Evaluation of *in vivo* effects on surfactant-irritated human skin, antioxidant properties and phenolic composition of five Ericaceae species extracts

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Arbutus unedo, *Bruckentalia spiculifolia*, *Calluna vulgaris*, *Erica arborea* and *Erica carnea* possess strong antioxidant activity and they are traditionally used for the treatment of various skin diseases. The present study investigated the total phenylpropanoid content, antioxidant properties and phenolic composition of dry leaf ethanol extracts of these species. Furthermore, the *in vivo* effects of gels, each containing 2% of a single extract were tested on the artificially irritated human skin using the objective methods of skin biophysical measurements (erythema index (EI), pH of the skin and electrical capacitance (EC) as a measure of skin hydration level were assessed). In total, 13 components were identified by RP-HPLC coupled with DAD detection; quercitrin, quercetin 3-O-glucoside and gallic acid were detected in all investigated samples while chlorogenic acid and quercetin were present in 4 samples. Regarding the *in vivo* study, all investigated gels significantly decreased the skin irritation level and reversed the pH of the skin disturbed by preirritation, while results were contradictory regarding skin hydration measurements.
In conclusion, the assessed *in vivo* topical effects of investigated extracts matched well with their phenylpropanoid content as well as with assessed antioxidant activities.
Keywords: Ericaceae, skin biophysical measurements, irritated human skin, antioxidant, phenylpropanoid, RP-HPLC

Valutazione *in vivo* degli effetti su pelle umana irritata da tensioattivi, proprietà antiossidanti e composizione fenolica di estratti di cinque specie della famiglia Ericaceae

Arbutus unedo, *Bruckentalia spiculifolia*, *Calluna vulgaris*, *Erica arborea* e *Erica carnea* possiedono una forte attività antiossidante e sono tradizionalmente utilizzati per il trattamento di varie malattie della pelle. Il presente studio ha analizzato e confrontato il contenuto totale di fenilpropanoidi, le proprietà antiossidanti e la composizione fenolica di estratti secchi di etanolo da foglie di queste specie. Inoltre, gli effetti *in vivo* di gel, ciascuno contenente il 2% di ciascun estratto sono stati testati in studio sulla pelle umana irritata artificialmente valutando l'eritema index (EI), il pH e la capacità elettrica (CE) come misura del livello di idratazione della pelle. In totale, 13 componenti sono stati identificati mediante RP-HPLC accoppiata alla rilevazione DAD; quercitrina, quercetina 3-O-glucoside e acido gallico sono stati rilevati in tutti i campioni esaminati mentre l'acido clorogenico e quercetina erano presenti in 4 campioni. Per quanto riguarda gli studi *in vivo*, tutti i gel indagati hanno ridotto significativamente il livello di irritazione cutanea e invertito il pH della pelle pre-irritata, mentre i risultati sono contraddittori per quanto riguarda le misurazioni di idratazione della pelle. In conclusione, la valutazione *in vivo* degli effetti topici degli estratti in esame sembra correlare bene con il loro contenuto in fenilpropanoidi nonché con l'attività antiossidante determinata.
Parole chiave: Ericaceae, misurazioni cutanee biofisiche, pelle umana irritata, antiossidanti, flavonoidi, RP-HPLC

1. INTRODUCTION

Various plant extracts have been widely used in wound-healing, skin-soothing, calming, anti-aging as well as in the topical treatment of skin diseases. Since the inflammation is the common pathway of most dermatoses, extracts with anti-inflammatory activity have received the special attention and they are commonly incorporated into the formulations intended for topical use [1,2]. Several reports from traditional medicine and phytotherapy indicate the efficacy of extracts of selected plants from Ericaceae family in the treatment of skin diseases. The leaves of *Arbutus unedo* are employed to prepare a decoction used as a vulnerary and as a refreshing bath against hemorrhoids [3, 4]. Since the Middle Ages, *Calluna vulgaris* was externally used for body washes, bandages and in the treatment of eczema; it possesses an antiseptic and anti-acne effect and it is also said to be antimicrobial, antirheumatic and wound healing active [5-7]. Different extracts of *Callunae herba* exerted significant inhibition of cyclooxygenase (due to its main flavonoid glycoside – kaempferol-3-O-β-D-galactoside) and lipooxygenase (ascribed to ursolic acid) activity, which support ethnobotanical and modern use of these plant extracts [8, 9].

