Alkyl polyglucoside-stabilized emulsion as a prospective vehicle for *Usnea barbata* CO_2-supercritical extract: Assessing stability, safety and efficiency of a topical formulation

Ana R. Žugić[1], Milica Z. Lukić[2], Marija Z. Tasić Kostov[3], Vanja M. Tadić[1], Ivana A. Arsic[3], Dušan R. Mišić[4], Slobodan D. Petrović[5], Snežana D. Savić[2]

[1]*Institute for Medicinal Plant Research "Dr Josif Pančić", Department of Pharmaceutical Research and Development, Belgrade, Serbia*
[2]*University of Belgrade, Faculty of Pharmacy, Department of Pharmaceutical Technology and Cosmetology, Belgrade, Serbia*
[3]*University of Niš, Faculty of Medicine, Department of Pharmacy, Niš, Serbia*
[4]*University of Belgrade, Faculty of Veterinary Medicine, Department of Microbiology, Belgrade, Serbia*
[5]*University of Belgrade, Faculty of Technology and Metallurgy, Department of Organic Chemistry, Belgrade, Serbia*

Abstract

Antimicrobial activity of *Usnea barbata* especially against bacteria involved in pathogenesis of various skin conditions has been well documented in literature. Nevertheless, there are no papers dealing with formulation of its isolates into topical preparations for treatment of skin infections. In present study, alkyl polyglucoside (APG)-based vehicle was developed as carrier of *U. barbata* CO_2-supercritical extract (U-SE) that demonstrated the best antimicrobial potential in preliminary screening. For comparison, chosen extract in the same concentration and using the same procedure was incorporated into a pharmacopoeial vehicle. Comparative evaluation of physicochemical stability, efficiency and safety proved APG-based vehicle to possess certain preferential features as carrier of U-SE compared to the reference one, composing a topical formulation with potential clinical relevance in treatment of skin infections.

Keywords: alkyl polyglucosides, *Usnea barbata* supercritical CO_2-extract, skin infections, physicochemical stability, antimicrobial activity, skin performance.

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Usnea barbata is one of the most studied lichens belonging to the genus *Usnea*, traditionally used in Asia, Africa and Europe for pain relief and fever control. According to literature data, *U. barbata* (UB) has allegedly been used by Hippocrates to treat urinary complaints and in folk medicine of South Africa for the treatment of wounds [1,2]. In addition to traditionally claimed properties of UB as a folk remedy, German Commission E approved its usage as an antimicrobial agent intended for the treatment of mild inflammation of the oral and pharyngeal mucosa in the form of lozenges [3].

Antimicrobial activity of UB has been fairly documented in several scientific studies [2,4,5]. Comparative testing of acetone, methanol and water extract of UB against 10 bacterial and 5 fungal strains using dilution method on solid agar medium revealed the best antimicrobial activity against Gram positive bacteria, with acetone extract being the most active among the investigated extracts [2]. Furthermore, an investigation of acetone extracts of several *Usnea* species including UB using agar disc diffusion method demonstrated antimicrobial activity to be proportional to usnic acid (UA) content in the investigative extracts [4]. Similar results were reported in a recent study screening nine plant extracts and isolated compounds for antimicrobial activity against bacteria and yeasts with dermatological relevance. Namely, supercritical CO_2-extract of UB in a form of a suspension with 4 mass% of UA and pure UA were the most active compounds, especially effective against anaerobic and Gram-positive bacteria as well as dimorphic yeast *Malassezia furfur*. Moreover, it was suggested that antimicrobial activity of the extract was mainly mediated by UA [5]. To the best of our knowledge, there are not papers regarding formulation of UB extracts into pharmaceutical preparations for the treatment of skin disorders.

Bearing in mind the above statements, the objective of our study was to develop and evaluate a pharmaceutical topical formulation with UB extract as an active ingredient for the treatment of skin infections. The current investigation consisted of two parts. The aim of the first part was screening of several UB ext-

Correspondence: S.D. Savić, University of Belgrade, Faculty of Pharmacy, Belgrade, Serbia.
E-mail: snexs@pharmacy.bg.ac.rs
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racts prepared using different protocols and/or solvents, in terms of their UA content and antimicrobial activity, as well as correlation thereof. The extract with the best antimicrobial potential against bacteria that commonly cause skin infections was further chosen for the second part of the study. In this part, our objective was to formulate selected extract into oil-in-water (O/W) emulsion and evaluate its physicochemical stability, as well as efficiency and safety features. We aimed at developing a vehicle with emollient properties as carrier for UB extract, being prospective topical antimicrobial, considering the potential of emollients (moisturizers) to restore skin barrier function accompanying skin infections, while soothing its concomitant symptoms [6]. In this regard, for stabilization of investigated emulsion we used alkyl polyglucoside (APG) emulsifiers, prepared from natural renewable raw materials, which have been gaining increasing attention in recent years due to their favorable ecological, toxicological and dermatological properties [7,8]. For comparison with the developed emulsion, chosen UB extract in the same concentration and using the same preparation procedure was incorporated into widely used conventional (pharmacopoeial) vehicle, used as a reference.

EXPERIMENTAL

Preparation of UB extracts

Lichen UB was collected in the Former Yugoslav Republic of Macedonia (GPS N41 53.5764 E21 33.6348) on 15th of October, 2009. The lichen was identified at the Faculty of Biology, University of Belgrade (voucher of specimen No. 16390/10.04.2010.). Ethanol 96.3 vol.% was purchased from Vranje, Serbia and ether from Carlo Erba Reagents, France.

