

SUPPORTING INFORMATION

Ephedra foeminea as a Novel Source of Antimicrobial and Anti-Biofilm Compounds to Fight Multidrug Resistance Phenotype

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Supporting information list

Supplementary Table S1. Minimal Inhibitory Concentration (MIC₁₀₀) values (mg/mL) determined for fractions obtained upon extraction in hexane and two sequential steps of column chromatography.

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Supplementary Figure S2. ¹H NMR spectrum of thymol, **compound 2** (CDCl₃, 400 MHz).

Supplementary Figure S3. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (MeOD, 400 MHz).

Supplementary Figure S4. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (MeOD, 400 MHz) in the range 8.1 to 5.4 ppm.

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Supplementary Figure S7. COSY spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

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Supplementary Figure S10. ¹³C NMR spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

Supplementary Figure S11. HSQC spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

Supplementary Figure S12. HMBC spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

Supplementary Figure S13. ESI MS spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** recorded in positive modality.

Supplementary Figure S14. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*E*-*p*-coumaroyl,4''-*Z*-*p*-coumaroyl)-rhamnoside, **compound 4** (MeOD, 400 MHz).

Supplementary Figure S15. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*E*-*p*-coumaroyl,4''-*Z*-*p*-coumaroyl)-rhamnoside, **compound 4** (MeOD, 400 MHz) in the range 8.0 to 5.3 ppm.

Supplementary Figure S16. COSY spectrum of kaempferol-3-*O*- α -L-(2''-*E*-*p*-coumaroyl,4''-*Z*-*p*-coumaroyl)-rhamnoside, **compound 4** (acetone-*d*₆, 400 MHz).

Supplementary Figure S17. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*E*-*p*-coumaroyl,4''-*Z*-*p*-coumaroyl)-rhamnoside, **compound 4** (acetone-*d*₆, 400 MHz).

Supplementary Figure S18. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*E*-*p*-coumaroyl,4''-*Z*-*p*-coumaroyl)-rhamnoside, **compound 4** (acetone-*d*₆, 400 MHz) in the range 8.0 to 5.4 ppm.

Supplementary Figure S19. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*Z*-*p*-coumaroyl,4''-*E*-*p*-coumaroyl)-rhamnoside, **compound 5** (MeOD, 400 MHz).

Supplementary Figure S20. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*Z*-*p*-coumaroyl,4''-*E*-*p*-coumaroyl)-rhamnoside, **compound 5** (MeOD, 400 MHz) in the range 8.0 to 5.4 ppm.

Supplementary Figure S21. ¹H NMR spectrum of kaempferol-3-O- α -L-(2",4"-di-Z-p-coumaroyl)-rhamnoside, **compound 6** (MeOD, 400 MHz).

Supplementary Figure S22. ¹H NMR spectrum of kaempferol-3-O- α -L-(2",4"-di-Z-p-coumaroyl)-rhamnoside, **compound 6** (MeOD, 400 MHz) in the range 7.9 to 5.5 ppm.

Supplementary Figure S23. ¹H NMR spectrum of kaempferol-3-O- α -L-(2",4"-di-Z-p-coumaroyl)-rhamnoside, **6** (acetone-*d*₆, 400 MHz).

Supplementary Figure S24. ¹H NMR spectrum of kaempferol-3-O- α -L-(2",4"-di-Z-p-coumaroyl)-rhamnoside, **compound 6** (acetone-*d*₆, 400 MHz) in the range 8.0 to 5.4 ppm.

Supplementary Table S2. MIC₁₀₀ values (μ g/mL) determined for the essential oils carvacrol and thymol purified from *E. foeminea* hexane extract.

Supplementary Table S3. MIC₁₀₀ values (μ g/mL) determined for the compounds kaempferol-3-O-(2",4"-di-E-p-coumaryl)- α -L-rhamno-piranoside, kaempferol-3-O-(2"-Z-p-coumaryl,4"-di-E-p-coumaryl)- α -L-rhamno-piranoside, kaempferol-3-O-(2"-E-p-coumaryl,4"-di-Z-p-coumaryl)- α -L-rhamno-piranoside and kaempferol-3-O-(2",4"-di-Z-p-coumaryl)- α -L-rhamno-piranoside purified from *E. foeminea* dichloromethane extract.

Supplementary Figure S25. Anti-biofilm activity of purified kaempferol-3-O-(2"-Z-p-coumaroyl,4"-E-p-coumaroyl)- α -L-rhamno-piranoside.

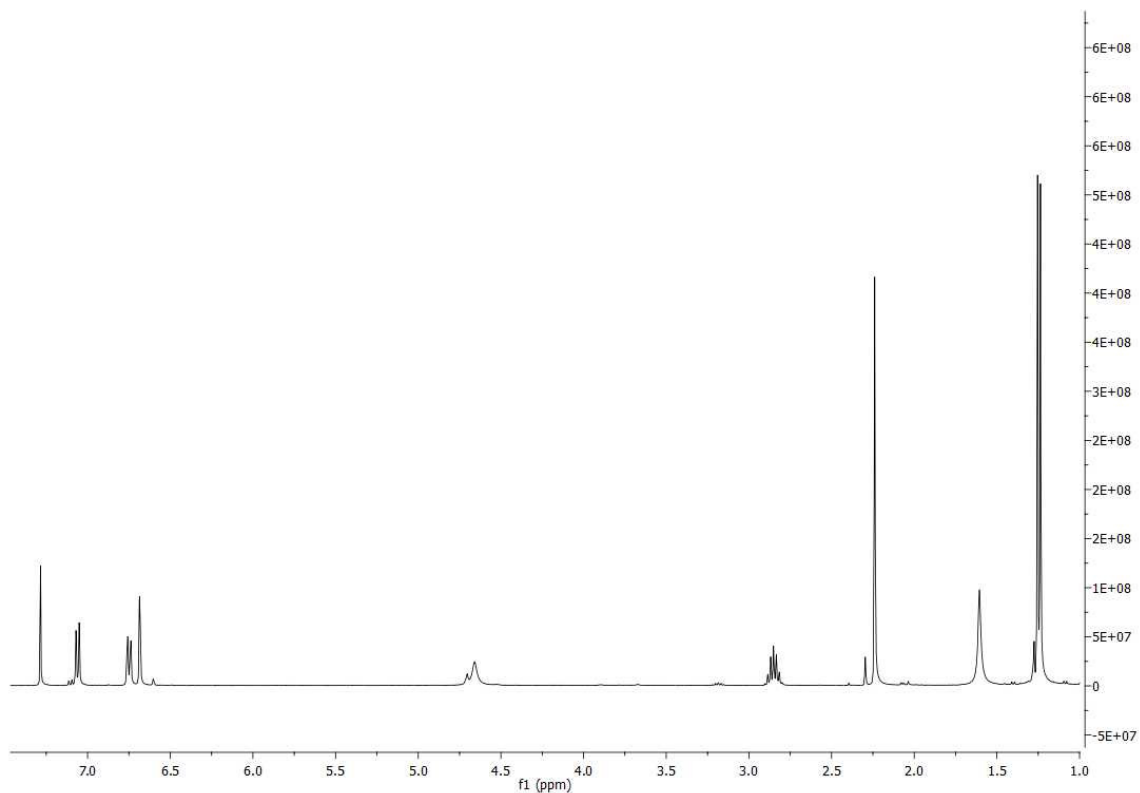
Supplementary Figure S26. Anti-biofilm activity of purified kaempferol-3-O-(2"-E-p-coumaroyl,4"-Z-p-coumaroyl)- α -L-rhamno-piranoside.

Supplementary Figure S27. Anti-biofilm activity of purified kaempferol-3-O-(2",4"-di-Z-p-coumaroyl)- α -L-rhamno-piranoside.

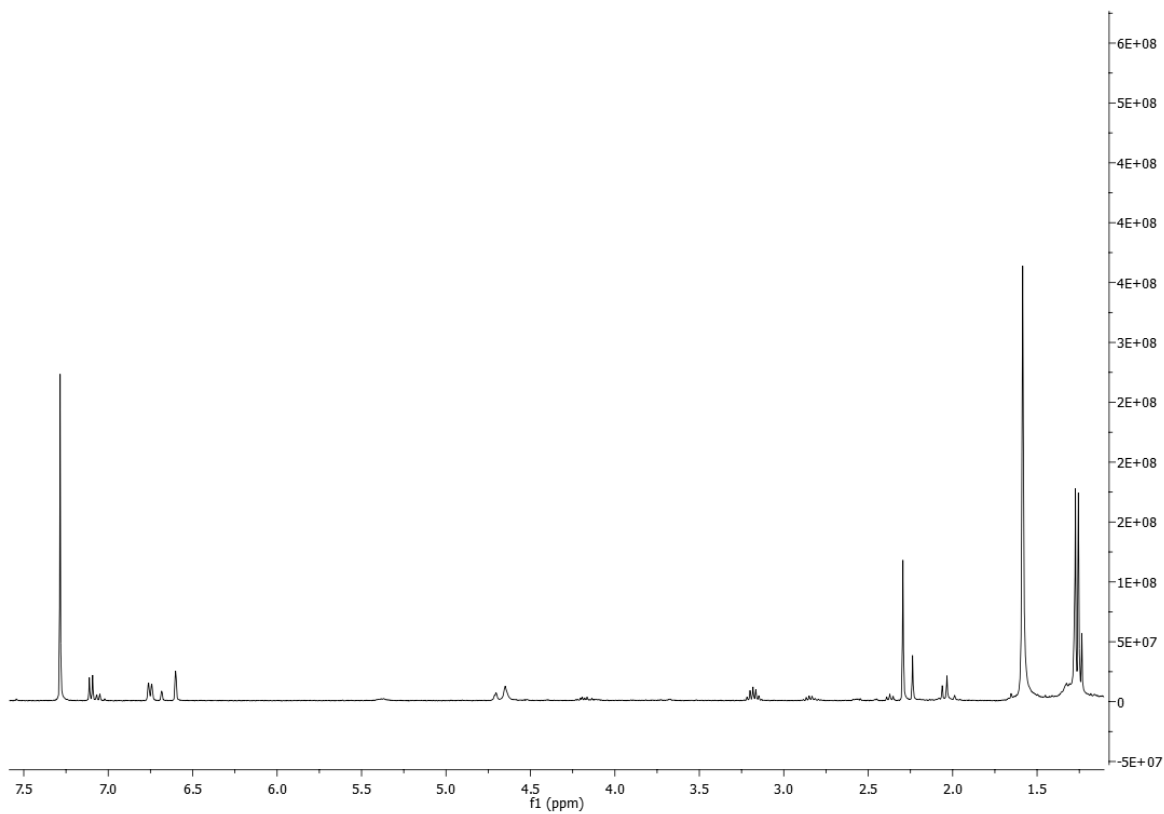
Supplementary Table S4. Details of the intermolecular interactions between the test ligand and *S. aureus* target enzymes tyrosyl tRNA synthetase and sortase A.

Supplementary Table S1. MIC₁₀₀ values (mg/mL) determined for fractions obtained upon extraction in hexane and two sequential steps of column chromatography.

