NASTAVNO-NAUČNOM VEĆU FARMACEUTSKOG FAKULTETA UNIVERZITETA U BEOGRADU

TO THE ACADEMIC COUNCIL OF THE FACULTY OF PHARMACY-UNIVERSITY OF BELGRADE

KOMISIJI ZA POSLEDIPLOMSKE STUDIJE

TO THE COMMITTEE FOR POSTGRADUATE STUDIES

Na sednici Nastavno-naučnog veća Farmaceutskog fakulteta u Beogradu, održanoj 9.6.2016. godine imenovani su članovi Komisije za ocenu i odbranu završene doktorske disertacije kandidata diplomiranog farmaceuta Ane Žugić, pod naslovom:

The Academic Council of the Faculty of Pharmacy at the University of Belgrade on the meeting held on June 9, 2016 has nominated the Commission for evaluation and defence of doctoral dissertation of dipl. pharm. Ana Žugić entitled:

Ekstrakt talusa *Usnea barbata* (L.) Weber ex F. H. Wigg., Parmeliaceae u emulzionim nosačima stabilizovanim biorazgradivim emulgatorima: preformulaciona i formulaciona istraživanja

Extract of the talus of Usnea barbata (L.) Weber ex F. H. Wigg., Parmeliaceae in the emulsion vehicles stabilized with biodegradable emulsifiers: preformulation and formulation investigations

Komisija u sastavu/*Commission including*:

1. **Dr Snežana Savić, vanredni profesor/*associate professor*, mentor**

Univerzitet u Beogradu–Farmaceutski fakultet/*University of Belgrade–Faculty of Pharmacy*

2. **Dr Gordana Vuleta, redovni profesor/*full professor***

Univerzitet u Beogradu–Farmaceutski fakultet/*University of Belgrade–Faculty of Pharmacy*

3. **Dr Vanja Tadić, naučni savetnik/*principal research fellow***

Institut za proučavanje lekovitog bilja „Dr Josif Pančić“, Beograd/*Institute for Medicinal Plant Research “Dr. Josif Pancic”, Belgrade*

4. **Dr Rolf Daniels, redovni profesor/*full professor***

Eberhard-Karls Univerzitet, Tibingen, Nemačka/*Eberhard-Karls Universität, Tübingen, Germany*

5. **Dr Ivana Arsić, vanredni profesor/*associate professor***

Univerzitet u Nišu-Medicinski fakultet/ *University of Nis-Faculty of Medicine*

pregledala je priloženu disertaciju i podnosi Nastavno-naučnom veću Farmaceutskog fakulteta Univerziteta u Beogradu sledeći/

based on detailed review of the submitted dissertation, presents to the Academic Council of the Faculty of Pharmacy-University of Belgrade the following

IZVEŠTAJ/*REPORT*

A. PRIKAZ SADRŽAJA DOKTORSKE DISERTACIJE/*THE CONTENT OF THE DOCTORAL DISSERTATION*

Doktorska disertacija pod nazivom: "**Ekstrakt talusa *Usnea barbata* (L.) Weber ex F. H. Wigg., Parmeliaceae u emulzionim nosačima stabilizovanim biorazgradivim emulgatorima: preformulaciona i formulaciona istraživanja**", sadrži šest poglavlja: Uvod, Cilj istraživanja, Eksperimentalni deo, Rezultati i diskusija, Zaključak i Literatura. Na početku rada je priložen Sažetak/Abstract, dok se na kraju rada nalazi spisak publikovanih/saopštenih radova koji čine deo doktorske disertacije, kratka biografija kandidata i potpisane izjave kandidata o autorstvu, istovetnosti štampane i elektronske verzije i korišćenju doktorske disertacije (obavezni Prilozi 1, 2 i 3).

*Doctoral dissertation entitled: "**Extract of the talus of Usnea barbata (L.) Weber ex F. H. Wigg., Parmeliaceae in the emulsion vehicles stabilized with biodegradable emulsifiers: preformulation and formulation investigations**", comprises six chapters: Introduction, The Aim of the research, Experimental section, Results and Discussion, Conclusion and Literature. In the beginning of the dissertation a short Abstract is given, while the material ends with the list of published papers that are a part of the dissertation, short biography of the candidate, and signed statements on the authorship, compatibility of the printed and electronic versions of the dissertation, and the usage of the doctoral dissertation (mandatory Appendix 1, 2 and 3).*

Disertacija je napisana na 123 strane i sadrži 24 slike (4 u uvodu i 20 u rezultatima i diskusiji), 17 tabela (3 u uvodu, 3 u eksperimentalnom delu i 11 u rezultatima i diskusiji). Pregled literature sadrži 205 navoda.

Dissertation is written on 123 pages and comprises 24 figures (4 in the introduction and 20 in the results and discussion section), 17 tables (3 in the introduction, 3 in the experimental part, and 11 in the results and discussion section). The literature section lists 205 references.

UVOD se sastoji iz tri dela. Svaki od njih sadrži informacije koje su od značaja za predmet proučavanja ove doktorske disertacije. U prvom delu uvoda kandidatkinja je izdvojila najznačajnije informacije o primeni antibiotika u lokalnoj terapiji nekomplikovanih/površinskih infekcija kože. Preciznije, kandidatkinja iznosi najnovije literaturne podatke o efektima dejstava uobičajeno korišćenih lokalnih antibiotika u terapiji i/ili prevenciji najčešćih nekomplikovanih/površinskih infekcija kože (impetigo, sekundarno inficirane dermatoze i sekundarno inficirane traumatske lezije kože). Nakon toga, kandidatkinja obrazlaže ograničenja primene lokalnih antibiotika, i, s tim u vezi, potrebu za razvojem novih lekova iz ove grupe, odnosno pronalaženjem novih lekovitih supstanci sa antimikrobnim delovanjem, ali i formulacijom savremenih nosača za navedene supstance, koji bi zamenili određene konvencionalne podloge.

*The **INTRODUCTION** is organized in three parts. Each part provides information relevant for the subject of the scientific research. In the first part of the introduction, the candidate presents the most relevant information on the use of antibiotics in the local treatment of uncomplicated/superficial skin infections. More precisely, the candidate states recent data on the effects of antibiotics in the local treatment and/or prevention of the most common uncomplicated/superficial skin infections (impetigo, secondarily infected*

dermatoses and secondarily infected traumatic lesions of skin). Thereafter, the candidate elaborates the limitations of local antibiotics and, in this connection, the need for the development of new preparations in this group, that is investigations of new antimicrobial substances as well as formulation of contemporary carriers of stated substances that could successfully replace conventional vehicles/bases.

U drugom delu ovog poglavlja opisani su lišajevi kao potencijalni izvor navedenih lekovitih supstanci. U tom smislu, u ovom delu Uvoda navedeni su literaturni podaci vezani za *Usnea barabata* (L.) Weber ex F.H. Wigg., Parmeliaceae (jevrejska ili lišaj brada), kao jednom od najbolje proučenih lišajeva, i dodatno, za usninsku kiselinu, jedinjenje koje je identifikovano kao glavni sekundarni metabolit jevrejske brade i nosilac njenih bioloških aktivnosti.

The second part of this chapter describes lichens as potential source of the stated drug substances. With respect to this, this part of the Introduction section states literature data about Usnea barabata (L.) Weber ex F.H. Wigg., Parmeliaceae (old man's beard) as one of the best studied lichens, and additionally, about usnic acid, a compound identified as the major second metabolite of old man's beard and the carrier of its biological activities.

