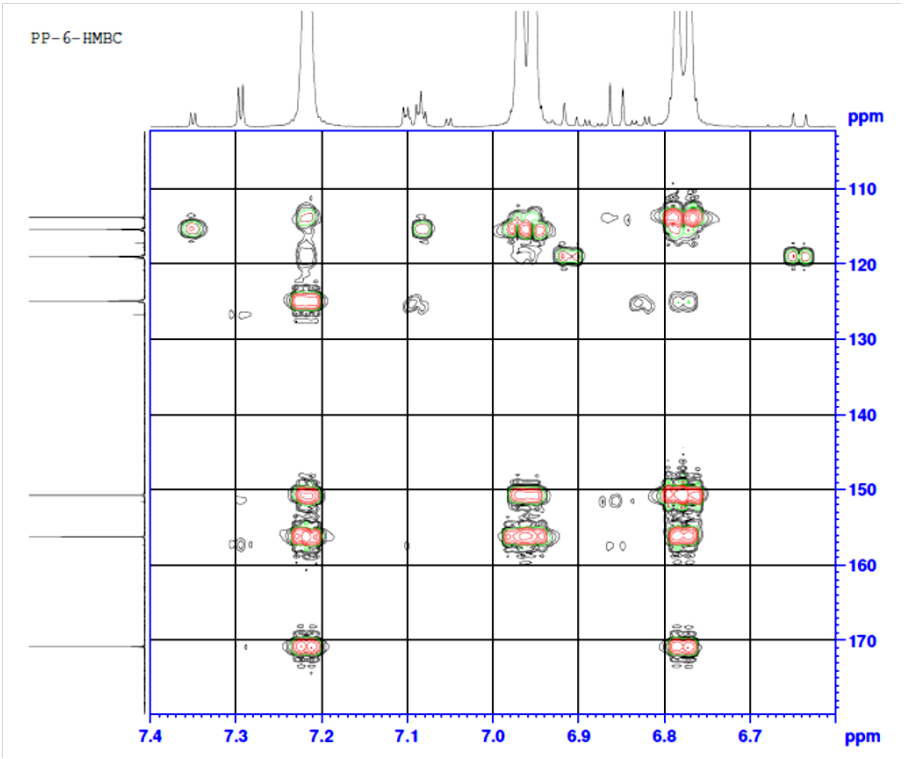

Figure S8. HSQC spectrum of compound **6**

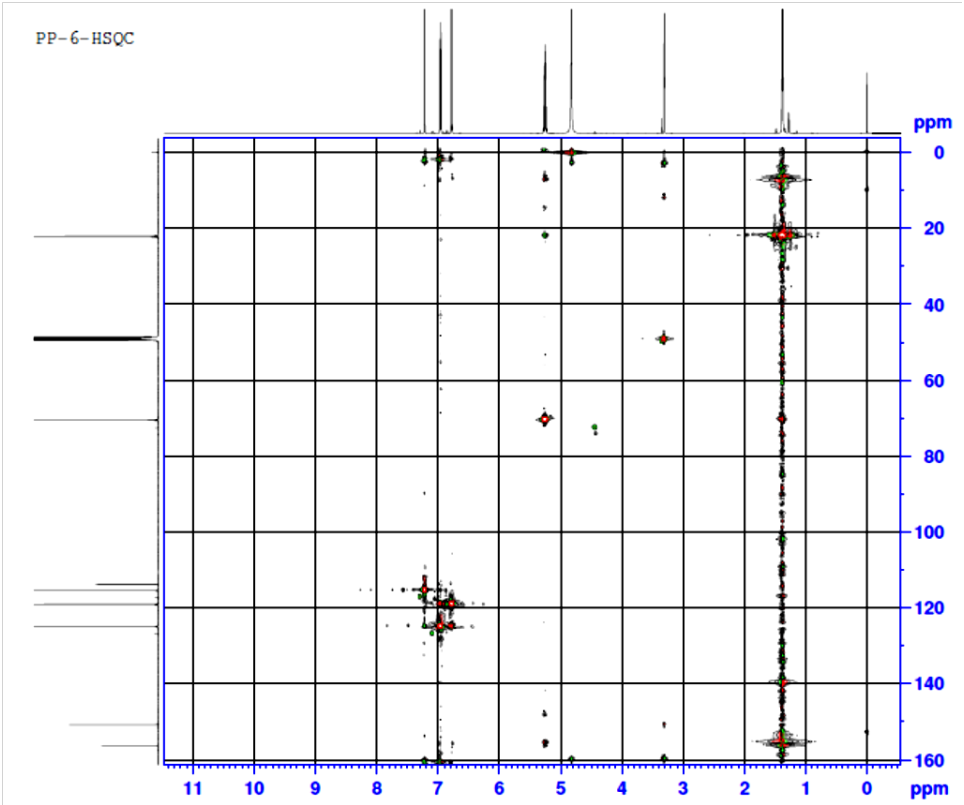

