

Senna alata leaves are a good source of propelargonidins

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SUPPLEMENTARY MATERIAL

Senna alata leaves are a good source of propelargonidins

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Proanthocyanidins (PA) in *Senna alata* leaves were investigated by thiolysis with benzyl mercaptan, LC-MS and NMR and consisted of rare, but almost pure propelargonidins with <6% procyanidins, had B-type linkages a mean degree of polymerisation of 3. Epiafzelechin was the major flavan-3-ol subunit (>94%) and epicatechin a minor constituent (6.4%) in residual PA and mainly detected as an extension unit.

Keywords: *Senna alata*, *Cassia alata*, proanthocyanidins, propelargonidins, LC-MS, NMR, thiolysis

1. Experimental

1.1 General

Acetone (analytical reagent grade), acetonitrile (HPLC grade), dichloromethane (HPLC grade) and hydrochloric acid (37%, analytical reagent grade), were purchased from ThermoFisher Scientific Ltd (Loughborough, U.K.); (±)-taxifolin (98%) and kaempferol (98%) from Apin Chemicals (Abingdon, U.K.); benzyl mercaptan (99%), epicatechin (EC) and quercetin (≥99% HPLC) from Sigma-Aldrich (Poole, U.K.). (+)-afzelechin (96-98%) from Plantech UK (Reading, UK). Sephadex™ LH-20 from GE Healthcare (Little Chalfont, UK). Deionised water was obtained from a Milli-Q System (Millipore, Watford, U.K.). Quercetin was dissolved in methanol/water (80/20 %) using six concentrations (n = 2) and peak areas were used for the calibration curve.

1.2 Samples

Senna alata leaves were harvested in December 2013 in private botanical garden in Trois-rivières, Guadeloupe, France. Any excess humidity was removed with kitchen paper, air-dried

for a few hours protected from direct light then immediately carefully packed and sent to the University of Reading, UK by airplane and arrived within 3 days. Upon arrival, leaves were freeze-dried and finely ground in an impeller SM1 cutting mill (Retsch, Haan, Germany) to pass a 1 mm sieve. The ground plant material was stored in the dark at room temperature.

1.3 Extraction and purification

1.3.1 Extractable proanthocyanidins

Finely ground leaves (10.2 g) were extracted for 1 h each with acetone/water (125 mL; 7:3; v/v). Acetone was removed under vacuum on a rotary evaporator; the remaining aqueous solution was centrifuged for 3 min at 4500 rpm (Jouan CR3i Multifunction Centrifuge, Thermo Electron Corporation, Basingstoke, UK) and freeze-dried to give the extract (extract = 1.5 g, yield = 15%). Acetone was allowed to evaporate from the plant residue in the fume cupboard overnight and protected from direct light before freeze-drying prior to analysis of unextractable proanthocyanidins.

1.3.2 Purification of proanthocyanidins

Dried extract (1.2 g) was dissolved in distilled water (500 mL) and passed through a Sephadex™ LH-20 resin (50 g), which had been conditioned with water. Distilled water was added to remove sugars and other impurities. The first fraction (F1-fraction) of proanthocyanidins was eluted with acetone/water (1 L; 3:7; v/v) and the second fraction (F2-fraction) with acetone/water (1 L; 1:1; v/v). Acetone was removed and the aqueous solutions were freeze-dried.

1.4 Analytical data

1.4.1 Thiolysis of extracted and fractionated proanthocyanidins

The freeze-dried extracts or fractions (8 mg) were placed into a screw cap glass tube with a stirring magnet, methanol (1.5 mL), methanol acidified with concentrated HCl (3.3%; 500 µL) and benzyl mercaptan (50 µL). The tubes were capped and placed into a water bath at 40°C for 1 h under vigorous stirring (ETS-D5, RCT basic, IKA®, GmbH, Staufen, Germany). Then distilled water (2.5 mL) and internal standard, taxifolin in methanol (500 µL; 0.1 mg/mL), was added and thoroughly mixed. The mixture was transferred into a vial (0.8 mL; Chromacol 08-CPV; ThermoFisher Scientific Ltd), closed with a crimp top and analysed by liquid chromatography-mass spectrometry (LC-MS) within 12 h.