MIC₁₀₀ (mg/mL) of fractions extracted in hexane							
Bacterial strains	1	2	3	4	5	6	7
<i>S. aureus</i> ATCC 29213	2.5	2.5	2.5	0.313	0.313	2.5	1.25
<i>E. coli</i> ATCC 25922	2.5	2.5	2.5	2.5	2.5	2.5	2.5
<i>S. typhimurium</i> ATCC 14028	2.5	2.5	2.5	2.5	2.5	2.5	2.5



Supplementary Figure S1. ¹H NMR spectrum of carvacrol, **compound 1** (CDCl₃, 400 MHz).



Supplementary Figure S2. ¹H NMR spectrum of thymol, **compound 2** (CDCl₃, 400 MHz).

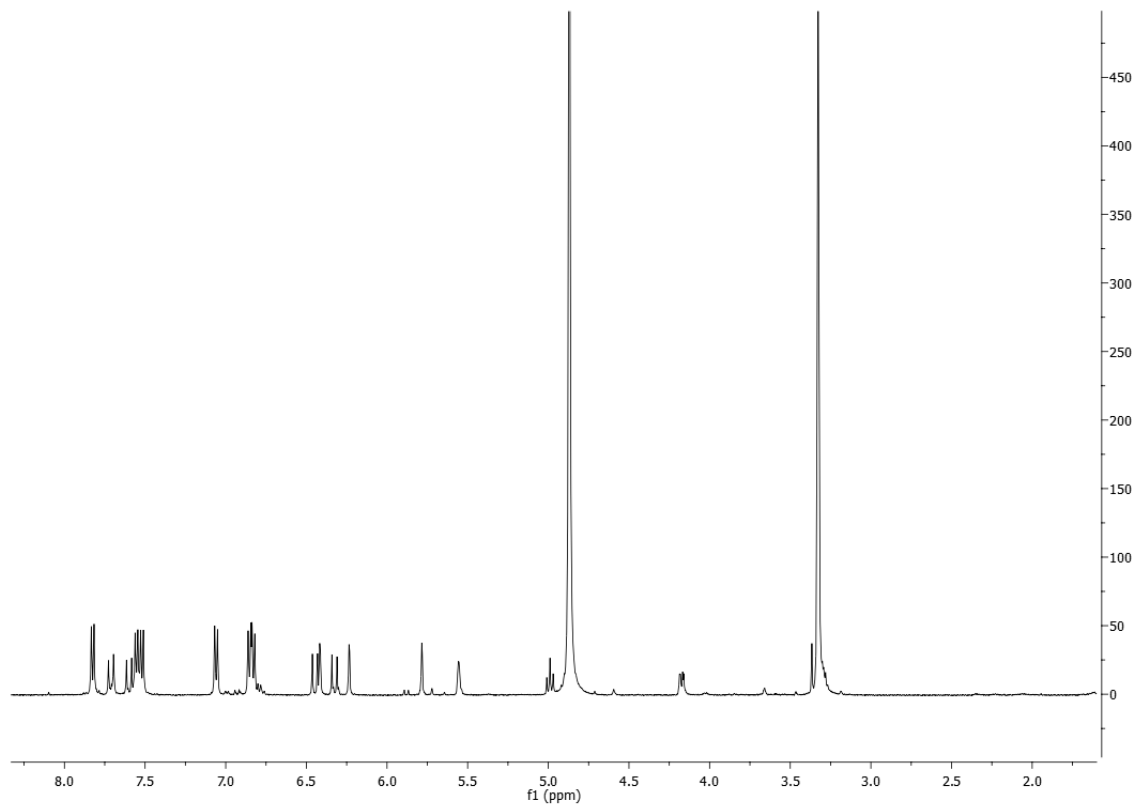


Figure S3. ^1H NMR spectrum of kaempferol-3- O - α -L-(2'',4'')-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (MeOD, 400 MHz).

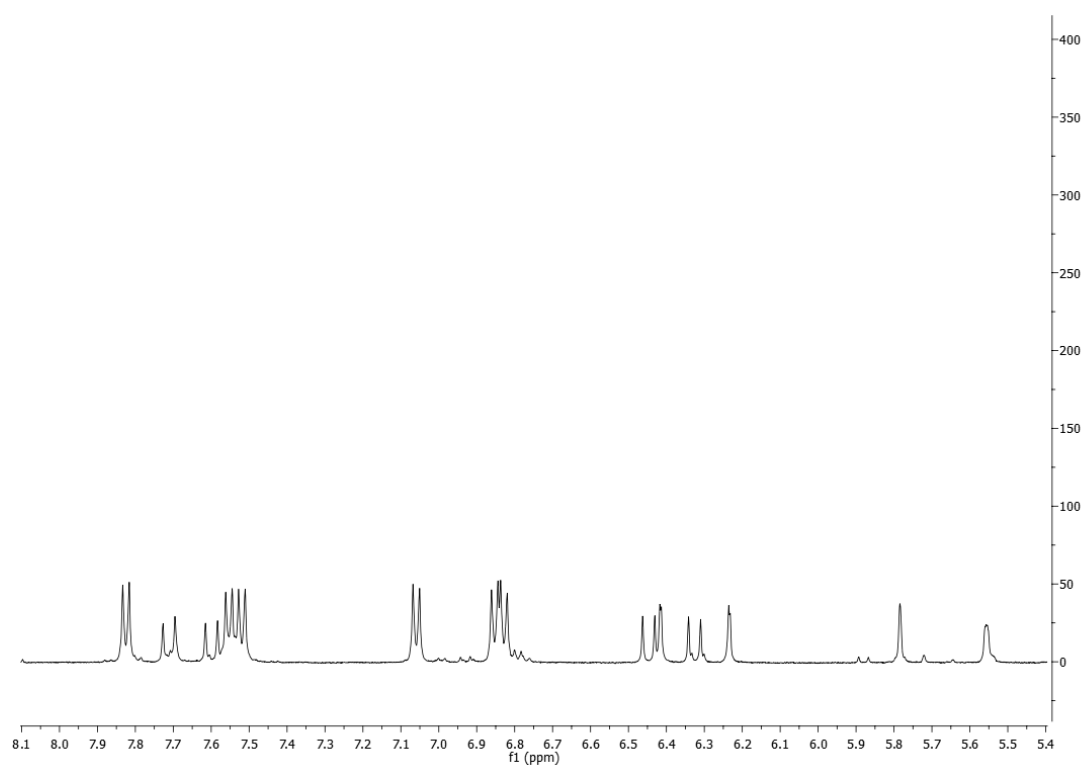


Figure S4. ^1H NMR spectrum of kaempferol-3- O - α -L-(2'',4'')-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (MeOD, 400 MHz) in the range 8.1 to 5.4 ppm.

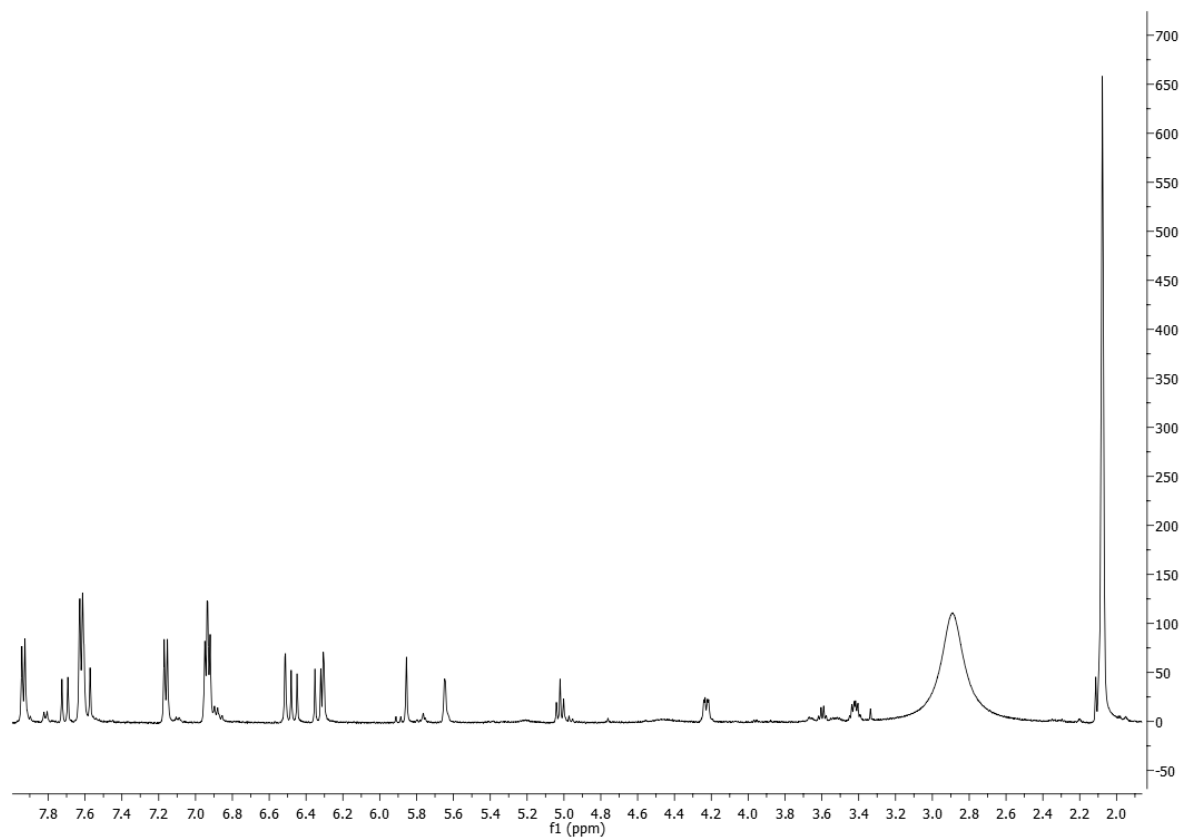


Figure S5. ^1H NMR spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone- d_6 , 400 MHz).