Investigated samples were following UB extracts: supercritical CO_2 extract (U-SE), Soxhlet extracts (ether fraction (U-SOX-E) and ethanol fraction (U-SOX-EtOH)) and macerate (U-MAC).

U-SE was purchased from Flavex, Germany. According to manufacturer's claims U-SE was obtained by the method of supercritical CO_2 extraction, and then purified in a second step to practical pure UA (drug:extract ratio (DER) 62–100:5). Samples U-SOX-E and U-SOX-EtOH were obtained by repeated continuous extraction using Soxhlet apparatus. 75.0 g of dried UB, grinded to the size of 180 mesh, was measured in each of five thimbles used, covered with appropriate amount of ether and extracted until ether discoloration. Thereafter, the same procedure as for U-SOX-E was utilized with ethanol 96.3 vol.% using thimbles with plant material already extracted with ether, in order to obtain U-SOX-EtOH. For the preparation of U-MAC, 93.91 g of dried UB grinded to the size of 180 mesh was covered with 600 mL of ethanol 70 vol.%, macerated for 24 h and then filtered. After all extractions, liquids were evaporated using rotary evaporator Buchi R 114, USA, yielding 2.04, 6.71 and 5.41 % residues for U-SOX-E, U-SOX-EtOH and U-MAC respectively, expressed as dry matter.

UA assay in UB extracts

UA was assayed using Hewlett Packard HPLC model 1200; column Zorbax Eclipse XDB-C18 600 Bar (4.6 mm×100 mm, 1.8 μm). The mobile phase A consisted of 99% H_2O and 1% H_3PO_4, while B was acetonitrile. Flow rate was 0.1 mL/min, and elution was as follows: 11–55% B, 0–5 min; 55–80% B, 5–10 min; 80% B, 10–12 min; 80–100% B, 12–20 min; 100% B, 20–35 min, 100––11% B, 35–40 min, 11% B, 40–55 min. Investigated samples (in triplicate) were prepared in a following procedure: 5.0, 7.1, 18.9 and 61.3 mg of U-SE, U-SOX-E, U-SOX-EtOH and U-MAC, respectively were dissolved in 50 mL methanol (analytical grade, purchased from Merck, Germany) then filtered through 0.45 μm PTFE syringe filters into glass HPLC vials and analyzed as described above. Commercially available UA, used as a reference standard was bought from Santa Cruz Biotechnology, USA, with the purity declared as > 98%.

Preparation of O/W creams

Test cream sample (labeled ML) was stabilized with mixed non-ionic APG emulsifier Montanov® L (INCI/$C_{12–14}$ glucoside and $C_{12–14}$ alcohol). A preliminary formulation study, conducted in accordance with manufacturer's recommendations, showed that the emulsifier concentration used (8 mass%) allowed the formation of creams with satisfactory organoleptic characteristics. For co-stabilization, a novel co-emulsifier Montanov® S (INCI/ cocoglucoside and coconut alcohol) was used in the concentration of 2 mass%, in accordance to manufacturer's recommendations. The emulsifiers were kindly provided by Seppic, France. The oil phase comprised caprylic-capric triglycerides (Sabo, Italy) and cetostearyl alcohol (Sabo, Italy), while the water phase was the mixture of 85% glycerol and water preserved using potassium sorbate.

Non-ionic hydrophilic cream (DAB 2011), used as a reference vehicle of pharmacopoeial quality (labeled P), was stabilized with mixed non-ionic emulsifier comprising Polysorbate 60 (Merck, Germany) and cetostearyl alcohol (Sabo, Italy). The oil phase contained white soft paraffin (Sigma Aldrich, Germany), while the water phase consisted of 85% glycerol and water preserved with potassium sorbate [9]. Detailed composition of investigated samples is given in Table 1.

Selection of the extract concentration was performed in the context of UA content, as a carrier of the stated biological activity of the extract. In accordance to our previous investigations (data not shown), chosen

concentration of the extract corresponded to 2 mass% of UA. In addition, selected extract concentration was supported by the literature data [10]. Upon the addition of extract, active samples were labeled ML-U and P-U. Double distilled water was used for the preparation of all samples. Compounds used were of pharmacopoeial quality (Ph. Eur.), whenever possible.

Table 1. Composition (mass%) of investigated vehicles (alkyl polyglucoside-stabilized vehicle (ML) and pharmacopoeial vehicle (P))

Component	ML	P
Caprylic-capric triglycerides	18.5	–
Cetearyl alcohol	1.5	10.0
C_{12-14} glucoside and C_{12-14} alcohol	8.0	–
Cocoglucoside and coconut alcohol	2.0	–
Polysorbate 60	–	5.0
White soft paraffin	–	25.0
Glycerol, 85%	5.0	10.0
Potassium sorbate	0.5	0.5
Water, double distilled	64.5	49.5

APG-stabilized vehicle was prepared by heating the emulsifiers and oil phase at 70-75 °C and then adding them to the preserved water phase at the same temperature by stirring (stirrer RW16 basic, IKA, Germany) at constant temperature for 3 min at 800 rpm and then 3 min at 500 rpm. Cooling was started at 500 rpm for 1 min and then at 300 rpm to the room temperature. Referent non-ionic hydrophilic cream was prepared according to DAB 2011 [9]. Briefly, white soft parrafin together with Polisorbate 60 and cetostearyl alcohol (oil phase) were heated to 70 °C. Components of the aqueous phase were heated to the same temperature, and then added to the oil phase and stirred until cooled to the room temperature. Active samples (ML-U and P-U) were prepared by suspending UB extract into appropriate vehicles. More precisely, the extract was rubbed with glycerol, using mortar and pestle, followed by addition of portions of vehicle. Prepared samples were allowed 7 days equilibration before being submitted to selected characterization techniques.