Figure S9. HSQC spectrum of compound **6** (Expansion)

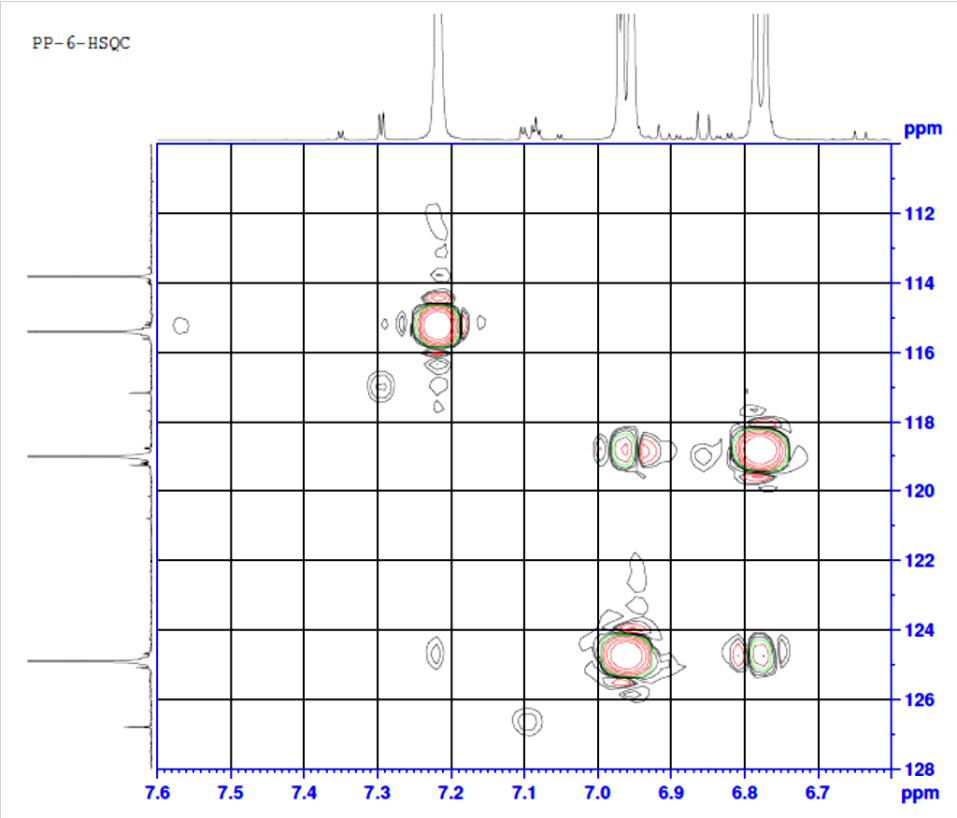

Figure S10. HMBC spectrum of compound **6** (Expansion)