Treći deo Uvoda posvećen je alkil poliglukozidima (APG), kao mogućim stabilizatorima savremenih nosača (emulzija i kremova) lekovitih/kozmetički aktivnih supstanci. Detaljan pregled radova najznačajnijih predstavnika ove grupe nejonskih mešanih emulgatora prirodnog porekla, pružio je uvid u njihove brojne prednosti (biorazgradivost, dobra podnošljivost na koži), ali i specifične karakteristike, važne za primenu u kozmetologiji, a potencijalno i u dermofarmaciji. Na osnovu navedenih podataka iz dostupne literature, kandidatkinja iznosi hipotezu da bi dva novija APG emulgatora: 1) $C_{14\text{-}22}$ alkohol i $C_{12\text{-}20}$ glukozid i 2) koko glukozid i alkohol kokosovog oraha mogla da omoguće formulisanje emulzionog sistema poboljšane/unapređene fizičko-hemijske stabilnosti, biofarmaceutskih karakteristika, senzornih osobina, kao i povoljnih efekata na koži, koji bi našao primenu kao nosač u dermofarmaceutskim preparatima za lokalnu primenu kod kutanih infekcija.

The third part of the Introduction is focused on the Alkyl Polyglucosides (APG), as stabilizers of contemporary carriers (emulsions and creams) of active pharmaceutical/cosmetic ingredients. Detailed review of the scientific studies investigating the most prominent representatives of this group of natural origin non-ionic emulsifiers has provided insight into their numerous advantages (biodegradability, skin compatibility), as well as specific properties important for their usage in cosmetology, and potentially in dermopharmacy. Based on the stated literature data, the candidate hypothesizes that two novel APG emulsifiers: 1) $C_{14\text{-}22}$ alcohol and $C_{12\text{-}20}$ glucoside and 2) cocoglucoside and coconut alcohol could allow formulation of the emulsion system having enhanced physical stability, biopharmaceutical and sensory properties, as well as beneficial skin performance, that could serve as the carrier in dermopharmaceutical preparations for the local treatment of cutaneous infections.

CILJ RADA je jasno definisan tako da se sagleda mogućnost upotrebe navedenih APG emulgatora kao stabilizatora emulzione podloge tipa hidrofilnog krema, koja bi našla primenu kao savremeni nosač za kvantifikovani ekstrakt talusa lišaja *U. barbata*, na usninsku kiselinu, supstancu dokazanog antimikrobnog delovanja, a u cilju razvoja finalnog farmaceutskog preparata potencijalno namenjenog lokalnoj terapiji infekcija kože. Uz osnovnu pretpostavku da navedeni emulzioni sistem, stabilizovan APG emulgatorima neće pokazivati nedostatke uobičajene za tradicionalne recapture podloga iz grupe krema, njegov potencijal da ostane fizički stabilan nakon opterećenja ekstraktom, sa posebnim osvrtom na rasvetljavanje uticaja variranja koncentracije/postupka inkorporiranja ekstrakta na višefaznu

koloidnu strukturu krema i njegov potencijal za isporuku aktivne supstance, procenjen je komparativnom primenom različitih fizičko-hemijskih i biofarmaceutskih ispitivanja, i konačno upotpunjen evaluacijom preliminarne efikasnosti i bezbednosti razvijene prototip formulacije finalnog proizvoda.

The AIM of the research is clearly defined–to assess the possible application of the stated APG emulsifiers as stabilizers of emulsion base of hydrophilic cream type that could serve as contemporary carrier for the extract of the talus of U. barbata, with quantified usnic acid, a substance of natural origin with well-documented antimicrobial activity, with the aim of developing a final pharmaceutical preparation potentially applied in the local treatment of skin infections. With the basic hypothesis that the APG-stabilized emulsion system will not demonstrate shortcomings typical for traditional formulations, its capacity to remain physically stable upon the extract addition, with a special interest in evaluating the influence of the variation of the concentration/procedure of incorporation of the extract on multiphase colloidal structure of the cream and its potential for the delivery of active substance was assessed by means of comparative application of different physicochemical and biopharmaceutical investigations and finally complemented by the preliminary efficacy and safety evaluation of the developed prototype formulation of the final product.

EKSPERIMENTALNI DEO-U ovom poglavlju prikazani su podaci o ispitivanim uzorcima i opisane sve metode i uređaji koji su korišćeni u eksperimentalnom radu. Eksperimentalni rad izveden je u tri faze.

THE EXPERIMENTAL PART*–This section contains information on the investigated samples and conducted methods, along with the description of the equipment used throughout the experiments which were organized in three phases.*

U I delu ovog poglavlja dati su detalji izrade ispitivanih ekstrakata talusa lišaja *U. barbata* (ekstrakt dobijen pomoću natkritičnog CO_2 (komercijalno dostupan) *vs.* ekstrakti dobijeni konvencionalnim metodama sa organskim rastvaračima), a zatim predstavljen način izvođenja (oprema i eksperimentalni uslovi) ispitivanja hemijskog profila i *in vitro* testova bioloških svojstava ovih ekstrakata (antimikrobna, citotoksična i antioksidativna aktivnost-u poređenju sa čistom usninskom kiselinom).

The first part of this chapter contains details of preparation of the extracts of the talus of U. barbata (supercritical CO_2 extract (commercially available) vs. conventional methods employing organic solvents), as well as data (equipment and experimental conditions) regarding the examinations of chemical profile and in vitro tests of biological properties of the stated extracts (antimicrobial, cytotoxic and antioxidative activity,compared to pure usnic acid).

U II delu ovog poglavlja opisani su detalji preformulacione studije ekstrakta, koji je u prethodnoj fazi odabran na osnovu najboljeg odnosa antimikrobnog potencijala protiv kutanih patogena i citotoksičnosti prema humanim keratinocitima. Dalje, dati su podaci vezani za sastav i način izrade emulzionih sistema sa izabranim APG mešanim emulgatorima, sa i bez dodatka ekstrakta, a zatim je opisana oprema i primenjeni uslovi ispitivanja fizičko-hemijske karakterizacije i *in vitro/in vivo* ispitivanja biofarmaceutskih svojstava ovih uzoraka, sa ciljem procene njihove koloidne strukture i fizičke stabilnosti, odnosno potencijala za isporuku usninske kiseline, kao aktivne supstance ispitivanih kremova sa ekstraktom, respektivno. Kao što je ranije naglašeno, pri inkoropriranju ekstrakta u podlogu stabilizovanu APG emulgatorima, variran je način njegovog dodavanja (suspendovanjem u gotovu podlogu-S uzorci i dispergovanjem u masnoj fazi krema, pre mešanja sa vodenom fazom-D uzorci), kao i koncentracija (koja je odgovarala sadržaju usninske kiseline od 1, 2 i 4 %

(m/m) u finalnoj formulaciji (koncentarcije su izabrane na osnovu literaturnih podataka i eksperimenata prve faze)).

The second phase of the experimental work describes the details of the preformulation study of the extract, which was selected in the previous phase of research on the basis of the best ratio of antimicrobial potential against cutaneous pathogens and cytotoxicity against human keratinocytes. Furthermore, the data regarding the composition and the method of preparation of the APG-stabilized emulsion systems with and without the extract are given, and thereafter, the equipment and experimental conditions of physicochemical characterization and in vitro/in vivo investigations of biopharmaceutical properties of these samples is described, with the aim of assessing their colloidal structure and physical stability, i.e. the potential for the delivery of usnic acid, as an active substance of the investigated active creams, respectively. As emphasized earlier, when the extract was added to APG-based emulsion vehicle, the method of its incorporation (by suspending it into the vehicle-S samples or by dispersing it into the oil phase of the cream, prior to the addition to the water phase-D samples) as well as extract concentration (corresponding to 1, 2 and 4 % (w/w) of usnic acid in the final formulation; these concentrations were selected in accordance to the literature data and the results of the first phase of experiments) were varied.