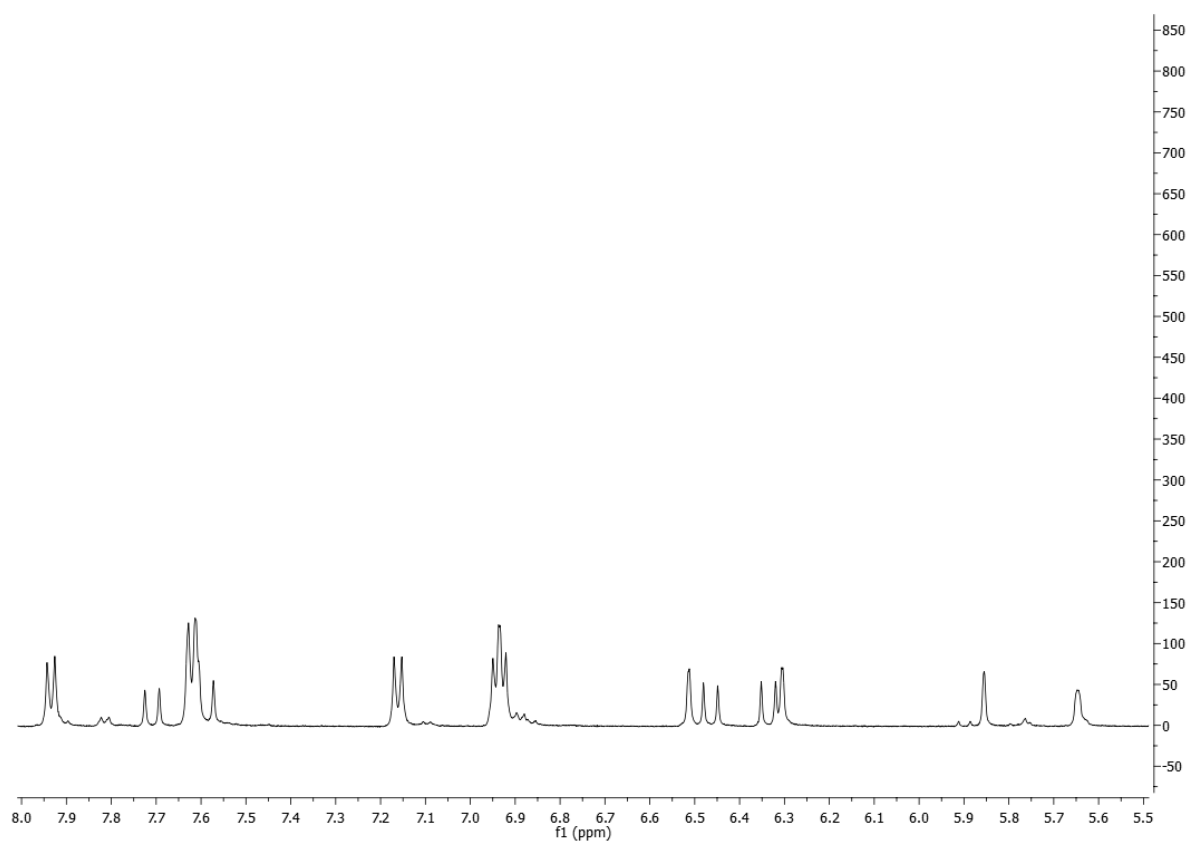


Figure S6. ^1H NMR spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone- d_6 , 400 MHz) in the range 8.0 to 5.5 ppm.

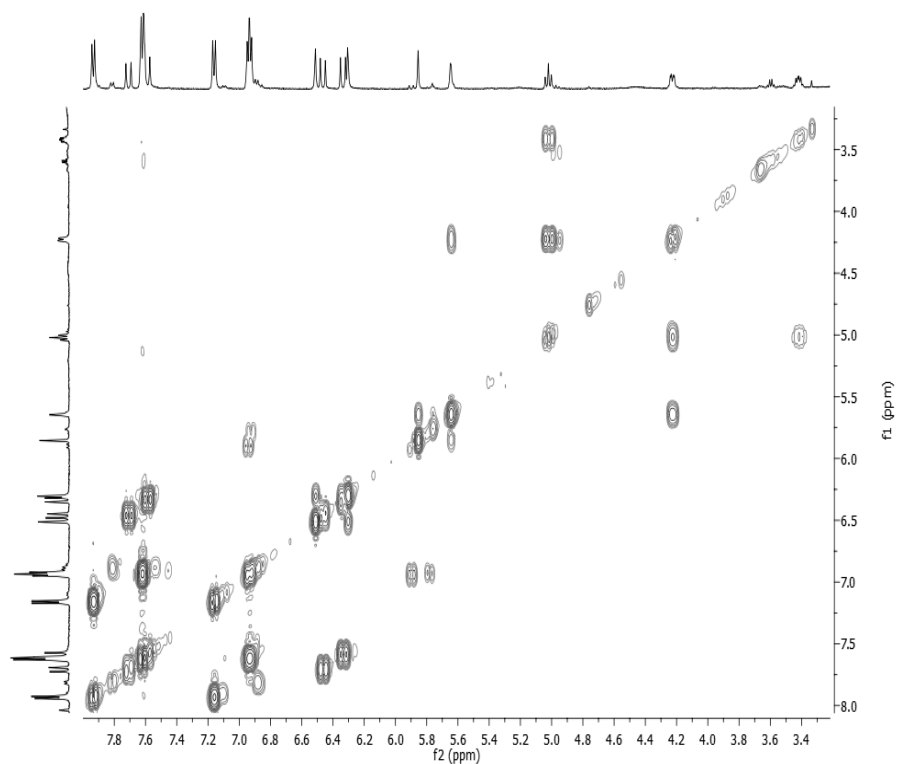


Figure S7. COSY spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

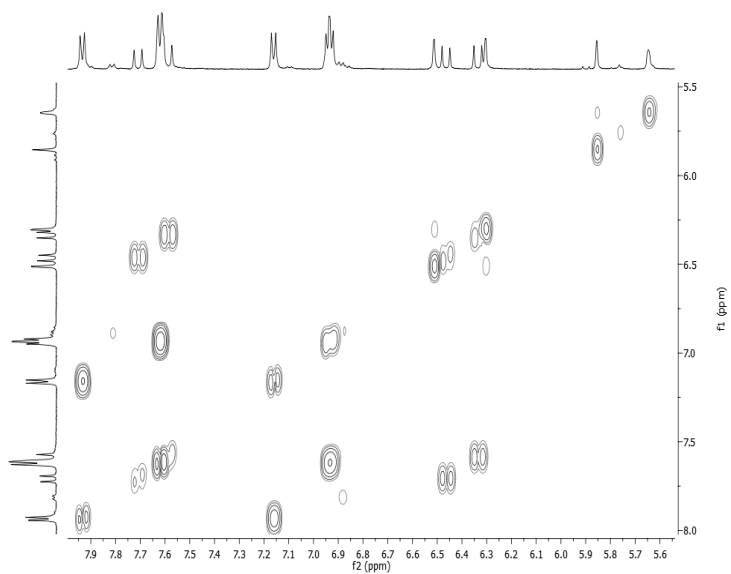


Figure S8. COSY spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz) in the range 8.0 to 5.6 ppm.