Rheological measurements

The rheological characterization was conducted in order to evaluate preliminary physical stability of investigated samples, especially in terms of the influence of UB extract addition to both types of investigated bases in a predetermined period of time. Additionally, flow properties were assessed as parameters known to exhibit influence on sensory properties of semisolids, crucial for patient acceptability [11].

Continual measurements were performed after 7, 30 and 90 days of storage at room temperature (Rheometer Rheolab MC 120, Paar Physica, Germany). All measurements were carried out using cone/ plate measuring system (diameter 50 mm, angle 1°), with 0.05 mm sample thickness, at 20±0.1 °C (in triplicate). Controlled shear rate procedure was applied (shear rate 0–200 s^{-1} and back again to the start point, each stage lasting 120 s).

Conductivity measurements

In order to assess both the emulsion type (mode of water distribution) and sample stability, conductivity measurements were performed in both placebo and active cream samples (conductivity meter CDM 230, Radiometer, Denmark). All measurements were performed after 7, 30 and 90 days of storage at room temperature.

pH Measurements

pH measurements were taken by direct immersion of pH meter glass electrode (Hanna instruments HI 9321, USA) in the investigated samples. In order to estimate impact of the extract addition to pH value of the placebo samples, pH value of the extract *per se* was assessed in its aqueous solution which was prepared by putting an excess amount of the extract into 100 mL of purified water in Erlenmeyer flasks, which were tightly closed and shaken on orbital shaker (KS 260 basic, IKA® Werke GmbH & Company KG Germany) at 250 rpm at 25 °C for 24 h. The samples were then centrifuged (Centrifuge MPW-56; MPW Med. Instruments, Poland) at 3000 rpm for 30 min to separate the undissolved extract. For the assessment of preliminary chemical stability, pH value of the tested cream samples was measured 7, 30 and 90 days after their preparation.

***In vitro* antimicrobial activity**

Antimicrobial activity was performed on Gram-positive (G+) and Gram-negative (G-) bacterial species––causative agents of skin infections in humans. Aside referential strains from American Type of Culture Collection (ATCC), investigated strains isolated from skin swabs taken from the diseased persons with skin infection symptoms were also used (clinical isolates, CI). The isolation was made from clinical material delivered to the Microbiology Department, Faculty of Veterinary Medicine, University of Belgrade, in accordance with conventional microbiological methods applied for the purpose of isolation and identification [12]. From the group of G+ microorganisms, *Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Staphylococcus epidermidis* (clinical isolate) and *Enterococcus faecalis* (clinical isolate) strains were chosen. From the group of G- bacteria, *Klebsiella pneumoniae* (clinical isolate) and *Escherichia coli* ATCC 25922 strains were selected.

For the investigation of antimicrobial activity and the determination of minimal inhibitory concentrations (MICs) of UB extracts (U-SE, U-SOX-E, U-SOX-EtOH and

U-MAC), investigated creams (ML-U and P-U) and UA, broth microdillution method was applied in accordance with the Clinical and Laboratory Standards Institute (CLSI) prescriptions for antimicrobial susceptibility testing [13,14].

Experiments were performed in 96-well microplate with "U" bottom (Spektar, Serbia) using cation adjusted Mueller Hinton II broth, CAMHB (Becton Dickinson, USA) with the addition of 1.6% bromocresol purple (Merck, Germany) in final concentration at 0.2 mL/200 mL for G+ and 1% phenol red (Merck, Germany) at 1 mL/200 mL for G- bacteria. Bromocresol purple and phenol red were added to obtain bacterial growth visibility. Dimethyl sulfoxide, DMSO (Merck, Germany) was used as a solvent for both extracts and creams. For the complete dissolving of the creams, DMSO was heated in water bath at 60 °C until deposit of creams disappeared. Investigated concentrations of both extracts and creams were 2560, 1280, 640, 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 μg/mL. The final bacterial inoculum density of 5×105 CFU/mL was achieved by adding 5 μL of 1–2×107 CFU/mL suspension of investigated strain in microtiter plate wells with 100 μL of previously added CAMHB. Microplates were incubated for 18–24 h at 37 °C. For MIC values determination the broth with lowest sample concentration, with no visible bacterial growth, was used.

In vivo skin irritation/performance study

In vivo skin irritation potential of the investigated samples was assessed in a 24-h study under occlusion. Thirteen healthy volunteers (mean age 21.92±1.6), which participated in the study, were thoroughly informed about the possible treatment effects and the protocol of the examination prior to signing written consents, in accordance with the Helsinki Declaration. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Niš, Serbia. The following parameters were evaluated: erythema index (EI), transepidermal water loss (TEWL) and electrical capacitance (EC) (quantifying the *stratum corneum* hydration) using Multi Probe Adapter MPA®9 (Courage & Khazaka Electronic GmbH, Germany). All measurements were conducted on flexor aspects of forearms at square application sites of 9 cm^2, leaving a site per each arm for untreated control under occlusion (UCO) and without occlusion (UC). After initial measurements, 0.016 g/cm^2 of investigative samples were applied, covered with silicone film and fixed with hypoallergenic adhesive tapes. Two hours upon removal of the 24-h occlusion, all parameters were reassessed.

