SUPPLEMENTARY MATERIAL

Increased synthesis of a new oleanane-type saponin in hairy roots of marigold (*Calendula officinalis*) after treatment with jasmonic acid

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Abstract

Native plant of marigold (*Calendula officinalis* L.) synthesizes oleanolic acid saponins classified as glucosides or glucuronides according to the first residue in sugar chain bound to C-3 hydroxyl group. Hairy root culture, obtained by transformation with *Agrobacterium rhizogenes* strain 15834, exhibit a potent ability of synthesis of oleanolic acid glycosides. The HPLC profile of saponin fraction obtained from *C. officinalis* hairy roots treated with plant stress hormone, jasmonic acid, showed the 10-times increase of the content of one particular compound, determined by NMR and MALDI TOF as a new bisdesmoside saponin, 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-galactopyranosyl-oleanolic acid. Such a diglycoside does not occur in native *C. officinalis* plant. It is a glucuronide, whereas in the native plant glucuronides are mainly accumulated in flowers, while glucosides are the most abundant saponins in roots. Thus, our results revealed that the pathways of saponin biosynthesis, particularly reactions of glycosylation, are altered in *C. officinalis* hairy root culture.

Keywords: Triterpenoid saponin; *Calendula officinalis*; hairy roots; NMR; jasmonic acid

Experimental:

Hairy root culture elicitation

Hairy roots of marigold (*Calendula officinalis* L.) *cv.* Persimmon Beauty were obtained previously by transformation with *Agrobacterium rhizogenes* strain ATCC 15834 (Długosz et al. 2013). Hairy root culture line CC16 (derived from cotyledon explant) was maintained in liquid Murashige and Skoog medium with half strength macronutrients concentrations (½ MS) on rotatory shaker (120 rpm) at 24°C in darkness. Nutrients for plant tissue culture were purchased from Chempur and Sigma Aldrich. Culture medium was sterilized by autoclaving at 121°C for 15 minutes. Roots were subcultured every 4-7 weeks (Figure S1).

Jasmonic acid (JA, Sigma J2500) stock solution was prepared by dissolving in 70% ethanol and the appropriate dilutions were sterilized using a 0,22 μm syringe filter (Millipore) prior to application to hairy roots. Explants obtained from existing cultures were pre-cultured for 17 days and then added to fresh medium.After 5 days, JA was added to the medium to final concentration of 100 μM. After elicitor application the roots were incubated for 10 days and harvested by filtration from the medium by Büchner funnel with the use of the vacuum pump.

Extraction of hairy roots

Air-dried hairy roots (4.57 g of jasmonate-treated roots obtained from five combined repetitions, and 4,56 g of five repetitions of control roots) were powdered, every portion was mixed with 50 mL ethanol and left at room temperature during 24h for extraction of saponins. The extracts were filtered under vacuum on fritted glass funnels.

Purification of the elicitated saponin

The filtrates were added to 50 mL of water and fractionated on a reversed phase silica gel column (500mm x 10mm, Polygoprep 60-50 C18, Macherey-Nagel GmbH & Co.). The column was first rinsed with pure water to eliminate the polar compounds (salts, sugars, amino acids). Saponin fraction was eluted with a linear gradient of ethanol/water solution (from 0% to 100% ethanol) distributed by a low pressure of liquid pump (Duramat, Merck). Organic solvents used for extraction: acetonitrile HPLC-S grade and ethanol absolute were purchased from Biosolve Chimie SARL and Sigma-Aldrich, respectively. Ultra high quality water was obtained with Elga system of purification. Fractions of 10 mL were collected. The presence of saponins was determined by TLC on silica gel plates in solvent system $CHCl_3$/MeOH/H_2O (4/1/5, v/v/v) and subsequent visualization with Liebermann-Burchard reagent. Fractions containing saponins were analyzed by HPLC.

HPLC analysis of saponin extracts

Analytical HPLC was performed on a Shimadzu chromatographic system (Kyoto, Japan) at 210 nm. HPLC saponins profiles were carried out on a reversed phase column Symmetry Shield, 250 mm x 4.7 mm, 5µm (Waters, Milford, MA). All data were acquired and processed with a Shimadzu CLASS-VP (Version 4.3) HPLC spectrophotometer at two simultaneous wavelengths 210nm and 254nm. Saponins were detected exclusively at 210nm, and 254nm wavelength was used for detection of eventual contaminations like phenolic compounds. The HPLC binary eluting system consisted in two HPLC pumps Shimadzu AT delivering a linear gradient of two solvents in withdrawn ionisation method i.e. with 0.1% *ortho*-phosphoric acidic solutions (pH 3): solvent A (sA): acetonitrile (0.1% *ortho*-phosphoric acid) and solvent B (sB): 0.1% *ortho*-phosphoric acid in water. HPLC gradient program started with 25% sA/75% sB followed by a linear gradient to 84% sA/16% sB for 30 min. The flow rate was 2 mL/min. The oven temperature was set at 40°C. The obtained chromatograms are presented in Figure S2.

