Correlation between chemical composition and topical safety and efficacy of *Alchemilla vulgaris* L. extract in emulsion vehicle

Marija Tasić-Kostov[1] ✉
Ivana Nešić [1]
VanjaTadić [2]
Dragana Pavlović [1]

[1] Faculty of Medicine
University of Niš
Department of Pharmacy
Niš, Serbia

[2] Institute for Medicinal Plant Research
"Dr Josif Pančić"
Department of pharmaceutical research
and development
Belgrade, Serbia

✉ CORRESPONDING AUTHOR:
Marija Tasić-Kostov, PhD,
Associate professor
Department of Pharmacy,
University of Niš-Faculty of Medicine
81 Blvd. Dr Zoran Djindjić,
18000 Niš, Serbia
Tel: (+38163) 1045488
Fax: (+38118) 4238770
e-mail: marijatk@medfak.ni.ac.rs

Received: February 2, 2023
Accepted: December 14, 2023

Medicinal plant extracts represent one of the largest categories of topical actives in the marketplace today due to the increasing consumer demand for natural products. Regarding the traditional application of Lady's mantle (*Alchemilla vulgaris* L., Rosaceae) in dermatological disorders, the aim of this study was to investigate its skin safety and efficacy as anti-irritant topical active incorporated into emulsion vehicle, and possible connection to the chemical composition of the extract. The total phenols, tannins and flavonoids content of leaf ethanol extract were determined. HPLC analysis revealed the significant presence of ellagic acid and its derivatives and quercetin derivatives. Further, we investigated the effects of creams formulated with chemically characterised *A. vulgaris* extract containing the natural alkyl polyglucoside (APG) emulsifier on skin. The safety and efficacy of creams was assessed estimating their antioxidant activity *in vitro* and *in vivo* safety. Then, *in vivo* sodium lauryl sulfate test was applied on artificially irritated skin of human volunteers. Lady's mantle extract *per se* showed satisfying lipid peroxidation inhibition potential. Creams based on APG emulsifiers with investigated extract showed significant anti-irritant effect in an *in vivo* double blind randomised study, and safety, suggesting that it could be used in anti-irritant topical phytopharmaceutical emulsions which protect skin against damage caused by free radicals and reactive oxygen species.
Keywords: Lady's mantle ethanol extract; quercetin derivatives; ellagic acid derivatives; antioxidant potential; safety and efficacy of topical phytopreparation.

INTRODUCTION

Due to the rising demands of modern consumers for natural products, plant extracts have become one of the largest categories of cosmetic actives found in the marketplace today [1]. At the same time, one of the most important goals of modern dermatology is to improve and strengthen prevention, diagnosis, and treatment of diseases, which could be accomplished by taking advantage of specific properties of the substances originated from plants. Aside consumers' requests, the popularity of phytopreparations for topical use (both cosmetics and dermopharmaceutics) probably lies in the fact that naturally occurring mixtures of active compounds in plants might be more effective than individual molecules and manufactured combinations of synthetic products [2, 3].

In recent years, an increasing scientific attention has been given to investigations of antioxidant activity of plant extracts [4], which might be closely related to their polyphenol content. Apart of the scavenging UV-induced radicals and inhibiting propagation of lipid peroxidative chain reactions, flavonoids, as a class of polyphenol compounds, might provide the protective effect against UV radiation by acting as strong UV-absorbing screens [5]. On the other hand, skin aging (both intrinsic and extrinsic), and some systemic diseases result in dry skin characterised by the inflammation and loss of elasticity.

These manifestations might be closely connected to oxidative stress and a skin-barrier defect which leads to a consequent loss of water from the *stratum corneum* (SC) [5, 6]. Treatment with moisturising emulsions (creams) and antioxidant agents is normally of great benefit to dry skin, helping to achieve a healthy SC which then is capable to form an effective permeability barrier, restricting the water loss from the body and blocking the penetration of harmful irritants and allergens [6, 7].

It has been widely accepted that topically applied antioxidants may be effective in the treatment of photo-aged skin [7]. However, in contrast to extensive scientific data on plant antioxidants' activity *per se*, there are only few studies dealing with the efficacy of these actives after they have been incorporated into emulsion vehicles. Most of these studies pointed out some problematic issues, such as potential loss of antioxidant activity which seemed to be dependent on the composition of the vehicle [8].

Skin irritation is one of the most common adverse effects of topicals [9]. Regulation (EC) 1223/2009 on cosmetics, which fully came into force in July 2013, requires evidence of safety profile of a cosmetic product when applied under normal and foreseeable conditions of use, as one of the main demands for such product to be placed onto the market. Hence, the importance of investigations of the potential for causing local (skin and eyes) adverse effects is emphasised [10, 11]. In the case of dermopharmaceutics, tests performed to predict skin irritation are regularly included during the new drug development and application process [12]. It is important to stress that mildness has become one of the most important features claimed for topical products [13]. Earlier, skin irritation profile of cosmetic actives, including plant extracts, and final products was assessed routinely and reliably using *in vivo* animal testing. But the ethical concerns prohibited the sale on the EU market of any cosmetic product that has been tested on animals or using any alternative methods other than validated ones [9]. *In vivo* tests on humans were used in our study [12].

