SUPPLEMENTARY MATERIAL

Proliferative and antioxidant activity of *Symphytum officinale* root extract

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Abstract

The root of *Symphytum officinale* L. is commonly used in folk medicine to promote the wound healing, reduce the inflammation and in the treatment of broken bones. The objective of our investigation was to analyze the extract from *S. officinale* in term of its antioxidant activity and the effect on cell viability and proliferation of human skin fibroblast (HSF). Moreover, the quantification of main phenolics and allantoin was conducted using HPLC-DAD method. Five compounds were found: rosmarinic, p-hydroxybenzoic, caffeic, chlorogenic and p-coumaric acid. DPPH, FRAP, and TPC assay showed the high antioxidant activity of the extract. MTT test proved the stimulatory effect on cell metabolism and viability of HSF cells. Moreover, no changes in cytoskeleton structure and cells shape were observed. The obtained results indicate that non-toxic extract from *S. officinale* root has strong antioxidant potential and a beneficial effect on human skin fibroblasts.

Keywords: *Symphytum officinale*; comfrey; cell viability; human skin fibroblast; antioxidant activity; phenolic acids; allantoin

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3. Experimental

Chemicals and reagents

Phenolic acid standards and allantoin were purchased from Sigma (St. Louis, MO, USA). Ethanol, methanol, HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). Water was deionized and purified by ULTRAPURE Milipore Direct-Q® 3UV–R (Merck, Darmstadt, Germany).

Trolox, the Folin-Ciocalteu phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), tetra-methyl-rhodamine-isothiocyanate-phalloidin (TRITC-phalloidin) were from Sigma-Aldrich. Medium cell culture (RPMI 1640), phosphate buffered saline (PBS), fetal calf serum (FCS) and antibiotics were supplied by GibcoTM (Paisley, UK).

Plant material

Root of *S. officinale* was collected at second half of September 2015, identified and deposited in Botanical Garden of UMCS in Lublin (Voucher specimen no. 2583-P). The plant material was dried at 30 ºC, pulverized, accurately weighted and extracted with use of ultrasonic bath at 40 ºC. Sample was extracted two times with 2 x 100 mL of ethanol/water mixture (50/50, v/v) (2 x 15 min.) to obtain the ethanol/water extract (EX1) and next, the fresh portion of plant material was extracted two times with 2 x 100 mL of water (2 x 15 min.) to prepare the water extract (EX2).

The extracts were lyophilized and dry residues were weighted. For HPLC analysis, antioxidant activity and biological assay the residues were dissolved in ethanol/water (50/50, v/v) or dimethyl sulfoxide (DMSO), respectively.

HPLC condition

Chromatographic determination was performed on VWR Hitachi Chromaster 600 chromatograph (Merck, Darmstadt, Germany) with a pump, a degasser, a thermostat, an autosampler, a DAD detector and EZChrom Elite software.

The elaborated gradient elution program provided the successful separation of allantoin and the most ubiquitous phenolic acids of plant origin. The extracts were analyzed on C18 reversed-phase column Kinetex (Phenomenex, Torrance, CA, USA) (10 cm × 4.0 mm i.d., 2.6 μm particle size), at temperature of 25°C. A mixture of acetonitrile with 0.025% of trifluoroacetic acid (solvent A) and water with 0.025% of trifluoroacetic acid (solvent B) was used as a mobile phase. The compounds were separated by gradient elution. The gradient program was as follows: 0-8 min A 0%, B 100%; 8-33 min A 0-11%, B 100- 89%; 33-38 min

A 11%, B 89% and 38-60 min A 11-70%, B 89-30%. The flow rate of the eluent was 1.0 mL min^{-1}. The data were collected in a wavelength range from 200 to 400 nm. The identity of compounds was established by comparison of retention times and spectra with the corresponding standards. The similarity factor calculated by the EZChrom Elite software was higher than 0.98. Additionally, the identity was confirmed by MS analysis using micrOTOF-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany). The quantitative analysis was conducted at analytical wavelength characteristic for investigated compounds using external calibration method

Antioxidant activity

The methodology was based on literature (Paduch et al., 2014). All spectrophotometric measurement were performed using E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

DPPH assay: 10 µL of the extract sample were diluted with methanol up to 1 mL and 1 mL of DPPH solution at concentration of 0.1 mg mL^{-1} was added. After 15 minutes, the absorbance was measured at 515 nm. The results were expressed as a trolox equivalent.