U III fazi eksperimentalnog dela opisani su uslovi izvođenja i oprema korišćena za ispitivanje preliminarne efikasnosti (procenjeno *in vitro* i *in vivo*) i bezbednosti/iritacionog potencijala (procenjeno *in vivo*) prototip formulacije gotovog proizvoda-krema stabilizovanog APG emulgatorima sa ekstraktom (dodatim u odgovarajućoj koncentraciji i odgovarajućim postupkom inkorporiranja), koja je izabrana na osnovu rezultata II faze eksperimenata (najbolja preliminarna fizičko-hemijska stabilnost i najbolji liberacioni profil).

The third phase of the experimental part focuses on the conditions and the equipment used for the investigations of preliminary efficiency (assessed in vitro and in vivo) and safety/irritation potential (assessed in vivo) of the prototype formulation of the finished product-APG-stabilized cream with the extract (added in the appropriate concentration and using the appropriate method of incorporation), chosen in accordance to the results of the second phase of experiments (the best preliminary physicochemical stability and the best liberation profile).

Sve *in vivo* studije su sprovedene u skladu sa Helsinškom deklaracijom i odobrenjem Etičkog komiteta Medicinskog fakulteta Univerziteta u Nišu.

All in vivo studies followed the principles of the Declaration of Helsinki, and were conducted in line with the obtained approval of the Ethical committee of the Faculty of Medicine in Nis.

Sve metode koje su korišćene u eksperimentalnom radu ove doktorske disertacije u skladu su sa savremenim zahtevima naučnoistraživačkog rada u relevantnoj oblasti, te su, s tim u vezi, omogućile dobijanje rezultata na osnovu kojih je moguće doneti adekvatne naučne zaključke. Dodatna objektivnost dobijenih rezultata dobijena je primenom istih metoda na poredbenim uzorcima (oficinalna podloga stabilizovana konvencionalnim nejonskim emulgatorima (Nejonski hidrofilni krem, DAB 2011) i odgovarajući krem sa ekstraktom koji sadrži navedenu podlogu).

All the methods used in the experimental work of this doctoral dissertation are in accordance to the contemporary requirements of the scientific research in this field, and, hence provided the results indispensable for reaching appropriate scientific conclusions. Additional objectivity of the obtained results is achieved by performing the same methods on

referent samples (pharmacopoeial base stabilized with conventional non-ionic emulsifiers (Non-ionic hydrophilic cream, DAB 2011) and the appropriate active cream, containing the stated base).

REZULTATI I DISKUSIJA–prikazani su na 53 stranice teksta, kroz 20 slika i 11 tabela, i prate prethodno opisane tri faze.

RESULTS AND DISCUSSION*–this section is written on 53 pages similarly organised in three phases, and comprises 20 figures and 11 tables.*

Kroz diskusiju dobijenih rezultata, kandidatkinja je, uz osvrt na relevantne nalaze drugih autora iz iste naučne oblasti, dala svoja tumačenja hipoteza navedenih u ciljevima rada, koja su izneta na razumljiv i sveobuhvatan način.

Through the discussion of the obtained results, the candidate has given rational explanations for hypothesis defined in the aim of the work brought up in a clear and comprehensive manner, while referring to the relevant findings of the authors in the same scientific field.

U poglavlju **ZAKLJUČAK** navedeni su najznačajniji zaključci koji su u skladu sa dobijenim rezultatima i navedenim ciljevima istraživanja.

*The **CONCLUSION** chapter contained the most significant conclusions that stemmed from the previous discussion, addressing each of the aims of the dissertation.*

U poglavlju **LITERATURA** navedeno je 205 referenci citiranih harvardskim stilom.

*The **LITERATURE** section lists 205 references cited using Harvard citation style.*

B. OPIS POSTIGNUTIH REZULTATA/*DESCRIPTION OF THE OBTAINED RESULTS*

Rezultati ove doktorske disertacije podeljeni su u tri celine, iznošenjem i tumačenjem rezultata svake od izvedenih faza eksperimentalnog rada. U okviru prvog dela izneti su rezultati uporedne hemijske karakterizacije i procene bioloških svojstava ekstrakata talusa *U. barbata* izrađenih primenom konvencionalnih metoda ekstrakcije (ekstrakcija po Soxhlet-u dietil-etrom i etanolom i maceracija etanolom) i ekstrakta talusa ovog lišaja koji je dobijen ekstrakcijom natkritičnim CO_2.

The results of this doctoral dissertation are presented and discussed in three parts, according to the phases of the conducted experimental work. Within the first phase, data regarding comparative chemical characterization and the assessment of biological properties of the extracts of the talus of U. barbata prepared using conventional extraction methods (Soxhlet extraction with diethyl ether and ethanol and maceration with ethanol) and the extract obtained using supercritical CO_2 are described.

Eksperimenti prve faze pokazali su da je ekstrakt talusa *U. barbata* koji je dobijen ekstrakcijom natkritičnim CO_2 imao najviši sadržaj usninske kiseline, što je dalje bilo u vezi i sa najboljom aktivnošću upravo ovog ekstrakta u svim ispitivanjima bioloških svojstava, tj. u testovima citotoksičnosti na tumorskim ćelijama (B16 mišji melanom i C6 pacovski gliom), kao i u ispitivanju antimikrobnog potencijala protiv bakterija koje izazivaju infekcije kože i FRAP testu, kojim je ispitivana antioksidativna aktivnost. Navedeni ekstrakt pokazao je ekvivalentnu aktivnost usninskoj kiselini u slučaju ispitivanja antimikrobnog i antioksidativnog potencijala. Sa druge strane, u testovima citotoksičnosti na tumorskim ćelijama, pokazana je bolja aktivnost ekstrakta dobijenog ekstrakcijom natkritičnim CO_2 u

odnosu na usninsku kiselinu *per se*, koja je dovedena u vezu sa oksidativnim stresom koju je ovaj ekstrakt (nasuprot čistoj supstanci) izazvao u ispitivanim ćelijama. Na osnovu rezultata ove faze, preciznije, na osnovu najboljeg odnosa antimikrobnog potencijala protiv bakterija koje su izazivači infekcija kože i citotoksičnosti prema humanim keratinocitima, ovaj kvantifikovani ekstrakt na usninsku kiselinu) je i izabran za sledeće faze istraživanja.

Experiments of the phase I of this doctoral disseration suggested the extract of the talus of U. barbata obtained using supercritical CO_2 to possess the highest usnic acid content, and hence the best activity in all the tests evaluating biological properties: cytotoxicity test against tumor cells (B16 mouse melanoma and C6 rat glioma), investigation of antimicrobial activity against bacteria that commonly cause skin infections and also in FRAP assay, as the common test used for the evaluation of antioxidant activity. The stated extract revealed equivalent activity related to usnic acid in the investigations of antimicrobial and antioxidant potential. On the other hand, in cytotoxicity tests against tumor cells, better activity of the extract obtained using supercritical CO_2 compared to usnic acid per se was observed, which was related to the oxidative stress in the tested cells, triggered by this extract, as opposed to usnic acid. Base on the results of this phase, i.e. based on the best ratio of antimicrobial potential against bacteria that cause skin infections and cytotoxicity against human keratinocytes, this extract (with quantified usnic acid) was selected for further phases of experiments.

U okviru druge faze dati su rezultati preformualcione studije odabranog ekstrakta, a zatim i procene koloidne strukture/fizičko-hemijske stabilnosti formulisanih emulzionih sistema stabilizovanih ispitivanim APG emulgatorima, njihovih biofarmaceutskih karakteristika i preliminarne efikasnosti i bezbednosti primene na koži, sa i bez dodatka ekstrakta, koji je inkorporiran u ove sisteme uz variranje načina dodavanja/koncentracije.