1.4.2 *In situ thiolysis of proanthocyanidins*

Freeze-dried leaves (200 mg) were reacted with the thiolysis reagent (2 mL methanol, 1 mL of 3.3% HCl in methanol, and 100 μ L benzyl mercaptan) as above. After the reaction, methanol (1 mL) was added to the mixture. The sample was mixed and centrifuged at 4000 rpm for 3 min (Centrifuge 5702, Eppendorf® GmbH, Hamburg, Germany) and supernatant (1 mL) was transferred into another empty and clean screw cap glass tube. Distilled water (9 mL) and internal standard, taxifolin in methanol (500 μ L; 0.1 mg/mL), was added and thoroughly mixed. The mixture was transferred into a vial (0.8 mL; Chromacol 08-CPV; ThermoFisher Scientific Ltd), closed with a crimp top and analysed by liquid chromatography-mass spectrometry (LC-MS) within 12 h.

1.4.3 *Liquid chromatography-mass spectrometry (LC-MS) analysis*

LC-MS was used for confirmation of the identity of flavan-3-ols in terminal and extension units using instrument specifications and conditions as previously described (Williams et al. 2014). Flavan-3-ols and their benzyl mercaptan adducts (-BM adducts) were identified by their retention times and characteristic UV-VIS spectra between 220 and 595 nm (Gea et al. 2011). Peak areas of flavan-3-ols at 280 nm were integrated and quantified using molar response factors relative to taxifolin: 0.30 for epicatechin; 0.26 for epicatechin-BM; 0.15 for epiafzelechin was determined with an authentic standard and the same was assumed for epiafzelechin-BM. This provided information on the proanthocyanidin composition in terms of % terminal and % extension flavan-3-ol units; it also allowed calculation of the mean degree of polymerisation (mDP), % procyanidins (PC) and propelargonidins (PP), and % *cis*- and *trans*-flavan-3-ols within the proanthocyanidins (Gea et al. 2011). Other phenolics concentrations compounds were determined in quercetin equivalent using four different concentrations (n = 2) for calibration curve.

1.4.4 *NMR analysis*

The F2-fraction (10 mg) was dissolved in 500 μ L DMSO-*d*₆ and transferred to a 5 mm NMR tube. ¹H-¹³C correlation 2D NMR (HSQC) spectra were recorded at 27 °C on a Bruker Avance III 500 (¹H 500.13 MHz, ¹³C 125.76 MHz) instrument equipped with TopSpin 2.4 software and a 5-mm BBI ¹H/¹³C gradient probe (Bruker, Coventry, U.K.). Spectral resonances were referenced to the residual signals of DMSO-*d*₆ (2.49 ppm for ¹H and 39.5 ppm for ¹³C spectra). For ¹H-¹³C HSQC experiments, spectra were obtained using 128 scans

and 1024 scans for ^{13}C experiments.

References

- Gea A, Stringano E, Brown RH, Mueller-Harvey I. 2011. *In situ* analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high-throughput germplasm screening. J. Agric. Food Chem. 59:495–503.
- Williams AR, Fryganas C, Ramsay A, Mueller-Harvey I, Thamsborg SM. 2014. Direct anthelmintic effects of condensed tannins from diverse plant sources against *Ascaris suum*. PLoS ONE. 9:1–16.

Table S1. Assignment of other phenolic compounds detected in *S. alata* leaves (mg/g of DW in quercetin equivalent, SD in parentheses; n = 3).

Peak numbers refer to Figure S1.

Peak number	Compounds	MW	t _R (min)	MS fragment <i>m/z</i>	Concentration (mg/g DW)	References
1	Aloe-emodin- <i>O</i> -glucoside	432	23.3	465.3 [M – H] [–] Cl [–]	1.8 (0.1)	Martin et al. 1998
2	Physcion- <i>O</i> -glucoside	446	26.5	479.3 [M – H] [–] Cl [–]	4.4 (0.1)	Smith et al. 1979
4	Kaempferol- <i>O</i> -gentiobioside	610	29.8	609.4 [M – H] [–]	6.9 (0.2)	Moriyama et al. 2003
6	Kaempferol- <i>O</i> -glucoside	448	32.9	447.3 [M – H] [–]	1.6 (0.1)	Hazni et al. 2008
8	Kaempferol	286	43.8	285.2 [M – H] [–]	6.6 (0.2)	Rahaman et al. 2006

