South African Journal of Botany 116 (2018) 175–184

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

# The biological activities of roots and aerial parts of *Alchemilla vulgaris* L.

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ARTICLE INFO

*Article history:*
Received 14 December 2017
Received in revised form 9 March 2018
Accepted 20 March 2018
Available online xxxx

Edited by AO Aremu

*Keywords:*
*Alchemilla vulgaris*
Lady's mantle
Antioxidant activity
Antimicrobial activity
Biocompatibility
Anti-inflammatory activity

ABSTRACT

Medicinal plants are considered to be a major source of biologically active compounds, which provides unlimited opportunities for their use either as medical treatments or as novel drug formulations.
The focus of our study was on basic phytochemical analysis and *in vitro* examination of the biological activity of *Alchemilla vulgaris* L. Methanolic extracts of above ground parts and roots of *A. vulgaris* (AVA and AVR, respectively) were prepared by maceration for 72 h. Phytochemical profile of extracts was evaluated by spectrophotometric determinations of phenolic compounds and HPLC-PDA analysis. AVA and AVR were analysed for their antioxidant efficacy as total antioxidant capacity, metal chelation and reducing power ability, inhibition of lipid peroxidation as well as their potential to neutralise DPPH, ABTS, and OH radicals. Microdilution method was employed to investigate the antibacterial and antifungal activity of extracts against nine ATCC and isolates of bacteria and ten fungal strains from biological samples. Anti-inflammatory activity of the extracts was evaluated using cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) assays and the assay for determination of COX-2 gene expression, while biocompatibility of extracts was assessed by MTT assay.
Our results revealed the high amount of phenolic compounds in both extracts; especially they were rich in condensed tannins. Ellagic acid and catechin were tentatively identified in AVA and AVR, respectively. Full biocompatibility as well as remarkable bioactivity were observed for both extracts in all employed assays, so our further investigations will be focused on the identification of active constituents in *A. vulgaris* and the molecular mechanisms of their action.

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## 1. Introduction

Several lines of evidence support the hypothesis that secondary metabolites from plants (*e.g.* flavonoids and phenolic acids) may play an antioxidant role and diminish the adverse effects of an imbalance between the production of enzymatic and non-enzymatic antioxidants and overproduction of free radicals in oxidative stress (Hussein and Khalifa, 2014). As a result of their antioxidant activity, either through their reducing capacity or through potential influences on intracellular redox processes, phenolic compounds manifest various beneficial effects, including anti-inflammatory and anticancerogenic activities (Han et al., 2007; Li et al., 2014).

*Alchemilla vulgaris* L. (Lady's mantle), an herbaceous perennial plant belonging to the Rosaceae family, is widely spread across Europe and Asia and commonly known in traditional medicine for treatment of ulcers, wounds, eczema, and digestive problems as well as a remedy for gynaecological disorders, such as heavy menstrual flow, menorrhagia and dysmenorrhoea (Jarić et al., 2015; Masullo et al., 2015; Ilić-Stojanović et al., 2017). *Alchemilla* species have been reported to exert a variety of biological activities, including antiviral, antioxidant, antiproliferative, and antibacterial activity as well as healing effects on cutaneous wounds in rats (Trouillas et al., 2003; Shrivastava and John, 2006; Filippova, 2017). Previous findings showed that aerial parts of *A. vulgaris* comprise mostly phenolic compounds – a large amount of tannins, phenolic acids (predominantly ellagic acid, gallic, and caffeic acids), flavonoids (quercetin), and flavonoid glycosides (isoquercetin, rutin, avicularin, and tiliroside) (Møller et al., 2009). To the extent of our knowledge, there is a scarce literature on phytochemical profile and biological activity of roots of *A. vulgaris*.

*Abbreviations:* AVA, *Alchemilla vulgaris* aerial parts methanolic extract; AVR, *Alchemilla vulgaris* roots methanolic extract; ROS, reactive oxygen species; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; $PGH_2$, prostaglandin $H_2$; NSAIDs, non-steroidal anti-inflammatory drugs; IL, interleukin; TNF-α, tumour necrosis factor α; iNOS, inducible nitric oxide synthase; NF-κB, the nuclear factor kappa-light-chain-enhancer of activated B cells; DEX, dexamethasone.

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https://doi.org/10.1016/j.sajb.2018.03.007
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Reactive oxygen species (ROS) encompass a broad range of reactive molecules triggering a multitude of ailments, such as rheumatoid arthritis, cardiovascular disorders, neurological disease, and cancer (Hitchon and El-Gabalawy, 2004; Valko et al., 2004; Melo et al., 2011). Increased ROS generation has been described as one of the key factors in the progression of inflammatory disorders (Mittal et al., 2014). Cyclooxygenases (COX-1 and COX-2) are enzymes involved in the inflammatory process and responsible for the conversion of arachidonic acid into pro-inflammatory mediators, like prostaglandin $H_2$ ($PGH_2$). COX-1 is a constitutive enzyme expressed in almost all cells providing homeostatic functions. Under normal conditions, COX-2 is unexpressed in most cells, but its expression can be induced by inflammatory stimuli. Hence, COX-2 is a major target for anti-inflammatory therapies. Since the use of non-selective COX inhibitors (non-steroidal anti-inflammatory drugs-NSAIDs) may lead to side effects, particularly evident in the gastrointestinal tract (Jones et al., 2008), novel COX-2-specific agents, with no or very little undesirable effects, are urgently needed.

The presented study was focused on assessment of biocompatibility, antioxidant, antimicrobial, and anti-inflammatory activities of methanolic extracts of aerial parts and roots of Lady's mantle (*A. vulgaris*) and their phytochemical profile as well.

## 2. Materials and methods

### 2.1. Chemicals and instruments

All spectrophotometric determinations were performed on UV–Vis double beam spectrophotometer Halo DB-20S (Dynamica GmbH, Dietikon, Switzerland). Ellagic acid, hyperoside, rutin and TRIS/HCl-buffer were obtained from Carl Roth (Karlruhe, Germany), trolox, epicatechin, catechin, gallic acid, vanillic acid, rutin, kaempferol, quercetin, DMSO (>99.98% purity), and formic acid from Sigma-Aldrich (Deisenhofen, Germany), caffeic acid and $Na_2EDTA$ (Titriplex III) from Merck KGaA (Darmstadt, Germany). HPLC-grade acetonitrile, water, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Resazurin was purchased from Acros Organics (Geel, Belgium), while all other chemicals used in antimicrobial experiments were purchased from Torlak Institute of Virology, Vaccines, and Sera (Belgrade, Serbia). Reagents used in COX-1 and -2 assays: purified prostaglandin H synthase (PGHS)-1 from ram seminal vesicles, human recombinant PGHS-2, NS-398, and arachidonic acid were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA), hematin (porcine) and indomethacin from ICN (Aurora, Ohio, USA), epinephrine hydrogen tartarate from Fluka (Buchs, Switzerland), and competitive $PGE_2$ EIA kit from Assay Designs Inc. (Ann Arbor, MI, USA). COX-2 gene expression kits, reagents and chemicals for this method: fetal bovine serum (FBS), N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), phosphate buffered saline (PBS), penicillin, and streptomycin human leukemic monocytic cell line THP-1 (European Collection of Cell Culture; Item No. 88081201), and RPMI1640 were obtained from Gibco® (NY, USA), phorbol 12-myristate 13-acetate (PMA), while dexamethasone, lipopolysaccharide (LPS) as well as GenElute™ Mammalian TotalRNA Miniprep Kit were from Sigma-Aldrich (MO, USA). High-Capacity cDNA Reverse Transcription Kit, Pre-developed TaqMan® Assay, COX-2 primers and COX-2 probe were from Applied Biosystems (NY, USA). BalbC-3 T3 fibroblasts (clone A31) were purchased from ATCC (Manassas, VA) and human epidermal keratinocytes (HaCaT) from Innoprot (Biscay, Spain).