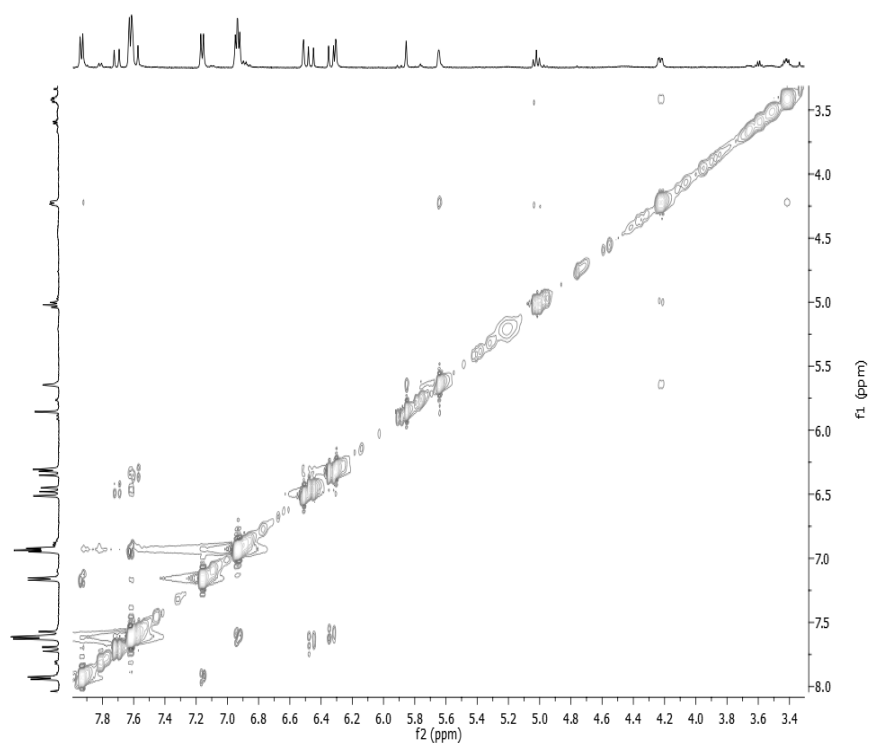


Figure S9. NOESY spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

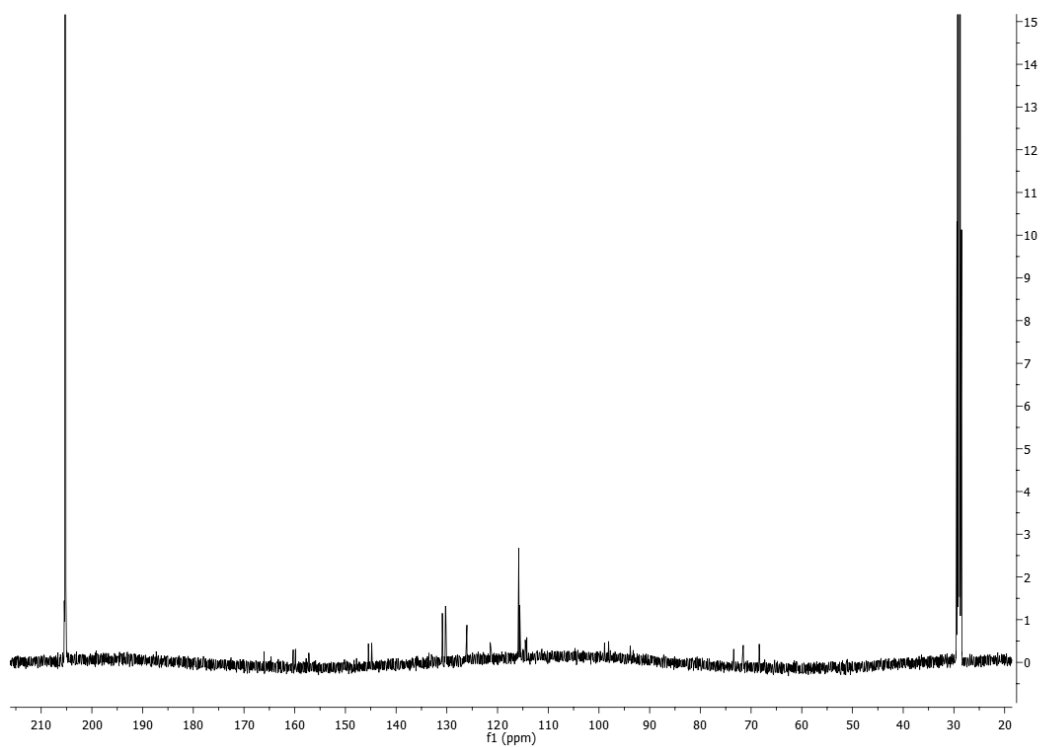


Figure S10. ¹³C NMR spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

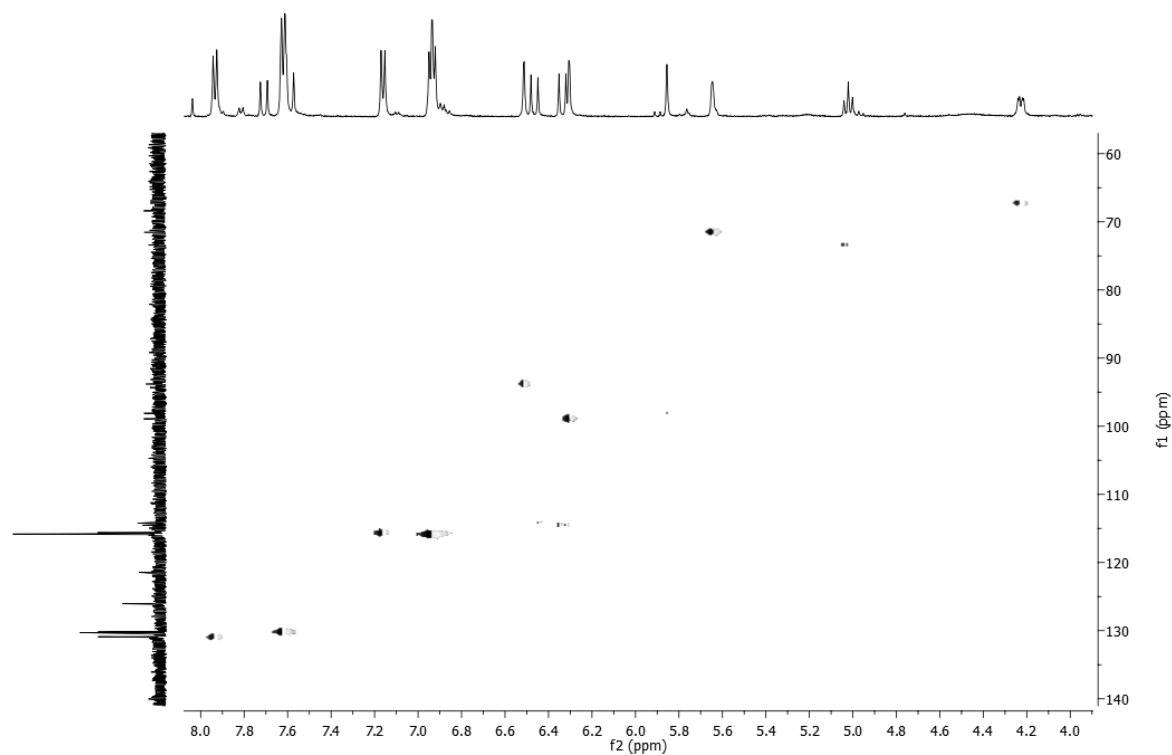


Figure S11. HSQC spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

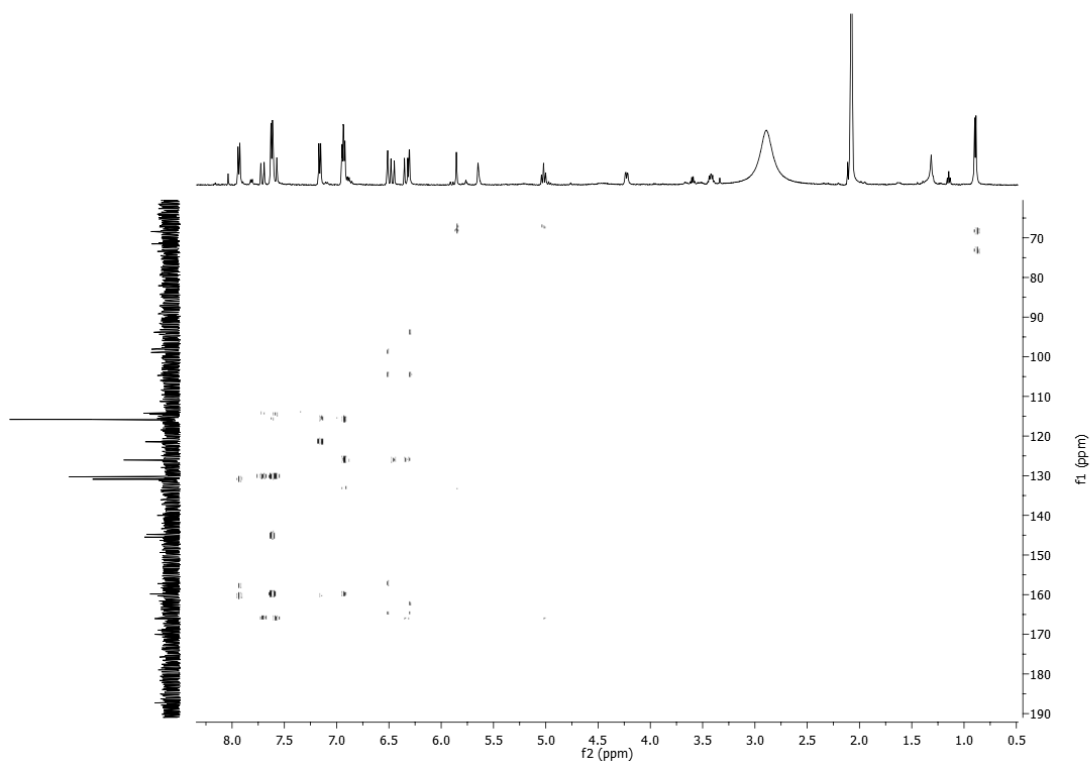


Figure S12. HMBC spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** (acetone-*d*₆, 400 MHz).

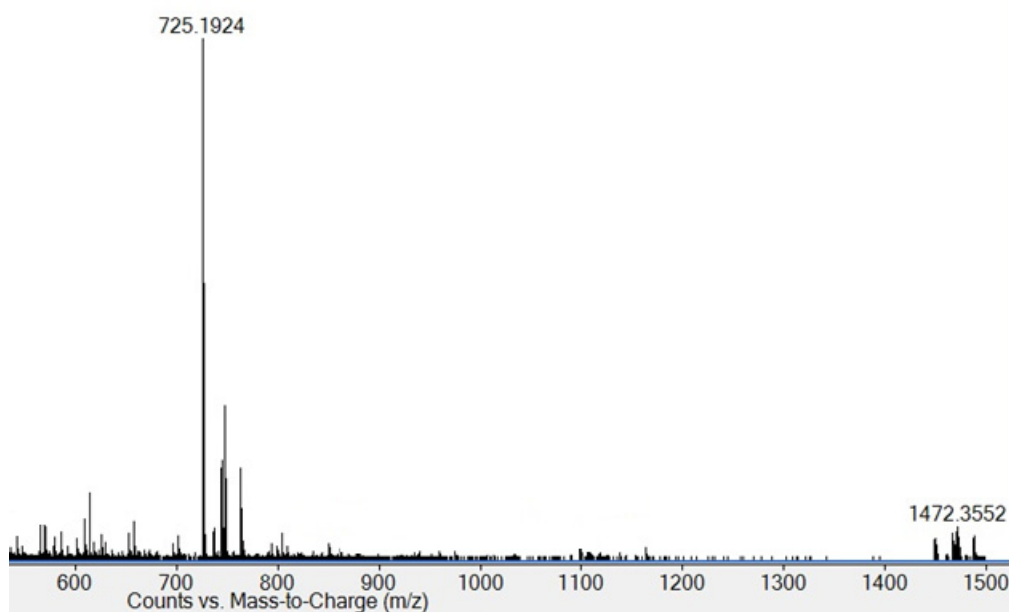


Figure S13. ESI MS spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*E*-*p*-coumaroyl)-rhamnoside, **compound 3** recorded in positive modality.

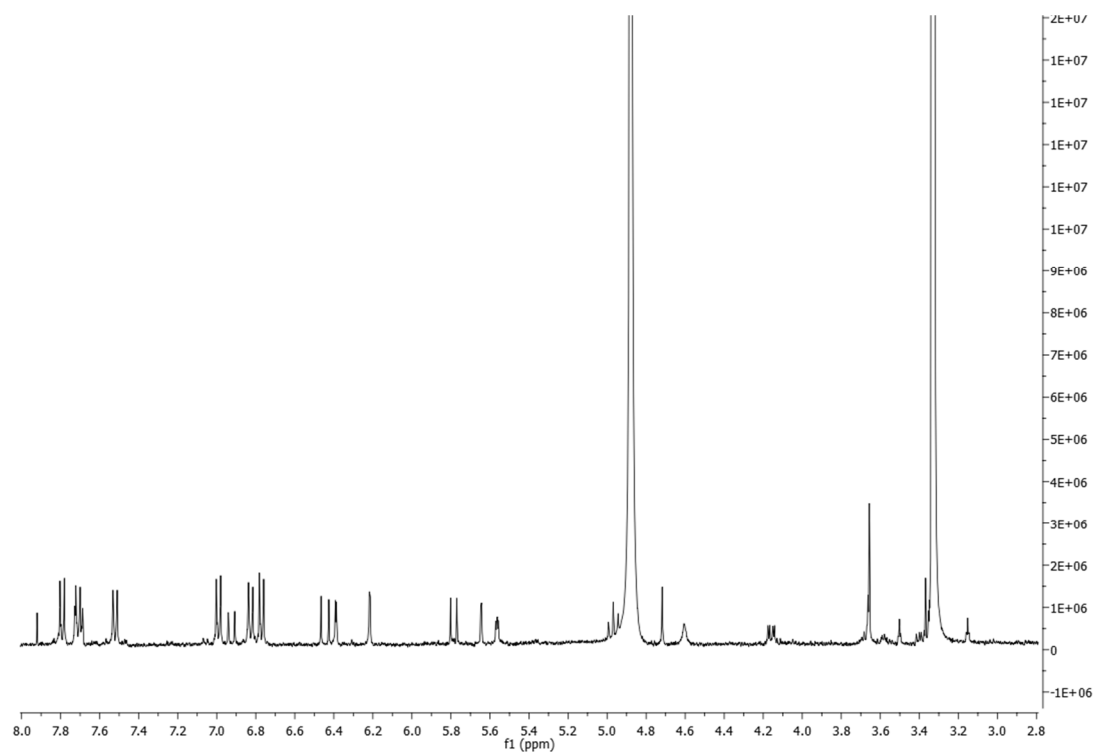


Figure S14. ¹H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*E*-*p*-coumaroyl,4''-*Z*-*p*-coumaroyl)-rhamnoside, **compound 4** (MeOD, 400 MHz).