Ferric reducing antioxidant power (FRAP): the extracts were mixed with an equal volume of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The solution was incubated at 37°C for 30 min and 10% trichloroacetic acid (w/v) was added. After centrifugation at 1000 g for 5 min, 1 mL of the upper layer was mixed with an equal volume of water and 0.1% ferric chloride. The measurement of absorbance was conducted at 700 nm. Ascorbic acid was used as a reference.

Total phenolic content (TPC): TPC was determined by the Folin-Ciocalteu assay (Szymczak et al., 2017). 200 µL of each extract were diluted to 5 mL with water and next 100 µL of the solution obtained were mixed with 100 µL of the Folin-Ciocalteu reagent and, after 3 minutes, with 1 mL of 7% sodium carbonate. Absorbance was measured after 45 min. at 760 nm and results were expressed as mg equivalents of gallic acid per 1 g of extract.

Biological experiments

The biological experiments were carried out according to Paduch et al. (2007). To evaluate significant differences between treatments Student's t-test was performed. The statistical differences were considered significant at $P < 0.05$.

HSF cell culture: The skin fibroblasts cells (HSF) were cultured in 25 cm^2 culture flasks in RPMI 1640 medium supplemented with 10% FCS and antibiotics (100 U mL^{-1} of penicillin, 100 µg mL^{-1} of streptomycin and 0.25 µg mL^{-1} of amphotericin B) incubated at 37ºC in a humidified 5% CO_2/95% air incubator.

MTT assay: HSF cells at a density of 2 x 10^4 cells/mL were placed in 96-well multiplates in 100 μL of medium and left for adhesion for next 24 h. Thereafter, to the wells plant extract was added and incubation was conducted for 24, 48 and 72 h. After each time, to the appropriate wells, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (25 μL/well) was added for 3 h. The crystals of formazan were solubilized overnight in a mixture consisting of 10% SDS in 0.01 M HCl. The absorbance of solution was measured at 570 nm wavelength.

Labeling of cytoskeleton F-actin: cells were incubated in 4-well Lab-Tek glass chamber slides in 1 mL of RPMI 1640 medium supplemented with 2% FBS and appropriate working concentrations of plant extract. After incubation, cells were rinsed with culture medium and exposed to paraformaldehyde solution (10%, v/v) for 20 min. Thereafter, cells were exposed to Triton X-100 (0.2%, v/v) for 5 min. TRITC-phalloidin (1 μg mL^{-1}) in PBS was added to each well and incubated in the dark at 37ºC/5% CO_2 for 30 min. Cells observation was conducted under a fluorescence microscope (Olympus, BX51).

References

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Table S1. Validation parameters of HPLC method and the results of allantoin and phenolic acids quantification in *S. officinale* root.

Component	Linear regression equation	Concentration range (mg mL^{-1})	RSD values of peak area (%)	Correlation coefficient (r)	Content* mg/g ± SD
Al	y = 2511248214x + 452214	0.2-2.0	0.51-0.82	0.9999	11.2±0.131
RA	y = 219477749x + 244527	0.025-0.25	0.91-1.21	0.9998	1.85±0.082
p-HA	y = 578895167x - 532556	0.002-0.02	1.51-1.73	0.9993	0.081±0.007
CA	y = 1526756125x - 810766	0.001-0.01	1.21-1.54	0.9995	0.021± 0.002
ChA	y = 115526222 x - 599112	0.0001-0.001	1.26-1.78	0.9998	0.009±0.001
p-CA	y = 121705953x - 172497	0.0001-0.001	1.43-2.01	0.9997	0.007±0.001

* Ethanol/water extract (EX1) was used to estimate the content of analytes in *S. officinale* root.
Al - allantoin; RA - rosmarinic acid; p-HA - p-hydroxybenzoic acid; CA – caffeic acid; ChA – chlorogenic acid; p-CA - p-coumaric acid.

