SUPPLEMENTARY MATERIAL

Comparison of metabolic profiles and bioactivities of the leaves of three edible Congolese *Hibiscus* species

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Abstract

Methanolic and dichloromethane extracts from the leaves of Congolese Hibiscus species were characterized by chromatographic and spectroscopic techniques and their *in vitro* biochemical activities against ROS production were evaluated in cellular models and on an enzyme, myeloperoxidase (MPO), involved in inflammation. *H. acetosella* has a chemical fingerprint different from *H. cannabinus* and *H. sabdariffa* both having similar fingerprints. Major compounds were polyphenols, represented mainly by caffeoyl-hydroxycitric acid for *H. acetosella* and neochlorogenic acid for the two other species. All extracts displayed high cellular antioxidant activity with IC_{50} values ranging from 0.5 to 3 µg mL^{-1} using lucigenin on neutrophils. Dichloromethane extracts showed more efficient effects on extracellular ROS production and MPO activity. Antioxidant and anti-inflammatory activities of caffeoyl-hydroxycitric acid were significantly higher than those of neochlorogenic acid. The bioactivities of Hibiscus species were positively correlated with their phytochemical content and could justify their use as local nutraceutical resources and medicines.

Keywords: Caffeoyl-hydroxycitric acid*; Hibiscus acetosella; Hibiscus cannabinus; Hibiscus sabdariffa;* Myeloperoxidase; Neochlorogenic acid.

Experimental

Plant Materials

The leaves of *Hibiscus acetosella* were collected from the areas of Kinshasa and Kisantu, while the leaves of *Hibiscus cannabinus* and *Hibiscus sabdariffa* were collected from the Kahemba and Kinshasa areas (DRC). They were dried at room temperature. The identities of the plants were confirmed by biologists from the University of Kinshasa (Democratic Republic of Congo) and the National Botanical Garden of Meise (Belgium). Voucher specimens were deposited at the herbarium of the National Botanical Garden of Meise, Belgium (*Hibiscus acetosella*: BR0000013601430; BR0000013601447; *Hibiscus sabdariffa*: BR000001360 and *Hibiscus cannabinus*: MUT 011 deposited at the herbarium of University of Kinshasa). Leaves were finely ground in a high-speed mill (Retsch ZM 100 Model) and sieved at 180 µm particle size.

Chemicals and Reagents

All solvents used were of analytical and HPLC grade and purchased from Merck VWR (Leuven, Belgium). 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2-Aminoethyldiphenylborat, Phorbol-12-myristate-13-acetate (PMA), Folin-Ciocalteu's phenol reagent, bis-N-methyl acridinium nitrate (lucigenin) and sodium persulfate were purchased from Sigma (Bornem, Belgium). 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) were purchased from Eastman Kodak (Rochester, NY, USA). Horseradish peroxidase (HRP) was obtained from Roche (Mannheim, Germany). Gallic acid (purity: 97%) was purchased from Sigma-Aldrich. Rutin (purity ≥99%), hyperoside (purity ≥98%); isoquercitrin (purity ≥99%) and, quercitrin (purity ≥98.5%) were HPLC grade and purchased from Extrasynthese. Water was treated using a Milli-Q water ultra-purification system before use.

Preparation of extracts

Methanolic extracts

Methanolic extracts were prepared by percolation with methanol from 10 g of leaf powder to obtain 200 mL of percolate. Evaporation of the solvent was performed under reduced pressure (at 40 °C) followed by 48-72 h stay in a vacuum chamber. The extracts were then weighed and kept in dark hermetic flasks at 4 °C.

Methylene dichloride extracts

10 g of leaf powder were sonicated 2 times for 15 minutes with 50 ml of methylene dichloride. The mixture was filtered through a filter paper (Whatman n^o 4). Evaporation of the solvent was performed under a reduced pressure (40 °C) followed by a 48-72 stay h in a vacuum chamber. The extracts were then weighed and kept in dark hermetic flasks at 4 °C.

Phytochemical analysis

Thin Layer Chromatography (TLC) analysis

Analytical TLC of solutions was carried out on normal phase Silica gel 60 F_{254} plates (Merck), using either:

A. Ethyl acetate/ Formic acid/ Glacial acetic acid/ Water (100:11:11:26; v/v/v/v) as eluent. Hyperoside, rutin, and quercitrin were used as standards. The plates were visualized using Natural Products-PEG reagent and observed at UV-365 nm light.

B. Toluene/ Ethyl acetate (90:10; v/v) and visualized in daylight with the anisaldehyde-sulfuric acid reagent. Terpenoids were detected as violet-grey, yellow to violet colored spots (Wagner et al. 2013).

High-Performance Liquid Chromatography (HPLC) analysis and purification

Analytical separation on HPLC-DAD was carried out on a Hypersil ODS ® RP18 column as described previously (Kapepula et al. 2016).

Preparative HPLC analysis was performed on a LiChrospher 100 RP 18 column (Merck®) (250 x 25 mm, 12 µm). Methanolic extract (500 mg) was dissolved in methanol (10 mL) and filtered through an Acrodisc PSF GXF/GHP 0.45 nm filter and injected in preparative HPLC Varian chain. The mobile phase consisted of trifluoroacetic acid (TFA) 0.05% in water (A) and acetonitrile (B), which was applied in the following gradient elution A/B (v/v) 100:0 (0 min), 97:3 (1 min), 60:40 (45 min), 60:40 (55 min), 40:60 (56 min) and 40:60 (66 min). Flow rate was 30 mL/min. Detection was performed at 350 nm and the collection time was 0.20 min. The isolated compounds were identified by Mass Spectroscopy (MS) and Nuclear Magnetic Resonance (NMR) analysis.

Mass Spectra

Ultra-Performance Liquid Chromatography –Mass Spectroscopy (UPLC-MS) for molecular identification of compounds was performed with an ACQUITY UPLC HSS T3 coupled to Mass spectrometer Time of flight XEVO-G2QTOF 2 with the Lock Spray option, , Waters, Altrincham(UK), using a column (2.1 x 100 mm, 1.8 µm dp) thermostated at 40 °C and a mobile phase of 15 mM ammonium acetate in water (A) and acetonitrile (B), in gradient profile A/B (v/v) 98:2 (0 min), 2:98 (0.75 and 1 min) and 98:2 (0.1 and 1.4 min). The flow rate was 0.60 mL/min and the injection volume 5 µL.

NMR analysis

NMR spectra were recorded in MeOH-d4 on a Bruker 500 MHz NMR AV II spectrometer equipped with a cryoprobe, with Tetramethylsilane (TMS) as an internal reference.

Cell-free, cellular and enzymatic assays

The extracts and molecules were solubilized in DMSO, thus their effect was compared to a control test performed with DMSO alone.