NMR experiments

NMR experiments were performed on a Bruker Avance III spectrometer operating at 9.4 Tesla (400Mhz and 100.6 MHz for ^{1}H and ^{13}C, respectively), using a Bruker 5 mm BBFO probe. Pulse widths were 14.1 and 10.5 µs for ^{1}H and ^{13}C, respectively. Samples were dissolved in a volume of 500 µL of ethanol-d_6 (the latter was used as reference: CH_2 signals at 3.56 ppm for ^{1}H and 56.96 ppm for ^{13}C), and all experiments were performed at 313K. Experiments were processed using the Bruker software package. The ^{1}H, ^{13}C decoupled from proton, ^{13}C JMOD, ^{1}H-^{1}H DQF-COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HSQC-TOCSY NMR experiments were run under standard conditions.

Acquisition parameters: ^{1}H spectral width 2400 Hz, ^{13}C spectral width 20120 Hz, repetition time 2 s. *Double Quantum Filtered COSY experiment*: 2K complex points x 256 increments; 8 scans per increment; *HSQC experiment*: 2K x 256 data set, 160 scans per increment; *HMBC experiment*: 50 ms used for the evolution of long-range coupling, 3.4 ms for the low-pass *J* filter. 2K x 512 data set, 192 scans per increment; in all above experiments the cardinal sine window function was used in both domains prior to Fourier transformations and final matrix size was 2K x 1K. *HSQC-TOCSY experiment*: DIPSI2 scheme for homonuclear Hartman-Hahn mixing (80ms). 1K x 256 data set, 192 scans per increment; cardinal sine window function used as previously; final matrix size: 2K x 512.

Obtained assignment of aglycone and sugar moieties is given in Tables S1 and S2.

Mass spectrometry

MALDI-TOF MS analyses were performed on a Bruker Ultraflex III instrument (BRUKER Daltonik GmbH) equipped with an UV Nd:YAG 200 Hz laser (λ = 355 nm). A solution (10 mg/mL) of α-cyano-4 hydroxycinnamic acid (Sigma) was prepared in a 1:1 (v/v) 0.1% TFA/CH3CN (Chromasolv Sigma Aldrich). 10 µL of sample was mixed with 10 µL of matrix. This mixture (1 µL) was spotted on MTP 384 target plate ground steel and air-dried. The data were obtained in positive ions reflectron TOF mode. Each spectrum represents the sum of 1 000 laser shots. External calibration was performed using Polyethylene glycol average M.W. 600 (Aldrich) as calibrant. Results are presented in Figure S3 and Table S3.

References

Długosz M, Wiktorowska E, Wiśniewska A, Pączkowski C. 2013. Production of oleanolic acid glycosides by hairy root established cultures of *Calendula officinalis* L. Acta Biochim Pol 60(3):467–473

Figures:

Figure S1 Hairy root culture of marigold *Calendula officinalis* after 21-days growth in liquid medium.

Figure S2 HPLC chromatograms of control (upper diagram) and JA-treated (lower diagram) hairy root ethanol extracts.

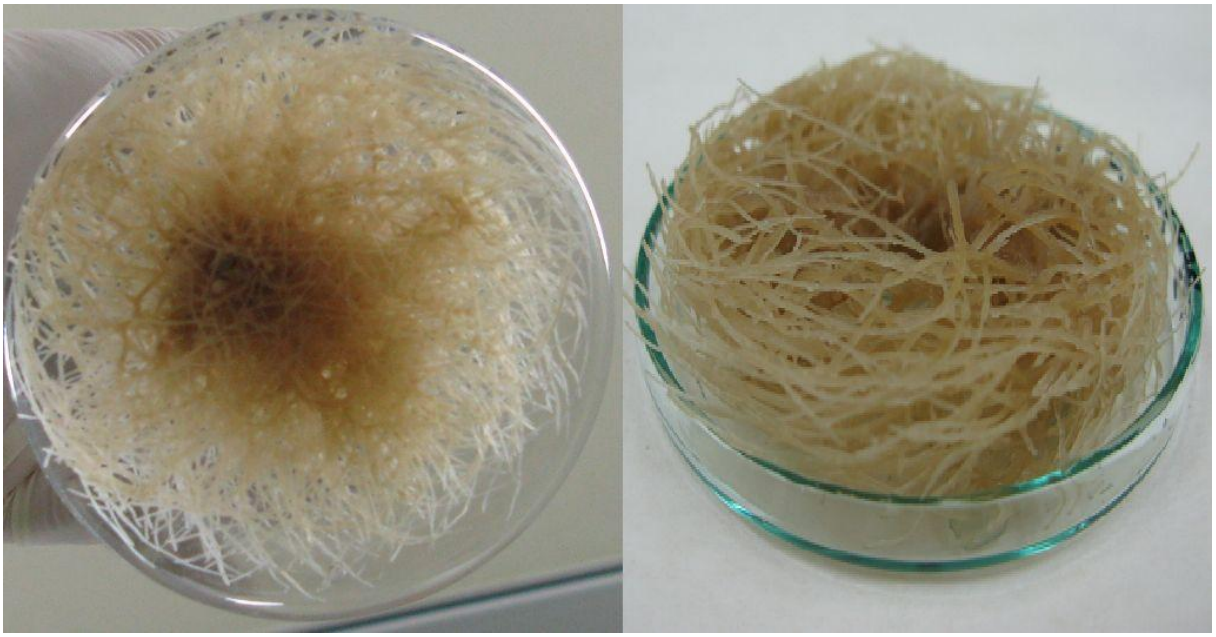
Figure S3 Positive-ion mode MALDI TOF MS spectrum of investigated saponin.