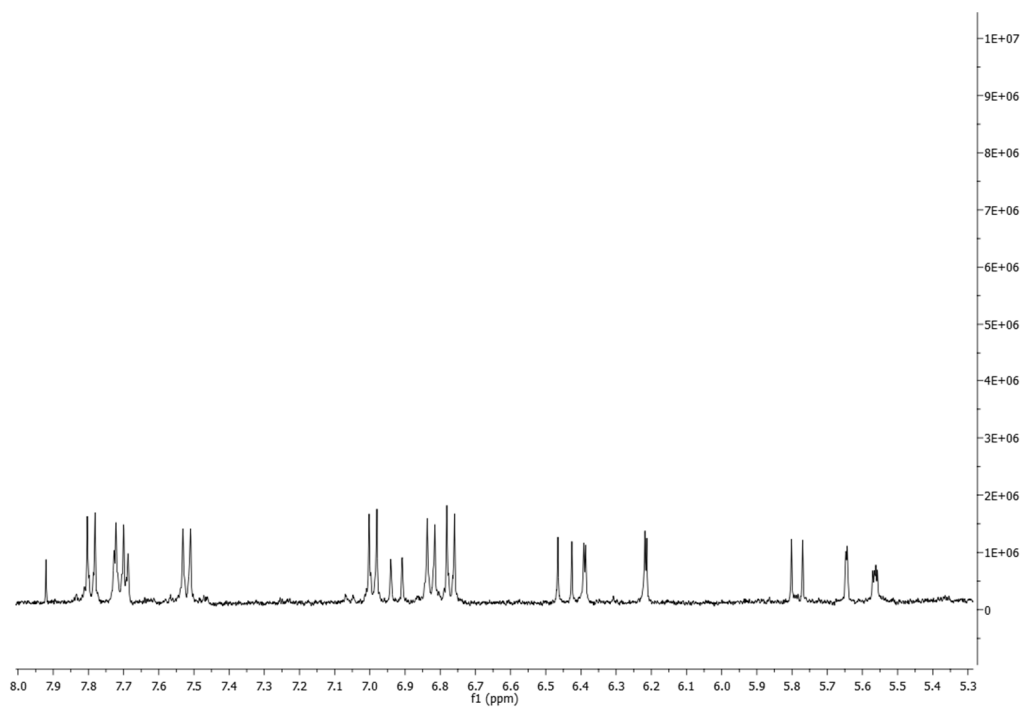


Figure S15. ^1H NMR spectrum of kaempferol-3- O - α -L-(2''- E - p -coumaroyl,4''- Z - p -coumaroyl)-rhamnoside, **compound 4** (MeOD, 400 MHz) in the range 8.0 to 5.3 ppm.

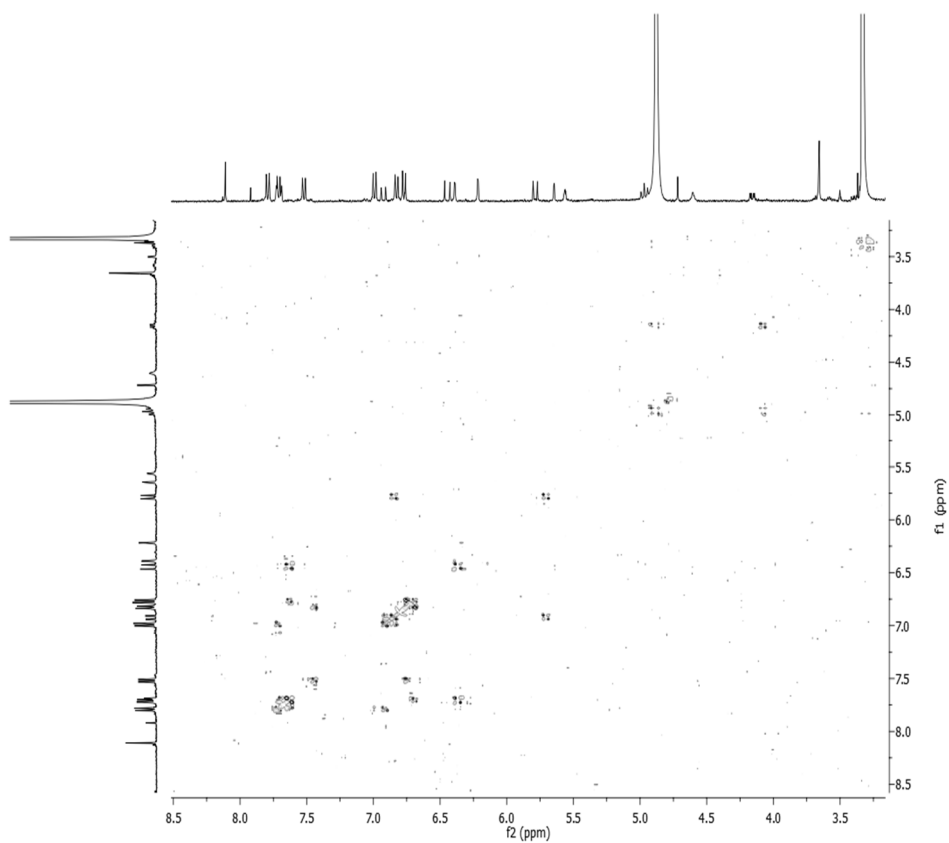


Figure S16. COSY spectrum of kaempferol-3- O - α -L-(2''- E - p -coumaroyl,4''- Z - p -coumaroyl)-rhamnoside, **compound 4** (acetone- d_6 , 400 MHz).

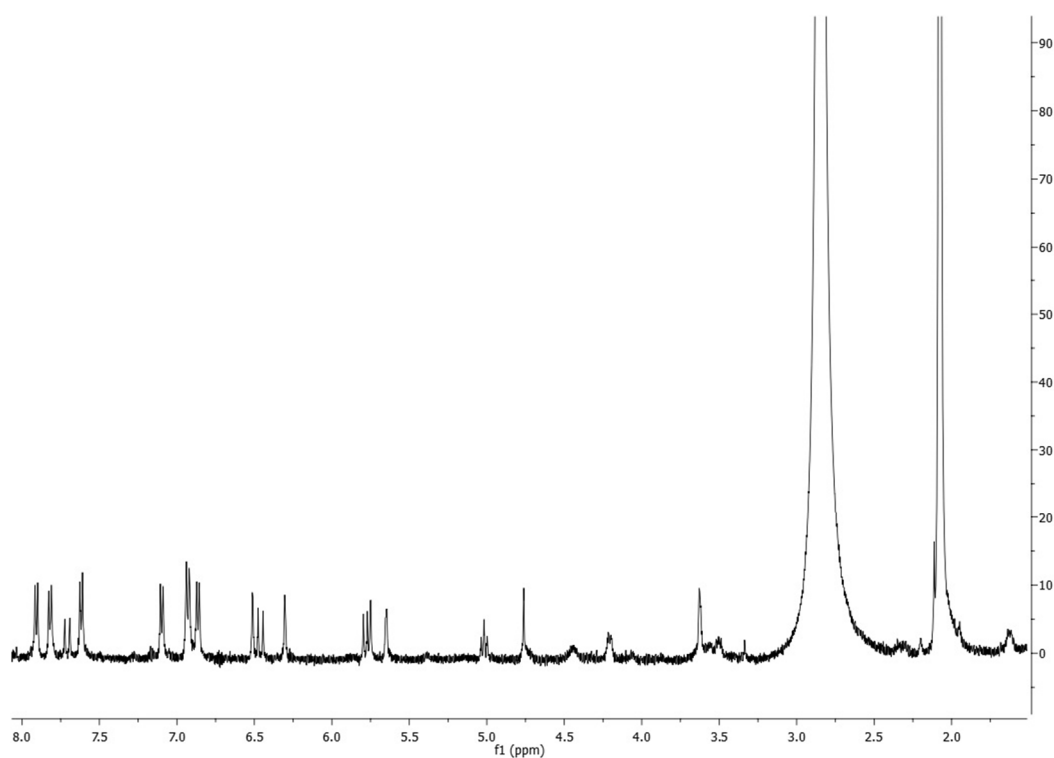


Figure S17. ^1H NMR spectrum of kaempferol-3- O - α -L-(2''- E - p -coumaroyl,4''- Z - p -coumaroyl)-rhamnoside, **compound 4** (acetone- d_6 , 400 MHz).

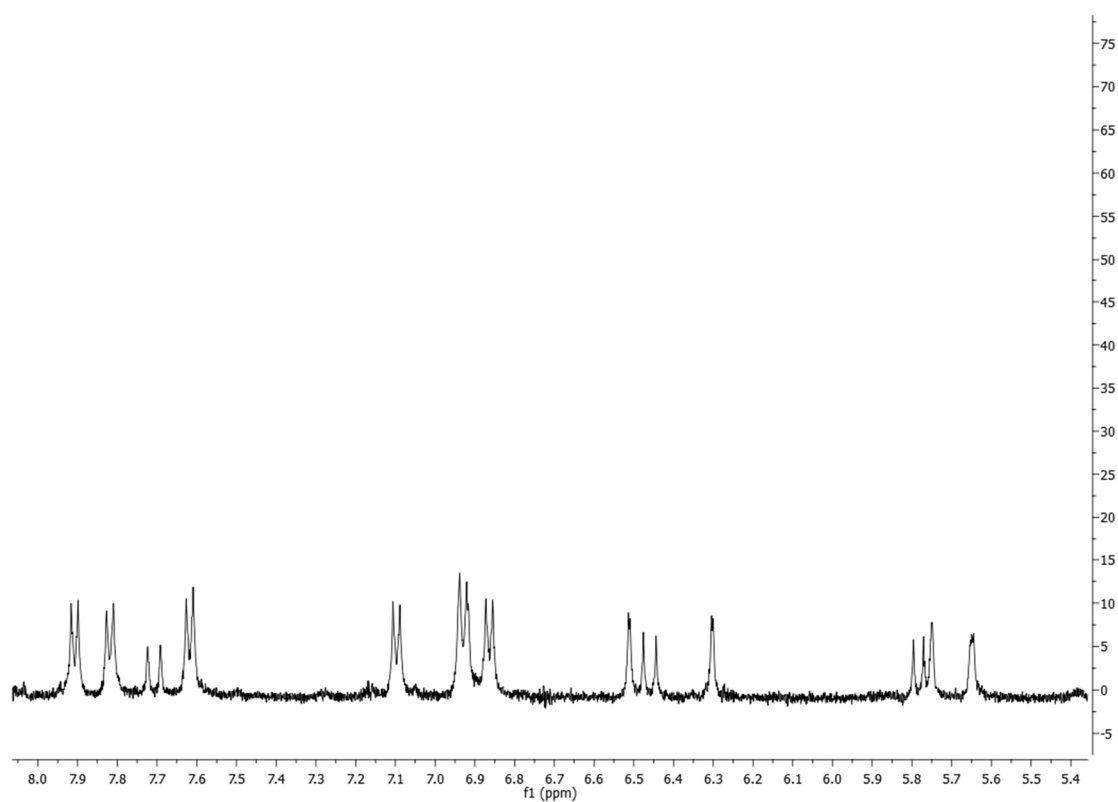


Figure S18. ^1H NMR spectrum of kaempferol-3- O - α -L-(2''- E - p -coumaroyl,4''- Z - p -coumaroyl)-rhamnoside, **compound 4** (acetone- d_6 , 400 MHz) in the range 8.0 to 5.4 ppm.