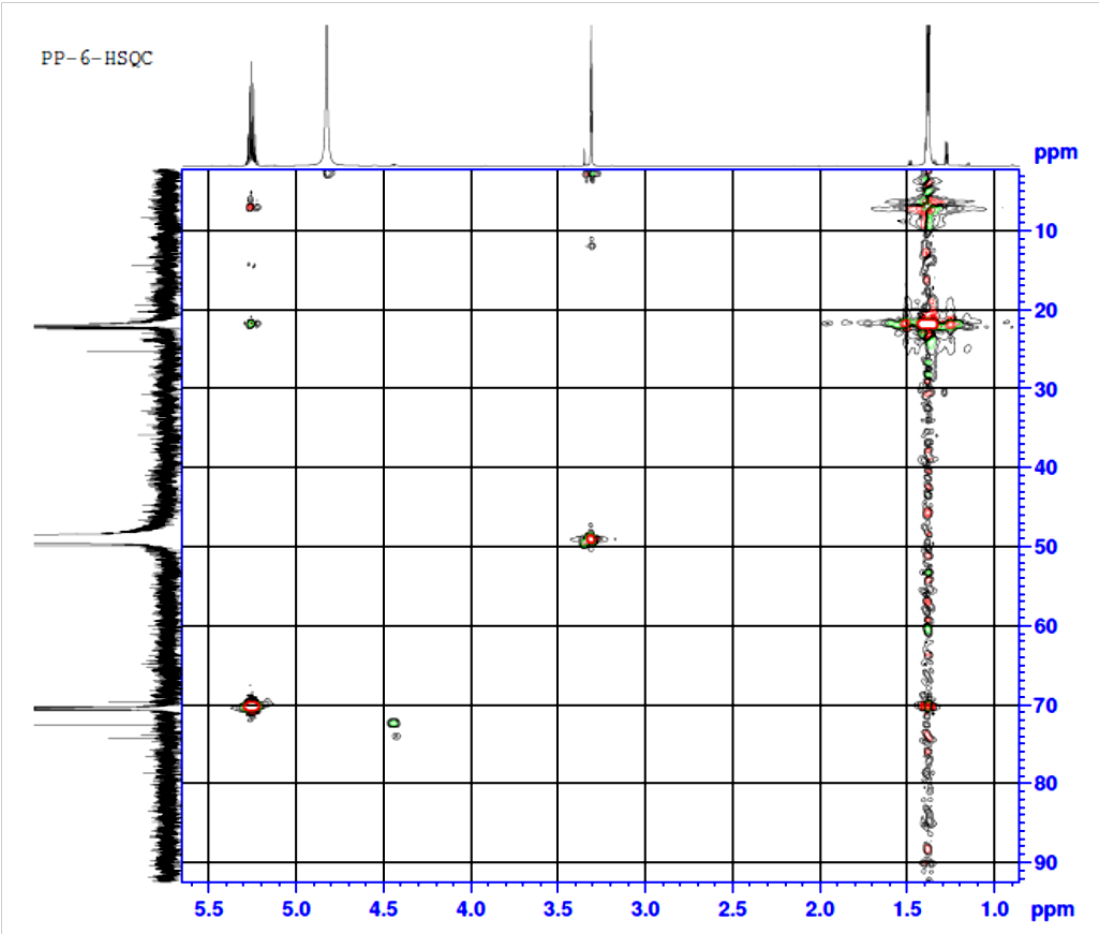

Figure S11. HMBC spectrum of compound **6** (Expansion)

