Sveučilište u Zagrebu

Farmaceutsko-biokemijski fakultet

Petar Ciganović

Optimizacija izrade i biološka aktivnost glicerolnih ekstrakata ljekovitoga bilja za primjenu u dermatofarmaciji

DOKTORSKI RAD

Zagreb, 2024.

Sveučilište u Zagrebu

Farmaceutsko-biokemijski fakultet

Petar Ciganović

Optimizacija izrade i biološka aktivnost glicerolnih ekstrakata ljekovitoga bilja za primjenu u dermatofarmaciji

DOKTORSKI RAD

Mentorica: prof.dr.sc. Marijana Zovko Končić

Zagreb, 2024.

University of Zagreb

Faculty of Pharmacy and Biochemistry

Petar Ciganović

Production optimisation and biological activity of glycerol extracts of medicinal plants for application in dermatopharmacy

DOCTORAL DISSERTATION

Supervisor: Professor Marijana Zovko Končić, PhD

Zagreb, 2024.

Doktorski rad predan je na ocjenu Fakultetskom vijeću Farmaceutsko-biokemijskog fakulteta Sveučilišta u Zagrebu radi stjecanja akademskog stupnja doktora znanosti u području biomedicine i zdravstva, polje farmacija, grana farmacija.

Rad je izrađen na Zavodu za Farmakognoziju Farmaceutsko-biokemijskog fakulteta Sveučilišta u Zagrebu pod stručnim vodstvom prof.dr.sc. Marijane Zovko Končić.

„Stay hungry, stay foolish.“

Steve Jobs

ZAHVALE

Doktorski rad je putovanje koje zahtijeva puno rada, upornosti i, prije svega, podrške. Iako moje ime stoji na koricama ovog rada, iza njega stojite svi vi koji ste me bodrili, savjetovali i gurali naprijed. Ovim vam putem želim izraziti svoju duboku zahvalnost što ste bili dio mog putovanja.

Prije svega, neizmjerno sam zahvalan svojoj mentorici Marijani koja je bila moj svjetionik kroz dinamične vode akademskog mora. Hvala Vam što ste vjerovali u mene. Vaša strpljivost, mudrost i smirenost bile su temelj ovome radu. Najveća stvar koju ste me naučili bila je spoznaja da pravog odgovora nema bez pravog pitanja.

Također zahvaljujem cijenjenim koautorima na velikom doprinosu kroz zajedničke znanstvene radove i podršku koja je bitno obogatila i usmjerila moj doktorski rad..

Veliko hvala mojim prijateljima. Vaša podrška podsjetnik je na pravu vrijednost prijateljstva..

Hvala mojoj baki i teti koje su bile uz mene tijekom pisanja ovog rada u Bihaću. Vaša podrška, briga i razumijevanje značili su mi više nego što riječi mogu opisati. Hvala vam što ste mi pružale miran prostor, toplinu doma i neodoljiv burek..

Hvala mojoj sestri Aneri. Svaki dan me podsjetiš da je moguće imati najboljeg prijatelja i sestru u jednoj osobi. Ti i ja smo dvije laste s velikim planovima.

Posebnu zahvalnost dugujem svojim roditeljima, koji su mi usadili ljubav prema učenju i znanosti, kao i vječnu znatiželju bez koje ne bih bio tu gdje jesam. Hvala vam što ste me učili da nikada ne odustajem i da je svaki korak naprijed, bez obzira koliko malen, vrijedan. Mama i tata, volim vas!

Najveće hvala ide mojoj supruzi Sari i djeci Aurori i Aleksandru, bez kojih bi ovaj rad bio moguć, ali ne bi imao nikakvog smisla. Hvala vam što ste me izabrali da vam budem suprug i tata. Ovaj rad posvećujem vama, uz podsjetnik da nije važno odredište, nego putovanje...

I na kraju, zahvaljujem sebi. Na svemu što sam nekad bio, što danas jesam i što ću tek biti.

S ljubavlju i neizmjernom zahvalnošću,
Petar.

SAŽETAK

Starenjem dolazi do niza vizualnih i strukturnih promjena u koži poput promjena u pigmentaciji, elastičnosti ili hidrataciji. Djelomično se takvi procesi mogu spriječiti primjenom dermatofarmaceutskih pripravaka pri čemu naročitu popularnost uživaju proizvodi s prirodnim bioaktivnim tvarima. U okviru ovog doktorskog rada razvijena je zelena metoda ultrazvučne ekstrakcije koja koristi mješavine glicerola/vode za ekstrakciju bioaktivnih sastavnica odabranih biljnih vrsta; *Glycyrrhiza glabra, Echinacea purpurea*, *Berberis vulgaris* i *Silybum marianum*. Za optimizaciju ekstrakcije korištena je metodologija odziva površine prema Box-Behnkenovom dizajnu. Postupak optimizacije ekstrakcije pokazao je da koncentracija glicerola, temperature i snaga ultrazvuka najviše utječu na njezinu učinkovitost. Priređeni su ekstrakti bogati odabranim bioaktivnim sastavnicama što je uključivalo skupine fenolnih spojeva (ukupni polifenoli i fenolne kiseline), specifične sastavnice karakteristične za odabrane biljne vrste, a provedena je i optimizacija ekstrakcije s obzirom na antiradikalnu aktivnost ispitanu pomoću 2,2-difenil-1-pikrilhidrazil slobodnog radikala. Priređenim ekstraktima ispitana je biološka aktivnost *in vitro* u vidu antiradikalnog učinka te djelovanja na enzime i procese koji utječu na izgled i zdravlje kože. Rezultati su pokazali da glicerolni ekstrakti odabranih biljnih vrsta djeluju kao antioksidansi, da učinkovito inhibiraju enzime tirozinazu, kolagenazu, elastazu, hijaluronidazu i lipoksigenazu kao i koagulaciju proteina. Posebno se ističu antiradikalno, ali i inhibitorno djelovanje *G. glabra* na enzime elastazu i tirozinazu, koje je usporedno ili jače izraženo od otopina standarda. Antihijaluronidazno djelovanje *E. purpurea* također je bilo izraženije od otopine standarda, dok je *S. marianum* pokazala značajnu aktivnost inhibicije oksidacije nezasićenih masnih kiselina. Inhibicija enzima lipoksigenaze bila je slično izražena kod *B. vulgaris* kao u otopini standarda. Ispitivanje na keratinocitima pokazalo je da je ekstrakt *E. purpurea* bio biokompatibilan s ispitivanim staničnim kulturama te da je potaknuo proces zacjeljivanja rana. Sve navedeno čini glicerolne ekstrakte odabranog ljekovitog bilja potencijalnim aktivnim sastavnicama u dermatofarmaceutskim proizvodima.

Ključne riječi: *Berberis vulgaris*, dermatopharmacy, *Echinacea purpurea*, glycerol, *Glycyrrhiza glabra,* green solvents, *Silybum marianum*, ultrasound-assisted extraction

EXTENDED SUMMARY

Introduction

The skin, the body's largest organ, has three layers: epidermis, dermis, and hypodermis. Constantly interacting with the environment, it plays a key role in maintaining homeostasis but is subject to aging due to internal and external factors like oxidative stress, UV exposure, and hydration loss. This aging manifests as skin sagging, wrinkles, and loss of elasticity. Skin aging theories highlight cellular DNA changes, particularly the impact of oxidative stress, which elevates reactive oxygen species levels. Although the body's endogenous antiradical systems counteract reactive oxygen species, they may become inadequate under external stress. UV radiation especially affects DNA; UV-B causes DNA dimerization, while UV-A inhibits repair, increasing risks for skin damage.

To combat these effects, exogenous antioxidants from diet or topical applications help by reducing reactive oxygen species impact, supporting skin structure, and offering additional anti-inflammatory, anti-carcinogenic, and anti-hyperpigmentation benefits. Plant-based antioxidants, such as polyphenols and alkaloids, are common in cosmetics for their antioxidant and antimicrobial properties.

As cosmetics grow more sophisticated, its role increasingly overlaps with that of pharmaceuticals, leading to creation of dermatopharmacy, where plant extracts are used to protect skin structure and enhance appearance. This shift also aligns with ethical and environmental demands for sustainable products. A pivotal aspect of producing active plant extracts is selecting eco-friendly extraction methods. Green extraction techniques like ultrasound assisted extraction achieve high yields with lower energy and solvent use by applying sound waves to break cell walls, expediting compound release. Solvent choice is critical; green solvents, like biodegradable glycerol, are valued for their non-toxicity and low flammability. Glycerol, in particular, is highly suitable for cosmetics due to its moisture-retaining properties, offering an eco-friendly option in plant-based extract production.

Methods

The extractions of 4 medicinal plants were optimized: *E. purpurea* aerial parts (optimized for the yield of total phenolic acids, caftaric and chicory acids and for antiradical activity), *G. glabra* root (optimized for the yield of total polyphenols and components of glabridin and isoliquiritigenin), *S. marianum* fruit (optimized for the yield of silymarin and antiradical activity) and *B. vulgaris* root bark (optimized for berberine yield and antiradical activity).

The identity of herbal drugs was confirmed by procedures described in the relevant pharmacopoeial monographs and other appropriate scientific literature. Prior to extraction, the plant material was pulverized and sieved on a 500 μm mesh site to obtain plant material of uniform particle size.

Herbal drugs were extracted using several selected methods. The procedure of maceration with mixtures of glycerol and water was performed and compared with the classic maceration with ethanol and water in order to select the optimal solvent. In addition to that, ultrasound assisted extraction with mixtures of glycerol and water was performed in order to compare the extraction effectiveness in regards to maceration. After obtaining optimal solvent and best extraction method, extractions were optimised in order to obtain extracts with desired chemical and biological characteristics. This was done by testing the influence of various parameters on the extraction efficiency, such as: glycerol concentration, extraction duration, ultrasound power, temperature and the ratio of herbal drug and solvent, as well as addition of ascorbic acid to the extraction mixture. The proportion of selected bioactive ingredients in plant drug extracts was determined by high-performance liquid chromatography using methods described in relevant pharmacopoeial monographs and other appropriate scientific literature.

The antioxidant potential of the main components of herbal drugs was tested using several methods. The antiradical activity of the extracts was tested spectrophotometrically in 2,2-diphenyl-1-picrylhydrazyl free radical assay. The inhibition of oxidative degradation of unsaturated fatty acids was tested in a test with β-carotene and linoleic acid by monitoring the kinetics of β-carotene degradation in the presence of linoleic acid at elevated temperature, whereas the ability to chelate iron was determined in the reaction with ferrozine. Butylated hydroxyanisole and EDTA were used as positive controls, respectively. Furthermore, the oxygen radical absorption capacity was determined fluorimetrically by monitoring emissions at 520 nm and excitation at 485 nm using fluorescein and 2,2'-azobis(2-methylpropionamidine) dihydrochloride radicals. A calibration curve was constructed using Trolox as a standard and an antioxidant-free blank.

Appropriate spectroscopic methods were used to determine the ability of plant extracts to inhibit specific enzymes that can affect the health and appearance of the skin. The activity of tyrosinase inhibition (obtained from *Agaricus bisporus*) was determined in the reaction of the formation of dopaquinone from 3,4-dihydroxy-L-phenylalanine. Kojic acid was used as a reference inhibitor. Elastase activity (obtained from pig pancreas) was tested with the substrate

N-succinyl-Ala-Ala-Ala-p-nitroanilide and oleanolic acid as a positive control, whereas collagenase activity (obtained from *Clostridium histolyticum*) with the substrate N- 3-2-furyl-acryloyl-Leu-Gly-Pro-Ala and gallic acid as a positive control. Anti-hyaluronidase activity was performed in an assay with p-dimethylaminobenzaldehide and tannic acid used as positive control. The anti-inflammatory activity of plant extracts was tested by measuring the concentration of conjugated diene resulting from the oxidation of linoleic acid by 5-lipoxygenase (obtained from soy) and in the reaction with ovalbumin. Nordihydroguaiaretic acid and diclofenac were used as positive controls, respectively.

The antioxidant potential and the ability to inhibit selected enzymes was calculated by regression analysis and expressed as an IC_{50} value. Measurements were performed in triplicate and expressed as arithmetic mean ± standard deviation. For RSM analysis and optimization of results, Box-Behnken design and two-factor interaction was used. Statistical differences were examined using ANOVA and corresponding post-hoc tests (Tukey's and Dunnett's post-hoc tests).

Results and discussion

Preliminary results showed that glycerol was an equally effective solvent in maceration extraction of total phenolic acids of *E. purpurea*. Taking this into account as well as aforementioned benefits of glycerol, glycerol was proposed as the solvent of choice in further investigations of this work.

Efficiency of classic maceration with UAE was compared. The results showed that the amount of total polyphenols and phenolic acids, as well as chicory and caftaric acid obtained by maceration of *E. purpurea* did not differ statistically significantly in relation to UAE, which can be explained by the better utilization of plant material by a whole series of phenomena that actively influence the outcome of UAE extraction in relation to a much more passive maceration. The results showed that the UAE outcome was most influenced by glycerol concentration, extraction time, temperature and ultrasound power. According to the results obtained, these independent variables were used during the optimization of the extraction conditions in the further work of this research.

As expected, the concentration of glycerol largely dictated the yield of total phenolic compounds and desired active ingredients. In all research conducted as part of this work, the extraction outcome (yield of selected phenolic compounds) was proportional to the negative square value of the glycerol concentration. Also, a negative linear effect of glycerol concentration was observed during the extraction of *E. purpurea*, *B. vulgaris* and *G. glabra*.

High concentrations of glycerol (90%) did not have a positive effect on the yield of total phenolic compounds, which is partly expected due to the increased viscosity of the solution, which hinders the diffusion of polyphenol molecules and reduces solvent penetration into the plant material.

In addition to the determination of selected phenolic compounds, individual active components of herbal drugs were also determined. Thus, *G. glabra* flavonolignans, as well as berberine, were best extracted at moderate concentrations of glycerol, while the extraction of *G. glabra* polyphenols glabridin and isoliquiritigenin required a higher concentration of glycerol.

In addition to glycerol concentration, temperature, another independent variable, strongly influenced UAE efficiency. In these studies, temperatures from 10 to 90 °C were used. As expected, the temperature had a linear influence on the outcome of the extraction (total phenolic compounds and individual active components). Thus, when optimizing the extraction for the highest yield of total polyphenols of *G. glabra*, *B. vulgaris*, *S. marianum* and *E. purpurea*, the optimal temperature of the extraction mixture was from 60 to 80 °C, while when optimizing the extraction for the highest yield of individual active components it was slightly higher, from 70 to 80 °C. These results indicate, among other things, the good thermostability of the extraction compounds. Also, by raising the temperature, extraction mixtures with a higher proportion of glycerol were less viscous, which further improved the outcome of the extraction, which, among other things, is also described in the literature.

As part of this work, the influence of ultrasound power on the extraction outcome was examined in two studies. The plant species used were *B. vulgaris* and *E. purpurea*, and the results show that the influence of ultrasound on the extraction outcome was proportional to the positive quadratic model, which is expected. Thus, a weak to moderate intensity of ultrasound (144 and 360 W) is optimal for the highest yield of total polyphenols, but also of individual active components of the mentioned plant species.

Extraction time is also a significant factor in all types of extraction since it determines the duration of contact of the plant material with the extraction solvent, which directly affects the outcome of the extraction. As part of this research, the influence of time was examined during the extraction of *S. marianum* and *E. purpurea*. The time range was from 20 to 60 minutes, and the obtained results unequivocally indicate that 60 minutes was the optimal extraction time for all tested outcomes.

As the last independent variable, the influence of the mass of plant material used for extraction was examined. In this study, the influence of the mass of the plant material was examined in the extraction of *G. glabra*. The obtained results indicate that a higher amount of the plant material had a more favourable effect on the extraction results, which is also seen in the obtained positive quadratic model of the influence of the mass of the plant material on the yield of glabridin and isoliquiritigenin. The results obtained on the yield of total phenolic compounds also show the same curve of the quadratic equation.

Based on the data obtained from the RSM methodology, optimized extracts were prepared, which were further used in biological activity tests. The RSA activity of the mentioned extracts, as well as the yields of individual active components, were determined by suitable tests and the results obtained were compared with the results obtained by the RSM methodology. Since the compared values did not differ statistically significantly, the validity of the proposed model was confirmed.

In addition to the above, the optimized extracts were also tested for their effect on enzymes that act on macromolecules that affect the appearance and function of the skin, as well as anti-inflammatory activity. Thus, the influence of the extracts on the enzymes tyrosinase, collagenase, elastase and hyaluronidase were tested, while the anti-inflammatory activity was tested with the inhibition test of heat-induced coagulation of ovalbumin and the enzyme lipoxygenase. The wound-healing activity of *E. purpurea* extract was also tested in the "scratch" test on HaCaT cell monolayer to confirm the claim that this extract is a suitable ingredient in herbal medicines to accelerate the healing of minor wounds and other skin damage. Active standards were used in each test, the activity of which was tested in relation to the activity of the extract.

Glycerol was also tested for biological activity in the above-mentioned tests at the concentrations in which it was used in the extracts in order to determine the potential influence of the solvent on the biological activity of the extract. The results of this research showed that glycerol has no statistically significant biological activity except in the exceptions described below.

As a rule, the antiradical activity of the optimized extracts was lower than the activity of the standard solution, BHA. Among all tested extracts, the activity of *G. glabra* extract was the closest to that of the standard. As expected, extracts of *B. vulgaris* and *E. purpurea* optimized for RSA had a slightly lower EC_{50} than extracts optimized for other components. Although determining the exact structure and amount of substances responsible for the observed in vitro activity is beyond the scope of this study, it is most likely that numerous antiradical compounds contributed to the observed activity.

Like the antiradical activity, the chelating properties of the optimized herbal drug extracts were lower than the standard solution, EDTA. It is interesting that the extracts of *B. vulgaris*, *E. purpurea* and *S. marianum* optimized for antiradical activity were approximately equally effective in chelating Fe^{2+} ions as well as the extracts optimized for individual components of the same herbal drugs, which indicates that most of the chelating properties are precisely contained in the components. This is not the case with *G. glabra* extract, where extracts optimized for total phenolic compounds showed the most prominent chelating ability, which was closest to the activity of the standard solution.

In the test of inhibition of the oxidative degradation of unsaturated fatty acids, the extracts of *S. marianum* and *E. purpurea* showed an extremely high inhibition capacity that was equal to or higher than the activity of the standard solution. This is especially important because most cosmetic products contain natural oils rich in linoleic acid, as well as many other polyunsaturated fatty acids. On the other hand, the activity of inhibiting unsaturated fatty acids in *B. vulgaris* and *G. glabra* extracts were more modest and lower than the activity of the standard.

Denaturation of tissue proteins is one of the causes of inflammatory processes in the skin. Therefore, the suppression of skin protein denaturation could slow down the further development of inflammatory skin changes, which is of particular importance when developing dermopharmaceutical grade cosmetics. In this study, all extracts showed good inhibition properties of heat-induced coagulation of ovalbumin. As the role of glycerol on the denaturation of proteins such as collagen is described in the literature, in this research an experiment was carried out on the role of glycerol solutions on the denaturation of egg ovalbumin. As expected, the results showed that glycerol significantly inhibits the denaturation of ovalbumin and most of the inhibitory activity of the extract is due to the role of the solvent. This ability of glycerol further confirms the importance of using glycerol extracts in cosmetics since it goes beyond the advantages of glycerol as a green solvent. Furthermore, inhibitory activity on lipoxygenase was obtained since it also plays important role in inflammatory processes in the skin. Although in the literature berberine from *Mahonia aquifolium* showed very low anti- lipoxygenase activity, the activity of *B. vulgaris* extract prepared in this study was significantly stronger. In conclusion, the described activity of extracts in inflammatory changes caused by lipoxygenase activity, as well as protein coagulation makes them good candidates for use in cosmetics.

In addition to the moisturizing effect and antiradical potential, modern cosmeceuticals are expected to have additional beneficial effects on the skin. Too strong enzymatic activity in the skin, which occurs due to numerous previously described changes caused by natural aging, as well as external influences, leads to premature or excessive degradation of important structural proteins in the skin such as elastase or collagenase. These changes also lead to the breakdown of important structural polysaccharides in the skin such as hyaluronic acid.

The results of this research show that the *E. purpurea* extract is a good inhibitor of collagenase and elastase. However, these activities are still lower than the activities of standard, gallic and ursolic acid solutions. In the case of inhibition of both enzymes, stronger activity was observed in the extract optimized for antiradical activity, which suggests that compounds other than phenolic acids play an important role in this process. In addition, *E. purpurea* extract is a strong inhibitor of hyaluronidase, which is in line with previous research describing caffeic acid derivatives with a strong antihyaluronidase effect. It should be noted that this effect was more pronounced than the solution of the standard, tannic acid. This is particularly important considering the ability of hyaluronic acid to retain moisture, which contributes to overall hydration in the skin.

Similar results are also visible with the extract of the *S. marianum* being good elastase inhibitor. On the other hand, all *G. glabra* extracts showed excellent elastase inhibition results, with special emphasis on glabridin and isoliquiritigenin optimized extract that exceeded the activity of the standard solution. It is also important to note that as part of this research, an experiment was conducted to determine whether the solvent (glycerol) affects elastase activity, and the results obtained indicate that this is not the case, which further confirms the beneficial effect of the selected extracts for use in cosmetics.

The effect of UV rays from sunlight was previously described, as well as its negative consequences on the skin, primarily due to the formation of melasma and hyperpigmentation spots. This is due to the activity of tyrosinase, an enzyme that catalyses the synthesis of the melanin protein. This research showed that all extracts are good inhibitors of tyrosinase, with special emphasis on extracts of *E. purpurea* and *G. glabra*, whose effects were comparable or better than those of kojic acid, used as a standard solution. It is important to note that even in these experiments glycerol did not show a significant effect on the inhibition of tyrosinase, which means that all the inhibitory activity belongs to the active components of the plants.

Wound healing is a process of dynamic cellular and molecular mechanisms, divided into several phases: hemostasis, inflammation, proliferation/migration, and maturation or remodeling. In the proliferation phase, the migration of keratinocytes and fibroblasts restores the network of blood vessels and participates in the granulation process. This characteristic is used for the in vitro "scratch" wound healing test method. In this procedure, a scratch that leaves an empty space (“wound”) at the bottom of the well is created in the HaCaT cell monolayer. If conditions are satisfactory, cell movement and proliferation occur, followed by gradual closure of the cell model of the wound. In this study, cells were treated with different dilutions of *E. purpurea* extract and glycerol. Hank's balanced salt solution was used as a negative control. Both extracts (optimized for yield of total phenolic acids and antiradical activity) accelerated wound closure in cells. The RSA optimized extract was particularly active. After 48 h, the scratch surface in the cell monolayer treated with this extract was barely visible, indicating excellent wound healing activity. The lack of solvent activity indicates that the active components of *E.purpurea* were responsible for stimulating the proliferation of HaCaT cells during the examined incubation time. This experiment confirms the earlier statement of the European Medicines Agency (EMA), which states that *E. purpurea* and its preparations can be used in herbal medicines to relieve skin diseases and promote the healing of minor wounds.

Conclusion

Glycerol proved to be an optimal green solvent for the extraction of the active components of *E. purpurea*, *G. glabra*, *B. vulgaris* and *S. marianum* due to its many advantages over conventional solvents, of which its non-toxicity, as well as the described humectant properties that contribute moisturizing effect on the skin, which eliminates the need to remove the solvent from the extract. UAE has proven to be the extraction method of choice due to its superiority over the alternative (maceration) which yields extracts rich in active constituents of interest. Optimized glycerol extracts showed good biological activity in the fields of antiradical effect, as an effect on selected enzymes important for the health and appearance of the skin. In conclusion, the optimized extracts show a promising role in products intended for the treatment and protection of the skin, with the aim of reducing skin changes due to skin aging. Additional research is needed to determine the method of incorporation of the mentioned extracts into dermopharmaceutical cosmetics, as well as their exact dosage.

SADRŽAJ

1. UVOD

1.1. Starenje kože

Koža je najveći organ ljudskog organizma, a čini više od 10% njegove tjelesne mase. Građena je od tri sloja: epidermisa, dermisa i hipodermisa. Kako se nalazi na površini organizma, koža je u neprekidnoj interakciji s okolinom, te kao takva ima važnu ulogu u održavanju homeostaze. Prvenstveno regulira tjelesnu temperaturu, ali sudjeluje i u regulaciji krvnog tlaka kao i izmjeni tvari organizma s okolišem. To je najvažniji osjetilni organ za osjete topline, dodira i boli, a predstavlja i barijeru ulasku toksina i mikroorganizama u organizam. Da bi ispunila svaku od ovih funkcija, koža mora biti čvrsta, robusna i fleksibilna, s učinkovitom komunikacijom između svake od svojih unutarnjih komponenti. Stoga je to organ koji je u stalnom stanju regeneracije i popravka (1).

Koža je izložena različitim unutarnjim i vanjskim utjecajima koji djeluju na njezin izgled i zdravlje te dovode do brojnih degenerativnih promjena poput hiperpigmentacije, promjena u strukturi, elastičnosti i stupnju hidratacije koje se očituju kao starenje kože (2). Ovisno o uzroku, nastale promjene se svrstavaju u intrinzično i ekstrinzično starenje. Intrinzično starenje je neizbježan fiziološki proces koji rezultira tankom, suhom kožom, finim borama i postupnom dermalnom atrofijom, dok je ekstrinzično starenje uzrokovano vanjskim čimbenicima okoliša poput izlaganja zagađenom zraku, pušenja, loše prehrane i posebice izlaganja sunčevom ultraljubičastom (UV) zračenju. Konačni rezultat ekstrinzičnog starenja je opuštena koža s grubim borama, bez elastičnosti i grubog izgleda. Postoje različita objašnjenja za molekularne osnove starenja kože, uključujući teoriju staničnog starenja i različitih promjena u staničnoj DNK i drugih. Među njima se svojom važnošću ističe oksidativni stres (3).

Oksidativni stres posreduje u velikom broju promjena koje se odvijaju tijekom starenja (2). To je stanje koje se očituje kao povećana razina reaktivnih kisikovih spojeva (engl. *reactive oxygen species*, ROS). ROS su normalni dio metabolizma te pri fiziološkim koncentracijama imaju važne funkcije u organizmu. Ipak, povećanjem njihove koncentracije izazivaju različita oštećenja u tkivima i stanicama. Koža je opskrbljena brojnim sustavima zaštite od ROS-a koji se zajednički nazivaju endogenim antiradikalnim sustavom, a sastoji se od enzimatskih i neenzimatskih antioksidansa. Primjeri enzimatskih antioksidansa su glutation peroksidaza, reduktaza i katalaza te superoksid dizmutaza. To su enzimi koji razgrađuju različite ROS poput lipidnih hidroperoksida, superoksidnog radikala i vodikovog peroksida. Među neenzimatske antioksidanse ubrajamo L-askorbinsku kiselinu (nalazimo je u vodenoj fazi), glutation (zastupljen u staničnom odjeljku), α -tokoferol (u membranama) i ubikinon (u mitohondrijima)

(4). Iako u fiziološkim uvjetima endogeni antiradikalni sustav učinkovito održava homeostazu, u situacijama izloženosti vanjskom oksidativnom stresu njegova učinkovitost postaje nedostatna. Primjerice, uslijed ozračivanja kožnih fibroblasta UV-A sunčevim zrakama, endogena aktivnost katalaze i superoksid dizmutaze reducira se, a ROS pokreću lančanu reakciju peroksidacije lipida u staničnim membranama i zadiru u kaskade prijenosa signala uključenih u ekspresiju određenih gena u stanicama kože. Dolazi do prekomjerne ekspresije matriksnih metaloproteinaza (MMP) poput MMP-1 (kolagenaza), MMP-3 (stromelizin) i MMP-9 (želatinaza). Ovi enzimi kataliziraju razgradnju vlakana kolagena što se očituje gubitkom čvrstoće kože. Genski je materijal također podložan oštećenjima djelovanjem sunčevih zraka budući da DNK može izravno apsorbirati UV-B zračenje, što dovodi do dimerizacije parova baza i greški u replikaciji DNK. Uz to, UV-A zrake posjeduju sposobnost inhibicije popravka DNK što povećava vjerojatnost nastanka brojnih kožnih oštećenja i bolesti (5). Osim toga, oksidativni stres izaziva blagu, ali kroničnu upalu u procesu koji se naziva „*inflammaging*". Oštećenje stanica i oksidacija lipida izazvani ROS-ovima dovode do aktivacije i infiltracije makrofaga zaduženih za njihovo uklanjanje. Makrofazi preopterećeni oksidiranim lipidima se talože na spoju dermisa i epidermisa, te oslobađaju proupalne citokine i dodatne ROS-ove (3). Sunčevo zračenje izaziva i nastanak melanina posredovan enzimom tirozinazom. Ukoliko je ta sinteza neravnomjerna, nastaju hiperpigmentacije i melazme, što značajno narušava izgled kože, a i jedna je od vidljivih značajki njezinog starenja (6).

Kako bi se takva oštećenja prevenirala ili postigli reparativni učinci mogu se koristiti i egzogeni antioksidansi. Oni se unose prehranom ili nanose izravno na kožu. Egzogeni antioksidansi mogu direktno ili indirektno dovesti do inhibicije nastanka ili učinka ROS-a i MMP-a što rezultira normaliziranom proizvodnjom kožnih strukturnih proteina (7). Najzastupljeniji izvori egzogenih antioksidansa za primjenu u kozmetici su prirodni produkti biljnog porijekla. Osim antiradikalnih učinaka, aktivne sastavnice biljnog materijala pokazuju protuupalne, antikancerogene, antihiperpigmentacijske i druge brojne učinke čime se proizvodi s egzogenim antioksidansima smještaju na granicu između kozmetičkih i ljekovitih tvari (8).

1.2. Dermatofarmacija

Zdrav i mladolik izgled kože oduvijek se smatrao simbolom ljepote, a kako bi se taj izgled održao, osmišljeni su brojni kozmetički pripravci. Osim za uljepšavanje i usavršavanje izgleda kože, kozmetika se rabila i za druge primjene poput prikrivanja nedostataka, maskiranja određenih dijelova tijela, pokazivanje socijalnog statusa pa čak i kao alat za zastrašivanje neprijatelja. Sve civilizacije imale su svoje oblike kozmetičkih pripravaka, pa tako nalazimo zapise o izradi kozmetičkih proizvoda koji datiraju od 3000 godina p.N.E., još iz doba starih Egipćana. Iako sastojcima, izradom, načinom uporabe i izgledom danas kudikamo drugačija, uporaba kozmetičkih proizvoda je i dalje jednako, ako ne i više, aktualna (9). Prema regulativi 1223/2009 Europske Unije, pod pojmom kozmetika smatra se svaki proizvod ili pripravak namijenjen korištenju na vanjskim dijelovima ljudskog tijela (epidermis, kosa, vlasište, nokti, usnice i vanjski spolni organi), zubima te sluznici. Primarna namjena kozmetičkih pripravaka na koži je čišćenje, parfimiranje, mijenjanje izgleda, zaštita i održavanje dobrog stanja (10). U kozmetičke pripravke ubraja se širok spektar proizvoda poput sapuna, šampona, proizvoda za tuširanje, sredstava za zaštitu od sunca, njegu kože i kose, dentalnu higijenu, te bojanje kose i kože (dekorativna kozmetika), dezodoransa i mnogih drugih, a među njima su najkorišteniji pripravci za njegu kože lica i tijela (11).

Zahtjevi potrošača prema kozmetičkim proizvodima postaju sve veći pa se tako od krema za lice, osim hidratantne funkcije, očekuje i da čisti, zaglađuje, obnavlja, jača i štiti kožu zahvaljujući svojim aktivnim sastavnicama (12). Time se granica između lijekova i kozmetike često briše. Dermatofarmacija je specijalizirano područje unutar farmacije koje se fokusira na proučavanje lijekova i tretmana povezanih s dermatološkim stanjima i njegom kože. Dermatofarmaceutski pripravci nerijetko sadržavaju prirodne produkte, najčešće u obliku biljnih ekstrakata, koji mogu djelovati antiradikalno i inhibirati enzime koji oštećuju kožne strukture te spriječiti neželjene promjene kože. Aktivne sastavnice biljnog porijekla pokazuju značajno protuupalno i antikancerogeno djelovanje (8), a mogu inaktivirati ROS-ove i MMP-aze što rezultira normalizacijom proizvodnje kožnih strukturnih proteina (7). Osim toga, brojne tvari biljnog porijekla djeluju kao učinkoviti antioksidansi i prezervativi štiteći tako ostale sastavnice dermatofarmaceutskih proizvoda od oksidacijske razgradnje tijekom skladištenja i korištenja (13).

Zabrinutost potrošača zbog nuspojava koje u određenim slučajevima može izazvati konvencionalna kozmetika, poput alergijskog kontaktnog dermatitisa uzrokovanog pomoćnim ili aktivnim sastavnicama te toksičnosti uzrokovanom teškim metalima prisutnim u proizvodu, dodatno potiče razvoj kozmetike bazirane na proizvodima prirodnog porijekla jer potrošači smatraju da upravo oni pokazuju željeni široki spektar djelovanja uz izvrstan sigurnosni profil (14). Stoga uporaba biljnih ekstrakata i njihovih sastavnica u lokalnim pripravcima za kozmetičku primjenu danas doživljava značajan porast (13).

Među mnogobrojnim biljnim sastavnicama svojim učinkom na kožu posebno se ističu polifenoli. Bilo uneseni prehranom ili naneseni na kožu, polifenoli djeluju antiradikalno tako što doniraju vodikove atome iz fenolne skupine. Osim toga mogu djelovati i kao posredni antioksidansi keliranjem teških metala poput željeza i bakra sprječavajući time njihov pro-oksidativni učinak (15). Ovisno o svojoj strukturi, prirodni polifenoli se ubrajaju u nekoliko skupina poput flavonoida, fenolnih kiselina, trijeslovina, antracenskih derivata i drugih. Ipak, svojom rasprostranjenošću i učincima na koži ističu se flavonoidi i fenolne kiseline. Tako flavonoid luteolin modulira upalne procese u koži, a brojne studije ukazuju na njegov potencijal da pozitivno utječe na brojna stanja poput starenja kože, melanoma, zacjeljivanja rana kao i upalne bolesti kože, uključujući psorijazu, kontaktni i atopijski dermatitis (16). Pokazalo se da flavonoidi mogu spriječiti i oksidativne promjene lipida stanične membrane. Tako katehin, epikatehin i kvercetin imaju snažan antiradikalni kapacitet u fosfolipidnom dvosloju izloženom ROS-u (15). I fenolne kiseline imaju značajan potencijal za primjenu u kozmetičkim proizvodima. Tako ferulična kiselina svojim antiradikalnim učinkom poboljšava kemijsku stabilnost drugih spojeva u topikalnim formulacijama, a svojim fotoprotektivnim učinkom prevenira fotooštećenja kože izazvanih UV zračenjem (17). K tomu, *in vivo* studije ukazuju da ružmarinska kiselina može ublažiti upalu psorijatične kože kod miševa blokiranjem interakcije između interleukina (IL) 17A i njegovog receptora. Osim fenolnih spojeva, i brojni se alkaloidi poput kapsaicina, berberina, piperina i spilantola koriste u kozmetičkim proizvodima gdje djeluju kao antimikrobna, protuupalna, antihiperpigmentacijska sredstva. Osim toga, ublažavaju znakove starenja i te smanjuju vidljivost celulita (18).

1.3. Zelena ekstrakcija

Razvoj novijih kozmetičkih proizvoda ima integrativni pristup, usmjeren ne samo na proces formulacije, već i na percepciju i zahtjeve potrošača kao dio razvojne, ali i marketinške strategije (11). Osim željenog djelovanja i sigurnosnog profila, suvremeni kupac očekuje da se prilikom proizvodnje i razvoja kozmetičkih proizvoda poštuju određena etička načela. Ona se ne odnose samo na korištenje pokusnih životinja u kozmetičkoj industriji već i na prekomjerno iskorištavanje prirodnih resursa i onečišćenje okoliša u proizvodnji, uporabi i odlaganju kozmetike (19). Sve to uvelike potiče potražnju za zelenom kozmetikom što dovodi do neprestanog razvoja novih, ekološki prihvatljivijih proizvoda (20), poput proizvoda baziranih na prirodnim pomoćnim i aktivnim tvarima (21). Jedan od ključnih koraka u primjeni biljnih tvari kao aktivnih kozmetičkih sastavnica je proces ekstrakcije aktivnih biljnih tvari iz sirovog biljnog materijala. U tom se procesu odvajaju željeni metaboliti poput alkaloida, flavonoida, terpena ili saponina od inertnog ili neaktivnog materijala korištenjem odgovarajućeg otapala i odabrane ekstrakcijske tehnike. Ekstrakcija aktivnih sastavnica iz biljnog materijala koristi se od najranije poznate povijesti. U današnje vrijeme gotovo da se ne može pronaći proizvodni proces u kozmetičkoj industriji koji ne koristi procese ekstrakcije, a odabir odgovarajućeg ekstrakta za uklapanje u kozmetički proizvod jedan je od ključnih koraka njegovog razvoja. (12).

Postupke ekstrakcije, prema korištenju tehnologije i načela ekstrakcije, možemo ugrubo podijeliti na konvencionalne (npr. maceracija, digestija, perkolacija) i nekonvencionalne (npr. ekstrakcija superkritičkim fluidom, ekstrakcija potpomognuta ultrazvukom ili mikrovalovima) (22). Sve te tehnike imaju neke zajedničke ciljeve, a to su: (a) ekstrahirati ciljane aktivne sastavnice iz složenog biljnog uzorka (matrici), (b) povećati selektivnost (c), povećati koncentraciju aktivnih sastavnica (d), pretvoriti aktivne sastavnice u prikladniji oblik za detekciju i odvajanje i (e) osigurati reproducibilnu metodu ekstrakcije koja je neovisna o varijacijama u matrici uzorka (23). U novije vrijeme sve se više vodi i računa o tome da ekstrakcijska metoda pridonosi očuvanju okoliša. Imajući to na umu, kontinuirano se razvijaju ekološki prihvatljive i održive metode ekstrakcije bioaktivnih prirodnih proizvoda iz ljekovitog bilja (24,25). Takve, zelene, metode obično postižu visoke prinose željenog metabolita, imaju nisku potrošnju energije i koriste prirodna, biorazgradiva i netoksična otapala koja se mogu dobiti iz obnovljivih izvora (26,27).

Ekstrakcija potpomognuta ultrazvukom (engl. *ultrasound-assisted extraction*, UAE) jedna je od tehnika ekstrakcije koja se zbog kratkog vremena ekstrakcije, te niske potrošnje energije i otapala smatra zelenom ekstrakcijskom tehnikom. Ultrazvuk je posebna vrsta zvučnog vala izvan percepcije ljudskog sluha. U zvučnom spektru, ultrazvuk se svrstava u frekvencijski raspon između 20 kHz i 100 MHz. Kao i drugi zvučni valovi, ultrazvuk prolazi kroz medij stvarajući kompresiju i ekspanziju. Ovaj proces u biljnom materijalu proizvodi fenomen koji se zove kavitacija, što znači stvaranje, rast i kolaps mjehurića. U tom procesu dolazi do pretvorbe dijela kinetičke energije gibanja u toplinu (28). Ultrazvučna energija za ekstrakciju također omogućuje učinkovitije miješanje, brži prijenos energije, smanjene toplinskog gradijenta i temperature ekstrakcije, selektivnu ekstrakciju, smanjenu kompleksnost ekstrakcijske opreme, brži odgovor na kontrolu procesa ekstrakcije, brzo pokretanje i povećanu proizvodnju (29). Ipak, glavna korist UAE može se uočiti u tome što ultrazvučna energija uzrokuje pucanje staničnih stijenki i time olakšava ispiranje organskih i anorganskih spojeva iz matrice biljke (30). Vjerojatni mehanizam je ultrazvučno pojačanje mase zbog fenomena kavitacije te prijenos i ubrzani pristup otapala unutrašnjosti stanice. Stoga mehanizam UAE-a uključuje dvije glavne vrste fizičkih fenomena: (a) difuziju preko stanične stijenke i (b) ispiranje sadržaja stanice nakon razbijanja stijenki. Neki od čimbenika koji utječu na učinkovitost ekstrakcije su sadržaj vlage u uzorku, veličina čestica, otapalo, temperatura, tlak, trajanje ekstrakcije i snaga (frekvencija) ultrazvučnih valova (31). Zbog svoje jednostavnosti i ekonomičnosti UAE se često koristi za ekstrakciju prirodnih produkata, a primjeri uključuju ekstrakciju kurkuminoida iz kurkume (32), polifenola iz lista masline (33) ili polisaharida iz smokve (34).

1.4. Zelena otapala

Osim ekstrakcijske tehnike, važan aspekt ekstrakcije je i ekološka prihvatljivost otapala. Idealno otapalo za zelenu ekstrakciju biljnog materijala trebalo bi biti netoksično, biološki razgradivo, nezapaljivo i imati visoku moć otapanja. Tradicionalna otapala dobivena iz fosilnih goriva, iako dobre moći otapanja, nisu povoljna za okoliš jer nisu biorazgradiva, lako su zapaljiva, lako hlapljiva i toksična. Upravo zbog toga poseban je naglasak stavljen na zelena ili alternativna otapala – otapala koja su dobivena iz obnovljivih izvora, netoksična su i neškodljiva te sigurna za okoliš. Etanol zbog svoje biorazgradivosti i prirodnog podrijetla ispunjava neke od uvjeta za zelena otapalo, te je još uvijek među najkorištenijim otapalima za ekstrakciju prirodnih spojeva. Međutim, etanol je vrlo zapaljiv i dovodi do iritacije kože. Stoga se pokušava zamijeniti drugim otapalima, po mogućnosti prirodnog podrijetla (26).

Glicerol (propan-1,2,3-triol) je prirodna, cjenovno pristupačna, netoksična i biorazgradiva viskozna tekućina. Proizvodi se iz obnovljivih izvora, npr. kao nusprodukt proizvodnje biodizela (35). Također ga je moguće proizvesti iz procesa fermentacije određenih kvasaca ili algi. Osim u kemijskoj industriji, glicerol se danas najviše koristi u prehrambenoj i farmaceutskoj industriji. Dodatna prednost glicerola je njegova higroskopnost, što ga čini jednim od najčešće korištenih sastojaka u kremama i losionima, gdje djeluje kao prirodni humektant, denaturant, mirisni sastojak, regenerator kose, zaštitnik kože i kao sredstvo za regulaciju viskoznosti (36). Budući da se glicerol koji se koristi za ekstrakciju može lako ugraditi u finalni proizvod, glicerolna ekstrakcija ljekovitog bilja vrlo je poželjna sa stajališta uštede energije (26). Zanimljivo je da je, unatoč brojnim prednostima glicerola kao ekstrakcijskog otapala, relativno nedovoljno iskorišten u proizvodnji ekstrakata za farmaceutske i dermatofarmaceutske svrhe. Neki noviji literaturni primjeri upotrebe glicerola za ekstrakciju prirodnih proizvoda uključuju ekstrakciju polifenola iz mekinja riže (37) i oraha (38).

1.5. Dizajn istraživanja

Aktivne sastavnice s povoljnim farmakološkim djelovanjima samo su djelić ukupnog sastava pojedinih biljnih vrsta. Njihova koncentracija u ekstraktima uvelike ovisi o kemijskim osobinama pojedinih sastavnica, izboru otapala, tipu ekstrakcije, kao i drugim brojnim čimbenicima koji utječu na ekstrakciju (ekstrakcijskim uvjetima). Zbog toga je jedan od ciljeva odabira ekstrakcijske metode ekstrakcije pronaći onaj postupak koji donosi najviši prinos bioaktivnih sastavnica, uz minimalnu količinu balastnih tvari. To je posebno važno prilikom razvoja postupaka zelene ekstrakcije kojim se hoće minimizirati potrošnja energije, biljnog materijala i ekstrakcijskog otapala uz zadržani kemijski sastav i bioaktivnost ekstrakta. Stoga se neprestano dizajniraju i razvijaju procesi ekstrakcije za smanjenje potrošnje energije i otapala uz korištenje obnovljivih prirodnih materijala koji proizvode sigurnu i visoku kvalitetu ekstrakta kako bi se razvijeni proces mogao prenijeti iz laboratorijskih eksperimenata u industrijske razmjere. Takva optimizacija metode igra važnu ulogu u procesu ekstrakcije (39).

Tradicionalne metode optimizacije uključuju proučavanje jednog po jednog čimbenika što je naporno i dugotrajno, a ne omogućuje proučavanje međudjelovanja pojedinih ekstrakcijskih uvjeta. Stoga se razvijaju statističke i kemometrijske metode kojima bi se taj postupak mogao pojednostaviti i ubrzati. Metodologija površine odziva (engl. *Response Surface Methodology*, RSM) zbirka je matematičkih i statističkih tehnika korištenih za konstrukciju modela i analizu problema u kojima nekoliko čimbenika koji se mogu kontrolirati (neovisne varijable) utječu na željeni ishod (ovisna varijabla) ili odgovor. Stoga je RSM prikladna metoda za optimizaciju ekstrakcijskih uvjeta za što se intenzivno i koristi (40). Prednosti RSM-a uključuju korištenje manjeg broja eksperimentalnih mjerenja, pružanje statističke interpretacije podataka i identificiranje potencijalnih interakcija među varijablama. RSM je uspješno primijenjena metoda u optimiziranju uvjeta ekstrakcije niza polifenola, antioksidansa i drugih metabolita u biljkama (41–43).

Među vrstama eksperimentalnog dizajna prikladnih za optimizaciju ekstrakcijskih procesa putem RSM-a svojom se jednostavnošću ističe Box-Behnkenov dizajn (BBD). To je vrsta faktorijalnog dizajna na tri razine. To znači da svaki faktor (ili nezavisna varijabla) varira na tri razine: niska, srednja i visoka (-1, 0, +1). Ukoliko se prostor dizajna predstavi kao kocka, točke dizajna sastoje se od njezine centralne točke i polovišta bridova. Ipak, iako se za opis dizajna često koristi analogija kocke, BBD je u svojoj naravi sferičan i okretljiv dizajn. Stoga su predviđanja koja BBD omogućuje jednako precizna u svim točkama unutar eksperimentalnog prostora. Zbog svoje jednostavnosti u usporedbi s drugim vrstama dizajna

BBD zahtijeva znatno manje eksperimenata za postizanje istog učinka što ga čini jednostavnijim i isplativijim (44). Stoga se učestalo koristi u optimizaciji ekstrakcije različitih skupina prirodnih biljnih sastavnica poput polisaharida (45), polifenola (46) ili alkaloida (47).

1.6. Korištene biljne droge

1.6.1 Echinaceae purpureae herba

Echinaceae purpureae herba (zelen purpurne rudbekije) dobiva se od vrste *Echinacea purpurea* L. Moench, Asteraceae, jedne od najpoznatijih ljekovitih biljaka na svijetu. Najpoznatija po svojem imunostimulirajućem i protuupalnom djelovanju, *E. purpurea* se najčešće koristi kod smanjenja simptoma obične prehlade (48). Ipak, prema Europskoj agenciji za lijekove, ekstrakti nadzemnih dijelova *E. purpurea* tradicionalno se koriste kod kožnih oboljenja i kao pomoć pri zacjeljivanju manjih rana (49). Smatra se da su tri skupine bioaktivnih sastavnica odgovorne za ljekovita svojstva *E. purpurea*, a to su derivati kavene kiseline, polisaharidi i alkilamidi (50). Među derivatima kavene kiseline najzastupljenija je cikorija kiselina (CIC), nakon koje slijedi kaftarna kiselina (CAF). CIC pokazuje široku lepezu povoljnih učinaka na koži, poput antivirusnog, antiradikalnog i protuupalnog učinka. Osim toga, CIC može ublažiti upalu izazvanu lipopolisaharidima u staničnim kulturama i kod miševa, kao i poboljšati starenje dermalnih fibroblasta izazvano UV-A zračenjem inhibicijom aktivnosti MMP-3. Time se otvara mogućnost blagotvornog djelovanja CIC na starenje kože (51). CAF također ima antiradikalno i protuupalno djelovanje, no pokazuje i antimutagenu i antikarcinogenu aktivnost (52). CAF je i kompetitivni inhibitor tirozinaze, što ju čini prikladnom za uključivanje u kozmetičke proizvode s antihiperpigmentacijskim učinkom (53). Osim toga, jedna manja dermatološka studija pokazala je da pripravci s ekstraktom *E. purpurea* mogu učinkovito poboljšati hidrataciju kože i smanjiti bore bez izazivanja iritacije (54), kao i smanjiti djelovanje ROS-a pomoću inhibicije ciklooksigenaze-1 (COX-1), ciklooksigenaze-2 (COX-2) i 5-lipoksigenaze (LOX) (55).

1.6.2 Liquiritiae radix

Liquiritiae radix (korijen sladića; *Glycyrrhiza glabra* L., Fabaceae) višegodišnja je biljka poznata po svom korijenu slatkog okusa. *G. glabra* se tradicionalno koristi za pospješivanje zacjeljivanja rana. Sadrži široku lepezu bioaktivnih prirodnih sastavnica potencijalno korisnih djelovanja u kozmetičkim i dermatološkim pripravcima. Ekstrakti *G. glabra* pokazuju snažan antibakterijski i antivirusni učinak, a djeluju i antiradikalno, antifungalno, antikancerogeno, protuupalno i citotoksično (56). Glicirizin, sastavnica koja *G.*

glabra daje slatki okus, je saponin triterpenskog tipa koji pokazuje antivirusne, protuupalne, antitumorske i antimikrobne značajke (57). Osim glicirizina i fenolne komponente *G. glabra*, kalkon izolikviritigenin (ISO), izoflavonoid glabridin (GLA) i flavon likviritin, također su važne za njegovu biološku aktivnost. ISO pokazuje snažan antiradikalni, protuupalni i antitumorski učinak (58). GLA djeluje kao antioksidans, fitoestrogen i protuupalno sredstvo (59). Taj izoflavonoid uzrokuje i depigmentaciju kože inhibicijom tirozinaze pa se uključuje u proizvode za topikalnu primjenu namijenjene posebno za tu svrhu (60). Likviritin je flavonoid koji ne djeluje na tirozinazu, ali uzrokuje depigmentaciju raspršivanjem melanina. Jedno je istraživanje pokazalo da je lokalna primjena kreme koja sadrži 2% i 4% likvirtina tijekom četiri tjedna bila učinkovito sredstvo za smanjenje pigmentacija nastalih kao posljedica melazme (61). Ekstrakti *G. glabra* štite kožu od ozljeda uzrokovanih oksidativnim stresom (62,63), ubrzavaju epitelizaciju rana, poboljšavaju remodeliranje na mjestu rane (64), a pojedine *in vivo* (65) i kliničke studije (65) ukazuju na to da učinkovito smanjuju simptome atopijskog dermatitisa te da djeluju antihiperpigmentacijski (61).

1.6.3 Silybi mariani fructus

Silybi mariani fructus ili plod sikavice (*Silybum marianum* (L.) Gaertn, Asteraceae) dvogodišnja je ljekovita biljka čiji se plod tradicionalno koristi u liječenju bolesti jetre (66). Najvažnija fitokemijska komponenta ploda *S. marianum* je kompleks flavonolignana zvani silimarin (SYL) koji se sastoji od dva stereoizomera nazvana silibinin A i silibinin B, s udjelom od oko 60-70%, a zatim slijede silikristin, silidianin i izosilibinin. SYL je zanimljiv i kao sastavnica za primjenu u kozmetici jer su mnoga njegova djelovanja povezana s djelovanjem na kožu. Flavonolignani *S. marianum* pokazuju antiradikalni učinak *in vivo*. SYL smanjuje edem kože, sprječava indukciju epidermalne hiperplazije i peroksidaciju lipida kod miševa uzrokovane 12-*O*-tetradekanoil-13 forbol acetatom, a posredovane oksidativnim stresom. Izomeri silibinina djeluju i protuupalno inhibicijom mijeloperoksidaze, LOX, COX, faktora nekroze tumora i IL-1α, a induciraju i apoptozu (67). SYL pokazuje snažan fotoprotektivni učinak na koži (68). Time silibinin posredno štiti od fotokarcinogeneze, opeklina i hiperplazije epiderme uzrokovane UV-B zračenjem. Silibinin štiti od oštećenja DNK uzrokovanih UV-B zrakama u stanicama epiderme kože, a neke studije upućuju i na to da bi silibinin mogao popravljati i oštećenja epidermalne DNK izazvanog UV-B zračenjem (67). Dodatno, ekstrakt *S. marianum* i SYL mogu inhibirati enzime koji sudjeluju u razgradnji izvanstanične matrice, a pokazuju i fotoprotektivni učinak na koži (68).