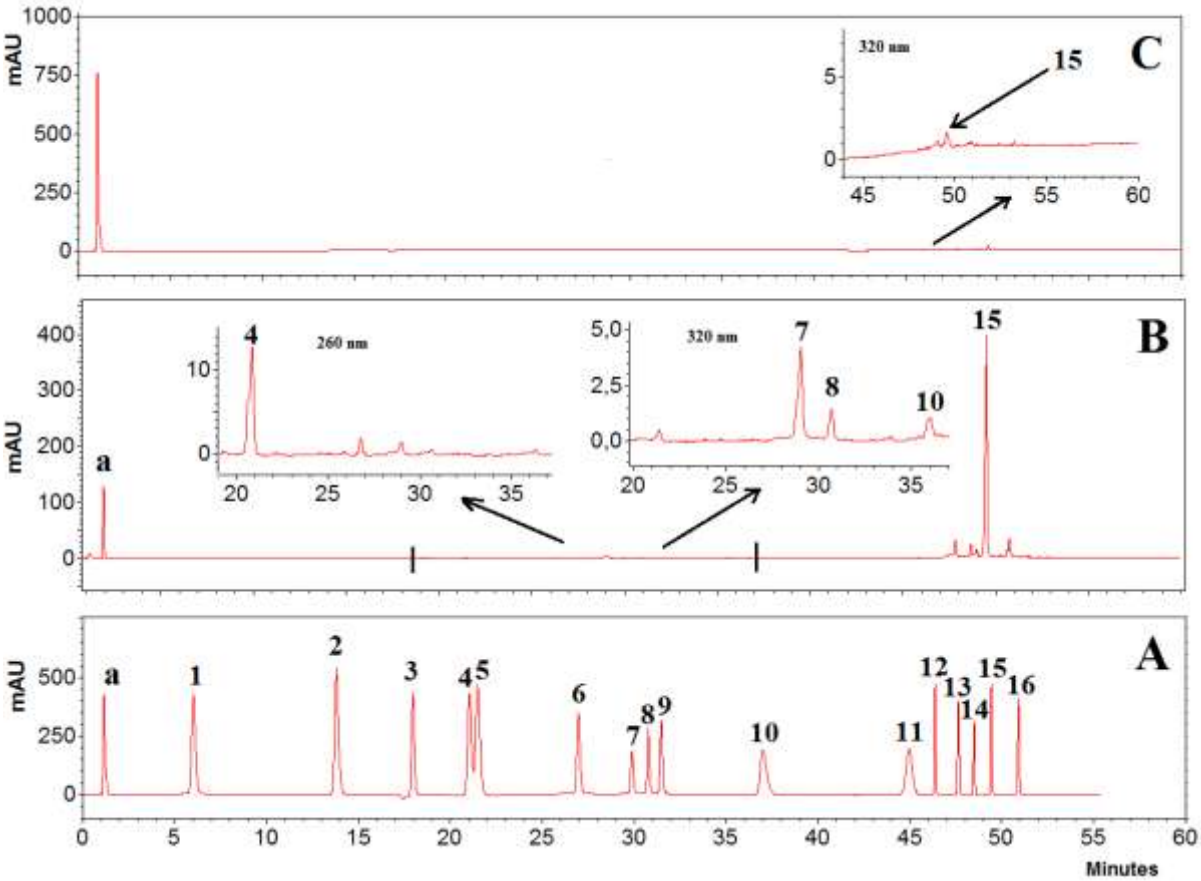

Figure S1. HPLC chromatogram of: standards (A) and *S. officinale* root extract (B); 1- gallic, 2- protocatechuic, 3- α-resorcylic, 4- p-hydroxybenzoic; 5- gentisic, 6- β-resorcylic, 7- caffeic, 8- chlorogenic; 9- syringic, 10- p-coumaric, 11- salicylic, 12- ferulic 13-isoferulic, 14 o-coumaric,15- rosmarinic, 16 cinnamic acid.

