SUPPLEMENTARY INFORMATION

Precision health targeting TMAO in postmenopausal women: Polyphenol effects modulated by urolithin A and equol metabotypes in a randomised, placebo-controlled crossover trial

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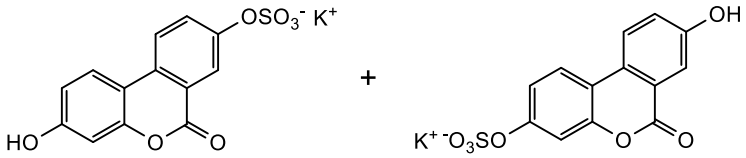
Supplementary Methods

Urolithin A sulfate was prepared as a mixture of regioisomers as previously described procedure (González-Sarrías et al., 2013). 4-Hydroxydibenzyl sulfate (4HDB-sulfate) was synthesised from 4HDB (4-Hydroxydibenzyl) using the SO3·pyridine complex as the sulfating reagent and pyridine as the basic solvent under reflux conditions. An analogous method, using $SO_3{\cdot}NMe_3$ and Et_3N, was applied to obtain a regioisomeric mixture of lunularin monosulfates (See Supp. Info.). The glucuronide derivatives of 4HDB and lunularin were obtained under conditions previously reported (Lucas et al., 2009). The phenolic precursors were treated with glucurosonyl donor (methyl-(3*S*,4*S*,5*S*,6*R*)-3,4,5-triacetoxy-6-(3,3,3-trichloroethanimidoyl)oxy-tetrahydropyran-2-carboxylate) and $BF_3{\cdot}OEt$ in anhydrous dichloromethane to obtain the protected 4HDB-*O*-glucuronide and the protected lunularin-*O*-glucuronide as a mixture of regioisomers. Subsequent ester hydrolysis with K_2CO_3 in $MeOH/H_2O$ yielded the corresponding glucuronide derivatives (4-HDB-*O*-glucuronide) and the regioisomers of (lunularin-*O*-glucuronide).

^{1}H NMR and ^{13}C NMR spectra

^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 300 and 400 MHz instruments. ^{1}H NMR chemical shifts are reported relative to Me_4Si and were referenced via residual proton resonances of the corresponding deuterated solvent, whereas ^{13}C NMR spectra are reported relative to Me_4Si using the carbon signals of the deuterated solvent. Abbreviations of coupling patterns are as follows: br, broad; s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet. Coupling constants (*J*) are expressed in Hz.

Regioisomeric mixture of urolithin A 3-*O*-sulfate and urolithin A 8-*O*-sulfate



The regioisomeric mixture of 3-*O*-sulfate urolithin A and 8-*O*-sulfate urolithin A was synthesised following the described procedure reported in González-Sarrías et al. (2013), showing identical spectroscopic data as those reported therein: ^{1}H NMR (400 MHz, DMSO, 298 K) δ 8.19 (d, *J* = 8.8 Hz, 2H), 8.11 (dd, *J* = 15.7, 8.8 Hz, 2H), 8.00 (d, *J* = 2.6 Hz, 1H), 7.61 (dd, *J* = 8.8, 2.6 Hz, 1H), 7.54 (d, *J* = 2.6 Hz, 1H), 7.35 (dd, *J* = 8.7, 2.6 Hz, 1H), 7.25 – 7.13 (m, 2H), 6.82 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.74 (d, *J* = 2.4 Hz, 1H) ppm; ^{13}C NMR (101 MHz, DMSO, 298 K) δ 160.5, 159.5, 157.9, 154.2, 152.8, 151.5, 150.0, 130.4, 128.3, 126.2, 124.5, 124.2, 124.2, 123.0, 122.9, 121.0, 119.6, 119.6, 117.0, 113.7, 113.2, 113.2, 109.3, 108.0, 102.9 ppm.

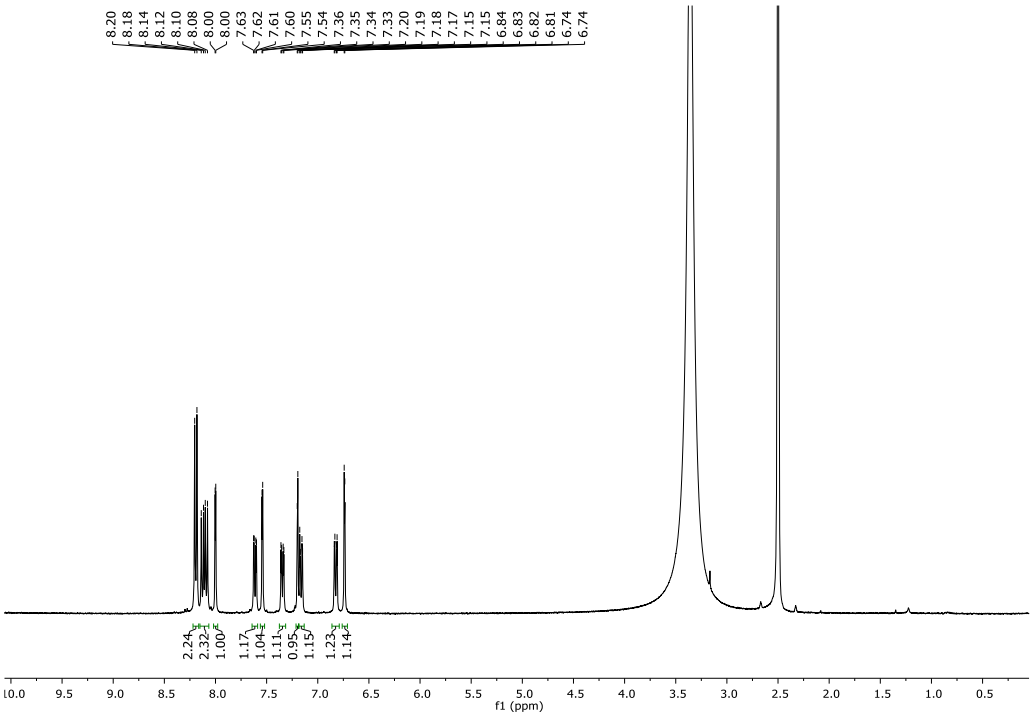


Fig. S1 ^{1}H NMR (400 MHz, DMSO, 298 K) of the mixture of regioisomeric urolithin monosulfates

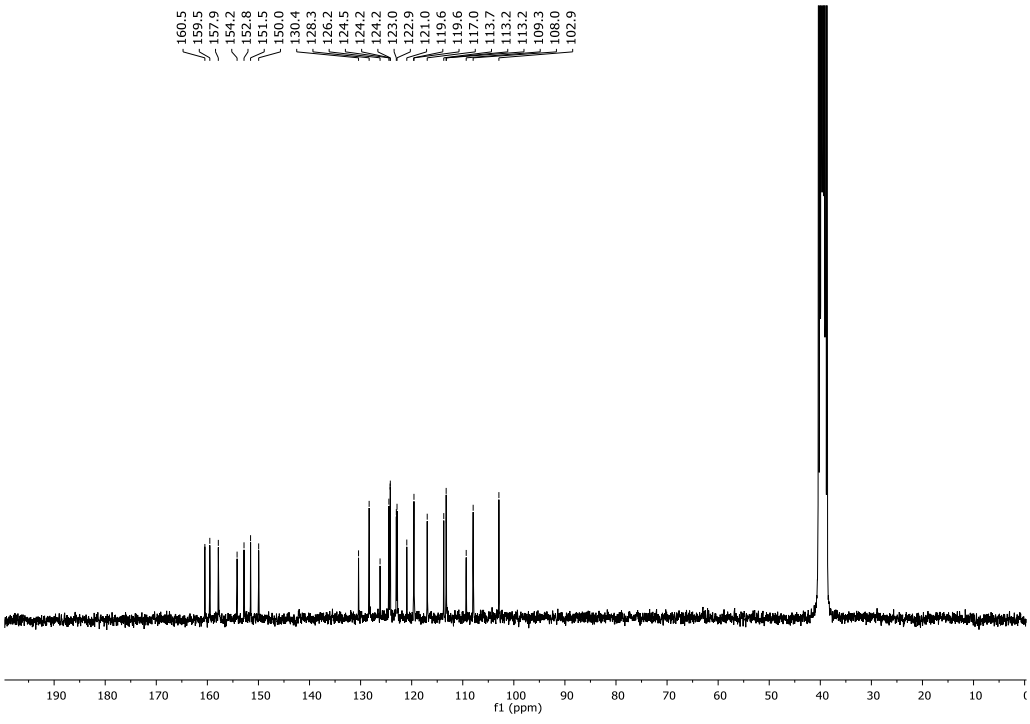
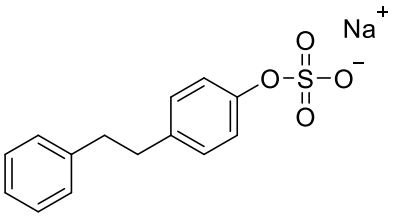