In order to evaluate applications of *Erica* species against inflammatory conditions, such as draining the edema, wounds and snake bites, Akkol et al. demonstrated that *Erica arborea* possesses remarkable anti-inflammatory and antinociceptive activities [10]. Recently, it has been shown that *Arbutus unedo* also has significant anti-inflammatory properties assessed in animal models of acute inflammation [11].

Therefore, the current study consisted of two parts. The first part of the study investigated antioxidant activity alongside content and phenolic composition of ethanol extracts of selected plants from the Ericaceae family (namely: *Arbutus unedo*, *Bruckentalia spiculifolia*, *Calluna vulgaris*, *Erica arborea* and *Erica carnea*). We aimed to confirm the *in vitro* antioxidant activity of selected Ericaceae species, which could be employed for evaluation of a potential protective effect in oxidative stress-mediated skin disorders [12]. Additionally, phenylpropanoids were included in this study, due to the fact that this class of secondary metabolites is getting more and more into the focus of skin researches because of their anti-inflammatory, antimicrobial, cytoprotective and wound healing activity [13-15]. They also promote skin regeneration and ameliorate skin inflammation due to ROS scavenging, antioxidant, iron chelating, and glutathione-S-transferase inducing properties [13].

Significant amounts of total polyphenolic compounds, including tannins and flavonoids, in selected species of Ericaceae family were reported in our previous papers [16, 17]. Since there have been insufficient data in the literature on the phenolic composition of selected species of the Ericaceae family, phenolic compounds present in ethanol extracts were analyzed by RP-HPLC with DAD.

Afterwards, the second part of the study assessed the *in vivo* effects of the hydrogels containing investigated extracts on the sodium lauryl sulfate (SLS)-irritated human skin [18-21]. We studied the potential of investigated extracts to effect the irritated skin, i.e. to reduce the skin erythema and regulate disturbed skin's pH alongside with its hydration level. These potential skin effects were evaluated by means of non-invasive biophysical measurements assessing the following skin parameters: erythema index (EI), pH of the skin and electrical capacitance (EC) as a measure of skin hydration level.

2. MATERIALS AND METHODS

2.1. PLANT MATERIAL

Plant materials were collected from wild growing species of the Ericaceae family in Serbia, Montenegro and Greece: *Arbutus unedo* L. (Luštica, Montenegro and Halkidiki, Greece); *Bruckentalia spiculifolia* Rchb. (Kopaonik – Pančićev vrh, Serbia); *Calluna vulgaris* (L.) Hull (Loznica – Gučevo, Serbia); *Erica arborea* L. (Luštica, Montenegro); *Erica carnea* L. (syn.: *Erica herbacea* L., *Erica saxatilis* Salisb.) (Mokra Gora, Serbia).

The taxonomic identification was performed by Prof. Dr. Branislava Lakušić and authenticated voucher specimens have been deposited in the Herbarium collection of the Faculty of Pharmacy, University of Belgrade: *Arbutus unedo* (from Serbia) HFF No. 1173 and (from Greece) HFF No. 2906, *Bruckentalia spiculifolia* HFF No. 1217, *Calluna vulgaris* HFF No. 1272, *Erica arborea* HFF No. 1430 and *Erica carnea* HFF No. 1431.

2.1.1. Determination of swelling index

The swelling indexes of powdered dry plant materials of selected species were measured according to procedure recommended in the European Pharmacopeia 6.0 [22].

2.2. EXTRACTS

Extracts were prepared with ethanol (70%, v/v) by percolation, as described in the European Pharmacopeia 5.0 [23]. Ethanol extracts of *Arbutus unedo* from Serbia (A1E), *Arbutus unedo* from Greece (A2E), *Bruckentalia spiculifolia* (BE), *Calluna vulgaris* (VE), *Erica arborea* (EE) and *Erica carnea* (CE) were obtained after evaporation to dryness under reduced pressure at 40°C and extraction yields were 45.05, 41.20, 32.35, 33.84, 38.98 and 38.61% (w/w), respectively.