### 2.2. Plant material and preparation of the extracts

The roots and aerial parts of *Alchemilla vulgaris* L. were collected in August 2014 at Goč Mountain (Central Serbia). Collection of plant material was carried out by the sampling of 20 representative individuals of the population in the full flowering period. The taxonomic and botanical identity was confirmed by Milan Stanković, PhD. A voucher specimen (No. 120/015) is kept in the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia. The collected plants were air-dried in darkness at ambient temperature. The dried aerial parts and roots separately from all individuals were cut up, mixed and powdered for extracts preparation.

Dried and powdered aerial parts (30.00 g) and roots (55.80 g) of *A. vulgaris* were macerated with methanol (150 and 280 mL, respectively) for 24 h three times, by continuous stirring at room temperature. Mass of the extracts was determined after filtration through Whatman No.1 filter paper and concentrating under reduced pressure at 45 °C. The obtained dry weights of the extracts were 3.70 g for *A. vulgaris* aerial parts (AVA) and 9.00 g for roots (AVR), (12% *w*/w and 16.13% w/w, respectively). The obtained extracts were kept at +4 °C until further use.

### 2.3. Chromatographic analysis

The identification of individual phenolic compounds in the extracts was performed using HPLC system (Shimadzu Prominence, Kyoto, Japan) as described previously (Mihailović et al., 2016). The detection wavelength of PDA was monitored at 260, 280, 325 and 330 nm. Methanolic solutions of ellagic acid, caffeic acid, gallic acid, vanillic acid, quercetin, rutin, kaempferol, (+)-catechin, and (−)-epicatechin were used as reference standards for the identification of compounds in the extracts, which was performed by comparing retention times and absorption spectra of the peaks with reference standards. The compounds identified in the extracts were confirmed by spiking the sample with the standard compound.

### 2.4. Phytochemical analysis

#### 2.4.1. Total phenolics

The method developed by Singleton et al. (1998) was used to determine the total phenolic content. 0.5 mL aliquots of the extracts diluted in methanol were mixed with 2.5 mL of Folin–Ciocalteu solution (previously diluted ten-fold with water) and 2 mL of 7.5% aqueous $NaHCO_3$ solution. The reaction mixture was incubated for 15 min at 45 °C. The absorbance was read at 765 nm. Mass concentrations of total phenols in plant material were determined using the standard curve for gallic acid and results were calculated as gallic acid equivalents (mg GAE/g dry weight of extract).

#### 2.4.2. Total flavonoids

The total flavonoid content was estimated by the method of Quettier-Deleu et al. (2000). The reaction mixture contained 0.5 mL 2% $AlCl_3$ in methanol and 0.5 mL of extracts solutions in methanol (1 mg/mL). The absorbance was measured at 415 nm after one hour of incubation at room temperature. Results were calculated as milligrammes of rutin equivalents per gram of dry weight of extract (mg RUE/g dry weight of extract).

#### 2.4.3. Phenolic acids

Determination of the total phenolic acids content in plant extracts was performed according to the method described in the The Polish Pharmacopoeia VIII (2009), with slight modifications. Briefly, 1 mL of plant extracts solutions was added to 5 mL of distilled water, followed by addition of 1 mL of 0.1 M HCl, 1 mL of Arnow's reagent (10% Na-molybdate and 10% Na-nitrite), 1 mL of 1 M NaOH, and 1 mL of distilled water. The absorbance was read immediately at 490 nm. Results are presented as caffeic acid equivalents (mg CAE/g dry weight of extract).

#### 2.4.4. Determination of tannins

The method suggested by Scalbert et al. (1989) was used to estimate the content of condensed tannins in plant extracts. In brief, the extracts were mixed with a certain amount of phloroglucinol (for each equivalent

of gallic acid in extracts 0.5 mol phloroglucinol was added). Subsequently, 1 mL of 4.8 M HCl solution and 1 mL of formaldehyde (13 mL of 37% formaldehyde diluted to 100 mL in water) were added. The reaction mixture was allowed to stand overnight at room temperature to precipitate the tannins. Total phenolics were determined in the solution above the precipitate using Folin–Ciocalteu method and this value was subtracted from the total phenolics' value to obtain the total tannin content, expressed as gallic acid equivalents (mg GAE/g dry weight of extract).

The gallotannin content was determined according to the procedure described by Haslam (1965). To 1.5 mL of a saturated $KIO_3$ solution, 3.5 mL of a methanol solution of the examined extracts were added. The absorbance of the red intermediate was sprectrophotometrically determined at 550 nm until the maximum absorbance was reached. The gallotannin content was determined as gallic acid equivalents (mg GAE/g dry weight of extract).

*2.4.5. Total anthocyanins content*

Determination of total and monomeric anthocyanins was conducted using single pH and pH differential methods (Cheng and Breen, 1991), based on the ability of anthocyanins to change their structure depending on the pH. The specified volume of the sample was mixed with pH 1.0 KCl -buffer (0.025 M) and pH 4.5 sodium-acetate buffer (0.4 M), respectively. After 30 min incubation, the absorbance was measured spectrophotometrically at 520 and 700 nm. The concentrations of the total and monomeric anthocyanins were determined as cyanidin-3-glycoside equivalents according to the following equation: $c = (A * M * F * 1000) / (\varepsilon * l)$, where c - concentration of total or monomeric anthocyanins; A - absorbance of total and monomeric anthocyanins, which is calculated as $(A_{520}\text{-}A_{700})_{pH\ 1.0}$ and $(A_{520}\text{-}A_{700})_{pH\ 1.0}\text{-}(A_{520}\text{-}A_{700})_{pH\ 4.5}$, respectively; M - molar weight of cyanidin-3-glycoside (449.2 g/mol); F - dilution factor; ε - molar absorptivity (26 900 L/mol * cm); l – cell length (1 cm).

*2.5. Antioxidant activity*

*2.5.1. $ABTS^{\cdot+}$ radical scavenging activity*

Radical scavenging activity against ABTS radical cation (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) was measured spectrophotometrically following the procedure of Re et al. (1999). The percentage of $ABTS^{\cdot+}$ decoloration is proportional to the ability of extract to neutralise radicals and has been calculated using the formula: % inhibition = ((Ac − As) * 100) / Ac, where: Ac – absorbance of the control (methanol instead of the sample); As – absorbance of the sample.