Fig. S2 ^{13}C NMR (101 MHz, DMSO, 298 K) of the mixture of regioisomeric urolithin monosulfates.

Sodium salt of 4HDB-*O*-sulfate



The SO_3·pyridine complex (181 mg, 1.13 mmol) was added to a stirred solution of 4HDB (150 mg, 0.76 mmol) in pyridine (5 mL). The mixture was refluxed for 16 h. After this time, an excess of Na_2CO_3 and 2 ml of water were added, and the suspension was stirred for 1 h. Then, the excess of Na_2CO_3 was removed by filtration, and the solvent was evaporated under reduced pressure. The crude was purified by column chromatography using silica gel as the stationary phase and $CHCl_3$/MeOH 2/1 as eluent to obtain the sodium salt of 4HDB sulfate as a white solid (90 mg, 40%). ^{1}H NMR (400 MHz, DMSO, 298 K) δ 7.30 – 7.21 (m, 4H), 7.20 – 7.14 (m, 1H), 7.14 – 7.10 (m, 2H), 7.08 – 7.03 (m, 2H), 2.83 (m, 4H) ppm; ^{13}C NMR (101 MHz, DMSO, 298 K) δ 151.6, 141.6, 136.0, 128.5, 128.4, 128.2, 125.8, 120.3, 37.3, 36.5 ppm; HRMS (ESI) calculated for $[C_{14}H_{13}O_4S]^-$ $[M]^-$ 277.0540, found 277.0549.

^{1}H NMR (400 MHz, DMSO, 298 K)

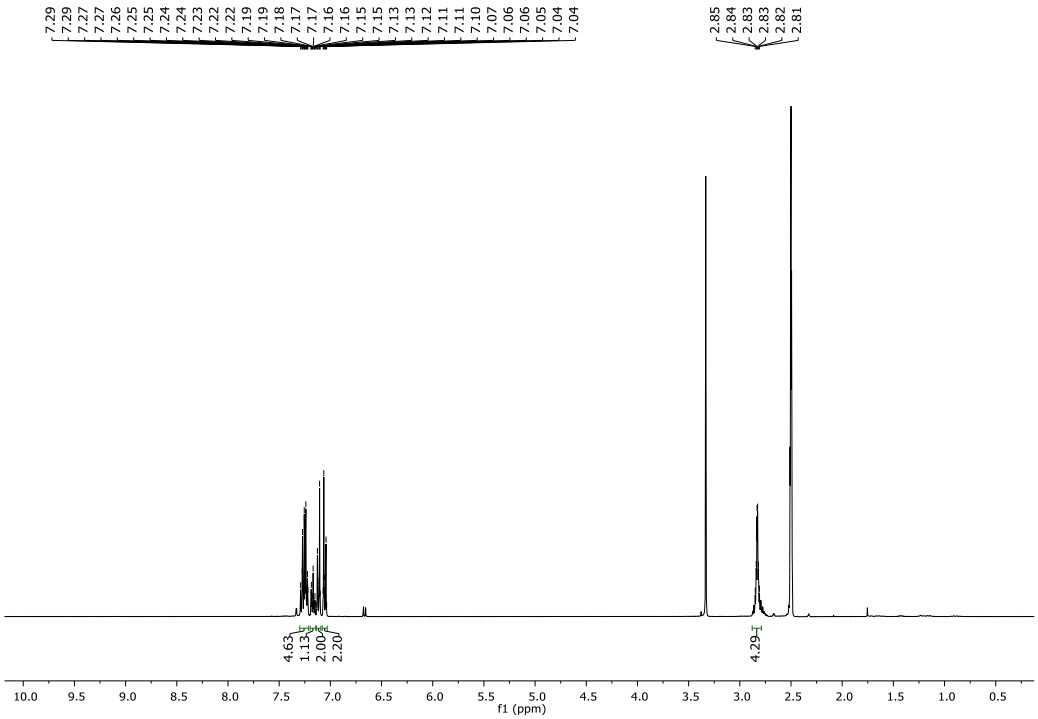


Fig. S3 ^{1}H NMR (400 MHz, DMSO, 298 K) of 4HDB-*O*-sulfate.

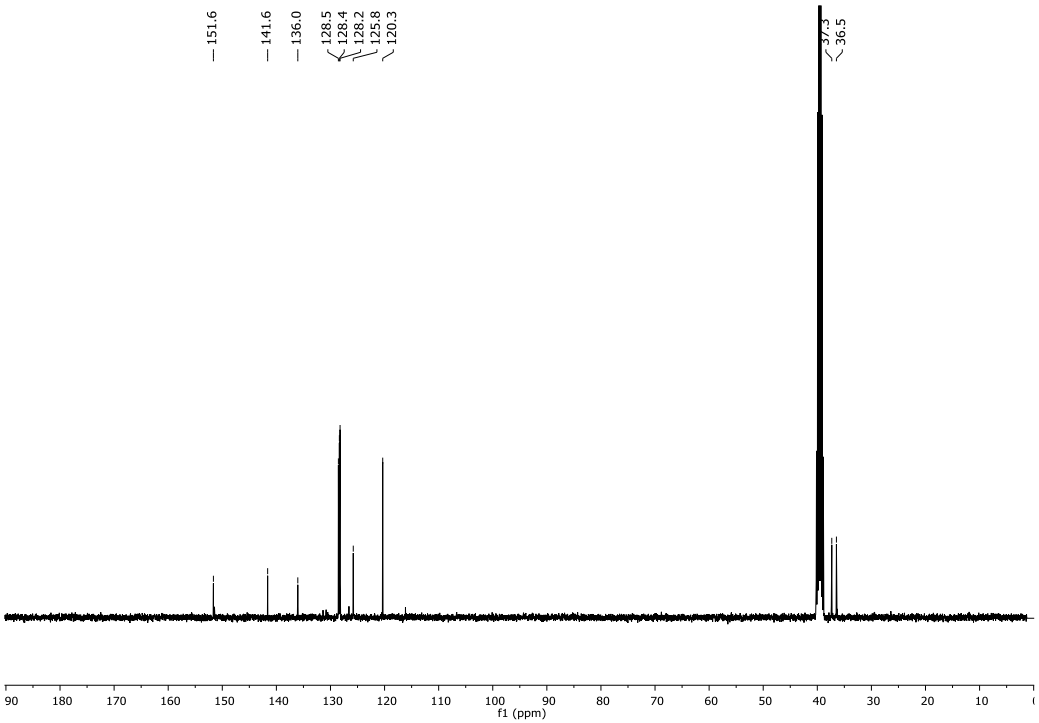
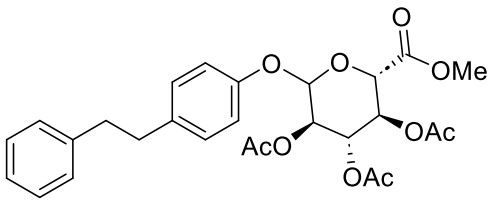


Fig. S4 ^{13}C NMR (101 MHz, DMSO, 298 K) of 4HDB-*O*-sulfate.