Statistical analysis

Statistical analysis was carried out in OriginPro 9.0 (Electronic Arts, USA). Parameters of *in vivo* measurements were assessed with a one-way ANOVA followed by Tukey post hoc test, whenever appropriate.

RESULTS AND DISCUSSION

Results of chemical analysis of UB extracts are presented in Table 2. UA content ranged from 1.39 % in U--MAC to 81.41% in U-SE, which could be expected, bearing in mind that its solubility increases with solvent polarity decrease [15]. Namely, the lowest UA content was found in the extracts prepared with ethanol, which was the most polar solvent used (Table 2). However, application of non-polar ether in the extraction process led to notable increase in UA content in the sample U-SOX-E (Table 2). The highest UA content was detected in the sample obtained by supercritical CO_2 extraction, U-SE (Table 2), known to be appropriate method for the isolation of low polarity compounds [16].

All of investigated extracts and pure UA showed antimicrobial activity against G+ strains, whereas there was no activity against G– bacteria (*MIC*s greater than 2560 μg/mL), which is in line with previous reports [2,4,5]. As expected, MIC values for the G+ bacteria were inversely proportional to the UA content, *i.e*., antimicrobial potential increased with the upsurge of UA percentage, thus reasserting its mediating role in demonstrated antimicrobial activity of UB extracts [4,5]. Precisely, among the investigated extracts, U-MAC revealed the highest *MIC* values (representing the weakest antimicrobial activity) against investigated bacteria, followed by U-SOX-EtOH and U-SOX-E (Table 2). U-SE was the most effective extract, revealing the same *MIC* values as the pure UA with *S. epidermidis*

Table 2. Usnic acid content and minimal inhibitory concentrations (MIC values) of the investigated isolates of U. barbata, expressed as μg/mL (usnic acid (UA), supercritical CO_2 extract (U-SE), Soxhlet extract-ether fraction (U-SOX-E), Soxhlet extract-ethanol fraction (U-SOX-EtOH) and macerate (U-MAC))

Extract/isolate	Usnic acid content, %	*S. aureus* ATCC 25923	MRSA ATCC 33591	*S. epidermidis* (CI)	*E. faecalis* (CI)	*K. pneumoniae* (CI)	*E. coli* ATCC 25922
UA	98.00	10	5	1.25	2.5	no effect	no effect
U-SE	81.41	10	5	1.25	2.5	no effect	no effect
U-SOX-E	67.09	40	20	5	5	no effect	no effect
U-SOX-EtOH	2.43	320	320	40	160	no effect	no effect
U-MAC	1.39	640	320	160	320	no effect	no effect

and *E. faecalis* clinical isolates being the most sensitive among the tested bacterial strains (Table 2).

Thus, U-SE, as the extract with the best antimicrobial potential, was incorporated into the developed APG-based (ML) and pharmacopoeial (P) vehicle in the same concentration corresponding to 2 mass% of UA, as a carrier of stated biological activity, by suspending the extract using mortar and pestle, yielding samples ML-U and P-U, respectively. Thereafter, their physicochemical stability, efficiency and safety were evaluated and compared both mutually and to their corresponding placeboes, in the second part of the study.

Both placebo samples were shiny, white semisolids, with ML having a softer consistency in comparison to P. Active samples (ML-U and P-U) were yellowish creams, somewhat thicker in comparison to their matching placebo samples.

A preliminary physical stability of investigated creams was carried out using continual rheology and electrical conductivity measurements, while pH value measurements gave an insight into their preliminary chemical stability. All measurements were carried out 7, 30 and 90 days after preparation, and tested parameters were compared as a function of time.

Taking into account shear stress–shear rate curves, all samples exhibited shear-thinning flow behavior with moderate (ML and ML-U) to pronounced (P and P-U) thixotropy, considered desirable for topically applied preparations (Fig. 1a) [17]. In addition, rheological measurements accentuated distinct sensory characteristics of APG-based samples in comparison to the reference ones, observed visually. Upon U-SE addition into both APG-based and pharmacopoeial vehicle, samples' yield stresses and hysteresis loops were increased, as