2.3. CHEMICALS AND INSTRUMENTATION

All reagents and solvents used herein were of analytical grade. Spectrophotometric measurements were performed using Evolution 60 Thermo scientific spectrophotometer (Fisher Scientific, UK) and Multiskan Ascent No354 (ThermoLabsystems, Finland) ELISA microplate reader. Incuterm Raypa® trade (Catalonia, Spain) was used for incubation, Vibramix 30 (Tehtnica, Slovenia) for shaking of microplates and stirrer RW16 basic (IKA®WERKE, Germany) for preparation of gels. Regarding non-invasive biophysical measurements, erythema index (EI) was measured using Mexameter® MX 18 probe, electrical capacitance (EC) as a measure of *stratum corneum* (SC) hydration was measured using Corneometer® CM 825, while skin surface pH was determined with pHmeter 900; all probes are the part of Multi Probe Adapter MPA®9 (Courage & Khazaka Electronic GmbH, Germany). HPLC analysis was performed using the Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump G1312A, a Zorbax Eclipse XDB-C18 column (4.6×250mm with 5 μm particle size) and a photodiode array (DAD) detector G1315B.

2.4. β-CAROTENE–LINOLEIC ACID ASSAY

Inhibition of lipid peroxidation in β-carotene–linoleic acid assay was determined according to the method described by Koleva et al. [24]. In brief, 200 μl of freshly prepared β-carotene–linoleic acid emulsion were added to 20 μl of the sample (concentrations of ethanol extracts in ethanol, 70% v/v, were 6.25, 12.5, 25, 50, 75, 100 or 125 μg/ml) in each well of 96-well microtitration plate. Samples were prepared in triplicate for each concentration used. The plate was shacked on a microplate shaker and read in a microplate reader using a 450 nm filter immediately (t = 0 min) and after 120 min (t = 120 min) of incubation at 55°C. The percentage inhibition of β-carotene bleaching by the samples was calculated according to formula [25]:

$$\% \text{ inhibition} = (A_{120} / A_0) \times 100$$

where A_{120} is the absorbance of the sample at t = 120 min and A_0 is the absorbance of the sample at t = 0 min.

2.5. DETERMINATION OF TOTAL PHENYLPROPANOIDS

The content of total phenylpropanoids (hydroxycinnamic acid derivatives) was determined spectrophotometrically in dry extracts according to the monograph of *Fraxini folium* in European Pharmacopoeia 6.0 [22]. The results were expressed as chlorogenic acid in % (w/w) of dry matter.

2.6. HPLC ANALYSIS

For HPLC analysis dry extracts were dissolved in acetonitrile (5 mg/ml). Samples were gradiently eluted with a two phase system, phase A = water/phosphoric acid (99.97:0.03, v/v), pH = 2.75 and phase B = 10% A in acetonitrile, flow rate of 0.8 ml/min, at 25°C. Gradient profile was: 0 min 90% A, 10% B; 5-15 min 75% A, 25% B; 20 min 70% A, 30% B; 25 min 50% A, 50% B; 30 min 30% A, 70% B and 35 min 90% A, 10% B. Quercetin, quercetin 3-O-glucoside, quercitrin, rutin, kaempferol, apigenin, apigenin 7-O-glucoside, gallic acid, chlorogenic acid, ferulic acid and ellagic acid were used as standards in concentration of 1 mg/ml in acetonitrile/water (1:1, v/v). Chromatograms of extracts and standards were recorded under the same conditions. Detection was performed at 350 nm. Identification of components was carried out by comparing their UV spectra and peak retention times with UV spectra and peak retention times of standards [26]. Quantification of components was performed from the calibration curves of representative standards.

2.7. THE *IN VIVO* STUDY

In the second part of the study, we investigated the *in vivo* effects of the test samples (hydrogels) containing investigated extracts on the SLS-irritated human skin. SLS in a closed patch test is frequently used for experimental induction of skin inflammation and irritation which can be evaluated by the use of non-invasive biophysical measurements [18-21, 27]; after an exposure to SLS, irritated skin sites were treated with test samples for one week in order to study and compare the modification of this irritant reaction by different samples.

2.7.1. The investigated samples

Gel samples based on xanthan gum in a fixed concentration of 0.5% (w/w) were prepared. The samples contained purified water and were adequately preserved using Euxyl K®300 (Schülke & Mayr, Germany) preservative blend.

We formulated and evaluated the following samples:

P – placebo sample consisted of purified water, preservative and xanthan gum. Each active sample was of the same composition as the placebo, added with 2% of a single extract. Active samples were marked as: A1 (sample containing extract A1E), A2 (extract A2E), B (extract BE), C (extract CE), E (extract EE) and V (extract VE).

For the purpose of preparation of the active samples, investigated dry ethanol extracts (2% (w/w)) were dissolved in water under an ultrasound bath. Then, gels were prepared by stirring dry ethanol extract in water (500 rpm); after 2 min xanthan gum was added and dispersed for 10 min (800 rpm). Samples were allowed to equilibrate for one week prior to their use in the study.