Protected 4HDB-*O*-glucuronide



A volume of 2.5 mL of a 0.1 M solution of $BF_3{\cdot}OEt_2$ (0.25 mmol) in anhydrous dichloromethane was added to a stirred solution of 4HDB (300 mg, 1.51 mmol) and methyl-(3*S*,4*S*,5*S*,6*R*)-3,4,5-triacetoxy-6-(3,3,3-trichloroethanimidoyl)oxy-tetrahydropyran-2-carboxylate (723 mg, 1.51 mmol) in anhydrous dichloromethane (6 mL). The reaction was stirred for 12 hours, and, after that time, the solvent was removed under reduced pressure. The product was purified by column chromatography using silica gel as the stationary phase and hexane/ethyl acetate 7/3 as the eluent to obtain the titled product as a white solid (495 mg, 64%). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ 7.32 – 7.23 (m, 2H), 7.23 – 7.14 (m, 3H), 7.13 – 7.05 (m, 2H), 6.97 – 6.87 (m, 2H), 5.41 – 5.21 (m, 3H), 5.10 (d, *J* = 7.1 Hz, 1H), 4.22 – 4.11 (m, 1H), 3.73 (s, 3H), 2.88 (s, 4H), 2.06 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 298 K) δ 170.3, 169.5, 169.4, 167.1, 155.2, 141.7, 137.1, 129.6, 128.6, 128.5, 126.1, 117.2, 99.6, 72.8, 72.1, 71.3, 69.3,

53.1, 38.1, 37.2, 20.7, 20.6 ppm; HRMS (ESI) calculated for $[C_{27}H_{34}NO_{10}]^+$ $[M+NH_4]^+$ 532.2177, found 532.2183.

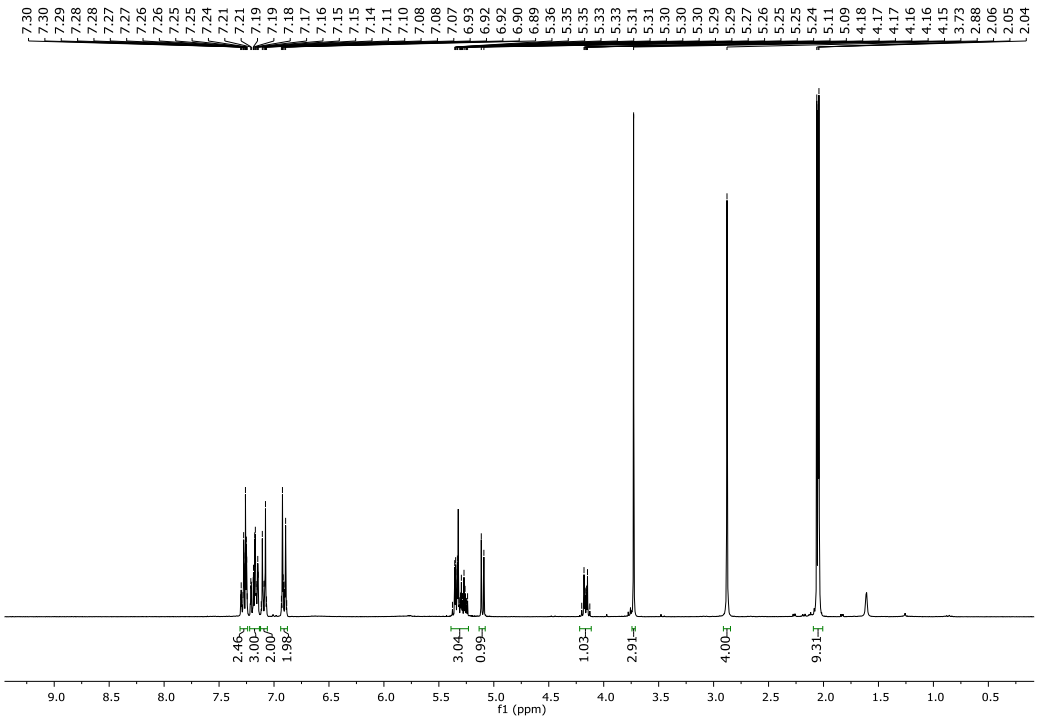


Fig. S5 ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) of protected 4HDB-*O*-glucuronide.

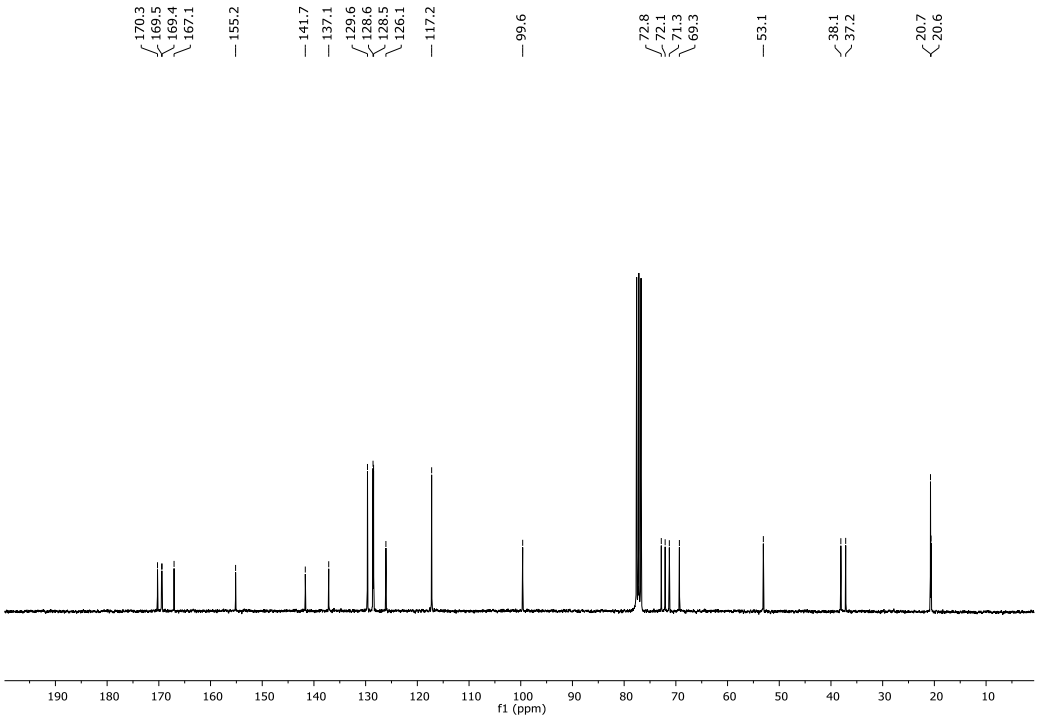
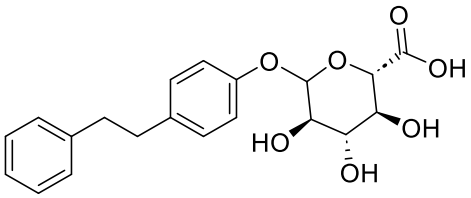


Fig. S6 ^{13}C NMR (75 MHz, $CDCl_3$, 298 K) of protected 4HDB-*O*-glucuronide.