Tables:

Table S1 ^{1}H and ^{13}C NMR data (δin ppm) of the aglycone part (oleanolic acid). Chemical shifts were referenced according to CH_2 signals of ethanol-d6 (3.56 ppm for ^{1}H and 56.96 ppm for ^{13}C)

Table S2 ^{1}H and ^{13}C NMR data (δin ppm) of the sugar moieties. Chemical shifts were referenced according to CH_2 signals of ethanol-d6 (3.56 ppm for ^{1}H and 56.96 ppm for ^{13}C)

Table S3 Molecular formulas proposition for the observed saponin peak



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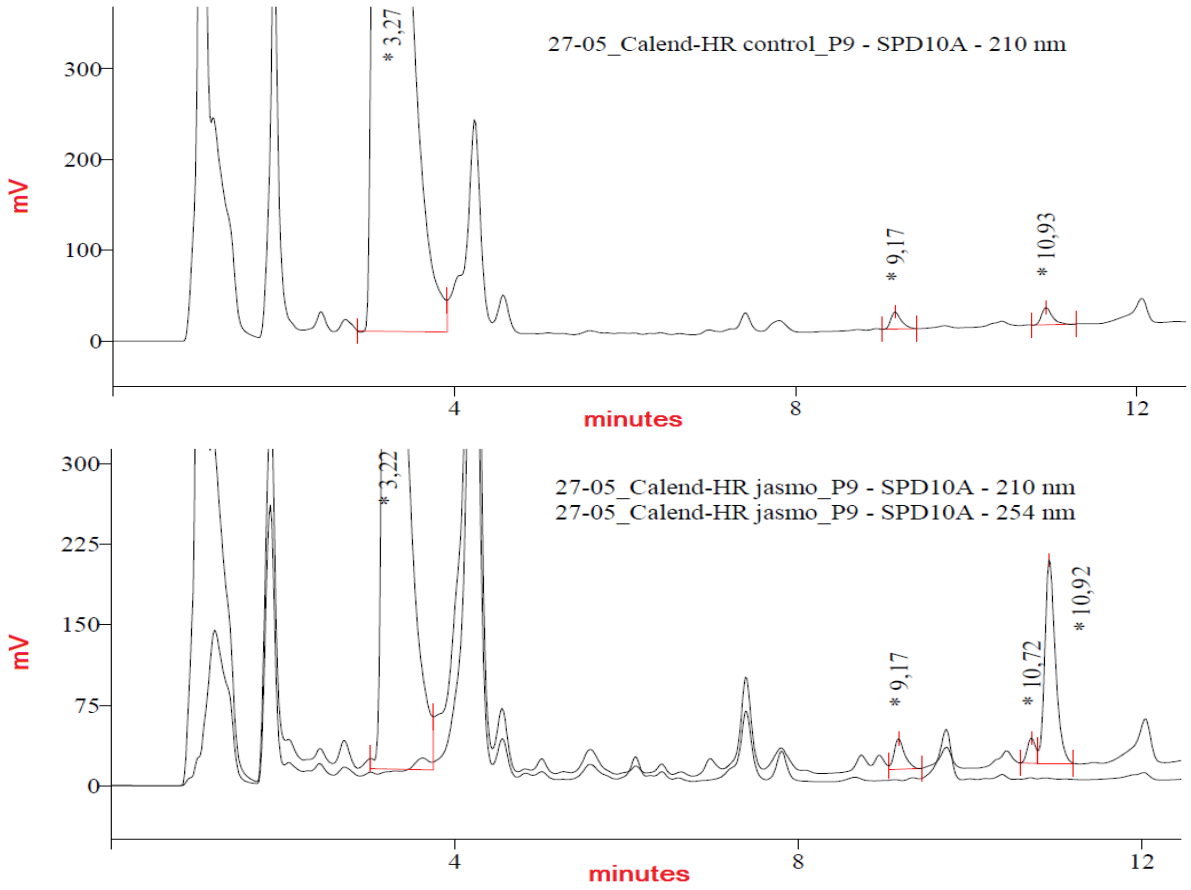