2.7.2. Subjects

The study was performed in a randomized, double-blind manner. In accordance with the Declaration of Helsinki, the study was approved by the local Ethical Committee. In total, 18 volunteers (17 female, 1 male; age 20 to 59 years, mean age 37.28±11.30 years) with no history of atopic eczema or other skin diseases were recruited and signed an informed consent. Participants were instructed not to use any skin care product on the test sites one week before the study, as well as during the experiment. None of the volunteers received systemic or topical drugs within the last 10 days. They were also asked to avoid sun exposure, but allowed to wash their forearms in a regular way. According to the published guidelines, all measurements were carried out in an environmentally controlled room (21±1°C and 50±5% RH). Participants were asked to acclimatize for 30 minutes before taking any measurements.

2.7.3. Test protocol

The effects of tested gels were studied on the volar forearms. Seventy-five micro liters of the irritant (aqueous SLS, 12%, purity of SLS >99%, Merck, Darmstadt, Germany) was applied under patch occlusion for 6h on five places of both forearms. Application was performed with 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 outcomes were measured 24 hours after the occlusion was removed.

Then, the volunteers were randomly divided into two test groups. First group (9 volunteers: 8 female, 1 male; mean age 39.78±10.11) was used to study the effects of the samples A1, A2 and P, while the second group (9 female volunteers, mean age 34.78±11.87) used the samples B, C, V and E. The samples were marked with differently colored 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 irritated under occlusion on the left forearm (UCO), and without irritation on the right (UCW).

The measurements of EC, EI and pH were performed before and 24h after SLS irritation, as well as after 3, 5 and 7 days of the treatment.

2.8. STATISTICS

All results are presented as mean ± standard error. *In vivo* measured parameters were expressed as a relative change to the baseline (%) and the results for each investigated sample were compared to the placebo sample as well as related to both controls (UCO and UCW). The values of measured parameters after SLS irritation, after 3, 5 and 7 days of application were compared to appropriate baseline values using 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.

Correlation between phenylpropanoid contents and antioxidant activity (% of inhibition of β-carotene bleaching reached at the highest tested concentrations) was established by regression analysis.

The differences were accepted as statistically significant at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. DETERMINATION OF SWELLING INDEX

Many medicinal plant materials are of specific therapeutic or pharmaceutical utility because of their swelling properties (i.e. those containing an appreciable amount of mucilage) [28]. Swelling indexes of dry plant materials of species *Arbutus unedo*, *Bruckentalia spiculifolia*, *Calluna vulgaris*, *Erica arborea* and *Erica carnea* were 3.33±0.24, 3.83±0.24, 7.83±0.24, 3.80±0.24, 4.5±0.00, respectively.

3.2. ANTIOXIDANT PROPERTIES

Topical application of plant extracts with strong antioxidant activity could protect damaged skin against the toxic effects of ROS [13]. Considering polyunsaturated fatty acids (PUFA) are accessible to peroxidation, the PUFA peroxidation is one of the most investigated consequences of ROS action on membrane structure and function [29]. Thus inhibition of lipid peroxidation seems to be the method of choice to determine antioxidant capacity of topically applied preparations.

All tested Ericaceae species possess excellent antioxidant activity [16, 17]. Since the measurement of antioxidant activity of a plant extract that is a complex mixture, cannot be evaluated satisfactorily by a single antioxidant test, several test procedures must be engaged. In order to supplement previous examinations, the same extracts were tested for inhibition of lipid peroxidation in β-carotene–linoleic acid assay.

All tested extracts showed notable activity in this assay (Table I). These results are similar to those found for the same extracts tested for inhibition of lipid peroxidation in liposomes [16, 17].

The addition of crude extracts at a level of 100 mg/ml decreased the β-carotene bleaching by more than 68%. Lower concentrations also showed good inhibition of β-carotene bleaching.

3.3. PHENYLPROPANOID CONTENT

Considerable amounts of phenylpropanoids,

Table I - Results of β-carotene bleaching assay and phenylpropanoid content (determined according to the method described in European Pharmacopoeia 6.0).