4HDB-*O*-glucuronide



Potassium carbonate (108 mg, 0.78 mmol) was added to a solution of protected 4HDB-*O*-glucuronide (200 mg, 0.39 mmol) in methanol/water 5/1 (10 mL). The reaction mixture was stirred for 1 hour at room temperature. After that time, Amberlyst 15 acid resin was added, and the reaction was stirred for 2 additional hours. Then, the reaction was filtered and the solvent removed under reduced pressure to obtain the titled product as a white solid (120 mg, 82%). ^{1}H NMR (400 MHz, DMSO, 298 K) δ 7.31 – 7.13 (m, 5H), 7.11 (d, *J* = 8.5 Hz, 2H), 6.91 (d, *J* = 8.5 Hz, 2H), 5.25 (s, 1H), 5.05 (s, 1H), 4.81 (d, *J* = 7.3 Hz, 1H), 2.87 – 2.76 (m, 4H) ppm (some signals are overlapped with the water signal); ^{13}C NMR (101 MHz, DMSO, 298 K) δ 171.1, 155.6, 141.6, 134.7, 129.2, 128.4, 128.2, 125.8, 116.1, 100.4, 76.5, 74.1, 73.1, 71.9, 37.4, 36.3 ppm; HRMS (ESI) calculated for $[C_{20}H_{21}O_7]^-$ [M-H]$^-$ 373.1293, found 373.1292.

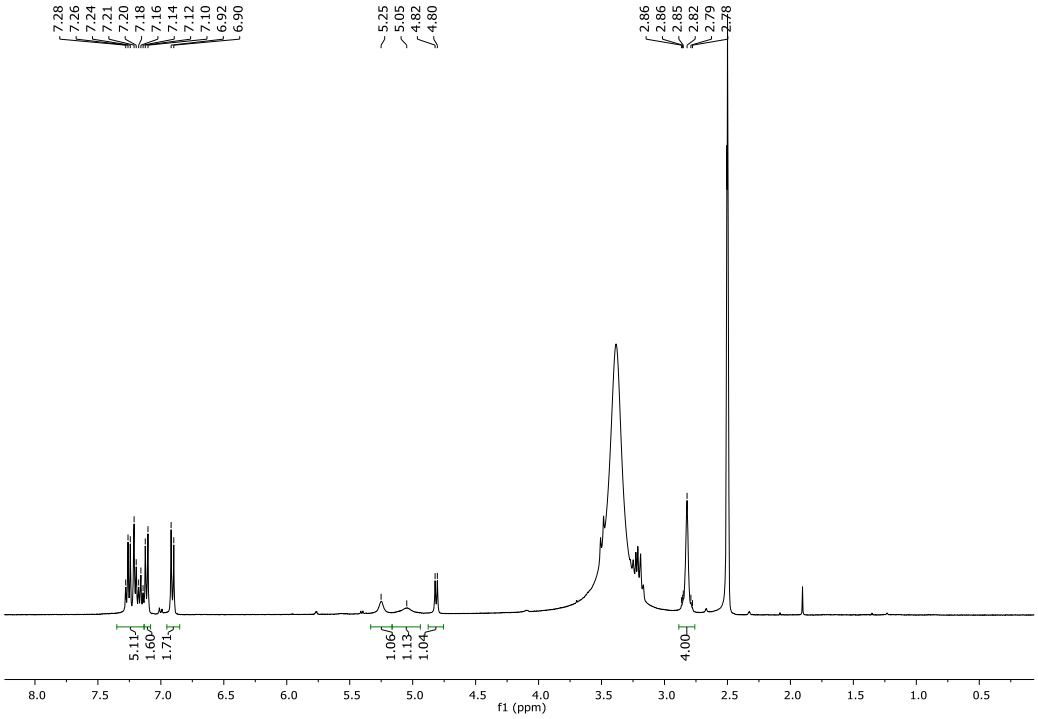


Fig. S7 ^{1}H NMR (400 MHz, DMSO, 298 K) of 4HDB-*O*-glucuronide.

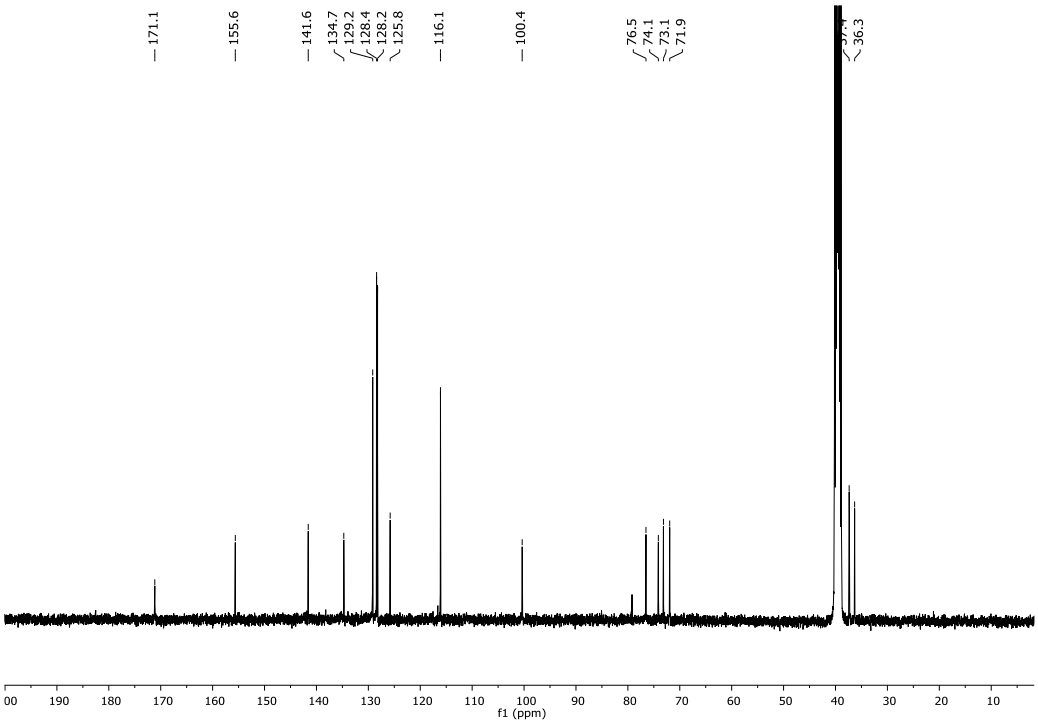
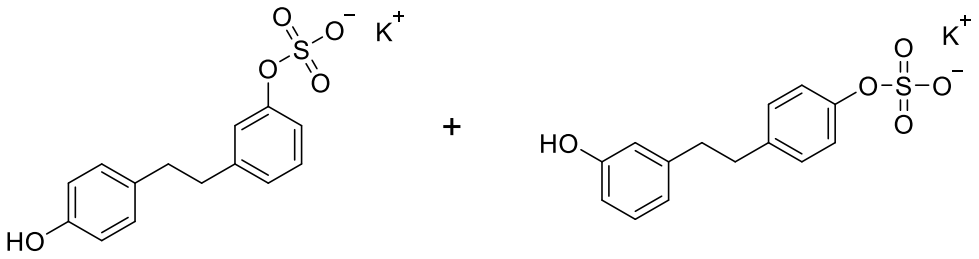


Fig. S8 ^{13}C NMR (101 MHz, DMSO, 298 K) of 4HDB-*O*-glucuronide.