Common Lady's mantle, *Alchemilla vulgaris* L., Rosaceae*,* is a perennial herbaceous plant widespread throughout Europe [14]. It is known for its astringent and anti-inflammatory properties and traditionally it has been used to treat ulcers, eczema, and skin rashes [15]. Published data indicated that the aerial part of the *A. vulgaris* contained the complex of diverse biologically active substances, dominated by phenolic substances (flavonoids, coumarols and polyphenols, including phenylcarbonic acids, up to 9.6%), depending on the developmental phase and site of the plant collection [15].

Taking into account the abovementioned challenges, the current study had the following objectives: a) chemical characterisation of *A. vulgaris* extract used in the investigation employing HPLC method, giving the insight in its total phenols, flavonoids and tannins content and b) *in vivo* assessment of skin irritation profile and *in vivo/in vitro* assessment of efficacy of the extract *per se* and after incorporation into emulsion vehicle based on natural alkyl polyglucoside (APG) emulsifier, as a starting point in evaluation of the potential protective effect of this extract in oxidative stress-mediated skin disorders [16]. To investigate *A. vulgaris* extract for cosmetic applications, we have followed the demands of new Regulation (EC) 1223/2009 on cosmetics. The efficacy of *A. vulgaris* extract in maintaining the normal skin properties was assessed as *in vivo* effects of O/W emulsions containing investigated extracts in two concentrations, 1 and 2% on the sodium lauryl sulphate (SLS)-irritated human skin [17, 18]. The potential of the investigated extract to affect the dry skin, i.e. to reverse skin barrier function and regulate disturbed skin's pH alongside with its hydration skin effects were accomplished by means of non-invasive biophysical measurements assessing the following parameters: transepidermal water loss (TEWL), pH of the skin and electrical capacitance (EC) as a measure of skin hydration level. We aimed to estimate whether the investigated plant extract in emulsion vehicles could be promoted as a safe and effective antioxidant topical active, taking into account the aspects of the skin safety of the final formulation and the influence of the emulsion vehicle on its topical activity, with regard to investigated chemical composition of the extract.

EXPERIMENTAL PART

GENERAL

Sodium bicarbonate (analytical grade), DPPH (1,1'-diphenyl-2-picrylhydrazyl) (analytical grade), trolox (analytical grade) and Folin-Ciocalteu reagent were purchased from Sigma Aldrich, Saint Luis, MO, USA. Analytical grade reagents 2,6-di-*tert*-butyl-4-methylphenol (BHT), absolute ethanol (96%, v/v) were purchased from Merck (Darmstadt, Germany). Acetonitrile (MeCN), water and methanol were of HPLC grade, and they were purchased from Merck (Darmstadt, Germany). Reference HPLC standards isoquercitrin, rutin, hyperoside, apigenin-7-*O*-glucoside, quercetin and ellagic acid were purchased from Sigma Aldrich, St. Louis, MO, or from Extrasynthese (Genay, France). Their purity was declared as >98%, based on the manufacturer's internal high-precision HPLC method. Linoleic acid, ammonium thiocyanate, Tween 20, SLS and phosphate buffer were purchased from Sigma-Aldrich.

Preparation of A. vulgaris extract

The extraction was performed in the Soxhlet apparatus, until the dried and powdered plant drug of *A. vulgaris folium* was completely exhausted, using 70 vol% of ethanol. Extraction was performed at the temperature of 60°C, with the continuous work of circulatory and uniform flow of the solvent. The extract

was put in the receiving container and the solvent was evaporated at the temperature of 65°C, under vacuum, to a volume giving the extract with dry residue with the value of more than 60%.

DETERMINATION OF TOTAL PHENOLS CONTENT (TPC)

The TPC was determined by the Folin-Ciocalteu method [29]. One hundred microliters of MeOH solution of the investigated sample (prepared concentration of stock solution was 2.5 mg/mL) was mixed with 0.75 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22°C for 5 min; 0.75 mL of sodium bicarbonate (60 g/L) solution was added to mixture. After 90 min at 22°C, absorbance was measured at 725 nm. Gallic acid (0-100mg/L) was used for calibration of a standard curve. The calibration curve showed the linear regression at $r > 0.99$, and the results are expressed as milligrams of gallic acid equivalents per gram of the extract dry weight (mg GAE/g DW). Triplicate measurements were taken, and data were presented as mean ± standard deviation (SD).

TOTAL FLAVONOIDS CONTENT (TFC)

The percentage of TFC was estimated using the method described in DAB 10 [30]. Briefly, the sample (502.7mg) was extracted with acetone/HCl under a reflux condenser; the absorbance of $AlCl_3$ complex of flavonoid fraction extracted by ethyl acetate was measured by UV-VIS Spectrophotometer HP 8453 at 425 nm. The content of flavonoid, expressed as hyperoside percentage, presented the mean ± standard deviation of three determinations.