1.6.4 Berberidis radicis cortex

Berberidis radicis cortex (kora korijena žutike) dobiva se od vrste *Berberis vulgaris* (L.), Berberidaceae. To je listopadni grm s dugom poviješću medicinske i prehrambene upotrebe u Europi, Aziji i Americi. Dok se plodovi *B. vulgaris* uglavnom koriste kao hrana, kora korijena i stabljike imaju ljekovita svojstva. Za njih je mahom zaslužan berberin (BER), izokinolinski alkaloid prisutan u tim organima (69). BER pokazuje brojne farmakološke učinke, uključujući protuupalno, antiradikalno (69), antibakterijsko (70) i antifungalno djelovanje (71) bez značajnijih nuspojava. U kozmetičkim se proizvodima koristi zbog svog antimikrobnog učinka (18). Smatra se da ga izvrsno antibakterijsko djelovanje širokog spektra čini prikladnijim od pojedinačnih kliničkih antibiotika za zacjeljivanje inficiranih rana na koži i sluznicama (72). BER sprječava ekspresiju MMP-1 izazvanu UV zračenjem i smanjenje ekspresiju prokolagena tipa I u ljudskim dermalnim fibroblastima (73). Ovisno o dozi inhibira bazalnu ekspresiju i aktivnost MMP-9 kao i onu nastalu indukcijom tkivnim aktivatorom plazminogena. K tomu, BER suprimira i kemijski induciranu ekspresiju IL-6 što upućuje na to da može spriječiti upalu kože i razgradnju proteina izvanstanične matrice, uključujući kolagen (74). BER značajno poboljšava preživljenje kožnih režnjeva promicanjem angiogeneze, inhibicijom upale, ublažavanjem oksidativnog stresa i smanjenjem apoptoze putem signalnog puta fosfoinozitid 3-kinaza/proteinska kinaza B/fosfo-endotelna sintaza dušikovog oksida (PI3K/Akt/eNOS) (75). Osim toga, BER može pospješiti i prolazak hidrofilnih lijekova kroz kožu (76). Topikalni učinak BER na zacjeljivanje rana potvrđen je i u kliničkim ispitivanjima. Pokazalo se da učinkovito ubrzava zacjeljivanje rana nastalih kao posljedica aftoznog stomatitisa te smanjuje simptome upale kod periodontitisa (77).

1.7. Cilj rada

Cilj rada je optimizacija glicerolne UAE ekstrakcije djelatnih biljnih vrsta: *G. glabra* , *E,purpurea*, *S. marianum* i *B.vulgaris*. Odredit će se antiradikalna aktivnost optimiziranih ekstrakata te ispitati njihova sposobnost inhibicije enzima koji utječu na izgled i zdravlje kože.

2. Rezultati

2.1. Comparison of Maceration and Ultrasonication for Green Extraction of Phenolic Acids from *Echinacea purpurea* Aerial Parts

molecules

MDPI

Article

Comparison of Maceration and Ultrasonication for Green Extraction of Phenolic Acids from *Echinacea purpurea* Aerial Parts

Plamen Momchev [1,2], Petar Ciganović [1], Mario Jug [1], Eva Marguí [3], Jasna Jablan [1,3] and Marijana Zovko Končić [1,*]

1 Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10000 Zagreb, Croatia; momchevplamen@gmail.com (P.M.); petar.ciganovic@me.com (P.C.); mjug@pharma.hr (M.J.); jjablan@pharma.hr (J.J.)
2 Faculty of Pharmacy, Medical University-Sofia, Bul. "Acad. Ivan Geshov" 15, 1000 Sofia, Bulgaria
3 Department of Chemistry, Faculty of Sciences, University of Girona, C/M. Aurèlia Campmany 69, 17003 Girona, Spain; eva.margui@udg.edu
* Correspondence: mzovko@pharma.hr; Tel.: +385-1-6394-792

Academic Editor: Elisa Ovidi
Received: 1 October 2020; Accepted: 30 October 2020; Published: 5 November 2020

check for updates

Abstract: *Echinacea purpurea* is used in herbal medicinal products for the prevention and treatment of the common cold, as well as for skin disorders and minor wounds. In this study, the efficiency of traditional maceration using water and ethanol was compared with the maceration using mixtures of water and glycerol, a non-toxic, biodegradable solvent from renewable sources. It was found that the glycerol–water mixtures were as effective as ethanol/water mixtures for the extraction of caffeic acid derivatives. All the prepared extracts demonstrated notable antiradical properties. Furthermore, an efficient ultrasound-assisted extraction using glycerol–water mixtures was developed using six independent variables. Their levels needed for the maximum extraction of caffeic acid derivatives were as follows: glycerol 90% (*m/m*), temperature 70 °C, ultrasound power 72 W, time 40 min, and ascorbic acid 0 mg/mL. Under the optimized conditions, ultrasound-assisted extraction was superior to maceration. It achieved significantly higher yields of phenolic acids in shorter extraction time. The presence of zinc in plant material may contribute to the beneficial effects of *E. purpurea* preparations. Since glycerol is a non-toxic solvent with humectant properties, the prepared extracts can be directly used for the preparation of cosmetics or oral pharmaceutical formulations without the need for solvent removal.

Keywords: antioxidant; *Echinacea purpurea*; glycerol; green extraction; phenolic acids

1. Introduction

The use of plants for medicinal and cosmetic applications is undergoing an unprecedented rise. For example, over 200 official monographs with scientific and regulatory standards related to the efficacy and safety of medicinal herbal preparations in the European Union have been published so far, and the number is constantly growing. The indications for such preparations include a variety of specific applications, such as skin, sleep, gastrointestinal, and circulatory disorders [1], as well as a broad spectrum of less specific activities, such as antioxidant and anti-inflammatory activities [2]. In addition to displaying the desired activity and safety profile, modern phytopharmaceuticals and cosmetics should fulfill additional requirements, such as the appropriate stability and sensory properties. Furthermore, new concerns about the environmental impact and animal welfare are constantly emerging and new products are being developed in order to meet such needs [3].

One of the emerging research areas of cosmetic and phytopharmaceuticals research is the design of green and sustainable methods for the extraction of bioactive natural products for medicinal and cosmetic purposes. Besides the high yield of the desired natural product, the ideal extraction procedure should have low energy consumption and employ biodegradable, non-toxic, and non-flammable solvents [4,5]. One such solvent is glycerol, a natural, low-cost, non-toxic, biodegradable liquid. It is manufactured from renewable sources, e.g., as a by-product of biodiesel production [6]. An additional advantage of glycerol is its hygroscopic nature, which makes it one of the most widely used ingredients in creams and lotions, where it acts as a natural humectant, denaturant, fragrance ingredient, hair conditioning agent, oral care agent, skin protectant, and viscosity decreasing agent [7]. Furthermore, it is often used in cough syrups, as a solvent, or as a thickening agent. However, glycerol also significantly contributes to the efficacy of cough syrups due to its special properties of lubrication, demulcent effects, sweetness, and humectant activity [8]. Since the glycerol used for the extraction may easily be incorporated into the final product, glycerolic extraction of medicinal plants is very desirable from an energy saving point of view [4]. Interestingly, in spite of the numerous advantages of glycerol as an extraction solvent, it is relatively underutilized in the production of extracts for pharmaceutical and cosmeceutical purposes. Some newer literature examples of glycerol use for extraction of natural products include the extraction of saponins and polyphenols from licorice [9], alkaloids from barberry bark [10], as well as polyphenols from bran rice [11] and walnut [12].

Echinacea purpurea (L.) Moench (Asteraceae) (purple coneflower) is a perennial medicinal herb with important immunostimulatory and anti-inflammatory properties. According to the European Medicines Agency, *E. purpurea* and preparations thereof may be used in herbal medicinal products for prevention and treatment of the common cold, as well as for alleviation of skin disorders and minor wounds [13]. In addition, numerous scientific studies have demonstrated antioxidant, antimicrobial, antianxiety, antidepression, cytotoxicity, and antimutagenicity activities of *E. purpurea* [14,15]. Some of those activities may be related to the anti-inflammatory activity of *Echinacea* extracts, which is based on cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and 5-lipoxygenase inhibition (LOX) [16]. *E. purpurea* aerial parts contain diverse bioactive phytochemicals, including essential oils, polysaccharides, nitrogen compounds (such as alkylamides and small amounts of alkaloids), and numerous bioactive phenolics. Among these, phenolic acids are among the most prominent ones [17]. Due to the numerous health benefits that phenolic acids display, they are used to estimate the quality of raw herbal materials and their preparations according to the European Pharmacopoeia [18].

The most important phenolic acids in *E. purpurea* are derivatives of caffeic acid. Cichoric acid is the most abundant among them. It exhibits a wide array of activities, such as antidiabetic, antiviral, antioxidant, anti-inflammatory, neuroprotective, and obesity prevention activities [19]. In particular, various studies on different models have found that cichoric acid may ameliorate inflammation induced by lipopolysaccharides (LPSs) in both cell cultures and mice. Reduced inflammation was associated with downregulation of nuclear factor κB (NF-κB) and tumor necrosis factor α (TNF-α), two major regulators of inflammation responses. Several other proinflammatory factors, including nitric oxide synthase, COX-2, prostaglandin E2, interleukin-1β (IL-1β), IL-12, and IL-18, have also been reported to be downregulated by chicoric acid [19]. In addition, cichoric acid may augment the immune response through the modulation of the CD28/CTLA-4 and Th1 pathways [20].

Caftaric acid, another caffeic acid derivative present in *E. purpurea,* acts as an antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic agent [21]. Caftaric acid was shown to be a competitive tyrosinase inhibitor, making it suitable for inclusion in cosmetic products with skin whitening properties [22]. Other phenolic acid derivatives may also add to the beneficial effect on wound healing. For example, chlorogenic acid, a caffeic acid derivative, plays several important therapeutic roles, such as having antioxidant activity, as well as antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, antiobesity, antiviral, antimicrobial, antihypertensive, and central nervous system stimulating effects [23].

Bearing in mind the importance of glycerol in the pharmaceutical and cosmetic industry, as well as the beneficial effects exerted by phenolic acids present in *E. purpurea*, the aim of this work was to compare and optimize maceration and ultrasound-assisted extraction (UAE) of phenolic acids from *E. purpurea* aerial parts using glycerol, a non-toxic and ecofriendly solvent.

2. Results

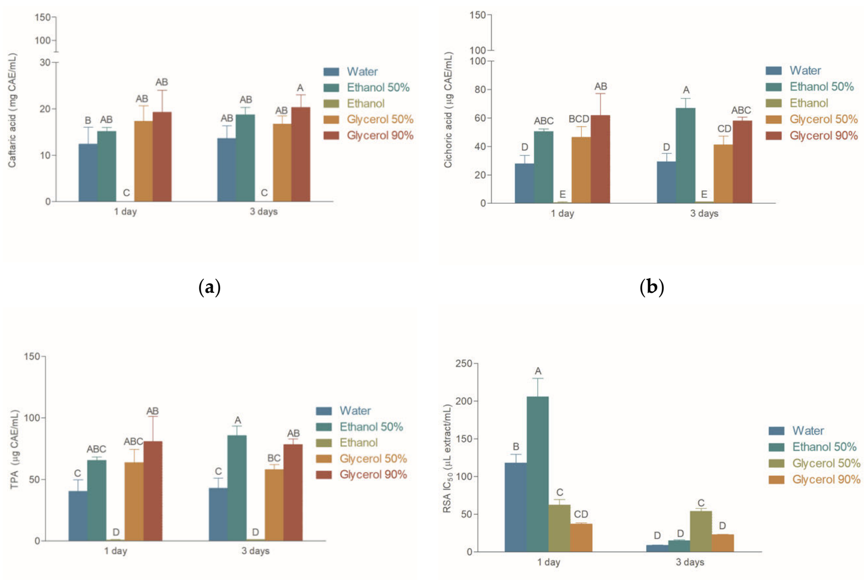
2.1. Macerations

The macerations were performed using protic solvents of different polarities and viscosities, allowing for the comparison of glycerol extraction with the more common ethanol/water extraction. The conditions used for the macerations are presented in Table 1.

Table 1. The conditions and the extracts prepared by maceration.

Extract	Solvent	Duration (Days)
W-1D	Water	1
E50-1D	Ethanol 50% (*m/m*)	1
E-1D	Ethanol	1
G50-1D	Glycerol 50% (*m/m*)	1
G90-1D	Glycerol 90% (*m/m*)	1
W-3D	Water	3
E50-3D	Ethanol 50% (*m/m*)	3
E-3D	Ethanol	3
G50-3D	Glycerol 50% (*m/m*)	3
G90-3D	Glycerol 90% (*m/m*)	3

While pure water and ethanol were suitable for the extraction, it was not possible to use pure glycerol due to its high viscosity. Therefore, 90% (*m/m*) glycerol was used instead. In order to investigate the influence of time on the composition of the extracts, macerations were performed for either 1 or 3 days. The contents of phenolic acids in the extracts are shown in Figure 1.



(**a**) (**b**)

(**c**) (**d**)

Figure 1. Contents of phenolic acids: (**a**) caftaric acid; (**b**) cichoric acid; (**c**) total phenolic acids (TPA); (**d**) radical scavenging activity (RSA) of the extracts prepared by maceration.

While the extracts were rich in cichoric and caftaric acids, the amount of chlorogenic acid in the extract was below limit of detection (LOD). Thus, it was omitted from Figure 1. Generally, cichoric acid was the most abundant phenolic acid in the extracts. Its concentration ranged between 2.1 (in W-3D)- and 3.3 (in E50-1D)-fold higher than the amount of caftaric acid in the corresponding extracts. Thus, the cichoric acid content was the main contributing factor to the total phenolic acid content (TPA), calculated as the sum of caftaric, chlorogenic, and cichoric acid contents. The concentrations of caftaric and cichoric acids correlated significantly (r^2 = 0.9346), implying that similar parameters affected their concentrations in the extracts.

The amounts of caftaric acids in the extracts varied greatly depending on the solvent used for the extraction. For example, E-1D and E-3D did not contain any detectable amounts of caftaric acid, while G90-1D and G90-3D contained about 20 µg/mL of phenolic acid. Similarly, in E-1D and E-3D, cichoric acid was present in very low amounts, while its content in G90-1D and E50-3D reached over 60 µg/mL. While the maceration duration did not significantly affect the contents of the target compounds, the influence of the solvent on the extraction efficiency was observable but rather low. The extraction efficiency levels for 50% ethanol, 50% glycerol, and 90% glycerol did not statistically differ from one another.

2.2. Radical Scavenging Activity

The radical scavenging activity (RSA) results for the extracts prepared by maceration are presented in Figure 1d. The extract prepared using ethanol did not display any observable RSA, and thus is not presented in the figure. Unlike the phenolic acid content, the radical scavenging activity of the extracts was influenced by the solvent, and in some cases the duration of the maceration. After one day of maceration, water and 50% ethanol yielded the extracts with the highest IC_{50} values (and thus the lowest RSA), while the activity levels of 50% and 90% glycerol extracts were more pronounced. However, the activity levels of the 50% ethanol and water extracts prepared by 3-day maceration were significantly better than their 1 day counterparts. On the other hand, the activity levels of 50% and 90% glycerol extracts did not significantly change with prolonged maceration. The 3 day water extract was the best radical scavenger in the study, with an RSA IC_{50} value of 8.79 µg/mL ± 0.44 µg/mL, followed by the 50% ethanol (RSA IC_{50} = 15.00 µg/mL ± 0.96 µg/mL) and 90% glycerol (RSA IC_{50} = 22.88 µg/mL ± 0.41 µg/mL) extract. The RSA activity levels of these three extracts did not statistically differ (Dunnett's test) from the activity of positive control, butylated hydroxyanisole (BHA), with an RSA IC_{50} value of 6.12 µg/mL ± 0.17 µg/mL. In the present study, the RSA and the contents of phenolic acids were not correlated (r^2 < 0.1).

2.3. Effects of UAE Variables on Phenolic Acid Extraction Yield

In this work, the efforts were undertaken to optimize the UAE of phenolic acids from *E. purpurea*. For this purpose, a two-level factorial design was used. The effects of the glycerol concentration (A), temperature (B), ultrasound power (C), time (D), ascorbic acid concentration (E), and the amount of solvent (F) were investigated. The independent variables and their levels are presented in Table 2.

Table 2. The independent variables and their levels for the two-level factorial design.

Factor Code	Factor	Units	Minimum (−1)	Maximum (+1)
A	Glycerol concentration	% (*w/w*)	10	90
B	Temperature	°C	20	70
C	Ultrasound power	W	72	720
D	Time	min	10	40
E	Ascorbic acid concentration	mg/g	0	2
F	Amount of solvent	g	10	30

The effects of the independent variables on the amount of target substances are presented in Table 3. The results clearly show that the extraction variables have a great impact on the success of the extraction. Depending on the extraction parameters, the concentrations of caftaric and cichoric acid changed from 6.64 to 50.26 μg/mL and from 7.49 to 155.31 μg/mL, which are approximately seven- and twenty-fold increases, respectively. The levels of independent variables needed for the maximum extraction of caffeic acid derivatives were as follows: glycerol 90% (*m/m*), temperature 70 °C, ultrasound power 72 W, time 40 min, and ascorbic acid 0 mg/mL.

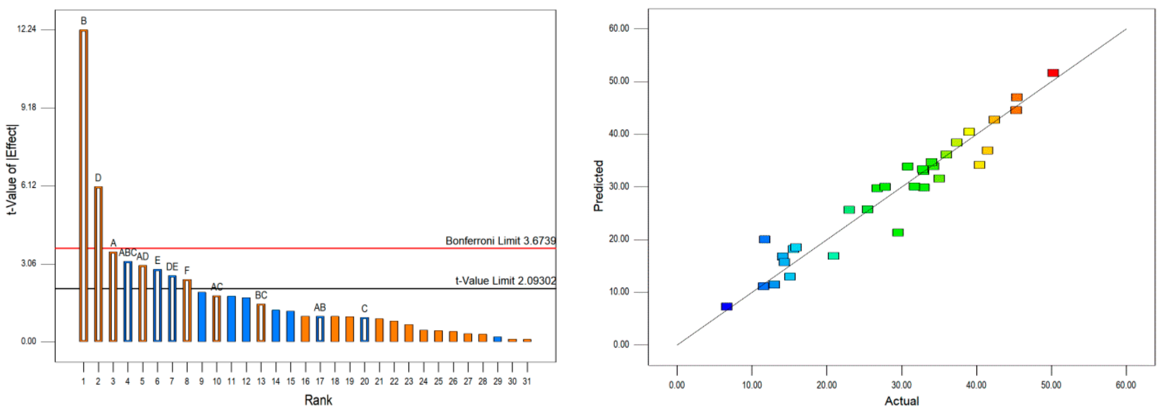
Table 3. Independent variables, their levels for the two-level factorial design, and the responses obtained.

Std	Run	A (%, *w/w*)	B (°C)	C (W)	D (min)	E (mg/g)	F (g)	CFTA (CAE μg/mL)	CLA (μg/mL)	CCA (CAE μg/mL)	TPA (CAE μg/mL)
26	1	90	20	72	40	2	30	23.01	<LD	55.32	78.33
17	2	10	20	72	10	2	30	29.54	0.63	55.62	85.79
14	3	90	20	720	40	0	30	41.47	0.56	136.71	178.74
24	4	90	70	720	10	2	10	31.71	0.35	86.96	119.02
31	5	10	70	720	40	2	10	32.83	0.52	77.46	110.81
19	6	10	70	72	10	2	10	27.82	0.29	53.77	81.88
25	7	10	20	72	40	2	10	15.90	<LD	26.59	42.49
5	8	10	20	720	10	0	30	11.55	<LD	19.98	31.53
3	9	10	70	72	10	0	30	30.83	<LD	84.48	115.31
8	10	90	70	720	10	0	30	34.29	0.33	99.25	133.87
22	11	90	20	720	10	2	30	11.71	<LD	32.86	44.57
21	12	10	20	720	10	2	10	6.64	<LD	7.49	14.13
32	13	90	70	720	40	2	30	42.38	0.49	114.59	157.46
6	14	90	20	720	10	0	10	20.90	<LD	63.53	84.43
18	15	90	20	72	10	2	10	15.08	<LD	41.48	56.56
30	16	90	20	720	40	2	10	25.45	0.43	71.45	97.33
1	17	10	20	72	10	0	10	15.57	<LD	31.40	46.97
9	18	10	20	72	40	0	30	26.73	0.37	50.66	77.76
23	19	10	70	720	10	2	30	35.97	0.6	90.77	127.34
29	20	10	20	720	40	2	30	13.01	<LD	20.4	33.41
16	21	90	70	720	40	0	10	45.38	0.94	145.02	191.34
7	22	10	70	720	10	0	10	32.96	0.48	98.06	131.5
27	23	10	70	72	40	2	30	40.41	0.56	88.48	129.45
20	24	90	70	72	10	2	30	34.01	0.57	89.1	123.68
2	25	90	20	72	10	0	30	14.14	<LD	44.3	58.44
15	26	10	70	720	40	0	30	45.31	0.61	132.99	178.91
11	27	10	70	72	40	0	10	37.39	0.58	103.09	141.06
28	28	90	70	72	40	2	10	39.05	0.74	109.48	149.27
4	29	90	70	72	10	0	10	35.04	0.56	107.71	143.31
12	30	90	70	72	40	0	30	50.26	0.63	155.31	206.2
10	31	90	20	72	40	0	10	32.99	0.59	101.58	135.16
13	32	10	20	720	40	0	10	14.34	<LD	30.98	45.32

Independent variables: A = glycerol concentration; B = temperature; C = ultrasound power; D = time; E = ascorbic acid concentration; F = amount of solvent. Abbreviations: <LOD = below level of detection; CAE = chlorogenic acid equivalents; CCA = cichoric acid; CFTA = caftaric acid; CLA = chlorogenic acid; TPA = total phenolic acids.

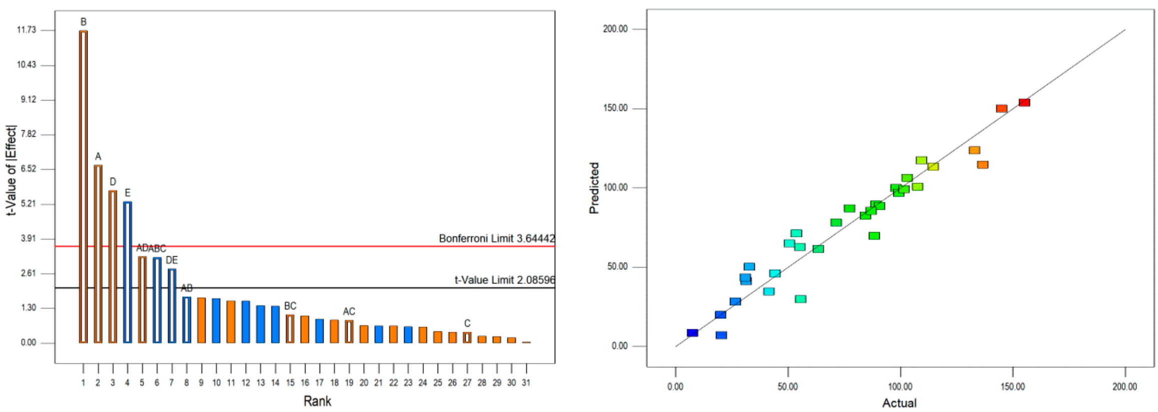
Cichoric acid accounted for about two-thirds of the total phenolic acids (TPA) present in the extracts. Similar to maceration findings, the concentrations of caftaric and cichoric acids correlated significantly (r^2 = 0.9169). In addition, their concentrations showed a week but significant correlation with chlorogenic acid (r^2 = approximately 0.7), indicating that similar factors affected their concentrations. The concentrations of chlorogenic acid ranged from below LOD to 0.94 μg/mL, and contributed to the TPA at less than 1%.

In order to characterize the significance of independent variables and to select the most significant variables based on their output responses, a Pareto chart was used. The Pareto chart depends on the standard deviation to estimate the sampling errors of variables. Two important limits in the Pareto chart are the Bonferroni limit and the *t*-value limit. Variables with coefficients above the Bonferroni limit are significant model factors. On the other hand, the terms that fall between the Bonferroni limit and the *t*-value limit are considered likely to be significant, while the coefficients below the *t*-value limit are insignificant [24]. The blue color on the charts indicates a negative and the orange color refers to a positive effect of independent variables. The ANOVA analysis confirmed that the selected models were highly significant ($P < 0.0001$), with high r^2 values (>0.92), as well as confirming that only the statistically significant effects and the terms supporting the hierarchy were included in the model (details are presented in the Supplementary Materials). The Pareto charts, along with the actual vs. predicted charts for the selected responses (caftaric acid, chlorogenic acid, and TPA), are presented in Figures 2–4. Due to the very low amounts of chlorogenic acid in the extracts, its chart was omitted from the analysis.



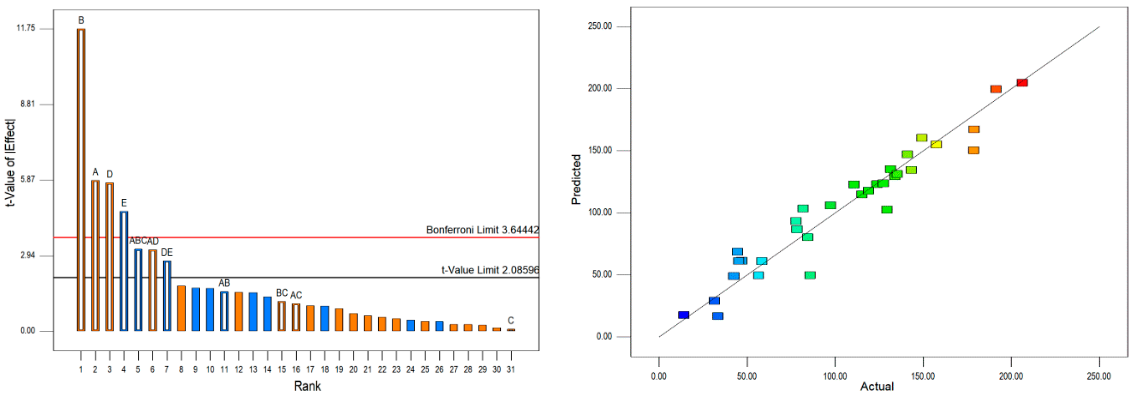
(**a**) (**b**)

Figure 2. Caftaric acid content model: (**a**) Pareto chart; (**b**) actual vs. predicted results. Independent variables: A = glycerol concentration; B = temperature; C = ultrasound power; D = time; E = ascorbic acid concentration; F = amount of solvent. Blue color on the chart (**a**) indicates a negative and the orange color refers to a positive effect of independent variables. The color points on the chart (**b**) represent the value of caftaric acid (blue: lowest value; red: highest value).



(**a**) (**b**)

Figure 3. Cichoric acid content model: (**a**) Pareto chart; (**b**) actual vs. predicted results. Independent variables: A = glycerol concentration; B = temperature; C = ultrasound power; D = time; E = ascorbic acid concentration. Blue color on the chart (**a**) indicates a negative and the orange color refers to a positive effect of independent variables. The color points on the chart (**b**) represent the value of cichoric acid (blue: lowest value; red: highest value).



(**a**) (**b**)

Figure 4. Total phenolic acid content model: (**a**) Pareto chart; (**b**) actual vs. predicted results. Independent variables: A = glycerol concentration; B = temperature; C = ultrasound power; D = time; E = ascorbic acid concentration. Blue color on the chart (**a**) indicates a negative and the orange color refers to a positive effect of independent variables. The color points on the chart (**b**) represent the value of total phenolic acid (blue: lowest value; red: highest value).

The Pareto chart of the effects of the extraction conditions on caftaric acid content (Figure 2a) shows that the factors B and D are above the Bonferroni limit (*t*-value of effect = 3.6739), and thus are significant model factors. Both of them exert positive influence on the caftaric acid content. On the other hand, not all the variables above the *t*-value limit (*t*-value of effect = 2.093) influence the content of caftaric acid positively. While its content increased together with A, F, and AD, the increase of variables E, DE, and ABC lead to the decrease of caftaric acid content. The actual vs. predicted result graph (Figure 2b) shows a good agreement between the actual values and the values predicted by the model.

The Pareto charts illustrating the effects of the extraction conditions on the contents of cichoric acid (Figure 3a) and TPA (Figure 4a) were rather similar due to the cichoric acid being the most abundant phenolic acid and contributing largely to TPA. The Bonferroni limit and the *t*-value limit were also rather similar, with the *t*-values of the effects being approximately 3.6 and 2.09, respectively. Variables with coefficients above the Bonferroni limit, such as A, B, D, and E, were significant model factors. Similarly, the terms AD, DE, and ABC, which fell between the Bonferroni limit and the *t*-value limit, were considered likely to be significant factors. The color codes indicate that A, B, D, and AD positively affected extraction efficiency, while E, DE, and ABC influenced the extraction negatively. The predicted and measured values were in good agreement (Figures 3b and 4b).

2.4. Metal Content in E. Purpurea Aerial Parts

The contents of selected transition and second group metals were determined (Table 4). It was found that the plant material contains several elements, the presence of which may influence the anti- or pro-oxidant behavior of ascorbic acid during extraction. On the other hand, the zinc present in the sample may beneficially affect the skin- and immunity-related properties of *E. purpurea* preparations.

Table 4. Contents of selected metals in *E. purpurea* aerial parts.

Element	C (mg/kg)
Mn	71.32 ± 6.65
Fe	255.48 ± 11.75
Cu	8.07 ± 4.70
Zn	37.74 ± 0.32

The plant material was especially rich in iron (255.48 mg/kg) and manganese (71.32 mg/kg). Copper, on the other hand, was present in significantly lower quantities.

3. Discussion

3.1. Phenolic Acid Contents in the Extracts Obtained by Maceration

Medicinal plants and plant extracts contain a myriad of secondary metabolites. While some of them have desirable pharmacological properties, the others may influence the overall activity of the natural extracts in either a positive or negative manner. Thus, medicinal plant extraction procedures aim to increase the amounts of desired metabolites while simultaneously decreasing the amounts of undesired or harmful ones. The amounts of secondary metabolites in the extracts depend on their physicochemical properties, extraction solvents, types of extraction, as well as on numerous extraction parameters related to the specific type of the extraction [5]. Finding the extraction procedures that yield the maximum amount of the target compound(s) with the minimum amount of the undesired ones may be a tedious, costly, and time consuming procedure.

In this work, efforts were undertaken to efficiently optimize the green extraction of bioactive phenolic acids from aerial parts of *E. purpurea* and to obtain the extracts ready to use in pharmaceutical and cosmetic products. In order to achieve this, classical maceration performed using glycerol or water mixtures was compared with maceration using ethanol, water, and mixtures thereof. Furthermore, the UAE of phenolic acids from *E. purpurea* using glycerol–water mixtures as the extraction solvent was developed.

Maceration is the oldest of the solid–liquid extraction methods and is characterized by the simplicity and low cost of the procedure, as well as by the long duration needed for the achievement of an equilibrium concentration of the extracted metabolite in the solvent [25]. The use of glycerol for maceration of phenolic secondary metabolites is relatively rare [11,26]. Bergeron et al. used glycerol for maceration of *E. purpurea* [27], but detailed and focused reports on the influence of glycerolic extraction conditions and comparisons with ethanol are still lacking.

In this work, the amount of phenolic acids extracted by using different solvents varied slightly. Although the previous research indicated that the *E. purpurea* phenolics are poorly extracted with ethanol [17], it was still interesting to note that the ethanol extracts did not contain any detectable amounts of caftaric acid, while the cichoric acid was present in very low amounts. In addition, chlorogenic acid was absent in the extracts prepared by maceration, even though Bergeron et al. [27] noted that unlike in glycerol extract, it should be present in ethanol extracts of *E. purpurea.* Previous reports indicated that 50% ethanol was the most efficient solvent for extraction of phenolic acids from potato peel (*Solanum tuberosum*) [26]. However, its extraction efficiency in this work did not statistically differ from the efficiency of the investigated glycerol–water mixtures. Bearing in mind the importance of glycerol in the cosmetic and pharmaceutical industry, as well as the aforementioned advantages of glycerol from ecological and biological points of view, this is an important finding with numerous practical implications.

3.2. Radical Scavenging Activity

Antioxidants in the pharmaceutical and cosmetic industry may be regarded as prophylactic and therapeutic agents. They prevent the damage caused by free radicals and other reactive oxygen species, thus hindering the pathogenesis of various disorders such as aging, cancer, diabetes, as well as cardiovascular, autoimmune, and neurodegenerative disorders [28]. Furthermore, antioxidants protect pharmaceutical and cosmetic products against the oxidation that occurs during their storage and use. Such influences include UV radiation [29], as well as free radicals- or metal-ion-induced peroxidation of polyunsaturated fatty acids, in which natural cosmetics and pharmaceuticals are especially rich [30]. In this work, the RSA of the extracts prepared by maceration was determined. The RSA of the extracts prepared by UAE was not determined, because those extracts contained ascorbic acid, a strong antioxidant, the activity of which would clearly outperform the activity of phytochemicals from *E. purpurea* and would indicate a falsely strong RSA. All the extracts prepared by maceration showed notable RSA. In general, 3 day maceration yielded the extracts with stronger RSA levels, with most 3 day extracts showing equal radical scavenging activity to BHA. The correlation between the phenolic content and the radical scavenging activity levels of the extracts was not significant. Similar observations were also reported by other authors [31]. This is not surprising, because caffeic acid derivatives are not the only substances with radical scavenging abilities in *E. purpurea*. Various other phytochemicals, which were not determined within scope of this work but are present in *E. purpurea*, such as phylloxanthobilins [32] and polysaccharides [33], may act as strong antioxidants and free radical scavengers.

3.3. Effect of UAE Variables on Phenolic Acid Extraction Yield

UAE is often used for extraction in solid–liquid systems because it is a simple and cost- and time-effective method, characterized by low CO_2 emissions and solvent consumption [34]. It is especially suitable for preparation of natural extracts due to its high reproducibility and short time of extraction. The cavitation, vibration, crushing, and mixing effects in media produced by ultrasound can break the cell wall and effectively increase the mass transfer process [35,36]. An efficient UAE process should maximize the recovery of target compounds with minimal degradation, resulting in an extract with high biological activity. Ideally, this should be accomplished using "green" environmentally friendly technologies and low-cost raw materials and solvents [10]. However, in order to determine the best UAE conditions for extraction of bioactive constituents, it is often necessary to perform multiple experiments and evaluate not only the direct influence of extraction variables, but their interactions as well. In order to achieve this, a two-level factorial design with six independent variables was employed.

The selection of the solvent greatly influences the UAE extraction efficiency due to the solvent's physical–chemical properties, such as the polarity, viscosity, and volatility. Therefore, the proportion of glycerol in water was used as the first independent variable. In accordance with their moderately polar nature, *E. purpurea* phenolic acids were best extracted using relatively high glycerol concentrations. Several studies reported that water–glycerol mixtures were more efficient extraction media than water. Examples include the UAE of chlorogenic acid and other caffeic acid derivatives from spent filter coffee [37] and polyphenols from red grape pomace [38].

In addition to the solvent, the temperature and ultrasound power may strongly affect the efficiency of UAE. High temperature and ultrasound power levels may improve the extraction process by reducing the viscosity of the solvent and by increasing the kinetic energy of the molecules in the solution. However, they may also lead to degradation of sensitive phytochemicals, including phenolic compounds [10]. Similarly, long extraction times may increase the amount of the extracted target compounds. However, long extraction times can also increase the chances of degradation of sensitive molecules. In this work, high temperature positively affected extraction. This is possibly related to the reduction of the glycerol viscosity and increase of the kinetic energy of the solvent molecules. Such an effect was observed in previous UAE glycerolic extractions of phenolics from *G. glabra* [9]. The extraction using the highest glycerol concentration seemed to be a relatively slow process. This was evidenced by the observation that the duration of the extraction exerted a positive influence on the extraction efficiency, as well as by the positive influence of the interaction of glycerol content and time. Other researchers also reported similar findings. For example, previous kinetic studies of eggplant peel extractions suggested that diffusion of phenolics in water–glycerol mixtures was slower compared with that attained with water–ethanol, but both systems had the ability to recover essentially the same levels of total polyphenols [39]. Similar results were presented in a study of the glycerolic UAE of caffeic acid derivatives from spent filter coffee [37]. It was noted in this work that the interaction of the glycerol concentration, temperature, and higher ultrasonication power exerted a negative effect on the phenolic acid extraction. This effect may be due to the generation of hydroxyl radicals, whose production is initiated by ultrasonication, especially at high temperatures [40], and their subsequent reaction with caffeic acid derivatives [41].

The influence of the amount of solvent used for the solid–liquid extraction of a fixed amount of herbal material was also assessed. In this study, the employed amounts of solvent did not significantly affect the extraction efficiency of cichoric acid or TPA. This indicates that the concentrations of the target phenolic acids did not significantly change, even when a larger volume of solvent was employed. This finding has numerous positive ecological and economical implications, because a larger amount of product may be obtained from a fixed amount of herbal material without compromising the quality of the extract. In this way, the expensive herbal material may be more efficiently utilized.

Finally, in order to impede the oxidative degradation of phenolic acids that may occur during the extraction, water-soluble antioxidant ascorbic acid was added to the reaction mixture and its influence on the phenolic acid concentration was investigated as the final independent variable. The intention of the ascorbic acid addition was to improve the extraction of phenolic acids by impeding oxidation processes that may occur during the extraction. Previous researchers have found that the addition of antioxidants to the previously prepared *E. purpurea* glycerol extracts may improve the stability of the phenolic acids present therein [27]. It is well known that ascorbic acid hinders the oxidation of chlorogenic acid [42]. Thus, it was expected that the addition of ascorbic acid to the glycerol–water extraction mixtures would have the same effect on other phenolic substances. Surprisingly, the presence of ascorbic acid in the reaction mixture had a negative influence on all of the phenolic acids analyzed in this work. The process seemed to be time-dependent, as evidenced by the negative influences of time and ascorbic acid interaction. A possible explanation is that ascorbic acid either reacted with the analyzed phenolic acids or enabled their reaction with other natural substances present in the extracts [43]. The presence of transition metal ions (e.g., ferro ions) in the solution may also be of importance, as discussed below.

In accordance with previous research [44], the results of this study showed that under the optimized conditions, UAE extraction was superior to classical maceration, because it achieved significantly higher yields of the desired phenolic acids within a much shorter extraction time than maceration. It was found that caffeic acid derivatives were best extracted using a high glycerol concentration without added ascorbic acid, a high temperature, and a low ultrasound power using a longer extraction time. This results correspond well with the described influences of independent variables. Application of those conditions led to an approximately 1.7-fold increase in caftaric acid concentration and up to a 2.6-fold increase in both the cichoric acid content and TPA in comparison with the best results achieved using the maceration protocol. Moreover, chlorogenic acid, which was absent from the extracts prepared by maceration, was present in the extracts prepared by UAE, albeit in rather low concentrations. The selected UAE variables affected the contents of targeted phenolic acids in a similar manner, which was rather expected due to their significant structural similarities. The results of this study may be used for direct preparation of the glycerol extracts suitable for use in the cosmetic and pharmaceutical industry, or for detailed investigation and optimization of the extraction using one of the designs suitable for response surface methodology, such as a Box–Behnken or central composite design.

3.4. Metal Contents in E. Purpurea Aerial Parts

In order to explain the observed degradation of caffeic acid derivatives in the presence of ascorbic acid, the contents of selected transition metal were assessed. Several elements, the presence of which may influence the anti- or pro-oxidant behavior of ascorbic acid during extraction, such as iron, copper, zinc, and manganese, were determined in *E. purpurea* aerial parts. It is known that ultrasonication may initiate the production of hydroxyl radicals, especially at high temperatures [40]. In addition, caffeic acid and its derivatives may react with hydroxyl radicals, forming an array of degradation products [41]. However, this effect was not pronounced enough to reduce the contents of phenolic acids in the extracts prepared without ascorbic acid. In addition to antioxidant activity, ascorbic acid in the presence of catalytic metal ions can also exert pro-oxidant effects. For example, in the Fenton reaction, ascorbic acid may enhance hydroxyl radical generation. Fe^{2+} reacts with H_2O_2 to generate Fe^{3+} and the hydroxyl radical. The presence of ascorbate can lead to recycling of Fe^{3+} back to Fe^{2+}, which in turn will catalyze the formation of highly reactive oxidants from H_2O_2 [45]. Furthermore, Mn^{2+} and Zn^{2+} catalyze the reaction of ascorbic acid with oxygen by increasing the rate of radical formation, while copper promotes the oxidation and formation of free radicals of ascorbic acid, even without the presence of oxidizing agents [46]. All of these processes may generate hydroxyl and other radicals, and consequently may cause degradation of caffeic acid and its derivatives [41].

Besides possible negative effects on the UAE of phenolic acids in the presence of ascorbic acid, some metals may also display beneficial bioactive properties. Since according to European legislation, *E. purpurea* may be used in traditional medicines for prevention and treatment of the common cold and alleviation of skin disorders and minor wounds [13], the content of zinc, the metal that may support skin- and immunity-related properties of *E. purpurea*, was also determined. The micronutrient zinc is important for maintenance and development of immune cells of both the innate and adaptive immune system [47]. Furthermore, zinc deficiency has detrimental effects on wound healing [48]. While the content of zinc in the plant material was not sufficient to grant the recommended dietary allowances [49], it may likely contribute to wound healing when applied locally, as it has been found that the amount of zinc in the wound increases during the healing process. This may induce the keratinocyte proliferation, as it has been shown that keratinocyte proliferation and differentiation are controlled by zinc [50].

4. Materials and Methods

4.1. Chemicals

Reagents and standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of the standards was as follows: BHA (≥98.5%), chlorogenic acid (European Pharmacopoeia Reference Standard), and gallium (99.99%). Acetonitrile was HPLC grade. Other reagents and chemicals were of analytical grade.

4.2. Plant Material

Plant material was supplied by the Suban company (Samobor, Croatia). The identity was confirmed by the authors using the EU pharmacopoeial monograph for *E. purpurea* [18]. A voucher specimen was deposited in the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia (FG-2018-EPS).

4.3. Maceration

Powdered plant material (0.1 g) was passed through a sieve of 850 µm mesh size and suspended in 30 g of appropriate solvent, namely water, ethanol, glycerol, or their mixtures, then macerated for either 1 or 3 days in the dark (details in Table 1). Upon extraction, the mixtures were filtered and stored in the dark at −20 °C until further analysis. For each set of conditions, three independent extracts were prepared and analyzed.

4.4. Radical Scavenging Activity

Radical scavenging activity (RSA) was evaluated using the sTable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical [10]. In short, DPPH solution (0.21 mg/mL, 70 µL) was added to the extract solution (130 µL). After 30 min, the absorbance was recorded at 545 nm (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). DPPH solution with methanol instead of the extract served as the negative control. RSA was calculated according to the following equation:

$$\text{RSA } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. The concentration of the extract, which scavenged 50% of the free radicals present in the solution (RSA IC_{50}), was calculated. BHA was used as the standard radical scavenger. The results were expressed as µL of extract in mL of reaction solution (µL extract/mL).

4.5. Preparation of the Extracts According to Two-Level Factorial Design

A preliminary extraction was carried out using a two-level factorial design with the following independent variables and their ranges: glycerol concentration (10%–90%, *w/w*), temperature (20–70 °C), ultrasound power (72–720 W), time (10–40 min), ascorbic acid concentration (0–2 mg/g), and amount of solvent (10–30 g). The numbers in brackets represent the low (−1) and high (+1) limits of the corresponding variables. Detailed conditions are presented in Table 3. Powdered plant material (0.1 g) was suspended in the appropriate amount and concentration of glycerol–water mixtures with or without addition of ascorbic acid in a 50 mL Erlenmeyer flask. The extraction was performed in an ultrasonic bath (Bandelin SONOREX® Digital 10 P DK 156 BP, Berlin, Germany) using the frequency of 35 Hz at various temperatures, ultrasonication strengths, and time intervals. Upon extraction, the mixtures were filtered and stored in the dark at −20 °C until further analysis.

4.6. RP-HPLC-DAD Determinations of Phenolic Acids

For determination of phenolic acids, the modified European Pharmacopoeia method [18] was used. Prior to the analysis, the extracts and the standard used for the construction of the calibration curve (chlorogenic acid, 0.025 mg/mL in 70% ethanol) were filtered through a 0.45 µm PTFE syringe filter. Quantifications were performed using an HPLC instrument (Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler and a DAD detector. Separation was performed on a Zorbax Eclipse XDB-C18 column (5 µm, 12.5 mm × 4.6 mm, Agilent, Santa Clara, CA, USA). A mixture of phosphoric acid and water (1:999 *V*/*V*) was used as mobile phase A, while acetonitrile was used as mobile phase B. Separation was performed at 35 °C using a flow rate of 1.5 mL/min according to the following protocol: 0–13 min (90%–78% A), 13–14 min (78%–60% A), and 14–20 min (60%–40% A). Quantification was carried out at 330 nm. The calibration curve of chlorogenic acid with the corresponding coefficient of determination (r^2) was y = 1834.03x + 7.12 (r^2 = 0.99964), where y is the absorbance at 330 nm and x is the weight of the analyte (µg). The limit of detection (LOD) and limit of quantification (LOQ), determined according to [51], were 0.0314 µ and 0.095 µg, respectively. Retention times (t_R) of the analytes were 6.40 ± 0.01, 7.03 ± 0.02, and 16.27 ± 0.01 min for caftaric, chlorogenic, and cichoric acids, respectively. The contents of caftaric and cichoric acids were calculated as chlorogenic acid equivalents (CAE). Total phenolic acids (TPA) were calculated as the sum of caftaric, chlorogenic, and cichoric acid contents. An example of a chromatogram is presented in Figure 5.

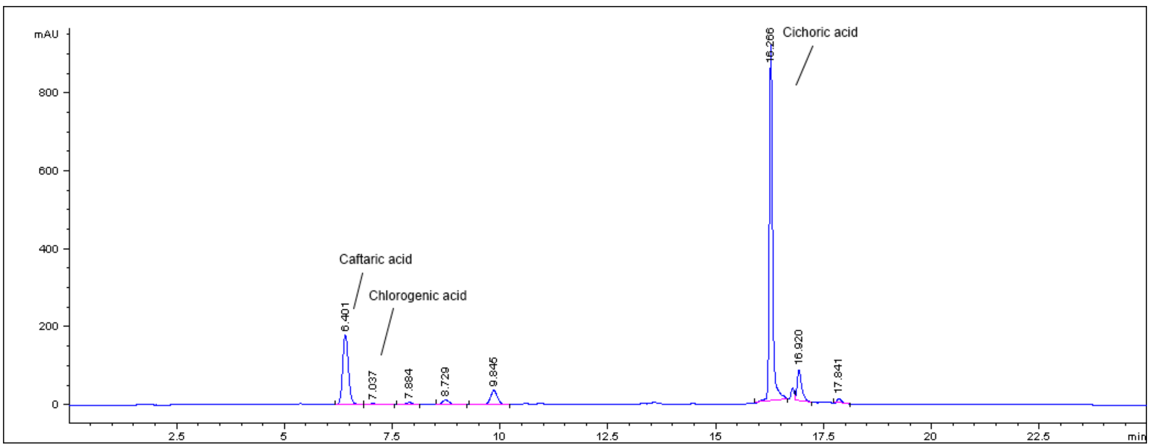


Figure 5. An example of a chromatogram (run 21) recorded at 330 nm.

4.7. TXRF Determination of Metals in the Plant Material

TXRF analysis was performed using a commercial benchtop S2 PICOFOX TXRF spectrometer (BrukerNano, GmbH, Berlin, Germany) equipped with a low-power tungsten X-ray tube (50 kV, 1 mA) and a silicon drift detector (SDD) with a resolution < 150 eV at Mn-K_(α). The evaluation of the TXRF spectra and calculation of the analyte net peak areas were performed using Spectra Plus 5.3 software (Bruker AXS Microanalysis GmbH, Berlin, Germany) linked to the equipment. The measurement time was established as 2000 s. The vegetation samples were sieved through a sieve (diameter less of 63 μm). Sample suspensions were prepared by weighing 20 mg of sample and adding 1 mL of de-ionized water containing 10 μg of Ga as an internal standard. Duplicates were prepared for each sample and 5 min sonication in an ultrasonic bath was applied. After this, an aliquot of 10 μL of the internal standardized sample was transferred onto a quartz glass sample carrier and dried using an infrared lamp, as described in [52].

4.8. Statistical Analysis

The extraction experiments were planned using Design Expert software v. 8.0.6 (Stat-Ease, Minneapolis, MN, USA). The validity of the model was confirmed by the analysis of variance (ANOVA). For macerations, measurements were performed in triplicate and the results were presented as the mean ± standard deviation. Statistical comparisons were made using one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons between extracts (JMP, SAS, San Diego, CA, USA) and Dunnett's test for comparison with the control. P values < 0.05 were considered statistically significant.

5. Conclusions

In this work, the extraction of bioactive phenolic acids from *E. purpurea* was performed using mixtures of water with glycerol, a biodegradable, safe, affordable solvent available from renewable sources. The extracts prepared by maceration were rich in phenolic acids and potent radical scavengers. The 3 day maceration with either water, 50% ethanol, or 90% glycerol afforded extracts with activity equal to the activity of synthetic antioxidant, BHA. The UAE method, on the other hand, showed superior extraction characteristics, yielding up to 2.6-fold higher phenolic acid contents within shorter extraction times. The composition of the solvent, the time, and the temperature of the extraction significantly affected the efficiency of the extraction. Furthermore, the presence of ascorbic acid in the extraction medium lead to decreased phenolic acid contents in the prepared extracts. In addition, the presence of zinc in the plant material may contribute to the beneficial effects of *E. purpurea* preparations. Since glycerol is a non-toxic solvent with humectant properties, the prepared extracts can be directly used for preparation of cosmetics or oral pharmaceutical formulations without the need for solvent removal.

Supplementary Materials: The following are available online. Tables S1–S3: Analysis of variance (ANOVA) for the caftaric, cichoric, and total phenolic acid content models, as well as post-ANOVA and prediction equations using Design Expert software.

Author Contributions: Conceptualization, M.Z.K.; methodology, P.M., P.C., M.J., E.M., J.J. and M.Z.K.; investigation, P.M., P.C, M.J., E.M., J.J.; writing—original draft preparation, M.Z.K. and J.J.; writing—review and editing, P.C. and M.Z.K.; supervision, M.Z.K.; project administration, M.Z.K.; funding acquisition, M.Z.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Croatian Science Foundation, as part of the project Bioactive Plant Principle Extraction using Green Solvents—A Step towards Green Cosmeceuticals, grant number IP-2018-01-6504.