Regioisomeric mixture of lunularin 3-*O*-sulfate and lunularin 4-*O*-sulfate



SO_3·NMe_3 (29 mg, 0.21 mmol) and Et_3N (29.2 µL, 0.21 mmol) were added to a stirred solution of lunularin (50 mg, 0.23 mmol) in MeCN (2 mL). The mixture was refluxed for 6 h. After this time, an excess of K_2CO_3 and 0.5 ml of water were added, and the suspension was stirred for 1 h. Then, the excess of K_2CO_3 was removed by filtration, and the solvent was evaporated under reduced pressure. The crude was purified by column chromatography using silica gel as the stationary phase and $CHCl_3$/MeOH 3/1 as eluent. The regioisomer mixture was obtained as a white solid (20 mg, 19%). ^{1}H NMR (400 MHz, MeOD, 298 K) δ 7.21 – 7.09 (m, 7H), 7.04 (t, *J* = 7.7 Hz, 1H), 6.99 – 6.92 (m, 3H), 6.72 – 6.54 (m, 5H,), 4.59 (s, 2H), 2.88 – 2.72 (m, 8H) ppm; ^{13}C NMR (101 MHz, MeOD, 298 K) δ 158.3, 156.4, 153.8, 152.0, 144.7, 144.5, 139.8, 133.9, 130.4, 130.3, 130.1, 129.8, 126.2, 122.5, 122.4, 120.8, 119.9, 116.3, 116.1, 113.8, 39.3, 39.1, 38.3, 38.0 ppm; HRMS (ESI) calculated for $[C_{14}H_{13}O_5S]^-$ $[M]^-$ 293.0489, found 293.0494.

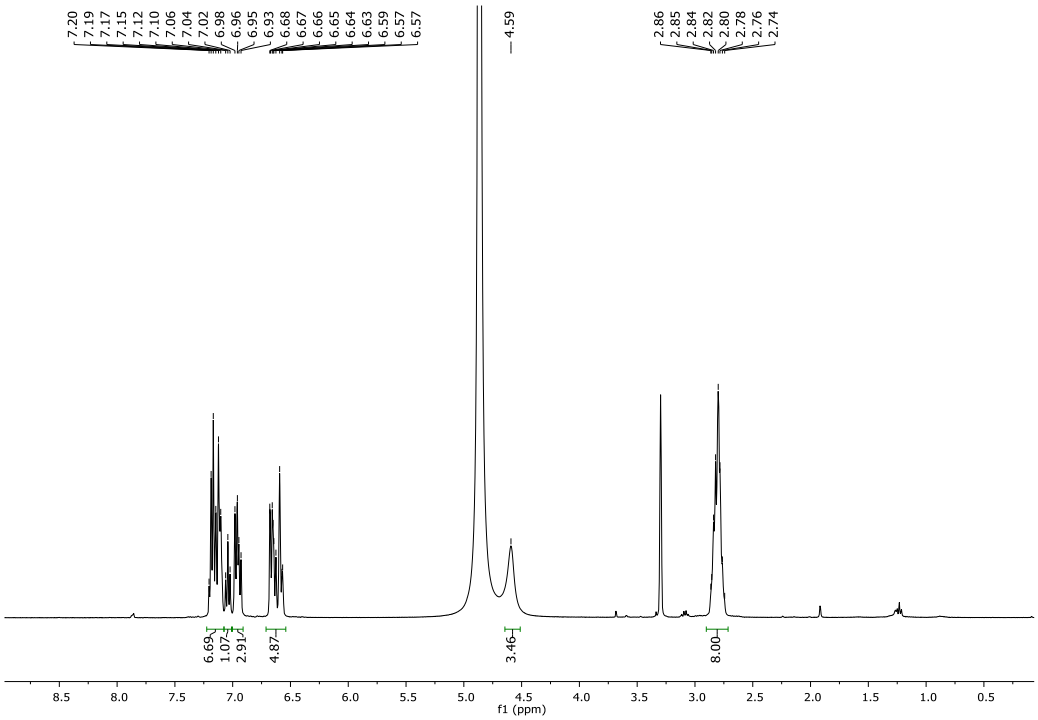


Fig. S9 ^{1}H NMR (400 MHz, MeOD, 298 K) of the mixture of regioisomeric lunularin monosulfates.

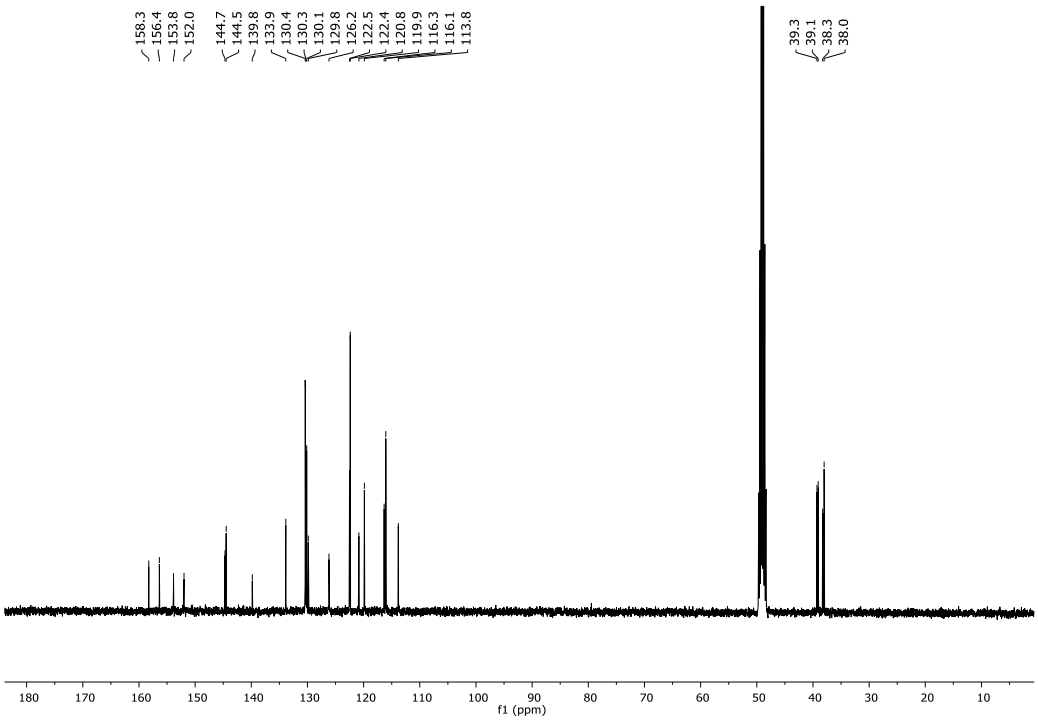
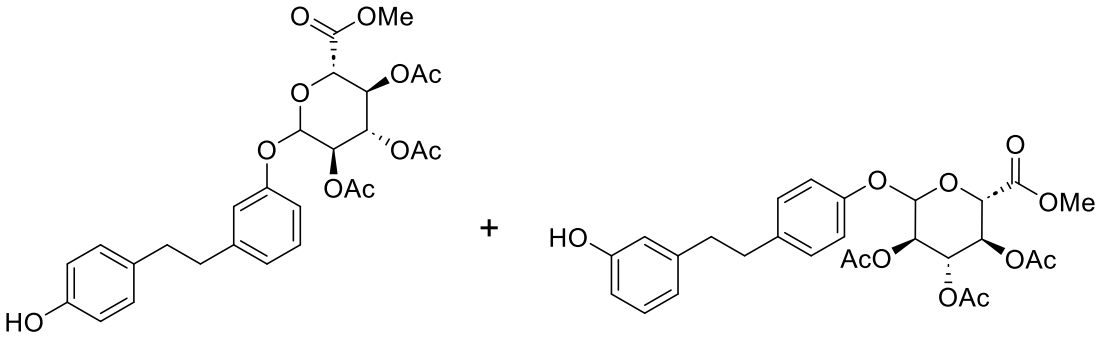


Fig. S10 ^{13}C NMR (101 MHz, MeOD, 298 K) of the mixture of regioisomeric lunularin monosulfates.