TANNINS CONTENT (TC)

The percentage of TC was achieved using the method described in the European Pharmacopoeia, Ph. Eur. 9.0 [31]. Shortly, decoctions, prepared from the investigated samples, were treated with phosphomolybdotungstic reagent in alkaline medium after and without treatment with hide powder. The absorbance was measured by UV-VIS Spectrophotometer HP 8453 (Agilent Technologies, USA), at λ_{max} 760 nm. From the difference in absorbance of total polyphenols and polyphenols not adsorbed by hide powder, the percentage content of tannins expressed as pyrogallol (%, w/w), were calculated from the expression:

$$\frac{62.5\,(A^1 - A_2) \times m_2}{A_3 \times m_1}$$

where m_1 represents mass of the sample to be examined, in grams; and m_2 is mass of pyrogallol, in grams. The results represent the mean ± SD of three determinations.

HPLC FINGERPRINT

HPLC fingerprint of the investigated sample was achieved by HPLC (Agilent Technologies 1200). Detection was performed using a Diode Array Detector (DAD), and the chromatograms were recorded at λ = 360 nm (isoquercitrin, rutin, hyperoside, apigenin-7-*O*-glucoside, quercetin and ellagic acid). HPLC separation of components was achieved using a LiChrospher 100 RP 18e (5 μm), 250 × 4 mm i.d. column, with a flow rate of 0.8 mL/min and mobile phase, A [1% H_3PO_4 (w/w) in water)], B (MeCN), elution, combination of gradient mode: 89-75% A, 0-35 min; 75-60% A, 35-55 min; 60-35% A, 55-60 min; 35-0% A, 60-70 min). A portion of the sample solution (15.9 mg/mL EtOH, DW = 63.2%), previously prepared as described, was filtered through 0.2 μm PTFE filters (Fisher, Pittsburgh, PA) prior to HPLC analysis. The injected volume was 4 μL for all prepared solutions of extract and the standards. Standard solutions for the determination of polyphenolic compounds were prepared in EtOH, with the concentrations for isoquercitrin 0.22 mg/mL, hyperoside 0.25 mg/mL, rutin 0.45 mg/mL, apigenin-7-*O*-glucoside 0.18 mg/mL, quercetin 0.1 mg/mL and ellagic acid 0.1 mg/mL. The identification was carried out thanks to retention time and spectra matching. Once spectra matching succeeded, results were confirmed by means of the so-called peak purity test, meaning that each peak was tested for purity by a three-point purity test and for the similarity by a library search comparing the peak spectrum to that of the standards. High similarity index and a common retention time with the standard were considered a positive identification. Those peaks not fulfilling the requirements were not quantified; a similar UV/VIS absorption spectrum but different retention time was considered as a partial identification (derivative of the phenolic compound with the similar absorption spectrum). Under the conditions employed in this study, the relative standard deviation for the retention times in three repetitive runs was in the range of 0.18 - 1.79%. Quantification was performed by external calibration with corresponding standards. The results represent the mean ± SD of three determinations.

PREPARATION OF EMULSION SAMPLES

Emulsion samples based on C14-22 Alcohols & C12-20 Alkyl Glucoside (Montanov L®, Seppic, France) as an emulsifier in a concentration of 8% (w/w) and Cocoglucoside & Coconut Alcohol (Montanov S®, Seppic, France) as a coemulsifier in a concentration of 2% (w/w). Emulsion samples were labelled as P-placebo (cream without extract), C1-cream containing 1% of *A. vulgaris* extract and C2- cream containing 2% of *A. vulgaris* extract. The samples also contained 18.5% (w/w) of caprylic-capric triglycerides and 1.5% of cetearyl alcohol as an oil phase, purified water, glycerol as a humectant in the concentration of 5% (w/w). Samples were adequately preserved using Dekaben C (Jan Dekker International, Netherlands) preservative blend.

The samples were manufactured in a manner that the

oil phase (which was heated to 70°C) was added to the water phase (75°C) and mixed with the laboratory stirrer (stirrer RW16 basic, IKA®WERKE, Germany) at 800 rpm for 3 min. Then, the mixing continued at a lower speed - 500 rpm for 3 min. During this process the temperature was constantly maintained at 70°C. After that, samples were mixed at 500 rpm for 1 min and at 400 rpm until they were cooled down, at room temperature.

A. vulgaris extract was mixed with glycerine and the mixture was incorporated into the cooled emulsions. Then, the samples were well homogenised and allowed to equilibrate for 24 h prior to use in the study.

DETERMINATION OF ANTIOXIDANT CAPACITY OF A. VULGARIS EXTRACT PER SE AND IN EMULSION SAMPLES

The antioxidant activity was estimated by determination of the inhibitory activity towards lipid peroxidation using the thiocyanate method [32]. The study was carried out with the stock solution (20 mg/ml) of the extract. The cream formulations containing 1.0 and 2.0% of *A. vulgaris* extract were first diluted 1:3 with the extraction solution (Tween 20/water 1:5, w/w) and mixed for 15 min at 400 rpm using the laboratory stirrer, at the temperature of 50°C. The obtained samples were used as stock solutions for further investigation and were kept for 20 min prior to the measurement of their antioxidant activity [25].