Acknowledgments: This work was financed in course of the project "Bioactive Plant Principle Extraction using Green Solvents—A Step Towards Green Cosmeceuticals" (IP-2018-01-6504), supported by the Croatian Science Foundation and in collaboration with Suban company.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Chinou, I. Monographs, list entries, public statements. *J. Ethnopharmacol.* **2014**, *158 Pt. B*, 458–462. [CrossRef]
2. Jiang, T.A. Health Benefits of Culinary Herbs and Spices. *J. AOAC Int.* **2019**, *102*, 395–411. [CrossRef]
3. Costa, R.; Santos, L. Delivery systems for cosmetics-From manufacturing to the skin of natural antioxidants. *Powder Technol.* **2017**, *322*, 402–416. [CrossRef]
4. Chemat, F.; Vian, M.A.; Cravotto, G. Green Extraction of Natural Products: Concept and Principles. *Int. J. Mol. Sci.* **2012**, *13*, 8615–8627. [CrossRef]
5. Chemat, F.; Abert Vian, M.; Ravi, H.K.; Khadhraoui, B.; Hilali, S.; Perino, S.; Fabiano Tixier, A.-S. Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects. *Molecules* **2019**, *24*, 3007. [CrossRef] [PubMed]
6. Wolfson, A.; Dlugy, C.; Shotland, Y. Glycerol as a green solvent for high product yields and selectivities. *Environ. Chem. Lett.* **2007**, *5*, 67–71. [CrossRef]
7. Becker, L.C.; Bergfeld, W.F.; Belsito, D.V.; Hill, R.A.; Klaassen, C.D.; Liebler, D.C.; Marks, J.G.; Shank, R.C.; Slaga, T.J.; Snyder, P.W.; et al. Safety Assessment of Glycerin as Used in Cosmetics. *Int. J. Toxicol.* **2019**, *38*, 6S–22S. [CrossRef] [PubMed]
8. Eccles, R.; Mallefet, P. Soothing Properties of Glycerol in Cough Syrups for Acute Cough Due to Common Cold. *Pharmacy* **2017**, *5*, 4. [CrossRef] [PubMed]
9. Ciganović, P.; Jakimiuk, K.; Tomczyk, M.; Zovko Končić, M. Glycerolic Licorice Extracts as Active Cosmeceutical Ingredients: Extraction Optimization, Chemical Characterization, and Biological Activity. *Antioxidants* **2019**, *8*, 445. [CrossRef]
10. Dulić, M.; Ciganović, P.; Vujić, L.; Zovko Končić, M. Antidiabetic and Cosmeceutical Potential of Common Barbery (*Berberis vulgaris* L.) Root Bark Extracts Obtained by Optimization of "Green" Ultrasound-Assisted Extraction. *Molecules* **2019**, *24*, 3613. [CrossRef]
11. Aalim, H.; Belwal, T.; Jiang, L.; Huang, H.; Meng, X.; Luo, Z. Extraction optimization, antidiabetic and antiglycation potentials of aqueous glycerol extract from rice (*Oryza sativa* L.) bran. *LWT* **2019**, *103*, 147–154. [CrossRef]
12. Vieira, V.; Calhelha, R.C.; Barros, L.; Coutinho, J.A.; Ferreira, I.C.; Ferreira, O. Insights on the Extraction Performance of Alkanediols and Glycerol: Using *Juglans regia* L. Leaves as a Source of Bioactive Compounds. *Molecules* **2020**, *25*, 2497. [CrossRef] [PubMed]
13. Committee on Herbal Medicinal Products Echinaceae Purpureae Herba. Available online: https://www.ema.europa.eu/en/medicines/herbal/echinaceae-purpureae-herba (accessed on 13 September 2019).
14. Aarland, R.C.; Bañuelos-Hernández, A.E.; Fragoso-Serrano, M.; Sierra-Palacios, E.D.C.; Díaz de León-Sánchez, F.; Pérez-Flores, L.J.; Rivera-Cabrera, F.; Mendoza-Espinoza, J.A. Studies on phytochemical, antioxidant, anti-inflammatory, hypoglycaemic and antiproliferative activities of Echinacea purpurea and Echinacea angustifolia extracts. *Pharm. Biol.* **2017**, *55*, 649–656. [CrossRef]
15. Manayi, A.; Vazirian, M.; Saeidnia, S. Echinacea purpurea: Pharmacology, phytochemistry and analysis methods. *Pharmacogn. Rev.* **2015**, *9*, 63–72. [CrossRef]
16. Maver, T.; Maver, U.; Kleinschek, K.S.; Smrke, D.M.; Kreft, S. A review of herbal medicines in wound healing. *Int. J. Dermatol.* **2015**, *54*, 740–751. [CrossRef]
17. Senica, M.; Mlinsek, G.; Veberic, R.; Mikulic-Petkovsek, M. Which Plant Part of Purple Coneflower (*Echinacea purpurea* (L.) Moench) Should be Used for Tea and Which for Tincture? *J. Med. Food* **2019**, *22*, 102–108. [CrossRef]
18. *European Pharmacopoeia*, 8th ed.; Council of Europe: Strasbourg, France, 2013.
19. Peng, Y.; Sun, Q.; Park, Y. The Bioactive Effects of Chicoric Acid as a Functional Food Ingredient. *J. Med. Food* **2019**, *22*, 645–652. [CrossRef]
20. Kour, K.; Bani, S. Augmentation of immune response by chicoric acid through the modulation of CD28/CTLA-4 and Th1 pathway in chronically stressed mice. *Neuropharmacology* **2011**, *60*, 852–860. [CrossRef]
21. Koriem, K.M.M. Caftaric acid: An overview on its structure, daily consumption, bioavailability and pharmacological effects. *Biointerface Res. Appl. Chem.* **2020**, *10*, 5616–5623. [CrossRef]
22. Honisch, C.; Osto, A.; Dupas de Matos, A.; Vincenzi, S.; Ruzza, P. Isolation of a tyrosinase inhibitor from unripe grapes juice: A spectrophotometric study. *Food Chem.* **2020**, *305*. [CrossRef]

23. Naveed, M.; Hejazi, V.; Abbas, M.; Kamboh, A.A.; Khan, G.J.; Shumzaid, M.; Ahmad, F.; Babazadeh, D.; FangFang, X.; Modarresi-Ghazani, F.; et al. Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomed. Pharmacother. Biomedecine Pharmacother.* **2018**, *97*, 67–74. [CrossRef]
24. Akbari, S.; Abdurahman, N.H.; Yunus, R.M.; Fayaz, F. Microwave-assisted extraction of saponin, phenolic and flavonoid compounds from Trigonella foenum-graecum seed based on two level factorial design. *J. Appl. Res. Med. Aromat. Plants* **2019**, *14*, 100212. [CrossRef]
25. Mosca, F.; Hidalgo, G.I.; Villasante, J.; Almajano, M.P. Continuous or Batch Solid-Liquid Extraction of Antioxidant Compounds from Seeds of Sterculia apetala Plant and Kinetic Release Study. *Mol. J. Synth. Chem. Nat. Prod. Chem.* **2018**, *23*, 1759. [CrossRef]
26. Paleologou, I.; Vasiliou, A.; Grigorakis, S.; Makris, D.P. Optimisation of a green ultrasound-assisted extraction process for potato peel (*Solanum tuberosum*) polyphenols using bio-solvents and response surface methodology. *Biomass Convers. Biorefin.* **2016**, *6*, 289–299. [CrossRef]
27. Bergeron, C.; Gafner, S.; Batcha, L.L.; Angerhofer, C.K. Stabilization of caffeic acid derivatives in *Echinacea purpurea* L. glycerin extract. *J. Agric. Food Chem.* **2002**, *50*, 3967–3970. [CrossRef]
28. Ratz-Łyko, A.; Arct, J. Resveratrol as an active ingredient for cosmetic and dermatological applications: A review. *J. Cosmet. Laser Ther. Off. Publ. Eur. Soc. Laser Dermatol.* **2019**, *21*, 84–90. [CrossRef]
29. Coelho, L.; Almeida, I.F.; Sousa Lobo, J.M.; Sousa, E.; Silva, J.P. Photostabilization strategies of photosensitive drugs. *Int. J. Pharm.* **2018**, *541*, 19–25. [CrossRef]
30. Mlakar, A.; Batna, A.; Dudda, A.; Spiteller, G. Iron (II) ions induced oxidation of ascorbic acid and glucose. *Free Radic. Res.* **1996**, *25*, 525–539. [CrossRef]
31. Moldovan, M.L.; Bogdan, C.; Iurian, S.; Roman, C.; Oniga, I.; Benedec, D. Phenolic content and antioxidant capacity of pomace and canes extracts of some Vitis vinifera varieties cultivated in Romania. *Farmacia* **2020**, *68*, 15–21. [CrossRef]
32. Karg, C.A.; Wang, P.; Vollmar, A.M.; Moser, S. Re-opening the stage for Echinacea research-Characterization of phylloxanthobilins as a novel anti-oxidative compound class in Echinacea purpurea. *Phytomed. Int. J. Phytother. Phytopharm.* **2019**, *60*, 152969. [CrossRef]
33. Russo, D.; Faraone, I.; Labanca, F.; Sinisgalli, C.; Bartolo, M.; Andrade, P.B.; Valentao, P.; Milella, L. Comparison of different green-extraction techniques and determination of the phytochemical profile and antioxidant activity of *Echinacea angustifolia* L. extracts. *Phytochem. Anal. PCA* **2019**, *30*, 547–555. [CrossRef]
34. Izadiyan, P.; Hemmateenejad, B. Multi-response optimization of factors affecting ultrasonic assisted extraction from Iranian basil using central composite design. *Food Chem.* **2016**, *190*, 864–870. [CrossRef]
35. Hou, M.; Hu, W.; Wang, A.; Xiu, Z.; Shi, Y.; Hao, K.; Sun, X.; Cao, D.; Lu, R.; Sun, J. Ultrasound-Assisted Extraction of Total Flavonoids from *Pteris cretica* L.: Process Optimization, HPLC Analysis, and Evaluation of Antioxidant Activity. *Antioxidants* **2019**, *8*, 425. [CrossRef]
36. Delgado-Povedano, M.M.; Luque de Castro, M.D. A review on enzyme and ultrasound: A controversial but fruitful relationship. *Anal. Chim. Acta* **2015**, *889*, 1–21. [CrossRef] [PubMed]
37. Michail, A.; Sigala, P.; Grigorakis, S.; Makris, D.P. Kinetics of Ultrasound-Assisted Polyphenol Extraction from Spent Filter Coffee Using Aqueous Glycerol. *Chem. Eng. Commun.* **2016**, *203*, 407–413. [CrossRef]
38. Eyiz, V.; Tontul, I.; Turker, S. Optimization of green extraction of phytochemicals from red grape pomace by homogenizer assisted extraction. *J. Food Meas. Charact.* **2020**, *14*, 39–47. [CrossRef]
39. Philippi, K.; Tsamandouras, N.; Grigorakis, S.; Makris, D.P. Ultrasound-Assisted Green Extraction of Eggplant Peel (*Solanum melongena*) Polyphenols Using Aqueous Mixtures of Glycerol and Ethanol: Optimisation and Kinetics. *Environ. Process.* **2016**, *3*, 369–386. [CrossRef]
40. Suslick, K.S.; Price, G.J. Applications of ultrasound to materials chemistry. *Annu. Rev. Mater. Sci.* **1999**, *29*, 295–326. [CrossRef]
41. Leopoldini, M.; Chiodo, S.G.; Russo, N.; Toscano, M. Detailed Investigation of the OH Radical Quenching by Natural Antioxidant Caffeic Acid Studied by Quantum Mechanical Models. *J. Chem. Theory Comput.* **2011**, *7*, 4218–4233. [CrossRef]
42. Wildermuth, S.R.; Young, E.E.; Were, L.M. Chlorogenic Acid Oxidation and Its Reaction with Sunflower Proteins to Form Green-Colored Complexes. *Compr. Rev. Food Sci. Food Saf.* **2016**, *15*, 829–843. [CrossRef]
43. Singleton, V.L.; Salgues, M.; Zaya, J.; Trousdale, E. Caftaric Acid Disappearance and Conversion to Products of Enzymic Oxidation in Grape Must and Wine. *Am. J. Enol. Vitic.* **1985**, *36*, 50–56.

44. Ghasemzadeh, A.; Jaafar, H.Z.E.; Juraimi, A.S.; Tayebi-Meigooni, A. Comparative Evaluation of Different Extraction Techniques and Solvents for the Assay of Phytochemicals and Antioxidant Activity of Hashemi Rice Bran. *Molecules* **2015**, *20*, 10822–10838. [CrossRef]
45. Du, J.; Cullen, J.J.; Buettner, G.R. Ascorbic acid: Chemistry, biology and the treatment of cancer. *Biochim. Biophys. Acta* **2012**, *1826*, 443–457. [CrossRef]
46. Davies, M.B. Reactions of L-ascorbic acid with transition metal complexes. *Polyhedron* **1992**, *11*, 285–321. [CrossRef]
47. Maares, M.; Haase, H. Zinc and immunity: An essential interrelation. *Arch. Biochem. Biophys.* **2016**, *611*, 58–65. [CrossRef]
48. Kogan, S.; Sood, A.; Garnick, M.S. Zinc and Wound Healing: A Review of Zinc Physiology and Clinical Applications. *Wounds Compend. Clin. Res. Pract.* **2017**, *29*, 102–106.
49. Institute of Medicine, Food and Nutrition Board. *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*; National Academy Press: Washington, DC, USA, 2001; ISBN 978-0-309-07279-3.
50. Coger, V.; Million, N.; Rehbock, C.; Sures, B.; Nachev, M.; Barcikowski, S.; Wistuba, N.; Strauß, S.; Vogt, P.M. Tissue Concentrations of Zinc, Iron, Copper, and Magnesium During the Phases of Full Thickness Wound Healing in a Rodent Model. *Biol. Trace Elem. Res.* **2019**, *191*, 167–176. [CrossRef]
51. Kleinschmidt, G. Case Study: Validation of an HPLC-Method for Identity, Assay, and Related Impurities. In *Method Validation in Pharmaceutical Analysis: A Guide to Best Practice*; Ermer, J., Miller, J.H.M., Eds.; Wiley-VCH Verlag: Weinheim, Germany, 2005; pp. 195–212.
52. Dalipi, R.; Marguí, E.; Borgese, L.; Depero, L.E. Multi-element analysis of vegetal foodstuff by means of low power total reflection X-ray fluorescence (TXRF) spectrometry. *Food Chem.* **2017**, *218*, 348–355. [CrossRef]

Sample Availability: Samples of the compounds are not available from the authors.

2.2. Extraction Optimisation, Antioxidant, Cosmeceutical and Wound Healing Potential of *Echinacea Purpurea* Glycerolic Extracts

molecules

MDPI

Article

Extraction Optimization, Antioxidant, Cosmeceutical and Wound Healing Potential of *Echinacea purpurea* Glycerolic Extracts

Petar Ciganović [1], Lejsa Jakupović [1], Plamen Momchev [1,2], Laura Nižić Nodilo [1], Anita Hafner [1] and Marijana Zovko Končić [1,*]

[1] Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10000 Zagreb, Croatia
[2] Faculty of Pharmacy, Medical University-Sofia, Boulevard "Akademik Ivan Evstratiev Geshov" 15, 1000 Sofia, Bulgaria
* Correspondence: mzovko@pharma.hr

Abstract: *Echinacea purpurea* is a plant with immunomodulating properties, often used in topical preparations for treatment of small superficial wounds. In the presented study, the best conditions for ultrasound-assisted extraction of caffeic acid derivatives (caftaric and cichoric acid) (TPA-opt extract), as well as the conditions best suited for preparation of the extract with high radical scavenging activity (RSA-opt extract), from *E. purpurea* aerial parts were determined. A Box–Behnken design based on glycerol content (%, *w*/*w*), temperature (°C), ultrasonication power (W) and time (min) as independent variables was performed. Antioxidant, antiaging and wound healing effects of the two prepared extracts were evaluated. The results demonstrate that glycerol extraction is a fast and efficient method for preparation of the extracts with excellent radical scavenging, Fe^{2+} chelating and antioxidant abilities. Furthermore, the extracts demonstrated notable collagenase, elastase and tyrosinase inhibitory activity, indicating their antiaging properties. Well-pronounced hyaluronidase-inhibitory activities, with IC_{50} values lower than 30 µL extract/mL, as well as the ability to promote scratch closure in HaCaT keratinocyte monolayers, even in concentrations as low as 2.5 µL extract/mL (for RSA-opt), demonstrate promising wound healing effects of *E. purpurea.* The fact that the investigated extracts were prepared using glycerol, a non-toxic and environmentally friendly solvent, widely used in cosmetics, makes them suitable for direct use in specialized cosmeceutical formulations.

Keywords: antioxidant; cosmeceutical; elastase; *Echinacea purpurea*; green extraction; tyrosinase; wound healing

Citation: Ciganović, P.; Jakupović, L.; Momchev, P.; Nižić Nodilo, L.; Hafner, A.; Zovko Končić, M. Extraction Optimization, Antioxidant, Cosmeceutical and Wound Healing Potential of *Echinacea purpurea* Glycerolic Extracts. *Molecules* **2023**, *28*, 1177. https://doi.org/10.3390/molecules28031177

Academic Editor: Toshio Morikawa

Received: 14 December 2022
Revised: 20 January 2023
Accepted: 24 January 2023
Published: 25 January 2023

1. Introduction

The use of plants in topical preparations for medicinal and cosmetic applications is experiencing an unprecedented rise. Such products often display a broad spectrum of activities such as antiaging, antioxidant, anti-inflammatory, antipigmenting and many others. Such efficacy, which surpasses that of cosmetic products and more closely resembles the efficacy of pharmaceutical agents, led to the introduction of the popular new term "cosmeceutical". Cosmeceutical is a topical preparation that is sold as a cosmetic product but has the performance characteristics that suggest a pharmaceutical action. As the term corresponds well with consumers' expectations, it is often used in lay language even though it has no regulatory meaning [1]. In addition to displaying the desired activity and safety profile, modern cosmeceutical products should also have a satisfactory stability and sensory properties. Furthermore, as consumers are increasingly aware of the environmental impact, new, eco-friendly products are constantly being developed in order to meet such needs [2]. As plant-derived products originate from natural sources, they are in special demand in the cosmetic market, due to consumers' perception of their extraordinary safety and bioactivity. It is widely considered that they can prevent and delay skin aging and deterioration. Indeed, numerous studies have shown that phenolics and other compounds, present in the plants and plant extracts, are desirable preservatives and functional ingredients in cosmetic products due to their antioxidant activity and their ability to impede numerous processes that negatively affect skin health and appearance [3].

One of the areas of cosmeceutical research is the design of green and sustainable extraction methods for bioactive natural products. For that purpose, the selection of an appropriate solvent is of utmost importance. Besides the high dissolving power, the ideal solvent should be safe, easy to handle and environmentally friendly [4,5]. One such solvent is glycerol, a natural, cost-efficient, non-toxic, biodegradable liquid with the additional benefit that it is manufactured from renewable sources, e.g., as a by-product of biodiesel production [6]. Furthermore, glycerol is one of the most widely used ingredients in cosmetic products, where it acts as humectant and viscosity-regulating agent [4]. Therefore, glycerol extracts of medicinal plants may have a dual role in cosmetic products, as active agents or excipients. Finally, the fact that glycerol may be incorporated into the final product makes glycerolic extraction highly attractive from the energy-saving point of view.

Echinacea purpurea (L.) Moench (Asteraceae) (purple coneflower) is a perennial medicinal herb with important immunostimulatory and anti-inflammatory properties. It is most frequently used for alleviation of common cold symptoms [7]. However, according to the European Medicines Agency, preparations of the aerial parts of *E. purpurea* are traditionally used for alleviation of skin disorders and minor wounds [8]. *E. purpurea* aerial parts contain diverse bioactive phytochemical constituents including essential oil, polysaccharides, phenolics, as well as nitrogen compounds, such as alkylamides, and small amounts of alkaloids. Among numerous constituents, caffeic acid derivatives and other phenolic acids are among the most prominent ones [9]. According to the European pharmacopoeial monograph for Echinaceae purpureae herba, caffeic acid derivatives are used for estimation of the quality of raw herbal material and its preparations [10].

The most abundant among caffeic acid derivatives in *E. purpurea* is cichoric acid, followed by caftaric acid. Cichoric acid displays a wide array of beneficial skin-related activities, such as antiviral, antioxidant and anti-inflammatory activity. In addition, cichoric acid may ameliorate inflammation induced by lipopolysaccharides (LPSs) in both cell culture and mice, as well as ameliorate UVA irradiation-induced dermal fibroblast senescence by inhibition of matrix metalloproteinase-3 activity. This opens the possibility of beneficial effects of cichoric acid on aging [11]. Caftaric acid may act as an antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic agent, which adds to the beneficial effect on the skin [12]. In addition, a small dermatological study has shown that *E. purpurea* preparations may effectively improve the hydration of the skin and decrease skin wrinkling without inducing skin irritation [13].

Having in mind the traditional use and phytochemical composition of *E. purpurea*, the aim of this work was to optimize extraction of phenolic acids from *E. purpurea* aerial parts using glycerol, a non-toxic and eco-friendly solvent. Skin-related biological activities (antioxidant, enzyme inhibiting and wound healing effects) of the prepared extracts were investigated with the aim of obtaining highly active extracts suitable for use in cosmeceutical products.

2. Results and Discussion

2.1. Box–Behnken Design

Natural materials often contain a myriad of secondary metabolites among whom only a selected few have desirable pharmacological properties. Their amount in the extracts depends on their physicochemical properties, extraction solvent, type of extraction, as well as on numerous extraction parameters related to the type of extraction. Thus, finding the extraction procedure which yields the maximum amount of the target compound(s) may be a tedious and time-consuming procedure. In this work, efforts were undertaken to optimize ultrasound-assisted extraction (UAE) of caftaric and cichoric acids from *E. purpurea*. UAE is often used for extraction in solid/liquid systems because it is a simple, cost- and time-effective method, characterized by low CO_2 emissions and solvent consumption.

Conditions for UAE were optimized using response surface methodology (RSM). It is a collection of mathematical and statistical techniques that enable building an empirical model between the response(s) of interest (dependent variables) and a number of associated independent variables. RSM is frequently applied for optimization of extraction of phenolic acids from various natural sources such as potatoes [14], birdsfoot trefoil [15] and *Rheum moorcroftianum* [16].

The results of a previous study have shown that, for the extraction of bioactive phenolic acids from *E. purpurea*, the concentration of glycerol used as solvent is of utmost importance. In that study, high glycerol content positively affected the extraction efficiency of phenolic acids [17]. Thus, for this study, glycerol concentrations higher than or equal to 50% (*w*/*w*) were used for the extraction. Furthermore, the temperature and the duration of the extraction, as well as the power of ultrasound, significantly affect the content of the target compounds. In order to fine-tune the extraction procedure and obtain the extracts with the maximum yields of phenolic acids and the most pronounced radical scavenging activity (RSA), RSM based on Box–Behnken design was used. The results are presented in Table 1. The extraction conditions greatly influenced the selected responses. For example, caftaric acid concentration varied greatly, from 13.07 µg/mL to 31.55 µg/mL in Run 4 and Run 5, respectively. Similarly, cichoric acid concentration spanned from 61.11 µg/mL to 103.26 µg/mL, again in Run 4 and Run 5, indicating that the same factors influence the extraction of both phenolic acids, which is expected due to their similar chemical structure. Similarly, the minimum and the maximum content of total phenolic acids (TPAs) was again reached in Run 4 and Run 5, respectively. Somewhat lower content of caffeic acid derivative in comparison with previous glycerol extraction of *E. purpurea* [17] may have contributed to the variability of plant material. RSA of the extracts varied greatly, from 8.17 µL extract/µL (Run 2) to 54.03 µL extract/µL (Run 18). While, expectedly, TPA correlated well with cichoric (r^2 = 0.9969) and caftaric (r^2 = 0.9773) acid content, no such correlation was found between TPA and RSA IC_{50}. This means that, even though caffeic acid derivatives are strong antioxidants [18], other substances also contribute to the observed antiradical activity of the prepared extracts.

Table 1. Levels of independent variables in the Box–Behnken design, concentration of phenolic acids and IC_{50} value of the radical scavenging activity (RSA IC_{50}) of the extracts.

Run	Std	X_1	X_2	X_3	X_4	Caf	Cic	TPA	RSA IC_{50}
1	14	70	70	72	40	28.17	91.39	119.56	14.53
2	19	50	55	360	40	27.58	88.74	116.32	8.17
3	6	70	55	360	20	28.68	92.21	120.89	8.65
4	2	90	40	216	40	13.70	61.11	74.81	19.9
5	5	70	55	72	20	31.55	103.26	134.81	18.29
6	1	50	40	216	40	21.23	65.28	86.51	19.2
7	21	70	40	216	20	23.19	79.58	102.77	41.58
8	26	70	55	216	40	29.24	96.86	126.1	14.9
9	13	70	40	72	40	25.82	85.41	111.23	15.51
10	11	50	55	216	60	29.25	94.15	123.4	10.75
11	3	50	70	216	40	23.93	77.59	101.52	11.07
12	12	90	55	216	60	21.37	75.9	97.27	16.33
13	25	70	55	216	40	27.38	90.33	117.71	8.33
14	17	50	55	72	40	29.03	90.58	119.61	8.32
15	7	70	55	72	60	29.62	98.63	128.25	9.16
16	20	90	55	360	40	22.36	78.6	100.96	23.53
17	29	70	55	216	40	27.41	86.64	114.05	14.3
18	10	90	55	216	20	20.37	73.95	94.32	54.03
19	16	70	70	360	40	28.39	92.58	120.97	9.76
20	8	70	55	360	60	30.82	103.02	133.84	8.27
21	24	70	70	216	60	28.46	94.37	122.83	9.43
22	23	70	40	216	60	29.13	97.92	127.05	23.53

Table 1. *Cont.*

Run	Std	X_1	X_2	X_3	X_4	Caf	Cic	TPA	RSA IC_{50}
23	15	70	40	360	40	22.72	78.13	100.85	23.52
24	28	70	55	216	40	21.91	74.26	96.17	8.67
25	9	50	55	216	20	26.76	84.36	111.12	19.66
26	22	70	70	216	20	27.4	89.79	117.19	8.71
27	4	90	70	216	40	20.06	65.24	85.3	17.28
28	18	90	55	72	40	20.74	76.8	97.54	19.1
29	27	70	55	216	40	28.7	91.82	120.52	12.79

X_1 = Glycerol concentration (%, *w*/*w*), X_2 = Temperature (°C), X_3 = Ultrasonication power (W), X_4 = Time (min), Caf = Caftaric acid concentration (μg/mL), Cic = Cichoric acid concentration (μg/mL), TPA = Total phenolic acid concentration (μg/mL), RSA IC_{50} = Radical scavenging activity IC_{50} (μL extract/μL).

2.2. Model Analysis

Multiple regression analysis was used to analyze the experimental results. Table 2 shows the relationship between the independent and dependent variables in the form of polynomial equations. The contents of phenolic acids (caftaric acid, cichoric acid and TPA) were influenced by all the selected independent variables as quadratic terms. Glycerol content and temperature were preceded with positive coefficients, while negative coefficients preceded the USP and time. This means that the extreme values of glycerol content and temperature negatively affect the content of phenolic acids, while the opposite is true for USP and time. Additionally, glycerol content and temperature influenced the phenolic acid content as negative and positive linear terms, respectively. Interestingly, high glycerol content positively affected the extraction efficiency of phenolic acids in an earlier study [17], while glycerol's influence was negative in this study. This apparent discrepancy is due to difference in lowest and highest glycerol contents. Namely, the first study used a much wider range of glycerol content (10–90%, *w*/*w*) and only the highest concentrations from that study (50–90%, *w*/*w*) were those selected for this investigation. On the other hand, independent variables influenced the antiradical activity's square root as linear terms preceded with either a positive (glycerol content) or negative (temperature and time) coefficient. This means that relatively high glycerol content, as well as lower temperature and extraction time, will produce extracts with high RSA IC_{50} and consequently low RSA activity.

Table 2. Coefficients of the models' polynomial equations ($a \times X_1^2 + b \times X_2^2 + c \times X_3^2 + d \times X_4^2 + e \times X_1 \times X_2 + f \times X_1 \times X_3 + g \times X_1 \times X_4 + h \times X_2 \times X_3 + i \times X_2 \times X_4 + j \times X_3 \times X_4 + k \times X_1 + l \times X_2 + m \times X_3 + n \times X_4 + o$) in terms of coded factors.

Response	Unit	The Equation Coefficients														
		a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*	*m*	*n*	*o*
Caf	μg/mL	−4.3 *	−2.4 *	1.8 *	1.9 *	0.9	0.8	−0.4	0.8	−1.2	1.0	−3.3 *	1.7 *	−0.4	0.9	26.9
Cic	μg/mL	−12.4 *	−6.6 *	6.0 *	7.0 *	−2.0	0.9	−2.0	2.1	−3.4	3.9	−5.8 *	3.6 *	−1.1	3.4	88.0
TPA	μg/mL	−18.4 *	−10.7 *	8.6 *	9.7 *	3.9	1.7	−2.3	2.9	−4.7	4.9	−10.7 *	7.0 *	−1.4	4.3	114.9
$(RSA\ IC_{50})^{-1/2}$	μL extract/μL	0	0	0	0	0	0	0	0	0	0	0.7 *	−0.7 *	−0.1	−0.6 *	3.9

X_1 = Glycerol concentration (%, *w*/*w*), X_2 = Temperature (°C), X_3 = Ultrasonication power (W), X_4 = Time (min), Caf = Caftaric acid concentration (μg/mL), Cic = Cichoric acid concentration (μg/mL), TPA = Total phenolic acid concentration (μg/mL), RSA IC_{50} = Radical scavenging activity IC_{50} (μL extract/μL). * = Significant model terms.

As demonstrated by ANOVA (Table 3), the relationship between the response variables and independent variables is satisfactorily expressed using the polynomial equations presented in Table 2. The statistical significance of each model was calculated using the *F*-test and *p*-values. The calculated *F*-values were higher than 5, while the *p*-values were 0.002 or lower. This indicates that the models are highly significant and that they can be used to optimize the extraction variables. Lack-of-fit in the models was statistically insignificant relative to the pure error which demonstrated that the fitting model is adequate to describe the experimental data. The determination coefficients (R^2) for phenolic acid content were approaching 0.9, showing that the observed values are well replicated by the model. However, in the case of antiradical activity, R^2 was rather low (0.4928). The predicted R^2 were in reasonable agreement with the adjusted ones, further confirming that the models may be used to predict and optimize the amount of target substances in the extracts.

Table 3. Analysis of variance (ANOVA) for the fitted quadratic models for optimization of *E. purpurea* extraction.

	Caf R^2 = 0.8958; R_a^2 = 0.79158; R_p^2 = 0.6933					**Cic** R^2 = 0.8765; R_a^2 = 0.7531; R_p^2 = 0.6634				
Source	SS	df	MS	*F* Value	*p*-value	SS	df	MS	*F* Value	*p*-value
Model	436.6	14	31.2	8.59	0.0001	4264.7	14	304.6	6.85	0.0005
LoF	16.7	10	1.6	0.20	0.9833	333.5	10	33.3	0.46	0.8535
PE	34.1	4	8.5			289.1	4	72.3		
	TPA R^2 = 0.8823; R_a^2 = 0.7647; R_p^2 = 0.5814					**IC_{50} RSA** R^2 = 0.4928; R_a^2 = 0.4083; R_p^2 = 0.2382				
Source	SS	df	MS	*F* Value	*p*-value	SS	df	MS	*F* Value	*p*-value
Model	7380.8	14	527.2	7.50	0.0003	15.9	4	4.0	5.83	0.0020
LoF	467. 9	10	46.8	0.36	0.9121	15.5	20	0.8	3.60	0.1111
PE	516.4	4	129.1			0.9	4	0.2		

SS = Sum of squares, df = Degrees of freedom, MS = Mean square, r_A^2 = Adjusted r^2, r_P^2 = Predicted r^2, LoF = Lack of fit, PE = Pure error, Caf = Caftaric acid concentration (μg/mL), Cic = Cichoric acid concentration (μg/mL), TPA = Total phenolic acid concentration (μg/mL), RSA IC_{50} = Radical scavenging activity IC_{50} (μL extract/μL).

2.3. Validation of Optimal Extraction Conditions

Based on the experimental results and statistical analysis, numerical optimizations were conducted to establish the optimum levels of independent variables (Table 4). As previously mentioned, the most important extraction factor for all the investigated parameters was glycerol concentration. It is well known that the extraction solvent greatly affects the extraction efficiency. In this work, the glycerol content needed for optimal extraction of specific phenolic compounds varied according to the response. In general, phenolic acids were best extracted using moderate glycerol concentration as reflected in the maximized TPA at 70%. The values for extraction temperature, USP and time were approaching the maximum values used in the Box–Behnken design, indicating their relatively good stability in the extraction medium. The best antiradical activity, on the other hand, was achieved using low glycerol concentration and lower ultrasonication power, indicating that, in addition to phenolic acids, other compounds of relatively lower polarity and higher sensitivity are partly responsible for the observed antiradical effects. The predicted results matched well with the experimental ones, with relatively low deviations from calculated values, indicating good suitability of the selected models (Table 4). The HPLC-DAD chromatograms of the two prepared extracts are shown in Figure 1.

Table 4. Predicted and observed values for the optimized extracts.

Extract	**Measured Response**	**X_1 (%. *w/w*)**	**X_2 (°C)**	**X_3 (W)**	**X_3 (min)**	**$Resp_{pred}$**	**$Resp_{ms}$**	**RD (%)**
TPA-opt	Caf	70	60	360	60	32.37	31.82	−1.7
TPA-opt	Cic	70	60	360	60	107.16	113.11	5.6
TPA-opt	TPA	70	60	360	60	139.53	144.93	3.9
RSA-opt	RSA IC_{50}	50	70	144	55	4.90	5.32	8.6

X_1 = Glycerol concentration (%, *w/w*), X_2 = Temperature (°C), X_3 = Ultrasonication power (W), X_4 = time (min), Caf = Caftaric acid concentration (μg/mL), Cic = Cichoric acid concentration (μg/mL), TPA = Total phenolic acid concentration (μg/mL), RSA IC50 = Radical scavenging activity IC50 (μL extract/μL), Rsppred/ms–RD = Response deviation, calculated as (Rspms − Rsppred)/Rsppred × 100.

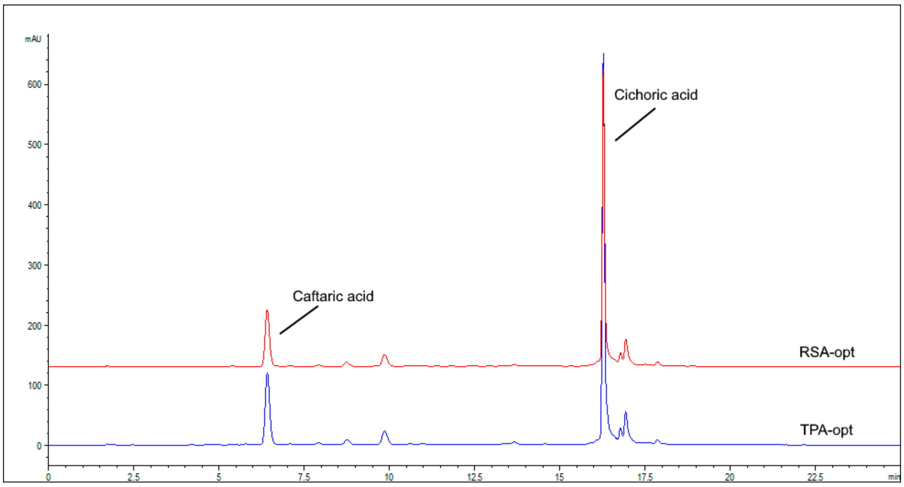


Figure 1. Chromatograms of RSA-opt and TPA-opt recorded at 330 nm.

2.4. Antioxidant Activity of the Optimized Extracts

Botanical ingredients represent one of the largest categories of natural active substances used in dermatology. In order to investigate *E. purpurea* extracts as potentially valuable cosmeceutical ingredients, their biological activity was determined using several methods. Antioxidant activity of cosmetic product ingredients is of utmost importance because they may act both as preservatives and active components in cosmeceutical products. Antioxidants may protect the cosmetic product against the oxidation that occurs during its storage and use by scavenging free radicals [19]. Chelation of metal, such as pro-oxidant Fe^{2+} and other ions, is also very important because they may induce peroxidation of polyunsaturated fatty acids that natural cosmetics are especially rich in [20]. Finally, functional cosmeceutical ingredients may have a more active role in such products. They offer protection against oxidative damage of skin macromolecules associated with the effects of free radicals and UV radiation on the skin [21,22]. Thus, in this work the influence of the prepared extracts on the free radicals (as modeled by DPPH free radical), chelating activity on Fe^{2+} ions and the activity in heat-induced unsaturated fatty acid degradation in a β-carotene–linoleic acid system were investigated and compared with the activity of standard antioxidants, butylated hydroxyanisole (BHA) and ethylenediaminetetraacetic acid (EDTA). Even though the activity of the extracts may not be directly compared to the activity of standard antioxidants, due to the fact that they are expressed in different measurements units (the activity of the extracts and standards was expressed as μL/mL and μg/mL, respectively), it is possible to regard the activity of the standards as volume equivalents of 1 mg/mL solutions. Thus, it was reported for general comparison purposes.

Figure 2a–c depict the results of the antioxidant assays performed in this work. Anti-radical and chelating activities of the extracts were lower than the activity of the standards solutions. However, in the β-carotene–linoleic acid assay, the extracts were notably stronger antioxidants than BHA. The activity of the individual extracts differed according to the assay. The prepared optimized extracts were similarly efficient Fe^{2+} ion chelators with IC_{50} values of approximately 120 μL of extract per mL of solution. However, expectedly, RSA-opt was a stronger radical scavenger than TPA-opt. Both extracts inhibited thermally induced degradation of the β-carotene–linoleic acid system. Since this assay is based on the ability of the mixture components to react with linoleic radical and other radicals formed in the solution, the high activity of RSA-opt, the extract optimized to display pronounced antiradical activity, is not surprising. Caffeic acid and its derivatives are strong antioxidants. For example, in many antioxidant assays caffeic acid shows activity that often surpasses the activity of standard antioxidants, ascorbic acid and trolox. Additional advantages of caffeic acid include higher stability than ascorbic acid and, unlike trolox, the possibility of extraction from natural sources [18]. Furthermore, cichoric acid, the main caffeic acid derivatives in *E. purpurea* extracts [23], as well as caftaric acid [12] also display potent antioxidant activity. However, it seems that caffeic acid derivatives are not the only antioxidant molecules in *E. purpurea* because RSA-opt, the most active radical-scavenger, contained lower amounts of caffeic derivatives than TPA-opt.

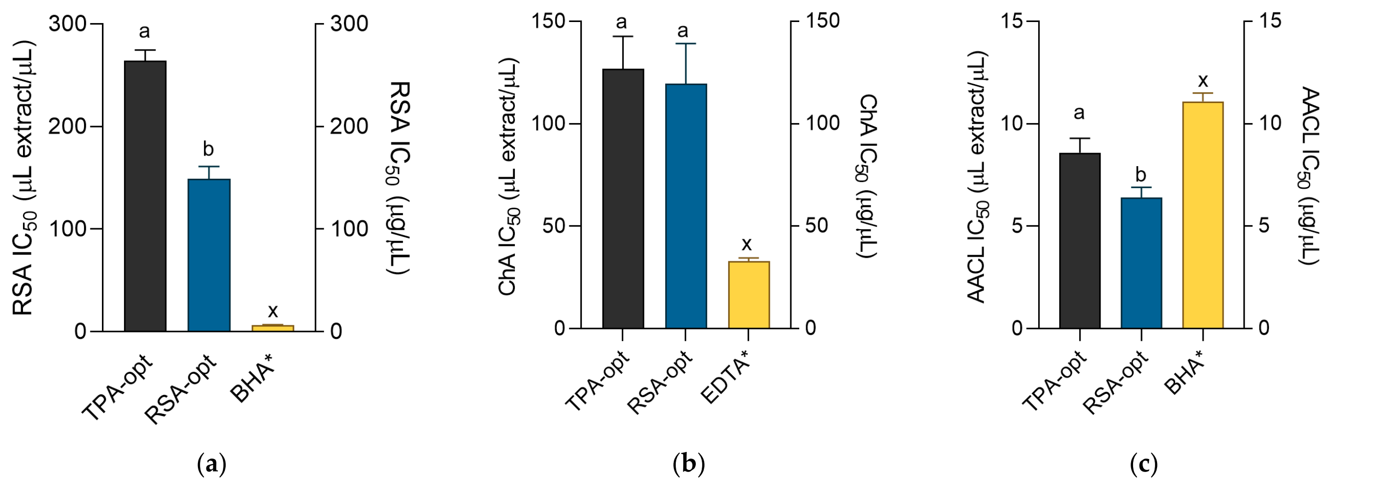
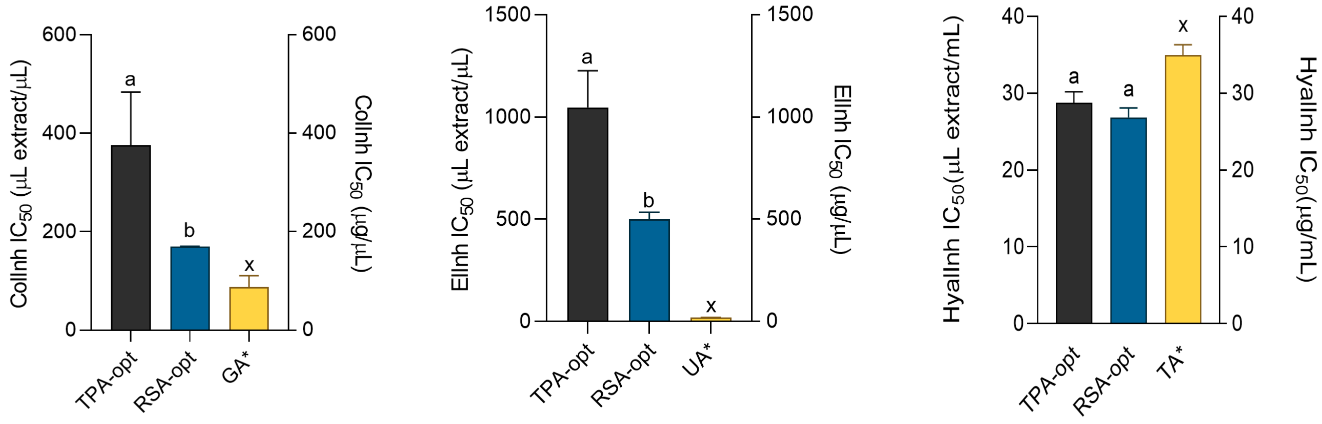


Figure 2. Antiradical activity (**a**), chelating activity (**b**) and the activity in β-carotene–linoleic acid assay (**c**) of the extracts and positive controls BHA (butylated hydroxyanisole) and EDTA (ethylenediaminetetraacetic acid). ^{a,b} = Differences between the extracts within a column (*t*-test, $p < 0.05$). ^{x} = differences from the positive control (Dunnett's post-test, $p < 0.05$). Columns not sharing the same letter are statistically different. Asterisk indicates that the unit is placed at the right ordinate.

2.5. Cosmeceutical Activity of the Optimized Extracts

In addition to hydration and antioxidant protection, contemporary cosmetic products are expected to have additional properties that beneficially affect skin appearance. Excessive enzymatic activity in the skin, caused by environmental factors and aging, can cause premature breakdown of skin proteins, such as elastin or collagen, as well as breakdown of polysaccharides, such as hyaluronic acid. By inhibiting the enzymes responsible for degradation of skin macromolecules, plant metabolites may decelerate the skin aging process and reduce its aesthetically visible effects, such as dehydrated skin, reduced elasticity, dark spots and the appearance of wrinkles [24].

Skin proteins play a pivotal role in maintaining not only the function and form, but also youthful appearance, of the skin. Fibrillar collagen is the most abundant skin protein that constitute three-quarters of skin's dry weight, while the amount of elastin fibers is substantially lower. Collagen is responsible for the strength and stability of skin tissue because sliding and realignment of collagen fibrils allows skin to deform while maintaining its integrity and preventing damage. On the other hand, elastin fibers contribute extensibility and reversible recoil to skin, which allows for skin to return to its resting state after external force is removed [25]. Collagenase is the enzyme active in the extracellular matrix that catalyzes degradation of collagen. As a reaction to aging or external influences (e.g., UV radiation), its activity increases. This leads to the formation of wrinkles and loss of skin tone [26]. Degradation of elastin is induced by the enzyme elastase, which is directly related to skin aging and oxidative stress [27]. Clinical trials confirm that natural products and other compounds that display inhibition of elastase have significant antiaging potential [28]. The collagenase- and elastase-inhibitory effects of the extracts are shown in Figure 3a,b. Even though the extracts were weaker collagenase and elastase inhibitors than the 1 mg/mL solutions of positive controls, gallic and ursolic acid, respectively, they still showed a significant degree of inhibition of these two enzymes. In both assays, RSA-opt was the more active extract. Previously, it was found that aqueous *E. purpurea* extract was a potent collagenase and elastase agent [29]. In addition, grape pomace extracts, rich in caftaric acid and other phenolic acids, showed inhibitory effects on both collagenase and elastase enzyme activities [30], indicating contribution of this phenolic acid to the observed activity of the extracts.

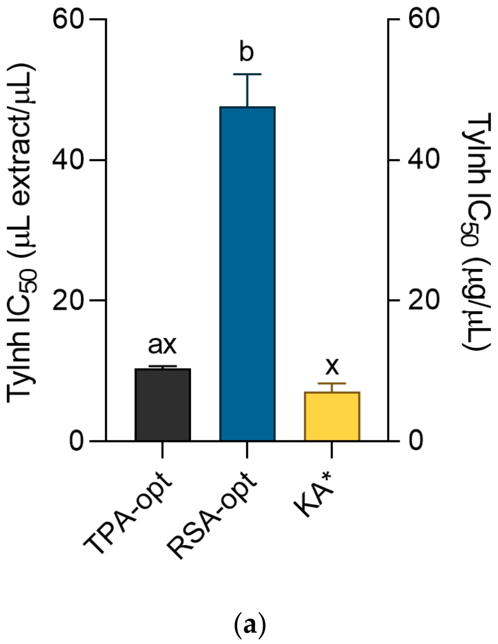


(**a**) (**b**) (**c**)

Figure 3. Collagenase (**a**), elastase (**b**) and hyaluronidase (**c**) inhibitory activity of the extracts and positive controls gallic acid (GA), ursolic acid (UA) and tannic acid (TA). ^{a,b} = Differences between the extracts within a column (*t*-test, $p < 0.05$). ^{x} = Differences from the positive control (Dunnett's post-test, $p < 0.05$). Columns not sharing the same letter are statistically different. Asterisk indicates that the unit is placed at the right ordinate.

Reduced hydration of skin is characterized by a reduced turgor, resilience and elasticity and loss of youthful appearance. Hyaluronic acid is a polysaccharide found in human skin that possesses extreme water retaining capacity. As such, it is one of the most important molecules responsible for skin hydration [31]. In various pathological processes, as well as during physiological skin aging, hyaluronic acid is increasingly degraded by hyaluronidase, the enzyme that controls the turnover of hyaluronic acid [32]. Thus, inhibition of hyaluronidase leads to retention of skin moisture and is one of the most promising approaches for the prevention of premature skin aging. As presented in Figure 3c, both extracts were excellent hyaluronidase inhibitors, with the activity surpassing that of the positive control, tannic acid. This is in line with a previous observation that aqueous *E. purpurea* extract possessed a significant antihyaluronidase activity [29]. This activity may be mediated by caffeic acid derivatives present in the extracts. Previous research shows that chicoric and caftaric acid have excellent antihyaluronidase activity [33]. Furthermore, chicoric acid, the main ingredient of TPA-opt, was found to inhibit human hyaluronidase 1, the enzyme that degrades high molecular weight hyaluronic acid [34]. Other caffeic acid derivatives may also add to the beneficial effect on wound healing. For example, echinacoside displays antihyaluronidase properties [35] and thus contributes to the observed effects of *E. purpurea* extracts.

Damage caused by UV radiation may be prevented by melanin, a photoprotective macromolecular pigment synthetized in the epidermis, with the enzyme tyrosinase catalyzing the first, rate-determining step. Most of the time, production of melanin is a beneficial or welcomed physiological reaction. However, in some cases, such as aging or melasma, irregularly distributed production of melanin results in uneven skin pigmentation and represents an esthetic problem for the affected individual. As tyrosinase inhibitors block melanogenesis and prevent hyperpigmentation of the skin, their inclusion in cosmetic products is desirable from an aesthetic point of view [36]. Although both investigated extracts showed notable antityrosinase activity (Figure 4a), the effectiveness of the TPA-opt extract was much more pronounced and statistically equal to the activity of the standard, kojic acid. Caftaric acid, present in TPA-opt, was shown to be a competitive tyrosinase inhibitor, and proposed as a promising ingredient in cosmetic products with skin whitening properties [37].



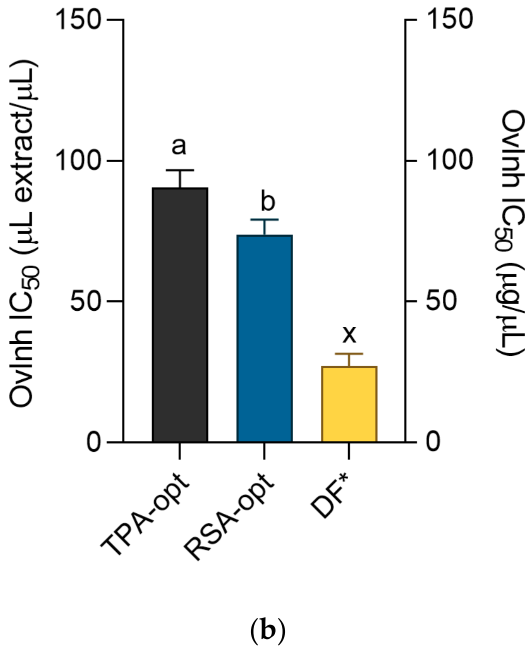


Figure 4. Tyrosinase inhibiting (**a**) and anti-inflammatory (**b**) activity of the extracts and positive controls kojic acid (KA) and diclofenac (DF). [a,b] = Differences between the extracts within a column (*t*-test, $p < 0.05$). [x] = Differences from the positive control (Dunnett's post-test, $p < 0.05$). Columns not sharing the same letter are statistically different. Asterisk indicates that the unit is placed at the right ordinate.

Denaturation of tissue proteins is one of the characteristics and causes of inflammatory processes in the body [38]. Therefore, the suppression of protein denaturation may impede the development of inflammatory skin changes which is another important aspect of anti-aging activity [39]. Although all the investigated extracts were able to inhibit heat-induced ovalbumin coagulation (Figure 4b), better anti-inflammatory activity was displayed by RSA-opt. Caftaric acid may be partly responsible for the observed effect because it was previously demonstrated that it acts as an anti-inflammatory agent [12]. It is important to say that glycerol probably plays a crucial role in this assay. Namely, the OVInh IC_{50} of glycerol in this assay was 19.55 ± 0.01 μL/mL, indicating that most of the observed activity in this assay is due to the presence of glycerol in the extracts. The role of glycerol as an active solvent that prevents the denaturation of proteins such as collagen has been previously established [40]. This experiment further confirms that the benefits of glycerol for the preparation of cosmeceutical extracts extend beyond its application as a green extraction solvent.

2.6. Evaluation of Cell Viability

In order to determine not only the toxicity of the prepared extracts, but also the concentration range in which the wound healing assay should be conducted, the influence of the prepared *E. purpurea* glycerol extracts and the solvents used for their preparation (glycerol in the appropriate dilutions) on cell viability was tested. An experiment was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT test) on HaCaT cells, a long-lived, spontaneously immortalized human keratinocyte line, able to differentiate in vitro [41]. HaCaT cells are often used as a suitable model for testing of wound healing activity [42]. Different concentrations (2.5–250 μL/mL) of the extracts and corresponding solvents, diluted in Hanks' balanced salt solution (HBSS), were used to estimate the toxicity of the extracts and solvents to HaCaT cell cultures.

The results are presented in Figure 5. Except for the difference between the highest and the lowest concentration of the extract, the viability of the cells treated with TPA-opt did not differ across concentrations. Among the RSA-opt dilutions, the cells treated with 25 µL/mL concentration showed the highest viability. Differences in dilution of the 70% (*w*/*w*) glycerol did not significantly affect viability. Among the different dilutions of 50% (*w*/*w*) glycerol, lower viability was recorded in the cells treated with the highest concentrations (one-way ANOVA followed by Tukey's post-test, $p < 0.05$, comparisons of different dilutions of the same extract/glycerol concentration). Relatively high viability of the cells treated with most extracts and solvent dilutions, recorded in this assay, indicates their low toxicity. The comparison of equal concentrations of the extracts and the solvents used for their preparation shows that the cells treated with the same concentrations of RSA-opt and 50% (*w*/*w*) glycerol most often show no statistically significant difference in their viability. On the other hand, HaCaT cells treated 70% (*w*/*w*) glycerol generally showed significantly lower viability than those treated with TPA-opt (paired *t*-test, $p < 0.05$). This could indicate the protective effect of *E. purpurea* extract and the phytochemicals contained in it. Based on the results of the MTT assay, 10 samples were tested for their wound healing activity: TPA-opt and 70% (*w*/*w*) glycerol (in concentrations 2.5 µL/mL and 12.5 µL/mL), as well as RSA-opt and 50% (*w*/*w*) glycerol (in concentrations 2.5 µL/mL, 12.5 µL/mL and 25 µL/mL).