The concentration of samples providing 50% of radical scavenging activity ($IC_{50}$) was calculated using dose–response sigmoidal curve plotted the percentage of inhibition against extract concentration (μg/mL).

*2.5.2. $DPPH^{\cdot}$ radical scavenging activity*

The ability of plant extracts to neutralise $DPPH^{\cdot}$ radical was estimated according to Kumarasamy et al. (2007). The reaction mixture containing 1 mL of $DPPH^{\cdot}$ solution in methanol (80 μg/mL) and 1 mL of each extract solution (serial dilutions in methanol, started from 0.25 mg/mL) was allowed to stand in the dark for 30 min. The absorbance was read spectrophotometrically at 517 nm and $IC_{50}$ values were calculated.

*2.5.3. Hydroxyl radical scavenging activity*

OH radical scavenging activity of extracts was determined using the method performed by Kunchandy and Rao (1990). Briefly, 200 μL of the extract was mixed with 200 μL of 10 mM iron (III) chloride solution, followed by 100 μL of 1 mM ascorbic acid solution, 100 μL of 1 mM EDTA solution, 200 μL of 10 mM 2-deoxy-ribose solution, and 100 μL of 10 mM hydrogen-peroxide solution. The reaction mixture was incubated at 37 °C for 1 h. Subsequently, 1 mL of TCA-TBA solution (0.5% TBA in 10% TCA water solution) was added and the final mixture was incubated at 80 °C for 30 min and cooled to room temperature. The absorbance of the cooled reaction mixtures was measured at 535 nm. On the basis of the obtained absorbance values, the percentage of inhibition and $IC_{50}$ values were calculated.

*2.5.4. Estimation of metal chelating ability*

The assessment of ability of plant extracts to inhibit the formation of $Fe^{2+}$-ferrozine complex was carried out according to the method by Chew et al. (2009). One mililitre of 0.125 mM iron (II) sulphate solution and 1 mL of 0.3125 mM ferrozine water solution were added to 1 mL of serial dilutions of extracts dissolved in methanol. The reaction mixture then allows standing at room temperature for 10 min. The $IC_{50}$ values were determined after reading the absorbance at 562 nm.

*2.5.5. Reducing power*

According to the method of Oyaizu (1986), to 2.5 mL of extracts solutions in methanol (0.5 mg/mL), 2.5 mL of sodium-phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide were added. The reaction mixture was left to stand for 20 min at 50 °C, followed by addition of 2.5 mL of 10% TCA. In 5 mL of this solution, 1 mL of 1% iron (III) chloride solution was added and absorbance was read promptly at 700 nm. Trolox, as a referent antioxidant, was used for the construction of calibration curve and the results of reducing capacity of tested extracts were expressed as Trolox equivalents (mg TE/g dry weight of extract).

*2.5.6. Total antioxidant activity*

Prieto et al. (1999) developed a method for the determination of the total antioxidant activity of plant extracts. In brief, 0.3 mL of the extracts dissolved in methanol was mixed with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate) and incubated for 90 min at 95 °C. Then, the reaction mixture was cooled to room temperature and the absorbance of the green-phosphate/Mo (V) complex was monitored at 695 nm. The results are expressed as mg ascorbic acid (AA) per gram of dry extract, using a standard curve for ascorbic acid.

*2.5.7. Oil-in-water emulsion*

Inhibitory activity of AVA and AVR towards lipid peroxidation was performed according to the procedure described by Hsu et al. (2008). To 0.5 mL of the serial dilutions of the extracts in methanol, 2.5 mL of linoleic acid emulsion (0.2804 g of linoleic acid and 0.2804 g of Tween-40 in 50 mL of 40 mM sodium phosphate buffer pH 7.0) was added. The emulsion was incubated for 72 h at 37 °C. Thereafter, 0.1 mL of this solution was mixed with 4.7 mL of ethanol, 0.1 mL of 30% ammonium thiocyanate solution, and 0.1 mL of 20 mM iron (II) chloride solution. Subsequently, the mixture was stirred for 3 min and afterwards the absorbance was read spectrophotometrically at 500 nm against the methanol (blank).

*2.6. Antimicrobial activity*

*2.6.1. Test microorganisms*

The antimicrobial properties of *A. vulgaris* were tested against nine bacterial and ten fungal strains. The employed bacteria were as follows: *Micrococcus lysodeikticus* (ATCC 4698), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 70063), *Pseudomonas aeruginosa* (ATCC 10145), *Salmonella typhimurium* (ATCC 14028), *Bacillus subtilis* (ATCC 6633), and isolated strains from biological samples *Bacillus mycoides* (FSB 1) and *Azotobacter chroococcum* (FSB 14). Antifungal activity was evaluated against ATCC cultures of *Aspergillus brasiliensis* (ATCC 16404) and yeast *Candida albicans* (ATCC 10259), whereas following fungi were isolated from biological samples: *Phialophora fastigiata* (FSB 81), *Penicillium canescens* (FSB 24), *Trichoderma viride* (FSB 11), *Trichoderma longibrachiatum* (FSB 13), *Aspergillus glaucus* (FSB 32), *Fusarium oxysporum* (FSB 91), *Alternaria alternata* (FSB 51), and *Doratomyces stemonitis* (FSB 41). The isolates of the bacteria and fungi were obtained from the Laboratory for Microbiology, Faculty of Science, University of Kragujevac, Serbia.

The ATCC strains were provided from Institute of Public Health, Kragujevac, Serbia.The bacteria and fungi cultures were subcultured prior to testing; bacterial strains were cultured for 24 h at 37 °C on nutrient agar (NA), *C. albicans* was cultured on Sabouraud dextrose broth (SDB) for 24 h at 35 °C, whereas fungi were grown on potato glucose agar (PDA).

*2.6.2. Microdilution method*

Microdilution method described by Sarker et al. (2007) was employed to evaluate the antimicrobial activity of the samples, with some modifications. Briefly, overnight-cultured bacterial cultures were suspended in small amount of 5% DMSO than adjusted to the 0.5 McFarland turbidity standard using sterile normal saline and diluted to obtain inoculum concentration of $5 \times 10^6$ CFU/mL for broth microdilution MIC testing (CLSI, 2012). The analysed extracts (40 mg/mL), ellagic acid and catechin (1 mg/mL) and antibiotic erythromycin (40 μg/mL) were also dissolved in 5% DMSO. Determination of minimum inhibitory concentrations (MIC) of extracts for bacteria was performed in sterile 96 well plates (Spektar, Čačak, Serbia). 50 μL of two-fold serial diluted extracts in Muller-Hinton broth (MHB) was added to each well, followed by addition of 10 μL of resazurin (indicator), 30 μL of MHB, and 10 μL of bacteria suspension. The final bacterial concentration of in each well was $5 \times 10^5$ CFU/mL (CLSI, 2012). Each plate also included positive (erythromycin at a concentration range 20–0.156 μg/mL), growth (MHB, resazurin, and bacteria suspension) and sterility (MHB and resazurin, without bacteria suspension) controls. The microplates were incubated for 24 h at 37 °C. The lowest concentration of the extracts containing blue-purple indicator's colour was considered as MIC.