A 0.5 mL aliquot of stock solution was added to linoleic acid emulsion (2.5 mL, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween 20 as emulsifier in 50mL 40mM phosphate buffer and the mixture was then homogenised. The final volume was adjusted to 5mL with 40mM phosphate buffer, pH 7.0.

After incubation at 37°C in the dark for 72 h, a 0.1 mL aliquot of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL $FeCl_2$ (20 mM), and 0.1 mL ammonium thiocyanate (30%). The absorbance of this mixture was measured at 500 nm, after it was stirred for 3 min. BHT was used as a reference compound. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion [8].

The inhibition percent of linoleic acid peroxidation was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\Delta Ac - \Delta As}{\Delta Ac} \times 100$$

where ΔAc represented the difference between absorbance values of the control before and after incubation, and ΔAs represented the difference between absorbance values of the sample before and after incubation.

IN VIVO SKIN PERFORMANCE

Two different *in vivo* studies were conducted – safety and efficacy study. *In vivo* skin effects of the samples P, C1 and C2 were assessed via three skin parameters - TEWL, pH and *stratum corneum* hydration (SCH) in both studies. SCH was measured as EC (electrical capacitance).

Since the increase in TEWL is observed after the application of skin irritants, TEWL measurements are often used to support the cosmetic claims of product mildness [27]. In the safety study, parameters were measured prior to (baseline values on the first day of the experiment) and 60 min upon cessation of 24h occlusive treatment (second day of the experiment). As many as 21 healthy female volunteers (28.4±5.9 years) were recruited. The flexor side of their left forearm was treated with the placebo (P sample) while the right forearm was treated with the C1 and C2 using a precisely delineated and marked cardboard ruler (with two empty spaces in the form of rectangles, 16 cm^2 each). Two additional sites were left as non-treated control under occlusion (UCO) on the right and without occlusion (UC) on the left forearm. Samples were applied in quantities of 0.016 g/cm^2, spread vigorously with a rubber glove, and immediately covered with Parafilm® and then with cotton adhesive tapes. All parameters were measured according to the published guidelines and documents [27]

To estimate the efficacy of the same samples in terms of their effects on biophysical parameters on artificially dried skin (i.e. on skin pretreated with SLS), an additional group of 16 healthy volunteers (24.2±1.6 years) was recruited. SLS in a closed patch test is frequently used for the experimental induction of skin inflammation and dryness which can be evaluated using non-invasive biophysical measurements [17]. Namely, 75µL of the irritant (aqueous SLS, 12%, purity of SLS >99%, Merck, Darmstadt, Germany) was applied under the patch occlusion for 6h on four places of both forearms. pplication was performed using filter paper (9 cm^2), covered with Parafilm® and then fixed with cotton adhesive Sensifix® tapes. Baseline values were taken prior to the sample application and the outcome was removed 24 hours after the occlusion.

Then, 24 hours after an exposure to SLS, the volunteers started to treat exposed skin sites with test samples to study and compare the modification of provoked dryness by different samples. The samples were marked with differently coloured labels, and the volunteers were given clear instructions regarding the amount, type of samples and frequency of applications (morning and evening). One site on each arm in both groups was left as an untreated control, whereby it was SLS-treated on the left forearm (UCO), and without treatment on the right (UC).

The measurements of EC, TEWL and pH were performed before and 24h after SLS irritation, as well as after 2 and 5 days of the treatment.

In vivo measurements were performed in accordance with the Declaration of Helsinki, and the volunteers signed a written consent. They were thoroughly in-

formed of the study and instructed not to use any skin cleansing or skin care products on the test sites the week prior to the study as well as throughout the experiment. The study was approved by the local Ethical Committee on Human Research (No 12-6316-2/7 from 16.6.2016.). All subjects had healthy skin and no known allergy to any ingredient of the samples. Before any measurements were taken, the subjects were asked to acclimatise for 30 min under controlled conditions (21±1°C and 50±5% RH). TEWL was measured using Tewameter®TM 210, pH using pHmeter®900 and EC by means of Corneometer®CM 825; all probes are part of Multi Probe Adapter MPA®9 (Courage & Khazaka Electronic GmbH, Germany).

STATISTICAL ANALYSIS

All data were presented as means +/- standard error of the means (SEM). When evaluating the safety of the samples, parameters (EC, TEWL, pH) were expressed as absolute changes to baseline (Δ values) second versus first day. *In vivo* effects of the samples were compared mutually and related to untreated controls, under and without occlusion (UC and UCO), using the one-way ANOVA, followed by Tukey's *t*-test, where appropriate. The same test was used for comparison of the antioxidant activities (measured by both methods) of the emulsion samples.