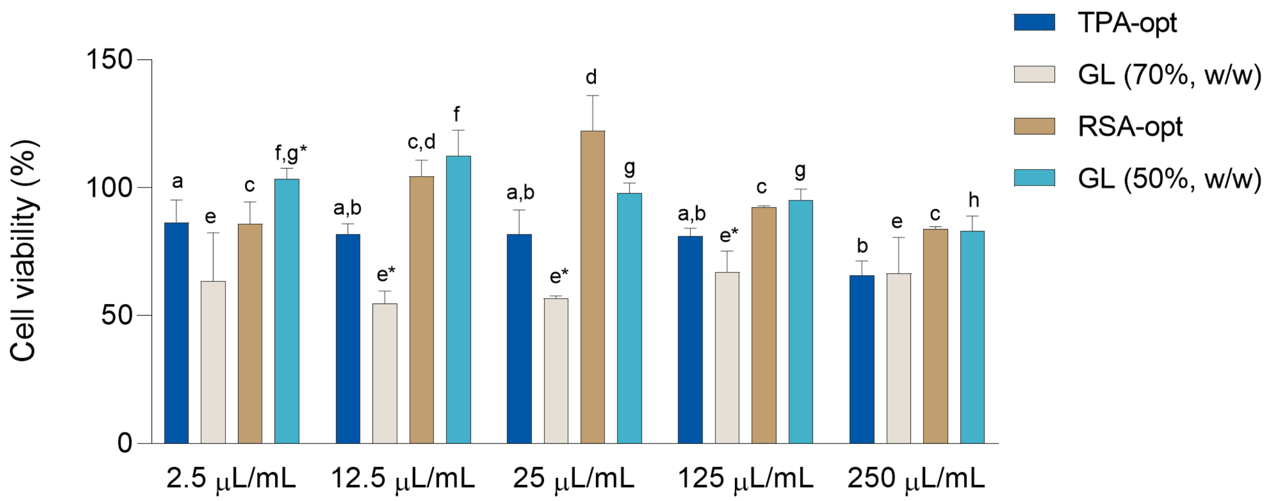


Figure 5. The influence of different extracts and glycerol (GL) dilutions on the survival of HaCaT cells. Cell survival is expressed as a percentage when compared to cells treated with HBSS. The results are shown as the mean ± SD (n = 3). ^{a,b} = Differences between the different dilutions of TP-opt extracts. ^{c,d} = Differences between the different dilutions of RSA-opt extracts. ^{e} = Differences between the different dilutions of 70% (*w*/*w*) glycerol. $^{f–h}$ = Differences between the different dilutions of 50% (*w*/*w*) glycerol (one-way ANOVA followed by Tukey's post-test, $p < 0.05$). Columns prepared from the same concentration of extract and glycerol not sharing the same letter are statistically different. * = Difference between the extract and the corresponding solvent (paired *t*-test, $p < 0.05$).

2.7. Wound Healing Effects of *E. purpurea*

Wound healing is a process of dynamic cellular and molecular mechanisms, divided into several stages, which may overlap over time: hemostasis, inflammation, proliferation/migration and maturation or remodeling, characterized by the formation of new tissue. In the proliferation phase, the migration of keratinocytes and fibroblasts recovers the network of blood vessels and participates in the granulation process. This characteristic is used for the in vitro "scratch" test method. In this procedure, a scratch that leaves an empty space ("wound") on the well bottom is created in a cell monolayer. If the conditions are satisfactory, cell movement and proliferation occur, followed by the gradual closure of the cell model wound [43].

In our research, cells were treated with different dilutions of *E. purpurea* extracts and glycerol. HBSS was used as a negative control. The wound-closure process was followed over 48 h. Figure 6 depicts the closure of the wounds treated with different samples. For this depiction, the extracts were used in a 2.5 μL/mL concentration and quantitatively compared with HBSS. Both extracts accelerated wound closure in a confluent cell layer (Figure 6a–c). The model wounds of the HaCaT cells treated with the extracts tended to be reduced over time. The RSA-opt sample was especially active. After 48 h, the scratch surface in the cell monolayer treated with that extract was barely visible, indicating excellent wound healing activity. On the other hand, the reduction of the wound surface in cells treated with the negative control was barely noticeable.

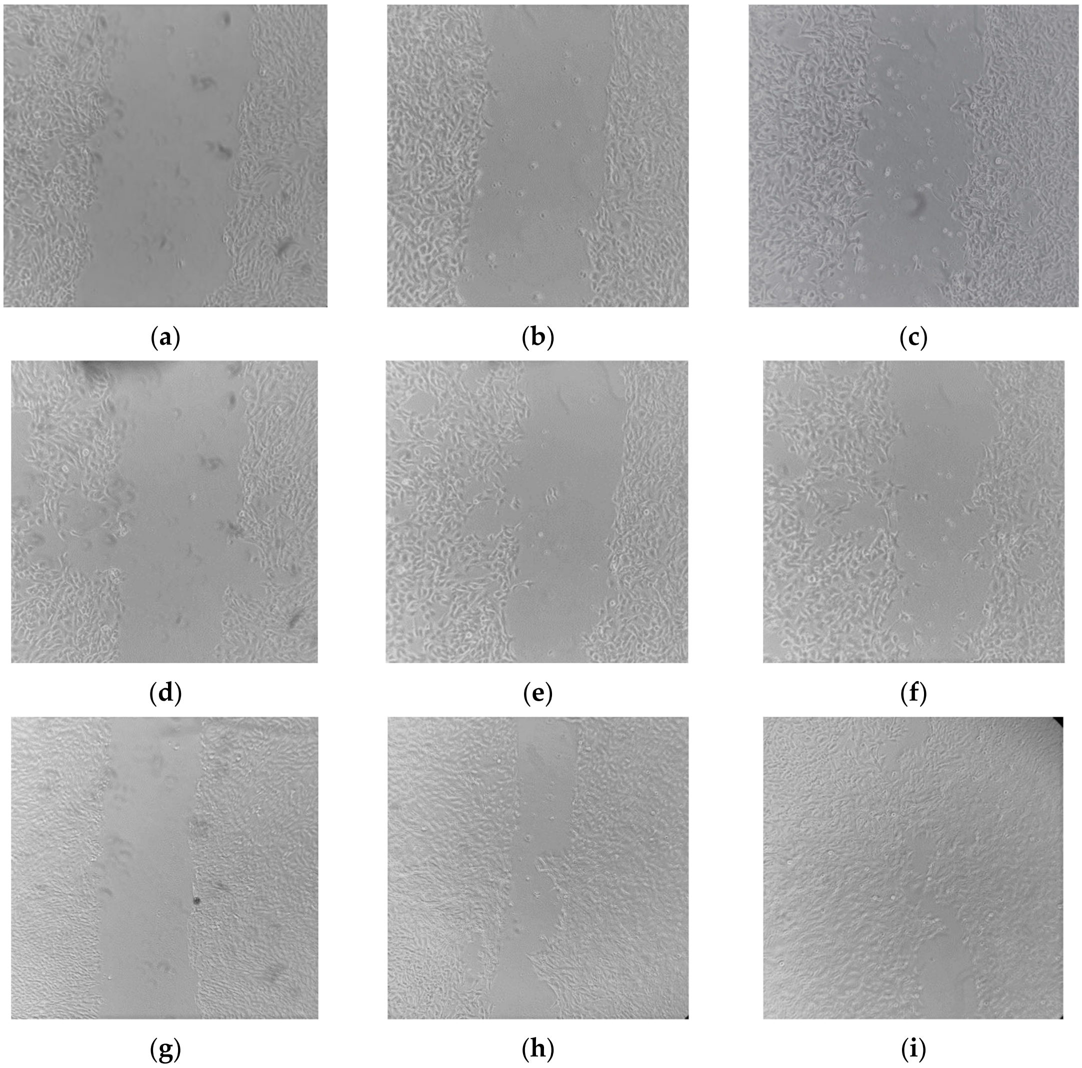


Figure 6. The influence of the HBSS (**a**–**c**), TPA-opt (**d**,**e**) and RSA-opt (**g**–**i**) in 2.5 μL extract/mL dilutions on the closure of scratch in HaCaT cell monolayer after 0 h (**a**,**d**,**g**), 24h (**b**,**e**,**h**) and 48 h (**c**,**f**,**i**) after being incubated with the extracts or HBSS for 2 h.

Figure 7 presents the percentage of wound closure (percentage of wound surface reduction relative to the wound surface at the beginning of the treatment at 0 h) after 48 h. The activity of the different glycerol dilutions was also tested, but since their activity was equal to or even lower than the activity of the HBSS control, they were omitted from the figure and the subsequent analysis. The lack of solvent activity also indicates that the phytochemical constituents were responsible for promotion of the proliferation of HaCaT cells during the tested incubation time. Interestingly, the wound healing activity of RSA-opt was not dose-dependent. For example, RSA-opt in a concentration of 2.5 µL extract/mL showed better wound healing activity than in a concentration of 12.5 µL extract/mL. Concentration-independent wound healing activity of herbal extracts is an occurrence that is not uncommon. The reason may be a complex interplay between the extracts' components, both those that accelerate wound healing and those that oppose it. Thus, one of the future research directions may be focused towards finding the components that are primarily responsible for the observed wound healing activity of the *E. purpurea* extracts, as well as the optimal dose range for the application of the extracts. Similar behavior of plant-based preparations has also been recorded in vivo, e.g., with ointment containing *Ocimum gratissimum* leaf extract [44]. The best activity was recorded in TPA-opt and RSA-opt extracts in the concentration of 12.5 µL extract/mL and 2.5 µL extract/mL, respectively. The activity among the other tested extracts and concentrations did not statistically differ (one-way ANOVA followed by Tukey's post-test, $p < 0.05$). This confirms the traditional indication of the European Medicines Agency, that *E. purpurea* and preparations thereof may be used in herbal medicinal products for alleviation of skin disorders and minor wounds [8].

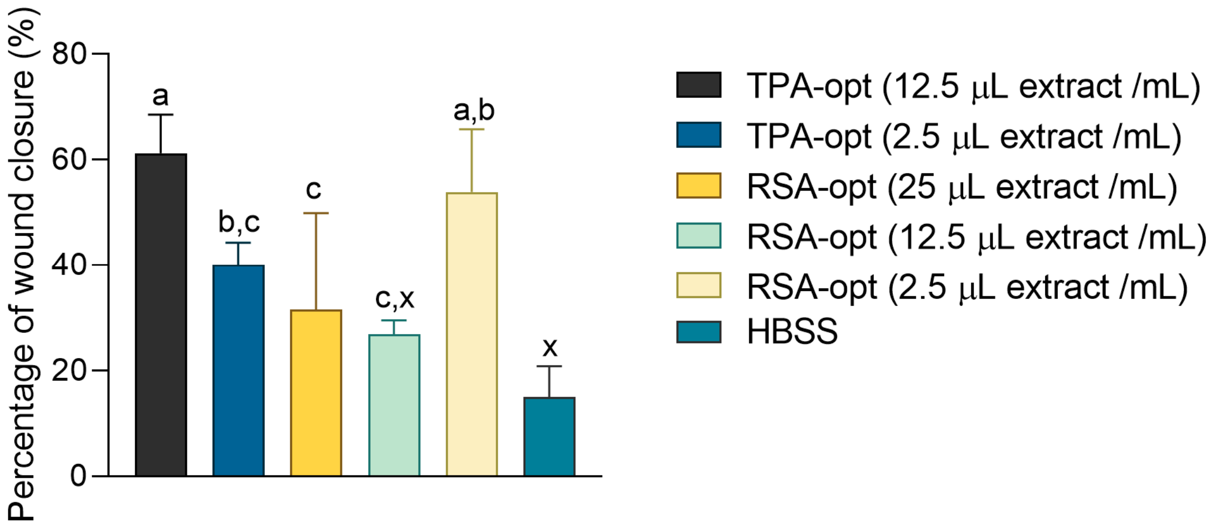


Figure 7. The influence of the different extract dilutions on 48 h wound closure in HaCaT cells. $^{a–c}$ = Differences between the extracts (one-way ANOVA followed by Tukey's post-test, $p < 0.05$). ^{x} = Differences from the negative control (one-way ANOVA followed by Dunnett's post-test, $p < 0.05$). Columns not sharing the same letter are statistically different.

3. Materials and Methods

3.1. Chemicals

Butylated hydroxyanisole (BHA, ≥98.5%), chlorogenic acid (European Pharmacopoeia Reference Standard), diclofenac (≥98%), kojic acid (≥98.5%), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, collagenase from *Clostridium histolyticum*, hyaluronidase from bovine testes, mushroom tyrosinase and porcine pancreas elastase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soybean LOX was a product from TCI chemicals (Tokyo, Japan). Acetonitrile was HPLC grade. Other reagents and chemicals were of analytical grade.

3.2. Plant Material

The commercially available *E. purpurea* aerial parts, consisting of leaves, stalks and flowers, were supplied by the company Suban. The identity was confirmed by the authors (P.C. and M.Z.K.) using the Echinaceae purpureae herba monograph of the European Pharmacopoeia [10]. Plant material was milled and passed through a sieve of 850 µm mesh size. A voucher specimen (EP-2021) was deposited in the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb.

3.3. Preparation of the Extracts According to Box–Behnken Design

For the Box–Behnken design, the following independent variables were used: glycerol concentration of 50–90%, *w*/*w*, temperature of 40–70 °C, ultrasound power of 72–360 W and time of 20–60 min. Powdered plant material (0.1 g) was suspended in 30 mL of a glycerol/water mixture in a 50 mL Erlenmeyer flask. The extraction was performed in an ultrasonic bath using frequency of 35 Hz at various temperatures, ultrasonication strengths and time intervals. The details are presented in Table 1. Upon the extraction, the mixtures were filtered and stored in the dark at −20 °C until analysis.

3.4. HPLC Determinations of Caffeic Acid Derivatives

The content of caffeic acid derivatives was determined according to the method described in the monograph of Echinaceae purpureae herba in the European Pharmacopoeia [10]. For the analysis, an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler and a DAD detector was used. Separation was performed on a Zorbax Eclipse XDB-C18 column (5 µm, 12.5 mm × 4.6 mm, Agilent, Santa Clara, CA, USA). The prepared extracts and the standard (0.025 mg/mL chlorogenic acid in 70% ethanol) were filtered through a PTFE syringe filter with pore size of 0.45 µm. Mobile phase A (phosphoric acid and water, 1:999 V/V) and mobile phase B (acetonitrile) were used according to the following protocol: 0-13 min (90–78% A), 13–14 min (78–60% A), 14-20 min (60–40% A). The analysis was performed at 35 °C using flow rate of 1.5 mL/min, and the chromatograms were recorded at 330 nm. TPA was calculated as the sum of caftaric and cichoric acid content.

3.5. Radical Scavenging Activity

Radical scavenging activity (RSA) was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical [45] method. To 130 µL of the extract or BHA (1 mg/mL) solution in methanol, 70 µL of DPPH (0.21 mg/mL) solution was added. After 30 min of incubation at room temperature, the absorbance was recorded at 545 nm. RSA was calculated according to Equation (1):

$$\mathrm{RSA}\ (\%) = \frac{A_0 - A_s}{A_0} \times 100 \tag{1}$$

where A_0 is the absorbance of the negative control which used methanol instead of the extract and A_s is the absorbance of the respective extract. Concentration of the extract which scavenged 50% of free radicals present in the solution (RSA IC_{50}) was calculated.

3.6. Fe^{2+} Chelating Activity

The chelating activity (ChA) was studied as described in [46]. To the solution of the extract in methanol (150 µL), 0.25 mM $FeCl_2$ solution (50 µL) was added. After 5 min of incubation, ferrozine solution was added (1.0 mM, 100 µL). Absorbance at 545 nm was recorded after 10 min. ChA was calculated using Equation (2):

$$\mathrm{ChA}\ (\%) = \frac{A_0 - A_s}{A_0} \times 100 \tag{2}$$

where A_0 is the absorbance of the negative control (which used methanol instead of the extract) and A_s is the absorbance of the respective extract. Concentration of the extract which chelates 50% of Fe^{2+} present in the solution (ChA IC_{50}) was calculated. EDTA (1 mg/mL) was used as positive control.

3.7. Antioxidant Activity in β-Carotene–Linoleic Acid Assay

The activity was evaluated according to a modified literature procedure [47]. The extract solution in methanol (50 µL) was added to 200 µL of emulsion containing β-carotene (6.7 µg/mL), linoleic acid (0.7 mg/mL) and Tween 40 (6.7 mg/mL). The reaction mixture was incubated at 50 °C. The antioxidant activity in the β-carotene–linoleic acid assay (AACL) was calculated based on the absorbances recorded after 60 min using Equation (3):

$$\text{AACL}\ (\%) = \frac{A_{sample}}{A_{control}} \times 100 \tag{3}$$

where $A_{control}$ and A_{sample} are the absorbances of the methanol control and the extract, respectively. Concentration of the extract that protects 50% of β-carotene present in the solution (AACL IC_{50}) was calculated. BHA (1 mg/mL) was used as positive control.

3.8. Collagenase Inhibitory Activity

In 50 mL of citrate buffer (0.2 M, pH 5.0), 80 mg of $SnCl_2 \times 2H_2O$ was dissolved [48]. Ninhydrin solution was prepared by dissolving 0.5 g of ninhydrin in 10 mL of DMSO. The ninhydrin reagent for color development was made by mixing $SnCl_2$ solution with an equal volume of ninhydrin solution before use. To the solution of the extract, gelatin (7 µL, 2 mg/mL) and collagenase (7 µL, 1 mg/mL) were dissolved in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$ and 1 µM $ZnCl_2$). Quench buffer contained 12% (w/v) PEG 6000 and 25 mM EDTA. The inhibition of collagenase (ColInh) was calculated by using the following Equation (4):

$$\text{ColInh}\ (\%) = \frac{A_0 - A_s}{A_0} \times 100 \tag{4}$$

where A_0 is the absorbance of the negative control (water) and A_s is the absorbance of the respective extract. Concentration of the extract which inhibits 50% of the ovalbumin coagulation (ColInh IC_{50}) was calculated. Gallic acid (1 mg/mL) was used as the positive control.

3.9. Elastase Inhibitory Activity

Elastase inhibitory activity was determined as described previously [49]. To the 100 µL of extract solution in Tris-HCl buffer (0.1 M, pH 8.0), 1 mM N-succinyl-$(Ala)_3$-nitroanilide in the same buffer was added. Elastase solution was added after 10 min and the absorbance was measured at 410 nm after an additional 10 min. Elastase inhibitory activity (ElInh) was calculated as follows (Equation (5)):

$$\text{ElInh}\ (\%) = \frac{A_0 - A_s}{A_0} \times 100 \tag{5}$$

where A_0 is the absorbance of the negative control (solution where instead of extract the Tris-HCL buffer was used) and A_s is the absorbance of the respective extract. Ursolic acid (1 mg/mL) was used as the standard elastase inhibitor.

3.10. Hyaluronidase Inhibitory Activity

For hyaluronidase (LOX) inhibitory activity [50], 25 µL of the extract solution and 20 µL of hyaluronidase solution (4 mg/mL) were mixed and incubated for 20 min at 37 °C. After 20 min, 40 µL of 12.5 mM $CaCl_2$ was added and incubated for an additional 20 min at 37 °C. Sodium hyaluronate (50 µL, 3.5 mg/mL) was added and incubated for at 37 °C with constant shaking. After 40 min, the reaction was stopped by adding 20 µL of 0.9 M NaOH and 40 µL of 0.2 M sodium tetraborate and heating for 3 min at 100 °C. Then, 160 µL of *p*-dimethylaminobenzaldehide reagent (DMABA) (0.25 g DMABA dissolved in 4.4 mL of acetic acid and 0.6 mL of 10 M HCl) was added and the reaction mixture was incubated at 37 °C for an additional 10 min. Absorbance was measured at 585 nm. Tannic acid was used as positive control. Hyaluronidase inhibitory activity (HyalInh) was calculated as shown in Equation (6):

$$\text{HyalInh}\ (\%) = \frac{A_0 - A_s}{A_0} \times 100 \tag{6}$$

where A_0 is the absorbance of the negative control and A_s is the absorbance of the corresponding extract. HyalInh IC_{50} was calculated as the concentration of the extract that inhibited 50% of hyaluronidase activity and is expressed as µL of extract/mL of solution.

3.11. Tyrosinase Inhibitory Activity

The activity was determined following the method described in [49]. To the extract solution (80 µL), 40 µL of tyrosinase solution (in 16 mM pH 6.8 phosphate buffer) was added. After 10 min in the dark at 25 °C, 80 µL of L-DOPA solution (0.19 mg/mL in phosphate buffer) was added. The absorbance was measured at 492 nm after 10 min. Tyrosinase inhibitory activity (TyInh) was calculated as (Equation (7))

$$\text{TyInh}\ (\%) = \frac{A_0 - A_s}{A_0} \times 100 \tag{7}$$

where A_0 is the absorbance of the negative control (where buffer was used instead of the extract) and A_s is the absorbance of the respective extract. Concentration of the extract which inhibits 50% of tyrosinase activity (TyInh IC_{50}) was calculated. Kojic acid (1 mg/mL) was used as positive control.

3.12. Inhibition of Heat-Induced Ovalbumin Coagulation

The activity was evaluated by the heat-induced ovalbumin coagulation method [39]. To 0.4 mL of fresh ovalbumin solution, 2.8 mL of phosphate buffered saline (pH 6.4) and 2 mL of the extract solution were added. After 15 min at 37 °C, the solutions were heated at 70 °C for 5 min. Upon cooling of the reaction mixture, the absorbance was recorded at 660 nm. The inhibition of denaturation (OvInh) was calculated by using the following Equation (8):

$$\text{OvInh}\ (\%) = \frac{A_0 - A_s}{A_0} \times 100 \tag{8}$$

where A_0 is the absorbance of the negative control (water) and A_s is the absorbance of the respective extract. Concentration of the extract which inhibits 50% of the ovalbumin coagulation (OvInh IC_{50}) was calculated. Diclofenac sodium (1 mg/mL) was used as the positive control.

3.13. Cell Culture Conditions

The HaCaT human keratinocyte cell line (CLS Cell Line Services, Heidelberg, Germany) was cultivated using Dulbecco's modified Eagle medium (DMEM) (St. Louis, MO, USA) supplemented with fetal bovine serum (10%, Biosera, Boussens, France), penicillin, streptomycin and amphotericin B (5%, Lonza, Basel, Switzerland). The cells were passaged at 80–90% confluence. The medium was changed approximately every 48 h. The cultures were maintained at 95% humidity and 37 °C in an atmosphere of 5% CO_2.

3.14. Cell Viability Study

Cell viability was determined with the colorimetric MTT assay. HaCaT cells were seeded onto 96-well plates at a density of 2×10^4 cells/well and allowed to reach confluence over 24 h. Solutions of the extracts were mixed with Hank's balanced salt solution (HBSS; pH 6.0, Capricorn Scientific, Ebsdorfergrund, Germany). Prior to the treatment with the extracts, the cell culture medium was withdrawn, and the cells washed with HBSS. The cells were then exposed to the solutions of the extracts in concentrations of 2.5–250 µL/mL for 2 h. Cells incubated in HBSS were used as a negative control. After 2 h of treatment with the extracts, the cells were washed twice with HBSS and incubated with fresh medium (500 µL/well) for 24 h. A total of 50 µL of the MTT solution (5 mg/mL) was added to each well. After 1 h at 37 °C, the medium was removed, and the cells were lysed. Formazan was dissolved with acidic isopropanol and its quantity quantified spectrophotometrically at 570 nm (1420 Multilabelcounter VICTOR3, PerkinElmer, Waltham, MA, USA). Metabolic activity was expressed as relative to control (untreated cells incubated in HBSS).

3.15. In Vitro Scratch Wound Healing Assay

In vitro scratch wound healing assay was performed according to Blažević et al., 2016 [42]. The HaCaT cells were seeded onto 24-well plates at a density of 10^5 cells/well and a volume of 500 µL/well and allowed to reach adequate confluence over 24 h in DMEM supplemented with 10% FBS and 5% antibiotic. Thereafter, the medium was removed and replaced with serum-free medium. After 24 h, a sterile 10 µL pipette tip was used to scrape across each well, creating a "wound" with a cell-free area. The cell monolayer was washed gently with HBSS (pH 6.0) to remove detached cells and cell debris. The wounds were exposed to the extracts' solutions in HBSS for 2 h. Each well was marked below the plate surface to allow the identification of the same scratched area. After a 2 h treatment, the cells were washed with HBSS and incubated with serum-free medium in a volume of 500 µL/well. Wounds exposed to HBSS were used as a negative control. In vitro wound epithelization was monitored over 48 h, every 24 h, using phase-contrast microscopy (10× magnification; Primovert, Carl Zeiss AG, Oberkochen, Germany). The scratch area was measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The percentage of wound closure (PWC) was expressed as the percentage of scratch closure in relation to the initial scratch area, according to Equation (9):

$$\mathrm{PWC}\ (\%) = \frac{\mathrm{A_0} - \mathrm{A_t}}{\mathrm{A_0}} \times 100 \quad (9)$$

where $\mathrm{A_0}$ is the scratch area at time 0 and $\mathrm{A_t}$ is the corresponding scratch area at 24 or 48 h.

3.16. Statistical Analysis

Design-Expert software v. 8.0.6 (Stat-Ease, Minneapolis, MN, USA) was used for the experimental design preparation (Box–Behnken) and validation (ANOVA) of Box–Behnken results. For evaluation of antioxidant and enzyme inhibiting activity, the results were presented as the mean ± standard deviation of three measurements. IC_{50} values were calculated using regression analysis. For wound healing assay, two independent experiments were performed, using three wells for each treatment. Statistical comparisons were made between the extracts using Students' *t*-test (GraphPad Prism) and Dunnett's post hoc test was used for comparison with the control. *p*-values < 0.05 were considered statistically significant.

4. Conclusions

E. purpurea aerial parts contain caffeic acid derivatives, potent cosmeceutical ingredients. In this work, the UAE method for preparation of *E. purpurea* bioactive extracts was developed. The extraction was performed using mixtures of water with glycerol, an environmentally friendly and safe solvent, used as a vehicle and active ingredient in cosmetic products. The extraction was optimized to obtain the extracts with the highest amount of phenolic acid and the best antiradical activity. The prepared extracts displayed excellent radical scavenging, Fe^{2+} chelating and antioxidant activity. In addition to that, collagenase, elastase and tyrosinase inhibitory activities, as well as their anti-inflammatory activity, indicate excellent antiaging properties of the extracts. The hyaluronidase inhibiting and wound healing effects were especially pronounced. The conducted research confirms a significant potential of *E. purpurea* extracts as valuable ingredients of cosmeceuticals with antiaging and wound healing properties.

Author Contributions: Conceptualization, M.Z.K.; methodology, P.C., L.J., P.M., L.N.N., A.H. and M.Z.K.; validation, M.Z.K.; investigation, P.C., L.J., P.M. and L.N.N.; resources, A.H. and M.Z.K.; writing—original draft preparation, L.J. and M.Z.K.; writing—review and editing, L.N.N., A.H. and M.Z.K.; supervision, L.N.N., A.H. and M.Z.K.; project administration, M.Z.K.; funding acquisition, M.Z.K. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financed in the course of the project "Bioactive plant principles extraction using green solvents—a step towards green cosmeceuticals" (IP-2018-01-6504) supported by the Croatian Science Foundation and in collaboration with the company Suban.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Pandey, A.; Jatana, G.K.; Sonthalia, S. Cosmeceuticals. In *StatPearls*; StatPearls Publishing: Treasure Island, FL, USA, 2020.
2. Costa, R.; Santos, L. Delivery Systems for Cosmetics—From Manufacturing to the Skin of Natural Antioxidants. *Powder Technol.* **2017**, *322*, 402–416. [CrossRef]
3. de Lima Cherubim, D.J.; Martins, C.V.B.; Fariña, L.O.; da Silva de Lucca, R.A. Polyphenols as Natural Antioxidants in Cosmetics Applications. *J. Cosmet. Dermatol.* **2020**, *19*, 33–37. [CrossRef]
4. Chemat, F.; Vian, M.A.; Cravotto, G. Green Extraction of Natural Products: Concept and Principles. *Int. J. Mol. Sci.* **2012**, *13*, 8615–8627. [CrossRef] [PubMed]
5. Chemat, F.; Vian, M.A.; Ravi, H.K.; Khadhraoui, B.; Hilali, S.; Perino, S.; Tixier, A.-S.F. Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects. *Molecules* **2019**, *24*, 3007. [CrossRef] [PubMed]
6. Wolfson, A.; Dlugy, C.; Shotland, Y. Glycerol as a Green Solvent for High Product Yields and Selectivities. *Environ. Chem. Lett.* **2007**, *5*, 67–71. [CrossRef]
7. Karsch-Völk, M.; Barrett, B.; Kiefer, D.; Bauer, R.; Ardjomand-Woelkart, K.; Linde, K. Echinacea for Preventing and Treating the Common Cold. *Cochrane Database Syst. Rev.* **2014**, *2014*, CD000530. [CrossRef]
8. Echinaceae purpureae Herba. Available online: https://www.ema.europa.eu/en/medicines/herbal/echinaceae-purpureae-herba (accessed on 13 February 2020).
9. Senica, M.; Mlinsek, G.; Veberic, R.; Mikulic-Petkovsek, M. Which Plant Part of Purple Coneflower (*Echinacea purpurea* (L.) Moench) Should Be Used for Tea and Which for Tincture? *J. Med. Food* **2019**, *22*, 102–108. [CrossRef] [PubMed]
10. *European Pharmacopoeia*, 8th ed.; Council of Europe: Strasbourg, France, 2013.
11. Peng, Y.; Sun, Q.; Park, Y. The Bioactive Effects of Chicoric Acid as a Functional Food Ingredient. *J. Med. Food* **2019**, *22*, 645–652. [CrossRef] [PubMed]
12. Koriem, K.M.M. Caftaric Acid: An Overview on Its Structure, Daily Consumption, Bioavailability and Pharmacological Effects. *Biointerface Res. Appl. Chem.* **2020**, *10*, 5616–5623. [CrossRef]
13. Yotsawimonwat, S.; Rattanadechsakul, J.; Rattanadechsakul, P.; Okonogi, S. Skin Improvement and Stability of *Echinacea purpurea* Dermatological Formulations. *Int. J. Cosmet. Sci.* **2010**, *32*, 340–346. [CrossRef]
14. Riciputi, Y.; Diaz-de-Cerio, E.; Akyol, H.; Capanoglu, E.; Cerretani, L.; Caboni, M.F.; Verardo, V. Establishment of Ultrasound-Assisted Extraction of Phenolic Compounds from Industrial Potato by-Products Using Response Surface Methodology. *Food Chem.* **2018**, *269*, 258–263. [CrossRef]
15. Fumić, B.; Jug, M.; Končić, M.Z. Optimization of Ultrasound-Assisted Extraction of Phenolic Antioxidants from Lotus Corniculatus. *Croat. Chem. Acta* **2019**, *92*, 369–377. [CrossRef]
16. Pandey, A.; Belwal, T.; Sekar, K.C.; Bhatt, I.D.; Rawal, R.S. Optimization of Ultrasonic-Assisted Extraction (UAE) of Phenolics and Antioxidant Compounds from Rhizomes of *Rheum moorcroftianum* Using Response Surface Methodology (RSM). *Ind. Crop. Prod.* **2018**, *119*, 218–225. [CrossRef]
17. Momchev, P.; Ciganović, P.; Jug, M.; Marguí, E.; Jablan, J.; Končić, M.Z. Comparison of Maceration and Ultrasonication for Green Extraction of Phenolic Acids from *Echinacea purpurea* Aerial Parts. *Molecules* **2020**, *25*, 5142. [CrossRef]
18. Spagnol, C.M.; Assis, R.P.; Brunetti, I.L.; Isaac, V.L.B.; Salgado, H.R.N.; Corrêa, M.A. In Vitro Methods to Determine the Antioxidant Activity of Caffeic Acid. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* **2019**, *219*, 358–366. [CrossRef]
19. Main Cosmetic Vehicles. In *Handbook of Cosmetic Science and Technology*; Barel, A.; Paye, M.; Maibach, H. (Eds.) Marcel Dekker: New York, NY, USA, 2001.
20. Mlakar, A.; Batna, A.; Dudda, A.; Spiteller, G. Iron (II) Ions Induced Oxidation of Ascorbic Acid and Glucose. *Free Radic. Res.* **1996**, *25*, 525–539. [CrossRef]

21. Ratz-Łyko, A.; Arct, J. Resveratrol as an Active Ingredient for Cosmetic and Dermatological Applications: A Review. *J. Cosmet. Laser Ther. Off. Publ. Eur. Soc. Laser Dermatol.* **2019**, *21*, 84–90. [CrossRef] [PubMed]
22. Thiele, J.; Elsner, P. *Oxidants and Antioxidants in Cutaneous Biology*; Current Problems in Dermatology; Karger: Basel, Switzerland, 2001; Volume 29.
23. Zhu, X.; Huang, F.; Xiang, X.; Fan, M.; Chen, T. Evaluation of the Potential of Chicoric Acid as a Natural Food Antioxidant. *Exp. Ther. Med.* **2018**, *16*, 3651–3657. [CrossRef]
24. Ganceviciene, R.; Liakou, A.I.; Theodoridis, A.; Makrantonaki, E.; Zouboulis, C.C. Skin Anti-Aging Strategies. *Dermato-Endocrinol.* **2012**, *4*, 308–319. [CrossRef]
25. Aziz, J.; Shezali, H.; Radzi, Z.; Yahya, N.A.; Kassim, N.H.A.; Czernuszka, J.; Rahman, M.T. Molecular Mechanisms of Stress-Responsive Changes in Collagen and Elastin Networks in Skin. *Skin Pharmacol. Physiol.* **2016**, *29*, 190–203. [CrossRef]
26. Uitto, J. Connective Tissue Biochemistry of the Aging Dermis: Age-Associated Alterations in Collagen and Elastin. *Clin. Geriatr. Med.* **1989**, *5*, 127–148. [CrossRef] [PubMed]
27. Ersoy, E.; Ozkan, E.E.; Boga, M.; Yilmaz, M.A.; Mat, A. Anti-Aging Potential and Anti-Tyrosinase Activity of Three Hypericum Species with Focus on Phytochemical Composition by LC–MS/MS. *Ind. Crop. Prod.* **2019**, *141*, 111735. [CrossRef]
28. Imokawa, G. Recent Advances in Characterizing Biological Mechanisms Underlying UV-Induced Wrinkles: A Pivotal Role of Fibrobrast-Derived Elastase. *Arch. Dermatol. Res.* **2008**, *300* (Suppl. 1), S7–S20. [CrossRef]
29. Chaiyana, W.; Charoensup, W.; Sriyab, S.; Punyoyai, C.; Neimkhum, W. Herbal Extracts as Potential Antioxidant, Anti-Aging, Anti-Inflammatory, and Whitening Cosmeceutical Ingredients. *Chem. Biodivers.* **2021**, *18*, e2100245. [CrossRef] [PubMed]
30. Wittenauer, J.; Mäckle, S.; Sußmann, D.; Schweiggert-Weisz, U.; Carle, R. Inhibitory Effects of Polyphenols from Grape Pomace Extract on Collagenase and Elastase Activity. *Fitoterapia* **2015**, *101*, 179–187. [CrossRef] [PubMed]
31. Papakonstantinou, E.; Roth, M.; Karakiulakis, G. Hyaluronic Acid: A Key Molecule in Skin Aging. *Dermato-Endocrinol.* **2012**, *4*, 253–258. [CrossRef] [PubMed]
32. Chaiyana, W.; Anuchapreeda, S.; Punyoyai, C.; Neimkhum, W.; Lee, K.-H.; Lin, W.-C.; Lue, S.-C.; Viernstein, H.; Mueller, M. *Ocimum sanctum* Linn. as a Natural Source of Skin Anti-Ageing Compounds. *Ind. Crop. Prod.* **2019**, *127*, 217–224. [CrossRef]
33. Facino, R.M.; Carini, M.; Aldini, G.; Marinello, C.; Arlandini, E.; Franzoi, L.; Colombo, M.; Pietta, P.; Mauri, P. Direct Characterization of Caffeoyl Esters with Antihyaluronidase Activity in Crude Extracts from *Echinacea angustifolia* Roots by Fast Atom Bombardment Tandem Mass Spectrometry. *Farm. Soc. Chim. Ital.* **1993**, *48*, 1447–1461.
34. Lengers, I.; Herrmann, F.; Le Borgne, M.; Jose, J. Improved Surface Display of Human Hyal1 and Identification of Testosterone Propionate and Chicoric Acid as New Inhibitors. *Pharmaceuticals* **2020**, *13*, 54. [CrossRef]
35. Pazyar, N.; Yaghoobi, R.; Rafiee, E.; Mehrabian, A.; Feily, A. Skin Wound Healing and Phytomedicine: A Review. *Skin Pharmacol. Physiol.* **2014**, *27*, 303–310. [CrossRef]
36. Chang, T.-S. An Updated Review of Tyrosinase Inhibitors. *Int. J. Mol. Sci.* **2009**, *10*, 2440–2475. [CrossRef]
37. Honisch, C.; Osto, A.; de Matos, A.D.; Vincenzi, S.; Ruzza, P. Isolation of a Tyrosinase Inhibitor from Unripe Grapes Juice: A Spectrophotometric Study. *Food Chem.* **2020**, *305*, 125506. [CrossRef] [PubMed]
38. Bessada, S.M.F.; Alves, R.C.; Oliveira, M.B.P.P. Coffee Silverskin: A Review on Potential Cosmetic Applications. *Cosmetics* **2018**, *5*, 5. [CrossRef]
39. Chandra, S.; Chatterjee, P.; Dey, P.; Bhattacharya, S. Evaluation of in Vitro Anti-Inflammatory Activity of Coffee against the Denaturation of Protein. *Asian Pac. J. Trop. Biomed.* **2012**, *2*, S178–S180. [CrossRef]
40. Penkova, R.; Goshev, I.; Gorinstein, S.; Nedkov, P. Stability of Collagen during Denaturation. *J. Protein Chem.* **1999**, *18*, 397–401. [CrossRef]
41. Colombo, I.; Sangiovanni, E.; Maggio, R.; Mattozzi, C.; Zava, S.; Corbett, Y.; Fumagalli, M.; Carlino, C.; Corsetto, P.A.; Scaccabarozzi, D.; et al. HaCaT Cells as a Reliable in Vitro Differentiation Model to Dissect the Inflammatory/Repair Response of Human Keratinocytes. *Mediat. Inflamm.* **2017**, *2017*, 7435621. [CrossRef]
42. Blažević, F.; Milekić, T.; Romić, M.D.; Juretić, M.; Pepić, I.; Filipović-Grčić, J.; Lovrić, J.; Hafner, A. Nanoparticle-Mediated Interplay of Chitosan and Melatonin for Improved Wound Epithelialisation. *Carbohydr. Polym.* **2016**, *146*, 445–454. [CrossRef] [PubMed]
43. Kawano, Y.; Patrulea, V.; Sublet, E.; Borchard, G.; Iyoda, T.; Kageyama, R.; Morita, A.; Seino, S.; Yoshida, H.; Jordan, O.; et al. Wound Healing Promotion by Hyaluronic Acid: Effect of Molecular Weight on Gene Expression and in Vivo Wound Closure. *Pharmaceuticals* **2021**, *14*, 301. [CrossRef] [PubMed]
44. Ibezim, E.C.; Momoh, M.A.; Onyishi, V.I.; Nwabunike, I.; Odimegwu, D.C.; Ibezim, N.E.; Nzekwe, N.J. Evaluating the Ethyl-Acetate Fraction of Crude Methanol Leaf Extract of Ocimum Gratissimum Formulated as Ointments for Wound Healing Properties Using the Excision Wound Model. *J. Pharm. Allied Sci.* **2018**, *15*. [CrossRef]
45. Jug, M.; Končić, M.Z.; Kosalec, I. Modulation of Antioxidant, Chelating and Antimicrobial Activity of Poplar Chemo-Type Propolis by Extraction Procures. *LWT-Food Sci. Technol.* **2014**, *57*, 530–537. [CrossRef]
46. Končić, M.Z.; Barbarić, M.; Perković, I.; Zorc, B. Antiradical, Chelating and Antioxidant Activities of Hydroxamic Acids and Hydroxyureas. *Molecules* **2011**, *16*, 6232–6242. [CrossRef] [PubMed]
47. Rajić, Z.; Končić, M.; Miloloža, K.; Perković, I.; Butula, I.; Bucar, F.; Zorc, B. Primaquine-NSAID Twin Drugs: Synthesis, Radical Scavenging, Antioxidant and Fe^{2+} Chelating Activity. *Acta Pharm.* **2010**, *60*, 325–337. [CrossRef] [PubMed]

48. Zhang, Y.; Fu, Y.; Zhou, S.; Kang, L.; Li, C. A Straightforward Ninhydrin-Based Method for Collagenase Activity and Inhibitor Screening of Collagenase Using Spectrophotometry. *Anal. Biochem.* **2013**, *437*, 46–48. [CrossRef]
49. Jabłonowska, M.; Ciganović, P.; Jablan, J.; Marguí, E.; Tomczyk, M.; Končić, M.Z. Silybum Marianum Glycerol Extraction for the Preparation of High-Value Anti-Ageing Extracts. *Ind. Crop. Prod.* **2021**, *168*, 113613. [CrossRef]
50. Jiratchayamaethasakul, C.; Ding, Y.; Hwang, O.; Im, S.-T.; Jang, Y.; Myung, S.-W.; Lee, J.M.; Kim, H.-S.; Ko, S.-C.; Lee, S.-H. In Vitro Screening of Elastase, Collagenase, Hyaluronidase, and Tyrosinase Inhibitory and Antioxidant Activities of 22 Halophyte Plant Extracts for Novel Cosmeceuticals. *Fish. Aquat. Sci.* **2020**, *23*, 6. [CrossRef]

2.3. Glycerolic Licorice Extracts as Active Cosmeceutical Ingredients: Extraction Optimisation, Chemical Characterisation, and Biological Activity

antioxidants

MDPI

Article

Glycerolic Licorice Extracts as Active Cosmeceutical Ingredients: Extraction Optimization, Chemical Characterization, and Biological Activity

Petar Ciganović [1], Katarzyna Jakimiuk [1,2], Michał Tomczyk [2] and Marijana Zovko Končić [1,*]

[1] Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10000 Zagreb, Croatia; petar.ciganovic@me.com (P.C.); katarzynajakimiuk1@gmail.com (K.J.)

[2] Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Białystok, ul. Mickiewicza 2a, 15-230 Białystok, Poland; michal.tomczyk@umb.edu.pl

* Correspondence: mzovko@pharma.hr; Tel.: +385-1-6394-792

Received: 30 June 2019; Accepted: 3 September 2019; Published: 1 October 2019

check for updates

Abstract: A green ultrasound-assisted extraction (UAE) method using glycerol/water mixtures for extraction of licorice (*Glycyrrhiza glabra*) bioactive constituents was developed in this study. The response surface method, according to the Box-Behnken design, was employed to optimize the extraction parameters: glycerol concentration (X_1), temperature (X_2), and the amount of herbal drug used in the production (X_3). The responses were content of total phenols (TP), TP extraction efficiency (TPy) and the content of licorice characteristic constituents, glabridin (Gla) and isoliquiritigenin (Iso). Response surface analysis predicted the optimal extraction conditions for maximized amounts of TP, Tpy, Gla, and Iso. The extracts were prepared using the calculated conditions. The analysis of the selected constituents confirmed the validity of the model. Furthermore, biological activity of the extracts was tested. The results demonstrate that UAE using glycerol is a fast and efficient method for preparation of extracts with excellent radical scavenging, Fe^{2+} chelating and antioxidant activity. Furthermore, the observed notable tyrosinase and elastase inhibitory activity of the extracts, as well as their anti-inflammatory activity, indicate the anti-aging properties of the investigated extracts. The fact that the extracts were prepared using the safe, cosmetically active solvent, glycerol, makes them suitable for direct use in specialized cosmeceutical formulations.

Keywords: licorice; anti-inflammatory; antioxidant; cosmetic; elastase inhibitory activity; green extraction; tyrosinase inhibitory activity

1. Introduction

The growing importance of physical appearance in the last century has led to an expansion of sophisticated beauty products purported to have high, almost pharmaceutical, efficacy, sensorial advantages, and safety. Such products, popularly called cosmeceuticals, are applied on the human skin, making it appear younger and healthier. Even though the word "cosmeceutical" is a marketing, rather than a legal term, it is often used in lay language because it reflects both the intended dual activity of such products, as well as the consumers' expectations. Furthermore, the products that are derived from natural sources, such as plants, are in special demand, not only due to the consumers preferences for natural skincare, but also because of their numerous beneficial effects on human skin [1].

Before being incorporated into cosmetic products, the bioactive principles of plants need to be extracted from crude plant material. The selection of an appropriate extraction method is one of the key steps to consider before proceeding to cosmeceutical formulation development. Failure to do so could lead to the loss of active compounds, hence resulting in the loss of biological activity.

However, in addition to displaying the desired biological properties, the extracts used in modern cosmetic products have to fulfill other requirements. Besides stability, safety, and sensory properties, new concerns about environmental impact or animal welfare with respect to the cosmetic development, manufacturing, and quality control are constantly emerging, and new products are being developed in order to meet such needs [2]. For example, the design of green and sustainable extraction methods for natural products is currently a hot research topic in the multidisciplinary area of applied chemistry, biology, and technology. Solvents used for extraction should ideally have a high dissolving power, be biodegradable, non-toxic, and non-flammable. Ethanol, due to its biodegradability and natural origin, fulfills some of the requirements for a green solvent, and it is still among the most used solvents for extraction of natural compounds. However, besides being a relatively good solvent for a wide range of natural products, ethanol is highly flammable and has skin-irritant properties. Thus, efforts are being made to replace ethanol with other solvents, preferably of natural origin [3].

One of the solvents that could effectively replace ethanol is glycerol, a natural, non-toxic, biodegradable liquid, manufactured from renewable sources [4]. Due to the hygroscopic nature of glycerol, it is already widely used for formulation of creams and lotions [3]. Therefore, the glycerol extracts of medicinal plants have a dual role in cosmetic products—as humectants and active agents [3]. Furthermore, the use of glycerol in the finished product means that the removal of the solvent from the cosmeceutical extract is redundant. This renders the glycerolic extraction of medicinal plants highly acceptable from an energy-saving point of view. Interestingly, in spite of all the aforementioned favorable characteristics of glycerol extraction, the use of this solvent for extraction of natural products is still under-researched. Relatively few examples include the use of glycerol for extraction of phenolic antioxidants from two *Artemisia* species [5], grapefruit peels [6], *Hypericum perforatum*, and olive (*Olea europaea*) leaves [7]; as well as stevioside from *Stevia rebaudiana* [8].

Licorice (*Glycyrrhiza glabra* L., Fabaceae) is a perennial plant, well-known for its sweet-tasting root. It contains a wide array of bioactive natural products. Glycyrrhizin, the sweet principle of licorice root is a triterpene-type saponin that displays antiviral, anti-inflammatory, antitumor, and antimicrobial properties [9]. Besides glycyrrhizin, phenolic components, such as chalcone isoliquiritigenin and isoflavonoid glabridin are also important for the observed biological activity of licorice root. *G. glabra* has been traditionally used for promotion of wound healing. Licorice root extracts protect the skin against oxidative stress injuries [10,11], accelerate wound epithelization, ameliorate remodeling at the wound site [12], and efficiently reduce the symptoms of atopic dermatitis (AD). Furthermore, isoliquiritigenin was also found to be beneficial for the treatment of AD-like skin lesions in mice, giving hope that it could be a potential therapeutic agent for the treatment of AD in humans [13]. Glabridin has many properties potentially beneficial in cosmeceutical products. It acts as antioxidant, estrogenic, anti-inflammatory, and skin-whitening agent [14]. It displays skin depigmentation activity and is being incorporated in topical products intended specifically for that purpose [15].

G. glabra extracts and its constituents display a wide array of activities potentially useful in cosmetic and dermatologic products. The aim of this work was extraction optimization of phenolic compounds from licorice root using glycerol, a non-toxic and eco-friendly solvent. Skin-related biological activities (antioxidant, enzyme inhibiting and anti-inflammatory) of the prepared extracts were investigated with the aim of obtaining highly active extracts suitable for use in cosmeceutical products.

2. Materials and Methods

2.1. Chemicals

Reagents, standards and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of the standards was butylated hydroxyanisole (BHA, ≥98.5%), glycyrrhizic acid ammonium salt (≥95.0%), glabridin (Gla) (≥98.0%), and isoliquiritigenin (Iso) (≥98.0%). Methanol and acetonitrile were HPLC grade. Other reagents and chemicals were of analytical grade.

2.2. Plant Material

The plant material (licorice root) was donated by the Suban company (Samobor, Croatia). The exact licorice species was determined using HPLC. The material was confirmed to be *G. glabra* based on the presence of Gla [16]. The presence of other related species was excluded by the absence of quercetine (*G. uralensis*) [17] and licochalcone A [16]. The identity was additionally confirmed using a pharmacopoeial monograph [18]. A voucher specimen is deposited in the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb.

2.3. Preparation of the Extracts

The root was milled and passed through a sieve of 850 µm mesh size. Powdered plant material of differing weights (0.6–1 g) was suspended in 10 g of the appropriate solvent (10–90% glycerol in water, *w*/*w*) in a 50 mL Erlenmeyer flask. The extraction was performed in an ultrasonic bath (Bandelin SONOREX® Digital 10 P DK 156 BP, Berlin, Germany) at ultrasonication power of 360 W and frequency of 35 Hz during 20 min. The bath was temperature-controlled (20–70 °C). Upon the extraction, the mixtures were filtered. All the extracts were stored at −20 °C, in the dark.

2.4. Spectrophotometric Determination of Total Phenol Content

Total phenols (TP) content was determined using the modified Folin–Ciocalteu colorimetric method [19], by mixing 80 µL extract solution, 80 µL of Folin–Ciocalteu reagent and 80 µL of 10% sodium carbonate solution. After 1 h, absorbance at 630 nm was measured (The FLUOstar® Omega, BMG Labtech, Offenburg, Germany and Stat Fax 3200 reader, Awareness Technologies, Palm City, FL, USA). TP was expressed as mg/g of dry weight from calibration curve recorded for gallic acid.

2.5. Spectrophotometric Determination of Total Flavonoid Content

Total flavonoid (TF) content was determined using modified Folin–Ciocalteu colorimetric method [20], by mixing 120 µL extract solution and 120 µL of 0.2% $AlCl_3$ solution. After 1 h, absorbance at 420 nm was measured. TF was expressed as mg/g of dry weight from calibration curve recorded for quercetin.

2.6. RP-HPLC-DAD Determinations of Glycyrrhizin, Glabridin and Isoliquiritigenin

Prior to the analysis, the extracts were filtered through a 0.45 µm PTFE syringe filter. Quantifications were performed using an HPLC instrument (Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler and a DAD detector. Injection volume was 10 µL. The peak assignment and identification was based on comparison of UV/VIS spectra and retention times of peaks in sample chromatogram with that of the standards. Quantification was performed using the respective standard calibration curve. The calibration curves, limit of detection (LD), and limit of quantification (LQ), were determined according to [21] (Table 1). For determination of glycyrrhizin, the modified European pharmacopoeia method [18] was used. Separation was performed on a Nucleodur 100-5 C18 column (Macheray-Nagel, Düren, Germany) column. A mixture of glacial acetic acid, acetonitrile, and water (6:30:64 V/V/V) was used as mobile phase. Separation was performed at 25 °C using flow rate of 2 mL/min. Glycyrrhizic acid ammonium salt was used as a standard for construction of calibration curve. The content of Gla and Iso was determined by a modified method described by Tada et al. [22] on the Zorbax Eclipse XDB-C18 (5 µm, 12.5 mm × 4.6 mm, Agilent, Santa Clara, CA, USA). Mobile phase (water:acetonitrile) was used according to the following protocol 0–3 min (7:3), 53-60 min (2:8). Flow rate was 1.0 mL/min. Gla and Iso were used as standards for the construction of calibration curves.