Ethanolic extract	Inhibition of β-carotene bleaching (% ± SEM)							Phenylpropanoid content (% ± SEM)
	6.25 µg/ml	12,5 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	125.0 µg/ml	
A1E	27.01±1.30	35.13±3.92	40.07±0.54	59.82±0.26	69.81±2.08	86.77±3.02	56.68±1.99	2.42±0.31
A2E	28.90±1.84	33.32±3.05	45.37±1.36	60.11±1.44	89.16±2.86	77.50±1.05	53.64±2.18	1.57±0.07
BE	36.79±1.48	40.05±0.89	42.81±1.77	55.87±2.78	72.78±2.50	86.43±1.42	96.54±3.25	6.53±0.30
VE	33.04±0.22	37.40±2.55	50.39±2.25	51.52±3.14	62.49±4.24	75.15±6.25	95.03±3.29	6.33±0.11
EE	37.97±0.04	40.87±1.72	52.08±2.04	51.93±0.81	69.18±0.29	84.94±1.43	82.32±3.91	3.86±0.18
CE	33.87±1.95	40.68±2.80	45.72±2.10	48.48±1.34	55.40±2.34	68.43±2.51	77.09±2.94	4.52±0.06

expressed as % of chlorogenic acid, were found in investigated extracts. Phenylpropanoids are represented in a range from 1.57 ± 0.07% (A2E) to 6.53 ±0.30% (BE) (as shown in Table I).

Antioxidant activity increased proportionally with the phenylpropanoid content: a linear relationship between results of β-carotene bleaching assay and total phenylpropanoid content was established (R = 0.97).

The results obtained in β-carotene bleaching assay were compared to corresponding values of previously reported antioxidant tests [16, 17].

BE (ethanol leaf extract of *Bruckenthalia spiculifolia*) showed the strongest dose-dependent activity in both tests based on inhibition of induced lipid peroxidation. In the concentration level 125 mg/ml, % of inhibition of β-carotene bleaching and lipid peroxidation in liposomes were 96.54 and 96.32, respectively. Among the tested extracts, BE had the highest phenilpropanoid content. Our previous study pointed out the highest flavonoid content and considerable amounts of tannins in BE [16].

Thus, pronounced inhibition of lipid peroxidation when BE is applied could be assigned to the highest quantity of herbal phenylpropanoids but also to the other classes of phenolic compounds present in BE.

Ethanol leaf extracts of *Arbutus unedo* (A1E and A2E), although the most potent in FRAP and DPPH tests [16], were less active in higher applied concentrations than other tested samples. These findings are in line with a lower total phenylpropanoid content and a remarkable positive phenylpropanoid/ β-carotene bleaching activity correlation.

3.4. QUALITATIVE AND QUANTITATIVE HPLC ANALYSIS

Thirteen phenolic compounds were identified in investigated extracts: results are indicated in Table II. Flavonoids quercitrin (trace-70.91 mg/g) and quercetin 3-O-glucoside (1.29-32.55 mg/g) and phenolic acid gallic acid (trace-13.44 mg/g) were present in all investigated samples.

The content of chlorogenic acid was 22.30, 29.51

Table II - HPLC analysis of BE, EE, CE, VE, A1E and A2E

RT (min)	Phenolic compound	BE	EE	CE	VE	A1E	A2E
4.4	Gallic acid	tr	tr	tr	tr	13.44	tr
7.4	Phenolic acid*	-	-	+	+	-	-
7.7	Chlorogenic acid	tr	22.30	29.51	36.67	-	-
10.1	Rutin	tr	tr	tr	-	-	-
10.9	Quercetin 3-O-glucoside	32.55	6.75	3.49	12.73	9.90	1.29
11.4	Ellagic acid	-	-	-	-	13.17	-
12.7	Quercetin glycoside*	+	+	-	-	-	-
14.0	Quercitrin	70.91	51.42	tr	1.10	26.91	1.46
14.3	Ferulic acid	-	-	-	0.90	-	-
15.5	Apigenin 7-O-glucoside	-	-	-	1.36	-	-
26.7	Quercetin	3.77	2.35	-	36.15	tr	-
28.8	Apigenin	-	-	-	tr	-	-
29.3	Kaempferol	-	-	-	4.81	tr	-

Results are expressed as mg/g dry extract or estimated from the areas of the HPLC peaks; +, present; tr, trace; -, not detected. * compound identified without representative standard

and 36.67 mg/g in EE, CE and VE, respectively. Chlorogenic acid is absent in A1E and A2E, while in BE is detected in trace. Phenolic composition of selected Ericaceae species is quite diverse among the same genus (EE and CE) and even among the same species (A1E and A2E).