When evaluating the efficacy of the samples on pre-irritated skin, the values of measured parameters after SLS irritation, after 2 and 5 days of application were compared to appropriate baseline values using a paired sample t-test. Data obtained from skin sites treated with different gels were compared mutually, to both untreated controls as well as to the placebo sample using t-test for unpaired data.

The differences were accepted as statistically significant at $p < 0.05$. Statistical analysis was performed with commercial statistical software SPSS for Windows 17.0.

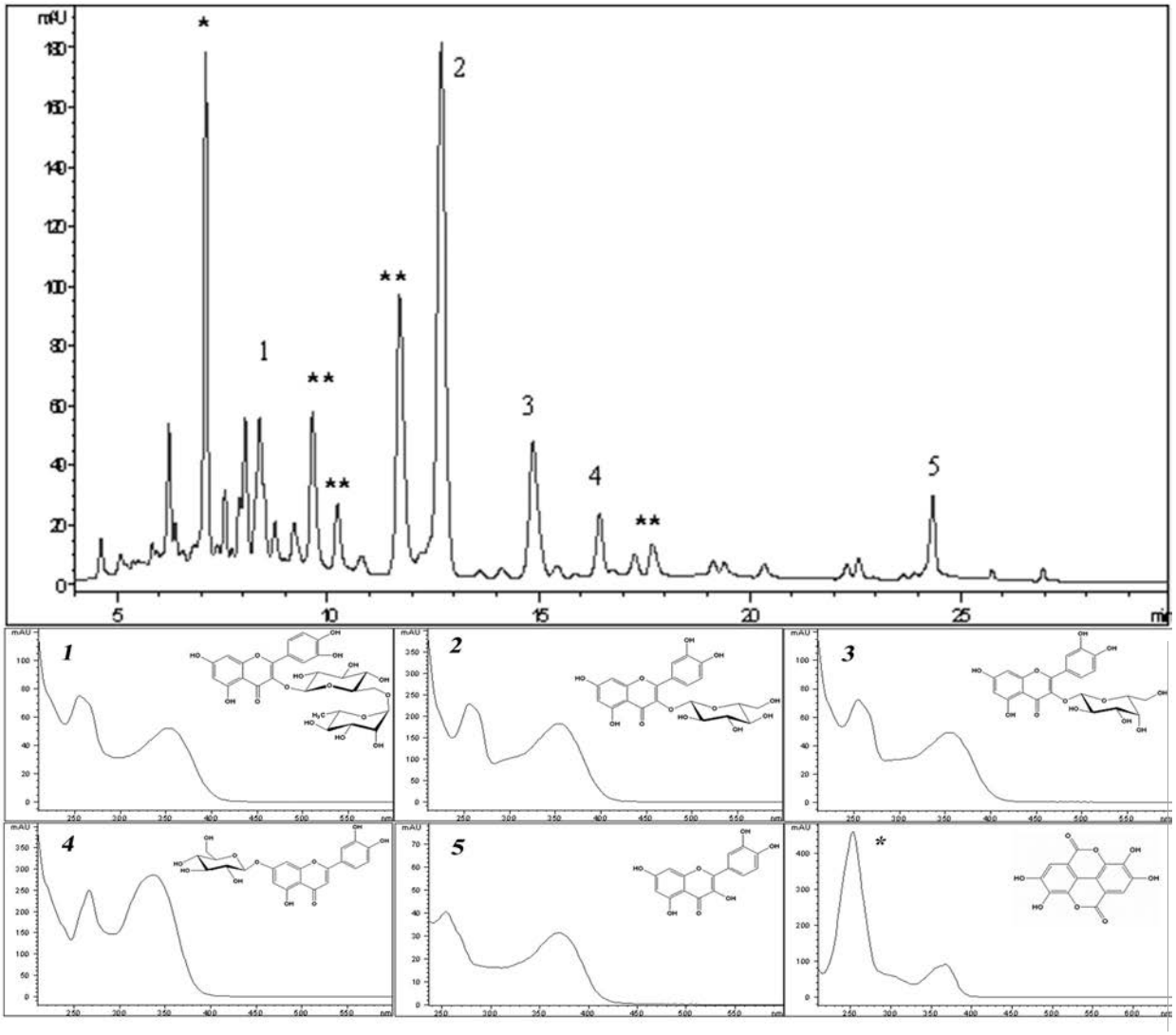

Figure 1 - Chemical profile obtained by HPLC analysis with the identified flavonoids in the lady's mantle (*Alchemilla vulgaris*) investigated extract: 1. rutin; 2. isoquercitrin; 3. hyperoside; 4. apigenin-7-*O*-glucoside; 5. quercetin; * ellagic acid derivatives

RESULTS AND DISCUSSION

HPLC profile of the investigated extract of A.vulgaris is presented in Table I and Figure 1. The following flavonoid compounds were identified: rutin, isoquercitrin, hyperoside, apigenin-7-O-glucoside, quercetin and ellagic acid and its derivatives.