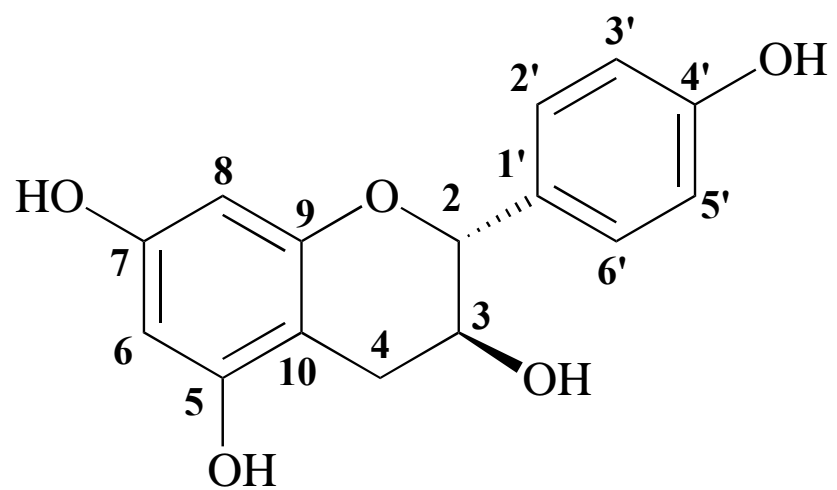


Figure S1: Epiafzelechin

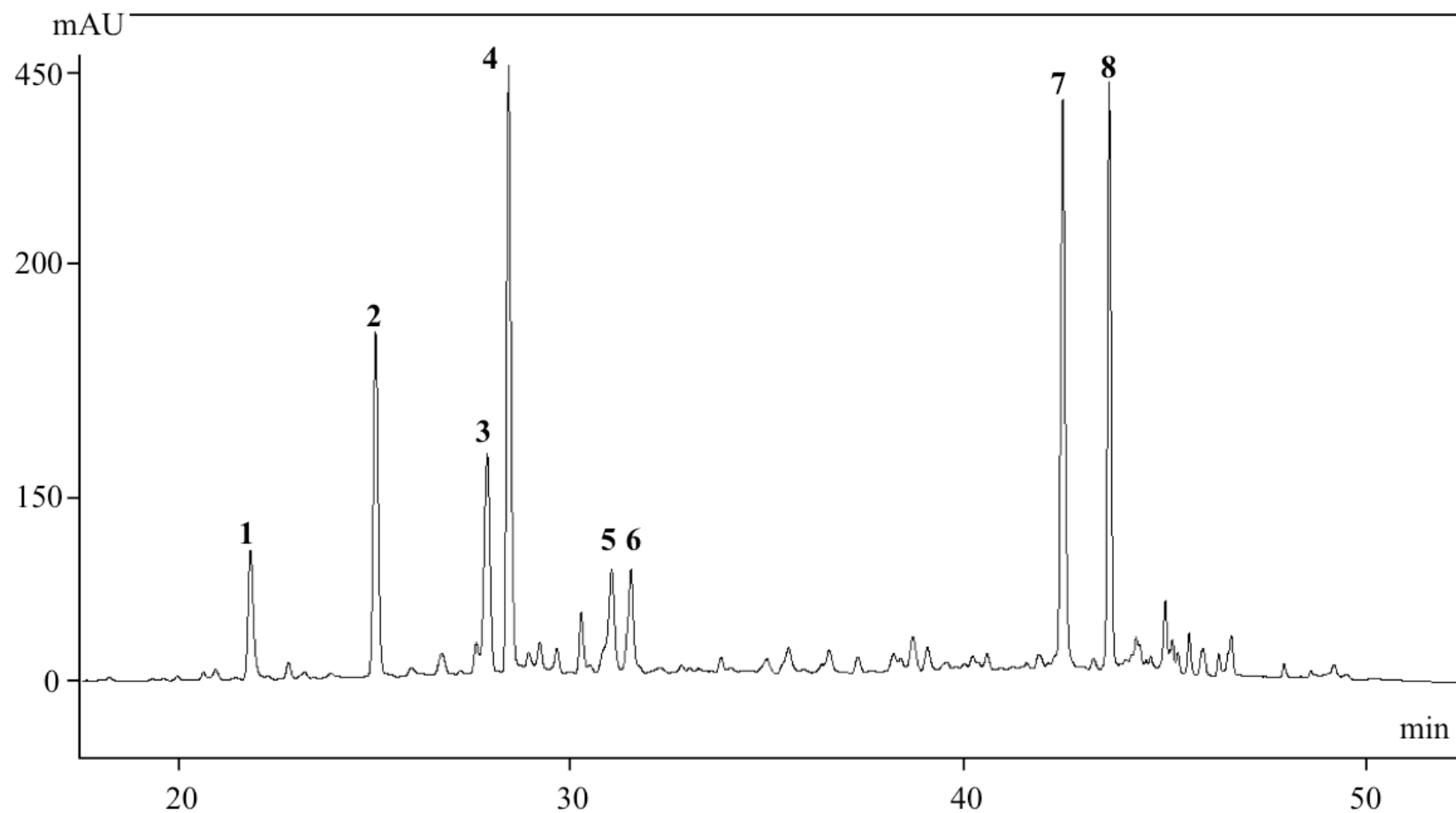


Figure S2. HPLC chromatogram after *in situ* thiolysis of proanthocyanidins from *S. alata* leaves: 1, aloe-emodin-*O*-glucoside; 2, physcion-*O*-glucoside; 3, epiafzelechin; 4, kaempferol-*O*-gentiobioside; 5, internal standard (taxifolin); 6, kaempferol-*O*-glucoside; 7, epiafzelechin-BM; 8, Kaempferol. BM: benzyl mercaptan (see Table S1 for retention times and MS fragments).