Within the second phase, the results of the preformulation study of the selected extract, and, thereafter results of the assessment of the colloidal structure/physicochemical stability of the formulated emulsion systems stabilized with the investigated APG emulsifiers (with and without the extract, which was incorporated in these systems by varying the method of its addition/concentration), their biopharmaceutical properties and preliminary efficiency and safety of their dermal usage are presented.

Preformulaciona studija ekstrakta talusa *U. barbata* koji je dobijen ekstrakcijom natkritičnim CO_2 ukazala su na njegovu slabu rastvorljivost u polarnim i nepolarnim rastvaračima, uz više izraženu hidrofobnu prirodu, potvrđenu merenjem particionog koeficijenta i kontaktnog ugla. Merenje kontaktnog ugla naglasilo je i svojstvo čestica ovog ekstrakta za uređenjem na granici faza ulje-voda, a skenirajuća elekronska mikroskopija na njihovu izraženu tendenciju ka agregaciji.

Preformulation study of the supercritical CO_2 extract of U. barbata pointed to its poor solubility in both polar and non-polar solvents, with more pronounced hydrophobic nature, confirmed by means of partition coefficient determination, as well as by contact angle measurement. Later investigation also accentuated the ability of extract's particles to arrange themselves at the oil-water border, while scanning electron microscopy pointed to their pronounced tendency toward aggregation.

Sveobuhvatna fizičko-hemijska karakterizacija ispitivanih kremova započeta je mikroskopskom analizom odgovarajućih uzoraka sa i bez ekstrakta, koja je, očekivano, ukazala na dobar potencijal upotrebljenih APG emulgatora da stabilizuju ispitivane emulzione sisteme, obrazovanjem lamelarnih mezofaza oba tipa (tečnokristalna i lamelarna gel faza). Suspendovanje ekstrakata u izrađenu oficinalnu krem podlogu (S uzorci) nije

imalo uticaja na intenzitet uočene anizotropije, dok je dispergovanje ekstrakta u masnu fazu krema (pre mešanja sa vodenom fazom-D uzorci) dovelo do njene redukcije, koja je rasla sa porastom koncentracije ekstrakta, sugerišući slabljenje formiranih lamelarnih mezofaza u ovim uzorcima. Pored toga, opisana polarizaciona, i dodatno, fluorescentna mikroskopija ukazale su na različitu distribuciju ekstrakta, shodno načinu inkorporiranja (u kontinualnoj fazi u S uzorcima, odnosno u unutrašnjosti kapi i, u manjoj meri, u kontinualnoj fazi u D uzorcima), kao i na veću homogenost uzoraka S serije u poređenju sa onima iz D serije. Ovi nalazi, koji su ukazali na bolju fizičko-hemijsku stabilnost S uzoraka, potvrđeni su i termoanalitičkim merenjima, kao i ponovljenim ispitivanjima pH, električne provodljivosti i reoloških karakteristika ovih kremova u tri vremenske tačke: nakon 7, 30 i 90 dana od izrade uzoraka, koji su čuvani na sobnoj temperaturi. Treba naglasiti i da su navedena ispitivanja, i dodatno Raman spektralna mikroskopija zajedno sa nalazima preformulacione studije ekstrakta, ukazala na uključivanje čestica ekstrakta u koloidnu strukturu ispitivanih kremova obe serije (S i D) putem istih mehanizama, koji su, bez obzira na ovu činjenicu, a kao što je već rečeno, imali različitu koloidnu strukturu, na koju je, osim načina inkorporiranja, dodatan uticaj imala i koncentracija ekstrakta.

Comprehensive physicochemical characterization of the investigated creams has commenced through microscopic analysis of the appropriate samples with and without the extract, which expectedly pointed to the good potential of the used APG emulsifiers to stabilize the investigated emulsion systems by forming lamellar mesophases of both types (liquid crystalline and lamellar gel phase). Suspending the extract into the emulsion vehicle (S samples) had no effect on the intensity of the detected anisotropy, while dispersing the extract into the oil phase of the cream (prior to the addition to the water phase-D samples) induced the reduction of anisotropy, which increased proportionally to the extract concentration increase, suggesting weakening of the formed lamellar mesophases in these samples. In addition to the described findings of polarization microscopy, fluorescence microscopy pointed to the different extract distribution pursuant to the method of its incorporation (the extract was observed in the continuous phase in S samples i.e. in the oil droplets, and in lesser extent, in the continuous phase in D samples), as well as to better homogeneity of the samples of S series in comparison to the ones belonging to D series. These findings, which suggested better physicochemical stability of S samples, were additionally confirmed by thermoanalytical measurements, as well as by the repeated examinations of pH, conductivity and rheological characteristics of these creams at three time points: 7, 30 and 90 days after preparation of the appropriate samples, which were stored at room temperature. Also, it should be emphasized that the stated investigations, and additionally, Raman spectral imaging alongside the findings of the preformulation study of the extract, accentuated extract's particles to participate in the colloidal structure of both groups of creams (S and D) via similar mechanisms, which, nevertheless, as previously stated had different colloidal structure that was, aside the method of extract incorporation, influenced by its concentration.

Biofarmaceutska karakterizacija sprovedena primenom sistema Franz-ove vertikalne difuzione ćelije, otkrila je da se veća količina ovog jedinjenja oslobodila iz svih S uzoraka u odnosu na korespodentne D uzorke, pri istoj koncentraciji ekstrakta, što je objašnjeno njegovom prethodno opisanom lokalizacijom u ovim kremovima (odnosno lakšom difuzijom iz kontinulane faze). Komparativno ispitivanje liberacije u grupi S uzoraka, shodno koncentraciji ekstrakta, izdvojilo je krem u kojem je ekstrakt suspendovan u APG emulzionu podlogu u koncentraciji koja odgovara sadržaju usninske kiseline od 2 % (m/m) u finalnoj formulaciji, kao uzorak sa najvećom brzinom i obimom liberacije aktivne supstance, na osnovu čega je ova formulacija odabrana za dalje faze istraživanja kao prototip gotovog

proizvoda. Ipak, uporedna analiza poredbenog uzorka (u kojem je ekstrakt istim postupkom inkorporiranja i u istoj koncentraciji dodat u farmakopejsku podlogu), pokazala je značajno bolju liberaciju usninske kiseline iz oficinalne, u odnosu na APG podlogu. Iako je količina usninske kiseline oslobođena iz obe emulzione baze bila relativno niska, a liberacija pratila kinetiku nultog reda, ukazujući na svojevrsno „zarobljavanje" najvećeg dela ovog jedinjenja u ispitivanim emulzionim sistemima i posledično produženo (engl. *sustained*) oslobađanje, na osnovu rezultata uporedne fizičko-hemijske karakterizacije APG i poredbenog uzorka, bolja liberacija usninske kiseline iz oficinalne podloge dovedena je u vezu sa specifičnom distribucijom ekstrakta/usninske kiseline u ispitivanim kremovima. Preciznije, zaključeno je da u oba uzorka ekstrakt stupa u hidrofobne interakcije sa alkil lancima surfaktanta i/ili masnog amfilila, koji vodi solubilizaciji usninske kiseline i posledično njenom boljem oslobađanju. Ipak, u APG uzorku, navedene interakcije se dešavaju prevashodno na nivou hidrofobnih domena tečnokristalne i hidrofilne gel faze, a u manjoj meri u lipofilnoj gel fazi, dok se u poredbenom uzorku ekstrakt, nakon suspendovanja u oficinalnu emulzionu podlogu, primarno uklapa upravo u ovu (lipofilnu gel) fazu, kao dominatno razvijenu, čineći usninsku kiselinu lakše dostupnom za difuziju i oslobađanje.