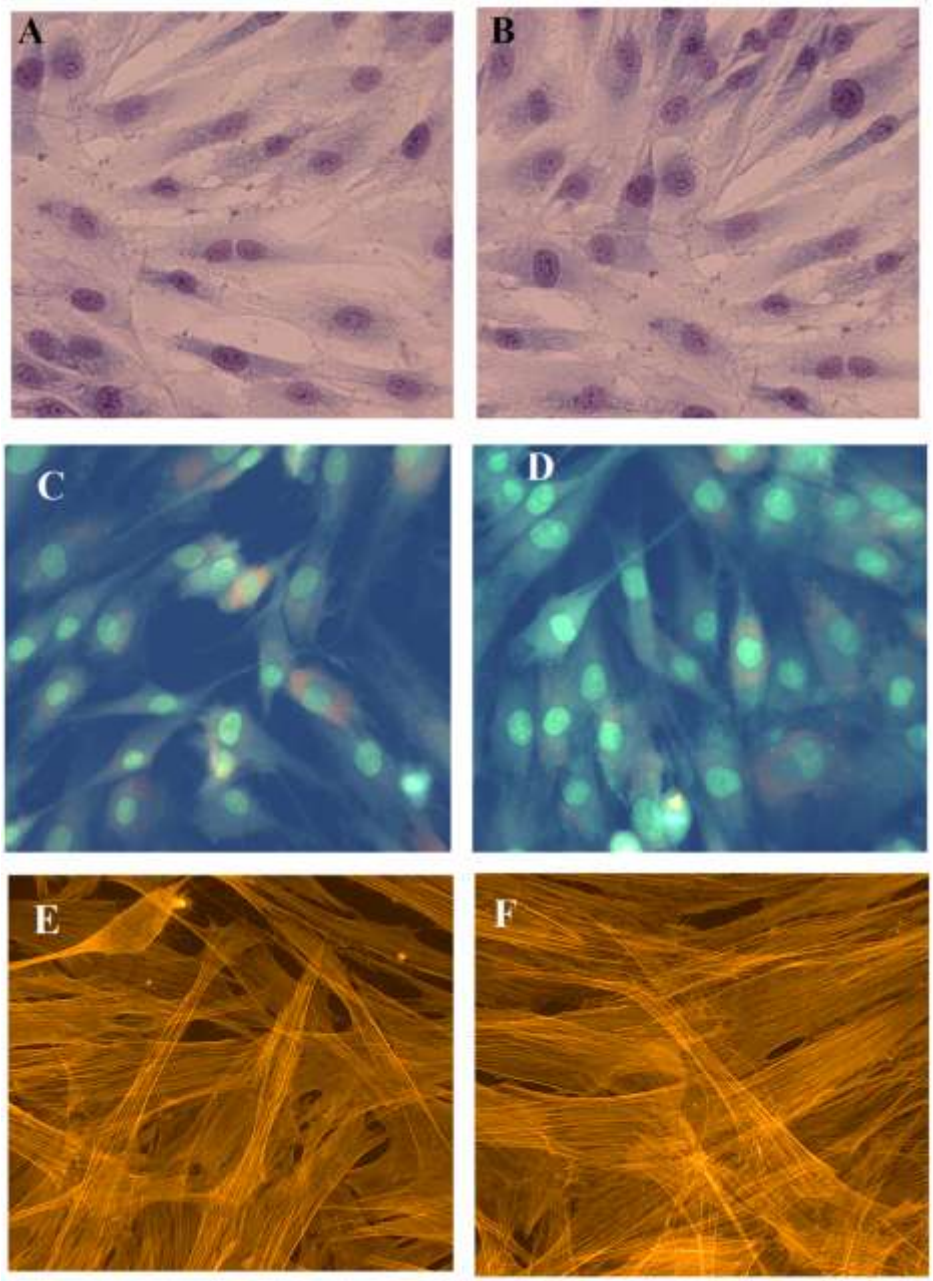

Figure S2. Human skin fibroblast cell (HSF) morphology. The control sample (A, C, E) and HSF cells after 48 h incubation with EX1 extract (B, D, F). A and B - May-Grünwald-Giemsa (MGG) staining; C and D - fluorescent staining with Hoechst 33342; E and F - cellular cytoskeleton organization after staining with fluorescent TRITC-phalloidine dye. Magnification 200x.