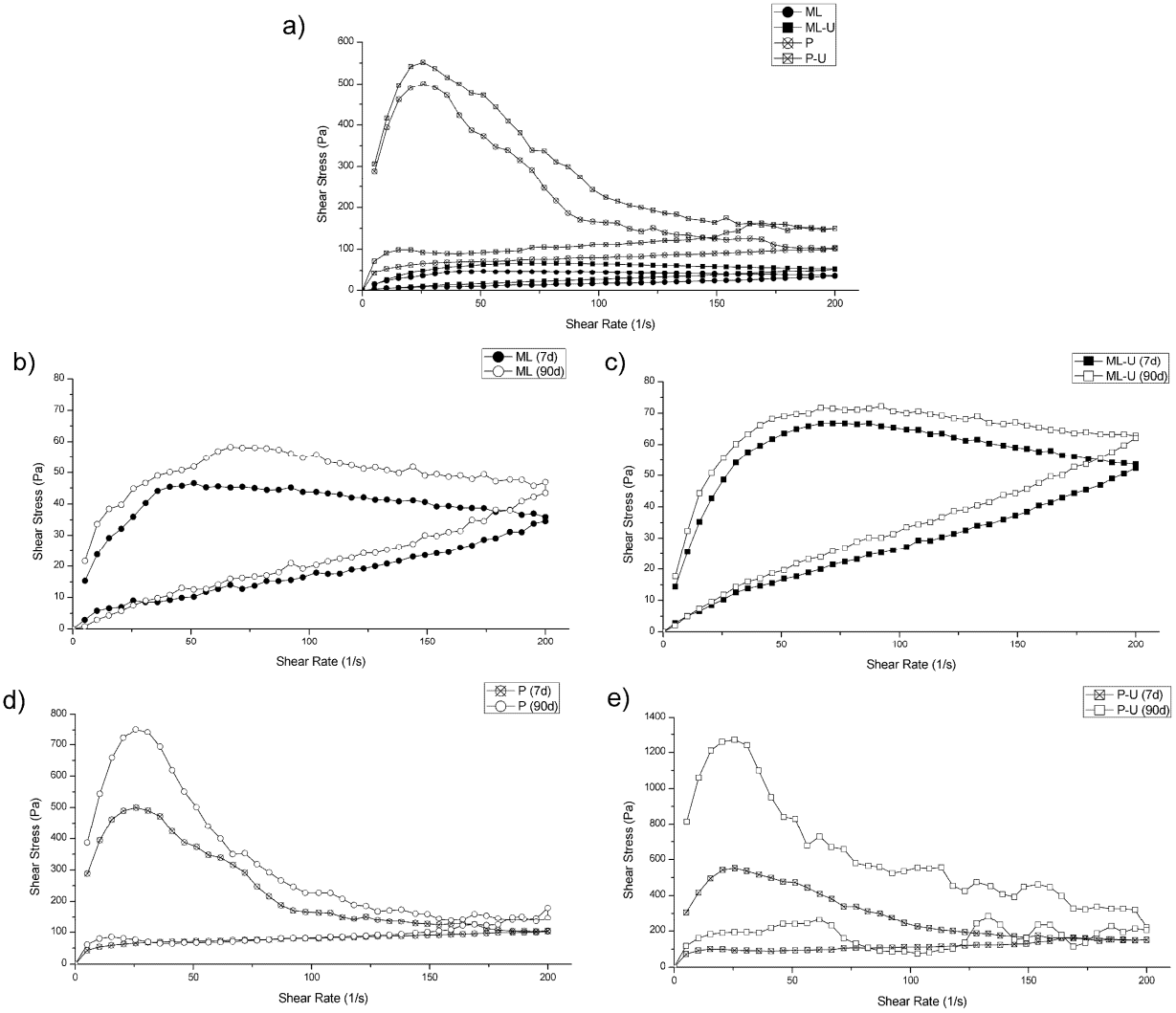

Figure 1. Flow curves of: a) all cream samples (alkyl polyglucoside-stabilized vehicle (ML) and pharmacopoeial vehicle (P)) and active cream samples with U. barbata supercritical CO_2 extract corresponding to 2 mass% of usnic acid in the final formulation incorporated into vehicle ML (ML-U and P (P-U)) 7 days after preparation, b) sample ML 7 days (ML(7d)) and 90 days (ML(90d)) after preparation; c) sample ML-U 7 days (ML-U(7d)) and 90 days (ML-U(90d)) after preparation, d) sample P 7 days (P(7d)) and 90 days (P(90d)) after preparation, e) sample P-U 7 days (P-U(7d)) and 90 days (P-U (90d)) after preparation.

expected, considering the fact that the extract was suspended in the investigated bases, substantiating findings of organoleptic inspection (Fig. 1a). It is a known fact that evolution of rheological properties can indicate instability of emulsions. Therefore, repeated measurements of rheological parameters may serve as a simple and convenient experimental method for estimating the state of an emulsion as a function of storage time [18]. In this accordance, samples ML, ML-U and P after 3 months did not show significant changes in rheological profiles (Fig. 1 b–d). More specifically, the lowest increase of hysteresis loop was recorded in APG-based samples, *i.e.*, in ML-U, followed by ML. On the other hand, a major upsurge of this rheological parameter for P-U, especially in respect to its corresponding placebo (P), could be connected to the dissatisfying preliminary physical stability, as well as unsatisfactory applicative characteristics of this sample (Fig. 1e) [19]. With this respect, continual rheological measurements indicated preferential physical stability of APG base, especially regarding U-SE incorporation potential, when compared to pharmacopoeial one. They also emphasized distinctively ameliorated sensory characteristics of APG-based samples, as an important property of topical preparations having a major impact on patients' compliance and consequent efficacy in the management of skin diseases [6].

Initial conductivity measurements of the placebo samples (ML and P) revealed the values being non-typical for the multiphase emulsion systems prepared with nonionic-mixed emulsifiers (0–50 μs/cm) and in the field of real O/W emulsions (Table 3). However, although conductivity measurements may indicate the degree of structuring and/or the type of emulsion, it is not possible to unequivocally interpret high conductivity solely as reduced structuring of the system, while it may also simply reflect the level of free ions present within the system [20,21]. Nevertheless, even though the only ionic moieties present in both systems originated from the preservative, potassium sorbate, used in the same concentration (Table 1) initially assessed specific conductivity detected in APG-based sample (ML) was notably higher in comparison to the pharmacopoeial one (P). This discrepancy might be attributed, at least partially, to the higher portion of water within the system and therefore probably higher percentage of free/bulk water (Table 1) but also the different time needed for achieving final microstructure of APG-based (ML) *versus* referent (P) emulsion [21,22]. Later assumption was broadly supported by conductivity measurements reasserted after 30 days of storage, that revealed discernible decrease in specific conductivity of APG-based vehicle (ML) pointing to its subsequent structuring [20], as opposed to pharmacopoeial one (P), having conductivity value at the same level as in initial measurements (Table 3). However, further measurement assessed 90 after preparation, disclosed an increase of conductivity for both placebo creams (ML and P). As for the active samples (ML-U and P-U), addition of the extract (U-SE) affected their matching vehicles (ML and P, respectively) in an inverse manner (Table 3). Namely, in the initial measurements, it led to certain decrease of specific conductivity in ML-U related to ML that is an increase in P-U connected to P (Table 3). In addition, both active creams followed the trend of conductivity change as the function of storage time pursuant to the corresponding vehicles (Table 3).