Biopharmaceutical characterization, conducted using the system of Franz vertical diffusion cells, revealed that the amount of released usnic acid was higher in all the S samples in comparison to the corresponding D samples, having the same extract concentration, which was related to the its previously described localization in these creams (facilititated diffusion from the continuous phse). Comparative release experiments of S samples, pursuant to extract concentration, singled out the cream in which the extract was suspended in the APG-based emulsion vehicle in the concentration corresponding to 2 % (w/w) of usnic acid in the final formulation, as the cream with the best rate and extent of active substance release, based on which this formulation was chosen for further investigations, as the prototype of the finished product. Nevertheless, comparative analysis of the referent sample (in which the extract was added to the pharmacopoeial vehicle in the same concentration and using the same incorporation method), revealed significantly better usnic acid liberation from the pharmacopoeial emulsion base in comparison to the APG-stabilized one. Even though the amount of released usnic acid was low from both emulsion bases, and the liberation profiles corresponded to zero-order kinetics, pointing to the entrapment of the majority of this substance within the investigated emulsion systems, and hence its sustained liberation, based on the comparative physicochemical characterization of the APG-based and referent sample, better liberation of usnic acid from the pharmacopoeial vehicle was related to the specific distribution of the extract/usnic acid within the tested emulsion systems. More precisely, in both samples, hydrophobic interactions of the extract and alkyl chains of the surfactant and/or fatty amphiphile, leading to solubilization of usnic acid and consequent enhancement of its release were proposed. However, in the APG-based sample, stated interactions were mostly present in the hydrophobic domains of liquid crystalline and hydrophilic gel phase, and in lesser extent, in the lipophilic gel phase, while after suspending the extract in the referent vehicle, its particles were primarily inserted in this (lipophilic gel) phase, as a predominantly developed one, making usnic acid more available for diffusion and liberation.

Sa druge strane, dalje sprovedena *in vivo* procena dermalne raspoloživosti usninske kiseline (što je preduslov za potencijalni terapijski efekat preparata sa ovim jedinjenjem) primenom *tape stripping* metode, ukazala je na obrnut trend u odnosu na prethodno navedene rezultate *in vitro* studije liberacije tj. poboljšanje stepena njene penetracije iz odabrane prototip fomulacije krema sa ekstraktom stabilizovanog APG emulgatorima u poređenju sa odgovarajućim poredbenim uzorkom. Ovakav nalaz doveden je u vezu sa fenomenima koji se

dešavaju pri realnim uslovima primene dermofarmaceutskih preparata, kao što su promene u strukturi samih nosača nakon aplikacije na kožu, odnosno interakcije komponenata formulacije sa *stratum corneum* (SC), koji mogu uticati na termodinamički potencijal aktivne susptance i sledstveno na njenu penetraciju. Preciznije, uočeno poboljšanje stepena penetracije usninske kiseline u kožu iz podloge stablizovane APG emulgatorima u odnosu na oficinalnu podlogu, pripisan je interakcijama tečnokristalne faze (prisutne u APG uzorku) sa intercelularnim lipidima SC, koje doprinose fluidnijem lipidnom pakovanju i posledičnom povećanju njegove permeabilnosti, odnosno olakšanoj difuziji ovog jedinjenja.

On the other hand, further conducted in vivo assessment of dermal availability of usnic acid (as a prerequisite for potential therapeutic effect of the preparations with this compound) by means of tape stripping method, pointed to the reverse trend compared to previously described in vitro release study i.e. to the enhancement of the extent of its penetration from the selected prototype formulation of APG-based active cream compared to the corresponding referent sample. Such finding was related to the phenomena that occur in the in the real-time conditions of dermopharmaceutical preparations usage, such as changes in the structure of the carriers after application to the skin and the interaction of the components of the formulation with the stratum corneum (SC), which may lead to the changes in the total thermodynamic potential of the active substance and consequently, affect its penetration. More precisely, detected enhancement in the extent of usnic acid penetration into the skin from APG-based vehicle compared to the referent one was attributed to the interactions of liquid crystalline phases in this sample with the intercellular SC-lipids, which contribute to more fluid lipid packaging and the consequent increase in its permeability, i.e. facilitated diffusion of this substance.

Rezultati predstavljeni u okviru treće faze obuhvatili su procenu preliminarne efikasnosti odabrane prototip formulacije gotovog proizvoda-krema sa ekstraktom stabilizovanog APG emulgatorima, kao i bezbednosti njegove dermalne primene. S obzirom na potencijalnu namenu ovog preparata-lokalnu terapiju infekcija kože, njegova preliminarna efikasnost procenjena je *in vitro* ispitivanjem antimikrobne aktivnosti, koja je ukazala na zadovoljavajući potencijal u inhibiranju rasta odabranih G+ bakterija koje su uzročnici ovih infekcija, kao i ispitivanjem kapaciteta njegove podloge da utiče na nespecifične simptome kožnih infekcija; ovo je procenjeno *in vivo* merenjem odgovarajućih biofizičkih parametara (transepidermalni gubitak vode, eritema indeks) na veštački iritiranoj koži, koja su pokazala potencijal APG podloge da popravi barijernu funkciju kože narušenu infekcijom, ali nisu uspela da pruže više podataka o uticaju ove emulzione podloga na eritem, kao jedan od simptoma koji prate infekcije kože.

Results of the third phase of experiments are describing the assessment of preliminary efficiency of the selected prototype formulation of APG-based active cream, as well as the safety of its dermal application. Bearing in mind the potential usage of this preparation (local treatment of skin infections), its preliminary efficiency was assessed by means of an in vitro investigation of its antimicrobial activity that pointed to its satisfactory potential in inhibiting the growth of the chosen G+ bacteria-causative agents of these infections. Preliminary efficiency of this preparation was additionally assessed by the evaluation of the capacity of its vehicle to affect non-specific symptoms of skin infections; this was investigated using in vivo measurements of the appropriate biophysical parameters (transepidermal water loss, erythema index) of SLS-irritated skin, which revealed the potential of the APG-based vehicle to repair skin barrier function disturbed by the infection, but failed to provide more data on the influence of this emulsion base on erythema, as one of the symptoms accompanying cutaneous infections.

Aspekt bezbednosti odabrane prototip formulacije gotovog proizvoda-krema sa kvantifikovanim ekstraktom talusa *Usnea barbata* stabilizovanog APG emulgatorima, ispitan *in vivo* tehnikama bioinženjeringa kože kojima su praćeni ključni biofizički parametri (eritema indeks, transepidermalni gubitak vode, stepen hidratacije *stratum corneum*), ukazao je na krajnje povoljnu dermalnu podnošljivost ovog preparata, kao i njegove podloge. Navedeni rezultati 3. faze eksperimenata navode na zaključak da odabrana prototip formulacija krema sa ekstraktom stabilizovanog APG emulgatorima poseduje zadovoljavajuću preliminarnu efikasnost i bezbednosni profil, naročito nakon uporedne analize poredbenih uzoraka.

Safety aspect of the selected prototype formulation of APG-based active cream, investigated using in vivo skin bioengineering techniques which monitored key biophysical parameters (erythema index, transepidermal water loss, stratum corneum hydration), suggested favorable skin tolerability of this preparation, and also its vehicle. The stated results of the experiments of the third phase imply that the selected prototype formulation of APG-based active cream possesses satisfactory preliminary efficiency and safety profile, especially when taking into account comparative analysis of the referent samples.