Evaluation of radical scavenging activity

ABTS radical scavenging capacity

ABTS assay was based on the method described by Franck et al (2013)(Franck et al. 2013).

DPPH radical scavenging capacity

DPPH assay was performed according to the method described previously by Floegel et al. (2011) with slight modifications (Floegel et al. 2011). A solution of 0.004% of DPPH in 80% (v/v) methanol was prepared one hour before use. The absorbance of the solution was adjusted to 0.75 ± 0.03 at 517 nm using fresh 80% (v/v) methanol. Then 0.02 mL of standard or sample were mixed with 1.98 mL of DPPH solution and incubated for 30 min in the dark. The decrease of absorbance was monitored at 517 nm with a Spectrophotometer Hewlett-Packard 8453.

The antiradical capacity analysis was performed on dichloromethane and methanolic dry extracts. Gallic acid was used as positive control and ABTS•+ and DPPH scavenging activities of extracts were expressed as IC_{50} values. Each sample was measured in triplicate.

Cellular Antioxidant Activity

Cell culture and treatment

Human promyelocytic leukemia cells (HL-60) were obtained from the American Type Culture Collection (ATCC, USA) and cultured in appropriate medium (Tsumbu et al. 2011). Equine Neutrophils were isolated as described previously (Zeraik et al. 2011).

Measurement of Cellular Antioxidant Activity (CAA)

The cellular antioxidant activity consists of evaluating the capacity of the extracts to modulate the production of ROS in cell models often used to study inflammation. The fluorescent

probe 2',7'-dichlorofluorescein diacetate was used to measure the intracellular ROS production in HL-60 cells and the lucigenin probes was used to measure the extracellular ROS production in PMN (Oddvar Myhre, Jannike M.Andersen, 2003; Girard-Lalancette. K, Pichette A., 2009; Derochette et al. 2013).

Measurement of the total ROS produced by PMA activated neutrophils (Chemiluminescence Assay: CL assay)

The ROS produced by activated neutrophils were measured by lucigenin-enhanced chemiluminescence (CL) with some adaptations from the method described by Benbarek et al. (1996) and recently reported by Franck et al. (2013).

Measurement of the ROS produced by PMA-Activated HL-60 Monocytes (Fluorescence Technique with Non-Fluorescent DCFH-DA)

This experiment was based on the method described by Amado et al. (1996) with adaptations (Tsumbu et al., 2011).

Anti-inflammatory activity: Myeloperoxidase activity

The SIEFED is a licensed method developed by Franck et al. for the specific detection of equine and human myeloperoxidase (MPO) and used for the screening of polyphenolic compounds or extracts that could modulate the activity of MPO (Franck et al. 2013).

Cell viability

The cell viability was investigated by the Trypan blue exclusion test with unstimulated neutrophils (10^6 cells/mL PBS) previously incubated for 1 h with the plant extracts at final concentrations of 1, 5 and 10 µg/mL (Tsumbu et al. 2012).

Statistical Analysis

Each concentration was tested in triplicate in each assay, and at least 3 different assays were performed. All results were expressed as mean values ± standard deviation (SD). The statistical analysis was performed with GraphPad 6.0 (GraphPad Software, San Diego California, USA). Two-way analysis (ANOVA) and Student's paired t-test were used; multiple comparisons of all data were performed using the "Sidak" Multiple Comparisons Test and the level of statistical significance was set at $p < 0.05$. The IC_{50} values were calculated with GraphPad Prism 6.0 under application of the function "log (inhibitor) vs. normalized response-variable slope" after converting the concentrations into their decimal logarithm.

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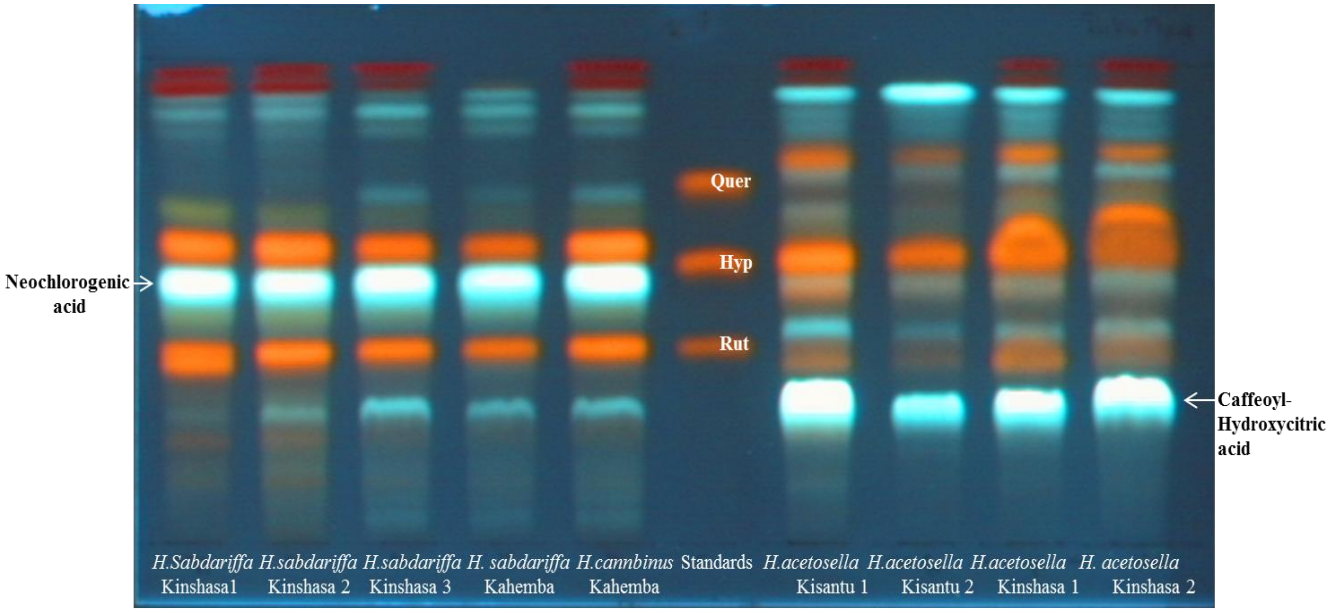

Figure S1. TLC chromatogram of methanolic extracts from Hibiscus species with hyperoside (Hyp), quercitrin(Quer) and rutin(Rut) as standards; developed with ethyl acetate/formic acid/glacial acetic acid/water (100:11:11:26; v/v/v/v) and visualized at 365 nm with Natural Products-PEG reagent. Flavonoids are detected as yellow-orange fluorescent spots and phenolic acids as blue fluorescent spots. TLC fingerprints of extracts of *H. cannabinus* and *H. sabdariffa* are similar and contained neochlorogenic acid as major phenolic compound but are different from those of *H.* acetosella which contained caffeoyl-hydroxycitric acid as major phenolic acid. The geographical origin (Kahemba, Kinshasa, Kisantu) does not seem to influence the TLC fingerprint.