Fungal species were cultured on PDA at 28 °C from 48 h to 5 days. Obtained colonies covered with a small volume of 5% DMSO to obtain the suspension, and then a final concentration of inoculum suspension was adjusted with sterile normal saline to $5 \times 10^4$ CFU/mL in accordance with NCCLS recommendation (NCCLS, 2002a, 2002b). The concentration of the extracts was 40 mg/mL, 40 μg/mL for antimycotic nystatin, and 2 mg/mL for ellagic acid and catechin. MICs for fungal species were also determined in sterile 96 well plates (NCCLS, 2002a, 2002b). 50 μL of serially diluted extracts in SDB, 40 μL of SDB, and 10 μL of fungal suspension were added to each well, whereupon microplates were incubated at 28 °C for 48 h. MICs were determined as the lowest concentration of extracts without visible fungal growth.

*2.7. Evaluation of anti-inflammatory activity*

*2.7.1. COX-1 and COX-2 in vitro assays*

The inhibition of COX-1 and COX-2 enzymes were evaluated using *in vitro* assays in a 96-well plate with prostaglandin H synthase (PGHS)-1 from ram seminal vesicles for COX-1 and human recombinant PGHS-2 for COX-2 as previously described (Fiebich et al., 2005) with modifications published by Katanić et al. (2016). Briefly, 10 μL of extracts (50 μg/mL) dissolved in DMSO were added to the incubation mixture containing 180 μL of 0.1 M TRIS/HCl-buffer (pH 8.0), 5 μM hematin, 18 mM epinephrine hydrogen tartarate, 0.2 U enzyme preparation and 50 μM $Na_2EDTA$ (only for COX-2 assay) and allowed to stand for 5 min. Positive controls, indomethacin (1.25 μM, for COX-1) and NS-398 (5 μM, for COX-2) were also dissolved in DMSO. To start the reaction, 10 μL of 5 μM arachidonic acid in ethanol was added to the reaction mixture. After 20 min of incubation at 37 °C, the reaction was terminated by adding 10 μL of 10% formic acid.

The competitive $PGE_2$ EIA kit was applied for the determination of the $PGE_2$, the main arachidonic acid metabolite in this reaction. The microplate reader (Tecan Rainbow, Switzerland) was used for evaluation of EIA and the $PGE_2$ concentration was determined according to the method described by Fiebich et al. (2005). All experiments were performed in at least three independent experiments run in duplicate. Inhibition of COX refers to the reduction of $PGE_2$ formation in comparison to a blank without inhibitor.

*2.7.2. COX-2 gene expression assay*

COX-2 gene expression analysis was performed in accordance with the previously described method (Livak and Schmittgen, 2001) and with slight modifications described in Katanić et al. (2016). The differentiated human leukemic monocytic cell line THP-1 were treated with plant extracts (25 μg/mL) for 1 h and stimulated with 7.5 ng/mL final concentration LPS (lipopolysaccharide). Cells treated with DMSO (dimethylsulfoxide ⩽ 0.1%) were used as calibrator sample.

*2.8. Biocompatibility of extracts*

BalbC-3 T3 fibroblasts and human epidermal keratinocytes were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (streptomycin and penicillin) in a 5% $CO_2$ humidified atmosphere at 37 °C.

For biocompatibility experiments, cells were seeded in 96-well plates at a density of $2 \times 10^3$/well (HaCaT cells) and $3 \times 10^3$/well (BalbC-3 T3 cells). 24 h after seeding, increasing concentrations of the extracts (from 10 to 50 μg/mL) were added to the cells. After 48 and 72 h incubation, cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described by Del Giudice et al. (2015). Cell survival was expressed as the percentage of viable cells in the presence of the extract compared to controls. Two groups of cells were used as a control, *i.e.* cells untreated with the extract and cells supplemented with identical volumes of buffer. The average of the two control groups was used as 100%. Each sample was tested in three independent analyses, each carried out in triplicates.

*2.9. Statistical analysis*

The standard deviation was calculated using Microsoft Office Excel 2007 software and all results were expressed as mean values ± SD. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) using the OriginPro8 software package (OriginLab, Northampton, Massachusetts, USA) for Windows. The statistical significance was set at $p < 0.05$.

## 3. Results

*3.1. Phytochemical results*

We analysed the total phenolic compounds by spectrophotometry and HPLC-PDA to identify major phenolic components in the *A. vulgaris* extracts. Our results of the analysis of phenolic compounds in methanolic extracts of aerial parts and roots of *A. vulgaris* revealed a high content in

**Table 1**
Total phenolic compounds in methanolic extracts of aerial parts and roots of *A. vulgaris*.

| | mg GAEs/g d.w. | | | mg RUEs/g | mg CAEs/g | mg C3Gs/g | |
|---|---|---|---|---|---|---|---|
| Plant extracts | Total phenolics | Condensed tannins | Gallotannins | Total flavonoids | Phenolic acids | Total anthocyanins | Monomeric anthocyanins |
| AVA | $558.19 \pm 4.83^{a}$ | $386.70 \pm 6.82^{c}$ | $97.98 \pm 0.01^{e}$ | $13.30 \pm 1.69^{f,g}$ | $33.43 \pm 1.15^{h}$ | $8.41 \pm 0.17^{f,g,j}$ | $8.00 \pm 0.18^{f,j}$ |
| AVR | $442.32 \pm 22.31^{b}$ | $360.88 \pm 2.17^{d}$ | n.d. | $19.80 \pm 0.35^{g}$ | $57.36 \pm 5.18^{i}$ | $1.36 \pm 0.06^{j}$ | $0.95 \pm 0.07^{j}$ |

Results are expressed as mean values ± SD from three measurements; GAEs – gallic acid equivalents, RUEs – rutin equivalents, CAEs – caffeic acid equivalents, and C3Gs – cyanidine-3-glycoside equivalents per gram of dry weight of extract; n.d. – not detected; means with different symbol in superscript are significantly different at $p < 0.05$.