Table 1. Slope, intercept and coefficient of determination (r^2) of the calibration curves *, limits of detection (LD) and quantification (LQ) for glycyrrhizin, glabridin, and isoliquiritigenin.

Analyte	Slope (a)	Intercept (b)	r^2	LD (μg)	LQ (μg)
Glycyrrhizin	257.96	1.54	0.99998638	0.006112	0.018522
Gla	3402.71	26.12	0.9999998	0.000741	0.002246
Iso	5079.81	21.21	0.9999931	0.005013	0.015191

* calibration curves are represented as $y = ax + b$, where y is the absorbance at the selected wavelength, and x is the weight of the analyte (μg).

2.7. Extraction Optimization

The experiment was planned using Box-Behnken design (BBD) in Design Expert software v. 8.0.6 (Stat-Ease, Minneapolis, MN, USA). The ranges of design parameters (independent variables) were: glycerol concentration (X_1, 10–90%, *w*/*w*), temperature (X_2, 20–70°C), and drug weight (X_3, 0.6–1g) used for the extraction. TP content, TP/X_3 ratio (TPy), as well as the Gla and Iso content of the extracts were dependent variables. Response-surface methodology was used to find the relationship between dependent and independent variables. Experimental data was analyzed by multiple regression analysis and fitted to the appropriate polynomial models. The validity of the model was confirmed by the analysis of variance (ANOVA). p values < 0.1 were considered statistically significant.

2.8. Radical Scavenging Activity

Radical scavenging activity (RSA) was evaluated using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical [23]. In short, DPPH solution (0.21 mg/mL, 70 μL) was added to the extract solution (130 μL). After 30 min, the absorbance was recorded at 545 nm. DPPH solution with methanol instead of the extract served as the negative control. RSA was calculated according to the following equation:

$$RSA\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which scavenges 50% of free radicals present in the solution (RSA IC_{50}), was calculated. BHA was used as the standard radical scavenger.

2.9. Fe^{2+} Chelating Activity

The chelating activity (ChA) of the investigated substances toward ferrous ions was studied, as described in [24]. To the solution of extract in methanol (150 μL), 0.25 mM $FeCl_2$ solution (50 μL) was added. After 5 min, 100 μL of 1.0 mM ferrozine solution was applied. Absorbance at 545 nm was recorded after 10 min. Reaction mixture containing methanol (150 μL) instead of extract served as a control. ChA was calculated using the following equation:

$$ChA\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which chelates 50% of Fe^{2+} present in the solution (ChA IC_{50}), was calculated. EDTA was used as the chelating standards.

2.10. Antioxidant Activity in β-Carotene-Linoleic Acid Assay

AOA was evaluated using the β-carotene-linoleic acid system according to modified literature procedure [25]. Aliquots (200 μL) of the emulsion containing β-carotene (6.7 μg/mL), linoleic acid (0.7 mg/mL), and Tween 40 (6.7 mg/mL) were added either to methanol (50 μL) (control) or to the solutions of the extract in methanol (50 μL). The reaction mixture was incubated at 50 °C. The antioxidant activity in β-carotene linoleic acid assay (AACL) was calculated based on the absorbances recorded after 60 min using the following equation:

$$AACL\,(\%) = \frac{A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ and A_{sample} are the absorbances of the water control and antioxidant, respectively. Concentration of the extract that protects 50% β-carotene present in the solution (AACL IC_{50}) was calculated. BHA was used as the standard antioxidant.

2.11. Tyrosinase Inhibitory Activity

Tyrosinase inhibition activity by the extracts was determined following a method described by [19] with some minor modifications. In 80 µL extract solution, 40 µL of tyrosinase solution (in 16 mM pH 6,8 phosphate buffer) was added. The solution was incubated in dark at 25 °C. After 10 min, 80 µL of L-DOPA solution (0.19 mg/mL in phosphate buffer) was added. After an additional 10 min, the absorbance at 492 nm was measured. Negative control contained a buffer instead of the extract solution. Tyrosinase inhibitory activity (TyInh) was calculated as:

$$TyInh\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which inhibits 50% of tyrosinase activity (TyInh IC_{50}), was calculated. Kojic acid was used as the standard inhibitor.

2.12. Elastase Inhibitory Activity

To 100 µL of plant extract solution, 1 mM *N*-succinyl-$(Ala)_3$-nitroanilide in Tris-HCl buffer (0.1 M, pH 8.0) was added. After 10 min, 25 °C, 25 µl of porcine pancreatic elastase solution was added. The mixture was further incubated at 25 °C for 10 min and absorbance was measured at 410 nm. A reaction mixture containing buffer instead of extract served as the control. Elastase inhibitory activity (ElInh) was calculated as:

$$ElInh\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which inhibits 50% of elastase activity (ElInh IC_{50}), was calculated. Ursolic acid was used as the standard inhibitor [26].

2.13. Anti-Inflammatory Activity

Anti-inflammatory activity was evaluated by the heat-induced ovalbumin coagulation method [27] using Perkin Elmer Lambda 25 spectrophotometer (Perkin Elmer, Waltham, MA, USA). The reaction mixture consisted of 0.4 mL of ovalbumin solution, 2.8 mL of phosphate buffered saline (pH 6.4), and 2 mL of the extract solution. The mixtures were incubated at 37 °C for 15 min and then heated at 70 °C for 5 min. After cooling, their absorbance was recorded at 660 nm. The percentage inhibition of ovalbumin denaturation (OvInh) was calculated using the following formula:

$$OvInh\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which inhibits 50% of the ovalbumin coagulation (OvInh IC_{50}), was calculated. Diclofenac sodium was used as the standard inhibitor.

2.14. Statistical Analysis

The measurements were performed in triplicate and the results presented as mean ± standard deviation. In order to establish the IC_{50} values, the experiments were performed using different concentrations (4–7 concentrations, depending on the assay). Statistical comparisons were made using one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons (GraphPad Prism, San Diego, CA, USA). p values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Response Surface Methodology

In this work, efforts were undertaken to optimize the extraction of bioactive phenolics from licorice root. Special attention was given to *G. glabra's* most prominent phenolic compounds, Gla and Iso. In order to develop a method that is not only efficient but also environmentally friendly, ultrasound-assisted extraction (UAE) was performed using glycerol/water mixtures.

UAE was used as the extraction technique due to its many advantages in comparison with conventional extraction methods, such as maceration and hot reflux extraction. It is characterized by shorter extraction time, reduced organic solvent consumption, and low energy costs [28]. During the UAE procedure, the extraction efficiency was influenced by numerous extraction parameters. These parameters interacted with each other, affecting the extraction efficacy in a more complex way. Therefore, it is important to evaluate the interactions among these parameters. Response surface methodology (RSM) could be adopted to optimize the parameters and obtain the maximum yields of target compounds [29]. In this work, RSM based on BBD was used to optimize the extraction conditions. Selection of solvent greatly influences extraction efficiency due to its physical-chemical properties, such as polarity, viscosity, and volatility. In this work, the proportion of glycerol in water was used as the first independent variable (X_1). In addition to solvent, temperature, the second independent variable (X_2), may strongly affect the efficiency of UAE. High temperature may improve the extraction process by reducing the viscosity of the solvent and increasing kinetic energy of the molecules in the solution. However, it may also lead to degradation of sensitive phytochemicals, including phenolic compounds. The influence of weight of the herbal material used for the extraction was investigated as the final independent variable (X_3). A higher weight of the drug used for the extraction may increase the content of target molecules in the extracts. However, when larger amounts of herbal drugs are extracted with organic/solvent water mixtures, swelling of the herbal material with water may change the proportions of the solvents in the mixture and consequently the polarity of the extraction mixture [30]. In addition, too high drug/solvent ratio may lead to unnecessary waste generation.

The aim of this study was to not only maximize the total extraction yield of the target compounds (TP, Gla and Iso) within the studied extraction parameters range, but also to achieve better utilization of the crude herbal drug. Therefore, maximized TP extraction yield (TPy) calculated as TP/X_3 was also investigated. The influence of the independent variables on the amount of target substances is presented in Table 2. The results clearly show that the extraction variables have a great impact on the success of the extraction. Depending on the extraction parameters, the amount of TP and Iso change approximately threefold and range from 279.5 µg/mL to 790.6 µg/mL, and 2.00 µg/mL to 5.76 µg/mL, respectively. However, the most dramatic change is observed in the more-than-fourfold increase of the Gla concentration (3.99–17.30 µg/mL). Keeping in mind the skin-related biological activities of Gla, this finding confirms the importance of careful selection of the extraction conditions for cosmeceutical ingredients. Detailed influence of extraction parameters on the selected responses will be presented later.

Table 2. Independent variables, their levels for the Box–Behnken design, and the responses obtained.

Run	X_1	X_2	X_3	TP	TPy	Gla	Iso
	(%. *w/w*)	(°C)	(g)	(µg/mL)	(µg/g mL)	(µg/mL)	(µg/mL)
1	50	70	0.6	605.3	1008.8	9.12	3.20
2	10	45	0.6	529.6	882.7	4.37	2.48
3	50	70	1.0	753.9	753.9	14.11	5.20
4	50	45	0.8	606.3	757.8	5.29	3.31
5	10	70	0.8	790.6	988.3	6.56	3.11
6	50	45	0.8	779.1	973.9	6.56	2.86
7	50	45	0.8	676.8	846.0	6.09	2.79
8	90	20	0.8	279.5	349.3	12.9	2.00
9	10	45	1.0	748.7	748.7	6.96	4.27
10	10	20	0.8	633.6	792.0	3.99	3.47
11	50	45	0.8	582.2	727.7	4.40	2.07
12	90	70	0.8	518.7	648.3	17.30	5.76
13	90	45	0.6	302.1	503.4	10.14	2.47
14	50	45	0.8	620.8	776.0	6.39	3.29
15	50	20	1.0	638.3	638.3	7.63	3.79
16	90	45	1.0	346.6	346.6	16.18	4.26
17	50	20	0.6	447.4	745.7	5.48	2.02

Independent variables: X_1 = glycerol content, X_2 = temperature, X_3 = weight of the plant material in 10 mL of solvent. TP, TPy, Gla, Iso: concentration of total phenols, TP/X_3 ratio, glabridin and isoliquiritigenin, respectively.

3.2. Fitting the Model

Multiple regression analysis was used to analyze the experimental results. Table 3 shows the relationship between the independent and dependent variables in the form of polynomial equations. Furthermore, Figure 1 shows the three-dimensional surface and contour plots of the models, which allowed visualizing the effects of the three selected parameters on dependent variables. It can be observed that the glycerol content influenced all the dependent variables as linear term. Furthermore, TP, TPy and Gla were influenced by glycerol content as quadratic term (Table 3). This is clearly visible in Figure 1(a1,2,b1,2), where relatively low glycerol concentration beneficially influenced both TP and TPy extraction efficiency. Gla and Iso concentration, on the other hand, were more favorably influenced by the higher glycerol content (Figure 1(c1,2,d1,2)). This indicates that, unlike the Gla and Iso, the majority of phenols in *G. glabra* root are relatively hydrophilic in nature. Known examples include flavonoid glycosides liquiritin, isoliquiritin, 5,8-dihydroxy-flavone-7-*O*-beta-D-glucuronide, and others [15].

Table 3. Polynomial equations of the models in terms of coded factors.

Response	Unit	The Equation Coefficients: $a \times X_1^2 + b \times X_2^2 + c \times X_3^2 + d \times X_1 \times X_2 + e \times X_1 \times X_3 + f \times X_2 \times X_3 + g \times X_1 + h \times X_2 + i \times X_3 + j$									
		a	*b*	*c*	*D*	*e*	*f*	*g*	*h*	*i*	*j*
TP	mg/mL	−113.482 ^{a}	16.020	−57.818 ^{b}	20.547	−43.631	−10.56	−156.966 ^{a}	83.702 ^{a}	75.393 ^{a}	653.045
TPy	mg/g mL	−144.084 ^{a}	22.256	−51.862	25.684	−5.703	−36.864	−195.495 ^{a}	109.235 ^{a}	−81.642 ^{a}	816.306
Gla	µg/mL	2.384 ^{a}	2.058 ^{a}	1.283 ^{a}	0.459	0.863	0.708	4.329 ^{a}	2.135 ^{a}	1.972 ^{a}	5.746
Iso	µg/mL	0.271	0.451	0.237 ^{b}	1.032 ^{a}	−0.003	0.055	0.146	0.748 ^{a}	0.919 ^{a}	2.864

X_1 = glycerol content, X_2 = temperature, X_3 = weight of the plant material in 10 mL of solvent. TP, Gla, Iso: concentration of total phenols, glabridin and isoliquiritigenin, respectively. ^{a,b} = The significant equation terms ^{a} = $p < 0.05$, ^{b} = $p < 0.1$.

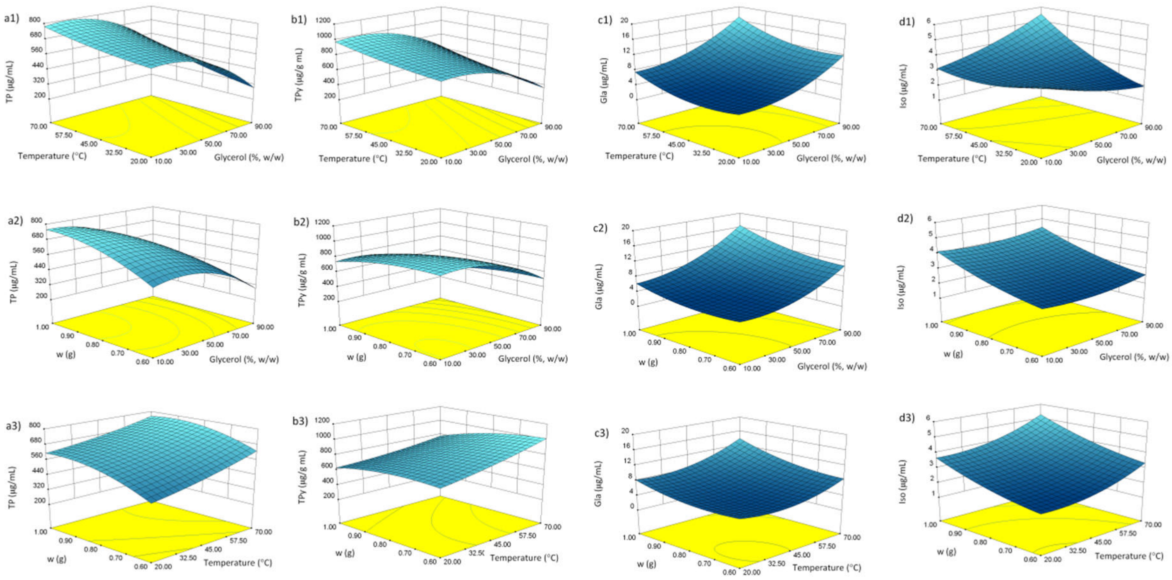


Figure 1. Response surface plots for content of phenols in licorice root extracts: Total phenols (TP) (**a1**–**3**), TP/X_3 (TPy) (**b1**–**3**), glabridin (Gla) (**c1**–**3**), and isoliquiritigenin (Iso) (**d1**–**3**). For significant model terms, see Table 3.

Table 3 shows that the influence of temperature was observed either as linear term (all the dependent variables), quadratic term (Gla), or as the interaction of temperature with glycerol content (Iso). In general, the elevated temperature positively influenced the extraction of phenolics from *G. glabra* root, indicating their good thermostability *G. glabra* (Figure 1(a1,3,b1,3,c1,3,d1,3)). This may be explained by the decreased viscosity of the solvent at high temperature, an effect particularly important in case of viscous solvents such as glycerol.

The positive influence of drug weight as linear term was, expectedly, observed in all the dependent variables (Table 3, Figure 1(a2,3,b2,3,c2,3,d2,3)). In addition, its mild positive influence as quadratic term was observed in Iso extraction. It is interesting to note that a negative, albeit weak, influence of drug weight as quadratic term was observed in case of TP concentration. This may be explained by the property of dry plant material to re-hydrate in a water solution. It may be postulated that the swelling of the material caused the increase in glycerol content, thus changing the composition and polarity of the solvent. This effect is less pronounced with smaller amounts of the herbal drug.

3.3. Model Analysis

ANOVA (Table 4) has shown that the relationship between the response variables and independent variables can be satisfactorily expressed using quadratic polynomial equations (Table 3, Figure 1). The statistical significance of each model was calculated using the *F*-test and *p*-values. The calculated *F*-values were higher than 10, while the *p*-values were lower than 0.003. This indicates that the models are highly significant and that they can be used to optimize the extraction variables. Lack-of-fit in the models was statistically insignificant, relative to the pure error which demonstrated that the fitting model is adequate to describe the experimental data. The determination coefficients (r^2) were relatively high ($0.9307 \leq r^2 \leq 0.9739$), showing that the observed values are well replicated by the model. The predicted r^2 were in reasonable agreement with the adjusted ones, further confirming that the models may be used to predict and optimize the amount of target substances in the extracts.

Table 4. Analysis of variance (ANOVA) for the fitted quadratic models for optimization of *G. glabra* extraction process.

	TP					TPy				
r^2	$r^2 = 0.9329$; $r_A{}^2 = 0.8467$; $r_P{}^2 = 0.8027$					$r^2 = 0.9325$; $r_A{}^2 = 0.8457$; $r_P{}^2 = 0.8389$				
Source	SS	df	MS	*F* Value	*p*-value	SS	df	MS	*F* Value	*p*-value
Model	379,961.7	9	42,218	10.82	0.0024	565,610.6	9	62,845.62	10.74213	0.0025
Lack of Fit	2607.6	3	869	0.14	0.9305	2337.216	3	779.072	0.0807	0.9671
Pure Error	24,713.9	4	6178			38,615.51	4	9653.877		
	Gla					**Iso**				
r^2	$r^2 = 0.9739$; $r_A{}^2 = 0.9403$; $r_P{}^2 = 0.7444$					$r^2 = 0.9307$; $r_A{}^2 = 0.8415$; $r_P{}^2 = 0.6810$				
Source	SS	df	MS	*F* Value	*p*-value	SS	df	MS	*F* Value	*p*-value
Model	277.3	9	30.811	29.01	<0.0001	17.23	9	1.915	10.44	0.0027
Lack of Fit	4.24	3	1.412	1.76	0.2926	0.27	3	0.09	0.36	0.7892
Pure Error	3.2	4	0.8			1.01	4	0.253		

SS = Sum of Squares; df = degrees of freedom; MS = Mean Square. $r_A{}^2$ = adjusted r^2; $r_P{}^2$ = predicted r^2. TP, TPy, Gla, Iso: concentration of total phenols, TP/X_3 ratio, glabridin and isoliquiritigenin, respectively.

3.4. Validation of Optimal Extraction Conditions

Based on the experimental results and statistical analysis, numerical optimizations were conducted in order to establish the optimum levels of independent variables (Table 4). As previously mentioned, the most important extraction factor for majority of the investigated parameters was glycerol concentration. It is well known that the extraction solvent greatly affects extraction efficiency. In this work, the glycerol content needed for optimal extraction of specific phenolic compounds varied according to the response. In general, TP were best extracted using moderate glycerol concentration, as reflected in the maximized TP at 20%. Similar solvent composition was the most suited for extraction of phenolics from grapefruit peels [6]. However, using a slightly higher percentage of ethanol (30% instead of 20%) resulted in better usage of the crude drug, albeit with somewhat lower TP. Gla and Iso, on the other hand, were most efficiently extracted using 85% glycerol. The extraction temperature of 70°C was the best suited for all the desired responses, while the amount of drug needed for the optimal extraction was, expectedly, lowest in the case of TPy. The selected conditions were applied for the preparation of extracts with the desired properties. The predicted results matched well with the experimental ones, with relatively low deviations from calculated values, indicating good suitability of the selected models (Table 5).

Table 5. Predicted and observed values for the optimized extracts.

Extract	Measured Response	X_1 (%, *w/w*)	X_2 (°C)	X_3 (g)	$Resp_{pred}$	$Resp_{ms}$	RD (%)
TP-opt	TP (µg/mL)	20	70	0.93	830.2	854.6	2.9
Tpy-opt	TP (µg/mL)	30	70	0.7	734.8	791.6	7.7
Gla-Iso-opt	Glabridin (µg/mL)	85	70	1	20.67	21.89	5.9
Gla-Iso-opt	Isoliquiritigenin (µg/mL)	85	70	1	6.51	6.23	−4.3

X_1 = glycerol content, X_2 = temperature, X_3 = weight of the plant material in 10 mL of solvent. $Resp_{pred/ms}$ − Predicted and measured response, respectively (units are as in the Measured response column). RD = Response deviation, calculated as ($Resp_{ms}$ − $Resp_{pred}$)/$Resp_{pred}$ × 100.

3.5. Chemical Composition of the Optimized Extracts

In order to test the hypothesis that the optimized extracts are potentially valuable cosmeceutical ingredients, their biological activity was determined using several methods. In addition to that, the prepared extracts were chemically characterized with respect to the main bioactive constituents (Table 6). In accordance with licorice root chemical composition, the prepared extracts were relatively rich in phenolics, especially flavonoids, with notable amounts of their most important representatives, glabridin and isoliquiritigenin. However, the most prominent constituent of the extracts was the saponin glycyrrhizin, the main constituent of licorice root [15]. It was well dissolved in all the applied solvents, and its concentration depended mostly on the weight of the drug used for the extraction (Table 5).

Table 6. Chemical composition of the optimized extracts.

Extract	TP (µg/mL)	TF (µg/mL)	Gla (µg/mL)	Iso (µg/mL)	Glycyrrhizin (mg/mL)
TP-opt	854.6 ± 42.7	667.5 ± 42.7	9.62 ± 0.72	4.02 ± 0.26	4.31 ± 0.22
Tpy-opt	791.6± 48.0	521.4 ± 8.9	8.38 ± 0.17	3.51 ± 0.18	4.20 ± 0.17
Gla-Iso-opt	535.4 ± 32.1	692 ± 32.4	21.89 ± 1.09	6.23 ± 0.16	4.67 ± 0.34

TP, TF, Gla, Iso: concentration of total phenols, total flavonoids, glabridin and isoliquiritigenin, respectively.

3.6. Antioxidant Activity of the Optimized Extracts

Antioxidant activity of the ingredients in cosmetic products is of utmost importance. Firstly, the right antioxidant protects the product against oxidation that occurs during its storage and use [31]. Such influences include free radicals- or metal ions-induced peroxidation of polyunsaturated fatty acids that natural cosmetics are especially rich in. For this reason, the presence of pro-oxidant Fe^{2+} and other ions may, in time, negatively impact not only quality but also safety of the product [32]. Finally, functional cosmeceutical ingredients may have a more active role in such products. They also offer protection against oxidative damage of skin macromolecules associated with the effects of free radicals and UV radiation on the skin [33,34]. Thus, in this work, the influence of the prepared extracts on the free radicals (as modeled by DPPH free radical), chelating activity on Fe^{2+} ions, and the activity in heat-induced unsaturated fatty acid degradation β-carotene-linoleic acid system, were investigated.

Figure 2 depicts the results of the antioxidant assays performed in this work. Even though the activity of the extracts may not be directly compared to the standard antioxidants due to the fact that the activity is expressed in different measurements units (the activity of the extracts and standards was expressed as µL/mL and µg/mL, respectively), it is interesting to note that the activity of the extracts and the standards solutions was rather similarly pronounced in all the assays, except for the β-carotene-linoleic acid assay, where BHA was a notably stronger antioxidant (Figure 2a–c). The activity of the individual extracts differed according to the assay. The prepared optimized extracts were similarly efficient radical scavengers with IC_{50} values of approximately 10 µL of extract per mL of solution. In addition, the extracts were able to efficiently chelate Fe^{2+} ions. Among the extracts, TP-opt was the most active ion chelator, followed by TPy-opt. Finally, the extracts inhibited thermally induced degradation of the β-carotene-linoleic acid system (Figure 2). TP-opt and TPy-opt also displayed the strongest, and statistically equal, AACL activity. Comparable activity of TP-opt and TPy-opt indicates that similar effects may be obtained with about 25% less crude drug, which is a finding important from both economical and ecological points of view. In order to test if the solvent contributed to the observed antioxidant activity, the solutions of glycerol, diluted in the same concentrations as it was present in the solutions of the TP-opt, TPy-opt and Gla-Iso-opt at their EC_{50}, was tested. However, glycerol displayed no measurable activity in any of the applied antioxidant assays.

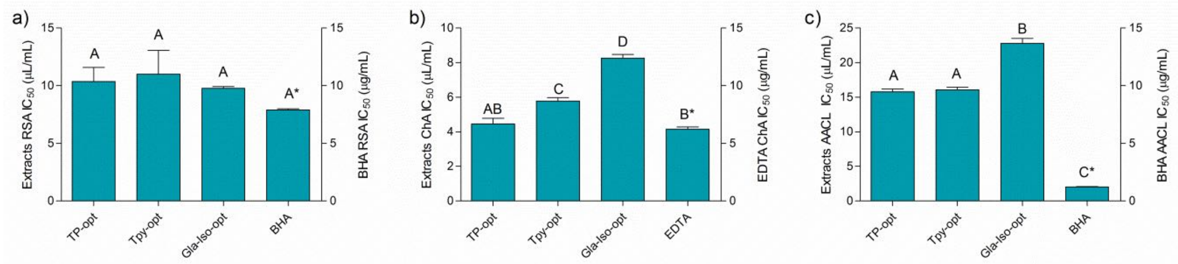


Figure 2. Antiradical (**a**), chelating (**b**), and activity in β-carotene-linoleic acid assay (**c**) and positive controls BHA (butylated hydroxyanisole) and EDTA (ethylenediaminetetraacetic acid). Different uppercase letters indicate statistical significance ($p < 0.05$). Asterisk (*) indicates that the IC_{50} unit is placed on the right *y*-axis.

3.7. Enzyme Inhibiting and Anti-inflammatory Activity of the Optimized Extracts

The activity of the plant extracts in cosmetic products extends beyond simple hydration and antioxidant protection. Therefore, in this work, tyrosinase and elastase inhibitory activity, as well as anti-inflammatory activity against protein coagulation, were investigated. Melanin is a macromolecular pigment that has a photoprotective function in human skin. However, the accumulation of an abnormal amount of melanin in specific skin parts results in hyperpigmented areas and represents an esthetic problem for the affected individual. Tyrosinase is the enzyme responsible for the first step of melanogenesis by catalyzing tyrosine oxidation to dopaquinone. The remainder of the reaction sequence proceeds spontaneously at a physiological pH value. Therefore, tyrosinase inhibitors block melanogenesis and prevent hyperpigmentation of the skin [35]. Specific plant metabolite may protect the skin macromolecules against enzymatic degradation. For example, skin aging and inflammation induced by exposure to UV radiation or other environmental stressors are related to the reduction of production of skin proteins and increased levels of elastase enzymes, which are responsible for elastin breakdown [36]. This damage results in distinctive degenerative changes of the upper dermal connective tissue [37]. Clinical trials confirm that the inhibition of elastase activity indicates the important anti-aging potential of the natural product and other compounds that display it [38]. Skin inflammation can be defined as a skin response to injury, infection, or destruction, normally characterized by heat, redness, pain, swelling or disturbed skin physiological functions [36]. One of the characteristic and causes of inflammatory processes is the denaturation of tissue proteins. Therefore, the suppression of protein denaturation hinders the development of inflammation-related skin changes, which is another important aspect of anti-aging activity [27].

As presented in Figure 3, the investigated extracts were excellent tyrosinase and elastase inhibitors, as well as anti-inflammatory agents. Similar to previously described antioxidant assays, the extracts displayed a notable activity in all the assays relative to the positive controls. Keeping in mind the well-established anti-tyrosinase activity of glabridin, the excellent activity of Gla-Iso-opt in this assay was not surprising. However, the other extracts displayed statistically equal activity in this assay. The anti-elastase activity of the Gla-Iso-opt extract, however, was much better pronounced and statistically higher than the activity of the other extracts. Although all the investigated extracts were able to inhibit heat-induced ovalbumin coagulation, the best anti-inflammatory activity was displayed by the Gla-Iso-opt. It is interesting to note that, in accordance with previous findings [39], glycerol itself has a role of an active solvent that prevents the denaturation of proteins such as collagen. Therefore, the influence of glycerol on the heat-induced protein denaturation was also investigated. In order to estimate the proportion of the glycerol activity in the overall activity of the extracts, glycerol was diluted to the same concentration as present in the solutions of the respective extract at its EC_{50}. The activity of glycerol, when tested in concentrations present in TP-opt, TPy-opt and Gla-Iso-opt extracts at their EC_{50}, was 4.69%, 8.53% and 23.07%, respectively. This means that, at the respective extract's EC_{50} (e.g., when the activity of the extracts was 50%), glycerol used for preparation of TP-opt only marginally influenced the assay outcome (less than 10%), while glycerol in TPy provided approximately 20%

of protection against protein denaturation. However, glycerol presence in the Gla-Iso-Opt extract, which was prepared using 85% glycerol, accounted for 46% of the observed stabilization, while the rest of the activity could be contributed to the specific compounds in the extract and/or their interaction with glycerol. Even though glycerol, when tested at concentrations present at EC_{50} of the extracts in the respective assays did not demonstrate any measurable elastase- or tyrosinase-inhibitory activity, its ability to hinder protein denaturation furthers confirms that the benefits of glycerol extraction for cosmeceutical production extend beyond its application as a green extraction solvent.

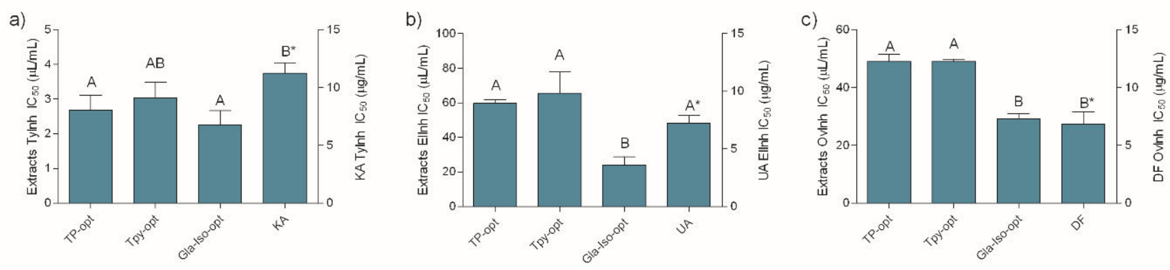


Figure 3. Tyrosinase (**a**) and elastase (**b**) inhibitory, and anti-inflammatory (**c**) activity of the extracts and positive controls KA (kojic acid), UA (ursolic acid) and DF (diclofenac). Different uppercase letters indicate statistical significance ($p < 0.05$). Asterisk (*) indicates that the IC_{50} unit is placed on the right *y*-axis.

4. Conclusions

Licorice root contains numerous bioactive natural products, many of which are potent cosmeceutical ingredients. In this work, the UAE method for preparation of licorice root bioactive extracts was optimized. The extraction was performed using mixtures of water with glycerol, a biodegradable, safe, cosmetically active solvent. The prepared extracts displayed excellent radical scavenging, Fe^{2+} chelating, and antioxidant activity. In addition, tyrosinase and elastase inhibitory activity of the extracts, as well as their anti-inflammatory activity, indicated excellent anti-aging properties. Such attractive array of skin-related biological activities makes glycerolic licorice extracts promising constituents of specialized cosmeceutical formulations.

Author Contributions: Conceptualization, M.T. and M.Z.K.; Investigation, P.C., K.J. and M.Z.K.; Methodology, P.C., K.J. and M.Z.K.; Supervision, M.T. and M.Z.K.; Writing—original draft, M.Z.K.; Writing—review & editing, P.C. and M.Z.K.

Acknowledgments: This work was financed in course of the project, "Bioactive plant principles extraction using green solvents - a step towards green cosmeceuticals" (IP-2018-01-6504), supported by Croatian Science Foundation. The authors are grateful to the company Suban for donation of plant material.

Conflicts of Interest: There is no conflicting interest to report.

References

1. Yahya, N.A.; Attan, N.; Wahab, R.A. An overview of cosmeceutically relevant plant extracts and strategies for extraction of plant-based bioactive compounds. *Food Bioprod. Process.* **2018**, *112*, 69–85. [CrossRef]
2. Costa, R.; Santos, L. Delivery systems for cosmetics—From manufacturing to the skin of natural antioxidants. *Powder Technol.* **2017**, *322*, 402–416. [CrossRef]
3. Chemat, F.; Vian, M.A.; Cravotto, G. Green extraction of natural products: Concept and principles. *Int. J. Mol. Sci.* **2012**, *13*, 8615–8627. [CrossRef] [PubMed]
4. Wolfson, A.; Dlugy, C.; Shotland, Y. Glycerol as a green solvent for high product yields and selectivities. *Environ. Chem. Lett.* **2007**, *5*, 67–71. [CrossRef]
5. Shehata, E.; Grigorakis, S.; Loupassaki, S.; Makris, D.P. Extraction optimisation using water/glycerol for the efficient recovery of polyphenolic antioxidants from two *Artemisia* species. *Sep. Purif. Technol.* **2015**, *149*, 462–469. [CrossRef]

6. El Kantar, S.; Rajha, H.N.; Boussetta, N.; Vorobiev, E.; Maroun, R.G.; Louka, N. Green extraction of polyphenols from grapefruit peels using high voltage electrical discharges, deep eutectic solvents and aqueous glycerol. *Food Chem.* **2019**, *295*, 165–171. [CrossRef] [PubMed]
7. Apostolakis, A.; Grigorakis, S.; Makris, D.P. Optimisation and comparative kinetics study of polyphenol extraction from olive leaves (*Olea europaea*) using heated water/glycerol mixtures. *Sep. Purif. Technol.* **2014**, *128*, 89–95. [CrossRef]
8. Rouhani, M. Modeling and optimization of ultrasound-assisted green extraction and rapid HPTLC analysis of stevioside from *Stevia rebaudiana*. *Ind. Crop. Prod.* **2019**, *132*, 226–235. [CrossRef]
9. Glória, M.B.A. SWEETENERS|Others. In *Encyclopedia of Food Sciences and Nutrition*, 2nd ed.; Caballero, B., Ed.; Academic Press: Oxford, MA, USA, 2003; pp. 5695–5702.
10. Castangia, I.; Caddeo, C.; Manca, M.L.; Casu, L.; Latorre, A.C.; Díez-Sales, O.; Ruiz-Saurí, A.; Bacchetta, G.; Fadda, A.M.; Manconi, M. Delivery of liquorice extract by liposomes and hyalurosomes to protect the skin against oxidative stress injuries. *Carbohydr. Polym.* **2015**, *134*, 657–663. [CrossRef]
11. Mostafa, D.M.; Ammar, N.M.; Abd El-Alim, S.H.; El-anssary, A.A. Transdermal microemulsions of *Glycyrrhiza glabra* L.: Characterization, stability and evaluation of antioxidant potential. *Drug Deliv.* **2014**, *21*, 130–139. [CrossRef] [PubMed]
12. Kotian, S.; Bhat, K.; Pai, S.; Nayak, J.; Souza, A.; Gourisheti, K.; Padma, D. The role of natural medicines on wound healing: A biomechanical, histological, biochemical and molecular study. *Ethiop. J. Health Sci.* **2018**, *28*, 759–770. [PubMed]
13. Yu, H.; Li, H.; Li, Y.; Li, M.; Chen, G. Effect of isoliquiritigenin for the treatment of atopic dermatitis-like skin lesions in mice. *Arch. Dermatol. Res.* **2017**, *309*, 805–813. [CrossRef] [PubMed]
14. Simmler, C.; Pauli, G.F.; Chen, S.N. Phytochemistry and biological properties of glabridin. *Fitoterapia* **2013**, *90*, 160–184. [CrossRef] [PubMed]
15. Pastorino, G.; Cornara, L.; Soares, S.; Rodrigues, F.; Oliveira, M.B.P.P. Liquorice (*Glycyrrhiza glabra*): A phytochemical and pharmacological review. *Phytother. Res.* **2018**, *32*, 2323–2339. [CrossRef] [PubMed]
16. Kondo, K.; Shiba, M.; Nakamura, R.; Morota, T.; Shoyama, Y. Constituent properties of licorices derived from *Glycyrrhiza uralensis*, *G. glabra*, or *G. inflata* identified by genetic information. *Biol. Pharm. Bull.* **2007**, *30*, 1271–1277. [CrossRef] [PubMed]
17. Liao, W.C.; Lin, Y.H.; Chang, T.M.; Huang, W.Y. Identification of two licorice species, *Glycyrrhiza uralensis* and *Glycyrrhiza glabra*, based on separation and identification of their bioactive components. *Food Chem.* **2012**, *132*, 2188–2193. [CrossRef]
18. European Pharmacopoeia Commission. *European Pharmacopoeia*, 8th ed.; Council of Europe: Strasbourg, France, 2013.
19. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1999; Volume 299, pp. 152–178.
20. Kumazawa, S.; Hamasaka, T.; Nakayama, T. Antioxidant activity of propolis of various geographic origins. *Food Chem.* **2004**, *84*, 329–339. [CrossRef]
21. Kleinschmidt, G. Case Study: Validation of an HPLC-Method for Identity, Assay, and Related Impurities. In *Method Validation in Pharmaceutical Analysis: A Guide to Best Practice*; Ermer, J., Miller, J.H., Eds.; Wiley-VCH: Weinheim, Germany, 2005; pp. 195–212.
22. Tada, A.; Ishizuki, K.; Sugimoto, N.; Yoshimatsu, K.; Kawahara, N.; Suematsu, T.; Arifuku, K.; Fukai, T.; Tamura, Y.; Ohtsuki, T.; et al. Determination of the plant origin of licorice oil extract, a natural food additive, by principal component analysis based on chemical components. *J. Food Hyg. Soc. Jpn.* **2015**, *56*, 217–227. [CrossRef]
23. Jug, M.; Končić, M.Z.; Kosalec, I. Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures. *LWT Food Sci. Technol.* **2014**, *57*, 530–537. [CrossRef]
24. Končić, M.Z.; Barbarić, M.; Perković, I.; Zorc, B. Antiradical, chelating and antioxidant activities of hydroxamic acids and hydroxyureas. *Molecules* **2011**, *16*, 6232–6242. [CrossRef]
25. Rajić, Z.; Končić, M.; Miloloža, K.; Perković, I.; Butula, I.; Bucar, F.; Zorc, B. Primaquine-NSAID twin drugs: Synthesis, radical scavenging, antioxidant and Fe^{2+} chelating activity. *Acta Pharm.* **2010**, *60*, 325–337. [CrossRef] [PubMed]

26. Bose, B.; Choudhury, H.; Tandon, P.; Kumaria, S. Studies on secondary metabolite profiling, anti-inflammatory potential, in vitro photoprotective and skin-aging related enzyme inhibitory activities of *Malaxis acuminata*, a threatened orchid of nutraceutical importance. *J. Photochem. Photobiol. B* **2017**, *173*, 686–695. [CrossRef] [PubMed]
27. Chandra, S.; Chatterjee, P.; Dey, P.; Bhattacharya, S. Evaluation of in vitro anti-inflammatory activity of coffee against the denaturation of protein. *Asian Pac. J. Trop. Biomed.* **2012**, *2*, S178–S180. [CrossRef]
28. Li, Y.; Cao, S.Y.; Lin, S.J.; Zhang, J.R.; Gan, R.Y.; Li, H.B. Polyphenolic profile and antioxidant capacity of extracts from *Gordonia axillaris* fruits. *Antioxidants* **2019**, *8*, 150. [CrossRef]
29. Zhou, Y.; Xu, X.Y.; Gan, R.Y.; Zheng, J.; Li, Y.; Zhang, J.J.; Xu, D.P.; Li, H.B. Optimization of ultrasound-assisted extraction of antioxidant polyphenols from the seed coats of red sword bean (*Canavalia gladiate* (Jacq.) DC.). *Antioxidants* **2019**, *8*, 200. [CrossRef] [PubMed]
30. Limwachiranon, J.; Jiang, L.; Huang, H.; Sun, J.; Luo, Z. Improvement of phenolic compounds extraction from high-starch lotus (*Nelumbo nucifera* G.) seed kernels using glycerol: New insights to amylose/amylopectin—Phenolic relationships. *Food Chem.* **2019**, *274*, 933–941. [CrossRef] [PubMed]
31. Buchmann, S. Main Cosmetic Vehicles. In *Handbook of Cosmetic Science and Technology*; Barel, A., Paye, M., Maibach, H., Eds.; Marcel Dekker: New York, NY, USA, 2001.
32. Mlakar, A.; Batna, A.; Dudda, A.; Spiteller, G. Iron (II) ions induced oxidation of ascorbic acid and glucose. *Free Radic. Res.* **1996**, *25*, 525–539. [CrossRef] [PubMed]
33. Ratz-Łyko, A.; Arct, J. Resveratrol as an active ingredient for cosmetic and dermatological applications: A review. *J. Cosmet. Laser Ther.* **2019**, *21*, 84–90. [CrossRef]
34. Thiele, J.; Elsner, P. *Oxidants and Antioxidants in Cutaneous Biology*; Karger: Basel, Switzerland, 2001; Volume 29.
35. Chang, T.S. An updated review of tyrosinase inhibitors. *Int. J. Mol. Sci.* **2009**, *10*, 2440–2475. [CrossRef]
36. Bessada, S.M.F.; Alves, R.C.; Oliveira, M.B. Coffee silverskin: A review on potential cosmetic applications. *Cosmetics* **2018**, *5*, 5. [CrossRef]
37. Akhma, Z.; Ibrahim, F.; Nor Rashid, N.; Mohamad Razif, M.; Yusof, R. The importance of some plant extracts as skin anti-aging resources: A Review. *Curr. Pharm. Biotechnol.* **2017**, *19*, 864–876.
38. Imokawa, G. Recent advances in characterizing biological mechanisms underlying UV-induced wrinkles: A pivotal role of fibrobrast-derived elastase. *Arch. Dermatol. Res.* **2008**, *300*, 7–20. [CrossRef] [PubMed]
39. Penkova, R.; Goshev, I.; Gorinstein, S.; Nedkov, P. Stability of collagen during denaturation. *J. Protein Chem.* **1999**, *18*, 397–401. [CrossRef] [PubMed]

2.4. Antidiabetic and Cosmeceutical Potential of Common Barberry (*Berberis vulgaris* L.) Root Bark Extracts Obtained by Optimisation og „Green“ Ultrasound-Assisted Extraction

molecules

MDPI

Article

Antidiabetic and Cosmeceutical Potential of Common Barbery (*Berberis vulgaris* L.) Root Bark Extracts Obtained by Optimization of 'Green' Ultrasound-Assisted Extraction

Marina Dulić [1], Petar Ciganović [1], Lovorka Vujić [2] and Marijana Zovko Končić [1,]*

1. Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, Marulićev trg 20, 10 000 Zagreb, Croatia; marinad4@net.hr (M.D.); petar.ciganovic@me.com (P.C.)
2. Department of Food Chemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb, Domagojeva 1, 10 000 Zagreb, Croatia; lvujic@pharma.hr

* Correspondence: mzovko@pharma.hr

Academic Editor: Gregory Chatel
Received: 1 August 2019; Accepted: 28 August 2019; Published: 8 October 2019

Abstract: *Berberis vulgaris* is rich in berberine, an isoquinoline alkaloid, with antidiabetic activity, often used topically for skin-related problems. The aim of this work was to develop a "green" method for berberine extraction using mixtures of water with glycerol, a non-toxic, environmentally-friendly solvent. Response surface methodology based on Box–Behnken design was used to optimize the experimental conditions for ultrasound-assisted extraction of berberine and anti-radical components from *B. vulgaris* root bark. The independent variables were temperature (X_1), glycerol concentration (X_2), and ultrasound power (X_3), while the responses were berberine concentration and DPPH radical scavenging activity of the extracts (RSA IC_{50}). The response values of the extracts prepared at optimum conditions were (response, X_1, X_2, X_3): berberine yield (145.5 μg/mL; 80 °C, 50%, 144 W) and RSA IC_{50} (58.88 μL/mL; 80 °C, 30%, 720 W). The observed values deviated from the predicted values by −3.45% and 6.42% for berberine and RSA IC_{50}, respectively, thus indicating the validity of the selected models. The prepared extracts demonstrated antioxidant, anti-melanogenic, and anti-inflammatory activity, as well excellent α-glucosidase and α-amylase inhibitory activity. The displayed biological properties and lack of glycerol toxicity makes the prepared extracts suitable for direct inclusion into antidiabetic and dermatologic food supplements and topical products.

Keywords: berberine; *Berberis vulgaris*; green extraction; glycerol; response surface methodology

1. Introduction

Berberis vulgaris L., Berberidaceae, is a deciduous shrub with a long history of medicinal and nutritional use in Europe, Asia, and America. While its fruit is mostly used as food, the root bark and stems of *B. vulgaris* have medicinal properties due to berberine, an isoquinoline alkaloid, mostly present in these organs [1]. Berberine and *Berberis* species display many pharmacological effects, including antidiabetic, anti-inflammatory, antioxidant [2], antibacterial [3], and antifungal effects [4]. Berberine is used in food supplements and dermatologic products. It is most commonly taken by mouth for diabetes, high cholesterol, and high blood pressure, or applied directly to the skin to treat burns and canker sores [5]. Although it is not suitable for use by children or during pregnancy and breastfeeding, berberine is considered safe for short-term use by adults when taken by mouth or applied to the skin [5].

Metabolic syndrome and type 2 diabetes are ailments that affect over 30–40% of the population older than 65 [6]. They are characterized by insulin resistance, hyperglycemia, as well as by overproduction of reactive oxygen species and a constant state of enhanced oxidative stress [7]. High blood glucose concentration in diabetes may cause polyol and hexosamine pathways, advanced glycation end-product formation, activation of protein kinase C, mitochondrial dysfunction, and consequently reactive oxygen species (ROS) accumulation. This leads to cellular damage and the development of diabetic complications, such as neuropathy, nephropathy, and retinopathy, as well as liver damage [7,8]. Berberine is well known for its anti-diabetic effects. For example, berberine lowers blood-glucose concentrations in healthy and diabetic people, and improves insulin secretion in healthy individuals [9]. In addition to this, it may reduce fasting and postprandial blood glucose, food, and water intake, as well as enhance antidiabetic effects of other drugs such as canagliflozin [10].

In addition to anti-diabetic activity, berberine has many properties that may be utilized for the development of cosmeceutical products. The word "cosmeceutical" is a marketing term often used in lay language to denote a topical product that possesses both cosmetic and dermatologic characteristics. In addition to hydrating properties, such products may display other activities, such as antioxidant, anti-wrinkle, skin-whitening, and anti-inflammatory activity among others. Among cosmeceuticals, the products that are derived from natural sources such as plants, are in special demand, not only because of the consumers' preferences for natural skin-care, but also because of their numerous beneficial effects on human skin [11]. The documented antioxidant [2] and anti-inflammatory activity of barberry extracts and berberine [12], as well as its notable anti-wrinkle properties [13] make them suitable for inclusion in cosmeceutical products.

In order to incorporate berberine and other bioactive plants principles into the final products they need to be extracted from crude plant material. Ultrasound-assisted extraction (UAE) is an inexpensive and simple extraction technique, appropriate for extraction in solid/liquid systems. UAE is characterized by relatively high reproducibility, short time of extraction, low solvent consumption, as well as low extraction temperature and energy input. Among the numerous factors that may influence the efficiency of UAE, solvent type selection has been recognized as the most important. An efficient UAE process should maximize the recovery of target compounds with minimal degradation, resulting in an extract with high biological activity. Ideally, this should be accomplished using "green" environmentally friendly technologies and low-cost raw materials and solvents [14]. Because of its wide availability and lack of toxicity, water is the most appropriate solvent for the extraction of medicinal plants' bioactive principles. It is often combined with ethanol to make it suitable for the extraction of non-polar bioactive molecules from plant material. However, in spite of its natural origin, the use of ethanol is limited by its flammability and skin-irritability. Furthermore, internal use of ethanol is not appropriate for children and members of certain religions. One of the solvents that could effectively replace ethanol for preparation of cosmetic products and food supplements is glycerol, a non-toxic, biodegradable liquid manufactured from renewable sources [15]. These characteristics make the extracts prepared using glycerol appropriate for the direct application in the formulation of the desired product, without the need for solvent removal. Interestingly, as opposed to optimization of ethanolic extraction, glycerol use for extraction of natural products is still under-researched. Few examples include the use of glycerol for extraction of polyphenolic antioxidants from two *Artemisia* species [16], olive (*Olea europaea*) leaves [17], and rice bran [18].

Seemingly similar extraction procedures can significantly affect the yield and the composition of plant extracts. Response surface methodology (RSM) is a statistical model-based methodology that determines the relationship between the extraction condition and one or more studied responses, thus decreasing the required time and cost of the experiments [19]. The aim of this work was to perform a comprehensive investigation of the influence of the extraction variables: temperature, glycerol concentration, and ultrasonication power (USP) on berberine content and radical scavenging activity (RSA) of *B. vulgaris* extracts using RSM. An additional goal was to test the biological activity of the prepared extracts using selected assays. Even though berberine can exert numerous beneficial effects on the human organism, its low oral bioavailability greatly limits its clinical application [20]. However, during the topical or oral application, the bioactive ingredients of *B. vulgaris* extracts may come into direct contact with skin or digestive tract enzymes, respectively. Therefore, in this work, the biological activity of *B. vulgaris* extracts was tested, targeting the activities relevant to cosmeceutical and digestive tract-related anti-diabetic applications.

2. Results

2.1. Response Surface Analysis of Berberine and RSA

UAE was employed to prepare *B. vulgaris* root bark extracts with high berberine content, as well as low RSA IC_{50} value. The independent variables selected for optimization were extraction temperature, concentration of glycerol in water, and USP. The research was carried out according to a three-factor Box–Behnken design (BBD) (Table 1). Preliminary experiments showed that water/glycerol mixtures are more suitable for *B. vulgaris* extraction than either pure water or glycerol. Therefore, 10–90% solutions of glycerol in water were used for the extraction in this work. Other extraction parameters were selected according to the maximum and minimum specifications of the ultrasonication bath.

Table 1. Independent variables and their levels for Box–Behnken design.

Independent Variables	Code	Levels		
		−1	0	1
Temperature °C	X1	20	50	80
Glycerol concentration (%, *w/w*)	X2	10	50	90
Ultrasonication power (USP) (W)	X3	144	432	720

Table 2 shows the process variables and experimental data of 17 runs. The amount of extracted berberine greatly differed among extracts (Table 2). For example, run 13 contained only 32.46 µg/mL of berberine while run 17 contained as much as 146.65 µg/mL of the alkaloid. The radical scavenging activity of the extracts, investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, also varied significantly. In the presented work, RSA IC_{50} values of *B. vulgaris* extracts ranged between 55.35 µL/mL and 262.95 µL/mL. Butylated hydroxyanisole (BHA), used as the positive control in this assay, presented an RSA IC_{50} of 5.13 ± 0.18 µg/mL. Even though the results are not directly comparable because of different units, this indicates rather modest radical scavenging abilities of the extracts.

Table 2. The Box–Behnken design and results of experiments.

Run	Standard	X_1 (°C)	X_2 (%, *w/w*)	X_3 (W)	Berberine	RSA IC_{50}
					µg/mL	µL/mL
1	1	20	10	432	57.11	108.73
2	15	50	50	432	75.06	103.47
3	9	50	10	144	84.58	134.14
4	16	50	50	432	69.00	90.84
5	14	50	50	432	83.33	83.79
6	4	80	90	432	89.79	74.37
7	10	50	90	144	68.53	128.59
8	12	50	90	720	59.98	113.51
9	5	20	50	144	69.27	98.35
10	17	50	50	432	84.28	83.63
11	2	80	10	432	111.03	72.98
12	13	50	50	432	72.56	86.46
13	3	20	90	432	32.46	262.95
14	8	80	50	720	120.8	55.35
15	7	20	50	720	95.58	87.74
16	11	50	10	720	69.62	72.59
17	6	80	50	144	146.65	78.86

X_1 = temperature, X_2 = glycerol content, X_3 = ultrasonication power; RSA = radical scavenging activity.

By applying multiple regression analysis on the experimental data, it was found that the relationship between the response variables and the independent variables can best be expressed by the quadratic polynomial equations. In order to achieve a better fit and thus observe the influence of the extraction conditions on RSA more clearly, the data were transformed using negative power (Table 3).