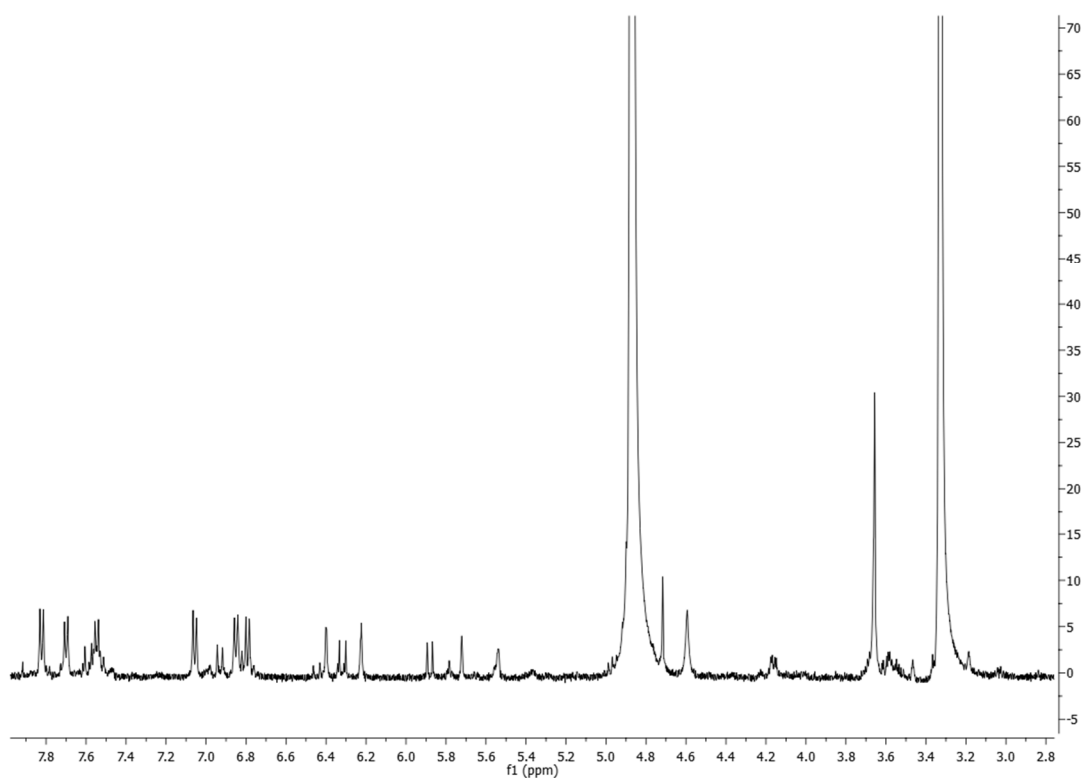


Figure S19. ^1H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*Z*-*p*-coumaroyl,4''-*E*-*p*-coumaroyl)-rhamnoside, **compound 5** (MeOD, 400 MHz).

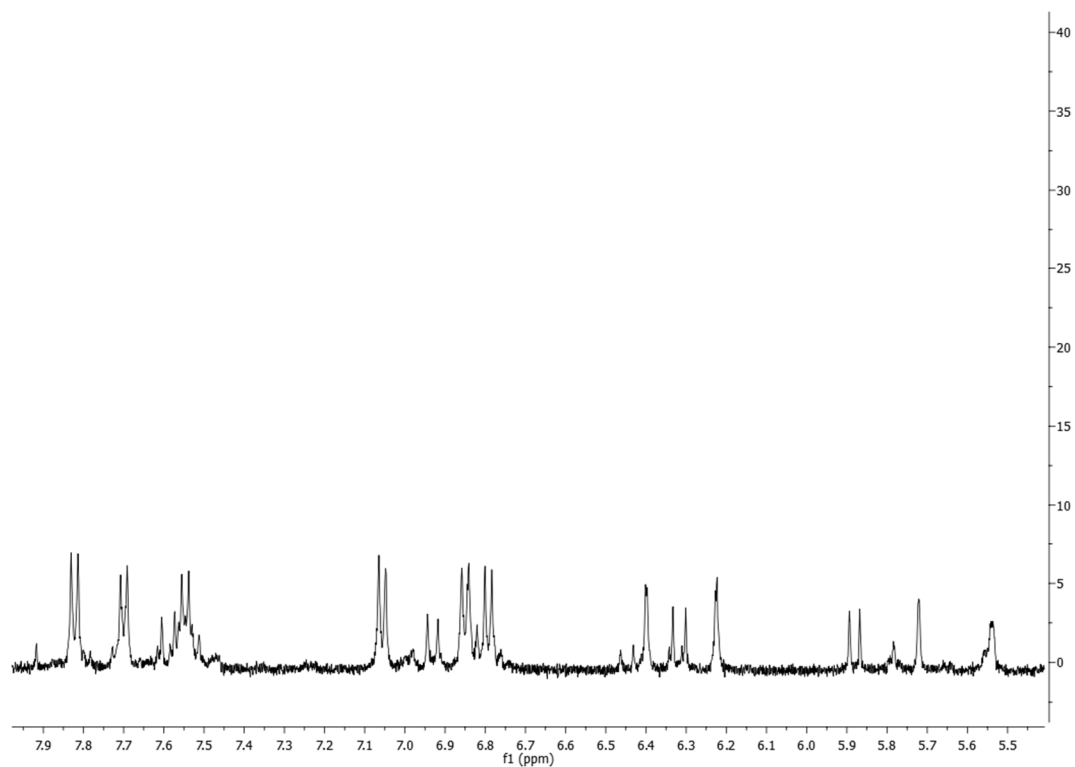


Figure S20. ^1H NMR spectrum of kaempferol-3-*O*- α -L-(2''-*Z*-*p*-coumaroyl,4''-*E*-*p*-coumaroyl)-rhamnoside, **compound 5** (MeOD, 400 MHz) in the range 8.0 to 5.4 ppm.

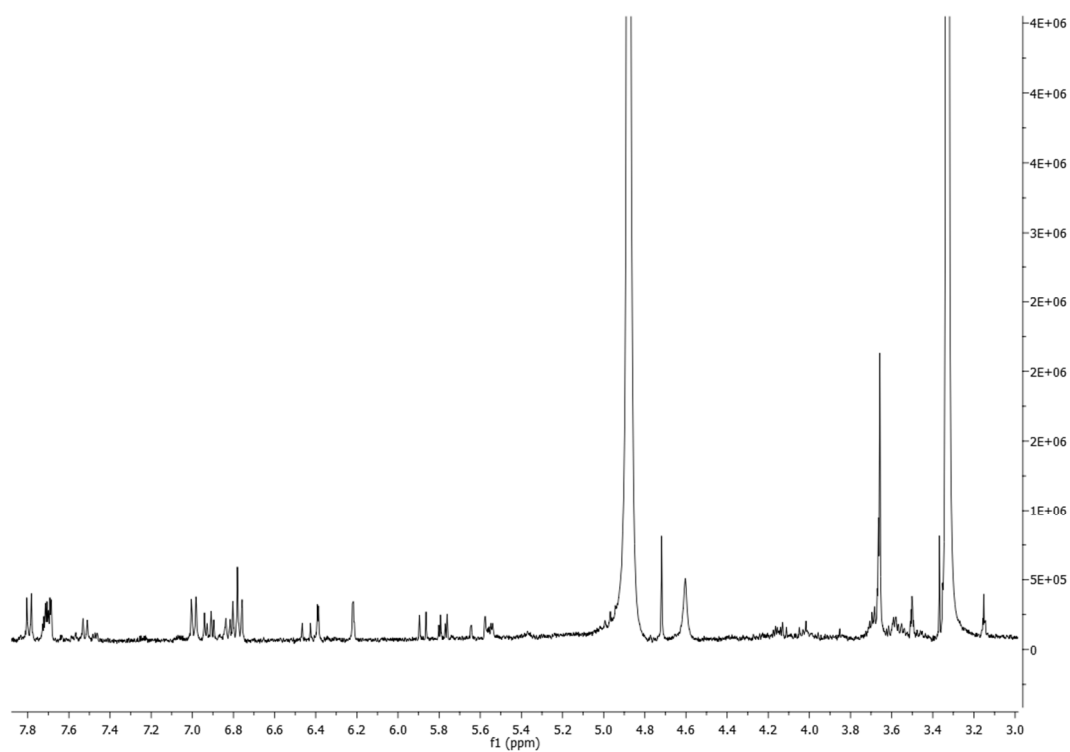


Figure S21. ^1H NMR spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*Z*-*p*-coumaroyl)-rhamnoside, **compound 6** (MeOD, 400 MHz).

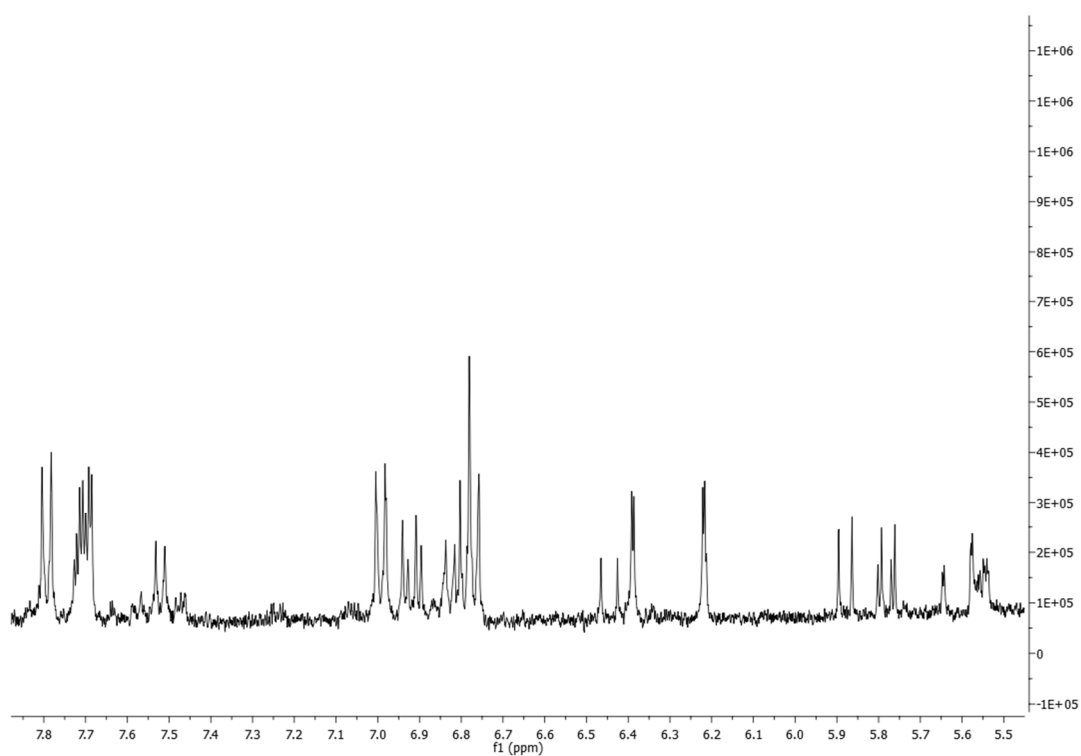


Figure S22. ^1H NMR spectrum of kaempferol-3-*O*- α -L-(2'',4''-di-*Z*-*p*-coumaroyl)-rhamnoside, **compound 6** (MeOD, 400 MHz) in the range 7.9 to 5.5 ppm.