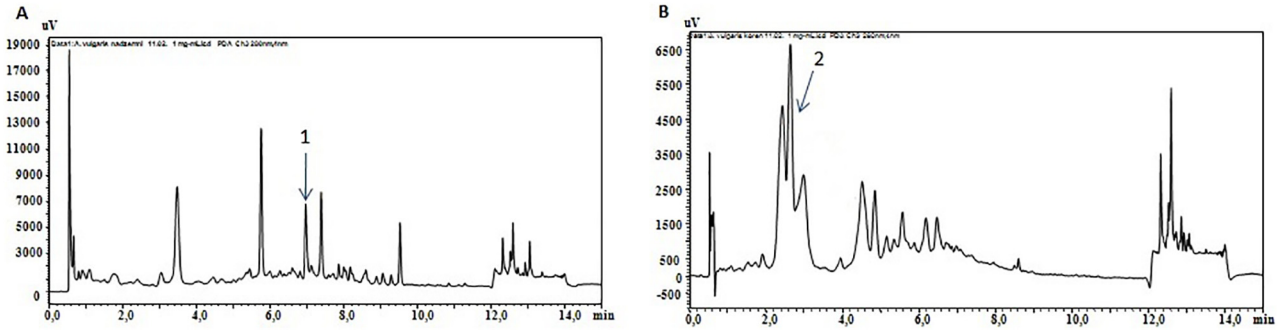


**Fig. 1.** HPLC-PDA chromatogram of *A. vulgaris* methanolic extract of aerial parts (A) and roots (B) recorded at 280 nm with tentatively identified ellagic acid (1) and (+)-catechin (2).

both extracts. As can be seen from Table 1, spectrophotometrical determination demonstrated significantly higher ($p < 0.05$) amount of total phenolics in AVA (558.27 mg GAEs/g) in comparison with AVR (442.32 mg GAEs/g). The most dominant phenolics in AVA were condensed tannins (proanthocyanidins) and gallotannins (386.70 and 97.80 mg GAEs/g), while AVR contained a slightly lower amount of condensed tannins (360.88 mg GAEs/g) and no detectable amount of gallotanins. On the contrary, the concentration of hydroxycinnamic acids derivatives was found to be significantly higher in the roots of *A. vulgaris,* while no significant difference in the content of flavonoids was observed. Anthocyanins content in the root extract was significantly lower ($p < 0.05$) when compared to all other examined classes of phenolic compounds. Fig. 1 presents HPLC-PDA chromatograms of AVA and AVR. Two of the peaks were tentatively identified as ellagic acid in the above ground parts and (+)-catechin in the roots extract by matching their retention times and comparing their UV-spectra at 260, 280, 325 and 330 nm. The identified compounds in the extracts were also confirmed by spiking the extracts with reference compounds.

### 3.2. Antioxidant activity

Because of the drawbacks of the individual use of any *in vitro* method for evaluation of antioxidant activity, several methods for screening the antioxidant potential of AVA and AVR have been employed and the results are reported in Table 2. The absorbance of green phosphate/Mo (V) complex formed at acidic pH was measured to evaluate the total antioxidant activity of the extracts. The obtained results showed that AVR exerts a higher total antioxidant activity than AVA (316.5 and 265.6 mg ascorbic acid/g, respectively). The potential ability of the extracts to neutralise free radicals was investigated using DPPH$^{\cdot}$, $ABTS^{\cdot+}$, and $^{\cdot}OH$ assays. In these assays, AVA showed better antiradical activity with significantly lower ($p < 0.05$) $IC_{50}$ values than AVR. Considering the reference antioxidants, AVA and AVR could scavenge DPPH$^{\cdot}$, $ABTS^{\cdot+}$, and $^{\cdot}OH$ radicals at significantly lower ($p < 0.05$) concentrations than the synthetic antioxidant butylated hydroxytoluene (BHT). Furthermore, there was no statistically significant difference in DPPH$^{\cdot}$ radical scavenging activities between AVA and the well-known phenolic

**Table 2**
Antioxidant capacity of the *A. vulgaris* aerial parts and roots methanolic extracts and standards: BHT, catechin, and ellagic acid.

| Plant extracts and standards | $IC_{50}$ value (μg/mL) | | | | Total antioxidant activity (mg AA/g of extract) | Reducing capacity (mg Trolox/g of extract) |
|---|---|---|---|---|---|---|
| | Radical scavenging activity | | | Inhibition of lipid peroxidation | | |
| | DPPH• | $ABTS^{+}$ | •OH | Oil-in-water system | | |
| AVA | 5.96 ± 0.21[a] | 14.80 ± 2.15[a] | 13.06 ± 0.97[a] | 31.91 ± 3.12[a] | 265.62 ± 12.10 | 632.99 ± 10.26 |
| AVR | 11.86 ± 0.56[b] | 32.49 ± 1.95[b] | 18.44 ± 1.11[b] | 475.13 ± 11.41[b] | 316.47 ± 18.71 | 607.52 ± 10.01 |
| BHT | 26.25 ± 1.9[c] | 44.67 ± 3.00[c] | 21.93 ± 0.47[c] | 4.53 ± 0.07[c] | n.t. | n.t. |
| Catechin | 7.52 ± 0.04[a] | 5.97 ± 0.16[d] | 6.32 ± 0.33[e] | 6.63 ± 0.11[c] | n.t. | n.t. |
| Ellagic acid | 3.54 ± 0.13[d] | 8.14 ± 0.18[d] | 7.18 ± 0.89[e] | 10.87 ± 0.50[c] | n.t. | n.t. |

AA – ascorbic acid; means in the same column with different symbol in superscript are significantly different at $p < 0.05$.

**Table 3**
Antibacterial activity of methanolic extracts of aerial parts and roots of *A. vulgaris.*

| Bacterial species | MIC values[a] | | | | |
|---|---|---|---|---|---|
| | AVA | AVR | Ellagic acid | Catechin | Erythromycin |
| *Micrococcus lysodeikticus* (ATCC 4698) | 0.156 | 0.156 | 0.5 | >1 | 20 |
| *Salmonella typhimurium* (ATCC 14028) | 0.625 | 0.625 | 0.5 | 0.5 | 20 |
| *Bacillus subtilis* (ATCC 6633) | 2.5 | 1.25 | 0.25 | 0.5 | 10 |
| *Enterococcus faecalis* (ATCC 29212) | 0.625 | 0.156 | 0.031 | >1 | 1.25 |
| *Escherichia coli* (ATCC 25922) | 1.25 | 1.25 | >1 | >1 | 5 |
| *Klebsiella pneumoniae* (ATCC 70063) | 5 | 10 | >1 | >1 | >20 |
| *Pseudomonas aeruginosa* (ATCC 10145) | 2.5 | 5 | 1 | 1 | 20 |
| *Bacillus mycoides* (FSB 1) | 0.625 | 0.156 | 0.016 | >1 | 1.25 |
| *Azotobacter chroococcum* (FSB 14) | 5 | 2.5 | 0.031 | >1 | 20 |

[a] MIC values, minimum inhibitory concentrations given as mg/mL for plant extracts, ellagic acid, and catechin, and as μg/mL for antibiotic erythromycin.

antioxidant catechin. The significant difference ($p < 0.05$) in antioxidant activity between the two extracts was also observed in an oil-in-water system, where AVR showed almost fifty times higher $IC_{50}$ value than AVA (475.1 and 31.9 μg/mL, respectively). Quite similar results for AVA and AVR were obtained in the reducing power assay (633.0 and 607.5 mg Trolox/g, respectively).

The ferrous ion chelating test was employed to estimate the ability of the extracts to chelate transition metals and to avoid the iron-overload and generation of free radicals. All tested samples failed to chelate $Fe^{2+}$ at concentration 1 mg/mL.