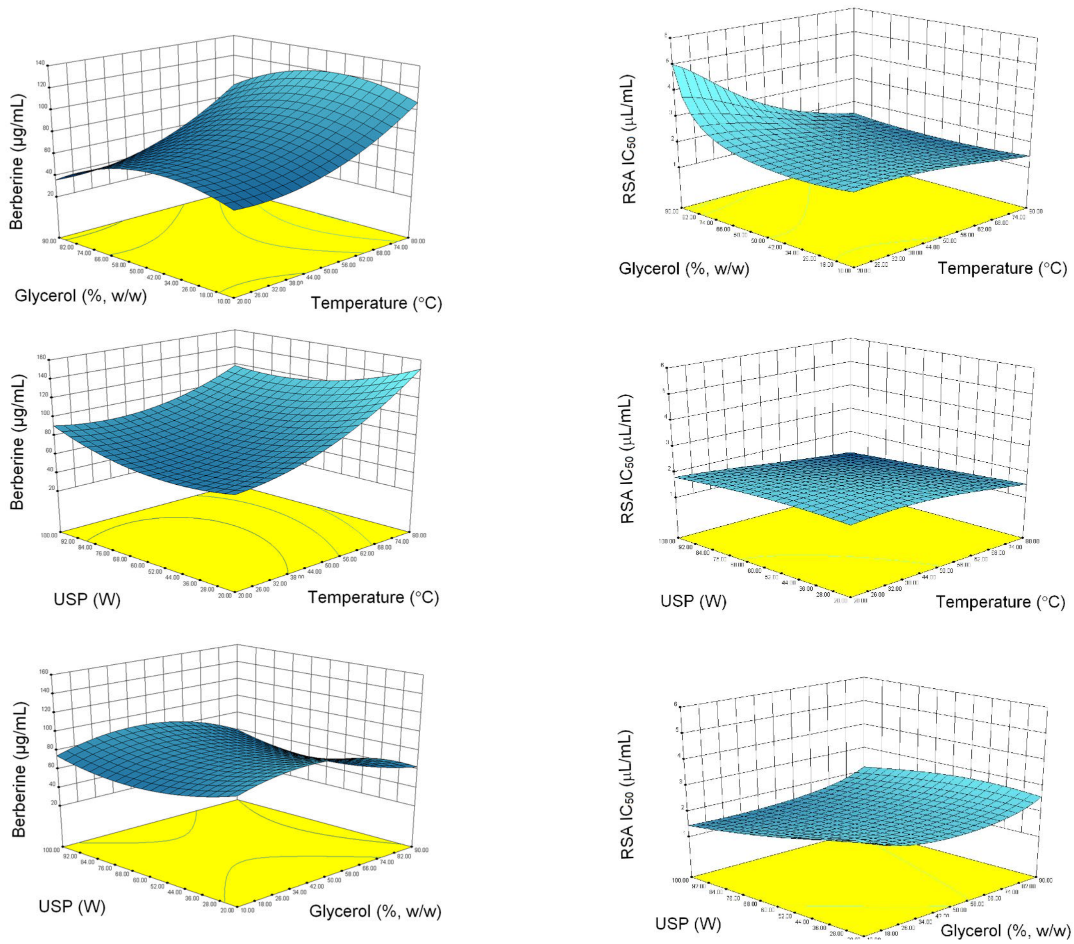
Table 3. Polynomial equations of the models in terms of coded factors.

Response	Unit	The Equation Coefficients: $a \times X_1^2 + b \times X_2^2 + c \times X_3^2 + d \times X_1 \times X_2 + e \times X_1 \times X_3 + f \times X_2 \times X_3 + g \times X_1 + h \times X_2 + i \times X_3 + j$									
		a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*
Berberine	(μg/mL)	16.57 *	−20.82 *	14.65 *	0.85	−13.04 *	1.60	26.73 *	−8.95 *	−2.88	76.85
RSA $IC_{50}^{-1.78}$	(mL/mL)	0.089 *	−0.12 *	0.039	0.045	0.081 *	−0.074 *	0.16 *	−0.061 *	0.11 *	0.36

X_1 = temperature (°C), X_2 = glycerol content (%, m/m), X_3 = ultrasonication power (W). * = the significant equation terms ($p < 0.05$).

In order to enable visualization of the interactions between the independent and independent variables, the results are also presented as response surface plots. Figure 1 shows the surface plots of the influence of investigated UAE parameters on the berberine content (Figure 1a) and RSA IC_{50} (Figure 1b). From the plots and from Table 3, it is evident that the independent variables influenced the extraction in different manner. For example, temperature positively influenced the berberine concentration and RSA $IC_{50}^{-1.78}$ value both as linear and quadratic factors. This means that both berberine and the substances responsible for radical scavenging activity are better extracted at higher temperature. However, the combination of strong ultrasound and high temperature seemed to affect the berberine concentration negatively, while the effect on RSA $IC_{50}^{-1.78}$ remained positive. As evidenced by negative coefficients before quadratic and linear glycerol concentration, both berberine and substances with antiradical properties are better extracted using medium to low glycerol content. On the other hand, USP alone enhanced both berberine extraction efficiency (as quadratic factor) and RSA $IC_{50}^{-1.78}$ (as quadratic and linear factors).

The analysis of variance (ANOVA) for the selected models (Table 4) has shown that the models are suitable for the description of the relationship of dependent and independent variables. The F-values of the models were higher than 26, while the *p*-values were lower than 0.0001. This indicates that both models are significant and that they were suitable for optimization of the extraction variables. Lack-of-fit test in both models was statistically insignificant relative to the pure error, meaning that the fitting model is adequate to describe the experimental data. The determination coefficients for both responses were relatively high ($r^2 > 0.97$) showing good predictability of the results by the selected models. The adjusted and predicted r^2 for both models were rather high and in good agreement. This further confirms the suitability of the models for the description of experimental data (Table 4).



(**a**) (**b**)

Figure 1. Response surface plots: influence of pairs independent variables on (**a**) berberine yield, and (**b**) radical scavenging activity (RSA IC_{50}).

Table 4. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of extraction parameters.

	Berberine $r^2 = 0.9720$; $r_{adj}^2 = 0.9359$; $r_{pr}^2 = 0.7850$					RSA (IC_{50}) $r^2 = 0.9740$; $r_{adj}^2 = 0.9406$; $r_{pr}^2 = 0.9034$				
Source	**SS**	**df**	**MS**	***F*-Value**	***p*-Value**	**SS**	**df**	**MS**	***F*-Value**	***p*-Value**
Model	10834.44	9	1203.83	26.957	0.0001	4.35×10^{-7}	9	4.84×10^{-8}	29.17	<0.0001
Lack of fit	132.178	3	44.06	0.977	0.4870	1.74×10^{-9}	3	5.79×10^{10}	0.23	0.8683
Pure error	180.436	4	45.11			9.87×10^{-9}	4	2.47×10^{-9}		

r_{adj}^2 = adjusted r^2; r_{pr}^2 = predicted r^2; RSA = radical scavenging activity; SS = sum of squares; df = degrees of freedom; MS = mean square.

2.2. Optimization of Extraction Parameters and Model Validation

The aim of this study was to maximize the berberine extraction yield, as well as to minimize the RSA IC_{50} of the *B. vulgaris* extracts. In order to establish the optimum levels of the independent variables, numerical optimizations have been conducted based on the experimental results and the statistical analysis. Two extracts, one with maximized berberin content (B-opt) and the other with minimized RSA IC_{50} value (RSA-opt) were prepared. Optimal extraction conditions and the predicted values of corresponding responses are presented in Table 5. As expected from the polynomial equations, high temperature beneficially affected both berberine concentration and RSA. Somewhat higher glycerol content and lower USP was needed for optimal berberine yield in comparison to those needed for optimal RSA of the extracts.

Table 5. Predicted and observed values for the optimized response variables.

Extract Name	Optimized Response	Aim of the Optimization	X_1 °C	X_2 %	X_3 W	Predicted	Observed	RD (%)
B-opt	Berberine (µg/mL)	maximized	80	50	144	150.7	145.5	−3.45
RSA-opt	RSA IC_{50} (µL/mL)	minimized	80	30	720	55.33	58.88	6.42

X_1 = temperature, X_2 = glycerol content, X_3 = ultrasonication power; RD = response deviation, calculated as (observed − predicted)/predicted × 100.

Berberine content and RSA IC_{50} value were determined in both extracts. In addition to the values presented in Table 5, B-opt had an RSA IC_{50} value of 77.37 µL/mL, while the berberine content in RSA-opt was 116.9 µg/mL.

2.3. Antioxidant Activity of the Extracts

RSA, chelating activity on Fe^{2+} ions, and the activity in heat-induced degradation of β-carotene-linoleic acid system were investigated (Figure 2). It is important to note that the activity of the extracts may not be directly compared to the standard antioxidants due to the fact that the activity was expressed in different measurements units (the activity of the extracts and standards were expressed as µL/mL and µg/mL, respectively). However, for comparison purposes, it is possible to regard the activity of the standards as volume equivalents of 1 mg/mL solutions.

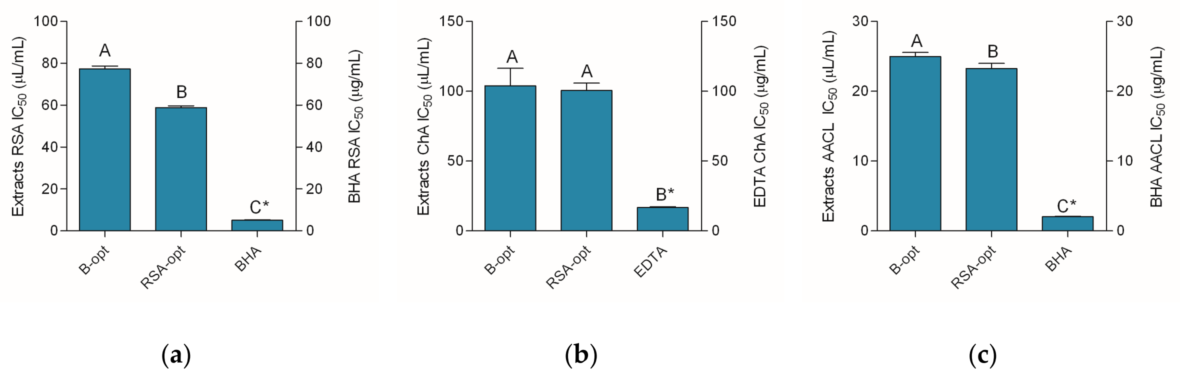
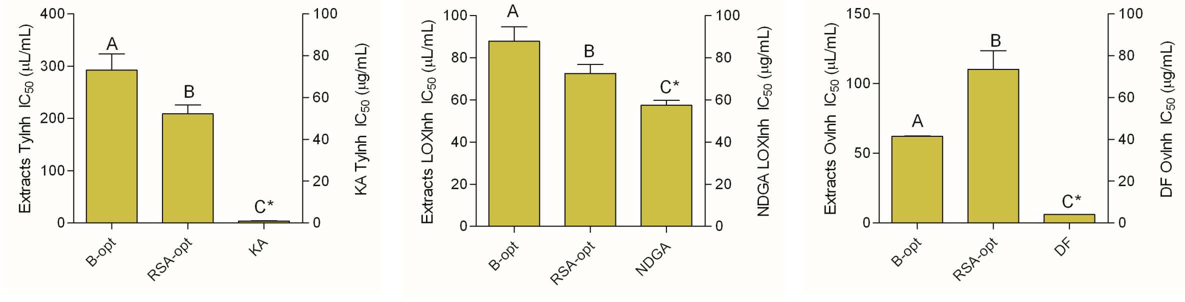


Figure 2. Antioxidant activity of the extracts and positive controls—BHA (butylated hydroxyanisole) and EDTA (ethylenediaminetetraacetic acid): (**a**) antiradical activity, (**b**) chelating activity, and (**c**) the activity in β-carotene-linoleic acid assay. Different uppercase letters indicate statistical significance ($p < 0.05$). Asterisks (*) indicate that the IC_{50} unit is placed on the right *y*-axis.

The data presented in Figure 2 indicate mild to moderate antioxidant activity of the extracts in comparison to the standards. Expectedly, RSA-opt was a stronger radical scavenger, while a somewhat lower level of scavenging activity is demonstrated by B-opt (Figure 2a). Similarly, RSA-opt was better capable of hindering oxidation of linoleic acid in a β-carotene-linoleic acid assay (Figure 2c), while the capability of the extracts to chelate Fe^{2+} ions was statistically equal (Figure 2b).

2.4. Tyrosinase-, Lipoxygenase-, and Coagulation-Inhibiting Activity

The cosmeceutical potential of the prepared extracts was investigated by studying their tyrosinase- and lipoxygenase (LOX)-inhibiting properties. Furthermore, the ability to inhibit heat-induced protein coagulation was also investigated. Both extracts were active in the performed assays but to varying degrees (Figure 3).



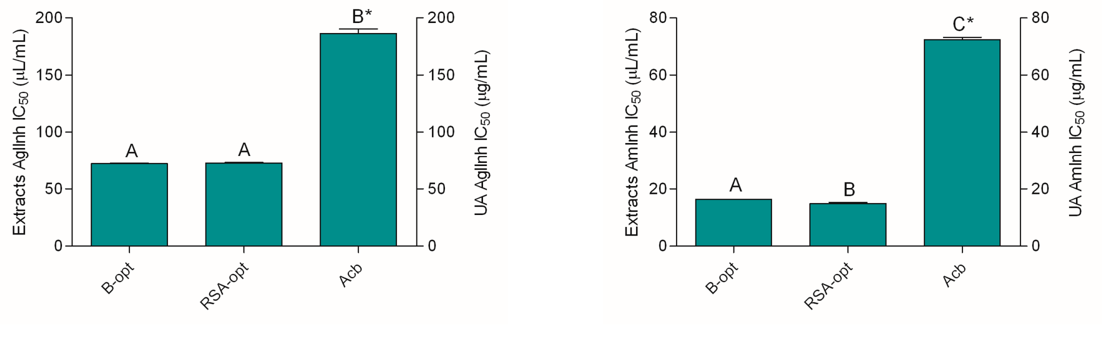
(**a**) (**b**) (**c**)

Figure 3. Cosmeceutical activity of the extracts and positive controls—kojic acid (KA), nordihydroguaiaretic acid (NDGA), and diclofenac (DF): (**a**) tyrosinase-inhibiting activity, (**b**) lipoxygenase (LOX)-inhibiting activity, and (**c**) coagulation-inhibiting activity. Different uppercase letters indicate statistical significance ($p < 0.05$). Asterisks (*) indicate that the IC_{50} unit is placed on the right *y*-axis.

Although the extracts displayed some level of anti-tyrosinase activity, their activity was rather low in comparison to kojic acid, the standard skin-whitening substance (Figure 3a). It may also be noted that the RSA-opt was significantly more active than B-opt in this assay. Furthermore, RSA-opt was a stronger LOX inhibitor than B-opt. Its activity was relatively close to the activity of 1 mg/mL nordihydroguaiaretic acid (NDGA) solution (Figure 3b). Even though both extracts were weaker inhibitors of heat-induced protein coagulation compared with diclofenac, B-opt displayed stronger activity than RSA-opt in this assay (Figure 3a).

2.5. α-Glucosidase- and α-Amylase-Inhibiting Activity

The anti-diabetic potential of the extracts was investigated by studying their potential to inhibit two enzymes involved in carbohydrate digestion: α-glucosidase and α-amylase. The extracts displayed a similar and rather notable inhibition of these two enzymes (Figure 4).



(**a**) (**b**)

Figure 4. Antidiabetic activity of the extracts and positive control acarbose (Acb): (**a**) α-glucosidase-inhibiting activity and (**b**) α-amylase-inhibiting activity. Different uppercase letters indicate statistical significance ($p < 0.05$). Asterisks (*) indicate that the IC_{50} unit is placed on the right *y*-axis.

The tested extracts were equally able to impair α-glucosidase activity. Interestingly, their activity was even higher than the activity of 1 mg/mL acarbose solution (Figure 4a). Similarly, the extracts were rather strong α-amylase inhibitors. The IC_{50} values of the extracts amounted to approximately one-half and one-third of the IC_{50} value of the acarbose solution in α-glucosidase and α-amylase assays, respectively. Although the activities of the extracts were rather similar, RSA-opt displayed slightly better inhibiting properties in the α-amylase assay.

3. Discussion

3.1. Response Surface Analysis and Optimization of Extraction Parameters

Berberis vulgaris root bark is a rich source of berberine, an isoquinoline alkaloid, with various beneficial health-related properties [3]. In this work, a "green" UAE extraction of bioactive principles from *B. vulgaris* root bark was optimized using RSM. Since this work was directed at the preparation of extracts suitable for direct use in cosmeceutical products and anti-diabetic food supplements, mixtures of water with glycerol, a non-toxic and environmentally friendly liquid of natural origin with humectant properties and a very low glycemic index, was chosen as the extraction solvent.

The extraction conditions for bioactive natural products must be carefully selected to ensure that the prepared extracts have the desired characteristics [21]. In order to achieve this, various RSM models are used. In this work, BBD was successfully applied for the optimization of berberine and antiradical compound extraction from *B. vulgaris* root bark. The first of the two optimized parameters, berberine concentration, was chosen because berberine is the most important and well-known constituent of *B. vulgaris* root. Most of the observed bioactivity of this herbal drug is attributed specifically to its berberine content [2,5]. The other dependent variable, the antioxidant activity of the extracts, was selected because it is important for both potential antidiabetic and cosmeceutical activity, as will be discussed later. Various phytochemicals present in *B. vulgaris* and other medicinal plants may display antioxidant activity. When investigating extraction with solvents of changing polarity, it is possible that one group of substances is the main responsible for the activity in predominantly hydrophilic extracts and the other in hydrophobic extracts. Therefore, the activity is not necessarily directly correlated either with the concentration of one antioxidant or even the sum of all antioxidants present in the solution (e.g., in case of synergism). Thus, instead of quantifying various phytochemicals, the target activity itself was determined and optimized. Among the many assays described in the literature, DPPH assay was selected as a simple, reliable and straightforward test, suitable for determination of antiradical activity of a large number of natural extracts in relatively short time. In spite of some limitations, these characteristics render the DPPH assay one of the most commonly used models for the determination of antioxidant activity in the scientific literature today [22].

While UAE may greatly improve the yield of the extraction in comparison to classical techniques such as maceration, it may also affect the composition and the biological activity of the prepared extracts, especially if target compounds are sensitive to degradation. It has been previously noted that the UAE extraction conditions strongly influence the biological activity of the extracts from different *Berberis* sp., such as in the antitumor activity of *B. amurensis* extracts [23].

In this work, high temperature positively affected the berberine content of the extracts. Similar to this, USP also positively influenced berberine concentration, while the influence of glycerol concentration was predominantly negative. While this is the first detailed analysis of the USP effect on berberine concentration, the positive influence of high temperature was previously recorded for ethanolic UAE of Rhizoma Coptidis [24]. High temperature and USP may improve the extraction process by reducing the viscosity of the solvent and increasing the kinetic energy of the molecules in the solutions. In addition to this, higher USP induces more damage to cell walls, thus releasing more intracellular components which can then partition into the extracting solvent [25]. It is interesting to note that, in this work, the combination of high USP and high temperature acted detrimentally on berberine concentration. The observed reduction of berberine concentration may be attributed to its degradation caused by hydroxyl radicals [26] whose production is initiated by ultrasonication, especially at high temperatures [27].

The negative influence of glycerol concentration on extraction of antiradical principles of *B. vulgaris* indicates relatively high polarity of the substances responsible for radical scavenging effects of the extracts. Temperature, USP, and their interaction, on the other hand, positively affected RSA of the extracts. Even though there are no specific studies investigating influence of those parameters on RSA of *B. vulgaris*, some reports show that low USP [28] or temperature [29] are beneficial for DPPH antiradical activity of plant extracts, while others demonstrate that the moderate-to-high temperature [30] or USP [31] may beneficially affect RSA. This is not surprising, as numerous plant components with different physicochemical properties may display RSA, and extraction conditions are do not influence all of them in the same manner. The overall positive influence of temperature and USP on RSA found in this work thus indicates good thermal and chemical stability of the radical scavengers present in the extracts. It is important to note that, even though berberine shows some degree of antiradical activity [32], the RSA IC_{50} of the extracts in this work was not in correlation with berberine content. This means that, besides berberine, other phytochemicals also contribute to the observed antiradical effects, as discussed below.

3.2. Antioxidant Activity of the Extracts

Antioxidant activity is a very important characteristic for the proper storage and function of cosmeticeutical products and anti-diabetic food supplements. Antioxidants may protect the product against the oxidation that occurs during its storage and use. Free radical or metal ions may induce peroxidation of polyunsaturated fatty acids in liquid and semi-solid dosage forms, thus impacting not only the quality but also the safety of the product [33]. In addition to this, antioxidant functional ingredients may have a more active role in such products. For example, antioxidants in topical products may offer protection against oxidative damage of skin macromolecules associated with the effects of free radicals and UV radiation [34]. Antioxidants have a very important role in the prevention and treatment of type 2 diabetes. It is well known that chronic exposure to high glucose concentration, as is the case in diabetes, depletes the levels of endogenous antioxidants and produces oxidative stress in various tissues. This may trigger irreversible damage of the affected cells, ultimately leading to apoptosis. Natural metabolites and extracts may prevent oxidative changes, normalize the concentration of intracellular antioxidants, and thus prevent or even reverse cell damage in vivo and in vitro [35]. The relatively modest antioxidant activity of the extracts observed in this work was not surprising. Some studies have demonstrated good RSA, chelating activities, and other types of antioxidant activity of *B. vulgaris* fruit [36] and leaves [37,38]. However, the activity of *B. vulgaris* root, although measurable, was always significantly lower than the activity of controls, such as ascorbic acid [39,40]. Previous studies have shown that the phenolics substances [37] (e.g., cannabisin G and (±)-lyoniresinol [41]) are the main substances responsible for RSA activity of the *B. vulgaris* root bark extracts. Furthermore, it was found that polysaccharides are the important radical scavengers in *B. dasystachya* [42]. In addition to this, berberine also displays some degree of DPPH radical scavenging and iron chelating activity. However the same work also reports that the activity in both assays was much lower than the activity of the used standard, ascorbic acid [32]. Although determining the exact structures and quantities of the substances responsible for the observed in vitro activity is outside of scope of this research, it was most likely that numerous antiradical compounds contributed to the observed RSA. This may have included berberine together with other phytochemicals present in the root bark, such as various phenolics and polysaccharides. Although the antioxidant activity of the extracts in the performed assays was rather modest, it still positively contributes to their potential use in various food supplements and cosmeceutical products.

3.3. Tyrosinase-, Lipoxygenase-, and Coagulation-Inhibiting Activity

Besides simple hydration and antioxidant protection, cosmeceutical products should also display other biological properties beneficial for skin. Previous research has shown that berberine and the plants and formulations that contain berberine may have an anti-inflammatory effect. Berberine can suppress the release of interleukins in the eosinophil culture and decrease the expression of tyrosinase [43]. In order to further assess the cosmeceutical potential of the prepared extracts, anti-tyrosinase and anti-LOX, as well as anti-inflammatory activity against protein coagulation, were investigated. Tyrosinase inhibitors block melanogenesis by inhibiting tyrosine oxidation to dopaquinone, thus preventing hyperpigmentation of the skin. They are used in treatment of skin discolorations, such as in the treatment of melasma or lentigo solaris [44]. Anti-inflammatory activity is also important for cosmeceutical products. Skin inflammation can be defined as the skin response to an injury, infection, or destruction. It is usually characterized by heat, redness, pain, swelling, or disturbed skin physiological functions. Many dermatologic diseases, such as atopic dermatitis or acne vulgaris, are characterized by inflammatory processes [45]. In this work, anti-inflammatory potential of the extracts was investigated using two assays. The first was the LOX-inhibition assay. LOX is the enzyme involved in arachidonic acid metabolism and the release of various pro-inflammatory eicosanoid substances, such as leukotrienes and lipoxins. LOX plays an important role in the elicitation of skin inflammation and mediates the inflammatory events that are developed as a result of various environmental factors, such as ultraviolet radiation, inflammation mediators, and allergens [46]. The second assay was the inhibition of protein coagulation. Denaturation of tissue proteins is one of the characteristics that causes inflammatory processes. Therefore, the suppression of protein denaturation hinders the development of inflammation-related skin changes, which is another important aspect of anti-aging activity [47]. While the direct tyrosinase-inhibiting and anti-inflammatory activity of *B. vulgaris* was not investigated before, the standardized *B. aristata* extracts were mixed-type tyrosinase inhibitors [48]. In addition to this, berberine from the antipsoriatic plant *Mahonia aquifolium* displayed only a very weak anti-LOX activity [49]. The extracts prepared in this study were active in all the applied assays. Their ability to inhibit the melanogenesis and inflammatory changes caused by LOX activity and protein coagulation makes the extracts potentially good candidates for inclusion into cosmeceutical products.

3.4. α-Glucosidase- and α-Amylase-Inhibiting Activity

Antidiabetic activity of plant extracts may be related to their influence on the enzymes that participate in polysaccharide digestion, thus impairing their degradation to glucose and other monosaccharides. The enzyme α-amylase is secreted in saliva and pancreatic juice. It catalyzes the hydrolysis of starch to a mixture of smaller oligosaccharides, which are then degraded to glucose by α-glucosidase, an enzyme located in the mucosal brush border of the small intestine. Therefore α-amylase and α-glucosidase inhibitors of natural origin can be of importance in the development of drug leads intended for the treatment of diabetes, obesity, and hyperlipemia. In accordance with some previous findings obtained using ethanolic [50] and methanolic [51] *B. vulgaris* extracts, the extracts used in this study demonstrated excellent inhibition of α-glucosidase and α-amylase. An increasing number of studies have shown that berberine significantly accumulates in the intestines [52]. Therefore α-glucosidase and α-amylase inhibitory activity of glycerol and other *B. vulgaris* extracts can certainly contribute to well-established antidiabetic properties of berberine and plants that contain it.

4. Materials and Methods

4.1. Plant Materials and Chemicals

Root bark of *Berberis vulgaris* was a gift from Suban (Samobor, Croatia). Berberine chloride (≥98.5%), BHA (≥98.5%), kojic acid, diclofenac, α-glucosidase, α-amylase, LOX, and tyrosinase were purchased from Sigma-Aldrich (St.Louis, MO, USA), while soybean LOX was from purchased from TCI chemicals (Tokyo, Japan). Acetonitrile was of HPLC grade. Other reagents and chemicals were of analytical grade.

4.2. Preparation of Extracts

Prior to the extraction, plant material was grinded and passed through a sieve of 850 µm mesh size. Powdered plant material (0.2 g) was suspended with 10 mL of the appropriate solvent in a 50 mL Erlenmeyer flask. The extraction was performed in an ultrasonic bath (Bandelin SONOREX Digital 10 P DK 156 BP, Berlin, Germany) at 35 Hz suitable USP. The bath was temperature-controlled. The extraction conditions were chosen according to the Box–Behnken design (Tables 1 and 2). Upon the extraction, the mixture was filtered using folded filter papers S&S 589/1 1/2 (Schleicher & Schuell, Keene, NH, USA). All the extracts were stored at +4 °C in the dark until use.

4.3. Experimental Design

Design Expert software version 8.0.6 (Stat-Ease, Minneapolis, MN, USA) was employed for the regression analysis and the optimization of the results. A three-level-three-factor BBD was employed to determine the best combination of independent extraction variables for the selected dependent variables. The coded values for design parameters (dependent variables) were chosen as presented in Table 1. Berberine concentration (Y_1) and RSA IC_{50} (Y_2) were selected as the responses (Table 2). Experimental data were fitted to a quadratic polynomial model, as described by the quadratic Equation (1):

$$Y = A_0 + \sum_{i=1}^{k} A_i X_i + \sum_{i=1}^{k} A_{ii} X_i^2 + \sum_{i=1}^{k-1} \times \sum_{j=1+1}^{k} A_{ij} X_i X_j \quad (1)$$

where Y is the dependent variable; A_0, A_i, A_{ii}, and A_{ij} are the regression coefficients for the intercept, linearity, square, and interaction, respectively; X_i and X_j are the independent variables.

4.4. Berberine Quantification

Berberine was quantified using an HPLC instrument (Agilent 1200 series, Agilent Technologies, USA) equipped with a diode array detector (DAD) and Zorbax Eclipse XDB C18 column (5 µm, 250 mm × 4.6 mm, Agilent Technologies, Santa Clara, CA, USA). The injection volume was 20 µL. Before the injections, the solutions of the standard (0.2 mg/mL solution of berberine) and the extracts were filtered through a 0.45 µm PTFE-syringe filter. Triethylamine-adjusted 0.02 mol/L H_3PO_4 (pH 4.82) with 25% acetonitrile was chosen as the mobile phase. The flow-rate was 1.0 mL/min. The peaks were observed and quantified at 254 nm. The peak assignment and identification was based on a comparison of retention times and the spectra of peaks in the sample chromatogram with those of the standard. Berberine was quantified according to its respective standard calibration curve. The calibration curve was plotted as area under curve (AUC) of berberine peak (y, arbitrary units) against the weight of berberine in the sample (x, µg). Limit of detection (LD) and limit of quantification (LQ) were determined according to [53]. LD and LQ were 0.0186 µg, 0.0564 µg, respectively, while the calibration curve is presented in Equation (2):

$$y = 2834.4x + 22.08 \ (r^2 = 0.9999) \quad (2)$$

4.5. Free Radical Scavenging Activity

RSA was evaluated as described by Fumić et al. [30]. Methanolic solution of DPPH (70 µL, 0.21 mg/mL) was added to 130 µL of either the methanolic solution of the extract (sample) or methanol (negative control). After 30 min in the dark at room temperature, the absorbance was read at 545 nm using microplate reader (Stat Fax 3200, Awareness Technologies, Palm City, FL, USA). RSA was calculated according to Equation (3):

$$\text{RSA}\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (3)$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the DPPH solution containing extract. Concentration of the extract, which scavenged 50% of DPPH free radicals present in the solution (RSA IC_{50}), was calculated and expressed as μL of extract/mL of solution (μL/mL). BHA was used as the standard radical scavenger.

4.6. Fe^{2+} Chelating Activity

The chelating activity (ChA) of the investigated substances toward ferrous ions was studied, as described by Bljajić et al. [8]. To the solution of the extract in methanol (150 μL), 0.25 mM of $FeCl_2$ solution (50 μL) was added. After 5 min, 100 μL of 1.0 mM ferrozine solution was applied. Absorbance at 545 nm was recorded after 10 min. Reaction mixture containing methanol (150 μL), instead of the extract, served as a negative control. ChA was calculated using Equation (4):

$$ChA\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (4)$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which chelated 50% of Fe^{2+} present in the solution (ChA IC_{50}), was calculated and expressed as μL of extract/mL of solution (μL/mL). Ethylenediaminetetraacetic acid (EDTA) was used as the chelating standard.

4.7. Antioxidant Activity in β-Carotene-Linoleic Acid Assay

Antioxidant activity in β-carotene-linoleic acid assay (AOA) was evaluated using the β-carotene-linoleic acid system according to modified literature procedure published by Rajić et al. [54]. Aliquots (200 μL) of the emulsion containing β-carotene (6.7 μg/mL), linoleic acid (0.7 mg/mL), and Tween 40 (6.7 mg/mL) were added either to water (50 μL) (control) or to the solutions of the extract in methanol (50 μL). The reaction mixture was incubated at 50 °C. The antioxidant activity in β-carotene linoleic acid assay (AACL) was calculated based on the absorbance recorded after 60 min using Equation (5):

$$AACL\ (\%) = \frac{A_{sample}}{A_{control}} \times 100 \quad (5)$$

where $A_{control}$ and A_{sample} are the absorbances of the water control and the antioxidant, respectively. Concentration of the extract, which protected 50% β-carotene present in the solution (*AACL* IC_{50}), was calculated and expressed as μL of extract/mL of solution (μL/mL). BHA was used as the standard antioxidant.

4.8. Tyrosinase Inhibitory Activity

Tyrosinase inhibition activity of the extracts was determined following the method described by Masuda et al. [55] with minor modifications. In 80 μL extract solution or water (negative control), 40 μL of mushroom tyrosinase solution (in 16 mM, pH 6, 8 phosphate buffer) was added. The solution was incubated in the dark at 25 °C. After 10 min, 80 μL of 3,4-dihydroxy-L-phenylalanine (L-DOPA) solution (0.19 mg/mL in phosphate buffer) was added. After the additional 10 min, the absorbance at 492 nm was measured. The negative control contained buffer instead of the extract solution. Tyrosinase inhibitory activity (TyInh) was calculated as described in Equation (6):

$$TyInh\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (6)$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which inhibited 50% of tyrosinase activity (*TyInh* IC_{50}), was calculated and expressed as μL of extract/mL of solution (μL/mL). Kojic acid was used as the standard inhibitor.

4.9. Lipoxygenase Inhibitory Activity

LOX inhibitory activity was determined spectrophotometrically [56]. A volume of 50 µL of different concentrations of extracts or water (negative control) was mixed with 150 µL phosphate buffer (pH 8, 100 µM) and 20 µL of soybean LOX solution. Finally, 30 µL of linoleic acid was added to initiate a reaction. The mixtureas incubated at 25 °C for 10 min and the absorbance was determined at 234 nm. LOX inhibitory activity (LOXInh) was calculated as presented in Equation (7):

$$LOXInh\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \tag{7}$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which inhibited 50% of LOX activity (*LOXInh* IC_{50}), was calculated and expressed as µL of extract/mL of solution (µL/mL). Nordihydroguaiaretic acid (NDGA) was used as a positive control.

4.10. Anti-Inflammatory Activity

Anti-inflammatory activity was evaluated using the heat-induced ovalbumin coagulation method [47]. The reaction mixture consisted of 0.4 mL of ovalbumin solution (50% fresh hen's albumen), 2.8 mL of phosphate buffered saline (pH 6.4), and 2 mL of the extract solution or water (negative control). The mixtures were incubated at 37 °C for 15 min and then heated at 70 °C for 5 min. After cooling, their absorbance was recorded at 660 nm. The percentage inhibition of ovalbumin denaturation (OvInh) was calculated by using the following Equation (8):

$$OvInh\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \tag{8}$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Concentration of the extract, which inhibits 50% of the ovalbumin coagulation (*OvInh* IC_{50}), was calculated and expressed as µL of extract/mL of solution (µL/mL). Diclofenac sodium was used as the standard inhibitor.

4.11. α-Glucosidase Inhibition Assay

Inhibition of α-glucosidase was determined spectrophotometrically [8] with slight modification. In brief, 20 µL of test samples or water (negative control) were incubated with 50 µL of α-glucosidase from *Saccharomyces cerevisiae* (0.2 U/mL dissolved in 0.1 M phosphate buffer, pH 6.8) for 10 min at 37 °C. Afterwards, 50 µL substrate (1 mM *p*-nitrophenyl-α-D-glucopyranoside prepared in same buffer) was added to the reaction mixture and the release of *p*-nitrophenol was measured at 405 nm after 5 min of incubation. Percentage of α-glucosidase inhibition (AglInh) was calculated as follows, according to Equation (9):

$$AglInh\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \tag{9}$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the reaction mixture containing extracts. Concentration of the extract, which inhibited 50% α-glucosidase activity (*AglInh* IC_{50}), was calculated and expressed as µL of extract/mL of solution (µL/mL). Standard reference acarbose was used.

4.12. α-Amylase Inhibition Assay

The assay was performed according to Apostolidis et al. [57]. Extracts (0.5 mL) at different concentrations, or water (negative control), and 0.5 mL of 20 mM phosphate buffer (pH 6.9) containing α-amylase from porcine pancreas (0.8 U/mL) were preincubated at 25 °C for 10 min. This was followed by the addition of 0.5 mL soluble starch (0.5% solution in the same buffer). The reaction mixtures were incubated at 25 °C for 10 min and then the reaction was stopped with 1 mL of 96 mM 3.5-dinitrosalicylic acid color reagent. Afterwards, the test tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixtures were diluted by adding 10 mL distilled water and absorbance was measured at 540 nm, and percentage of α-amylase inhibition (AmInh) was calculated, as shown in Equation (10):

$$AmInh\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \tag{10}$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the reaction mixture containing extracts. Concentration of the extract, which inhibited 50% amylase activity (*AmInh* IC_{50}), was calculated and expressed as μL of extract/mL of solution (μL/mL). Acarbose was used as the positive control.

4.13. Statistical Analysis

The measurements were performed in triplicate and the results are presented as mean ± standard deviation. In order to establish the IC_{50} values, the experiments were performed using different concentrations (four to seven concentrations, depending on the assay). Statistical comparisons were made using one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons (GraphPad Prism, San Diego, CA, USA). $p < 0.05$ was considered statistically significant. IC_{50} values were calculated by applying the appropriate regression analysis.

5. Conclusions

Glycerolic UAE procedure of berberine and antiradical components from *B. vulgaris* was developed using RSM. The prepared extracts were efficient radical scavengers and Fe^{2+} ion chelators. Furthermore, they were able to impair heat-induced degradation proteins and linoleic acid and. In addition to this, the prepared extracts were efficient tyrosinase, LOX, α-glucosidase, and α-amylase inhibitors. Because of their excellent cosmeceutical and anti-diabetic properties, as well as the non-toxicity of the solvent used for the extraction, the prepared extracts are suitable candidates for direct use in antidiabetic and dermatologic food supplements and topical products.

Author Contributions: Conceptualization, M.Z.K.; methodology, P.C., L.V., and M.Z.K.; investigation, M.D., L.V., and P.C.; writing—original draft preparation, M.Z.K.; writing—review and editing, P.C. and M.Z.K.; supervision, M.Z.K.; project administration, M.Z.K.; funding acquisition, M.Z.K.

Funding: This research was funded by Croatian Science Foundation, project Bioactive plant principles extraction using green solvents—a step towards green cosmeceuticals, grant number IP-2018-01-6504.

Acknowledgments: We kindly thank the Suban Company for the donation of plant material.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Bruneton, J. *Pharmacognosy, Phytochemistry, Medicinal Plants*, 2nd ed.; Lavoisier: Cachan, France, 1999.
2. Neag, M.A.; Mocan, A.; Echeverría, J.; Pop, R.M.; Bocsan, C.I.; Crisan, G.; Buzoianu, A.D. Berberine: Botanical occurrence, traditional uses, extraction methods, and relevance in cardiovascular, metabolic, hepatic, and renal disorders. *Front. Pharmacol.* **2018**, *9*, 557. [CrossRef] [PubMed]
3. Kosalec, I.; Gregurek, B.; Kremer, D.; Zovko, M.; Sanković, K.; Karlović, K. Croatian barberry (*Berberis croatica* Horvat): A new source of berberine–analysis and antimicrobial activity. *World, J. Microbiol. Biotechnol.* **2009**, *25*, 145–150. [CrossRef]

4. Da Silva, A.R.; de Andrade Neto, J.B.; da Silva, C.R.; de Sousa Campos, R.; Costa Silva, R.A.; Freitas, D.D.; do Nascimento, F.B.S.A.; de Andrade, L.N.D.; Sampaio, L.S.; Grangeiro, T.B.; et al. Berberine antifungal activity in fluconazole-resistant pathogenic yeasts: Action mechanism evaluated by flow cytometry and biofilm growth inhibition in *Candida* spp. *Antimicrob. Agents Chemother.* **2016**, *60*, 3551–3557. [CrossRef] [PubMed]
5. Berberine: Uses, Side Effects, Interactions, Dosage, and Warning. Available online: https://www.webmd.com/vitamins/ai/ingredientmono-1126/berberine (accessed on 31 July 2019).
6. Han, T.S.; Lean, M.E. A clinical perspective of obesity, metabolic syndrome and cardiovascular disease. *JRSM Cardiovasc. Dis.* **2016**, *5*. [CrossRef] [PubMed]
7. Fiorentino, T.V.; Prioletta, A.; Zuo, P.; Folli, F. Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Curr. Pharm. Des.* **2013**, *19*, 5695–5703. [CrossRef] [PubMed]
8. Bljajić, K.; Petlevski, R.; Vujić, L.; Čačić, A.; Šoštarić, N.; Jablan, J.; Saraiva de Carvalho, I.; Zovko Končić, M. Chemical composition, antioxidant and α-glucosidase-inhibiting activities of the aqueous and hydroethanolic extracts of *Vaccinium myrtillus* leaves. *Molecules* **2017**, *22*, 703. [CrossRef] [PubMed]
9. Cicero, A.F.G.; Tartagni, E. Antidiabetic properties of berberine: From cellular pharmacology to clinical effects. *Hosp. Pract. 1995* **2012**, *40*, 56–63. [CrossRef] [PubMed]
10. Tian, C.-M.; Jiang, X.; Ouyang, X.-X.; Zhang, Y.-O.; Xie, W.-D. Berberine enhances antidiabetic effects and attenuates untoward effects of canagliflozin in streptozotocin-induced diabetic mice. *Chin. J. Nat. Med.* **2016**, *14*, 518–526. [CrossRef]
11. Azzanizawaty Yahya, N.; Attan, N.; Wahab, R. An overview of cosmeceutically relevant plant extracts and strategies for extraction of plant-based bioactive compounds. *Food Bioprod. Process.* **2018**, *112*, 69–85. [CrossRef]
12. Li, Z.; Geng, Y.-N.; Jiang, J.-D.; Kong, W.-J. Antioxidant and anti-inflammatory activities of berberine in the treatment of diabetes mellitus. *Evid. Based Complement. Alternat. Med.* **2014**, *2014*, 1–12. [CrossRef]
13. Jang, Y.-A.; Lee, J.-T. Anti-wrinkle effect of berberine by inhibition of MMP-2 and MMP-9 activity in fibroblasts. *J. Appl. Biol. Chem.* **2018**, *61*, 9–15. [CrossRef]
14. Izadiyan, P.; Hemmateenejad, B. Multi-response optimization of factors affecting ultrasonic assisted extraction from Iranian basil using central composite design. *Food Chem.* **2016**, *190*, 864–870. [CrossRef] [PubMed]
15. Wolfson, A.; Dlugy, C.; Shotland, Y. Glycerol as a green solvent for high product yields and selectivities. *Environ. Chem. Lett.* **2007**, *5*, 67–71. [CrossRef]
16. Shehata, E.; Grigorakis, S.; Loupassaki, S.; Makris, D.P. Extraction optimisation using water/glycerol for the efficient recovery of polyphenolic antioxidants from two *Artemisia* species. *Sep. Purif. Technol.* **2015**, *149*, 462–469. [CrossRef]
17. Apostolakis, A.; Grigorakis, S.; Makris, D.P. Optimisation and comparative kinetics study of polyphenol extraction from olive leaves (*Olea europaea*) using heated water/glycerol mixtures. *Sep. Purif. Technol.* **2014**, *128*, 89–95. [CrossRef]
18. Aalim, H.; Belwal, T.; Jiang, L.; Huang, H.; Meng, X.; Luo, Z. Extraction optimization, antidiabetic and antiglycation potentials of aqueous glycerol extract from rice (*Oryza sativa* L.) bran. *LWT* **2019**, *103*, 147–154. [CrossRef]
19. Babamoradi, N.; Yousefi, S.; Ziarati, P. Optimization of ultrasound-assisted extraction of functional polysaccharides from common mullein (*Verbascum thapsus* L.) flowers. *J. Food Process Eng.* **2018**, *41*, e12851. [CrossRef]
20. Cui, H.-X.; Hu, Y.-N.; Li, J.-W.; Yuan, K.; Guo, Y. Preparation and evaluation of antidiabetic agents of berberine organic acid salts for enhancing the bioavailability. *Molecules* **2019**, *24*, 103. [CrossRef] [PubMed]
21. Mandal, S.C.; Mandal, V.; Das, A.K. Chapter 6 - Classification of Extraction Methods. In *Essentials of Botanical Extraction*; Mandal, S.C., Mandal, V., Das, A.K., Eds.; Academic Press: Boston, MA, USA, 2015; pp. 83–136.
22. Schaich, K.M.; Tian, X.; Xie, J. Hurdles and pitfalls in measuring antioxidant efficacy: A critical evaluation of ABTS, DPPH, and ORAC assays. *J. Funct. Foods* **2015**, *14*, 111–125. [CrossRef]
23. Wu, J.; Yu, D.; Sun, H.; Zhang, Y.; Zhang, W.; Meng, F.; Du, X. Optimizing the extraction of anti-tumor alkaloids from the stem of *Berberis amurensis* by response surface methodology. *Ind. Crops Prod.* **2015**, *69*, 68–75. [CrossRef]
24. Teng, H.; Choi, Y.H. Optimization of ultrasonic-assisted extraction of bioactive alkaloid compounds from Rhizoma coptidis (*Coptis chinensis* Franch.) using response surface methodology. *Food Chem.* **2014**, *142*, 299–305. [CrossRef] [PubMed]

25. Hossain, M.B.; Tiwari, B.K.; Gangopadhyay, N.; O'Donnell, C.P.; Brunton, N.P.; Rai, D.K. Ultrasonic extraction of steroidal alkaloids from potato peel waste. *Ultrason. Sonochem.* **2014**, *21*, 1470–1476. [CrossRef]
26. Marszalek, M.; Wolszczak, M. Radiolysis of berberine or palmatine in aqueous solution. *Radiat. Phys. Chem.* **2011**, *80*, 94–99. [CrossRef]
27. Suslick, K.S.; Price, G.J. Applications of ultrasound to materials chemistry. *Annu. Rev. Mater. Sci.* **1999**, *29*, 295–326. [CrossRef]
28. Aware, C.B.; Patil, R.R.; Vyavahare, G.D.; Gurme, S.T.; Jadhav, J.P. Ultrasound-assisted aqueous extraction of phenolic, flavonoid compounds and antioxidant activity of *Mucuna macrocarpa* beans: Response surface methodology optimization. *J. Am. Coll. Nutr.* **2019**, *38*, 364–372. [CrossRef] [PubMed]
29. Jagadeesan, G.; Muniyandi, K.; Manoharan, A.L.; Thamburaj, S.; Sathyanarayanan, S.; Thangaraj, P. Optimization of phenolic compounds extracting conditions from *Ficus racemosa* L. fruit using response surface method. *J. Food Meas. Charact.* **2019**, *13*, 312–320. [CrossRef]
30. Fumić, B.; Jug, M.; Končić, M.Z. Multi-response optimization of ultrasound-assisted extraction of bioactive components from *Medicago sativa* L. *Croat. Chem. Acta* **2017**, *90*, 481–491. [CrossRef]
31. Sharmila, G.; Nikitha, V.S.; Ilaiyarasi, S.; Dhivya, K.; Rajasekar, V.; Kumar, N.; Muthukumaran, K.; Muthukumaran, C. Ultrasound assisted extraction of total phenolics from *Cassia auriculata* leaves and evaluation of its antioxidant activities. *Ind. Crops Prod.* **2016**, *84*, 13–21. [CrossRef]
32. Shirwaikar, A.; Shirwaikar, A.; Rajendran, K.; Punitha, I.S.R. In vitro antioxidant studies on the benzyl tetra isoquinoline alkaloid berberine. *Biol. Pharm. Bull.* **2006**, *29*, 1906–1910. [CrossRef] [PubMed]
33. Mlakar, A.; Batna, A.; Dudda, A.; Spiteller, G. Iron (II) ions induced oxidation of ascorbic acid and glucose. *Free Radic. Res.* **1996**, *25*, 525–539. [CrossRef] [PubMed]
34. Ratz-Łyko, A.; Arct, J. Resveratrol as an active ingredient for cosmetic and dermatological applications: A review. *J. Cosmet. Laser Ther. Off. Publ. Eur. Soc. Laser Dermatol.* **2019**, *21*, 84–90. [CrossRef] [PubMed]
35. Chandrasekaran, K.; Swaminathan, K.; Chatterjee, S.; Dey, A. Apoptosis in HepG2 cells exposed to high glucose. *Toxicol. In Vitro* **2010**, *24*, 387–396. [CrossRef] [PubMed]
36. Hassanpour, H.; Alizadeh, S. Evaluation of phenolic compound, antioxidant activities and antioxidant enzymes of barberry genotypes in Iran. *Sci. Hortic.* **2016**, *200*, 125–130. [CrossRef]
37. Zovko Končić, M.; Kremer, D.; Karlović, K.; Kosalec, I. Evaluation of antioxidant activities and phenolic content of *Berberis vulgaris* L. and *Berberis croatica* Horvat. *Food Chem. Toxicol.* **2010**, *48*, 2176–2180. [CrossRef] [PubMed]
38. Hadaruga, D.I.; Hadaruga, N.G.; Bandur, G.N.; Rivis, A.; Costescu, C.; Ordodp, V.L.; Ardelean, A. *Berberis vulgaris* extract/β-cyclodextrin nanoparticles synthesis and characterization. *Rev. Chim.* **2010**, *61*, 669–675.
39. El khalki, L.; Tilaoui, M.; Jaafari, A.; Ait Mouse, H.; Zyad, A. studies on the dual cytotoxicity and antioxidant properties of *Berberis vulgaris* extracts and its main constituent berberine. *Adv. Pharmacol. Sci.* **2018**, *2018*. [CrossRef]
40. Mezouar, D.; Lahfa, F.B.; Djaziri, R.; Boucherit-Otmani, Z. Évaluation de l'activité antioxydante de *Berberis vulgaris* L. *Phytothérapie* **2014**, *12*, 297–301. [CrossRef]
41. Tomosaka, H.; Chin, Y.-W.; Salim, A.A.; Keller, W.J.; Chai, H.; Kinghorn, A.D. Antioxidant and cytoprotective compounds from *Berberis vulgaris* (barberry). *Phytother. Res.* **2008**, *22*, 979–981. [CrossRef]
42. Han, L.; Suo, Y.; Yang, Y.; Meng, J.; Hu, N. Optimization, characterization, and biological activity of polysaccharides from *Berberis dasystachya* Maxim. *Int. J. Biol. Macromol.* **2016**, *85*, 655–666. [CrossRef]
43. Yonghwa, L.E.E.; Lichao, L.I.U.; Yongsub, Y.I. Inhibitory effect of berberine from *Coptidis rhizoma* on melanin synthesis of murine malignant melanoma. *Pharmazie* **2018**, *73*, 300–303.
44. Chang, T.-S. An updated review of tyrosinase inhibitors. *Int. J. Mol. Sci.* **2009**, *10*, 2440–2475. [CrossRef] [PubMed]
45. Bessada, S.M.F.; Alves, R.C.; Oliveira, P.P.; Beatriz, M. Coffee silverskin: A review on potential cosmetic applications. *Cosmetics* **2018**, *5*, 5. [CrossRef]
46. Aruna, V.; Amruthavalli, G.V.; Gayathri, R. Use of cosmetic products for treating certain diseases—Know the science. *J. Cosmet. Dermatol.* **2019**, *18*, 221–225. [CrossRef] [PubMed]
47. Chandra, S.; Chatterjee, P.; Dey, P.; Bhattacharya, S. Evaluation of in vitro anti-inflammatory activity of coffee against the denaturation of protein. *Asian Pac. J. Trop. Biomed.* **2012**, *2*, S178–S180. [CrossRef]
48. Biswas, R.; Mukherjee, P.K.; Chaudhary, S.K. Tyrosinase inhibition kinetic studies of standardized extract of *Berberis aristata*. *Nat. Prod. Res.* **2016**, *30*, 1451–1454. [CrossRef]

49. Müller, K.; Ziereis, K. The Antipsoriatic *Mahonia aquifolium* and its active constituents; I. Pro- and antioxidant properties and inhibition of 5-lipoxygenase. *Planta Med.* **1994**, *60*, 421–424. [CrossRef]
50. Abd El-Wahab, A.E.; Ghareeb, D.A.; Sarhan, E.E.M.; Abu-Serie, M.M.; El Demellawy, M.A. In vitro biological assessment of *Berberis vulgaris* and its active constituent, berberine: Antioxidants, anti-acetylcholinesterase, anti-diabetic and anticancer effects. *BMC Complement. Altern. Med.* **2013**, *13*. [CrossRef]
51. Boudjelthia, K.; Hammadi, K.; Kouidri, M.; Djebli, N. Evaluation of antidiabetic activity of two plants *Berberis vulgaris* and *Zygophyllum geslini*. *J. Phys. Chem. Biophys.* **2017**, *7*, 236. [CrossRef]
52. Wang, H.; Guan, L.; Li, J.; Lai, M.; Wen, X. The Effects of berberine on the gut microbiota in Apc min/+ mice fed with a high fat diet. *Molecules* **2018**, *23*, 2298. [CrossRef]
53. Kleinschmidt, G. Case Study: Validation of an HPLC-method for identity, assay, and related impurities. In *Method Validation in Pharmaceutical Analysis: A Guide to Best Practice*; Ermer, J., Miller McB, J.H., Eds.; Wiley-VCH: Weinheim, Germany, 2005; pp. 195–212.
54. Rajić, Z.; Končić, M.; Miloloža, K.; Perković, I.; Butula, I.; Bucar, F.; Zorc, B. Primaquine-NSAID twin drugs: Synthesis, radical scavenging, antioxidant and Fe_{2+} chelating activity. *Acta Pharm.* **2010**, *60*, 325–337. [CrossRef]
55. Masuda, T.; Yamashita, D.; Takeda, Y.; Yonemori, S. Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica*. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 197–201. [CrossRef] [PubMed]
56. Chekir, S.; Debbabi, M.; Regazzetti, A.; Dargère, D.; Laprévote, O.; Ben Jannet, H.; Gharbi, R. Design, synthesis and biological evaluation of novel 1,2,3-triazole linked coumarinopyrazole conjugates as potent anticholinesterase, anti-5-lipoxygenase, anti-tyrosinase and anti-cancer agents. *Bioorganic Chem.* **2018**, *80*, 189–194. [CrossRef] [PubMed]
57. Apostolidis, E.; Kwon, Y.-I.; Shetty, K. Inhibitory potential of herb, fruit, and fungal-enriched cheese against key enzymes linked to type 2 diabetes and hypertension. *Innov. Food Sci. Emerg. Technol.* **2007**, *8*, 46–54. [CrossRef]

Sample Availability: Samples of the compounds are not available from the authors.