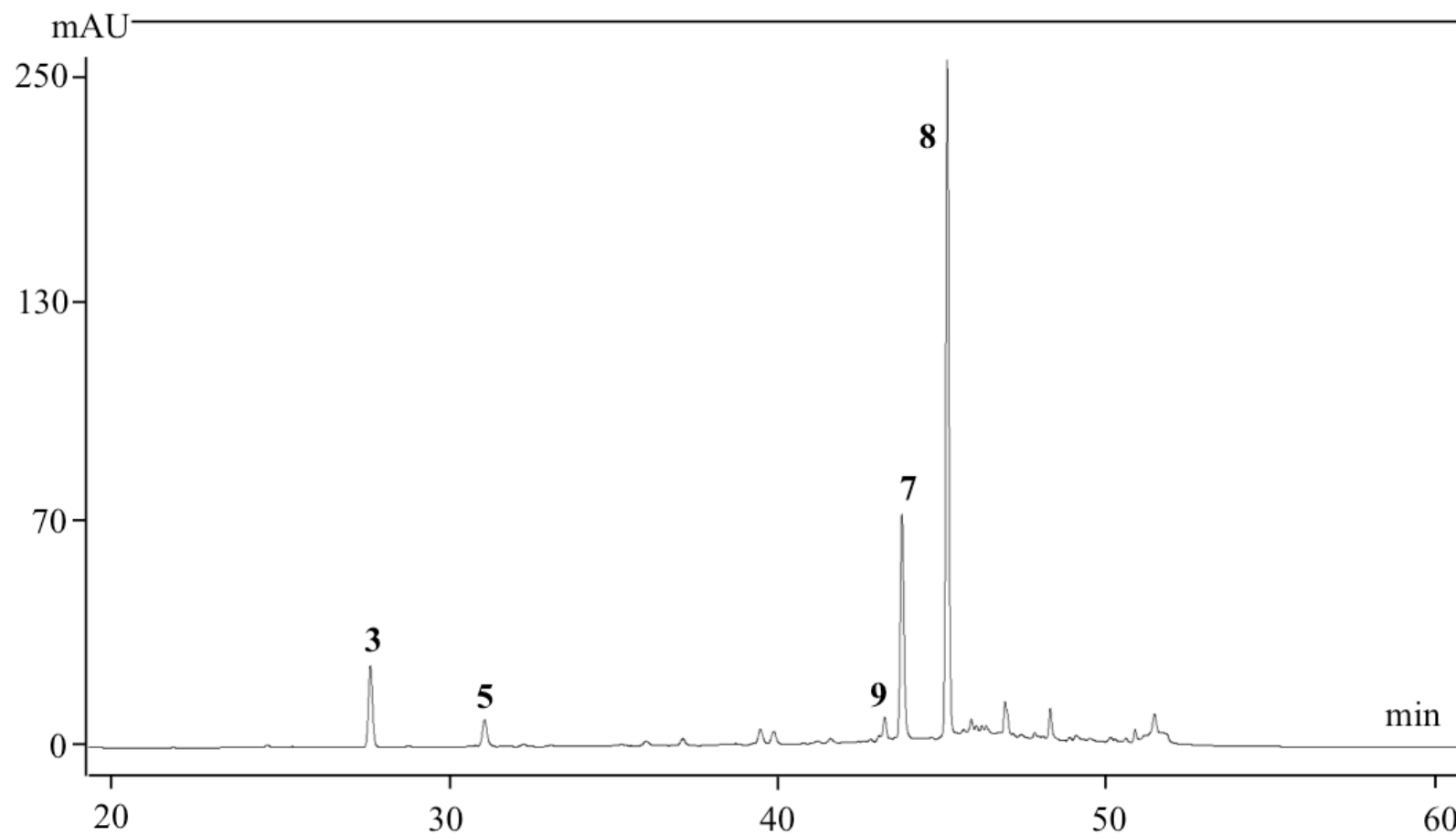


Figure S3. HPLC chromatogram after thiolysis of proanthocyanidins in the F2-fraction: 3, epiafzelechin; 5, internal standard (taxifolin); 7, epiafzelechin-BM; 