### 3.3. Antimicrobial activity

The results obtained for antimicrobial activity are shown in Tables 3 and 4. *Enterococcus faecalis*, *Salmonella typhimurium, Micrococcus lysodeikticus*, and *Bacilus mycoides* were the most sensitive examined bacterial species to the tested *A. vulgaris* extracts, with MICs between 0.156 and 0.625 mg/mL. On the contrary, *Klebsiella pneumoniae* was the most resistant bacteria in our study (MIC = 5 mg/mL for AVA and 10 mg/mL for AVR). MICs values above 1 mg/mL for AVA and AVR were also observed for *Pseudomonas aeruginosa, Bacillus subtilis, Azotobacter chroococcum*, and *Escherichia coli*. Ellagic acid and catechin were used as reference compounds. Catechin failed to inhibit the growth of all tested bacteria at concentrations lower than 0.5 mg/mL. We observed that ellagic acid has antibacterial potential against the same bacteria as the extracts, but MICs obtained for it were lower than those for the extracts for the majority of bacteria. The commercially available antibiotic erythromycin was more active against all tested bacteria than the investigated extracts, ellagic acid, and catechin, with MICs ranging from 1.25 to 20 μg/mL.

The investigated extracts showed similar activity against most of the tested fungi, with MICs from 2.5 to above 20 mg/mL (Table 4). AVA and AVR exhibited negligible antifungal activity against *Doratomyces stemonitis* (2.5 and 5 mg/mL, respectively) and *Aspergillus glaucus* (5 and 10 mg/mL, respectively). On the contrary, AVA failed to inhibit both *Trichoderma* species, while AVR did not show any antifungal effect against *Aspergillus brasiliensis* and *Alternaria alternata* at a concentration of 20 mg/mL. Moreover, both extracts did not inhibit the growth of *Candida albicans* at the highest tested concentration. Ellagic acid failed to inhibit the growth of the majority of the employed fungi at a concentration lower than 1 mg/mL, except for *P. canescens* (MIC = 0.25 mg/mL), while catechin did not exhibit any antifungal activity at the same concentration. Our results showed the lower activity of AVA, AVR, and reference compounds against all tested fungi in comparison with the antimycotic nystatin, which has demonstrated antifungal activity at concentrations from 0.078 up to 5 μg/mL.

**Table 4**
Antifungal activity of the methanolic extracts of aerial parts and roots of *A. vulgaris*.

| Fungal species | MIC[a] values | | | | |
|---|---|---|---|---|---|
| | AVA | AVR | Ellagic acid | Catechin | Nystatin |
| *Phialophora fastigiata* (FSB 81) | 10 | 20 | >1 | >1 | 1.25 |
| *Penicillium canescens* (FSB 24) | 20 | 20 | 0.25 | >1 | 2.5 |
| *Trichoderma viride* (FSB 11) | >20 | 20 | 1 | >1 | 0.078 |
| *Trichoderma longibrachiatum* (FSB 13) | >20 | 20 | >1 | >1 | 0.078 |
| *Aspergillus brasiliensis* (ATCC 16404) | 20 | >20 | >1 | >1 | 5 |
| *Aspergillus glaucus* (FSB 32) | 5 | 10 | 1 | >1 | 5 |
| *Fusarium oxysporum* (FSB 91) | 10 | 20 | 1 | >1 | 2.5 |
| *Alternaria alternata* (FSB 51) | 20 | >20 | >1 | >1 | 0.625 |
| *Doratomyces stemonitis* (FSB 41) | 2.5 | 5 | >1 | >1 | 2.5 |
| *Candida albicans* (ATCC 10259) | >20 | >20 | >1 | >1 | 0.625 |

[a] MIC values, minimum inhibitory concentrations given as mg/mL for plant extracts, ellagic acid, and catechin, and as μg/mL for antimycotic nystatin.

### 3.4. Anti-inflammatory activity

Fig. 2. represents results of COX-1 and COX-2 inhibition assays as well as COX-2 gene expression. Our results revealed that at a concentration of 50 μg/mL, AVA was capable of inhibiting the activity of COX-1 enzyme by 44.4%, whereas the inhibition of COX-2 was higher (63.6%). Similar results were observed for AVR (44.1% for COX-1 and 40.4% for COX-2). The tested extracts at a concentration of 25 μg/mL did not inhibit COX-2 gene expression.

### 3.5. Biocompatibility results

Finally, we analysed the biocompatibility of the extracts by performing a cell survival assay. The extracts were tested on immortalised murine BalbC-3 T3 fibroblasts and human normal HaCaT keratinocytes in a dose- and time-response test. As shown in Fig. 3, no significant differences in cell survival between control group and groups treated with extracts were observed. Indeed, both extracts showed total biocompatibility with the two cell lines after 48 and 72 h.

## 4. Discussion

Notwithstanding that species from genus *Alchemilla* have been used for many years in traditional medicine and are widely spread across Europe, only a few reports have focused on their chemical composition analysis. We tentatively identified ellagic acid in AVA and (+)-catechin in AVR by HPLC-PDA analysis. Our results supported previously published research, which indicated that ellagic acid is the major phenolic component in the aerial parts of *A. vulgaris* (Møller et al., 2009; Neagu et al., 2015; Ilić-Stojanović et al., 2017). To the best of our knowledge, phytochemical screening of *A. vulgaris* underground parts was performed only by Geiger et al. (1994). They found condensed tannins as major components (50% of the total tannins) of the 80% methanolic extract of *A. vulgaris* roots and confirmed the presence of ellagitannins (agrimoniin, pedunculagin, and laevigatin F) in the fresh aerial and underground parts as well. By spectrophotometric determination, we observed a high amount of phenolic compounds in both extracts; the extracts were especially rich in condensed tannins. Maier et al. (2017) found that the Lady's mantle herb extract contains about 30% w/w of tannins, which is in agreement with our findings. Ellagic acid manifests a broad spectrum of biological activity (Khanduja et al., 1999; Beserra et al., 2011). With regard to the high amount of phenolic compounds with proven health benefits, *A. vulgaris* can be considered as a promising medicinal plant.

Free radicals and other oxidants are responsible for the emergence of a large number of diseases, such as Parkinson's disease, cancer, cardiovascular, and obesity-related diseases (Lobo et al., 2010). There is increasing evidence that phenolics and other natural antioxidants from plants in the human diet may prevent, postpone and control the development of degenerative diseases (Consolini and Sarubbio, 2002; Dastmalchi et al., 2012; Costa et al., 2013; Batista et al., 2014). The results we obtained for antioxidant activity of examined extracts through seven methods undoubtedly demonstrated the strong antioxidant potential of extracts, comparable with reference compound catechin and even better than synthetic preservative (BHT) in certain antioxidant assays. AVA showed significantly better ($p < 0.05$) antioxidant activities in all employed methods in our study, except for total antioxidant activity. Furthermore, AVA demonstrated particularly better antioxidant activity in the inhibition of lipid peroxidation than AVR. The lowest $IC_{50}$ values for the extracts were recorded in DPPH assay. $IC_{50}$ values for the extracts were lower in comparison with those for BHT and they are in common with the results reported by Ilić-Stojanović et al. (2017), while no statistically significant difference ($p < 0.05$) was observed between AVA and catechin. Since numerous plant phenolics have been found to be responsible for biological properties, it can be assumed that antioxidant activities of these extracts are