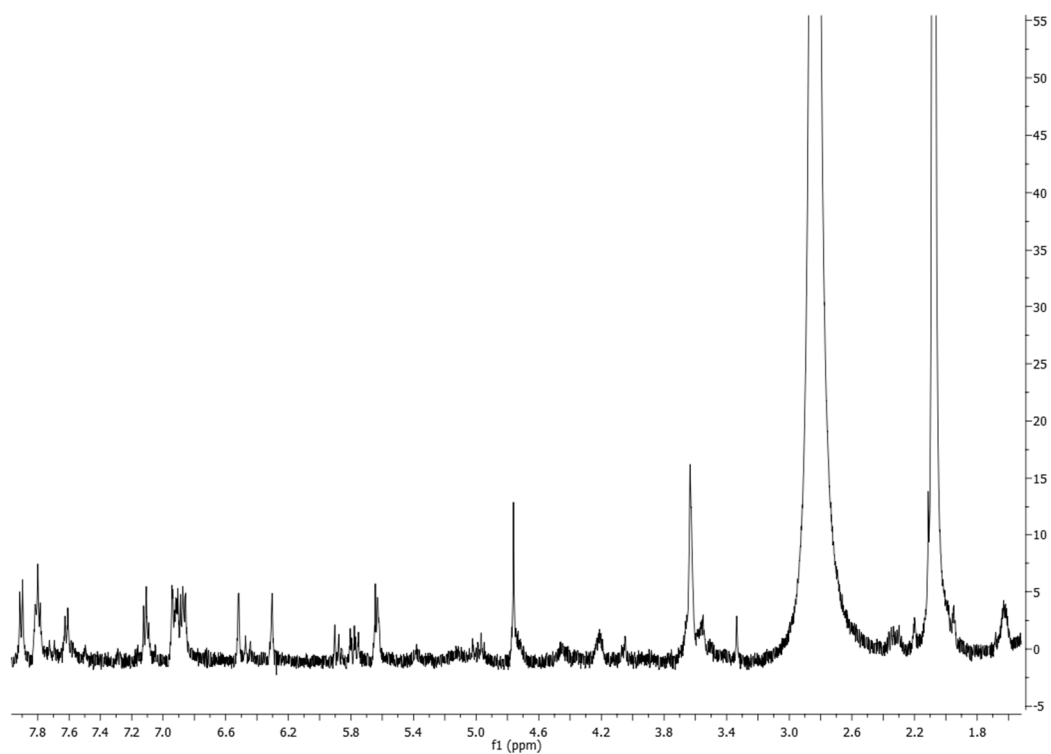


Figure S23. ^1H NMR spectrum of kaempferol-3- O - α -L-(2'',4''-di-Z- p -coumaroyl)-rhamnoside, **compound 6** (acetone- d_6 , 400 MHz).

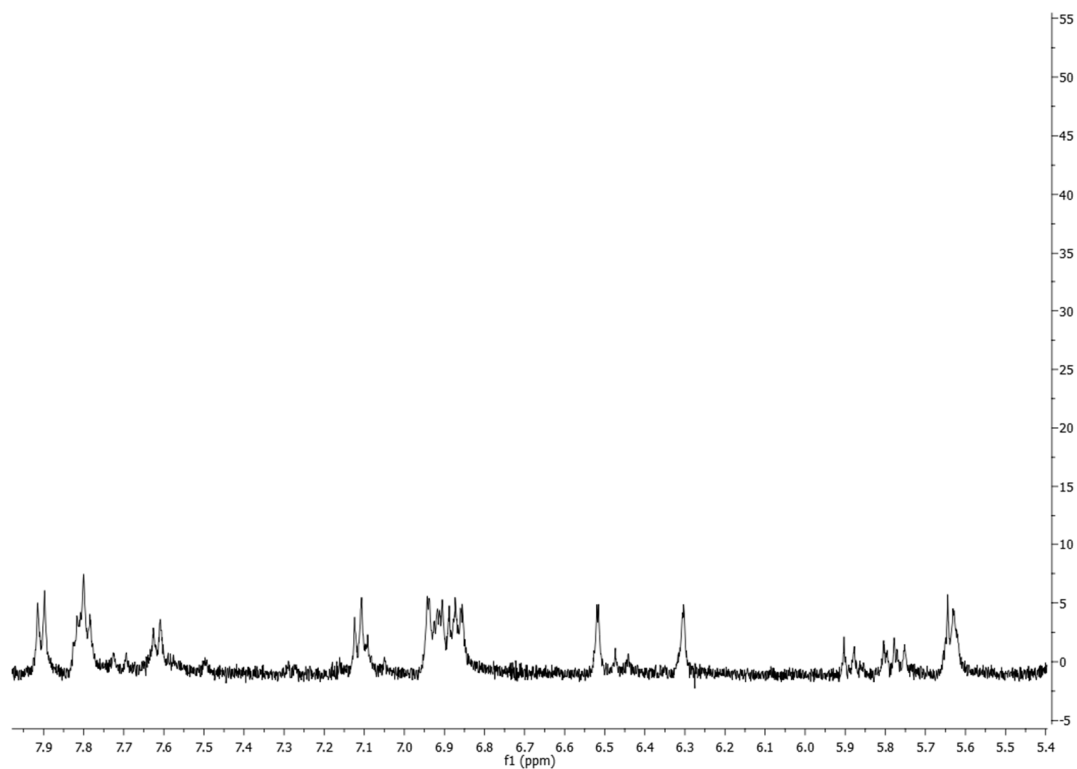


Figure S24. ^1H NMR spectrum of kaempferol-3- O - α -L-(2'',4''-di-Z- p -coumaroyl)-rhamnoside, **compound 6** (acetone- d_6 , 400 MHz) in the range 8.0 to 5.4 ppm.

Supplementary Table S2. MIC₁₀₀ values (µg/mL) determined for the essential oils carvacrol and thymol purified from *E. foeminea* hexane extract.

	MIC ₁₀₀ (µg/mL)	
Gram-positive strains	Carvacrol	Thymol
<i>S. aureus</i> ATCC 29213	100	600
<i>S. aureus</i> MRSA WKZ-1	50	2,400
<i>E. faecalis</i> ATCC 29212	100	1,200
Gram-negative Strains		
<i>E. coli</i> ATCC 25922	200	600
<i>S. typhimurium</i> ATCC 14028	100	300
<i>A. baumannii</i> ATCC 17878	100	1,200

Supplementary Table S3. MIC₁₀₀ values (µg/mL) determined for the compounds kaempferol-3-O-(2",4"-di-E-p-coumaryl)-α-L-rhamno-piranoside, kaempferol-3-O-(2"-Z-p-coumaryl,4"-di-E-p-coumaryl)-α-L-rhamno-piranoside, kaempferol-3-O-(2"-E- p-coumaryl,4"-di-Z-p-coumaryl)-α-L-rhamno-piranoside and kaempferol-3-O-(2",4"-di-Z-p-coumaryl)-α-L-rhamno-piranoside purified from *E. foeminea* dichloromethane extract.

	MIC ₁₀₀ (µg/mL)	
	<i>S. aureus</i> MRSA WKZ-1	<i>A. baumannii</i> ATCC 17878
kaempferol-3-O-(2",4"-di-E-p-coumaryl)-α-L-rhamno-piranoside	0.49	1,000
kaempferol-3-O-(2"-Z-p-coumaryl,4"-di-E-p-coumaryl)-α-L-rhamno-piranoside	>1,000 MIC ₉₅ = 1,000	1,000
kaempferol-3-O-(2"-E- p-coumaryl,4"-di-Z-p-coumaryl)-α-L-rhamno-piranoside	>1,000 MIC ₉₈ = 1,000	1,000
kaempferol-3-O-(2",4"-di-Z-p-coumaryl)-α-L-rhamno-piranoside	>1,000 MIC ₉₈ = 1,000	1,000

S. aureus MRSA WKZ-2

kaempferol-3-O-(2''-Z-p-coumaroyl,4''-E-p-coumaroyl)- α -L-rhamno-piranoside

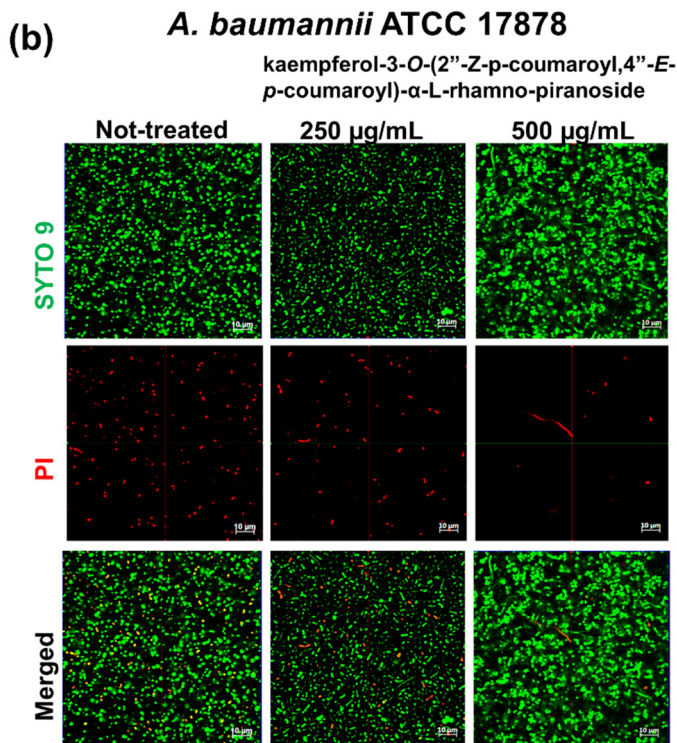
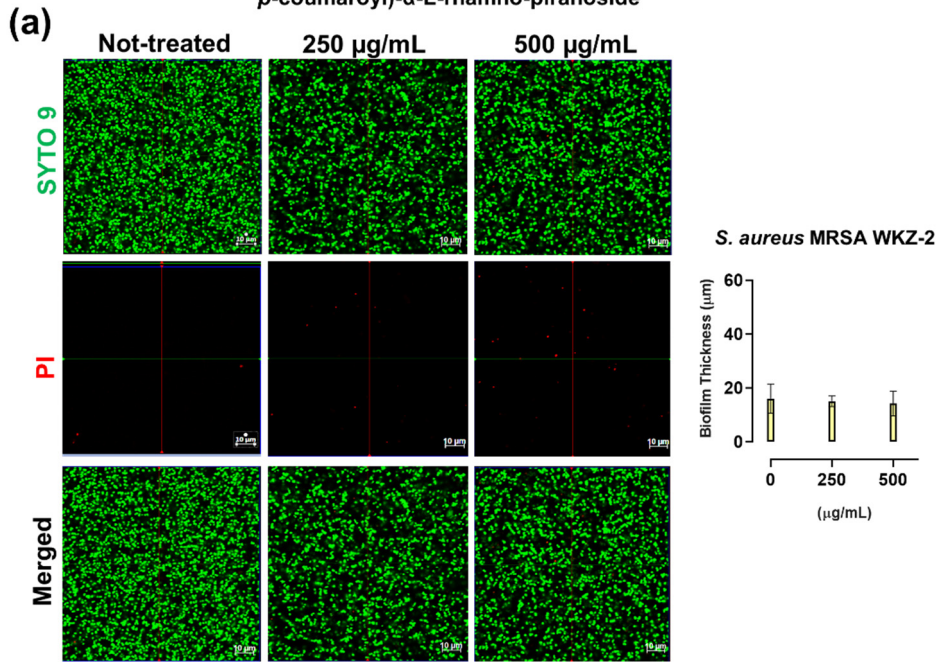


Figure S25. Anti-biofilm activity of purified kaempferol-3-O-(2''-Z-p-coumaroyl,4''-E-p-coumaroyl)- α -L-rhamno-piranoside. Effects of kaempferol-3-O-(2''-Z-p-coumaroyl,4''-E-p-coumaroyl)- α -L-rhamno-piranoside have been evaluated on biofilm formation in the case of *S. aureus* MRSA WKZ-2 (a), and *A. baumannii* ATCC 17878 (b). Biofilm cells were stained with LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR) containing a 1:1 mol/mol ratio of SYTO-9 (green fluorescence, all cells) and propidium iodide (PI; red fluorescence, dead cells). The biofilm images were obtained by confocal z-stack using Zen Lite 2.3 software. All the images were taken under identical conditions. Significant differences were indicated as (* P < 0.05) or (** P < 0.001) for treated versus control samples. Each experiment was carried out in triplicate.