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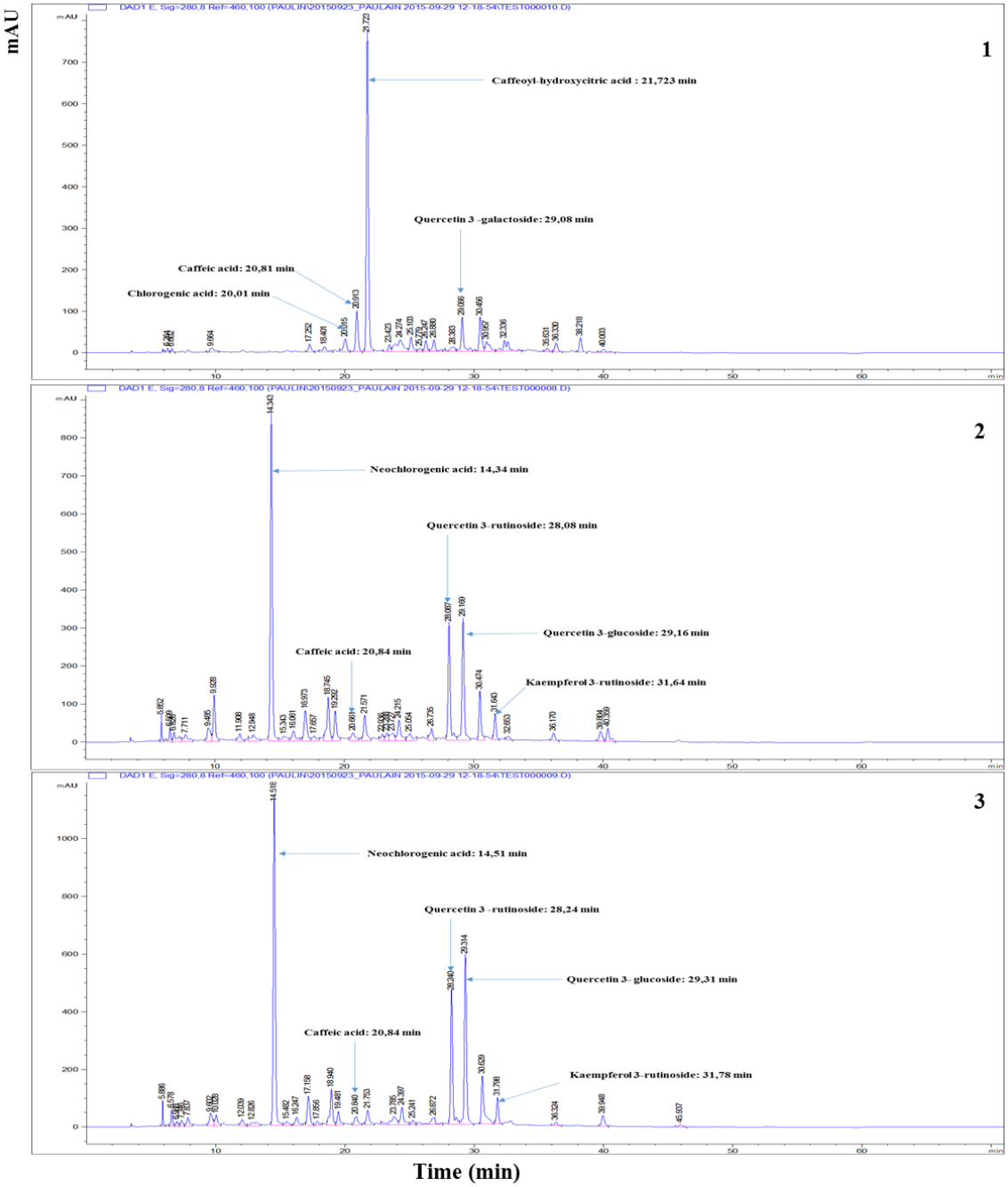

Figure S2. HPLC chromatogram of methanolic extracts of *H. acetocella* (1), *H. cannabinus* (2) and *H. sabdariffa* (3) using a nonlinear gradient of 0.05% trifluoroacetic acid in ultra-pure water (A) and acetonitrile (B): 0 min, 0:100 (A:B), 1 min, 3:97 (A:B); 45 min, 40:60 (A:B); 55min, 40:60 (A:B); 56 min, 0:100 (A:B)and 60 min, stop at 1 mL/min on Hypersil ODS column (4 mm × 250 mm) with detection at 340 nm. The elution time is given in minutes (horizontal axis). On the y-axis, the height of the elution peak, expressed in arbitrary milli-units (mAU) corresponds to the concentration of the eluted compound in the sample analyzed.

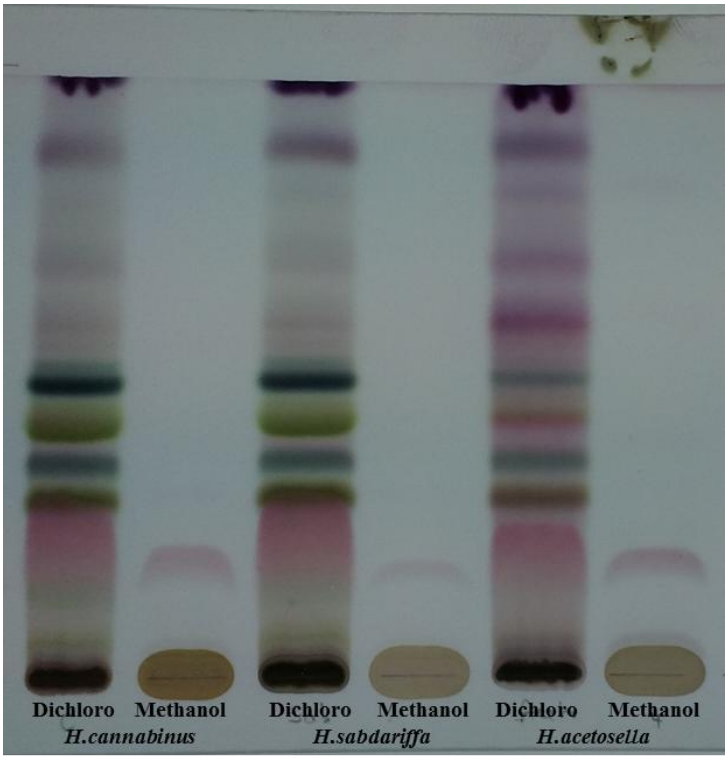

Figure S3. TLC chromatogram of dichloromethane and methanolic extracts from Hibiscus species developed with toluene/ethyl acetate 90:10 and visualized in daylight with anisaldehyde sulfuric acid reagent. Terpenoids are detected as violet-grey, yellow to violet colored spots.