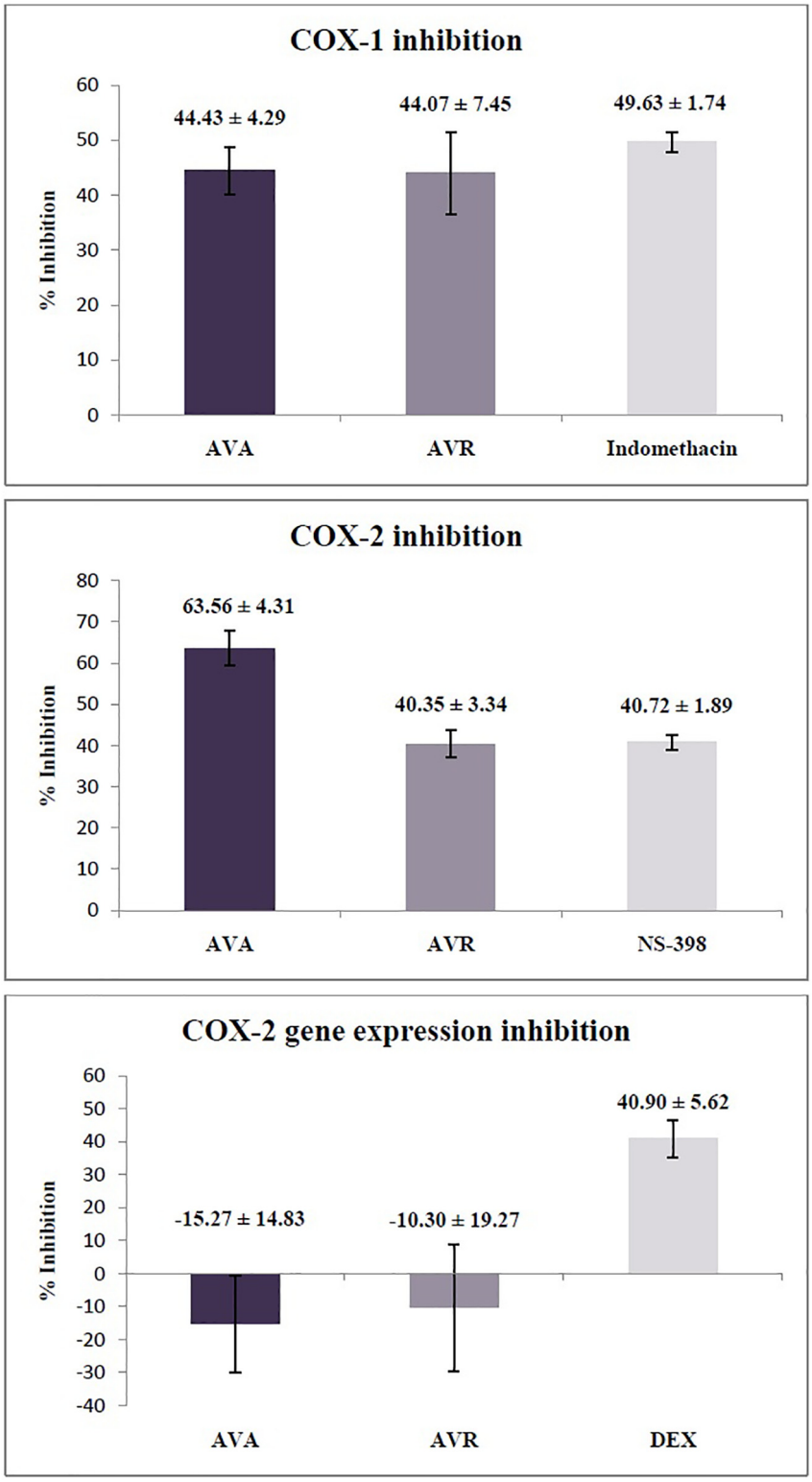


**Fig. 2.** COX-1 and COX-2 inhibitory activities and COX-2 gene expression on THP-1 of *A. vulgaris* extracts (50 and 25 μg/mL, respectively). Indomethacin, NS-398 and dexamethasone (DEX) were used as positive controls, according to Katanić et al. (2016). The graph represents compiled data (% inhibition) of two independent experiments (mean ± SD).

related to their phenolic profile. With respect to the high amount of polyphenols, strong antioxidant activity under *in vitro* and *in vivo* conditions was reported for other species from the Rosaceae family (Katanić et al., 2015; Jiménez-Aspee et al., 2016).

One of the main reasons to find novel natural sources of antioxidants is the fact that a large number of reactive oxygen species is produced during the inflammatory process (Conner and Grisham, 1996). Serious side effects of existing anti-inflammatory drugs are burning pharmaceutical concern worldwide. Therefore, research goes on to find new highly effective and harmless anti-inflammatory remedies of natural origin which can be alternatives to NSAID's. The undertaken study demonstrates, for the first time, the effects of *A. vulgaris* methanolic extracts on COX-1 and COX-2 enzymes inhibition, with the preferential COX-2 inhibitory activity of AVA with AVR approximately the same