8, kaempferol; 9, epicatechin-BM. BM: benzyl mercaptan.

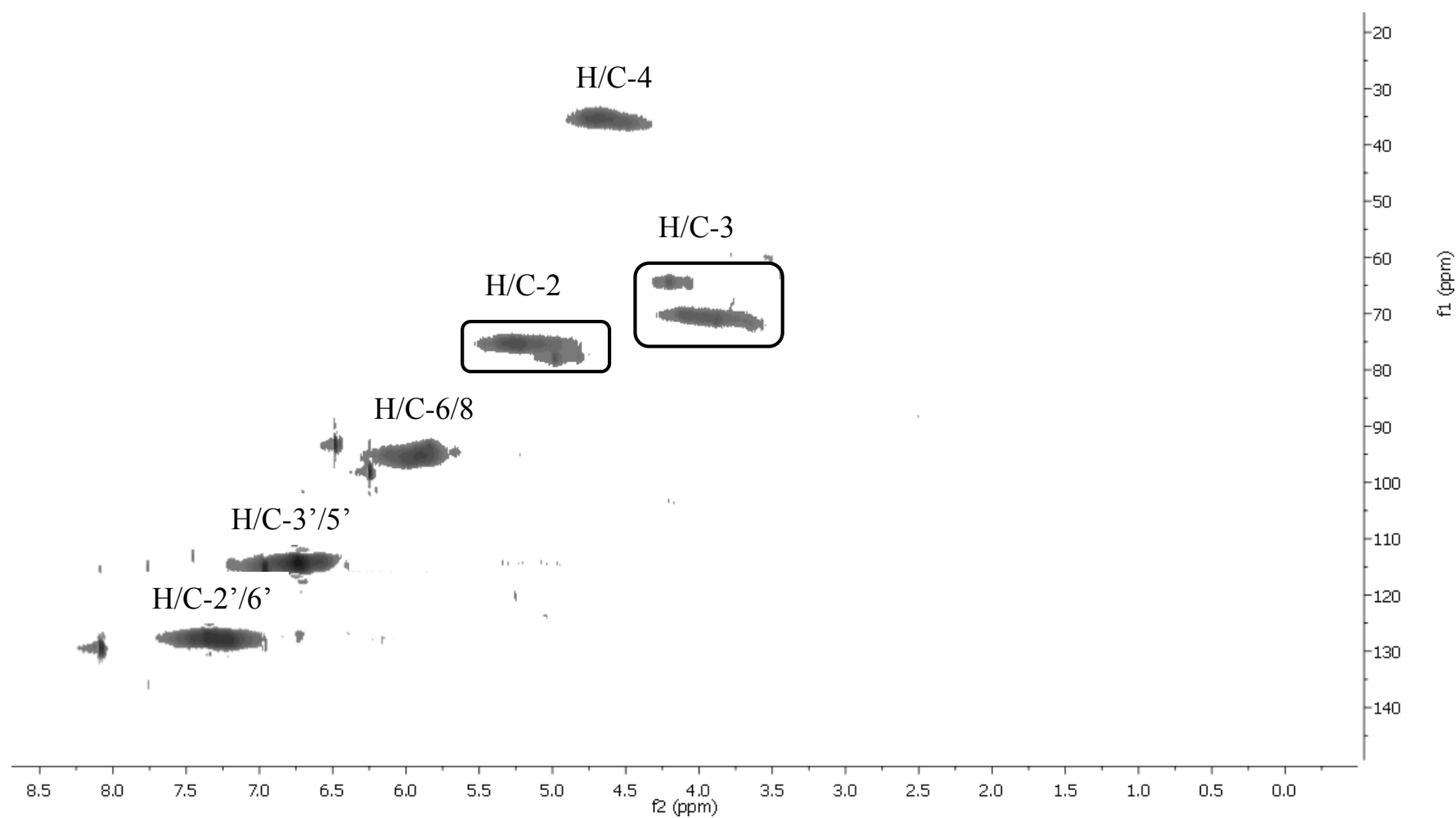


Figure S4. ^1H – ^{13}C HSQC NMR spectrum of F2-fraction PA from *S. alata* leaves (DMSO- d_6 , 500 MHz).