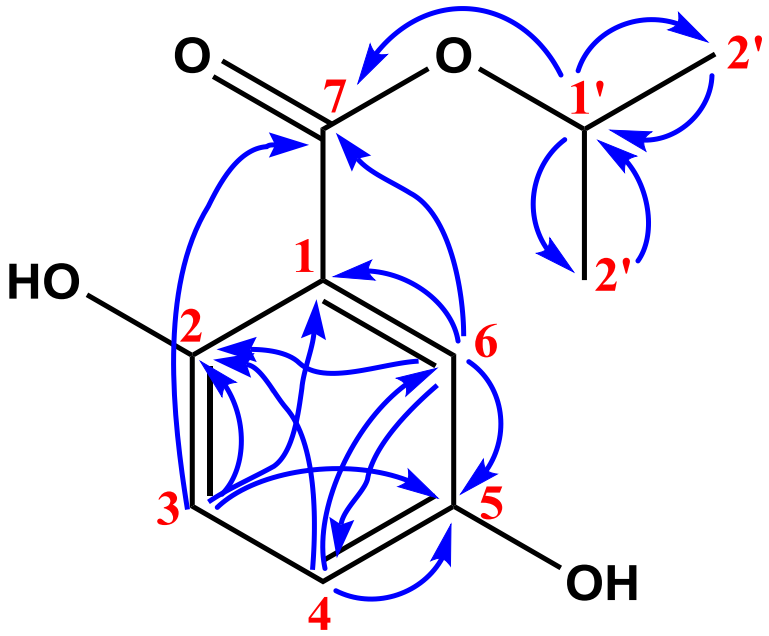

Figure S12. ORAC assay result of compound **6**

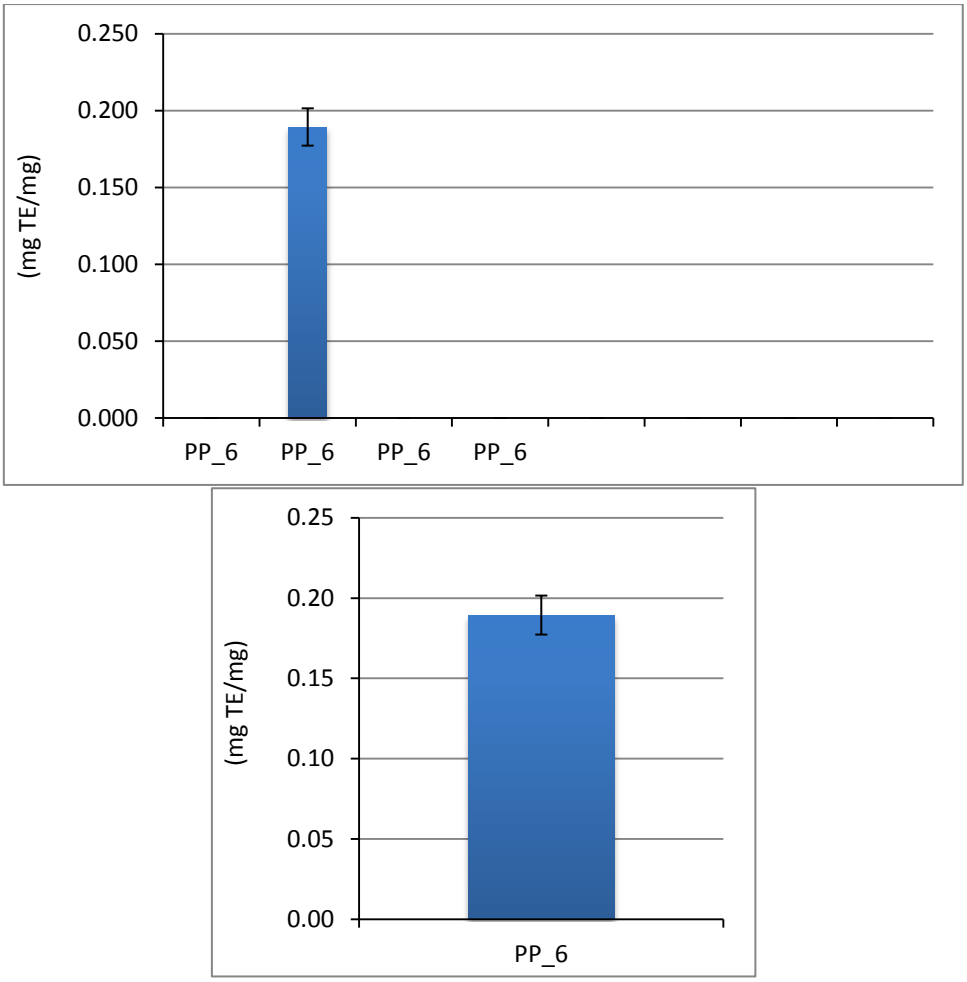

Figure S13. HFDPC assay result of compound **6**