2.5. *Silybum marianum* Glycerol Extraction for the Preparation of High-Value Anti-Aging Extracts

Industrial Crops & Products 168 (2021) 113613

Contents lists available at ScienceDirect

Industrial Crops & Products

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

Silybum marianum glycerol extraction for the preparation of high-value anti-ageing extracts

Magda Jabłonowska [a,b], Petar Ciganović [a], Jasna Jablan [a,c], Eva Marguí [c], Michał Tomczyk [b], Marijana Zovko Končić [a,*]

[a] University of Zagreb, Faculty of Pharmacy and Biochemistry, A. Kovačića 1, 10000 Zagreb, Croatia
[b] Medical University of Białystok, Faculty of Pharmacy, ul. Mickiewicza 2a, 15-230 Białystok, Poland
[c] University of Girona, Department of Chemistry, C/M. Aurèlia Campmany, 69, 17003 Girona, Spain

ARTICLE INFO

Keywords:
Antioxidant
Cosmeceutical
Glycerol
Green extraction
Silybum marianum
Silymarin

ABSTRACT

Silybum marianum fruit is rich in silymarin, a flavonolignan mixture with hepatoprotective properties, whose favourable effects on skin make it a valuable ingredient in cosmetic products. The scope of the present study is to compare the efficiency of classic ethanol/water maceration with the maceration using mixtures of water with glycerol, a non-toxic, biodegradable, and affordable solvent, available from renewable sources. Furthermore, a glycerol-based ultrasound-assisted extraction (UAE) method was developed and optimized for silymarin content and antiradical activity. The prepared extracts were tested for their antioxidant and cosmeceutical activity. Maceration with glycerol/water mixtures was somewhat less efficient than the ethanol/water maceration. However, the extraction efficiency was significantly improved by means of ultrasonication. When UAE was conducted with 40 % (w/w) glycerol during 60 min at 80 °C the amount of extracted silymarin in the extracts was similar to that in the ethanol/water macerates (107.19 μg/mL *vs.* 116.17 μg/mL, respectively). Antiradical activity of the extracts was related to the silymarin content. The prepared extracts displayed mild Fe^{2+} chelating activity and hindered heat-induced degradation of polyunsaturated fatty acids, as well as elastase activity. Glycerol showed additive effects on the anti-tyrosinase activity of the extracts and was responsible for anti-inflammatory activity in the heat-induced coagulation assay. The presence of zinc, magnesium and other minerals with beneficial skin-related properties in *S. marianum* fruit further substantiate its use in cosmetics. The results indicate that glycerol extraction of *S. marianum* eliminates the need and energy necessary for solvent removal and enhances the desired functional and anti-ageing properties of the prepared extracts.

1. Introduction

The growing global concern for the future of our planet has led to a rise in public environmental awareness, including in relation to nature preservation, as well as reducing carbon emissions and pollution. This resulted in an expansion of the market for products consisting solely of natural ingredients and produced using eco-friendly materials and procedures. Cosmetic users in particular show strong preference for products derived from plant extracts and other natural sources because they judge them to be environmentally friendly, safe, and able to beneficially affect health and appearance of human skin (Yahya et al., 2018).

Environmentally friendly and sustainable methods of extracting bioactive natural products from medicinal plants are continuously being developed (Fu et al., 2020; Huang et al., 2019). Such methods are typically achieving high yields of the desired metabolite, have low energy consumption and use natural, biodegradable, non-toxic solvents, obtainable from renewable sources (Chemat et al., 2019, 2012). Glycerol is one of the few solvents that fully fits this description. In addition

Abbreviations: AACL, Antioxidant activity in β-carotene linoleic acid assay; ANOVA, Analysis of variance; BHA, Butylated hydroxyanisole (BHA); ChA, Chelating activity; DF, Degrees of freedom; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, Ethylenediaminetetraacetic acid; ElInh, Elastase inhibitory activity; ICP-OES, Inductively Coupled Plasma Optical Emission Spectrometry; HRS, Herbal reference standard; IL-1α, Interleukin 1 alpha; MS, Mean Square; OvInh, Inhibition of ovalbumin denaturation; RSA, Radical scavenging activity; SS, Sum of Squares; TNF, Tumour necrosis factor; TXRF, Total Reflection X-ray Fluorescence Spectroscopy; TyInh, Tyrosinase inhibitory activity; UAE, Ultrasound-assisted extraction.

* Corresponding author.
E-mail address: mzovko@pharma.hr (M. Zovko Končić).

https://doi.org/10.1016/j.indcrop.2021.113613
Received 12 January 2021; Received in revised form 30 April 2021; Accepted 5 May 2021
Available online 11 May 2021
0926-6690/

to its natural origin, safety, and biodegradability, glycerol is manufactured at a very low-cost, as a by-product of biodiesel production (Wolfson et al., 2007). Glycerol is a ubiquitous ingredient in topical formulations due to its hygroscopic nature and humectant properties. It is employed as a light fragrance, skin protectant, and as a viscosity-decreasing, hair-conditioning and oral care agent (Becker et al., 2019). Thus, the preparation of glycerolic extracts for pharmaceutical and cosmetic purposes has an additional advantage in that such extracts may be easily incorporated into the final product. This makes the extraction of medicinal plants with this solvent ecologically acceptable due to low waste, reduced carbon footprint, and energy consumption. Unlike the use of ethanol, another natural solvent widely used for extraction of plant bioactive principles, the use of glycerol in cosmetics and pharmaceuticals is acceptable in all cultural, religious, and age groups (Chemat et al., 2012). Glycerol/water mixtures were shown to be effective solvents for natural polyphenols from e.g. *Echinacea purpurea* (L.) Moench aerial parts (Momchev et al., 2020), bran rice (Aalim et al., 2019), and leaves of walnut trees (*Juglans regia* L.) (Vieira et al., 2020). However, despite the obvious advantages of glycerol as an extraction solvent, it remains relatively underutilised in the production of extracts for pharmaceutical and cosmeceutical purposes.

Ultrasound-assisted extraction (UAE) is one of the prevailing techniques for extraction of secondary metabolites due to its simplicity, high reproducibility, low CO_2 emissions, limited solvent use, as well as low cost- and high time-effectiveness (Caldas et al., 2018; Delgado-Povedano and Luque de Castro, 2015; Hou et al., 2019). The efficiency of UEA may be explained by the capillary effects, cavitation, and cell disruption which enables an improved mass transfer, penetration of the solvent into the cell, as well as the release of cellular components into the surrounding medium (Drouet et al., 2019). Despite its wide availability and low price, the use of glycerol as a solvent for UAE of secondary metabolites is relatively uncommon. Nevertheless, good extraction yields and mild reaction conditions that characterised glycerolic UAE extraction of natural products in the examples published to date (Ciganović et al., 2019; Dulić et al., 2019; Katsampa et al., 2015) give hope that the use of this eco-friendly solvent for extracting natural products will only increase in the future.

Silybum marianum (L.) Gaertn (Asteraceae) (milk thistle) is a biennial medicinal herb whose fruit (*Silybi mariani fructus*) is traditionally used for therapy of liver disorders. According to the European Medicines Agency, *S. marianum* and preparations thereof may be used to relieve digestive disorders, the sensation of fullness and indigestion, and support the liver function (European Medicines Agency, 2018). The most important phytochemical component of the fruit is a flavonolignan complex called silymarin. The greatest proportion of silymarin consists of two stereoisomers called silibinin A and silibinin B (about 60–70 %), followed by silicristin, silidianin, and isosilibinin. Silymarin and its components are safe and powerful antioxidants with detoxifying, preventive, protective, and regenerative properties. Silibinin isomers, for example, down-regulate lipoxygenase, cyclooxygenase, tumour necrosis factor (TNF), and interleukin 1 alpha (IL-1α), as well as induce apoptosis (Singh and Agarwal, 2009). Silymarin is also very interesting as a cosmetic and dermatologic ingredient because many of its activities are related to its action on the skin. It seems that silymarin and the milk thistle fruit extract may inhibit the enzymes involved in the cleavage of extracellular matrix components, as well as offer powerful antioxidant and UV-protective effects for the skin (Drouet et al., 2019).

The multiple biological activities of milk thistle fruit and silymarin are of particular interest to those researching cosmetic applications and point to the necessity of developing efficient green extraction protocols for its extraction. Therefore, this work aimed to compare the efficacy of the extraction of bioactive flavonolignans and other antioxidants from *S. marianum* fruit using glycerol/water maceration with ethanol/water maceration. Furthermore, glycerol/water UAE of *S. marianum* fruit was optimised using response surface methodology based on Box-Behnken design. The anti-ageing activity of the optimized extracts was assessed with the aim of preparing extracts ready to use in topical formulations.

2. Materials and methods

2.1. Chemicals

Butylated hydroxyanisole (BHA), ethylenediaminetetraacetic acid (EDTA), gallium, kojic, and ursolic acid were purchased from Sigma-Aldrich (St. Louis, MO, US), while diclofenac was purchased from TCI Chemicals (Tokyo, Japan). The purity of the employed standards was higher than 98.5 %. For quantification of silymarin, European pharmacopoeia milk thistle dry extract herbal reference standard (HRS) was used. Mushroom tyrosinase and porcine pancreas elastase were purchased from Sigma-Aldrich (St. Louis, MO, USA). For chromatographic separations, HPLC grade solvents were used. Other reagents and chemicals were of analytical grade.

2.2. Plant material

The identity of plant material, supplied by the Suban company (Samobor, Croatia), was confirmed according to the EU pharmacopoeial monograph for *S. marianum* fruit (European Pharmacopoeia, 2013). A voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia (FG-2018-SM).

2.3. TXRF and ICP-OES determination of metals in the plant material

Microwave acid digestion, based on the United States Environmental Protection Agency method 3052, was employed for inductively coupled plasma optical emission spectrometry (ICP-OES) sample preparation. A sample of about 70 mg was added to PTFE vessel with 6 mL of nitric acid and 0.6 mL of hydrogen peroxide. The vessels were closed, heated for 5 min to reach 180 °C and then for additional 10 min at constant temperature. The solutions were then transferred to a 10 mL flask and brought to volume with ultrapure de-ionized water. For ICP-OES measurement, Agilent ICP-OES 5100 spectrometer (Agilent Technologies, Santa Clara, CA, USA) with a pneumatic concentric nebuliser, radio frequency power of 1200 W, plasma flow of 12 L min^{-1} radial torch configuration and multichannel charge transfer detector, was used. The metals were quantified at the following wavelengths: 259.372 nm (Mg), 202.548 nm (Zn) and 238.204 nm (Fe). The strontium and calcium content was determined using total reflection x-ray fluorescence (TXRF). For the TXRF analysis plant material was sifted through a sieve with a diameter of 63 μm. Then, 20 mg of sample were suspended in 1 mL of de-ionized water containing 10 μg of Ga as an internal standard and the mixture was homogenized by means of ultrasonication. Finally, an aliquot (10 μL) of prepared mixture was placed onto the carrier made of quartz glass and dried using an infrared lamp (Dalipi et al., 2017). Spectrometer (S2 PICOFOX TXRF BrukerNano, GmbH, Berlin, Germany) with a tungsten X-ray tube (50 kV, 1 mA) and a silicon drift detector with resolution <150 eV at Mn-K_{α}, was used for the analysis. The measurement time was set at 2000s. Spectra Plus 5.3 software (Bruker AXS Microanalysis GmbH, Berlin, Germany) was used to evaluate the TXRF spectra.

2.4. Maceration

An aliquot (0.1 g) of dry plant material, previously sieved through the mesh size of 850 μm, was suspended in 30 g of the appropriate solvent and left to stand in the dark. After either 1 or 3 days (details in Table 1), the macerates were filtered and stored in the dark at −20 °C until further analysis. Three independent extraction procedures were performed for each extract.

Table 1
Maceration conditions.

Extract	Extraction solvent	Extraction time (Days)
E100-1D	Ethanol	1
E50-1D	Ethanol 50 % (*w/w*)	1
G50-1D	Glycerol 50 % (*w/w*)	1
G90-1D	Glycerol 90 % (*w/w*)	1
W100-1D	Water	1
E100-3D	Ethanol	3
E50-3D	Ethanol 50 % (*w/w*)	3
G50-3D	Glycerol 50 % (*w/w*)	3
G90-3D	Glycerol 90 % (*w/w*)	3
W100-3D	Water	3

2.5. Ultrasound assisted extraction

Box-Behnken design with three independent variables: glycerol concentration (20 %–80 %, *w/w*), temperature (40–80 °C) and time (20–60 min) was performed. Aliquots (0.1 g) of powdered *S. marianum* fruit were placed in 50 mL Erlenmeyer flasks, suspended in 30 g of glycerol–water mixtures, and placed in an ultrasonic bath (Bandelin SONOREX® Digital 10 P DK 156 BP, Berlin, Germany). The ultrasonication frequency and power of 35 Hz and 350 W, respectively, were employed. Extraction details are presented in Table 2 (Box Behnken design) and Table 5 (optimized extracts). Upon the extraction, the mixtures were filtered and stored in the dark at −20 °C until further analysis.

2.6. RP-HPLC-DAD determination of silymarin

For quantification of silymarin flavonolignans, the modified European Pharmacopoeia method was used (European Pharmacopoeia, 2013). Analysis was performed on EC 125/4 Nucleodur 100−5 column (Macherey-Nagel, Düren, Germany) using an Agilent 1200 series HPLC instrument equipped with an autosampler and DAD detector. Mobile phase A was a mixture of phosphoric acid, methanol, and water (0.5:35:65 V/V/V), while mobile phase B consisted of phosphoric acid, methanol, and water (0.5:50:50 V/V/V). A 0.8 mL/min flow rate was used as follows: 0–28 min (100 %–0% A), 28–35 min (0% A), 35–36 min (0%–100 %A), and 36–51 min (100 % A). Quantification was carried out at 288 nm. Total silymarin content was calculated as described in European Pharmacopoeia from the areas under peaks (AUCs) of flavonolignans (silicristin, silidianin, silibinin A and B, isosilibinin A and B) present in the chromatogram of milk thistle dry extract HRS, and expressed as silibinin content. Example of the extract's and the standard's chromatogram are presented in Fig. 1.

2.7. Radical scavenging activity

Aliquot of the extract solution (130 μL) and 70 μL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.21 mg/mL) were mixed (Dulić et al., 2019) and the absorbance at 545 nm recorded after 30 min (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). Negative control was the mixture where methanol (130 μL) was used instead of the extract. RSA was calculated as:

Table 2
Mineral content of *S. marianum* fruit.

Element	Concentration (mg/kg)
Fe	116 ± 5
Zn	106 ± 4
Mg	180 ± 10
Sr	33 ± 2
Ca	9020 ± 50

$$\mathrm{RSA}\ (\%) = (A_{\mathrm{control}}\text{-}A_{\mathrm{sample}})/A_{\mathrm{control}} \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the solution with the extract. BHA (1 mg/mL) was used as the standard. The results were expressed as μL of extract in mL of the reaction solution (μL extract/mL).

2.8. Fe^{2+} chelating activity

To determine the chelating activity (ChA) (Končić et al., 2011), $FeCl_2$ solution (0.25 mM, 50 μL) was added to the extract solution in methanol (150 μL). After 5 min incubation at room temperature, ferrozine solution (100 μL, 1.0 mM) was added and the resulting absorbance measured at 545 nm after 10 min. A reaction mixture with 150 μL methanol used instead of extract served as the negative control. ChA was calculated as follows:

$$\mathrm{ChA}\ (\%) = (A_{\mathrm{control}}\text{-}A_{\mathrm{sample}})/A_{\mathrm{control}} \times 100 \quad (2)$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the solution containing the respective extract. The iron chelator EDTA (1 mg/mL) was used as the standard.

2.9. Antioxidant activity in β-carotene-linoleic acid assay

The activity in β-carotene linoleic acid assay (AACL) was determined according to (Ciganović et al., 2019). The emulsion (200 μL) containing 91 μg/mL β-carotene, 0.46 μg/mL linoleic acid, and 4.1 mg/mL Tween 40, was added to the methanolic extract solution (50 μL). Negative control was the reaction mixture where 50 μL of methanol was used instead of the extract. After 60 min, the absorbance at 520 nm was measured and AACL calculated as:

$$\mathrm{AACL}\ (\%) = A_{\mathrm{sample}}/A_{\mathrm{control}} \times 100 \quad (3)$$

where $A_{control}$ and A_{sample} are the absorbances of the water control and antioxidant, respectively. The antioxidant BHA (1 mg/mL) was used as the standard.

2.10. Tyrosinase inhibitory activity

Extract solution (80 μL) and tyrosinase solution (40 μL) prepared in 16 mM pH 6.8 phosphate buffer were mixed (Dulić et al., 2019). After 10 min in the dark, 80 μL of L-3,4-dihydroxyphenylalanine (L-DOPA) solution (0.19 mg/mL in the same buffer) was added and the absorbance at 492 nm measured after 10 min. The negative control contained a buffer instead of the extract solution. Tyrosinase inhibitory activity (TyInh) was calculated using the equation:

$$\mathrm{TyInh}\ (\%) = (A_{\mathrm{control}}\text{-}A_{\mathrm{sample}})/A_{\mathrm{control}} \times 100 \quad (4)$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the solution containing respective extract. Kojic acid (1 mg/mL) was used as the standard tyrosinase inhibitor.

2.11. Elastase inhibitory activity

For elastase inhibitory activity determination (Bose et al., 2017), 100 μL of extract solution in Tris-HCl buffer (0.1 M, pH 8.0) was added to 1 mM *N*-succinyl-$(Ala)_3$-nitroanilide in the same buffer. Elastase solution was added after 10 min and the absorbance was measured at 410 nm after additional 10 min. Elastase inhibitory activity (ElInh) was calculated as follows:

$$\mathrm{ElInh}\ (\%) = (A_{\mathrm{control}}\text{-}A_{\mathrm{sample}})/A_{\mathrm{control}} \times 100 \quad (5)$$

where $A_{control}$ is the absorbance of the negative control (solution where instead of extract the Tris-HCL buffer was used) and A_{sample} is the

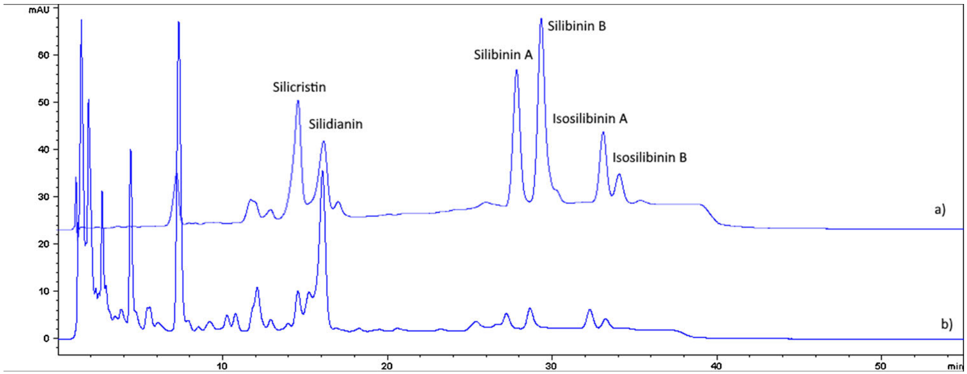


Fig. 1. Chromatogram of milk thistle dry extract reference standard a) and extract example (Run 5) b) recorded at 288 nm.

absorbance of the respective extract. Ursolic acid (1 mg/mL) was used as the standard elastase inhibitor.

2.12. Anti-inflammatory activity

The reaction mixture to determine the anti-inflammatory activity by the heat-induced ovalbumin coagulation method (Chandra et al., 2012) consisted of ovalbumin solution (0.4 mL), phosphate buffered saline (pH 6.4, 2.8 mL), and the extract solution (2 mL). After 15-min incubation at 37 °C, the temperature was increased to 70 °C and the heating continued for additional 5 min. Perkin Elmer Lambda 25 spectrophotometer (Perkin Elmer, Waltham, MA, USA) was used for the absorbance measurement at 660 nm. The inhibition of ovalbumin denaturation (OvInh) was calculated using the following formula:

$$\text{OvInh}\ (\%) = (A_{\text{control}}\text{-}A_{\text{sample}})/A_{\text{control}} \times 100 \qquad (6)$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the respective extract. Diclofenac sodium (1 mg/mL) was used as the standard inhibitor.

2.13. Statistical analysis

Design Expert software v. 8.0.6 (Stat-Ease, Minneapolis, MN, USA) was used for the experimental design preparation (Box Behnken) and validation (ANOVA). The results were presented as the mean ± standard deviation of three measurements. The activities were calculated as half maximal inhibitory concentrations (IC_{50}) using regression analysis as the concentration of the extract that displayed 50 % of the measured activity. Statistical comparisons were made using one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons between the extracts and Dunnett's test for comparison with the control. Paired *t*-test was used for comparison between the extracts prepared using different maceration times. PrismGraphPad 9 (GraphPad Software, Inc., san Diego, USA). P values<0.05 were considered statistically significant. The differences between 1-day and 3-day macerations were investigated using a paired *t*-test.

3. Results and discussion

3.1. Metal content of S. marianum fruit

Metals in the plant material were determined using two methods: ICP-OES (Fe, Zn, and Mg) and TXRF (Sr and Ca). As it is shown in Table 2, the plant material contained iron, the presence of which may have dual influence in cosmetic products. While high iron levels in cosmetic products may negatively affect their stability, this mineral also has a beneficial influence on the skin. It plays an essential role in the regulation of the inflammatory response, wound healing process, and maturation of skin collagen (Coger et al., 2019). *S. marianum* fruit contained several other minerals that may beneficially affect the skin. The most abundant among them was calcium, a mineral that serves as a modulator of keratinocyte proliferation and differentiation. It has also been postulated that calcium is a central regulator of wound healing. In wound repair, calcium is predominantly involved as Factor IV in the haemostatic phase, but it is expected to be required in epidermal cell migration and regeneration patterns in the later stages of healing (Lansdown, 2002). Magnesium, either alone or combined with calcium, accelerates skin barrier recovery (Denda et al., 1999).

One of the most important minerals for skin health, also rather abundant in *S. marianum* fruit, is zinc. It is well known that numerous skin disorders, such as acrodermatitis enteropathica, pellagra, alopecia, and delayed wound healing, are accompanied by the dysregulation of Zn metabolism (Ogawa et al., 2018). It has been repeatedly shown that differentiation and proliferation of keratinocytes are closely related to zinc concentration, and it is well-accepted that the presence of this mineral in a cosmetic product may contribute to wound healing (Coger et al., 2019). Another interesting mineral in the plant material was strontium, the metal whose salts are often used in dentistry for reducing the sensitivity of gums in periodontal disease. Dermal use of strontium salts, namely strontium chloride hexahydrate, may cause a reduction in inflammation related to lower TNF levels (Berksoy Hayta et al., 2018).

3.2. Maceration

Maceration was used as a screening step in the development of the green extraction of bioactive flavonolignans from *S. marianum* fruit. Maceration is the simplest of solid-liquid extraction methods, characterised by long durations due to the low kinetic energy of solvent molecules and slow achievement of an equilibrium concentration between the solid and the liquid phase (Mosca et al., 2018). However, as it does not require any specialised equipment, it remains one of the most commonly used extraction procedures. The most usual maceration solvent is ethanol, and it has been successfully used for the preparation of extracts of numerous medicinal plants, including *S. marianum* (Wallace et al., 2005). However, to the best of our knowledge, despite its numerous advantages compared with ethanol, the use of glycerol for the extraction of bioactive metabolites from *S. marianum* has never been described in the scientific literature.

In order to make a preliminary observation of the influence of solvent and extraction duration on the content of silymarin in the extracts, maceration was performed using several mixtures of glycerol or ethanol with water. Ethanol and water are fairly common solvents for the extraction of natural compounds and, together with their mixtures of different proportions, offer a spectrum of extraction media of different polarities. For the ethanol/water extraction, pure solvents, as well as their 1:1 (*w/w*) mixture was used. On the other hand, pure glycerol is a highly viscous solvent and thus unsuitable for preparation and subsequent filtration of the extracts in its pure form. Therefore, in addition to the 1:1 glycerol/water (*w/w*) mixture, 90 % glycerol was used. Maceration is usually performed by adding the solvent to the required amount of the drug, which is allowed to soak at room temperature for the required amount of time before it is strained (Gurib-Fakim, 2006). The time required for maceration may take as much as 21 days (Turrini et al., 2019). However, it usually ranges from 6 h (Gurib-Fakim, 2006) to 24 h (Masota et al., 2020). As the glycerol's viscosity, as well as the size of its molecules may negatively affect the extraction kinetics, macerations, aimed to assess the influence of the extraction time on the composition of the extracts, were performed for either 1 or 3 days (Table 2).

The content of flavonolignans in the extracts is shown in Fig. 2a. Even though silymarin is generally considered the most represented flavonolignan in *S. marianum* fruit (European Medicines Agency, 2018; Singh and Agarwal, 2009), silidianin was the most abundant flavonolignan in the analyzed sample. It was followed by silicristin, silibinin A and silibinin B, that were present in similar amounts, with some variations depending on the solvent. Silymarin content ranged from 9.24 μg/mL to 106.67 μg/mL in G90-1D and E50-3D, respectively. This represents an almost tenfold difference in the quantity of silymarin, indicating the strong influence of solvent and duration on the extraction effectiveness. Statistical analysis has shown that the amount of silymarin in the 3-day macerates was significantly higher than in those prepared during one day (paired *t*-test, $P < 0.05$). As shown in Fig. 2, 50 % ethanol was the most efficient solvent for extracting flavonolignans from *S. marianum*, followed by pure ethanol and 50 % glycerol. The content of individual flavonolignans followed a similar pattern. For example, after 3-day maceration, the content of silidianin was almost 7 fold greater (7.43 μg/mL *vs.* 50.67 μg/mL) if 50 % ethanol was used for extraction instead of 90 % glycerol.

While the extraction conditions exhibited remarkable influence on the silymarin content, they influenced RSA relatively mildly (Fig. 2). The RSA activity of all the prepared extracts was lower than the activity of positive control, BHA, which displayed the RSA IC_{50} value of 8.25 μg/mL ±0.55 μg/mL. The extraction time did not influence the RSA of the extracts (paired *t*-test, $P > 0.05$). In general, extraction using 50 % ethanol yielded the two most active radical scavengers, E50-1D and E50-3D. Nonetheless, it is important to note that G50-1D was just as active as a radical scavenger as the two extracts prepared using 50 % ethanol. Aqueous extraction, on the other hand, yielded the extracts with rather low RSA. While the maceration time did not influence 50 % ethanol and aqueous extraction, the RSA activity of the extracts prepared using glycerol was better if a shorter maceration time was employed. On the other hand, the RSA of ethanol extracts decreased with prolonged maceration time. The silymarin content and RSA correlated significantly ($r^2 = 0.3285$, $P = 0.0009$), indicating that silymarin flavonolignans play an important role in the overall antiradical activity of the prepared extracts.

When used at their boiling temperatures, pure ethanol was shown to be best solvent for extraction silibinin A and B from *S. marianum* fruit, surpassing water, methanol, acetonitrile and acetone (Wallace et al., 2005). The results presented herein indicate that glycerol maceration is a viable alternative to ethanol extraction, characterised by similar yields and lower solvent-induced toxicity of the prepared extracts. Ethanol presence in cosmetic and dermatologic products may have a disadvantage that this alcohol may remove the protective lipid barrier on the surface of the skin. Glycerol is, therefore, often added into alcoholic hand sanitizers to prevent skin dryness (Gold et al., 2020). In addition, glycerol is frequent ingredient of topical formulations of *S. marianum,* where it acts as a humectant (Sampatrao et al., 2011). In that light, the use of glycerol for the extraction has an important advantage that the extraction solvent can be directly used in the final cosmetic product as a functional ingredient.

3.3. Ultrasound-assisted extraction

Different procedures for extraction of *S. marianum* constituents were employed to make the process of silymarin extraction efficient and sustainable. The examples include UAE, microwave-assisted extraction, pressurized liquid extraction, enzyme-assisted extraction, supercritical fluid extraction, and subcritical water extraction. Among them, UAE was distinguished by its lower cost and higher quality of extracts because it avoids flavonolignans degradation caused by the high-temperature treatment (Wianowska and Gil, 2017). With the aim of developing a sustainable, time- and cost-efficient procedure for silymarin extraction, glycerol UAE, based on a three factor Box-Behnken design, was used. Various factors may influence the effectiveness of the UAE and other types of extraction. Different solvents, with their characteristic physical-chemical properties, such as the polarity, viscosity, and volatility, may extract different types and proportions of secondary metabolites from plant material. Glycerol content, employed in the UAE with glycerol/water mixtures, was found to be an important extraction variable in several studies investigating the influence of the solvent on the phenolic antioxidants content (Ciganović et al., 2019; Katsampa et al., 2015). Two other important parameters were the extraction duration and temperature. By increasing the kinetic energy of the molecules in the solution, high temperature enhances the interaction of the solvent and target molecules. While this may lead to the more rapid dissolution process, it also increases the chances of degradation of sensitive phytochemicals, such as phenolic compounds. Thus, chances for both, dissolution and degradation, increase with the elevated temperature and prolonged extraction time. Temperature and time are consistently identified as the important factors affecting the UAE. Glycerol-based UAE are no exception and previous studies demonstrate that time and temperature should be carefully selected to achieve the optimal extraction conditions (Ciganović et al., 2019; Momchev et al., 2020). The maceration yields in this study also indicate that the extraction time and solvent composition may significantly influence the silymarin content, and to a lesser extent the RSA of the extracts. Thus, the glycerol content, temperature and time were selected as independent variables for the Box-Behnken design. Apart from the silymarin content, the RSA

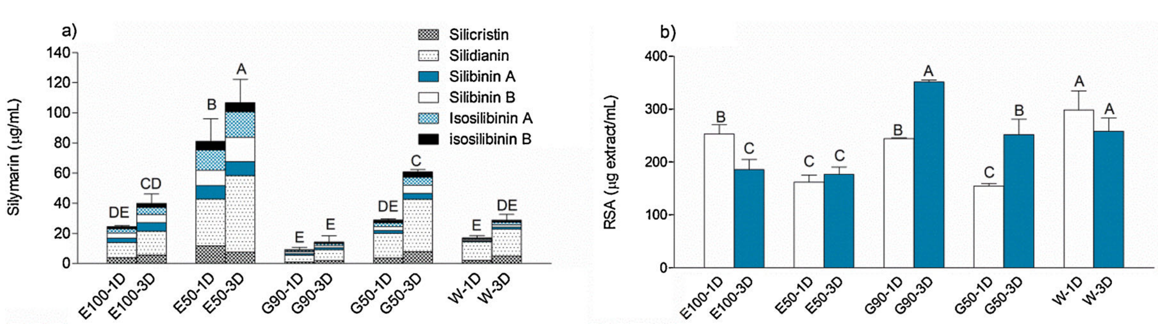


Fig. 2. Content of Individual flavonolignans and their total content (silymarin, expressed as silibinin) (a) and radical scavenging activity (RSA) of the extracts (b) prepared by maceration. Values are average of three replicates ± SD. The abbreviations are presented in Table 2. A-E = differences between the extracts (extracts not connected with the same capital letter are statistically different, Tukey post-test, $P < 0.05$).

of the extracts was also subjected to the optimisation procedure. The extraction conditions, the content of individual flavonolignans, their combined content (silymarin), as well as RSA are presented in Table 3.

The selected independent variables significantly affected silymarin content. Depending on the extraction conditions, its concentration changed fourfold, from 22.81 μg/mL (Run 17) to 100.51 μg/mL (Run 8). The comparison between the most silymarin-rich extract prepared by glycerol maceration (G50-3D) and UAE (Run 8) showed that UAE at higher temperatures was able to increase the efficiency of 50 % glycerol extraction by almost twofold, almost reaching the efficacy of 50 % ethanol (100.51 *vs*. 106.67 μg/mL), despite the much shorter extraction time (60 min *vs*. three days). Other studies reported similar findings. For instance, kinetic studies of phenolics extraction from aubergine peel suggested that extraction of phenolics by water-glycerol mixtures was slower than the extraction using water-ethanol, but both mixtures recovered similar levels of total polyphenols (Philippi et al., 2016). As it could be expected from maceration results, silidianin was the most represented flavonolignan in the extracts, followed by silicristin and silibinin A and B. Content of each flavonolignan was the highest in Run 8, while Run 5 and Run 17 contained similarly low amounts of the individual isomers.

In addition to silymarin content, extraction conditions affected RSA, although to a lesser extent. The IC_{50} value of the strongest radical scavenger (Run 6) was approximately two-and-a half-fold lower than the IC_{50} value of the weakest radical scavenger (Run 5) (Table 3). It seems that the ultrasound did not have a positive impact on RSA because the activity of the UAE extracts was similar or even slightly less pronounced than the antiradical activity of the glycerol/water or ethanol/water macerates.

In order to assess the relationship between the extraction conditions and the two selected response variables, silymarin content and RSA of the extracts, multiple regression was used. Statistical analysis resulted in polynomial equations of degree two and degree one, for the silymarin content and RSA, respectively. The polynomial equations for the silymarin content and the RSA IC_{50} values are presented in the Eqs. (7) and (8), respectively. Significant model factors are denoted with an asterisk.

$$\text{Silymarin } (\mu g/mL) = -6.41\times X_1^2(*) + 4.55\times X_2^2 - 1.40\times X_3^2 - 1.02\times X_1\times X_2 - 3.06\times X_1\times X_3 + 7.79\times X_2\times X_3(*) - 2.47\times X_1 + 26.90\times X_2(*) + 7.62\times X_3(*) + 50.93 \quad (7)$$

$$\text{RSA } (\mu L\ \text{extract}/mL) = 26.57\times X_1 - 75.21\times X_2(*) - 19.85\times X_3 + 303.92 \quad (8)$$

In order to calculate the statistical significance of the obtained models, *F*-test and *P*-values were used (ANOVA, Table 4). While the calculated *F*-values of models were higher than 5, the *P*-values for the models were lower than 0.05. On the other hand, *P*-values for the lack-of-fit in the models were higher than 0.05. This demonstrates the significance of the models, as well as their suitability for the description of the experimental data. The predicted r^2 were in reasonable agreement with the adjusted ones, further confirming the ability of the models to predict and optimise the selected responses. The determination coefficients (r^2) for silymarin extraction were relatively high (0.9815), showing that the observed values are well replicated by the model. However the RSA model had a relatively low r^2 value indicating that the selected extraction conditions can only partially predict the observed IC_{50} values.

In order to allow for easy visualisation of the extraction conditions on the dependent variables, three-dimensional surface plots of the models are constructed and presented in Fig. 3. Whereas the equation describing silymarin concentration contained quadratic factors, RSA activity could be satisfactorily described only using a linear equation. The temperature was the most important variable for glycerol extraction of *S. marianum* as it influenced both, silymarin concentration and RSA, as a linear term. The relationship between the RSA IC_{50} and temperature was negative, meaning that higher temperatures produce extracts with lower RSA IC_{50} values and thus higher activity (Table 3, Fig. 3b1,3). The effect of temperature on silymarin concentration is even more pronounced because temperature influences silymarin concentration positively, both as the linear factor and through its positive interaction with another significant factor, time. As it may be observed in Fig. 3a1–3, the extracts produced by long extraction times at high temperature have the highest silymarin concentrations. On the other hand, temperature did not seem affect the efficiency of 50 % (*v/v*) ethanol to extract silymarin from *S. marianum* fruit (Drouet et al., 2019). Unlike 50 % ethanol, glycerol is a relatively viscous solvent. Therefore, such a strong influence of the temperature on the glycerol extraction outcomes may be primarily related to the reduced viscosity of glycerol/water mixtures at higher temperatures. Such an effect was observed in previous UAE glycerolic extractions of phenolics from *Glycyrrhiza glabra* L. (Ciganović et al., 2019).

Similarly to the observation that the extracts prepared by 3-day maceration were richer in silymarin than 1-day maceration extracts, the time of UAE extraction positively affected the extraction efficiency. This was evidenced by the positive and statistically significant influence of the extraction time on silymarin concentration. The increased silymarin concentrations with an increased extraction time also indicate that the extracted flavonolignans are stable in the applied extraction conditions. This is in contrast with the observation that the silymarin

Table 3
Levels of independent variables in the Box-Behnken design, silymarin concentration and half maximal inhibitory concentrations (IC_{50}) value of the radical scavenging activity (RSA IC_{50}) of the extracts.

Run	Standard	X_1 % (w/w)	X_2 (°C)	X_3 (min)	Silicristin[a] (μg/mL)	Silidianin[a] (μg/mL)	Silibinin A[a] (μg/mL)	Silibinin B[a] (μg/mL)	Isosilibinin A[a] (μg/mL)	Isosilibinin B[a] (μg/mL)	Silymarin[a] (μg/mL)	RSA IC_{50} (μL extract/mL)
1	3	20	80	40	9.66	46.39	4.46	6.95	6.08	3.81	77.35	77.35
2	17	50	60	40	6.63	29.81	4.37	5.78	5.38	3.31	55.28	55.27
3	4	80	80	40	8.32	36.99	5.06	6.54	7.31	4.58	68.8	68.79
4	16	50	60	40	5.66	26.70	3.37	4.83	4.91	3.08	48.55	48.56
5	9	50	40	20	2.71	12.54	2.07	2.54	2.18	1.19	23.23	23.23
6	7	20	60	60	6.39	33.55	3.16	4.38	4.22	2.58	54.28	54.27
7	15	50	60	40	5.85	28.49	3.99	4.84	5.05	3.14	51.36	51.36
8	12	50	80	60	12.87	56.42	6.69	9.04	9.47	6.02	100.51	100.50
9	8	80	60	60	5.71	21.18	4.38	4.94	5.34	3.23	44.78	44.77
10	11	50	40	60	2.92	13.44	2.54	2.74	2.39	1.30	25.33	25.32
11	6	80	60	20	4.41	23.88	2.36	3.24	2.69	1.50	38.08	38.08
12	10	50	80	20	8.42	37.13	4.68	6.30	6.62	4.10	67.25	67.25
13	5	20	60	20	4.26	18.67	2.71	3.50	3.88	2.31	35.33	35.33
14	14	50	60	40	6.06	25.12	3.53	4.66	4.57	2.77	46.71	46.72
15	13	50	60	40	6.37	28.26	3.87	5.86	5.20	3.15	52.71	52.71
16	1	20	40	40	2.90	17.07	2.04	2.47	1.85	0.96	27.29	27.29
17	2	80	40	40	2.56	12.03	2.14	2.55	2.32	1.21	22.81	22.82

X_1 = glycerol content, X_2 = temperature, X_3 = time. [a] = Expressed as silibinin.

Table 4
Analysis of variance (ANOVA) of the models for the optimisation of *S. marianum* extraction.

	Silymarin					RSA				
r^2	$r^2 = 0.9815$; $r^2_A = 0.9576$; $r^2_P = 0.7976$					$r^2 = 0.5396$; $r^2_A = 0.4333$; $r^2_P = 0.2382$				
Source	SS	DF	MS	*F* Value	*P*-value	SS	DF	MS	*F* Value	*P*-value
Model	6846.48	9	760.72	41.17	<0.0001	54050.92	3	18016.97	5.08	0.0152
Lack of Fit	83.80	3	27.93	2.45	0.2030	26145.56	9	2905.06	0.58	0.7707
Pure Error	45.53	4	11.38			19973.38	4	4993.34		

SS = Sum of Squares, DF = degrees of freedom, MS = Mean Square.
r^2_A = adjusted r^2; r^2_P = predicted r^2; RSA = radical scavenging activity.

Table 5
Comparison of the predicted and experimental values for the optimised extracts.

Extract	Response	Optimisation goal	X_1 (%, w/w)	X_2 (°C)	X_3 (min)	Rsp_{pred}	Rsp_{ms}	RD (%)
SM-S	Silymarin[a] (μg/mL)	maximise	40	80	60	98.1	99.6	+1.5
SM-R	RSA (μL extract/mL)	minimise	20	80	60	185.3	192.3	+3.8

X_1 = glycerol content, X_2 = temperature, X_3 = time, RSA = radical scavenging activity.
Rsp_{pred} = predicted response, Rsp_{ms} = measured response. The units for Rsp_{pred} and Rsp_{ms} are given in the respective Response column.
RD = Response deviation, calculated as $(Rsp_{ms} - Rsp_{pred})$ / $Rsp_{pred} \times 100$.
SM-S = the extract optimized to the highest silymarin content, SM-R = the extract optimized to the best antiradical activity.
[a] = Expressed as silibinin.

yield of 50 % ethanol UAE is decreased when the extraction time exceeded 45 min (Drouet et al., 2019). The favourable effects of time on extraction yields recorded in this study, as well as in the study of the glycerolic UAE of phenolics from spent filter coffee (Michail et al., 2016), indicate good stability of silymarin flavonolignans and other phenolic compounds in glycerol/water mixtures.

The silymarin content was dependent on glycerol concentration as a quadratic term. This is visible in Fig. 3a1–2, where changes in glycerol concentration produced a curvature of silymarin content surface. These results agree with several studies that reported that water–glycerol mixtures of similar proportions were more efficient extraction media than those with predominant content of one of those solvents. Examples include the UAE of chlorogenic acid and other caffeic acid derivatives from spent filter coffee (Michail et al., 2016) and polyphenols from *Echinacea purpurea* (Momchev et al., 2020).

Concentrations of individual flavonolignans were significantly influenced by temperature and time as linear terms (all flavonolignans), as well as by their interaction (all except silibinin A). While those influences were positive, glycerol concentration affected all flavonolignans as a negative quadratic term. The exception again was silidianin whose content was influenced by glycerol as a negative linear term. In addition, interaction of glycerol and time exerted a positive influence on isosilibinin A and B content, and a negative influence on silidianin content. The relationships between the concentration of individual flavonolignans and extraction conditions could be satisfactorily described by quadratic equations. The only exception was silidianin whose content followed a two-factor-interaction equation. All the relationships had relatively high determination coefficients ($r^2 \geq 0.95$) with good agreements between r^2 adjusted and r^2 predicted (Supplement).

The silymarin content and the observed IC_{50} values RSA of UAE extracts correlated significantly ($r^2 = 0.4802$, $P = 0.0020$). It seems that silymarin plays one of the most important roles in the RSA of UAE of glycerolic extracts. However, a relatively low coefficient of determination may indicate that other antioxidants present in *S. marianum* fruit such as flavones apigenin, chrysoeriol, eriodictyol, and flavonols taxifolin, quercetin, dihydrokaempferol, and kaempferol (European Medicines Agency, 2018), or tocopherols (Fathi-Achachlouei et al., 2019), may also significantly contribute to the observed radical scavenging activity. Strong influence of the temperature on the RSA may confirm this hypothesis because the extraction of the more polar phenolic constituents of *S. marianum*, such as taxifolin and silychristin, was efficiently conducted with boiling water, while the extraction at 50 °C resulted in 75 % lower yields (Wallace et al., 2005).

3.4. Validation of the models

In order to prepare the extracts for further biological evaluation and confirm the validity of the obtained models, two optimised extracts were prepared, one calculated to have the highest silymarin content (SM-S), and the other to display the best antiradical activity (SM-R) (Table 5). Silymarin content and RSA of the prepared extract were determined and they only slightly deviated from the calculated values, indicating the validity of the obtained models. The approximate yield of the extraction, expressed as silymarin/herbal material, may be calculated using the weight of the solvent and the herbal material used for the extraction, as well as the density of the 40 % glycerol (w/w) (1.10 mg/mL) employed for preparing the SM-S extract. The calculated result (2.7 % silymarin in herbal material, w/w), is well above the pharmacopoeial limit of minimum 1.5 % of total silymarin in dried drug (European Pharmacopoeia, 2013). However, it is important to note that the calculated value is the ideal yield that may be obtained only if all the extraction solvent is recovered in form of the extract. The actual yields, calculated when solvent recovery is taken into account, may vary significantly (e.g. because the filtration speed decreases with time, herbal material absorbs one part of the extraction solvent etc.). Thus, 100 % recovery is unlikely even when the extraction is performed with a less viscous solvents such as ethanol (He et al., 2019). In the case of SM-S, the amount of the extract obtained from 30 g solvent was 26.5 g, indicating that 2.4 g of silymarin was actually obtained from 1 g of herbal material, a value still in accordance with pharmaceopoeial requirements.

3.5. Antioxidant activity of the optimised extracts

Oxidative changes that occur during the storage and use of cosmetic products negatively affect not only chemical stability and appearance of the product but also its biological activity. Natural extracts and metabolites are especially rich in various antioxidants which makes them especially valuable in the cosmetic industry (Mlakar et al., 1996). Such ingredients in cosmetic products may be regarded as both preservatives and therapeutic agents. They protect both, fatty acid present in the cosmetic product and those naturally present in the skin, against UV

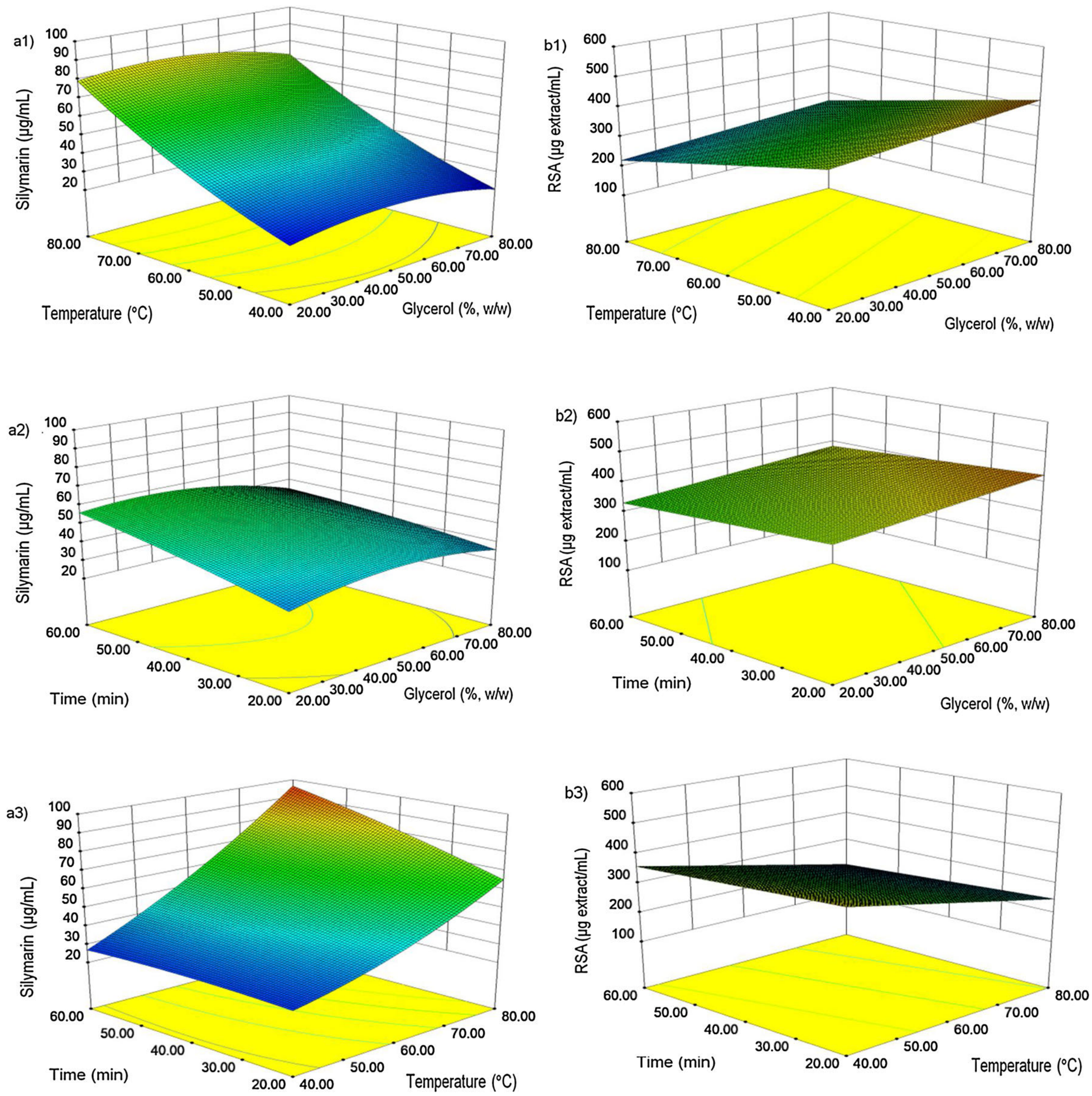


Fig. 3. Response surface plots of relationships between extraction conditions and a) silymarin content (a1–3) and b) radical scavenging activity (RSA) (b1–3). For significant model terms, see Eqs. (7) and (8).

radiation, free radicals- or metal-ions (Coelho et al., 2018). Furthermore, oxidative stress is known to be involved in pathogenesis and progression of numerous skin diseases such as contact-, seborrhoeic- and atopic dermatitis, as well as vitiligo, acne, psoriasis, and many others. Thus, it has been postulated that the use of antioxidants may serve as simple and effective strategy for improving these conditions (Baek and Lee, 2016).

In order to assess the antioxidant activity of the prepared extracts, factors such as the DPPH free radicals scavenging activity, Fe^{2+} ions chelating activity, and the activity in heat-induced degradation of

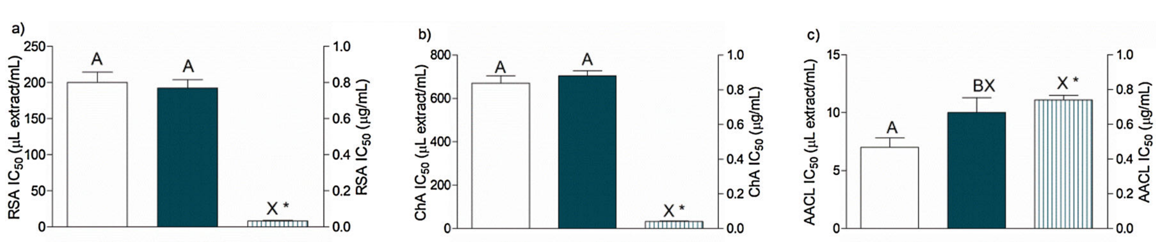


Fig. 4. Antiradical (**a**), chelating (**b**), and activity in β-carotene-linoleic acid assay (**c**) of the extracts SM-S (the extract optimized for silymarin content) SM-R (the extract optimized for radical scavenging activity) and the standards BHA (butylated hydroxyanisole) and EDTA (ethylenediaminetetraacetic acid). Values are average of three replicates ± SD. Asterisk (*) indicates that the IC_{50} unit is placed on the right y-axis. Columns not connected with the same capital letter are statistically different: A,B = differences between the extracts (Students *t*-test, $P < 0.05$), X = differences with the 1 mg/mL standard solution (Dunnet's post ANOVA test, $P<0.05$).

unsaturated fatty acid in β-carotene-linoleic acid system were investigated. Since it was not possible to evaporate glycerol due to its high boiling point, the unit for IC_{50} values of the extracts is expressed μL extract/mL (reaction solution). Thus, a direct comparison of the extracts' IC_{50} values with the IC_{50} values of standard antioxidants, expressed as μg /mL, was not possible. However, as this IC_{50} value of the standards was numerically equal to the IC_{50} value of the tested 1 mg/mL standard solution the comparison is presented herein for informative purposes. Fig. 4a-c depicts the activity of the extracts and the standards assessed using the three assays. Glycerol/water mixtures used for the preparation of the optimized extracts did not display the activity in the performed antioxidant assays.