Conductivity increase could point to certain changes in physical characteristics of investigated systems and might be interpreted as a sign of physical instability, and it has been suggested that the later this rise occurred and the smaller the growth was, the stability of the cream increased [22]. In our experiments, all of the investigated cream samples, with the exception of ML, revealed an increase of conductivity after 90 days of storage at room temperature in comparison to the values assessed 7 days after their preparation (Table 3). However, when considering an initial decrease of specific conductivity of APG-based samples (ML and ML-U) within first 30 days of room temperature storage, an overall growth of conductivity may be considered more accentuated in these samples in relation to the reference ones (P and P-U, Table 3). It is well established that for the definitive evaluation of physical stability of an emulsion system, parameters assessed using different methods are considered relevant. In this regard, greater extent of conductivity increase in APG-based samples related to the reference ones was not in line with previously discussed rheological findings. On the other side, P-U was the only sample disclosing perm-

Table 3. Conductivity and pH values of placebo (alkyl polyglucoside-stabilized vehicle (ML) and pharmacopoeial vehicle (P)) and active cream samples (with U. barbata supercritical CO_2 extract corresponding to 2 mass% of usnic acid in the final formulation incorporated into vehicle ML (ML-U) and P (P-U)) assessed 7, 30 and 90 days after preparation

Sample	Conductivity, μS/cm			pH		
	7 days	30 days	90 days	7 days	30 days	90 days
ML	204.40	91.50	181.00	7.04	6.95	6.65
ML-U	199.50	107.30	248.00	6.96	6.98	6.90
P	73.70	73.30	110.70	7.37	7.15	7.47
P-U	83.60	85.90	131.60	7.30	7.32	7.47

anent increase of conductivity throughout 90-day period of storage at room temperature. Connected to previously discussed changes in rheological behavior of this sample as a function of the stated storage time, such observations should be taken into account in the long-term predictions of physical stability of this sample. Overall, on the basis of certain dissidences in findings of rheological and conductivity measurements, additional investigations are needed for the final assessment on physical stability of all investigated cream samples.

pH measurements showed all examined samples to be within ranges suitable for skin application (Table 3) [6]. Addition of the U-SE in the active samples (ML-U and P-U) led to a slight decrease in pH value in comparison to corresponding vehicle (ML and P, respectively), which could be expected considering the measured pH value of the extract *per se* (5.80). In addition, obtained values remained similar throughout the 3-month observation period, indicating satisfying preliminary chemical stability of investigated samples.

In accordance to the results obtained for the extract of UB incorporated into the investigative creams (U--SE), their antimicrobial activity assessment was performed only against G+ bacterial species (the same as used for the extracts). Results of antimicrobial activity are presented in Table 4. ML and P served as negative controls for the appropriate active creams (ML-U and P-U, respectively), while ampicillin was used as a positive control for UA. *MIC*s are presented for UA as a part of ML-U and P-U and as intact substance [23]. None of the placebo creams (neither ML nor P) showed activity against tested microorganisms (MICs greater than 2560 μg/mL), as seen in Table 4. On the other hand, both active creams (ML-U and P-U) revealed the potential to inhibit growth of investigated bacteria (Table 4). Accordingly, UA as the active substance of active creams followed the same pattern (Table 4). Moreover, obtained MIC values were consistent with the ones determined for UA *per se*, revealing strong antimicrobial activity against tested G+ strains, especially in the case of UA as a part of ML-U. With this respect, comparison of antimicrobial activity of ML-U *versus* P-U revealed the same inhibition spectrums of both creams against MRSA ATCC 33591 and clinical isolate of *E. faecalis*, whereas ML-U was more active against *S. aureus* ATCC 25923 and clinical isolate of *S. epidermidis* (Table 4). In contrast, ampicillin showed no effect against most of the tested strains. It was, however, more effective than UA against clinical isolate of *E. faecalis* (Table 4).

Investigated samples showed overall satisfying preliminary safety profiles (Fig. 2). Two hours after occlusion removal, there was no significant change in EI, which was even decreased for all investigated samples, indicating well-tolerated skin formulations (Fig. 2a). TEWL was increased for the samples ML and P-U related to corresponding baseline, but not the controls. Nevertheless, this increase might be attributed predominantly to the occlusion effect, regarding the fact that TEWL obtained for UCO was also significantly higher in comparison to the baseline (Fig. 2b). As for EC, all samples led to an increase of this parameter (Fig. 2c). The increase could be considered significant in the case of both APG-based samples (ML and ML-U) compared to baselines and both controls, revealing skin hydration potential probably related to the vehicle itself, considering that there was no statistically significant differences in EC increase between these two samples (Fig. 2c). The same finding regarding EC was noted for the pharmacopoeial vehicle (P), but not the active cream (P-U), which EC increase was statistically significant compared to UC, but not UCO.