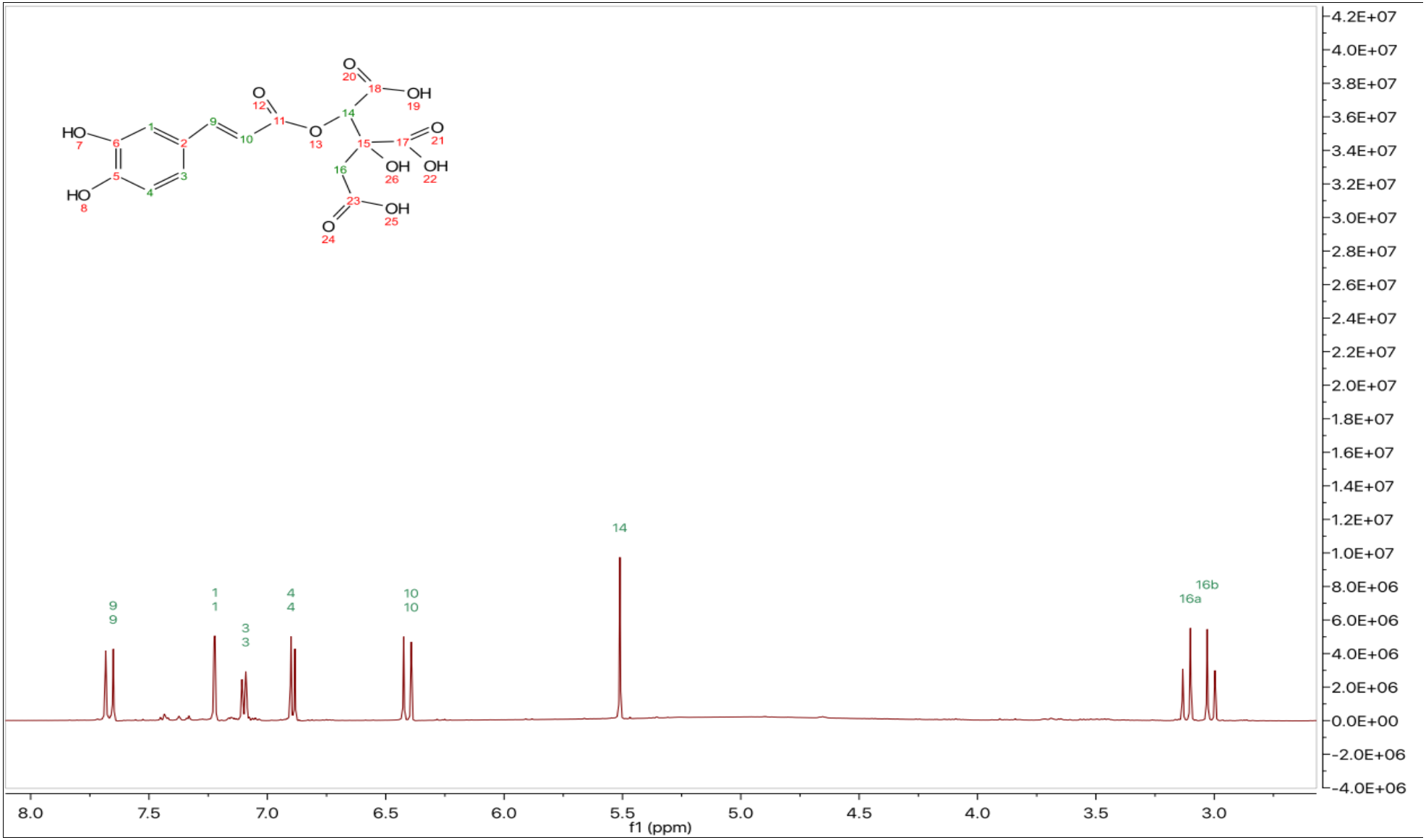

Figure S4. ^{1}H –NMR spectrum of caffeoyl-hydroxycitric acid

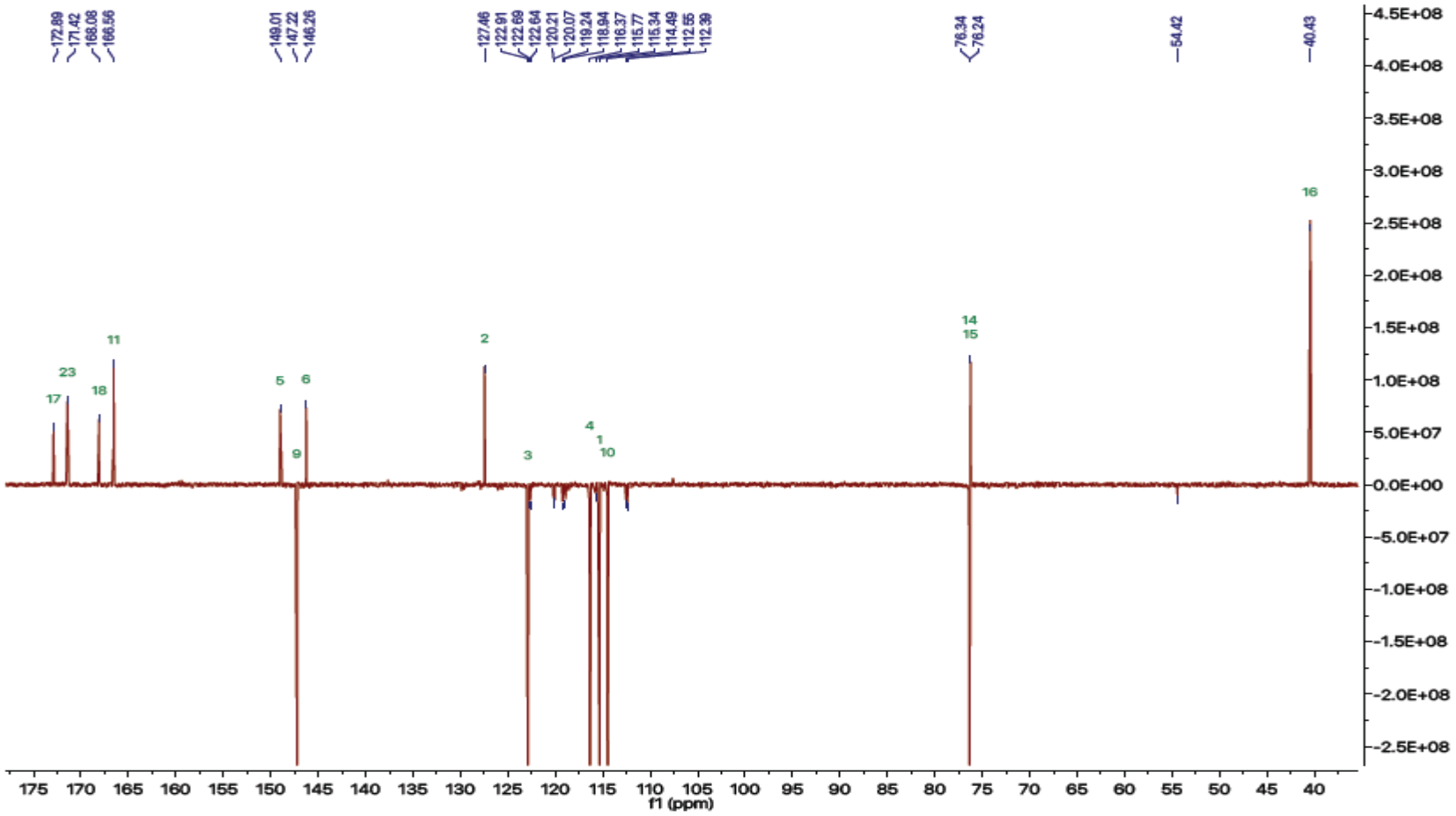

Figure S5. ^{13} **C APT spectrum of caffeoyl-hydroxycitric acid**

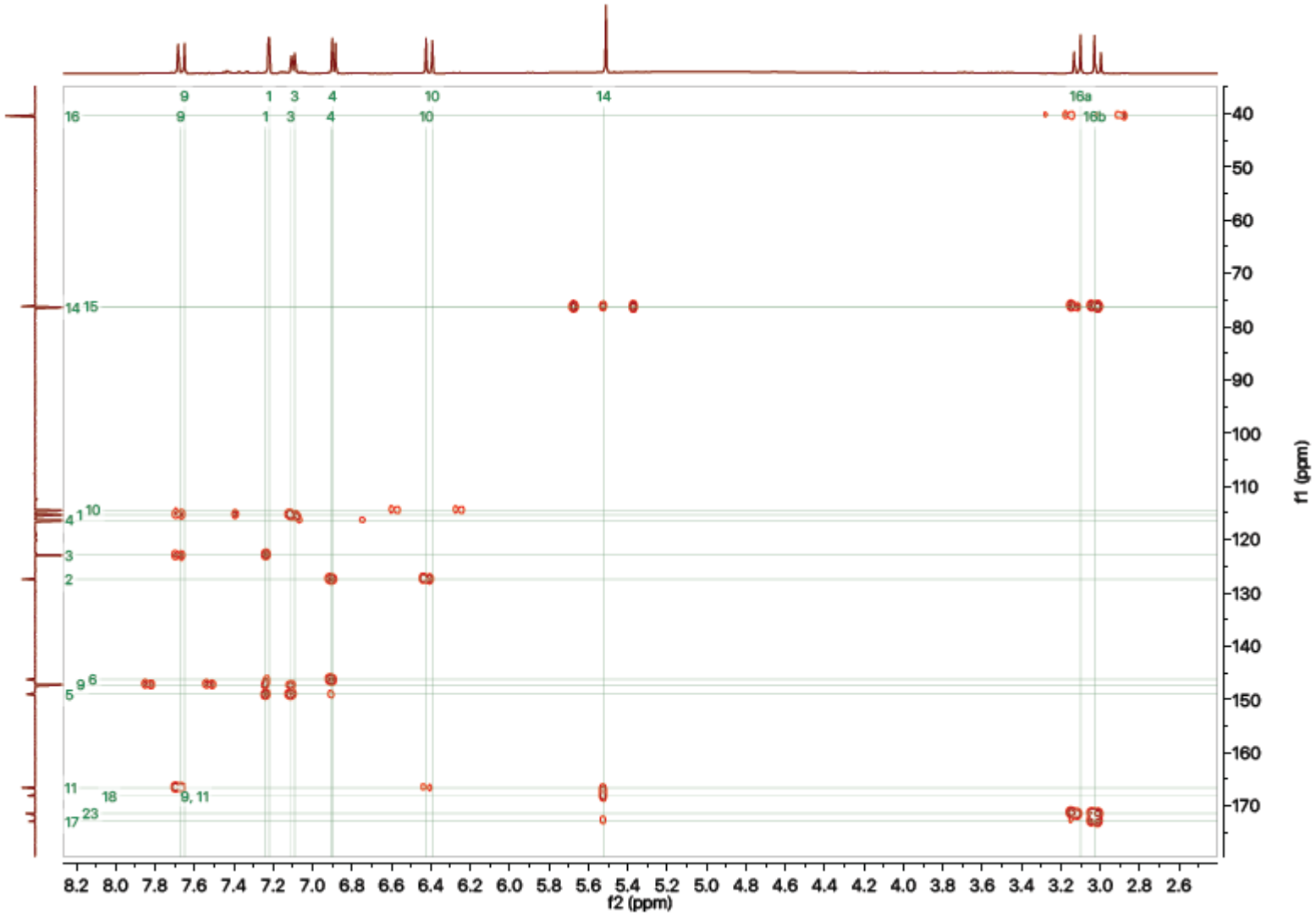

Figure S6. ^{1}H, ^{13}C- HMBC spectrum of caffeoyl-hydroxycitric acid

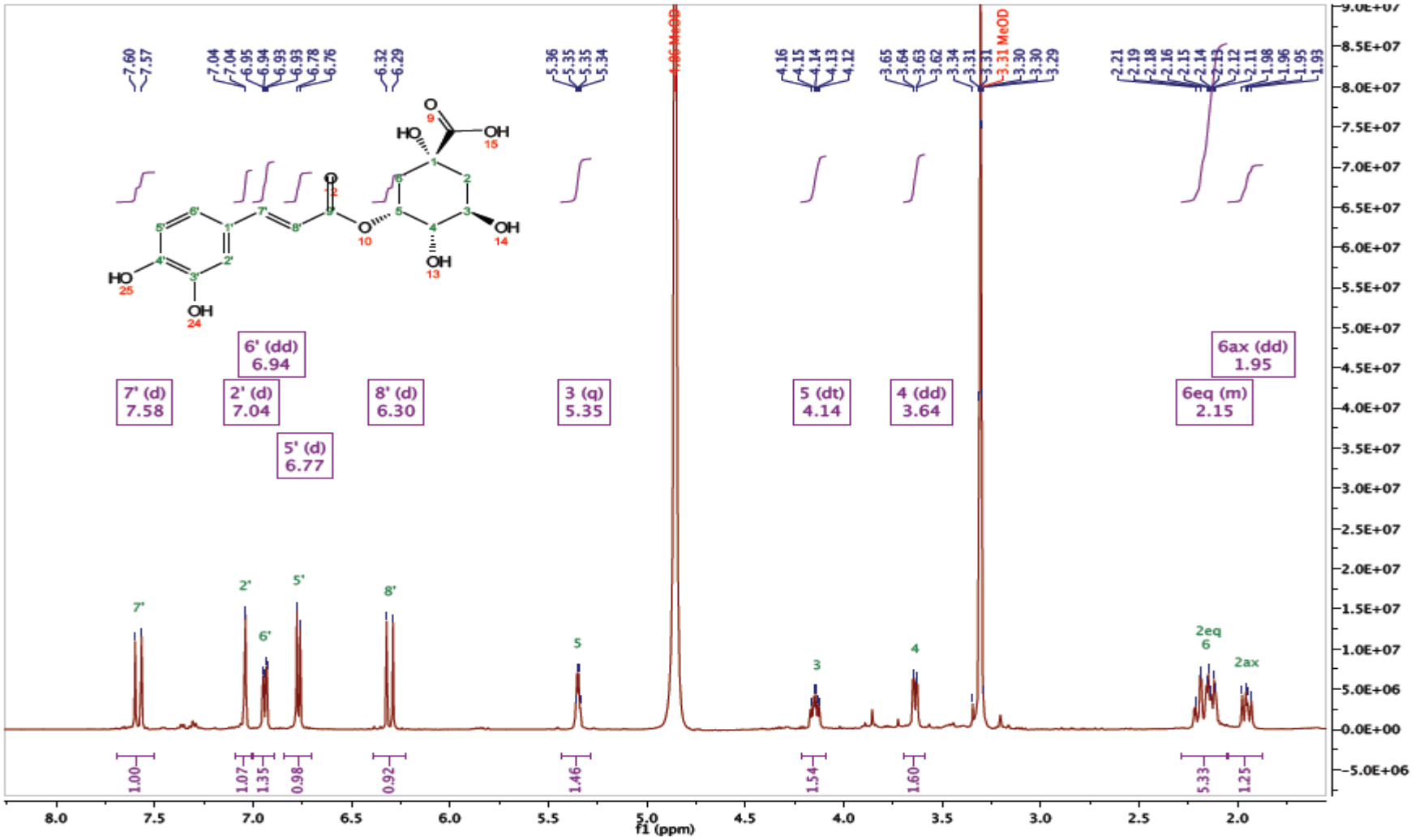

Figure S7. ^{1}H –NMR spectrum of neochlorogenic acid

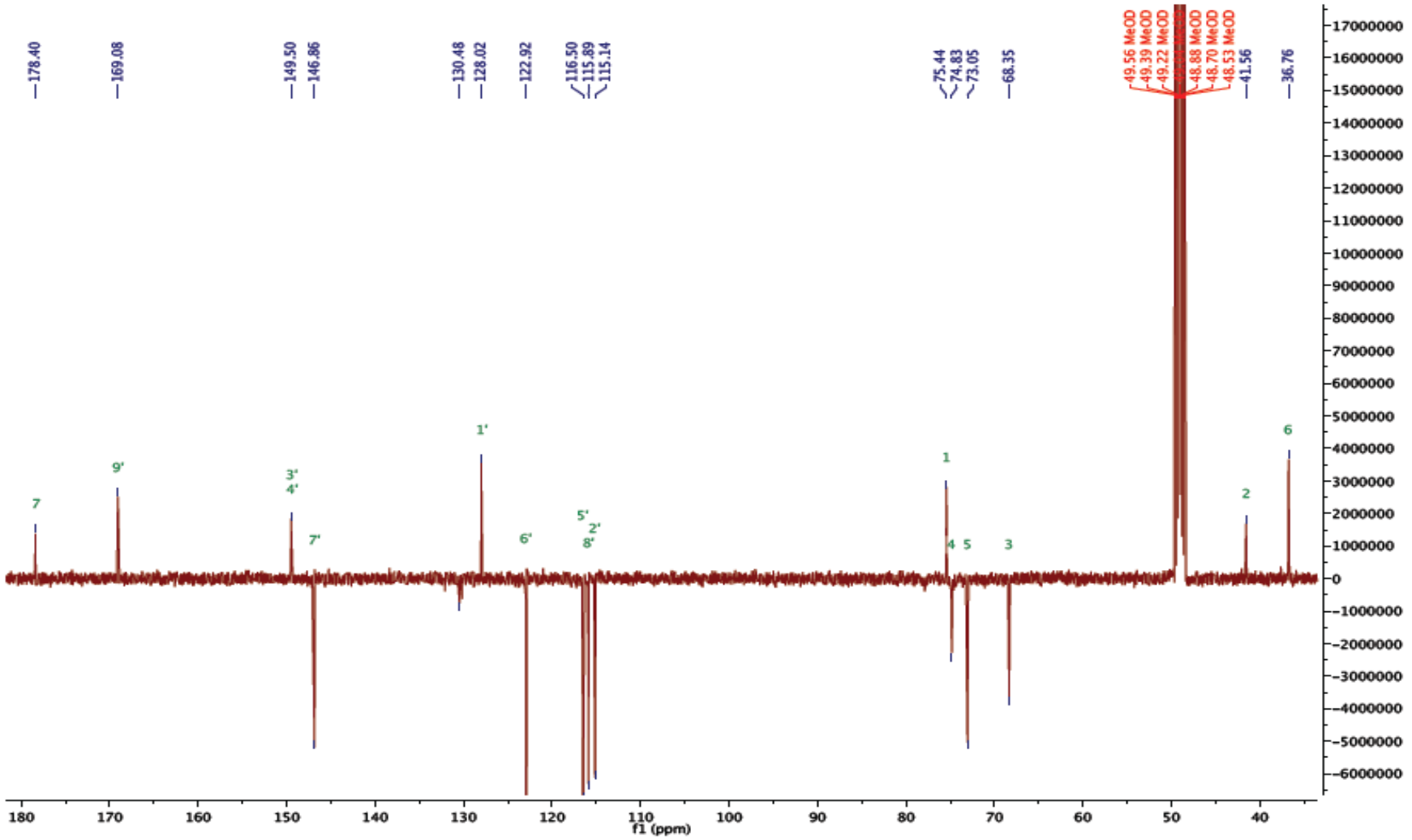

Figure S8. ^{13}C APT spectrum of neochlorogenic acid

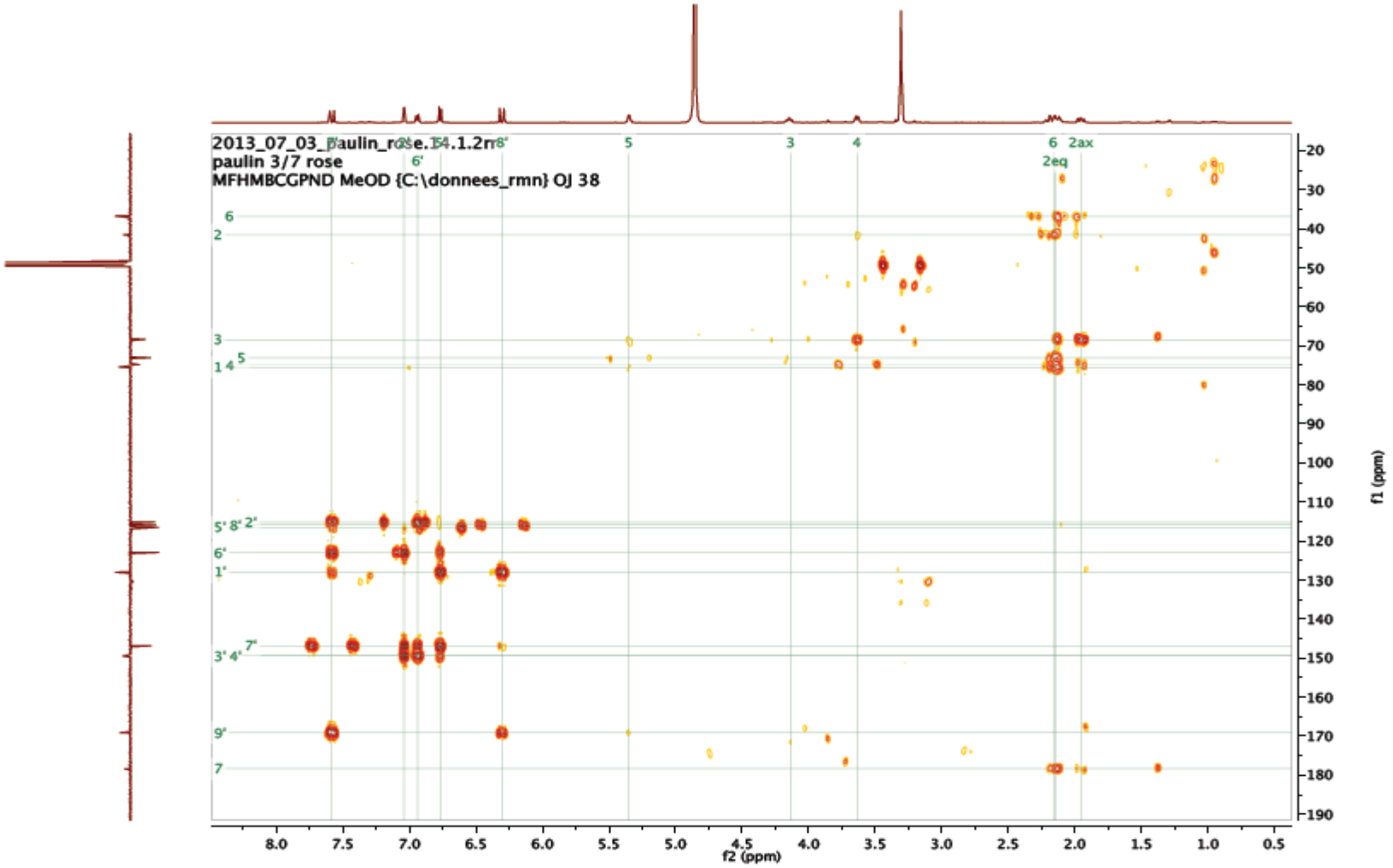

Figure S9. ^{1}H, ^{13}C- HMBC spectrum of neochlorogenic acid

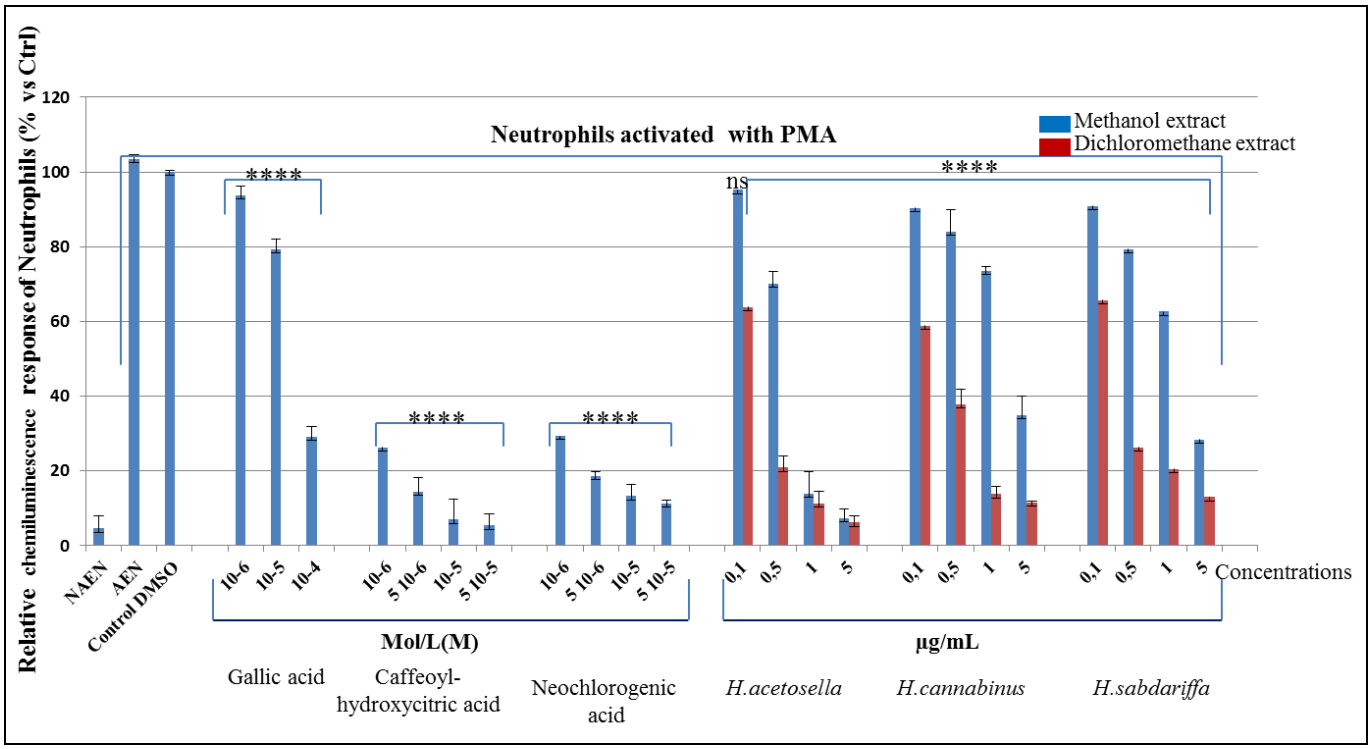

Figure S10. Effects of gallic acid, caffeoyl-hydroxycitric acid, neochlorogenic acid and dichloromethane and methanolic extracts of *H. acetosella, H. cannabinus* and *H. sabdariffa* on the CL response produced by PMA activated