3.5. *IN VIVO* STUDY

There are no previous studies concerning the *in vivo* effects of selected species of Ericaceae family on human skin, despite the fact that the extracts of these plants are traditionally used in the treatment of various dermatological disorders. *Calluna vulgaris* water extract is even commercially available as a cosmetic anti-aging active [9], although it has not been extensively investigated for that purpose.

Thus, in the second part of this study, we have investigated the *in vivo* effects of the hydrogels (A1, A2, B, C, E, and V) on human skin pretreated with SLS. This anionic surfactant and model irritant in the field of the skin irritation testing [19] is frequently used to estimate the anti-inflammatory potential of topical preparations, knowing the fact that exposure to surfactants induces irritant contact dermatitis (ICD), a non-immunological local inflammatory reaction [30]. ICD is clinically visible as erythema and could be measured using the biophysical measurements [18-20, 31]. In this study, we have measured EI, EC and pH of the skin in order to estimate the level of skin irritation before and after the treatment with the test gels containing investigated extracts.

The disruption of the SC barrier by surfactants includes transient swelling of the corneocytes and the increase in EC (i.e. hydration level) of irritated skin immediately after irritation [30]. The duration of the hyper-hydrated state of the skin is not determined precisely furthermore it is followed by sub-basal hydration levels [32]. Figure 1 reveals that occlusive application of 12% SLS solution resulted in a significant decrease of the skin hydration level 24h after its use, compared to baseline values, on all test sites except on the skin treated with placebo and samples A1, A2 as well as on UCO. That could be explained by prolonged water uptake by the SC after irritation on this skin sites, as a consequence of inter-individual differences of the skin. However, the expected decrease of the skin hydration level was observed later in the treatment with untreated as well as with skin sites treated with the samples A1 and A2, while skin treated with the placebo showed a further increase of EC. It could be presumed that the application of xanthan gum gel (an effective humectant *per se* [33]) did not allow the expected drying of the skin.

The treatment of the skin with A1 and A2 led to an improvement of the skin hydration level after 7 days of application compared to the baseline values, although this improvement was not statistical-

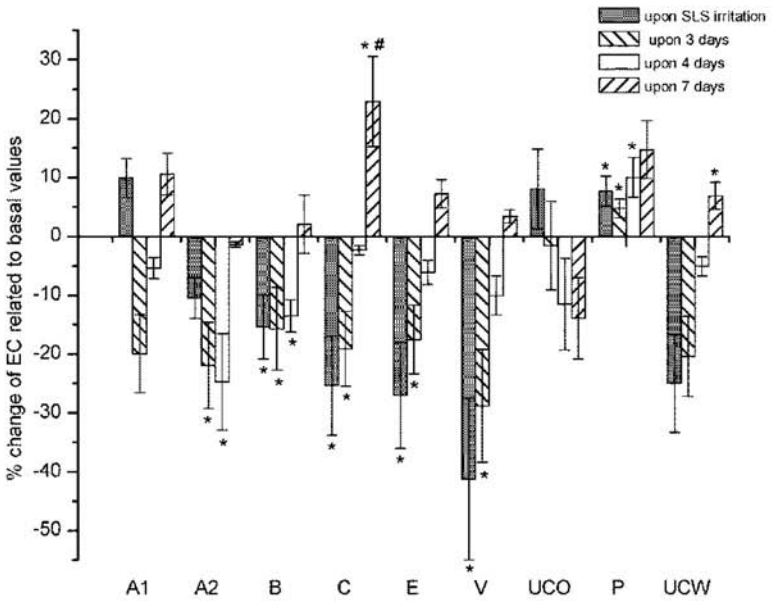

Figure 1 - The influence of the irritation *per se* and investigated samples after irritation on EC. Parameters were expressed as absolute changes to baseline at distinct time points. Differences to baseline were checked using paired sample t-test, significant differences being marked with (*); differences of the effects of different formulations to UCO as well as to effect of placebo (P) were checked using t-test for unpaired data, marked with (#) for significant difference to UCO.