Regioisomeric mixture of protected lunularin 3-*O*-glucuronide and lunularin 4-*O*-glucuronide



A volume of 0.5 mL of a 0.1M solution of $BF_3{\cdot}OEt_2$ (0.05 mmol) in anhydrous dichloromethane was added to a solution of lunularin (91 mg, 0.43 mmol) and methyl (3*S*,4*S*,5*S*,6*R*)-3,4,5-triacetoxy-6-(3,3,3-trichloroethanimidoyl)oxy-tetrahydropyran-2-carboxylate (134 mg, 0.28 mmol) in anhydrous dichloromethane (2 mL). The reaction was stirred for 12 hours, and, after that time, the solvent was removed under reduced pressure. The product was purified by column chromatography using silica gel as the stationary phase and hexane/ethyl acetate 7/3 as eluent. The regioisomer mixture was obtained as a colourless oil (59 mg, 26%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ 7.20 – 7.05 (m, 4H), 6.97 – 6.59 (m, 12H), 6.15 – 5.85 (m, 2H), 5.61 – 4.89 (m, 8H), 4.61 – 4.09 (m, 2H), 3.74 – 3.72 (m, 6H), 2.83 – 2.79 (m, 8H), 2.07 – 2.03 (m, 12H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$, 298 K) δ 170.5, 170.3, 169.8, 169.6, 169.5(5), 169.5, 168.4(9), 167.1, 167.0(9), 156.6, 155.9, 154.8, 154.1, 143.6, 143.3 137.0, 133.2, 129.7, 129.6, 129.5, 129.4, 126.2, 123.8, 120.6, 117.4, 117.1, 115.5, 115.2, 114.6, 112.9, 99.3, 96.2, 90.2, 72.5, 72.0, 71.1, 70.8, 69.6, 69.2, 69.16, 68.0, 53.0, 52.9, 37.9, 37.8, 36.8, 36.7, 20.6, 20.6, 20.5 ppm; HRMS (ESI) calculated for $[C_{27}H_{34}O_{11}N]^+$ $[M+NH_4]^+$ 548.2132, found 548.2136.

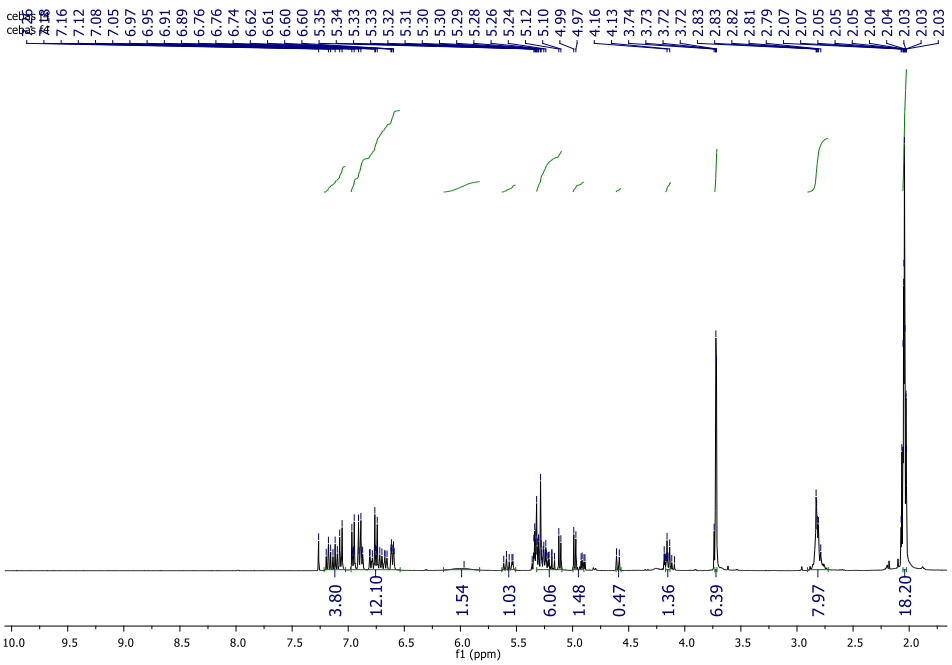


Fig. S11 ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) of the mixture of protected regioisomeric lunularin monoglucuronides.

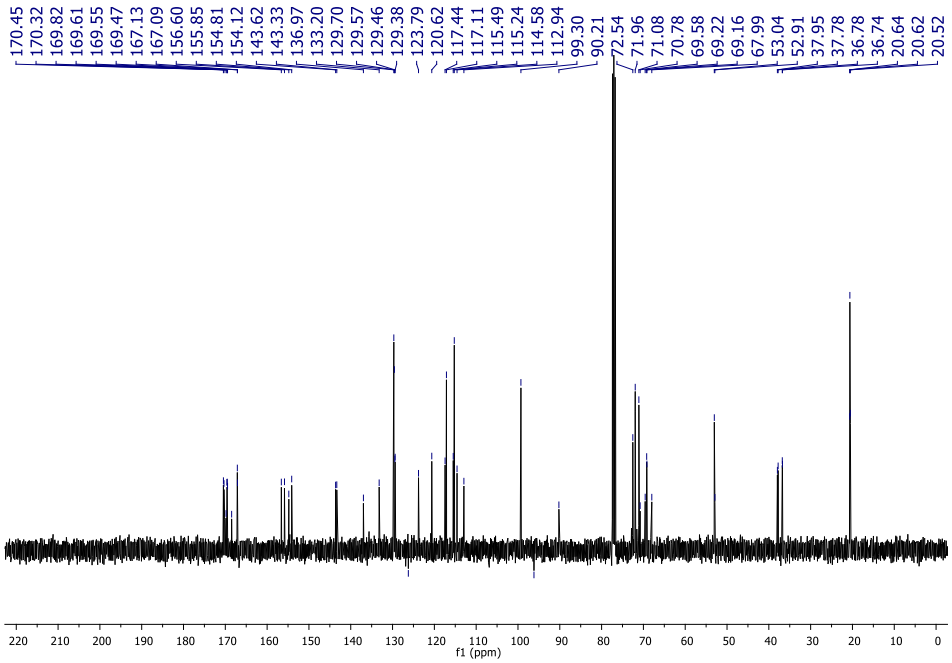
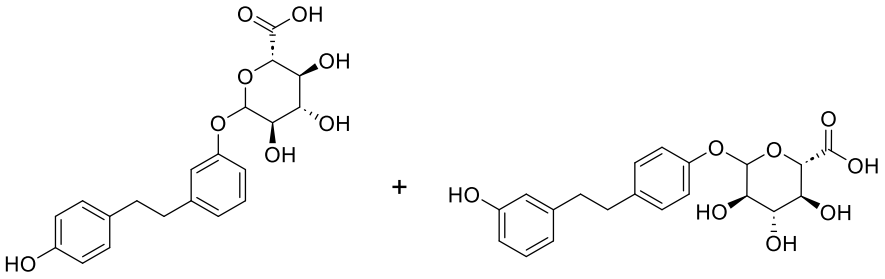


Fig. S12 ^{13}C NMR (101 MHz, $CDCl_3$, 298 K) of the mixture of protected regioisomeric lunularin monoglucuronides.

Regioisomeric mixture of lunularin 3-*O*-glucuronide and lunularin 4-*O*-glucuronide



Potassium carbonate (31 mg, 0.22 mmol) was added to a solution of the mixture of regioisomers in the tetraester form (59 mg, 0.111 mmol) in methanol (3.5 mL) and water (0.7 mL). The reaction mixture was stirred for 1 hour at room temperature. After that time, Amberlyst 15 acid resin was added, and the reaction was stirred for 2 additional hours. Then, the reaction was filtered and the solvent removed under reduced pressure. The regioisomer mixture was obtained as a pale yellow oil (30 mg, 69%). ^{1}H NMR (400 MHz, CD_3OD, 298 K) δ 8.74 – 8.36 (m, 11H), 8.27 – 8.16 (m, 5H), 5.54 – 5.50 (m, 2H), 5.33 – 5.04 (m, 8H), 4.36 – 4.31 (m, 8H) ppm (some signals are overlapped with the water signal); ^{13}C NMR (101 MHz, CD_3OD, 298 K) δ 172.5, 158.5, 157.8, 156.8, 155.9, 144.7, 144.5, 137.4, 133.9, 130.5, 130.4, 130.2, 130.1, 124.1, 121.0, 118.1, 117.7, 116.3, 116.0, 115.3, 113.73, 102.4, 102.3, 77.1, 76.2, 74.3, 72.8, 39.0, 38.9, 37.9, 37.7 ppm; HRMS (ESI) calculated for $[C_{20}H_{21}O_8]^-$ [M-H]$^-$ 389.1245, found 389.1236.