Table 4. Minimal inhibitory concentrations (MIC values), expressed as μg/mL, for the investigated samples: placebo creams (alkyl polyglucoside-stabilized vehicle (ML) and pharmacopoeial vehicle (P), negative control); active creams (with U. barbata supercritical CO_2 extract corresponding to 2 mass% of usnic acid in the final formulation incorporated into vehicle ML (ML-U) and P (P-U)); active substance usnic acid (UA, as a part of active cream (ML-U and P-U) and per se) and ampicillin (positive control)

Sample	*S. aureus* ATCC 25923	MRSA ATCC 33591	*S. epidermidis* (CI)	*E. faecalis* (CI)
ML	No effect	No effect	No effect	No effect
ML-U	1280	320	40	80
UA (AP ML-U)[a]	25.6	6.4	0.8	1.6
P	No effect	No effect	No effect	No effect
P-U	2560	320	80	80
UA (AP P-U)[b]	51.2	6.4	1.6	1.6
UA (*per se*)	10	5	1.25	2.5
Ampicillin	No effect	No effect	No effect	0.125

[a]*MIC* of UA as a part of ML-U (AP ML-U) was calculated as the concentration of UA in the cream multiplied by *MIC* for ML-U; [b]*MIC* of UA as a part of P-U (AP P-U) was calculated as the concentration of UA in the cream multiplied by *MIC* for P-U

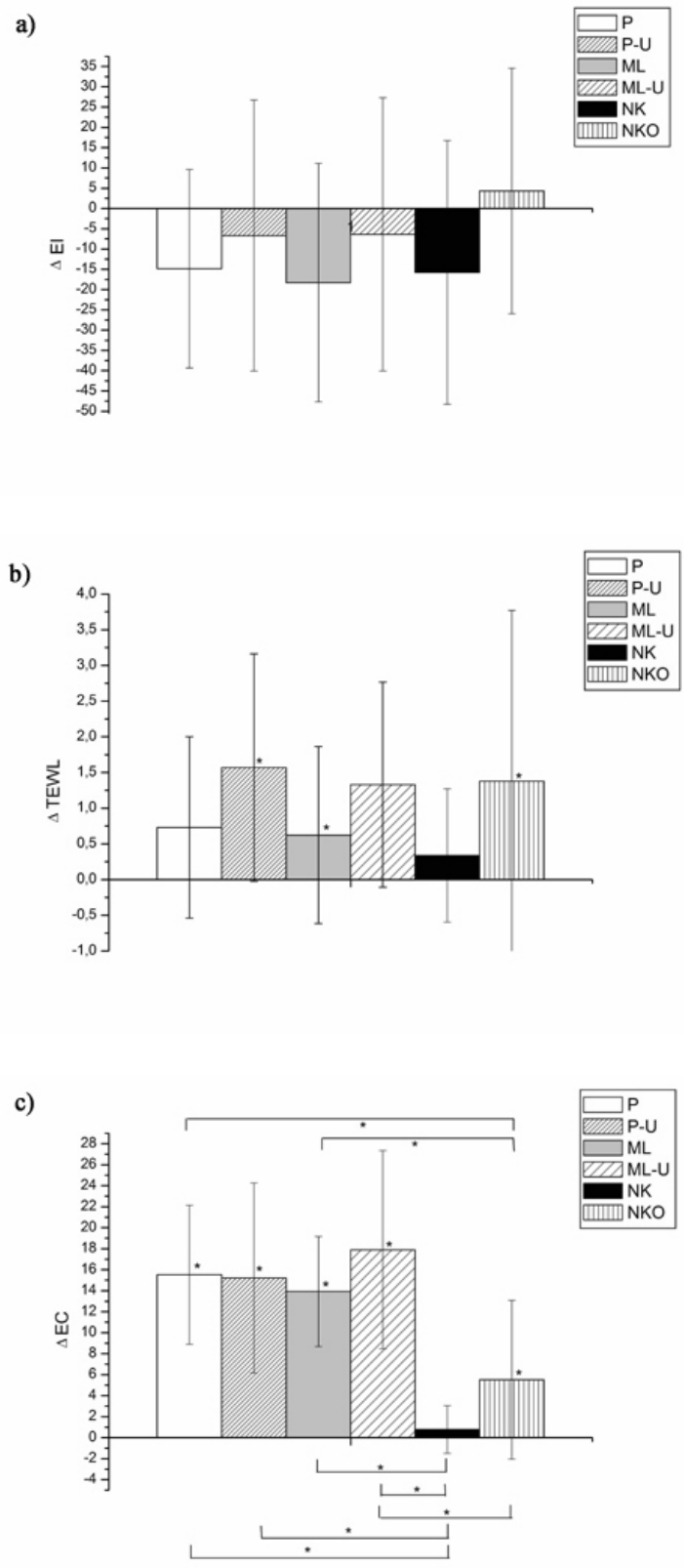

Figure 2. The effects of placebo (alkyl polyglucoside-stabilized vehicle (ML) and pharmacopoeial vehicle (P) and active cream samples (cream samples with U. barbata supercritical CO_2 extract corresponding to 2 mass% of usnic acid in the final formulation incorporated into vehicle ML (ML-U) and P (P-U)) on: a) erythema index (EI), b) transepidermal water loss (TEWL) and c) electrical capacitance (EC). The results are shown as absolute changes of mean values and standard error of mean to the baseline. The effects of tested formulations were compared mutually and to both controls (under occlusion, UCO and without occlusion, UC). Significant differences are marked with ($P < 0.05$).*

CONCLUSION

Results of our study proved the developed alkyl polyglucoside (APG)-based vehicle to be a promising carrier for *Usnea barbata* CO_2-supercritical extract (U--SE) in the concentration corresponding to 2 mass% of usnic acid, composing a topical formulation with potential clinical relevance in the treatment of skin infections. Investigations on rheological behavior of the investigated cream samples indicated APG-stabilized emulsion to possess highly satisfactory ability to remain physically stable upon the addition of U-SE, when compared to pharmacopoeial one. However, conductivity measurements revealed certain dissidences related to rheological findings, imposing the need for additional investigations for the final assessment on physical stability of the investigated emulsion systems. Satisfying preliminary chemical stability was shown for all tested cream samples. Further efficiency and safety evaluation revealed stronger antimicrobial potential of APG-containing pharmaceutical vehicle when compared to traditionally used pharmacopoeial base of proven quality. Overall satisfying preliminary safety profiles were demonstrated for both APG-based and reference cream samples.