ly significant.
12% SLS occlusion resulted in a significant decrease in the skin hydration level compared to baseline values on all other test sites, while treatment with sample C (hydrogel loaded with ethanol leaf extract of *Erica carnea*) led to a significant improvement of the skin hydration compared to the baseline after 7 days of application, however with no significant difference in comparison to other investigated samples (Fig. 1.). It is obvious that, except in the case of C, the addition of investigated extracts had no additive effect on the inherent ability of xanthan gum gel to efficiently hydrate the skin and even to suppress reduction in the water binding capacity of hyper-hydrated skin immediately after the SLS irritation. Although the application of all samples resulted in an improvement of skin moisture (increase of EC), compared to UCO (it was significant only for gel loaded with C), the advantageous skin hydrating potential of actives *vs*. placebo is unlikely to be achieved. On the contrary, it could be presumed that the presence of the extracts in active samples decreased the ability of xanthan gum gel to efficiently hydrate the skin at the beginning of the treatment of SLS-irritated skin. It is likely that some kind of competition for water occurred between the skin and some compounds of the investigated extracts (probably mucilage). Further physico-chemical investigations are however necessary to prove more evidence and deeper insight into the mechanisms included in the ability of investigated herbal extracts to bound and withhold the water when incorporated in xanthan gum-based gel, which could be attributed to substantial swelling properties *i.e.* humectant capacity – hygroscopicity of selected species (as previously stated in 3.1.).
As expected, EI, as a measure of skin erythema, was significantly increased after SLS-induced irritation on all test sites compared to the baseline values (Fig. 2), including control site (UCO).
Application of all investigated samples led to the significant decrease of erythema of the irritated skin in the seven-day treatment, as measured by EI (Fig. 2). Since the significant EI recovery to the baseline values was observed after the treatment with the placebo as well, it could be presumed that efficient hydration alongside the protective effects of the xanthan gum *per* se [34] led to the amplification of the skin's own capacity to calm irritation after 7 days of treatment. It is observed that the effective hydration of the healthy skin makes it less susceptible to irritation and also accelerates the recovery of the inflicted skin barrier [35]. However, gels with BE, CE, EE and VE, which did not show good skin moisturizing capacity (Fig. 1), pointed to a significantly greater potential in decreasing EI of the irritated skin in comparison with the UCO probably

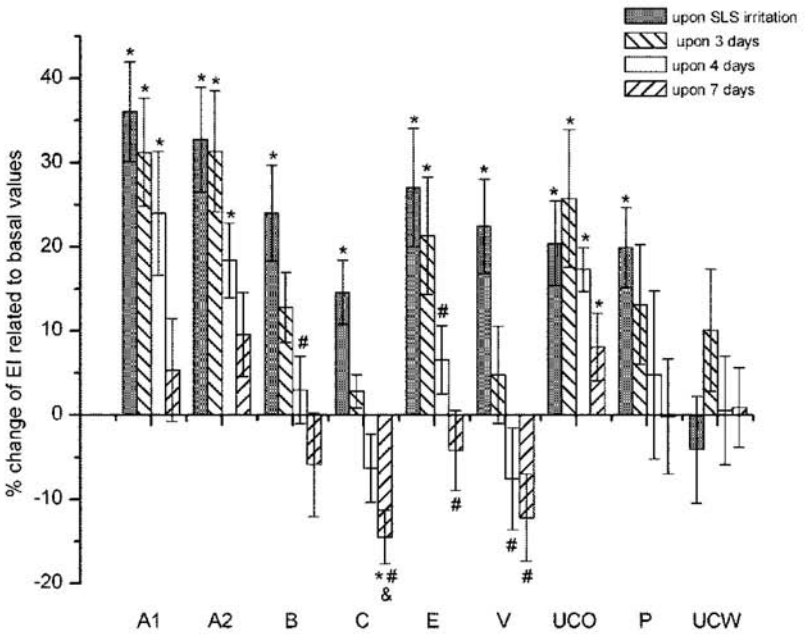

Figure 2 - The influence of the irritation *per se* and investigated samples after irritation on EI. Parameters were expressed as absolute changes to baseline at distinct time points. Differences to baseline were checked using paired sample t-test, significant differences being marked with (*); differences of the effects of different formulations to UCO as well as to effect of placebo (P) were checked using t-test for unpaired data, marked with (#) for significant difference to UCO and with (&) for significant difference to P.

due to their composition. These findings are in line with the results obtained from the evaluation of the content of chlorogenic acid (Table I). Chlorogenic acid exerts anti-inflammatory [36], anti-edematogenic and antinociceptive activities [37] and exhibits excellent inhibitory activity on microsomal lipid peroxidation [15].

Pronounced activity of investigated gels in decreasing EI of the irritated skin (Fig. 2) is also in line with antioxidant activity of the extracts (Table I). Considering the fact that oxidative stress is involved in chemically-induced cutaneous reactions (such as acute ICD), topical application of antioxidants could inhibit inflammation and ameliorate the skin conditions [38]. It is worth pointing out that xanthan gum gel *per se* exhibits antioxidant activity [33].