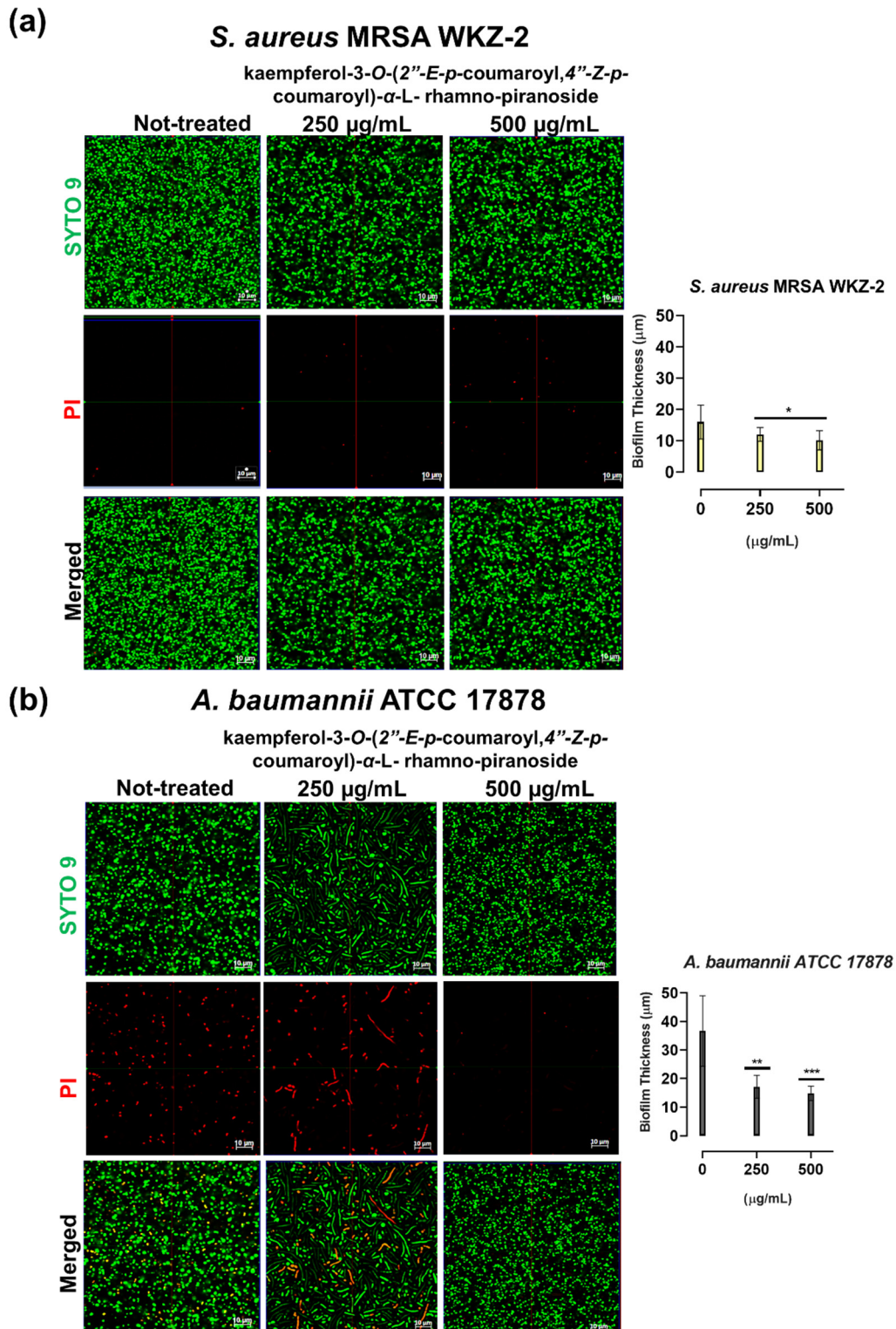


Figure S26. Anti-biofilm activity of purified kaempferol-3-O-(2''-E-p-coumaroyl,4''-Z-p-coumaroyl)- α -L-rhamno-piranoside. Effects of kaempferol-3-O-(2''-E-p-coumaroyl,4''-Z-p-coumaroyl)- α -L-rhamno-piranoside have been evaluated on biofilm formation in the case of *S. aureus* MRSA WKZ-2 **(a)**, and *A. baumannii* ATCC 17878 **(b)** by CLSM. Biofilm cells were stained with LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR) containing a 1:1 mol/mol ratio of SYTO-9 (green fluorescence, all cells) and propidium iodide (PI; red fluorescence, dead cells). The biofilm images were obtained by confocal z-stack using Zen Lite 2.3 software. All the images were taken under identical conditions. Significant differences were indicated as (* P < 0.05), (** P < 0.001) or (***) P < 0.0001) for treated *versus* control samples. Each experiment was carried out in triplicate.

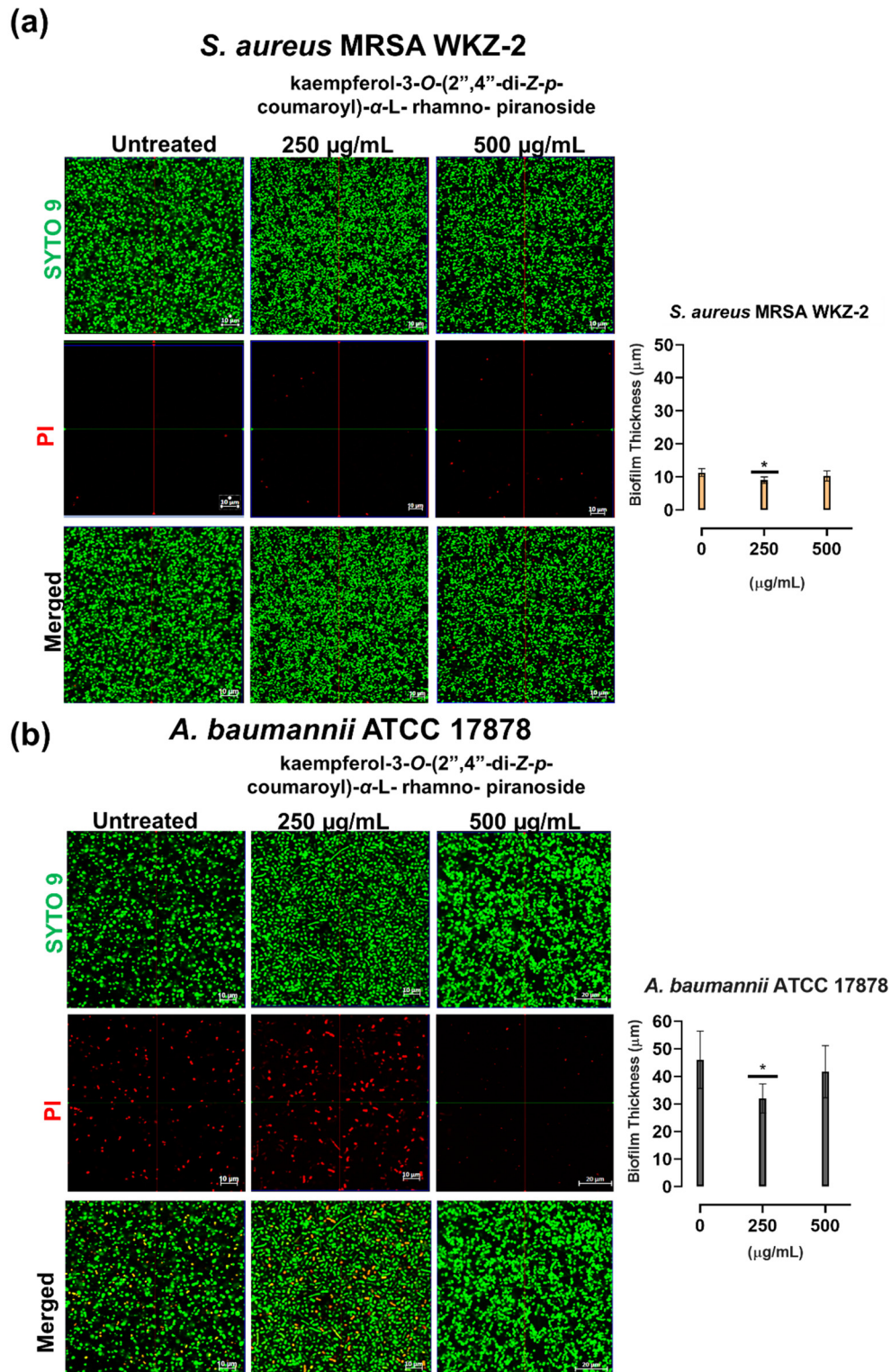


Figure S27. Anti-biofilm activity of purified kaempferol-3-O-(2'',4''-di-Z-p-coumaroyl)- α -L-rhamno-piranoside. Effects of kaempferol-3-O-(2'',4''-di-Z-p-coumaroyl)- α -L-rhamno-piranoside have been evaluated on biofilm formation in the case of *S. aureus* MRSA WKZ-2 (a), and *A. baumannii* ATCC 17878 (b) by CLSM. Biofilm cells were stained with LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR) containing a 1:1 mol/mol ratio of SYTO-9 (green fluorescence, all cells) and propidium iodide (PI; red fluorescence, dead cells). The biofilm images were obtained by confocal z-stack using Zen Lite 2.3 software. All the images were taken under identical conditions. Significant differences were indicated as (* $P < 0.05$) for treated versus control samples. Each experiment was carried out in triplicate.

Supplementary Table S4. Details of the intermolecular interactions between the test ligand and *S. aureus* target enzymes tyrosyl tRNA synthetase and sortase A.

Interaction between antimicrobial compound and tyrosyl tRNA synthetase			Interaction between antimicrobial compound and sortase A		
Interacting amino acid	Type of interaction	Bond length (Å)	Interacting amino acid	Type of interaction	Bond length (Å)
His 50	Carbon-hydrogen bond	3.57	Val 168	Hydrogen bond	2.21
His 47	Pi-Sigma	3.92	Arg 197	Hydrogen bond	2.35
His 47	Pi-Pi T shaped	5.02	Ala 92	Hydrogen bond	2.66
His 47	Pi-Pi T shaped	4.96	Val 168	Hydrogen bond	2.70
Gly 38	Amide Pi Stacked	3.52	Thr 180	Hydrogen bond	2.49
Ala 43	Pi -Alkyl	5.00	Val 193	Pi-Sigma	3.79
Leu 223	Pi -Alkyl	5.37	Ala 92	Pi-alkyl	5.11
			Ala 104	Pi-Alkyl	5.07
			Val 168	Pi-Alkyl	4.65
			Ile 199	Pi-Alkyl	4.54