In all, investigated APG base revealed certain preferential features as a vehicle for U-SE compared to the conventional one, especially considering its improved sensory characteristics (confirmed by organoleptic and rheological assessment), as a property often decisive for the patient compliance.

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IZVOD

EMULZIJA STABILIZOVANA ALKIL POLIGLUKOZIDNIM EMULGATOROM KAO POTENCIJALNI NOSAČ ZA CO_2-NATKRITIČNI EKSTRAKT VRSTE *Usnea barbata*: PROCENA STABILNOSTI, BEZBEDNOSTI I EFIKASNOSTI TOPIKALNE FORMULACIJE

Ana R. Žugić[1], Milica Z. Lukić[2], Marija Z. Tasić Kostov[3], Vanja M. Tadić[1], Ivana A. Arsic[3], Dušan R. Mišić[4], Slobodan D. Petrović[5], Snežana D. Savić[2]

[1]*Institut za proučavanje lekovitog bilja "Dr Josif Pančić", Odsek za farmaceutska istraživanja i razvoj, Beograd, Srbija*
[2]*Univerzitet u Beogradu, Farmaceutski fakultet, Katedra za farmaceutsku tehnologiju i kozmetologiju, Beograd, Srbija*
[3]*Univerzitet u Nišu, Medicinski fakultet, IAS Farmacije, Niš, Srbija*
[4]*Univerzitet u Beogradu, Fakultet veterinarske medicine, Katedra za mikrobiologiju, Beograd, Srbija*
[5]*Univerzitet u Beogradu, Tehnološko-metalurški fakultet, Katedra za organsku hemiju, Beograd, Srbija*

(Naučni rad)

Antimikrobna aktivnost vrste *Usnea barbata* naročito protiv bakterija koje učestvuju u patogenezi različitih bolesti kože, dobro je dokumentovana u naučnoj literaturi. Uprkos tome, ne postoje radovi koji se bave formulacijom topikalnih preparata na bazi ovog lišaja namenjenih lečenju kožnih infekcija. U ovoj studiji, razvijena je podloga stablizovana alkil poliglukozidnim (APG) emulgatorom, kao potencijalni nosač za ekstrakt vrste *U. barbata* koji je pokazao najbolji antimikrobni potencijal u preliminarnom istraživanju nekoliko ekstrakata dobijenih upotrebom različitih ekstragenasa/postupaka. Radi poređenja, odabrani CO_2-natkritični ekstrakt je inkorporiran u istoj koncentraciji (koja odgovara 2 mas.% usninske kiseline) i istim postupkom izrade u često korišćenu podlogu farmakopejskog kvaliteta, a zatim je sprovedeno uporedno istraživanje fizičko-hemijske stabilnosti, efikasnosti i bezbednosti na obe grupe uzoraka. Rezulati našeg istraživanja pokazali su da se razvijena podloga stabilizovana APG emulgatorom može smatrati pogodnim nosačem za CO_2-natkritični ekstrakt vrste *U. barbata*, čineći topikalnu formulaciju sa potencijalnim kliničkim značajem u terapiji kožnih infekcija. Reološka istraživanja pokazala su zadovoljavajuću sposobnost emulzije stabilizovane APG emulgatorom da ostane fizički stabilna nakon dodatka CO_2-natkritičnog ekstrakta vrste *U. barbata*, u poređenju sa farmakopejskom. Međutim, konduktometrijska merenja su pokazala određena neslaganja sa reološkim nalazima, namećući potrebu za dodatnim istraživanjima radi konačne procene fizičke stabilnosti testiranih emulzionih sistema. Izmerene vrednosti pH tokom perioda od 90 dana čuvanja uzoraka na sobnoj temperaturi ukazale su na zadovoljavajuću preliminarnu hemijsku stabilnost svih uzoraka. Dalja istraživanja efikasnosti i bezbednosti pokazala su bolji antimikrobni potencijal uzoraka sa podlogom stabilizovanom APG emulgatorom u odnosu na tradicionalno upotrebljavanu farmakopejsku bazu poznatog kvaliteta. Zadovoljavajući preliminarni bezbedonosni profili su pokazani kako za uzorke stabilizovane APG emulgatorom tako i za referentne krem uzorke. Na osnovu navedenog, može se zaključiti da je istraživana podloga bazirana na APG emulgatoru pokazala određene povoljnije karakteristike kao nosač za CO_2-natkritični ekstrakt vrste *U. barbata* u poređenju sa konvencionalno korišćenom podlogom, naročito uzimajući u obzir njene poboljšane senzorne karakteristike, kao osobinu koja je često odlučujuća za komplijansu pacijenata.

Ključne reči: Alkil poliglukozidi • *Usnea barbata* natkritični CO_2-ekstrakt • Infekcije kože • Fizičko–hemijska stabilnost • Antimikrobna aktivnost • Performanse na koži