C. UPOREDNA ANALIZA REZULTATA SA PODACIMA IZ LITERATURE/*COMPARISON OF THE OBTAINED RESULTS WITH THE PUBLISHED DATA*

Dobijeni i predstavljeni rezultati ove doktorske disertacije delom su saglasni sa podacima iz literature, a rezultati određenih ispitivanja u okviru disertacije nisu mogli biti provereni publikovanim izvorima, te se smatraju doprinosom istraživačkog rada kandidatkinje.

Results obtained and presented in this doctoral dissertation are partially in accordance with the published data, while the results of certain experiments conducted in the scope of the dissertation could not be verified with available sources, and are thus considered the candidate's contribution to the scientific research in the field.

Iako u svom hemijskom sastavu sadrži različita jedinjenja, brojna istraživanja pokazala su da se biološka svojstva jevrejske brade (*U. barbata*) mogu pripisati usninskoj kiselini, kao njenom glavnom hemijskom konstituentu i, generalno, jednom od najbolje proučenih sekundarnih metabolita lišajeva (Cansaran i sar., 2006; Engel i sar., 2007; Weckesser i sar., 2007; Rankovic i sar., 2012). Istraživanja ove doktorske disertacije potvrdila su literaturne navode o posredničkoj ulozi usninske kiseline u ispitivanim biološkim aktivnostima jevrejske brade. Preciznije, nađena je dobra korelacija sadržaja usninske kiseline u ispitivanim ekstraktima talusa lišaja *U. barbata* i njihove antimikrobne aktivnosti protiv ispitivanih bakterija koje su uobičajeni izazivači bakterijskih infekcija kože, odnosno sposobnosti redukcije feri jona, kao mere antioksidativne aktivnosti. I u slučaju ispitivanja citotoksičnosti protiv tumorskih ćelija, navedena aktivnost ovih ekstrakata bila je u dobroj korelaciji sa sadržajem usninske kiseline, sa izuzetkom ekstrakta talusa jevrejske brade koji je dobijen ekstrakcijom natkritičnim CO_2, za koji su utvrđene niže IC_{50} vrednosti nego za ovo jedinjenje *per se*. Kao što je već naglašeno, dalja istraživanja nagovestila su da je ovakav nalaz u vezi sa oksidativnim stresom, koji ovaj ekstrakt, nasuprot usninskoj kiselini, izaziva u navedenim ćelijama. S tim u vezi, navedeni rezultati nametnuli su potrebu za daljim istraživanjima koja bi razjasnila tačan mehanizam uključen u citotoksičnost ovog ekstrakta talusa lišaja *U. barbata* posredovanu slobodnim kiseoničnim radikalima (engl. *Reactive oxygen species, ROS*).

Despite the presence of different chemical constituents in the composition of old man's beard (U. barbata), numerous investigations have shown that its biological properties could be attributed to usnic acid, as the major chemical compound identified in this lichen, and generally, one of the most studied lichen secondary metabolites (Cansaran et al., 2006; Engel et al., 2007; Weckesser et al., 2007; Rankovic et al., 2012). Investigations of this doctoral dissertation have confirmed literature data implying mediating role of usnic acid in the investigated biological properties of man's beard. More precisely, good correlation of usnic acid content in the investigated extracts of U. barbata and their antimicrobial activity against bacteria that commonly cause skin infections i.e. ferric reducing power, as the measure of their antioxidant activity, was found. In the case of cytotoxicity test against tumor cells, stated activity of these extracts was also in accordance with the content of usnic acid, with the exception of supercritical CO_2 extract having lower IC_{50} values than the ones determined for this compound per se. As already mentioned, additional analysis suggested such finding to be related to oxidative stress in the tested cells, triggered by this extract, as opposed to usnic acid. In this connection, stated results imposed the need for furher investigations that would elucidate precise mechanisms involved in cytotoxicity of the stated extract induced by reactive oxygen species (ROS).

Potreba za sveobuhvatnom karakterizacijom koja je sprovedena u II fazi eksperimentalnog rada pokazala se kao opravdana, uzimajući u obzir činjenicu da je objašnjenje mnogih prikazanih rezultata zahtevalo pozivanje na relevantne literaturne izvore sa jedne strane, i povezivanje rezultata nekoliko sprovedenih metoda, sa druge strane. Tako je, primera radi, zadovoljavajuće tumačenje fizičko-hemijskih karakteristika ispitivanih emulzionih sistema stabilizovanih APG emulgatorima, koje je nagovestilo uključivanje čestica ekstrakta u osnovni mehanizam stabilizacije uzoraka obe serije (u kojima je ipak uočena drugačija koloidna struktura), postignuto tek nakon uporednog sagledavanja rezultata mikroskopske analize (polarizaciona, fluorescentna i Raman spektralna mikroskopija), kontinulanih reoloških i termoanalitičkih merenja. Dalje, nalazi ovih ispitivanja potvrdili su zaključke drugih istraživača da se kod kremova koji su stabilizovani i surfaktantima i čvrstim česticama postupak izrade (u ovom slučaju-način inkorporiranja ekstrakta) odražava na njihovu koloidnu strukturu (Binks i Desforges, 2007). Takođe, dalje sprovedeno *in vitro* ispitivanje oslobađanja usninske kiseline iz kremova stabilizovanih APG emulgatorima, ukazalo je da upravo način inkorporiranja ekstrakta (i sledstveno, različita koloidna struktura korespodentnih sistema) ima odlučujuću ulogu na profile oslobađanja ovog jedinjenja, što je u skladu sa uobičajenom upotrebom ovog ispitivanja kao validnog testa za procenu kritičnih atributa kvaliteta polučvrstih preparata za primenu na koži, u kontekstu odabira završne (prototip) formulacije prilikom razvoja ovih proizvoda, pre pristupanja odgovarajućim *in vivo* ispitivanjima (Csoka i sar., 2005; Chang i sar., 2013). S tim u vezi, u ispitivanjima ove doktorske disertacije uočen je izostanak korelacije između rezultata *in vitro* studije oslobađanja usninske kiseline i stepena njene penetracije (ispitanog *in vivo*) iz testirane APG i poredbene podloge. Dodatno, rezultati navedenog ispitivanja dermalne raspoloživosti usninske kiseline potvrdili su literaturne navode o inherentnom potencijalu podloga stabilizovanih APG emulgatorima za poboljšanje isporuke aktivnih supstanci u kožu (Makai i sar., 2003; Hosmer i sar., 2011; Pantelic, 2014).

The need for such a comprehensive characterization conducted in the second phase of the experimental work was shown to be justified, taking into account that the correct interpretation of some results required review of the relevant literature, on one hand, and simultaneous discussion of results provided by two or more methods, on the other. For instance, satisfactory discussion of physicochemical characteristics of the investigated emulsion systems, which pointed to the inclusion of particles of the extract into the basic

stabilization mechanisms of both series of active creams (in which, nevertheless, different colloidal structure has been observed), was accomplished only after comparative evaluation of the results of microscopic analysis (polarization, fluorescence and Raman spectral microscopy) and continual rheological and thermogravimetric measurements. In addition, findings of these investigations confirmed preparation protocol (in this case-method of extract incorporation) to influence colloidal structure of emulsion systems stabilized with both surfactants and particles, which was previously highlighted by other authors (Binks and Desforges, 2007). Also, further conducted in vitro release experiments of usnic acid from APG-based creams, suggested that it is the method of extract incorporation (and, consequently, different colloidal structure of the corresponding systems) that had a decisive role on release profiles of this compound, which is in line with the common use of the this examination as a valid test for the evaluation of the critical quality attributes of dermatological semisolid preparations in the context of the selection of the final (prototype) formulation, during the development of these products, prior to the appropriate in vivo investigations (Csoka et al., 2005; Chang et al., 2013). In this connection, in the investigations of this doctoral dissertation, absence of correlation between usnic acid liberation profiles (assessed in vitro) and the extent of its penetration (assessed in vivo) from the investigated APG and referent vehicles was observed. In addition, the results of the stated evaluation of usnic acid dermal availability confirmed literature data suggesting APG-based vehicles to possess inherent potential for the enhancement of active ingredients delivery into the skin (Makai et al., 2003; Hosmer et al., 2011; Pantelic, 2014).