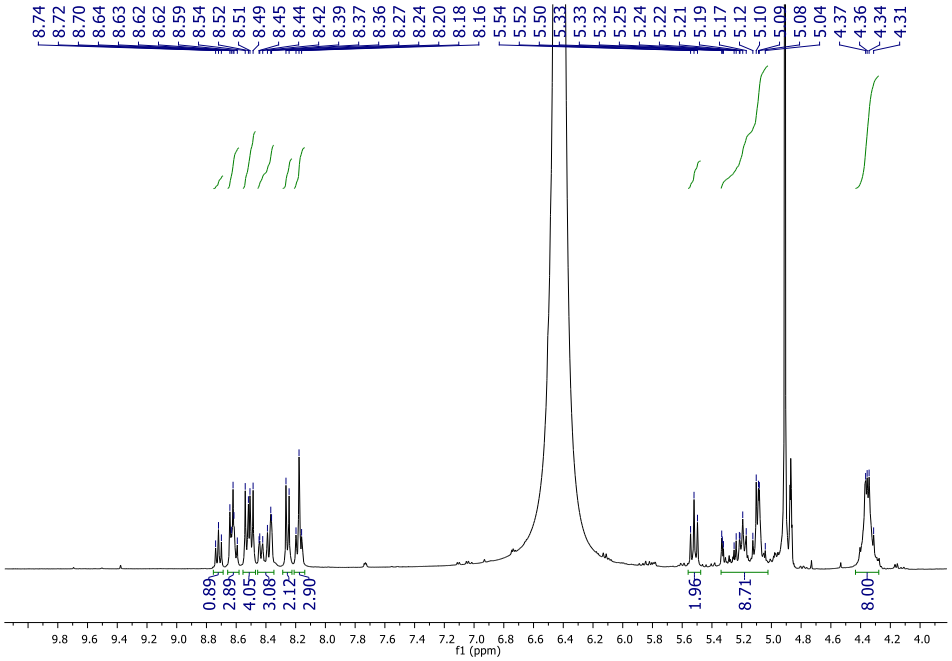


Fig. S13 ^{1}H NMR (400 MHz, CD_3OD, 298 K) of the mixture of regioisomeric lunularin monoglucuronides.

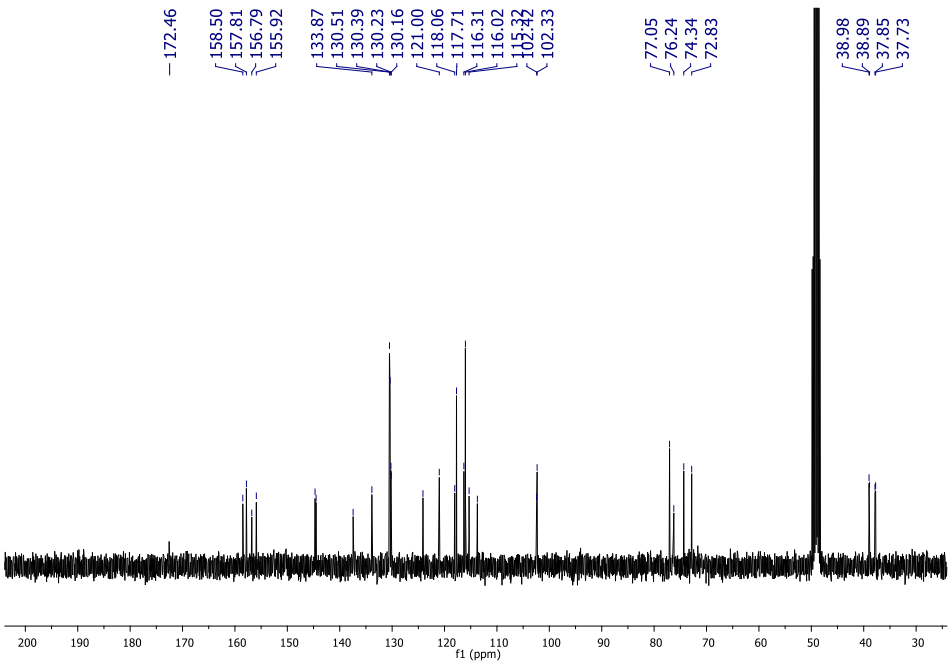


Fig. S14 ^{13}C NMR (101 MHz, CD_3OD, 298 K) of the mixture of regioisomeric lunularin monoglucuronides.

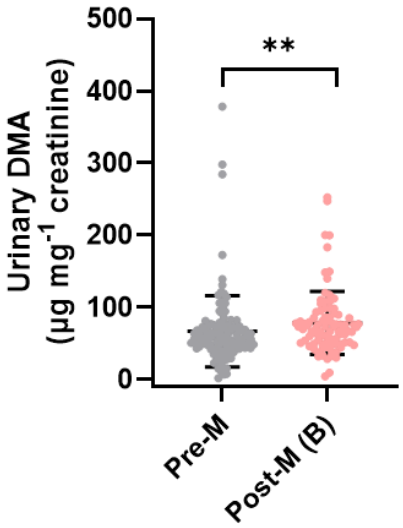


Fig. S15 Comparison of urinary DMA levels between pre-menopausal (Pre-M; n = 120) and post-menopausal women at baseline (Post-M (B); n = 90). ** $p < 0.01$.

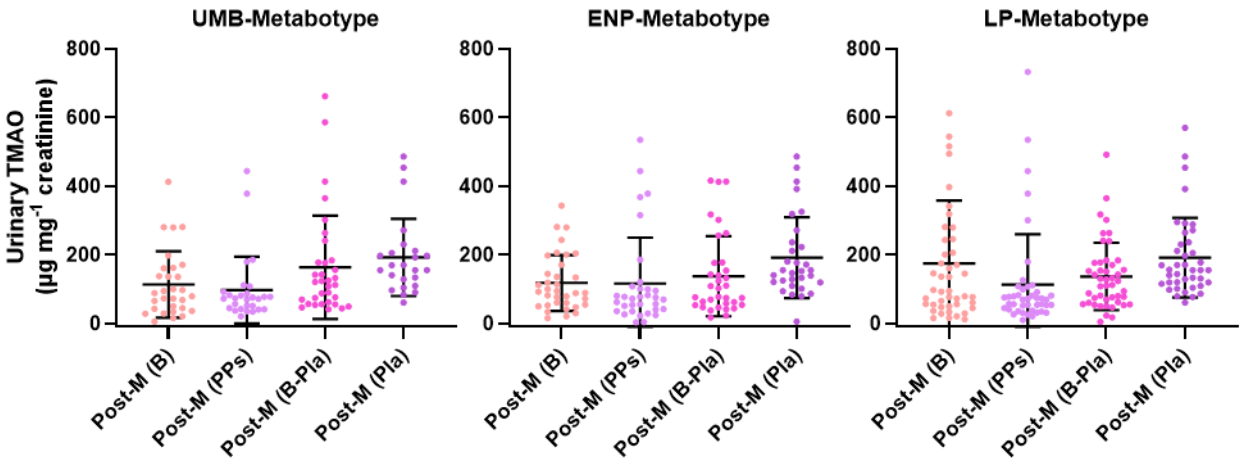


Fig. S16 Urinary TMAO concentrations in Post-M women stratified by metabotypes (showing no significant changes). Post-M (B), baseline; Post-M (PPs), after PPs intervention; Post-M (B-Pla), placebo baseline; and Post-M (Pla), after placebo consumption. UMB, urolithin B metabotype (n = 29); ENP, equol non-producers metabotype (n = 32); LP, lunularin producers metabotype (n = 34).