Polyphenolic compounds are an important group of plant bioactives which exhibit various biological activities, including antioxidative and anti-inflammatory [19-21]. The presence of a relatively high content of ellagic acid and its derivatives, polyphenol compounds in the investigated extract of A.vulgaris, (Figure 1) might contribute to the in vivo effects observed in this study. Moreover, the presence of quercetin is of particular importance regarding that flavonoid with its analogue, isoquercitrin, are among the most potent antioxidants [19]. Moreover, it was shown that topical formulations containing quercetin are highly effective in the protection from oxidative stress-induced skin damages [20, 21].

Namely, ellagic acid and its derivatives have recently received attention as the agents that may have potential bioactivities preventing chronic diseases. Bae et al. [22] examined the effects of ellagic acid on collagen breakdown and inflammatory responses in UV (ultraviolet)-B irradiated human skin cells and hairless mice and revealed its photoprotective effects on the skin wrinkle formation. Moreover, ellagic acid may be effective against inflammation, may have a prolonged onset and duration of action, and may interact with cyclooxygenase inhibitors.

The topical application of plant extracts with a strong antioxidant activity could protect the skin against the toxic effects of reactive oxygen species (ROS), chemically reactive species containing oxygen [23]. Regarding that polyunsaturated fatty acids (PUFA) are accessible to peroxidation; it is one of the most investigated consequences of ROS action on cell membrane structure and function [24]. In fact, inhibition of lipid peroxidation seems to be the method of choice to determine the antioxidant capacity of topically applied preparations.

The results obtained for the evaluation of antioxidant activity of the investigated extract and creams C1 (cream containing 1% of *Alchemilla vulgaris* extract) and C2 (cream containing 2% of *Alchemilla vulgaris* extract) were given in Table II. The capacity of the *A. vulgaris* extract to inhibit lipid peroxidation was notable; it remained almost unchanged after its incorporation in APG-based vehicle in both concentrations (Table II), showing that a satisfying efficacy in oxidative stress-mediated skin disorders could be reached at 1% of extract in vehicle, without need for a further increase of concentration. That could be of great benefit, having in mind that even the vitamin E, one of the most effective and frequently used antioxidants active in commercial formulations, often lose its activity after incorporation into an emulsion carrier (cream) [25].

It was previously shown that the emulsions and creams with antioxidants reached a low degree of inhibition of the oxidative reaction in the experimental system if they had not been correctly solubilised in the mixture. Proper homogenisation with an adequate extraction solution is necessary for measuring the antioxidant capacity of emulsion samples [8, 25]. In this study, we used the extraction solution (Tween-20/ H_2O 1:5, w/w) to provide better solubilisation of the actives.

So, the following could be assumed from our antioxidant activity evaluation: cetearyl glucoside and cetearyl alcohol-based emulsion could be an eligible vehicle for *A.vulgaris* in order to reach its antioxidant activity; but also the methodologies in this study (used on *A.vulgaris* extract) are appropriate to analyse the antioxidant activity of herbal extracts when incorporated in the emulsion vehicle. Naturally, interferences with the colloidal structure of the vehicle might occur, but they affected neither the antioxidant activity of extract nor its determination.

Regarding the *In vivo* skin performance study, all participants reported their strict compliance with the instructions given initially.

Irritants have been indicated to induce the release of ROS to the skin even at non-cytotoxic concentrations

Table I - The chemical characterization of the investigated extract of *A.vulgaris*

Extract *Alchemilla vulgaris*		
Total flavonoids (expressed as hyperoside percentage**)**		4.1
Total phenol content (mg GAE/g DW)		314.2
Tannins (%)		4.12
DW (dry weight, %)		63.2
Identified compounds by HPLC (their abundance expressed in mg/g DW of the investigated extract)		
1	Rutin	4.8
2	Isoquercitrin	17.8
3	Hyperoside	4.1
4	Apigenin-7-*O*-glucoside	3.4
5	Quercetin	1.4
*	Ellagic acid an dits derivatives expressed as ellagic acid	21.1

Table II - Antioxidant activities of *Alchemilla vulgaris* L. extract and creams C1 and C2; results are given as means ± S.D.[a]

Sample	Antioxidant activity ±SD (%)
Alchemilla vulgaris L. extract	60,0 ± 3,27
C1	58,82 ± 0,37
C2	58,83 ± 0,50

[26], so it is extremely important to establish the safety profile of formulations intended for an application on skin, particularly if it is already damaged. The *in vivo* skin safety profile was investigated in this study; absolute changes of the *in vivo* measured parameters (TEWL, pH and EC) after 24h occlusion related to the baseline values for the investigated samples, as well as for both controls, were presented in Figure 2.

Creams containing *A. vulgaris* extract 1% and 2% investigated in this study are not expected to affect the skin barrier and cause an immediate irritation of the skin. That is, the application of all test samples (Figure 2) increased the TEWL values after 24h, but no statistically significant change was found. The increase of TEWL, which normally indicate the skin barrier damage, could be here attributed to the actual occlusion [27]. This could support the claim that creams with *Alchemilla vulgaris* L. extract are not expected to affect the skin barrier and cause an immediate irritation of the skin.