Uporedni prikaz minimalnih inhibitornih koncentracija (MIK) za usninsku kiselinu kao aktivni sastojak ispitivane prototip formulacije krema sa ekstraktom stabilizovanog APG emulgatorima (teorijske vrednosti izračunate na osnovu MIK vrednosti dobijenih za ovaj krem i masenog udela usninske kiseline u njemu) i za usninsku kiselinu kao intaktnu supstancu (MIK dobijene u prvoj fazi eksperimenata) po ugledu na ovakav pristup opisan u studiji Asterholma i saradnika (2010) pokazao se opravdanim, s obzirom na pokazanu dobru korelaciju ovih vrednosti, koja je ukazala na zadovoljavajuću antimikrobnu aktivnost ovog jedinjenja protiv testiranih G+ bakterija (koje izazivaju infekcije kože), nakon inkorporiranja ekstrakta u ispitivani krem. Pored toga, poznato je da se natrijum-laurilsulfat koristi za ciljano izazivanje iritativnog kontaktnog dermatitisa, radi procene antiiritantnih svojstava dermalnih preparata, odnosno ispitivanja njihovog zaštitnog dejstva na barijernu funkciju kože (Engel i sar., 2008; Korponyai i sar., 2011). U ovoj doktorskoj disertaciji, test sa natrijum-laurilsulfatom poslužio je kao korisno ispitivanje za evaluaciju efikasnosti odabrane prototip formulacije krema sa ekstraktom sa APG emulgatorima kroz procenu kapaciteta njegove podloge da utiče na nespecifične simptome infekcija kože.

Comparative evaluation of minimal inhibitory concentrations (MICs) of usnic acid as an active ingredient of the investigated prototype formulation of APG-based active cream (theoretical values, calculated on the basis of MICs for this cream and the mass fraction of usnic acid in it) and as intact substance (MICs from the phase I of experimental work), based on the approach previously described by Asterholm and colleagues (2010) seemed to be justified, considering good correlation of these values, which pointed to the satisfactory antimicrobial activity of this compound against tested G+ strains (causative agents of skin infections) upon extract addition into the investigated cream. Additionally, it is well-known that sodium lauryl sulfate (SLS) is used for the targeted induction of irritant contact dermatitis aiming to assess anti-irritant properties of topical preparations i.e. to estimate their barrier protective features (Engel et al., 2008; Korponyai et al., 2011). In this doctoral dissertation, SLS-test served as useful tool for the investigation of the efficiency of the

selected prototype formulation of APG-based active cream through the assessment of the capacity of its vehicle to affect non-specific symptoms of skin infections.

Jedna od prednosti upotrebe APG emulgatora jeste njihova blaga priroda i sledstvena dobra podnošljivost na koži (Holmberg, 2001; Pantelic, 2014). Ipak, poznato je da u nekim slučajevima dolazi do pojave neželjenih reakcija na koži tek nakon primene gotovih preparata, usled aditivnog efekta više prisutnih supstanci. Stoga je u III fazi eksperimentalnog rada opravdano sprovedeno *in vivo* ispitivanje bezbednosti primene odabrane prototip fomulacije krema sa ekstraktom stabilizovanog APG emulgatorima i njegove korespondentne podloge, koje je potvrdilo krajnje povoljnu podnošljivost datih uzoraka na koži.

One of the advantages of the utilization of APG emulsifiers is their mild nature and subsequent good skin tolerability(Holmberg, 2001; Pantelic, 2014). Nevertheless, it is known that adverse skin reactions sometimes occur only after the application of the final formulation, due to the additive effect of several compounds. Therefore, the third phase of the experiments encompassed in vivo safety assessment of the prototype formulation of APG-based active cream and its corresponding vehicle, which confirmed their favorable skin performance.

Može se reći da su rezultati ove doktorske disertacije zasnovani na detaljnom pregledu literature, koji je, zajedno sa upotrebom odgovarajućih metoda, dao jasan doprinos u oblasti ispitivanja APG emulgatora kao potencijalnih stabilizatora emulzione podloge koja bi našla primenu kao savremeni nosač za ekstrakt talusa lišaja *U. barbata*, sa ciljem razvoja finalnog farmaceutskog preparata potencijalno namenjenog lokalnoj terapiji infekcija kože.

It could be stated that the results of this doctoral dissertation are based on a detailed review of the literature, which, alongside utilization of the appropriate characterization methods, gave a clear contribution in the field of investigations of APG emulsifiers as prospective stabilizers of the emulsion vehicle that could serve as contemporary carrier for the extract of the talus of U. barbata, with the aim of developing a final pharmaceutical preparation potentially applied in the local treatment of skin infections.

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D. OBJAVLJENI REZULTATI KOJI ČINE DEO DISERTACIJE/*PUBLISHED RESULTS WHICH ARE PART OF THE DOCTORAL DISSERTATION*

Rezultati dobijeni u okviru ove doktorske disertacije do sada su objavljeni u vidu tri rada u časopisima koji su na SCI listi (jedan rad kategorije M21 i dva rada kategorije M23), kao i u obliku većeg broja saopštenja na međunarodnim i domaćim naučnim skupovima štampanih u celini ili u izvodu. Podaci o publikovanim radovima dati su u nastavku:

Results presented in this doctoral dissertation were published in three papers in SCI list journals (one paper in the journal of category M21 and two papers in journals of category M23), as well as several abstracts presented in international and national meetings. Papers published in journals are given below:

1) Zugic A, Jeremic I, Isakovic A, Arsic I, Savic S, Tadic V. Evaluation of anticancer and antioxidant activity of a commercially available CO_2 supercritical extract of old man's beard (*Usnea barbata*). Plos One 2016; 11(1): e0146342 (IF 3,057/2015).

2) Zugic A, Lunter DJ, Daniels R, Pantelic I, Tasic Kostov M, Tadic V, Misic D, Arsic I, Savic S. *Usnea barbata* CO_2-supercritical extract in alkyl polyglucoside-based emulsion systems: Contribution of Confocal Raman imaging to the formulation development of a natural product. Pharm Dev Technol, 2016; 21 (5): 563-575 (IF 1,566/2015).

3) Žugić AR, Lukić MZ, Tasić Kostov MZ, Tadić VM, Arsic IA, Mišić DR, Petrović SD, Savić SD. Alkyl polyglucoside-stabilized emulsion as a prospective vehicle for *Usnea barbata* CO_2-supercritical extract: assessing stability, safety and efficiency of a topical formulation. Hem Ind 2015; 69 (6) 703–712 (IF 0,437/2015).

E. ZAKLJUČAK-OBRAZLOŽENJE NAUČNOG DOPRINOSA DISERTACIJE/*CONCLUSION–JUSTIFICATION OF SCIENTIFIC CONTRIBUTION OF THE DOCTORAL DISSERTATION*

Rezultati ove doktorske disertacije predstavljaju dopunjena saznanja o hemijskom profilu i biološkim svojstvima ekstrakta talusa lišaja *U. barbata* (jevrejska ili lišaj brada) koji je dobijen pomoću natkritičnog CO_2, kao i nova saznanja o upotrebi dva do sada neispitana emulgatora iz grupe alkil poliglukozida (APG) (C_{14-22} alkohol i C_{12-20} glukozid/koko glukozid i alkohol kokosovog oraha) kao stabilizatora savremenih emulzionih sistema-kremova sa preparatom biljne droge kao aktivnom supstancom, potencijalno korišćenih u dermofarmaciji.