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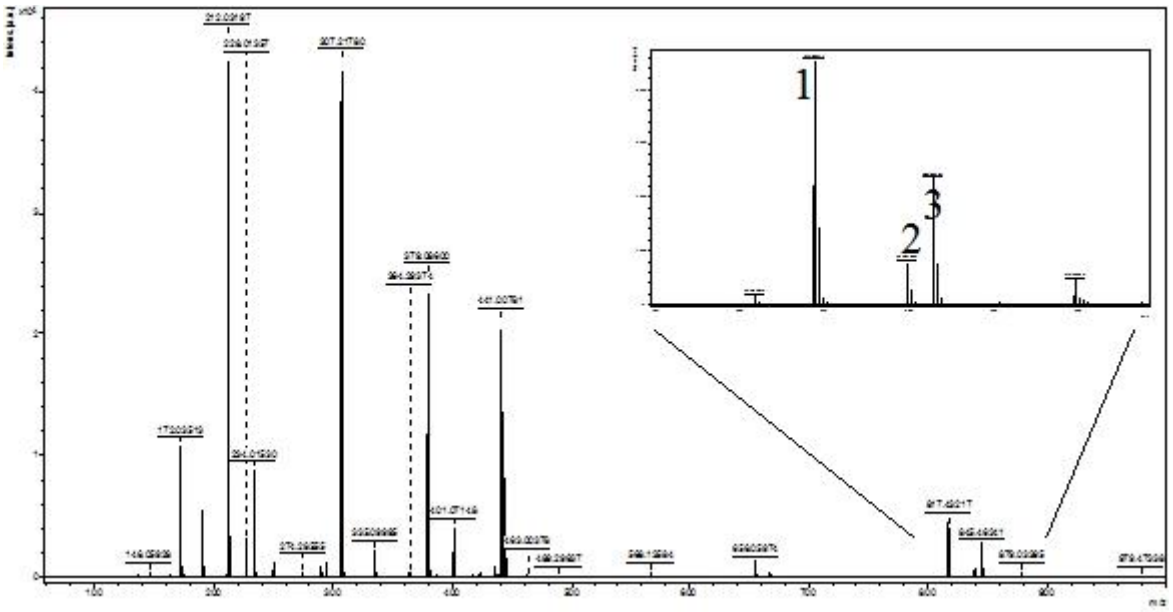

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Atoms No.	^{1}H (ppm)	^{13}C (ppm)
1	1.60/0.95	39.7
2	1.87/1.69	26.7
3	3.14	90.6
4	-	40.0
5	0.77	56.9
6	1.54/1.40	19.3
7	1.48/1.35	33.8
8	-	40.6
9	1.57	48.9
10	-	37.7
11	1.90	24.4
12	5.26	123.6
13	-	144.5
14	-	42.7
15	1.85/1.09	28.7
16	2.02/1.73	23.8
17	-	47.7
18	2.88	42.4
19	1.70/1.17	47.1
20	-	31.4
21	1.38/1.21	34.9
22	1.75/1.33	33.0
23	1.05	28.6
24	0.85	16.9
25	0.95	16.0
26	0.82	17.6
27	1.15	26.3
28	-	177.7
29	0.91	33.6
30	0.94	24.1

Table S2 ^{1}H and ^{13}C NMR data (δin ppm) of the sugar moieties. Chemical shifts were referenced according to CH_2 signals of ethanol-d6 (3.56 ppm for ^{1}H and 56.96 ppm for ^{13}C)

Atoms No.	^{1}H (ppm)	^{13}C (ppm)
GlcAc-1	4.36	106.7
GlcAc-2	3.28	74.9
GlcAc-3	3.39	77.6
GlcAc-4	3.57	72.8
GlcAc-5	3.76	76.5
GlcAc-6	-	170.6
Gal-1	5.38	96.5
Gal-2	3.35	73.6
Gal-3	3.44	78.2
Gal-4	3.36	71.2
Gal-5	3.36	78.3
Gal-6	3.81/3.66	62.48

Table S3 Molecular formulas proposition for the observed saponin peak

Peak number	Molecular formulas proposition	m/z calculated	m/z observed	δ ppm
1	$C_{42}H_{66}O_{14}Na^{+}$	817.43448	817.43217	-2.8
2	$C_{42}H_{65}O_{14}Na_2^{+}$	839.41642	839.41493	-1.8
3	$C_{44}H_{70}O_{14}Na^{+}$	845.46578	845.46241	-4.0