equine neutrophils (Means ± SD, n = 6). The CL intensity results from the reaction between lucigenin and the ROS produced by the non-activated (NAEN) and activated equine neutrophils (AEN). The chemiluminescence response of stimulated neutrophils in presence of DMSO used to solubilize the extracts was defined as 100%. P-values (**** $p < 0.0001$) calculated by two-way ANOVA followed by Sidak Multiple Comparisons Test indicated a significant effect of the extracts vs. DMSO control; ns: no significance. There is a statistical difference between activities of dichloromethane and methanolic extracts for all the Hibiscus species. Dichloromethane extracts are more active than methanolic extracts, caffeoyl-hydroxycitric and neochlorogenic acids are more active than gallic acid used as positive control. Extracts of *H. acetosella* are the most active, followed by those of *H. cannabinus* and *H. sabdariffa*.

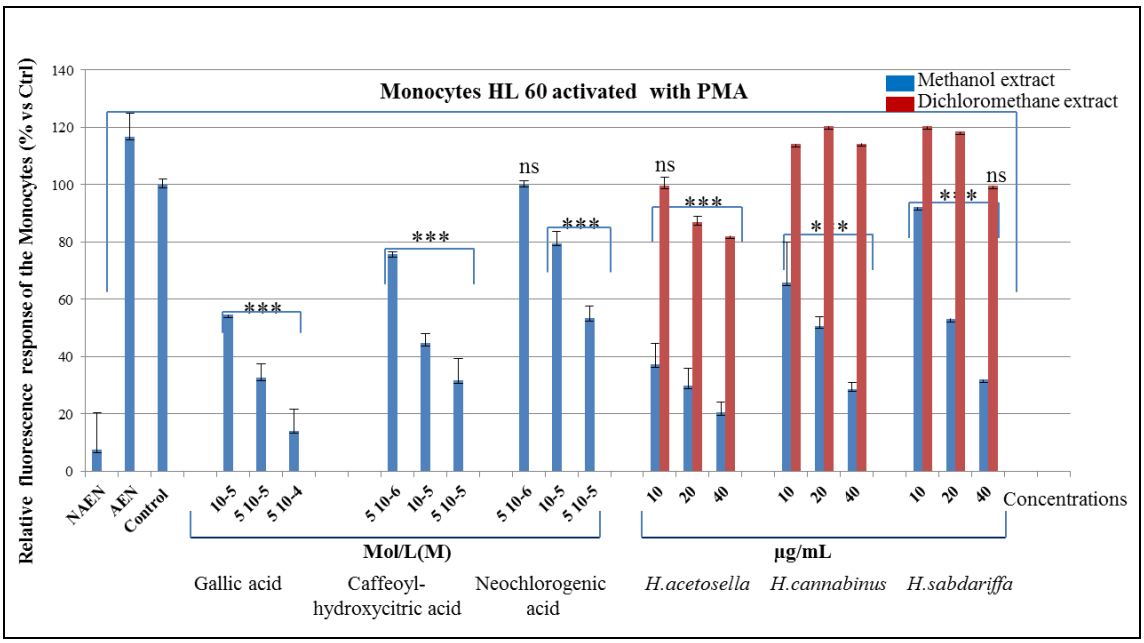

Figure S11. Effect of gallic acid, caffeoyl-hydroxycitric acid, neochlorogenic acid and methanolic and dichloromethane extracts of *H. acetosella*, *H. cannabinus* and *H. sabdariffa* on the intracellular ROS production by PMA activated HL-60 monocytes (Means ± SD, n = 6). Fluorescence intensity of Dichlorofluorescein results from the oxidation of DCFH2 by intracellular ROS produced by non-activated monocytes (NAEN) and activated monocytes (AEN). The fluorescence observed in the presence of DMSO was taken as 100%. P-values (**** $p < 0.0001$) calculated by two-way ANOVA followed by Sidak Multiple Comparisons Test indicated a significant effect of the extracts vs. DMSO control. ns: non-significant. There is a statistical difference between activities of dichloromethane and methanolic extracts of all Hibiscus species.