Results of this doctoral dissertation represent additional information on the chemical profile and biological properties of the extract of the talus of U. barbata (old man's beard) obtained using supercritical CO_2, as well as new data on the use of two so far unexamined emulsifiers of alkyl polyglucoside (APG) type (C_{14-22} glucoside and C_{12-20} alcohol; cocoglucoside and coconut alcohol), as stabilizers of contemporary emulsion systems-creams with herbal drug preparation as an active ingredient, potentially utilized in dermopharmacy.

Naime, eksperimentima ove doktorske disertacije potvrđeni su literaturni navodi o višem sadržaju usninske kiseline i sledstveno, boljoj antimikrobnoj aktivnosti ekstrakta talusa lišaja *U. barbata* dobijenog pomoću natkritičnog CO_2, (kao novijom metodom ekstrakcije koja poslednjih godina privlači pažnju naučne javnosti) u odnosu na izolate ovog lišaja dobijene pomoću konvencionalnih metoda sa organskim rastvaračima. Pored toga, po prvi put je pokazan antioksidativni potencijal (pokazan FRAP testom), kao i citotoksična aktivnost (protiv B16 mišjeg melanoma i C6 pacovskog glioma) ovog ekstrakta, koja je, štaviše, bila bolja u odnosu na čistu usninsku kiselinu (koja je korišćena kao pozitivna kontrola); ovakav nalaz nametnuo je potrebu za budućim naučnim istraživanjima koja bi obuhvatila detaljna ispitivanja mehanizama ispoljene toksičnosti prema navedenim tumorskim ćelijama.

Namely, the experiments of this doctoral dissertation confirmed literature data claiming the extract of the talus of U. barbata obtained using supercritical CO_2 (as the novel extraction method that has recently received increasing attention) to possess higher usnic acid content and consequently, better antimicrobial activity compared to the islolates of this lichen prepared by means of conventional extractions employing organic solvents. Beside this, antioxidative potential (assessed by means of FRAP assay) as well as cytotoxic activity (against B16 mouse melanoma i C6 rat glioma) of this extract have been reported for the first time; moreover, cytotoxic activity of this extract was shown to be better compared to pure usnic acid (used as positive contol), imposing the need for future investigations, which would minutely evaluate mechanisms of the exerted toxicity against stated tumor cells.

Ispitivani APG emulgatori uspešno su stabilizovali emulzionu podlogu koja se može smatrati pogodnim nosačem za ekstrakt talusa *U. barbata* dobijen primenom natkritičnog CO_2, čineći prototip formulaciju gotovog proizvoda u obliku hidrofilnog krema, zadovoljavajuće fizičko-hemijske stabilnosti, biofarmaceutskih karakteristika, efikasnosti i bezbednosti za dermalnu primenu, uz potencijalni klinički značaj u lokalnoj terapiji infekcija kože, kao moguća alternativa lokalnim antibioticima. Dodatno treba naglasiti da je ispitivana emulziona baza stabilizovana APG emulgatorima pokazala određene povoljnije karakteristike kao nosač za navedeni ekstrakt u poređenju sa poredbenom (oficinalnom) podlogom, pre svega poboljšane senzorne karakteristike, kao osobinu koja je često odlučujuća za komplijansu pacijenata.

The investigated APG emulsifiers successfully stabilized the emulsion vehicle, which could be considered as suitable carrier for the extract of the talus of U. barbata obtained using supercritical CO_2, composing a prototype formulation of the final product in the form of a hydrophilic cream shown to possess satisfactory physicochemical stability, biopharmaceutical characteristics, efficiency and safety for dermal application, as well as potential clinical relevance in the local treatment of skin infections, as a possible alternative to local antibiotics. In addition, it should be emphasized that the investigated APG-stabilized base revealed certain preferential features as the carrier for the stated extract compared to the referent (pharmacopoeial) one, primarily reflected through its improved sensory characteristics, as a property often decisive for the patient compliance.

F. PREDLOG KOMISIJE ZA OCENU ZAVRŠENE DOKTORSKE DISERTACIJE/*THE COMMISSION'S OPINION ON THE COMPLETED DOCTORAL DISSERTATION*

Doktorska disertacija pod nazivom **"Ekstrakt talusa *Usnea barbata* (L.) Weber ex F. H. Wigg., Parmeliaceae u emulzionim nosačima stabilizovanim biorazgradivim emulgatorima: preformulaciona i formulaciona istraživanja"**, kandidata dipl. farm. Ane R. Žugić, po svom sadržaju i formi, dobro napisanom uvodnom delu, jasno postavljenim istraživačkim ciljevima, zadovoljavajuće osmišljenoj metodologiji, precizno iznetim rezultatima rada, razložnoj diskusiji i dobro formulisanim zaključcima ispunjava sve kriterijume adekvatno napisanog naučnog dela.

*Doctoral dissertation entitled **"Extract of the talus of Usnea barbata (L.) Weber ex F. H. Wigg., Parmeliaceae in the emulsion vehicles stabilized with biodegradable emulsifiers: preformulation and formulation investigations"**, written by the candidate dipl. pharm. Ana R. Žugić, fulfils all the criteria of a successfully written scientific work, reflected through its contents and form, well written introduction section, clearly defined research aims, adequate experimental design, clearly presented results, detailed discussion and well formulated conclusions.*

Komisija, stoga, sa zadovoljstvom predlaže Nastavno-naučnom veću Farmaceutskog fakulteta u Beogradu da prihvati pozitivan izveštaj o izrađenoj doktorskoj disertaciji pod nazivom **"Ekstrakt talusa *Usnea barbata* (L.) Weber ex F. H. Wigg., Parmeliaceae u emulzionim nosačima stabilizovanim biorazgradivim emulgatorima: preformulaciona i formulaciona istraživanja"** i kandidatu dipl. farm. Ani R. Žugić odobri javnu odbranu doktorske disertacije po dobijanju saglasnosti Veća naučnih oblasti medicinskih nauka Univerziteta u Beogradu.

Napomena: Na osnovu tehničkog previda u predloženom naslovu doktorske disertacije „Ekstrakt talusa *Usnea barbata*-e (L.) Weber ex F.H. Wigg., Parmeliaceae u emulzionim nosačima stabilizovanim biorazgradivim emulgatorima: preformulaciona i formulaciona istraživanja“, uočenom na sednici Komisije za poslediplomske studije Nastavno-naučnog veća Farmaceutskog fakulteta Univerziteta u Beogradu, održanoj 9.6.2016. godine, isti je u priloženoj doktorskoj disertaciji korigovan u naslov „Ekstrakt talusa *Usnea barbata* (L.) Weber ex F. H. Wigg., Parmeliaceae u emulzionim nosačima stabilizovanim biorazgradivim emulgatorima: preformulaciona i formulaciona istraživanja“.

*Therefore, the Commission advises the Academic Council of the Faculty of Pharmacy in Belgrade to accept this positive Report on the completed doctoral dissertation entitled **"Extract of the talus of Usnea barbata (L.) Weber ex F. H. Wigg., Parmeliaceae in the***

***emulsion vehicles stabilized with biodegradable emulsifiers: preformulation and formulation investigations"**, and permit the candidate Ana R. Žugić to defend her doctoral dissertation after being given the appropriate approval by the University of Belgrade.*

Beograd, 08.7.2016./*Belgrade*, July, 8th, 2016

Članovi Komisije/*Commission Members*:

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Univerzitet u Beogradu-Farmaceutski fakultet/
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