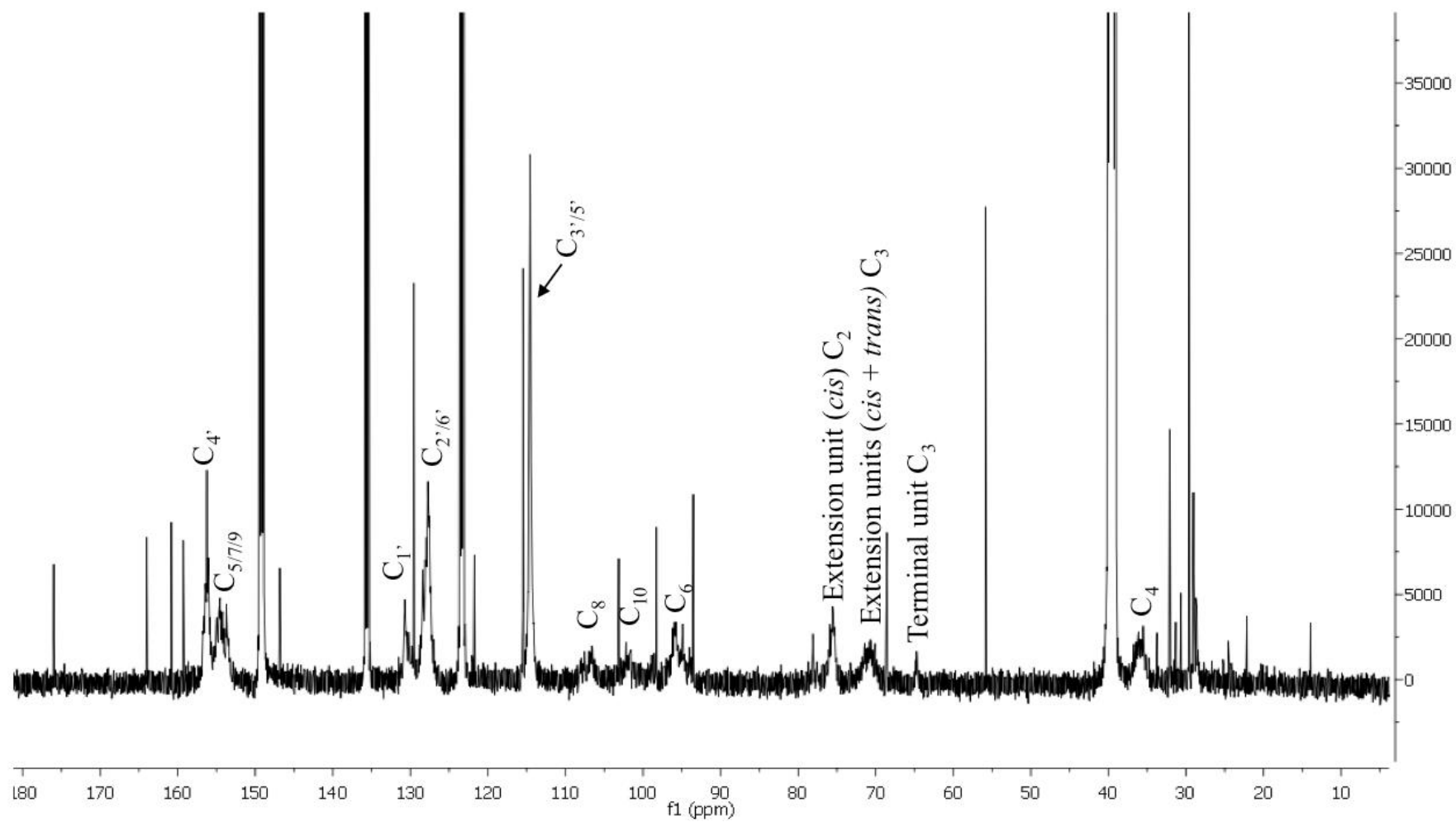


Figure S5. ^{13}C NMR spectrum of F2-fraction PA from *S. alata* leaves ($\text{DMSO-}d_6$, 125 MHz).