Regarding the efficacy investigations, the in vivo effects of the creams on the human skin pretreated with SLS were investigated. The results are shown in Table III.

Bearing in mind that oxidative stress can alter the cellular hydration state [28], it could be a great additional benefit for antioxidant cosmetics to exhibit a satisfying skin moisturising potential. The results obtained for skin hydration (EC) of the investigated samples in the safety study (Figure 2) revealed a good correlation with the EC values obtained for the treatment with investigated samples of previously SLS dried skin (Table III). Skin treated with test sample C2 exhibited a significant increase in EC after 24h in comparison with both controls. Since the increase in this parameter was not significant for skin treated with a sample containing 1% of the extract (sample C1), it could be presumed that the *A. vulgaris* extract beneficially affects the skin moisturising potential of creams containing it in 2%.

TEWL, as a measure of the level of damage to the skin barrier, showed a statistically significant increase at all skin sites treated with SLS. Then, there was a decrease in TEWL after two days on all treated skin sites regardless the treatment (P, C1 or C2) while on

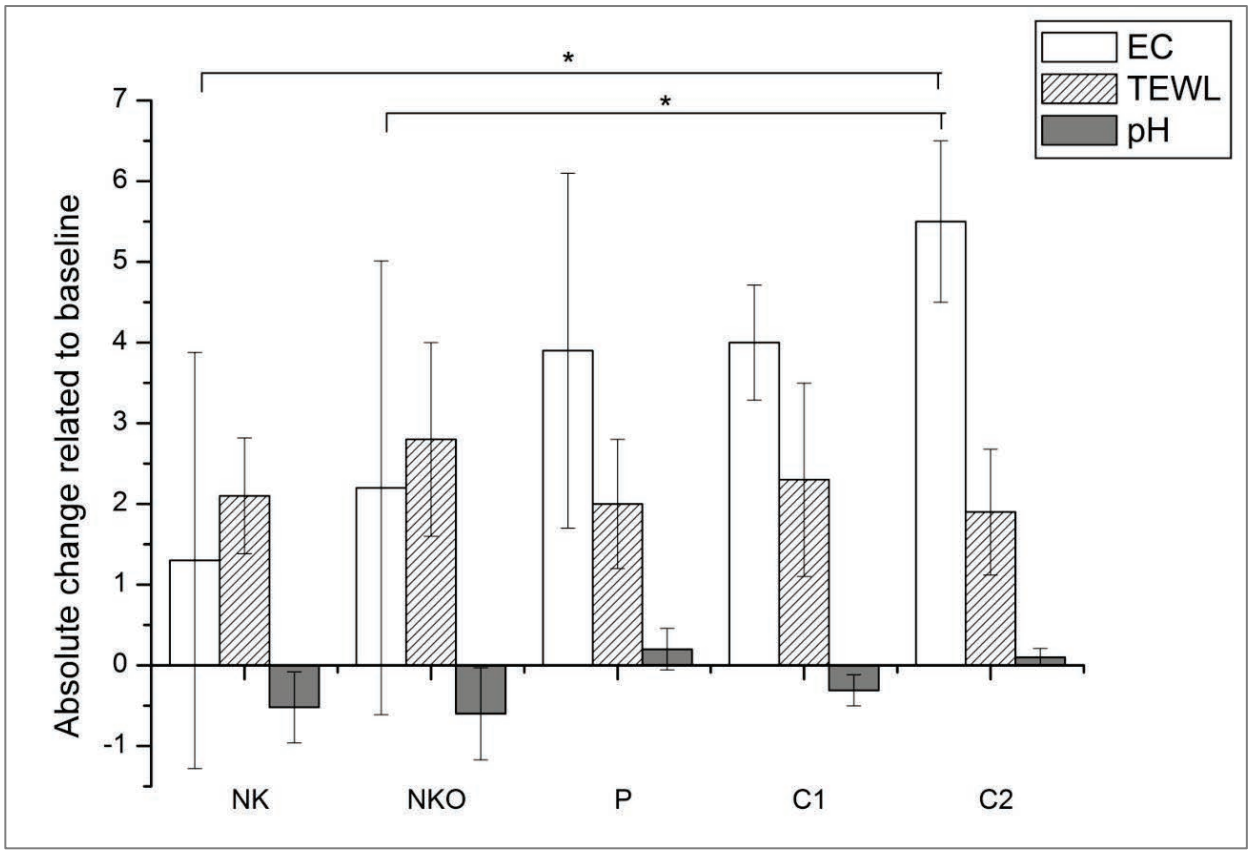

Figure 2 - The influence of the investigated samples on *in vivo* measured skin parameters: EC (as a measure of SCH), TEWL and pH. The effects of different samples were compared mutually and related to baseline as well as to UC and UCO. Significant differences were marked with *p<0.05.