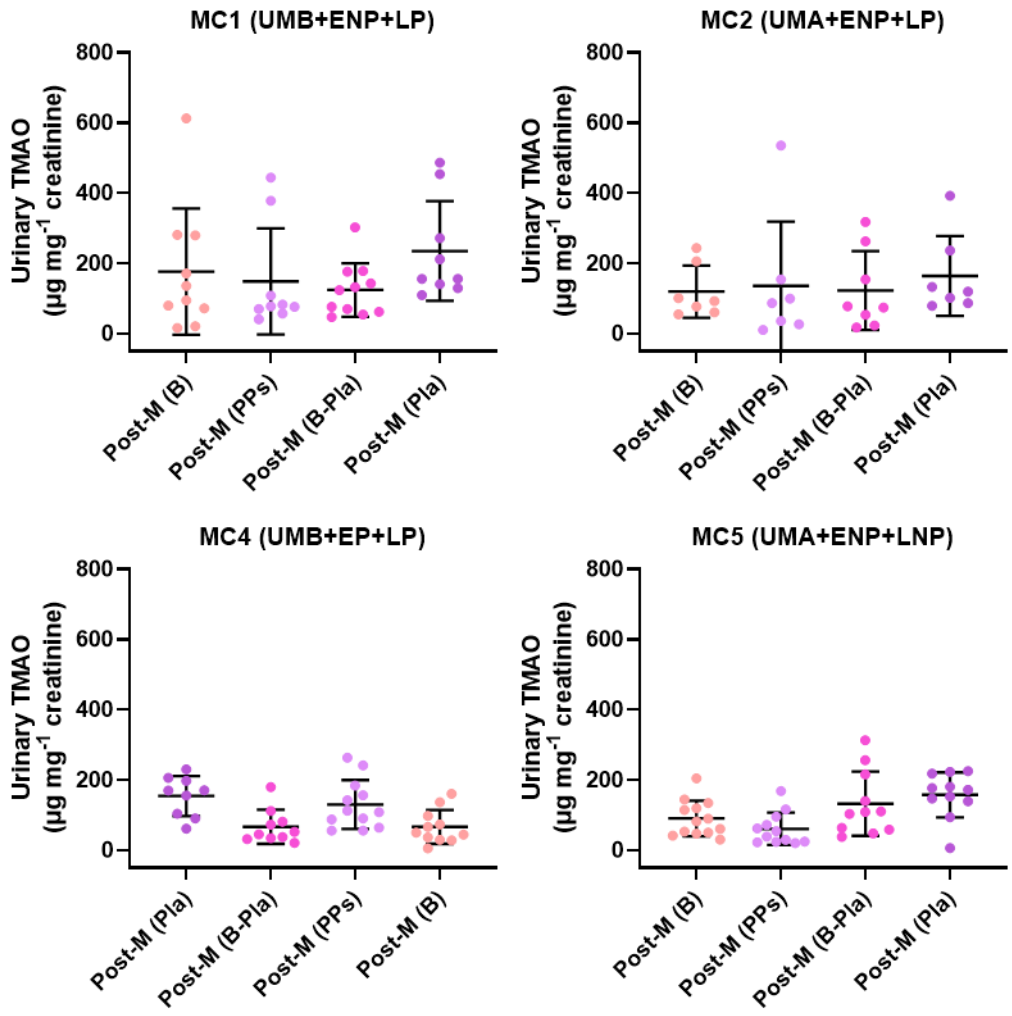


Fig. S17 Urinary TMAO concentrations by polyphenol supplementation (PPs) in Post-M women stratified by metabotype clusters (MC). (MC1, MC2, MC4 and MC5). Post-M (B), baseline; Post-M (PPs), after PPs intervention; Post-M (B-Pla), placebo baseline; and Post-M (Pla), after placebo consumption. The percentage per cluster is detailed in Table 1.

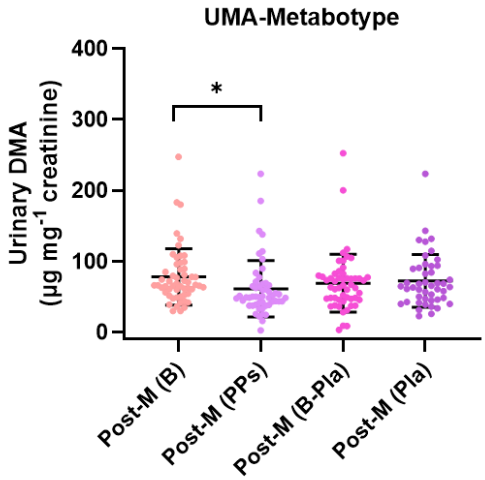


Fig. S18 Urinary DMA changes after stratification by UMA metabotype. UMA, urolithin A metabotype (n = 49). *$p < 0.05$

Table S1. Phenolic compounds in the plant extract mixture (PPs).

Compound	RT	*m/z*⁻	MS/MS	λ_{max}	mg/g extract	mg/capsule
Pomegranate						
Punicalin	12.76	781	721/601/299	258/380	11.47±0.92	8.03±0.65
Punicalagin α	15.08	1083	781/721/601	258/372	48.15±8.75	33.71±6.13
Punicalagin β	17.08	1083	781/721/601	258/372	48.20±6.24	32.16±2.28
Ellagic acid	25.68	301	257/229/185	254/360	42.81±1.81	29.97±1.27
Σ Ellagitannins and ellagic acid						**103.9±10.32**
Polygonum cuspidatum						
trans-Resveratrol	30.84	227	185/157	306	63.42±4.80	44.39±3.36
Red clover						
Daidzein	32.33	253	225/209	248/304	5.20±0.61	3.64±0.43
Genistein	37.15	269	241/225	260/324	4.86±0.15	3.41±0.10
Formononetin	40.80	267	252/235/211	250/304	43.81±1.60	30.67±1.12
Biochanin A	43.15	283	268/251/227	260/332	25.34±10.63	17.74±7.44
Σ Isoflavones						**55.45±9.10**
Total phenolics					**203.7±22.8**	

The PPs mixture was analysed by High-Performance Liquid Chromatography coupled to Electrospray Ionisation-Ion Trap Tandem Mass Spectrometry (HPLC-ESI-IT-MS/MS), as previously described (Ávila-Gálvez et al., 2019). The identification of compounds was based on their elution order, UV spectra, *m/z*⁻ values, MS/MS fragmentation patterns, and comparison with authentic standards when available. *trans*-Resveratrol (quantified at 310 nm), ellagic acid (360 nm), punicalagin α and β (360 nm), daidzein (270 nm), and genistein (270 nm) were identified by direct comparison with authentic standards. Punicalin was quantified as the sum of its α and β isomers at 360 nm. Formononetin (270 nm) and biochanin A (270 nm) were identified and quantified using daidzein as the reference standard. Values are expressed as mean ± SD (n = 5). RT, retention time.

Table S2. MRM parameters for the analysis of TMAO, TMA, DMA, and creatinine in urine (U) and serum (S).

Metabolite	Precursor Ion	Quantifier Product Ion (m/z^+)	Collision Energy (V)	Qualifier Product Ion (m/z^+)	Collision Energy (V)	Matrix
TMAO	76.1	58.1	8	42.2	10	U, S
TMA	60.1	44.1	30	45.1	5	U
DMA	46.1	30.1	25	28.1	50	U
Creatinine	114	44	20	55	35	U

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