Dichloromethane extracts are not active in this cell model and exhibited a pro-oxidant effect at tested concentrations. Caffeoyl-hydroxycitric and neochlorogenic acids are more active than gallic acid used as positive control.

Table 1 IC_{50} (µg/mL) and R^2 (between brackets) values of gallic acid, caffeoyl-hydroxycitric acid, neochlorogenic acid, dichloromethane extracts and methanolic extracts on ABTS, DPPH and chemiluminescence (CL) assays (Means ± SD, n=6). nf: not found. ABTS, DPPH radical scavenging, cellular antioxidant activities of *H. acetosella*, *H. cannabinus* and *H. sabdariffa* are significantly different ($p < 0.05$). There is also a significant difference between dichloromethane and methanolic extracts of Hibiscus species in CL assay ($p < 0.0001$). Activities of caffeoyl-hydroxycitric acid and neochlorogenic acid are significantly different. *H. acetosella* is the most active and caffeoyl-hydroxycitric acid is more active compared to neochlorogenic acid.

Samples	**IC_{50} (µg/mL)**					
	ABTS		**DPPH**		**CL**	
	Methanol extract	Dichloromethane extract	Methanol extract	Dichloromethane Extract	Methanol extract	Dichloromethane Extract
Hibiscus acetosella	43.05 ± 4.05 (0,961)	116.4 ± 24.50 (0,942)	59.43 ± 7.10 (0,944)	nf	0.62 ± 0.02 (0,986)	0.16 ± 0.02 (0,989)
Hibiscus cannabinus	58.97 ± 8.7 (0,982)	123.65 ± 19.80 (0,960)	73.79 ± 17.20 (0,891)	nf	2.79 ± 0.20 (0,971)	0.17 ± 0.03 (0,921)
Hibiscus sabdariffa	64.72 ± 6.17 (0,972)	186.3 ± 83.50 (0,981)	86.04 ± 4.32 (0,976)	nf	1.84 ± 0.15 (0,983)	0.19 ± 0.03 (0,954)
Caffeoyl-hydroxycitric acid	4.32 ± 0.11 (0.938)		10.23 ± 0.97 (0.973)		0.59 ± 0.06 (0.733)	
Neochlorogenic acid	6.40 ± 0.11 (0.938)		16.33 ± 1.58 (0.973)		1.22 ± 0.712 (0.751)	
Gallic acid	0.71 ± 0.08 (0.954)		1.07 ± 0.10 (0.998)		6.6 ± 1.2 (0.992)	