Phenylpropanoids from the selected species (Table I) can be assessed to be at least partly responsible for the influence on EI. To what extent the specific classes of polyphenols, which are present in the material, contribute to the skin activity has to be evaluated in further studies.

According to our result, it could be stated that the sample C (hydrogel loaded with CE) displayed the best potential to decrease the erythema index in experimentally irritated human skin. Although CE showed a high phenylpropanoid content (though lower than BE and VE) and a good potential to stop lipid peroxidation, it is probable that the presence of chlorogenic acid (36.67 mg/g) is a predominant factor affecting the C impact on the skin irritatation, *i.e.* EI values measured during the study.

The pH values obtained were as expected: under occlusion, SLS increased the skin pH significantly compared to the pre-irritation values. The initial trend of pH increase after the irritation was followed with the return to the baseline values immediately following the application of each sample including the placebo as well as on untreated irritated skin, probably due to the skin's own buffering potential (Fig. 3). On the other hand, samples B, C, E and V significantly decreased pH of the skin after 7 days of treatment, compared to the baseline, *i.e.* the values before the irritation.

A decrease in pH of the skin is commonly connected to an increase in skin irritation [39]. According to this study, samples B, C, E and V are able to decrease the pH of the skin (Fig. 3), but without concomitant irritation (Fig. 2.). That could be of great benefit in the treatment of some pathological skin conditions.

Generally, the present study brings forward the new data regarding the phenolic composition of *Arbutus unedo*, *Bruckentalia spiculifolia*, *Calluna*

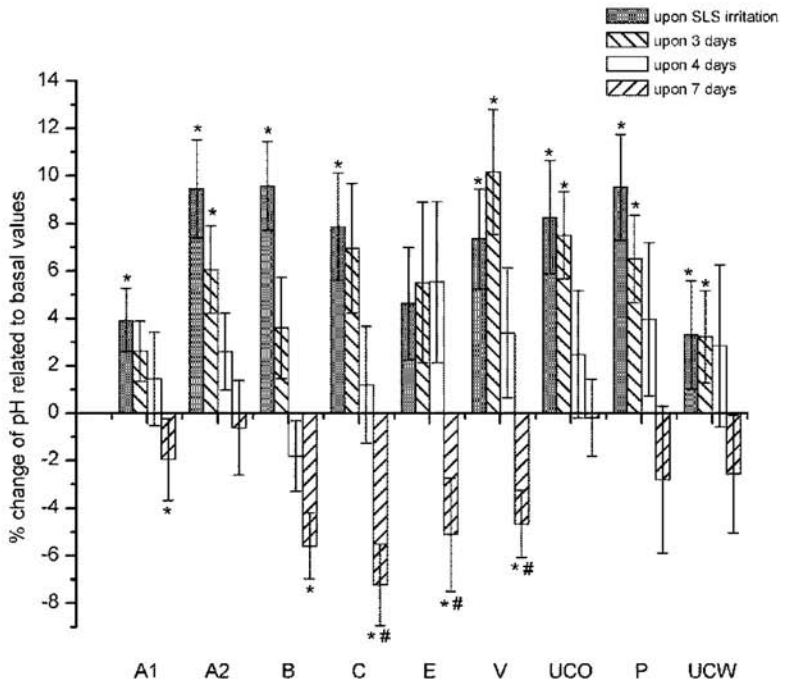

Figure 3 - The influence of the irritation *per se* and investigated samples after irritation on pH. Parameters were expressed as absolute changes to baseline at distinct time points. Differences to baseline were checked using paired sample t-test, significant differences being marked with (*); differences of the effects of different formulations to UCO as well as to effect of placebo (P) were checked using t-test for unpaired data, marked with (#) for significant difference to UCO.

vulgaris, *Erica arborea* and *Erica carnea.* On the other hand, the results of the *in vivo* study on human skin confirmed the effects of the traditional application of selected species in the treatment of skin disorders [3-7].

Samples with extracts containing high levels of total phenylpropanoids exhibited slightly better effects. Antioxidant activity, phenolic composition and especially the presence of chlorogenic acid probably contribute to their effects on human skin. Our observations that hydrogels containing the investigated extracts with potent antioxidant activity, but also the 0.5% xanthan gum gel *per se,* ameliorated the surfactant-induced irritation, emphasizing the fact that the topical treatment with antioxidant formulations alongside the effective moisturizing treatment could be an effective way to enhance the skin's own recovery potential.

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