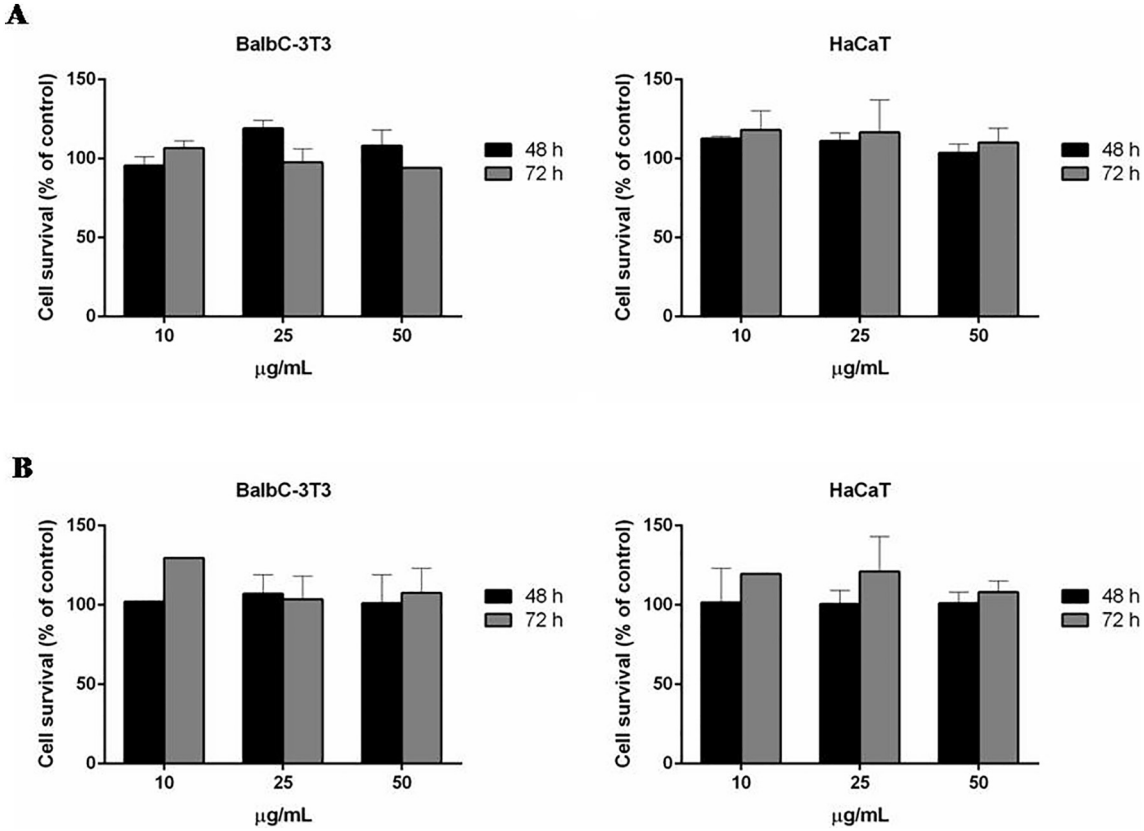


**Fig. 3.** Effects of *A. vulgaris* aboveground parts (A) and roots (B) methanol extracts on the viability of mouse immortalised BalbC-3 T3 fibroblasts and human normal HaCaT keratinocytes. Dose- and time-response curves of cells after 48 h (black bars) and 72 h (grey bars) incubation in the presence of increasing concentrations of the extracts. Cell viability was assessed by the MTT assay; the cell survival percentage was defined as described in Materials and Methods section. Values are given as means ± S.D. ($n \geq 3$).

inhibitory activity on both COX isoforms. Notwithstanding that AVR is not COX-2 specific, the relatively high percentage of the inhibition of COX enzymes is confirming the presence of anti-inflammatory compounds in this extract. Therefore, these results can be of great importance for further testing of Lady's mantle as a potential anti-inflammatory remedy. NF-κB is a nuclear transcription factor regulating the expression of various genes, including IL-1β, IL-6, TNF-α, and iNOS, which play critical roles in inflammation, apoptosis, and tumour genesis (Lawrence et al., 2001). Negative results obtained for NF-κB production in our study indicate that neither AVA nor AVR exert their anti-inflammatory activity through the inhibition of NF-κB. Anti-inflammatory activity of *A. vulgaris* has already been tested for the inhibition of 15-lipoxygenase activity (Trouillas et al., 2003), and the results provided a presumption that anti-inflammatory action of *A. vulgaris* may be related to the inhibitory activity of phenolic compounds on arachidonic acid metabolism through the lipoxygenase pathway. According to Şeker-Karatoprak et al. (2017), methanolic and water extracts of *A. mollis* decreased the nitrite as well as inhibited TNF-α production in LPS-induced macrophages. In support of the traditional use of *Alchemilla* species in wound treatment, Shrivastava et al. (2007) displayed acceleration in wound healing and a significant reduction in the size of dorsal skin lesions in rats by the second day of the treatment with 3% *A. vulgaris* in glycerine. Also, *A. vulgaris* enhanced proliferation of epithelial, liver and myofibroblasts cells as well. It is worth noting that no cytotoxic effect or any morphological changes were observed in the cells exposed to *A. vulgaris* extract in the above mentioned study. We obtained the same results in our study through biocompatibility assays on immortalised fibroblasts BalbC-3 T3 and normal epidermal HaCaT cells during 48 and 72 h. Fibroblasts and keratinocytes facilitate the protective role of normal skin and play a crucial role in cutaneous reparation process. When a wound occurs, then natural skin barrier is disrupted, which promotes the proliferation as well as the maturation of fibroblasts and keratinocytes. They migrate into the wound site and communicate *via* certain signalling loops, which support the restoration and regeneration of tissue homeostasis after wounding (Wojtowicz et al., 2014). Biocompatibility of plant extracts may prevent differentiation, proliferation, and attaching of these skin cells. The results of our study indicate that the application of AVA and AVR at doses ranging from 10 to 50 μg/mL provides good biocompatibility, with no toxic or injurious effects on the healthy cells.

According to Kuete (2010), an extract can be considered as a potent antibacterial agent with significant antibacterial activity with MICs below 0.1 mg/mL, while MICs between 0.1 and 0.625 pointed to moderate activity against bacterial growth. MICs above 0.625 mg/mL referred to weak activity. The results in Table 3 revealed that Gram-positive bacteria are more susceptible in comparison with Gram (−) ones. These findings are not surprising, considering the fact that Gram (−) bacteria are more resistant than Gram (+) ones to plant extract treatment, because of the porins and lipopolysaccharides present in their outer membrane, which provides a protective barrier and prevents intracellular penetration of antibiotics, especially lipophilic ones (Apetrei et al., 2011). To the extent of our knowledge, this is the first study covering antimicrobial activity of *Alchemilla* roots. Additionally, there are no literature data related to the antifungal activity of *Alchemilla* species, while only a few scientific reports provide information about activities of the aboveground parts of *Alchemilla* species against bacteria and *C. albicans*. Our results displayed negligible antifungal activity of *A. vulgaris* with MICs from 2.5 to above 20 mg/mL. Our results are in accordance with the results published by Keskin et al. (2010), indicated that ethanol extract of *A. vulgaris* at a concentration of 4 mg/mL exhibited moderate antibacterial activity against ten bacterial species, whereby the most sensitive ones were Gram-positive bacteria, including *E. faecalis*. Krivokuća et al. (2015) reported that extracts of four *Alchemilla* species exerted anti-*Helicobacter pylori* effect with MICs

ranging from 4 to 256 μg/mL. The same pattern of antibacterial activity as for AVR and AVA has been observed for ellagic acid, where the most sensitive strains were Gram positive bacteria *M. lysodeikticus*, *E. faecalis,* and *B. mycoides* and Gram negative – *A. chrococcum*. Our results showed low susceptibility of tested bacteria to catechin. Antibacterial effects of *A. vulgaris* may be attributed to the high content of tannins presented in the extracts (Djipa et al., 2000). With respect to the results of antibacterial activity of phenolic standards, our findings indicate that use of *A. vulgaris* extracts may be more beneficial than the application of individual compounds, due to the possible synergistic effects of the other components of the extracts.

## 5. Conclusion

Our results have demonstrated that methanolic extracts of aerial parts and roots of *A. vulgaris* are rich in phenolic compounds. Through the evaluation of antioxidant, antibacterial, antifungal, and anti-inflammatory activities, we observed the remarkable biological activity of the tested extracts, as well as their full biocompatibility with fibroblasts and keratinocytes. Taking into account promising biological activity and safety of *A. vulgaris*, our further research will be aimed to investigate its biological activities under *in vivo* conditions. We hope to discover the potential mechanism of biological action and to elucidate whether the specific biological activity is a result of the activity of individual components or synergistic action of several constituents.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgments

This research is financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant No. 43004).

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