Table III - The influence of the irritation *per se* and investigated samples after irritation on biophysical skin parameters: TEWL, pH and EC

Sample	Baseline(a)	Upon SLS irritation(b)	Upon 2 days treatment(c)	Upon 5 days treatment(d)	p-values (repeated measures ANOVA)
TEWL					
Control (UCO)	7,5±1,5	11,5±3,2^{UC}	10,7±3,2^{UCO}	7,5±1,8^{UC,P,C1,C2}	0,05*;a:b;a:c;b:d;c:d
Control (UC)	6,7±2,5	7,0±1,3^{UCO,C2}	7,2±1,7^{UC}	6,9±2,3	0,841
P	6,7±2,0	9,7±2,9	8,5±2,5	6,8±2,6	0,005*;a:b;b:d
C1	6,4±1,9	10,2±3,1^{UC}	7,8±2,0	6,3±2,5	0,005*;a:b;b:c;b:d
C2	6,6±1,7	9,7±2,8	8,7±2,9	6,7±1,5	0,01*;a:b; b:d
p-values (one-way ANOVA)	0,679	0,004#	0,020#	0,676	-
pH					
Control (UCO)	5,0±0,3	5,3±0,4	5,1±0,3	5,1±0,4	0,404
Control (UC)	4,9±0,4	5,2±0,4	5,1±0,2	5,3±0,6	0,276
P	5,0±0,3	5,3±0,4	5,2±0,3	5,4±0,5	0,084
C1	5,1±0,3	5,4±0,4	5,2±0,2	5,1±0,5	0,03*;a:b
C2	5,1±0,3	5,3±0,4	5,3±0,3	5,4±0,4	0,290
p-values (one-way ANOVA)	0,790	0,777	0,088	0,779	-
EC					
Control (UCO)	29,1±5,1	30,7±7,1	32,1±6,7^{1,3}	32,6±5,$^{P, C1,C2}$	<0,025*; a:d
Control (UC)	28,1±5,6	28,2±5,0	29,9±5,8^{C1}	31,2±6,5$^{P, C1,C2}$	0,054
P	27,5±6,6	26,8±6,9	42,4±9, ^{UC,UCO}	42,6±5,2 ^{UC,UCO}	<0,001*; a:c; a:d;b:c;b:d
C1	26,6±7,9	23,4±6,4	36,6±8,3	41,6±6,2 ^{UC,UCO}	<0,001*; a:c; a:d; b:c;b:d
C2	27,1±7,3	29,6±9,1	41,9±7,8	44,8±6,1 ^{UC,UCO}	<0,001*; a:c; a:d; b:c;b:d
p-values (one-way ANOVA)	0,943	0,103	0,000#	0,000#	-

the skin site that was artificially dried and irritated, but not treated afterwards (UCO), TEWL returned to baseline values only after five days. This indicates that all investigated samples could be effective in the repair of the interrupted skin barrier function which might be essential in achieving properly hydrated skin.

The values of EC measured on the skin before and after the artificially provoked dryness, as well as after the treatment with investigated samples were expressed as absolute changes to the baseline and shown in Table III. It can be concluded that, although SLS treatment significantly interrupted the skin barrier function (which was expressed as TEWL increase after irritation), there was no significant decrease in the value of the electrical capacitance as a reflection of the state of hydration of SLS dried skin. However, all investigated samples significantly increased the degree of hydration of the skin during treatment, and in particular C2 cream which contained 2% of A. vulgaris extract.

The results of the pH measurements of the skin were as expected. The initial upward trend in pH values after irritation was followed by a return to baseline values after two days of treatment by any of the samples. Return to pre-irritation, but slower, was observed in the untreated skin sites, which could be attributed to a certain buffering capacity of the skin itself.

Our study showed that *A. vulgaris* extract in topical products (both cosmetics and dermopharmaceutics) could play an ameliorative role in improving the overall health of the skin barrier. Our goal was to emphasise the fact, confirmed by this study, that topical treatment with herbal antioxidant formulations containing flavonoid compounds (with focus on ellagic acid), alongside the effective moisturising treatment could be an effective way to enhance the skin's own recovery potential.

Besides showing beneficial effects on the skin, the extract provided the fulfilment of the demand for the satisfactory safety profile i.e. succeeded in meeting the safety requirements of the Regulation (EC) 1223/2009 on cosmetics what was of the particular importance. Therefore, the desirable effect of the traditional application of *A. vulgaris* in the treatment of skin disorders alongside its safety was confirmed by this work. The results indicated that *A. vulgaris* might be a candidate as a natural and safe topical active in phytopharmaceuticals and cosmetics as its extracts in a proper natural emulsion vehicle possess a strong antioxidant, skin hydrating and anti-irritant potential.

Acknowledgments

This research was supported by the Ministry of Science, Technological
Development and Innovation of the Republic of Serbia (Grant No. 451-03-65/2024-03/200113 and 451-03-66/2024-03/200113) and the Faculty of Medicine University of Niš Internal Scientific Project No. 15.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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