From Fig. 4a it is evident that, although the RSA IC_{50} values of the extracts were higher than RSA IC_{50} of 1 mg/mL BHA solution, the extracts demonstrated a notable antiradical activity indicating that they may offer a significant degree of protection against free radicals. It is also important to note that the IC_{50} value of the extract SM-S (200.00 μL/mL) was equal to the IC_{50} value predicted by the model, further confirming the model's validity.

The extracts have shown a mild but observable chelating activity (Fig. 4b), indicating that they were able to chelate ions of transition metals and thus retard the oxidation processes. It has been recently suggested that metal chelators in cosmetic products may help prevent photoageing (Kitazawa et al., 2006). It seems that exposure of the skin to UV radiation leads to an increase in cutaneous intracellular catalytic iron levels and subsequently to the generation of free radicals. By binding the free iron, metal chelators may thus prevent UV-induced photodamage to the skin. The observed antiradical activity may add additional beneficial effects to the products containing *S. marianum* glycerol extracts, especially considering the iron content observed in the plant material.

The β-carotene linoleic acid assay gives an insight into the behaviour of the extracts in the mixtures with polyunsaturated fatty acids (PUFAs). The activity in the β-carotene linoleic acid was rather well pronounced (Fig. 4c) and statistically equal to the activity of 1 mg/mL BHA solution. This is important because cosmetic products often contain natural oils rich in linoleic and other polyunsaturated fatty acid. Such products are often used to treat atopic dermatitis, seborrheic dermatitis, and other skin diseases (Lin et al., 2017). The extracts that impede unsaturated fatty acid degradation beneficially affect the shelf life and activity of products that contain them.

3.6. *Enzyme inhibiting and anti-inflammatory activity of the optimised extracts*

Apart from their antioxidant properties, plant extracts in cosmetic products may act as functional ingredients and delay or prevent processes that negatively influence skin health and appearance. In order to assess the potential influence of *S. marianum* glycerol extracts on the skin pigmentation and elasticity, their tyrosinase- and elastase-inhibitory activity were investigated. Furthermore, the anti-inflammatory activity against heat-induced protein coagulation was determined.

Melanin is a photoprotective substance responsible for the pigmentation of human skin. However, the uneven accumulation of melanin in specific skin parts, such as in melasma, liver spots or even freckles, results in hyperpigmented areas and represents an aesthetic problem for the affected individual. Tyrosinase is an enzyme that catalyses tyrosine oxidation to dopaquinone, which is the rate-limiting first step of melanogenesis. As a result, tyrosinase inhibitors impede production of melanin and prevent hyperpigmentation of the skin (Pillaiyar et al., 2017). Skin-whitening formulations containing *S. marianum* have been clinically tested (Rasul et al., 2011), and it seems that silymarin mixture is responsible for tyrosinase-inhibiting properties (Zhao and Li, 2015). Tyrosinase-inhibiting properties of the prepared extracts are presented in Fig. 5a. Even though its activity was not as pronounced as the activity of the standard tyrosinase inhibitor, kojic acid, the SM-S extracts presented a favourable tyrosinase-inhibiting activity, while the activity of SM-R was significantly lower. It is important to note that, when tested parallel to the extract, glycerol was found to be responsible for 10 % and 7% of the observed activity of SM-S and SM-R, respectively. Thus, it seems that *S. marianum* bioactive constituents and glycerol present in the solution show additive effects on the tyrosinase-inhibiting properties of the investigated extracts. This finding may further support the use of *S. marianum* glycerol extracts in cosmetic products.

Plants and natural products derived thereof may protect the macromolecules of skin extracellular matrix against the activity of hydrolytic enzymes. Inflammation, caused by UV radiation, injury or chemicals, reduces synthesis of skin proteins and increases the concentration of proteolytic enzymes, such as elastases, enzymes responsible for the breakdown of elastin fibres. Elastin is a vital protein of the extracellular matrix, responsible for the firmness and shape of the skin. Thus, damage to elastin macromolecules results in typical degenerative changes of the upper dermal connective tissue. Clinical trials confirm that the possession of elastase-inhibitory activity indicates a substantial anti-ageing potential of the plant extracts and natural products they contain (Yasin et al., 2017). While neither of the extracts has shown the activity comparable to the activity of the standard, ursolic acid, SM-R was the stronger elastase inhibitor (Fig. 5b). Mild elastase-inhibiting properties of *S. marianum* ethanolic extracts have been recorded previously (Drouet et al., 2019). It seems that elastase inhibition was unaffected by the glycerol presence because, unlike its effects on tyrosinase, the effects of glycerol on elastase in the tested concentrations were negligible.

Chronic, low-grade inflammation is recognised as a significant characteristic of the ageing process in all organs, including the skin. Inflammatory processes in the skin may lead to denaturation of tissue proteins, which further exacerbate the inflammation progression. Thus, the inhibition of protein denaturation delays the development of inflammation-induced skin changes, and contributes to the anti-ageing activity of the product (Chandra et al., 2012). Both SM-S and SM-R were able to inhibit heat-induced ovalbumin coagulation (Fig. 5c), but SM-R displayed significantly better activity. It is important to note that

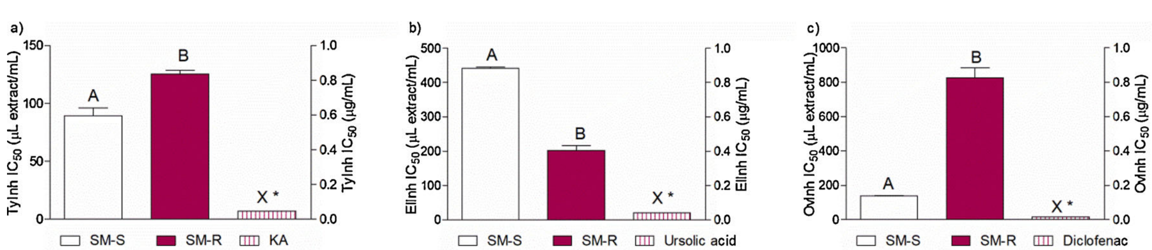


Fig. 5. Tyrosinase inhibitory (a), elastase inhibitory (b), and anti-inflammatory (c) activity of the extracts SM-S (the extract optimized for silymarin content) and SM-R (the extract optimized for radical scavenging activity) and standards KA (kojic acid), UA (ursolic acid) and DF (diclofenac). Asterisk (*) indicates that the IC_{50} unit is placed on the right y-axis. Columns not connected with the same capital letter are statistically different: A,B = differences between the extracts (Students *t*-test, $P < 0.05$), X = differences with the 1 mg/mL standard solution (Dunnet's post ANOVA test, $P<0.05$).

glycerol may have an active role in preventing denaturation of proteins such as collagen (Penkova et al., 1999). With this in mind, the influence of glycerol on the heat-induced albumin denaturation was also investigated. When appropriate glycerol concentrations were tested in parallel with the extracts, it was found that the glycerol present in the extracts accounted for most of the observed activity. Thus, even though it is well known that silymarin acts as an anti-inflammatory agent through the inhibition of lipoxygenase, cyclooxygenase, and TNF (Singh and Agarwal, 2009), it seems that the presence of silymarin or other antioxidants present in the extract did not enhance the activity of glycerol in this assay. The ability of glycerol to hinder protein denaturation further confirms that the benefits of glycerol extraction for production of anti-ageing *S. marianum* extracts extend well beyond its application as a green extraction solvent.

4. Conclusions

The UAE using glycerol was superior to maceration, yielding up to 2.6-fold higher silymarin content within shorter extraction times. The UAE extracts prepared using 40 % (w/w) glycerol during 60 min at 80 °C extraction contained a comparable amount of silymarin to the most silymarin-rich ethanolic extracts prepared by maceration. Silymarin content correlated well with the RSA of the extracts. The excellent toxicological and bioactivity profile of glycerol, good silymarin yields, as well as the observed antiradical, antioxidant, Fe^{2+} chelating, anti-elastase, anti-tyrosinase, and anti-inflammatory activity indicate that the prepared glycerol extracts may be used for the preparation of high-value anti-ageing products. The presence of zinc and other minerals with favourable skin-related properties further contribute to the potential use of *S. marianum* in cosmetics. Glycerol extraction of *S. marianum* not only reduces the time and energy necessary for solvent removal but also enhances the desired functional properties of such extracts.

CRediT authorship contribution statement

Magda Jabłonowska: Investigation, Formal analysis. **Petar Ciganović:** Investigation, Validation, Formal analysis, Methodology. **Jasna Jablan:** Investigation, Conceptualization, Methodology, Writing - review & editing. **Eva Marguí:** Methodology, Resources, Writing - review & editing, Supervision. **Michał Tomczyk:** Writing - review & editing, Project administration, Funding acquisition, Writing - review & editing, Formal analysis. **Marijana Zovko Končić:** Conceptualization, Methodology, Writing - original draft, Visualization, Project administration, Formal analysis, Visualization, Supervision, Project administration, Funding acquisition, Resources.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Acknowledgements

The preliminary phase of extraction studies was funded by Suban company within collaboration “Analysis of glycerin preparation of medicinal plants extracts”. Bioactivity and extraction studies were funded by the Croatian Science Foundation, as part of the project Bioactive Plant Principle Extraction using Green Solvents—A Step towards Green Cosmeceuticals, grant number IP-2018-01-6504.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.113613.

References

Aalim, H., Belwal, T., Jiang, L., Huang, H., Meng, X., Luo, Z., 2019. Extraction optimization, antidiabetic and antiglycation potentials of aqueous glycerol extract from rice (*Oryza sativa* L.) bran. LWT 103, 147–154. https://doi.org/10.1016/j.lwt.2019.01.006.

Baek, J., Lee, M.-G., 2016. Oxidative stress and antioxidant strategies in dermatology. Redox Rep. 21, 164–169. https://doi.org/10.1179/1351000215Y.0000000015.

Becker, L.C., Bergfeld, W.F., Belsito, D.V., Hill, R.A., Klaassen, C.D., Liebler, D.C., Marks, J.G., Shank, R.C., Slaga, T.J., Snyder, P.W., Gill, L.J., Heldreth, B., 2019. Safety assessment of glycerin as used in cosmetics. Int. J. Toxicol. 38, 6S–22S. https://doi.org/10.1177/1091581819883820.

Berksoy Hayta, S., Durmuş, K., Altuntaş, E.E., Yildiz, E., Hisarcıklıo, M., Akyol, M., 2018. The reduction in inflammation and impairment in wound healing by using strontium chloride hexahydrate. Cutan. Ocul. Toxicol. 37, 24–28. https://doi.org/10.1080/15569527.2017.1326497.

Bose, B., Choudhury, H., Tandon, P., Kumaria, S., 2017. Studies on secondary metabolite profiling, anti-inflammatory potential, in vitro photoprotective and skin-aging related enzyme inhibitory activities of *Malaxis acuminata*, a threatened orchid of nutraceutical importance. J. Photochem. Photobiol. B 173, 686–695. https://doi.org/10.1016/j.jphotobiol.2017.07.010.

Caldas, T.W., Mazza, K.E.L., Teles, A.S.C., Mattos, G.N., Brígida, A.I.S., Conte-Junior, C. A., Borguini, R.G., Godoy, R.L.O., Cabral, L.M.C., Tonon, R.V., 2018. Phenolic compounds recovery from grape skin using conventional and non-conventional extraction methods. Ind. Crop. Prod. 111, 86–91. https://doi.org/10.1016/j.indcrop.2017.10.012.

Chandra, S., Chatterjee, P., Dey, P., Bhattacharya, S., 2012. Evaluation of in vitro anti-inflammatory activity of coffee against the denaturation of protein. Asian Pac. J. Trop. Biomed. 2, S178–S180. https://doi.org/10.1016/S2221-1691(12)60154-3.

Chemat, F., Vian, M.A., Cravotto, G., 2012. Green extraction of natural products: concept and principles. Int. J. Mol. Sci. 13, 8615–8627. https://doi.org/10.3390/ijms13078615.

Chemat, F., Abert Vian, M., Ravi, H.K., Khadhraoui, B., Hilali, S., Perino, S., Fabiano Tixier, A.-S., 2019. Review of alternative solvents for green extraction of food and natural products: panorama, principles, applications and prospects. Molecules 24, 3007. https://doi.org/10.3390/molecules24163007.

Ciganović, P., Jakimiuk, K., Tomczyk, M., Zovko Končić, M., 2019. Glycerolic licorice extracts as active cosmeceutical ingredients: Extraction optimization, chemical characterization, and biological activity. Antioxidants 8, 445. https://doi.org/10.3390/antiox8100445.

Coelho, L., Almeida, I.F., Sousa Lobo, J.M., Sousa E Silva, J.P., 2018. Photostabilization strategies of photosensitive drugs. Int. J. Pharm. 541, 19–25. https://doi.org/10.1016/j.ijpharm.2018.02.012.

Coger, V., Million, N., Rehbock, C., Sures, B., Nachev, M., Barcikowski, S., Wistuba, N., Strauß, S., Vogt, P.M., 2019. Tissue Concentrations of zinc, iron, copper, and magnesium during the phases of full thickness wound healing in a rodent model. Biol. Trace Elem. Res. 191, 167–176. https://doi.org/10.1007/s12011-018-1600-y.

Dalipi, R., Marguí, E., Borgese, L., Depero, L.E., 2017. Multi-element analysis of vegetal foodstuff by means of low power total reflection X-ray fluorescence (TXRF) spectrometry. Food Chem. 218, 348–355. https://doi.org/10.1016/j.foodchem.2016.09.022.

Delgado-Povedano, M.M., Luque de Castro, M.D., 2015. A review on enzyme and ultrasound: a controversial but fruitful relationship. Anal. Chim. Acta 889, 1–21. https://doi.org/10.1016/j.aca.2015.05.004.

Denda, M., Katagiri, C., Hirao, T., Maruyama, N., Takahashi, M., 1999. Some magnesium salts and a mixture of magnesium and calcium salts accelerate skin barrier recovery. Arch. Dermatol. Res. 291, 560–563. https://doi.org/10.1007/s004030050454.

Drouet, S., Leclerc, E.A., Garros, L., Tungmunnithum, D., Kabra, A., Abbasi, B.H., Lainé, É., Hano, C., 2019. A green ultrasound-assisted extraction optimization of the natural antioxidant and anti-aging flavonolignans from milk thistle*Silybum marianum* (L.) Gaertn. fruits for cosmetic applications. Antioxidants 8, 304. https://doi.org/10.3390/antiox8080304.

Dulić, M., Ciganović, P., Vujić, L., Zovko Končić, M., 2019. Antidiabetic and cosmeceutical potential of common barbery (*Berberis vulgaris* L.) root bark extracts obtained by optimization of “green” ultrasound-assisted extraction. Molecules 24. https://doi.org/10.3390/molecules24193613.

European Medicines Agency, 2018. Silybi mariani fructus (accessed 11 March 2021). https://www.ema.europa.eu/en/medicines/herbal/silybi-mariani-fructus.

European Pharmacopoeia, 2013. European Pharmacopoeia, 8th ed. Council of Europe, Strasbourg.

Fathi-Achachlouei, B., Azadmard-Damirchi, S., Zahedi, Y., Shaddel, R., 2019. Microwave pretreatment as a promising strategy for increment of nutraceutical content and extraction yield of oil from milk thistle seed. Ind. Crop. Prod. 128, 527–533. https://doi.org/10.1016/j.indcrop.2018.11.034.

Fu, X., Belwal, T., Cravotto, G., Luo, Z., 2020. Sono-physical and sono-chemical effects of ultrasound: Primary applications in extraction and freezing operations and influence on food components. Ultrason. Sonochem. 60, 104726 https://doi.org/10.1016/j.ultsonch.2019.104726.

Gold, N.A., Mirza, T.M., Avva, U., 2020. Alcohol sanitizer. StatPearls. StatPearls Publishing, Treasure Island (FL).

Gurib-Fakim, A., 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. Mol. Aspects Med. 27, 1–93. https://doi.org/10.1016/j.mam.2005.07.008.

He, Y., Huang, Z., Zhong, C., Guo, Z., Chen, B., 2019. Pressurized liquid extraction with ethanol as a green and efficient technology to lipid extraction of *Isochrysis* biomass. Bioresour. Technol. 293, 122049 https://doi.org/10.1016/j.biortech.2019.122049.

Hou, M., Hu, W., Wang, A., Xiu, Z., Shi, Y., Hao, K., Sun, X., Cao, D., Lu, R., Sun, J., 2019. Ultrasound-assisted extraction of total flavonoids from *Pteris cretica* L.: process optimization, HPLC analysis, and evaluation of antioxidant activity. Antioxidants 8, 425. https://doi.org/10.3390/antiox8100425.

Huang, H., Xu, Q., Belwal, T., Li, L., Aalim, H., Wu, Q., Duan, Z., Zhang, X., Luo, Z., 2019. Ultrasonic impact on viscosity and extraction efficiency of polyethylene glycol: A greener approach for anthocyanins recovery from purple sweet potato. Food Chem. 283, 59–67. https://doi.org/10.1016/j.foodchem.2019.01.017.

Katsampa, P., Valsamedou, E., Grigorakis, S., Makris, D.P., 2015. A green ultrasound-assisted extraction process for the recovery of antioxidant polyphenols and pigments from onion solid wastes using Box–Behnken experimental design and kinetics. Ind. Crop. Prod. 77, 535–543. https://doi.org/10.1016/j.indcrop.2015.09.039.

Kitazawa, M., Iwasaki, K., Sakamoto, K., 2006. Iron chelators may help prevent photoaging. J. Cosmet. Dermatol. 5, 210–217. https://doi.org/10.1111/j.1473-2165.2006.00218.x.

Končić, M.Z., Barbarić, M., Perković, I., Zorc, B., 2011. Antiradical, chelating and antioxidant activities of hydroxamic acids and hydroxyureas. Molecules 16, 6232–6242. https://doi.org/10.3390/molecules16086232.

Lansdown, A.B.G., 2002. Calcium: a potential central regulator in wound healing in the skin. Wound Repair Regen. 10, 271–285. https://doi.org/10.1046/j.1524-475x.2002.10502.x.

Lin, T.-K., Zhong, L., Santiago, J.L., 2017. Anti-inflammatory and skin barrier repair effects of topical application of some plant oils. Int. J. Mol. Sci. 19 https://doi.org/10.3390/ijms19010070.

Masota, N.E., Vogg, G., Heller, E., Holzgrabe, U., 2020. Comparison of extraction efficiency and selectivity between low-temperature pressurized microwave-assisted extraction and prolonged maceration. Arch. Pharm. 353, e2000147 https://doi.org/10.1002/ardp.202000147.

Michail, A., Sigala, P., Grigorakis, S., Makris, D.P., 2016. Kinetics of ultrasound-assisted polyphenol extraction from spent filter coffee using aqueous glycerol. Chem. Eng. Commun. 203, 407–413. https://doi.org/10.1080/00986445.2015.1004667.

Mlakar, A., Batna, A., Dudda, A., Spiteller, G., 1996. Iron (II) ions induced oxidation of ascorbic acid and glucose. Free Radic. Res. 25, 525–539. https://doi.org/10.3109/10715769609149074.

Momchev, P., Ciganović, P., Jug, M., Marguí, E., Jablan, J., Zovko Končić, M., 2020. Comparison of maceration and ultrasonication for green extraction of phenolic acids from *Echinacea purpurea* aerial parts. Molecules 25, 5142. https://doi.org/10.3390/molecules25215142.

Mosca, F., Hidalgo, G.I., Villasante, J., Almajano, M.P., 2018. continuous or batch solid-liquid extraction of antioxidant compounds from seeds of*Sterculia apetala* plant and kinetic release study. Molecules 23. https://doi.org/10.3390/molecules23071759.

Ogawa, Y., Kinoshita, M., Shimada, S., Kawamura, T., 2018. Zinc and skin disorders. Nutrients 10. https://doi.org/10.3390/nu10020199.

Penkova, R., Goshev, I., Gorinstein, S., Nedkov, P., 1999. Stability of collagen during denaturation. J. Protein Chem. 18, 397–401. https://doi.org/10.1023/a:1020632424142.

Philippi, K., Tsamandouras, N., Grigorakis, S., Makris, D.P., 2016. Ultrasound-assisted green extraction of eggplant peel (*Solanum melongena*) polyphenols using aqueous mixtures of glycerol and ethanol: optimisation and kinetics. Env. Proc. 3, 369–386. https://doi.org/10.1007/s40710-016-0140-8.

Pillaiyar, T., Manickam, M., Namasivayam, V., 2017. Skin whitening agents: medicinal chemistry perspective of tyrosinase inhibitors. J. Enzyme Inhib. Med. Chem. 32, 403–425. https://doi.org/10.1080/14756366.2016.1256882.

Rasul, A., Naveed, A., Khan, B.A., Mahmood, T., Zaman, S.uz, Ali, A., Khan, H.M.S., Parveen, R., 2011. Assessment of anti erythmic and skin whitening effects of milk thistle extract. Afr. J. Pharm. Pharmacol. 520, 2306–2309.

Sampatrao, K.A., Bhalchandra, K.J., Ravindra, J., Manohar, P., 2011. Formulation development and evaluation of Silymarin gel. Res. J. Pharm. Tech. 4, 1633–1636.

Singh, R.P., Agarwal, R., 2009. Cosmeceuticals and silibinin. Clin. Dermatol. 27, 479–484. https://doi.org/10.1016/j.clindermatol.2009.05.012.

Turrini, F., Donno, D., Beccaro, G.L., Zunin, P., Pittaluga, A., Boggia, R., 2019. Pulsed ultrasound-assisted extraction as an alternative method to conventional maceration for the extraction of the polyphenolic fraction of*Ribes nigrum* buds: A new category of food supplements proposed by the FINNOVER project. Foods 8, 466. https://doi.org/10.3390/foods8100466.

Vieira, V., Calhelha, R.C., Barros, L., Coutinho, J.A.P., C. F. R. Ferreira, I., Ferreira, O., 2020. Insights on the extraction performance of alkanediols and glycerol: Using*Juglans regia* L. leaves as a source of bioactive compounds. Molecules 25, 2497. https://doi.org/10.3390/molecules25112497.

Wallace, S.N., Carrier, D.J., Clausen, E.C., 2005. Batch solvent extraction of flavanolignans from milk thistle (*Silybum marianum* L. Gaertner). Phytochem. Anal. 16, 7–16. https://doi.org/10.1002/pca.803.

Wianowska, D., Gil, M., 2017. Chapter 15 - silymarin extraction from *Silybum marianum* L. Gaertner. In: Dominguez González, H., González Muñoz, M.J. (Eds.), Water Extraction of Bioactive Compounds. Elsevier, pp. 385–397. https://doi.org/10.1016/B978-0-12-809380-1.00015-2.

Wolfson, A., Dlugy, C., Shotland, Y., 2007. Glycerol as a green solvent for high product yields and selectivities. Environ. Chem. Lett. 5, 67–71. https://doi.org/10.1007/s10311-006-0080-z.

Yahya, N.A., Attan, N., Wahab, R.A., 2018. An overview of cosmeceutically relevant plant extracts and strategies for extraction of plant-based bioactive compounds. Food Bioprod. Process. 112, 69–85. https://doi.org/10.1016/j.fbp.2018.09.002.

Yasin, Z.A.M., Ibrahim, F., Rashid, N.N., Razif, M.F.M., Yusof, R., 2017. The importance of some plant extracts as skin anti-aging resources: a review. Curr. Pharm. Biotechnol. 18, 864–876. https://doi.org/10.2174/1389201019666171219105920.

Zhao, F., Li, X., 2015. Ultrasonic-assisted enzymatic extraction of silymarin from the *Silybum marianum* seed shell and evaluation of its antioxidant activity *in vitro*. EXCLI J. 14, 861–874. https://doi.org/10.17179/excli2015-257.

3. RASPRAVA

Povećanjem brige potrošača za dobrobit i zdravlje kože, kozmetička industrija raste iz godine u godinu te intenzivno radi na razvijanju novih oblika proizvoda koji pozitivno utječu na izgled i zdravlje kože. Važne sastavnice takvih proizvoda često su sekundarni metaboliti ljekovitih biljaka poznatih po svojem blagotvornom učinku na kožu. U ovom radu opisana je optimizacija ultrazvukom potpomognute glicerolne ekstrakcije četiri ljekovite biljne vrste s ciljem izrade ekološki prihvatljivih ekstrakata s najvećim prinosom odabranih biljnih sastavnica i najizraženijim biološkim učinkom.

3.1. Odabir ekstrakcijskog otapala

Pored učinkovitosti, etička načela primijenjena u proizvodnji kozmetike od velikog su značaja za suvremenog potrošača. Etička načela se, naime, ne odnose samo na korištenje pokusnih životinja u kozmetičkoj industriji već i na prekomjerno iskorištavanje prirodnih resursa i onečišćenje okoliša u proizvodnji, uporabi i odlaganju kozmetike. Stoga je u prvom koraku istraživanja odabrano otapalo koje najbolje odgovara zadanom cilju izrade biološki učinkovitih i ekološki prihvatljivih ekstrakata. Za tu svrhu uspoređena je ekstrakcijska učinkovitost dvaju zelenih otapala, etanola i glicerola. Različite fizikalno-kemijske značajke otapala, poput polarnosti, te viskoznosti i hlapljivosti značajno utječu na učinkovitost ekstrakcije (78). Kako bi se odabralo otapalo najprikladnije za daljnje ekstrakcije odabranih ljekovitih droga zbog svoje je jednostavnosti korištena metoda maceracije. Preliminarni pokusi provedeni su na *E. purpurea* i *S. marianum*. Korištena otapala bila su voda, te različite koncentracije vodenih otopina glicerola i etanola. Kako su etanol i voda otapala koja se relativno često koriste za ekstrakciju biljnih sastavnica, vodeno/etanolna ekstrakcija ploda *S. marianum* (79) i *E. purpurea* (80) opisane su u literaturi. S druge strane, glicerolna ekstrakcija aktivnih biljnih sastavnica je, usprkos brojnim prednostima glicerola u odnosu na etanol, relativno rijetko zastupljena u literaturi.

Preliminarni rezultati su pokazali da je učinkovitost glicerola i etanola u ekstrakciji ukupnih fenolnih kiselina iz *E. purpurea* maceracijom bila usporediva. Slični su rezultati dobiveni i prilikom ekstrakcije flavonolignana iz *S. marianum*. Glicerol, pored uloge otapala, ima i aktivnu ulogu u kozmetičkim proizvodima. Naime, zbog svoje higroskopne prirode te na koži može djelovati kao humektant te sredstvo za regulaciju viskoznosti (36). Dodatna je prednost netoksičnost gotovog ekstrakta koji se odmah može uklopiti u kozmetički proizvod bez potrebe uklanjanja viška otapala (27). Time se štedi energija koja bi se inače trošila za uparavanje otapala što postupak ekstrakcije čini i cjenovno prihvatljivim i manje štetnim za okoliš (29). Uzevši to u obzir, glicerol je predložen kao otapalo izbora u daljnjim istraživanjima.

3.2. Ispitivanje utjecaja UAE ekstrakcijskih uvjeta

U sljedećem koraku istraživanja provedena je usporedba učinkovitosti UAE s klasičnom maceracijom. Maceracija, jedna od najstarijih tradicionalnih ekstrakcijskih metoda, a karakterizirana je jednostavnošću i pristupačnošću, ali i dugim trajanjem postupka koje u nekim slučajevima iznosi i do 21 dan (81). Stoga su u sklopu istraživanja provedeni napori da se maceracija zamjeni s nekom od metoda kojom bi se vrijeme ekstrakcije moglo značajnije skratiti. Jedna od novijih, nekonvencionalnih metoda ekstrakcije koju krasi kraće vrijeme ekstrakcije, niska emisija CO_2, mala potrošnja otapala i relativno niska cijena je UAE (82). Zbog toga, u ovom je radu uspoređena učinkovitost klasične maceracije s UAE. Zbog složenosti UAE na učinak ekstrakcije može utjecati i znatno veći broj ekstrakcijskih varijabli. Iako je ovo ekstrakcijska metoda čija je primjena u literaturi relativno rijetko opisana, neka novija istraživanja spominju ekstrakciju polifenola iz mekinja riže, kao i oraha upotrebom UAE (37,38). Stoga je u ovom radu prije detaljnog postupka optimizacije ekstrakcije određeno koji faktori značajno utječu na učinkovitost glicerolne ekstrakcije. Ovo je učinjeno pomoću preliminarnih ekstrakcija koje su utvrđene pomoću dvorazinskog faktorskog dizajna (engl. *two-level factorial design*, 2LFD). U njima je ispitan utjecaj slijedećih ekstrakcijskih varijabli: koncentracija glicerola, temperatura, snaga ultrazvuka, vrijeme ekstrakcije, dodatak askorbinske kiseline u reakcijsku smjesu te omjer droga/otapalo. Kao model (korištena je ekstrakcija fenolnih kiselina iz *E. purpurea* , odnosno antiradikalna aktivnost (RSA) izrađenih ekstrakata određena pomoću 2,2-difeni-1-pikrilhidrazil slobodnog radikala (DPPH) slobodnog radikala i izražena kao IC_{50} vrijednost. Rezultati su pokazali da su na ishod najviše utjecali koncentracija glicerola, vrijeme ekstrakcije, temperatura i snaga ultrazvuka.

Različiti udjeli otapala (glicerola i vode), daju ekstrakcijske smjese različite polarnosti te je i očekivano kako je taj parametar utjecao na učinkovitost ekstrakcije. I u literaturi se ovaj faktor pokazao važnim. Neki od primjera su glicerolne UAE pri određivanju klorogenske kiseline i ostalih derivata kavene kiseline u ostatku filter kave, gdje se pokazalo kako je smjesa glicerola i vode učinkovitija pri ekstrakciji TP od vode (83). Također, prilikom određivanja polifenola u komini crvenog grožđa pokazalo se kako je viša koncentracija glicerola u otapalu doprinijela višim vrijednostima TP, što su autori pripisali nižoj polarnosti otapala, povišenoj aktivnosti vezivanja dušika, kao i steričkim efektima (84).

Vrijeme ekstrakcije je također igralo ključnu ulogu u učinkovitosti i ishodu procesa ekstrakcije biljnih spojeva. U načelu, dulje vrijeme ekstrakcije može povećati prinos bioaktivnih tvari, jer omogućuje veću interakciju između otapala i biljnog materijala, što olakšava otapanje i prijenos spojeva u otapalo. Međutim, produženo trajanje ekstrakcije također povećava rizik od degradacije osjetljivih biljnih sastavnica, poput fenolnih spojeva, flavonoida i vitamina (85). Primjerice, istraživanja ekstrakcije polifenola iz masline su pokazala da dulje vrijeme ekstrakcije može dovesti do značajnog povećanja prinosa fenolnih spojeva u početnim fazama procesa, ali je također zabilježeno da nakon određene točke produžena ekstrakcija može uzrokovati degradaciju tih spojeva. Degradacija je bila rezultat oksidativnih reakcija ili toplinske razgradnje fenolnih kiselina i flavonoida, što je ujedno smanjilo i antiradikalnu aktivnost ekstrakta (86). Osim toga, predugo trajanje ekstrakcije također može uzrokovati oslobađanje neželjenih spojeva, poput tanina i lignina, koji mogu negativno utjecati na okus, boju ili stabilnost ekstrakta, osobito u prehrambenim i kozmetičkim proizvodima (87). To je posebice važno kod ekstrakcije polifenola iz biljnih izvora, gdje prekomjerno vrijeme ekstrakcije može rezultirati ekstraktom lošije kvalitete, s većim udjelom nepoželjnih spojeva (88).

Visoka temperatura i snaga ultrazvuka mogu značajno poboljšati učinkovitost ekstrakcije smanjujući viskoznost otapala i povećavajući kinetičku energiju molekula u otopini. Povećanjem temperature dolazi do smanjenja površinske napetosti otapala, što omogućuje bolji kontakt između otapala i čestica biljnog materijala, čime se ubrzava prijenos bioaktivnih tvari iz biljne matrice u otapalo (85). Nadalje, visoke temperature olakšavaju difuziju molekula, omogućujući bolju penetraciju otapala u biljne stanice i brže otapanje ciljnih spojeva (89). Snaga ultrazvuka, s druge strane, generira mehaničke efekte u otopini zbog fenomena kavitacije. Kavitacija, koja nastaje zbog brzog stvaranja i kolapsa mjehurića u tekućini pod djelovanjem ultrazvučnih valova, generira mikro-mlazove i visoki pritisak koji mehanički razbijaju biljne stanice, olakšavajući oslobađanje bioaktivnih tvari (90). Navedeni procesi poboljšavaju otapanje ciljanih spojeva, što može značajno skratiti vrijeme ekstrakcije i povećati prinos. Ipak, kao što je to slučaj s vremenom ekstrakcije, visoka temperatura i snaga ultrazvuka također nose određene rizike. Visoke temperature mogu uzrokovati termalnu degradaciju osjetljivih spojeva što može rezultirati smanjenjem antiradikalne aktivnosti ekstrakta (91,92). Također, ultrazvučni valovi mogu uzrokovati oksidaciju spojeva tijekom ekstrakcije, osobito kod dugotrajne izloženosti ultrazvuku, što može dodatno smanjiti kvalitetu ekstrakta (93).

Suprotno očekivanjima, dodatak askorbinske kiseline u ekstrakcijsku smjesu negativno je utjecao na količinu TPA. Prijašnja su istraživanja, naime, pokazala kako dodatak antioksidansa u prethodno pripremljenu ekstrakcijsku smjesu *E. purpurea* usporava oksidacijsku degradaciju fenolnih spojeva te je očekivano kako će askorbinska kiselina imati utjecaja na ekstrakcijski ishod (94,95). Kako to nije slučaj, jedno od potencijalnih objašnjenja je što askorbinska kiselina, iako djeluje kao antioksidans, može pod određenim uvjetima pokazati prooksidativno djelovanje, osobito u prisutnosti metalnih iona poput željeza ili bakra. Ti metali kataliziraju Fentonovu reakciju, što dovodi do stvaranja slobodnih radikala koji mogu oksidirati fenolne kiseline (96). Ovaj proces oksidacije može smanjiti ukupnu količinu fenolnih spojeva u ekstraktu. Dodatno, askorbinska kiselina može međusobno reagirati s fenolnim kiselinama, stvarajući kompleksne spojeve koji nisu lako detektabilni standardnim metodama kvantifikacije, što rezultira prividno nižim prinosom fenola (97).

Rezultati ovog istraživanja su pokazali kako se količina TPA, kao i koncentracija CIC i CAF dobivenih maceracijom *E. purpurea* nisu statistički značajno razlikovali u odnosu na UAE iako je vrijeme ekstrakcije bilo znatno kraće (40 min u odnosu na 1 i 3 dana u slučaju maceracije). Navedeno se može objasniti cijelim nizom fenomena koji aktivno utječu na ishod UAE ekstrakcije u odnosu na mnogo pasivniju maceraciju. Samo neki od njih su kavitacija, povišena kinetička energija, bubrenje, razbijanje stanične stijenke i izljev sadržaja stanice u otapalo (98–100). Stoga se u daljnjim istraživanjima pristupilo optimizaciji glicerolne ekstrakcije potpomognute ultrazvukom.

3.3. Optimizacija ekstrakcijskih uvjeta

Po završetku preliminarnih istraživanja provedena je detaljna optimizacija UAE ekstrakcije aktivnih sastavnica odabranog ljekovitog bilja s ciljem izrade ekstrakata visoke biološke učinkovitosti. Ekstrakcija *E. purpurea* optimizirana je na prinos TPA, te CAF i CIC, ekstrakcija *B. vulgaris* optimizirana je na prinos BER a ekstrakcija *S. marianum* optimizirana je na prinos SYL. Osim toga. ekstrakcija tri navedene biljne doge optimizirana je i tako da su priređeni i ekstrakti s najjačom na antiradikalnom aktivnosti (RSA). S druge strane ekstrakcija *G. glabra* optimizirana je na koncentraciju ukupnih polifenola u ekstraktu (TP), kao i učinkovitost ekstrakcije ukupnih polifenola (TPy) te koncentraciju sastavnica GLA i ISO. Za određivanje optimalne vrijednosti ekstrakcijskih parametara, primijenjena je RSM metodologija temeljena na Box-Benhken dizajnu. RSM uključuje stvaranje matematičkog modela koji predviđa odgovor na temelju eksperimentalnih faktora i njihovih razina (40). RSM se često primjenjuje za optimizaciju ekstrakcije fenolnih spojeva iz raznih prirodnih izvora kao što su krumpir (101), ptičja šapa (102) i *Rheum moorcroftianum* (103).

3.3.1. Utjecaj glicerola

Kao prva neovisna varijabla u optimizaciji ekstrakcija korišten je glicerol čije su koncentracije u istraživanjima provedenim u ovom radu u pravilu bile između 10 i 90%. Očekivano, koncentracija glicerola uvelike je diktirala prinos fenolnih spojeva i drugih biljnih sastavnica prema kojima je optimizirana ekstrakcija. U svim istraživanjima provedenim u sklopu ovoga rada ishod ekstrakcije bio je proporcionalan negativnoj kvadratnoj vrijednosti koncentracije glicerola. Također, primijećen je i negativno linearan utjecaj koncentracije glicerola i to prilikom ekstrakcija *E. purpurea* , *B. vulgaris* i *G. glabra* . Udio glicerola potreban za maksimalni prinos TP u ekstrakciji *E. purpurea* , *S. marianum* i *B. vulgaris* bio je umjeren (50% do 70%), što pokazuju i navodi iz literature poput ekstrakcije fenolnih skupina iz kore patlidžana u čijoj je ekstrakcijskoj smjesi korišten 50% glicerol (104). S druge strane, koncentracija glicerola za maksimalni prinos TP u *G. glabra* bila je niska (20%) što se može objasniti prisutnošću polarnijih polifenola u *G. glabra*. Sličan sastav otapala zabilježen je i u ekstrakciji fenola iz kore grejpfruta (105). Visoke koncentracije glicerola (90%) nisu pozitivno utjecale na prinos TP što je dijelom i očekivano zbog povećane viskoznosti otopine, koja otežava difuziju molekula polifenola i smanjuje penetraciju otapala u biljni materijal. Također, visoka viskoznost glicerola može ograničiti prijenos topline, smanjujući učinkovitost ekstrakcije, dok smanjena topljivost polifenola u visoko koncentriranom glicerolu dodatno utječe na ukupni prinos (87).

Pored određivanja koncentracije pojedinih skupina fenolnih spojeva, određivane su i pojedine aktivne sastavnice biljnih droga. Tako su se flavonolignani u *S. marianum*, kao i BER najbolje ekstrahirali pri umjerenim koncentracijama glicerola (40% do 50%), dok je za ekstrakciju polifenola *G. glabra* GLA i ISO bila potrebna viša koncentracija glicerola (85%). To ukazuje da je, za razliku od GLA i ISO, većina polifenola u *G. glabra* relativno hidrofilne prirode. Poznati primjeri uključuju flavonoidne glikozide likviritin, izolikviritin, 5,8-dihidroksi-flavon-7-O-beta-d-glukuronid i druge (60). Slične rezultate pokazuje i ekstrakcija *E. purpurea* gdje otapalo s visokim postotkom glicerola daje najviši prinos derivata kavene kiseline, CAF i CIC.

3.3.2. Utjecaj temperature

Osim koncentracije glicerola, temperatura, druga neovisna varijabla, snažno je utjecala na učinkovitost UAE. U ovim su istraživanjima primijenjene temperature od 10 do 90 °C. Očekivano, na ishod ekstrakcije (TP i pojedine aktivne sastavnice) temperatura je imala linearan utjecaj. Tako je prilikom optimizacije ekstrakcije za najviši prinos TP *G. glabra*, *B. vulgaris*, *S. marianum* i *E. purpurea* optimalna temperatura ekstrakcijske smjese iznosila od 60 do 80 °C, dok je pri optimizaciji ekstrakcije za najviši prinos pojedinih aktivnih sastavnica bila nešto viša, od 70 do 80 °C. Ovi rezultati ukazuju, između ostalog, na dobru termostabilnost ekstrakcijskih spojeva. Dodatno, RSM je pokazao kako je utjecaj temperature na ishod ekstrakcije *E. purpurea*, pored linearne, imao i komponentu negativnog kvadratnog modela što ukazuje kako, nakon određene (optimalne) vrijednosti temperature, prinos ukupnih fenolnih kiselina opada, vjerojatno zbog toplinske degradacije biljnog materijala. Također, povišenjem temperature ekstrakcijske smjese s višim udjelom glicerola bile su manje viskozne što je dodatno poboljšalo ishod ekstrakcije što je, između ostalog, opisano i u literaturi (106).

3.3.3. Utjecaj jakosti UZV

U UAE, pored izbora otapala važan je čimbenik i snaga ultrazvučnih valova koja uzrokuje ranije spomenute procese u staničnoj strukturi biljnih vrsta koji posljedično dovode do efikasnije ekstrakcije. U sklopu ovog rada u dva je istraživanja ispitan utjecaj snage ultrazvuka na ishod ekstrakcije. Korištene biljne vrste bile su *B. vulgaris* i *E. purpurea* , a u rezultatima se vidi kako je na ishod ekstrakcije utjecaj ultrazvuka bio proporcionalan pozitivnom kvadratnom modelu, što je i očekivano. Tako je slaba do umjerena jakost ultrazvuka (144 i 360 W) optimalna za najviši prinos ukupnih polifenola, ali i pojedinih aktivnih sastavnica spomenutih biljnih vrsta (BER, CIC te CAF). Ovaj pronalazak u skladu je s očekivanjima, ali i opisima u literaturi gdje je navedeno kako je optimalna snaga ultrazvuka umjerena. Naime, preniska snaga neće prouzročiti dovoljne promjene u biljnom materijalu kako bi se isti optimalno ekstrahirao, dok će prejaka snaga uzrokovati degradaciju biljnog materijala što negativno utječe na ishod ekstrakcije (107).

3.3.4. Utjecaj vremena

Vrijeme ekstrakcije također je značajan faktor u svim vrstama ekstrakcije budući da određuje trajanje kontakta biljnog materijala s ekstrakcijskim otapalom, što direktno utječe na ishod ekstrakcije. U sklopu ovog istraživanja, utjecaj vremena ispitan je prilikom ekstrakcije *S. marianum* i *E. purpurea*. Vremenski raspon bio je od 20 do 60 minuta, a dobiveni rezultati jednoznačno ukazuju kako je 60 minuta bilo optimalno vrijeme ekstrakcije po svim ispitivanim ishodima što je u skladu i s rezultatima dobivenim RSM analizom koji kazuju kako je odnos određivanih fenolnih spojeva proporcionalan pozitivnom kvadratnom modelu duljine ekstrakcije.

3.3.5. Utjecaj mase biljnog materijala

Kao posljednja neovisna varijabla ispitivan je utjecaj mase biljnog materijala korištenog za ekstrakciju. Veća masa biljnog materijala koja se koristi za ekstrakciju može povećati sadržaj traženih aktivnih sastavnica u ekstraktima. Međutim, kada se veće količine biljnih materijala ekstrahiraju mješavinama otapala i vode, bubrenje biljnog materijala zbog prisutnosti vode može promijeniti udjele otapala u smjesi, a posljedično i polaritet ekstrakcijske smjese. Osim toga, previsok omjer biljnog materijala i otapala može dovesti do nepotrebnog stvaranja otpada (106). Utjecaj mase biljne droge u ovom je istraživanju ispitivan u ekstrakciji *G. glabra*. Korištene mase varirale su od 0,6 g/10 ml otapala do 1,0 g/10 ml otapala. Dobiveni rezultati ukazuju kako je viša količina biljne droge povoljnije utjecala na ishode ekstrakcije, što se također vidi i u dobivenom pozitivnom kvadratnom modelu utjecaja mase biljne droge na prinos GLA i ISO. Rezultati dobiveni na prinos TP također pokazuju istu krivulju kvadratne jednadžbe. Tako za maksimalni prinos TP bilo potrebno 0,93 g, dok je za maksimalni prinos GLA i ISO potrebno 1,0 g biljne droge na 10 ml otapala.

3.3.6. Validacija modela i izrada optimiziranih ekstrakata

Kako bi se priredili ekstrakti za daljnje određivanje biološke aktivnosti i potvrdila valjanost dobivenih modela, pripremljeni su optimizirani ekstrakti. Na osnovi podataka dobivenih RSM metodologijom, odnosno korištenjem uvjeta za koje su modeli predvidjeli da će imati najveću koncentraciju odabranih bioaktivnih sastavnica, te najvišu RSA, priređeni su optimizirani ekstrakti. Tako su ekstrakti *E. purpurea* optimizirani na RSA aktivnost, kao i na najviši prinos TPA, kao i pojedinih derivata kavene kiseline – CAF i CIC. Ekstrakti *G. glabra* optimizirani su na prinos TP te sastavnica GLA i ISO, ekstrakti *S. marianum* na prinos SYL te RSA aktivnost, dok su ekstrakti *B. vulgaris* optimizirani na prinos BER i RSA aktivnost. RSA aktivnost spomenutih ekstrakata, kao i prinosi pojedinih aktivnih sastavnica (TP, TPA, te pojedinačnih sastavnica) su određeni prikladnim testovima i dobiveni rezultati uspoređeni su s rezultatima predviđenim RSM modelima. Odstupanje stvarnih od vrijednosti predviđenih modelima je u pravilu bilo manje od 5%, što ukazuje na valjanost dobivenih modela. Priređeni ekstrakti korišteni su za istraživanje biološke aktivnosti u daljnjem radu.

3.4. Biološka aktivnost optimiziranih ekstrakata

U zadnjem dijelu istraživanja određena je aktivnost priređenih ekstrakata na inhibiciju enzima i procesa koji utječu na izgled i zdravlje kože. Tako je ispitan utjecaj ekstrakata na enzime tirozinazu, kolagenazu, elastazu i hijaluronidazu, dok je protuupalna aktivnost ispitana testom inhibicije toplinom inducirane koagulacije ovalbumina, kao i testom inhibicije enzima lipoksigenaze. Također je ispitana aktivnost ekstrakta *E. purpurea* na sposobnost zacijeljivanja rana u testu "grebanja" na HaCaT staničnom monosloju kako bi se potvrdila tvrdnja da je ovaj ekstrakt prikladan sastojak biljnih lijekova za ubrzanje cijeljenja manjih rana i drugih kožnih oštećenja (49). U svakom su testu korišteni aktivni standardi čija je aktivnost ispitana u odnosu na aktivnost ekstrakta. Iako se ispitana aktivnost standarda ne može direktno usporediti s aktivnošću ekstrakta zbog toga što su spomenute aktivnosti izražene u drugačijim vrijednostima (aktivnost standarda izražena je u µg/mL, dok je aktivnost ekstrakta izražena u µL/mL), moguće je usporediti ih zbog toga što se aktivnost standarda izrazila kao volumni ekvivalenti otopine koncentracije standarda 1 mg/mL.

Glicerol je također podvrgnut ispitivanju biološke aktivnosti u gore navedenim testovima u koncentracijama u kojima je bio korišten u ekstraktima kako bi se utvrdio potencijalni utjecaj otapala na biološku aktivnost ekstrakta. Rezultati ovog istraživanja pokazali su kako glicerol nema statistički značajnu biološku aktivnost osim u iznimkama opisanim niže u tekstu.

3.4.1. Antiradikalna aktivnost

Antiradikalno djelovanje pojedinih sastojaka kozmetičkih proizvoda od iznimne je važnosti jer mogu djelovati i kao konzervansi i kao aktivne komponente. Antioksidansi mogu zaštititi kozmetički proizvod od oksidacije do koje dolazi tijekom njegovog skladištenja i korištenja hvatanjem slobodnih radikala (108). Keliranje metala, kao što je prooksidans Fe^{2+} i drugi ioni, također je vrlo važno jer ti metali mogu izazvati peroksidaciju višestruko nezasićenih masnih kiselina kojima je prirodna kozmetika posebno bogata (109). Nedavno je sugerirano da kelatori metala u kozmetičkim proizvodima mogu pomoći u sprječavanju UV zrakama uzrokovanog starenja kože. Čini se da izloženost kože UV zračenju dovodi do povećanja intracelularne razine željeza i posljedično stvaranju slobodnih radikala. Vezivanjem slobodnog željeza, kelatori metala mogu tako spriječiti sunčevim zrakama inducirano oštećenje kože (110).

Upravo zato su aktivne biljne sastavnice zanimljive pri izradi dermatofarmaceutskih pripravaka budući da, pored zaštitnog i blagotvornog djelovanja na kožu, djeluju i kao svojevrsni prezervativi štiteći gotove proizvode od razgradnje utjecajem slobodnih radikala. Tako se u ovom istraživanju proučavaju učinci pripremljenih ekstrakata na slobodne radikale (prema modelu slobodnih radikala DPPH), kelirajuća aktivnost na Fe^{2+} ione i aktivnost u toplinski induciranoj razgradnji nezasićenih masnih kiselina u sustavu β-karoten-linolenska kiselina. Rezultati su uspoređeni s aktivnošću otopina standarda, butiliranog hidroksianisola (BHA) i etilendiamintetraoctene kiseline (EDTA).

Antiradikalna aktivnost optimiziranih ekstrakta u pravilu je bila manja od aktivnosti otopine standarda, BHA. Među svim ispitivanim ekstraktima, aktivnost ekstrakata *G. glabra* bila je najbliža aktivnosti standarda. Očekivano, ekstrakti *B. vulgaris* i *E. purpurea* optimizirani na RSA imali su nešto manju EC_{50} od ekstrakata optimiziranih na druge sastavnice. Prethodne studije pokazale su da su polifenoli poput kanabisina G i (±)-lionirezinola glavne tvari odgovorne za antiradikalnu aktivnost ekstrakata *B. vulgaris* kao i određeni polisaharidi (111–113). Iako je određivanje točne strukture i količine tvari odgovornih za uočenu *in vitro* aktivnost izvan dosega ovog istraživanja, najvjerojatnije je da su sekundarni metaboliti različitih struktura pridonijeli uočenoj RSA. To je možda uključivalo BER zajedno s drugim sastavnicama prisutnim u kori korijena, poput raznih fenola i polisaharida. Spagnol i sur. pokazali su kako u ekstraktima *E. purpurea* derivati kavene kiseline (CAF i CIC) posjeduju iznimno visoko antiradikalno djelovanje, nadmašujući aktivnost korištenih standarada, askorbinske kiseline i Troloksa. Dodatne prednosti derivata kavene kiseline su viša stabilnost od askorbinske kiseline i mogućnost dobivanja iz prirodnih izvora (za razliku od Troloksa) (114). S druge strane, oba ispitivana ekstrakta *S. marianum* bila su jednako učinkovita što govori o važnosti uloge SYL u aktivnosti.

Poput antiradikalne aktivnosti, i kelirajuća svojstva optimiziranih ekstrakata biljnih droga bila su niža od otopine standarda, EDTA. Zanimljivo je kako su RSA optimizirani ekstrakti biljnih droga *B. vulgaris*, *E. purpurea* i *S. marianum* bili otprilike izjednačene učinkovitosti u keliranju Fe^{2+} iona kao i ekstrakti optimizirani na pojedine sastavnice istih biljnih droga, što ukazuje kako je većina kelirajućih svojstava upravo sadržana u sastavnicama. Takav slučaj nije kod ekstrakta *G. glabra* , gdje su upravo TP optimizirani ekstrakti pokazali najistaknutiju sposobnost keliranja, koja je bila najbliža aktivnosti otopine standarda. Sukladno tomu, TP optimizirani ekstrakti *G. glabra* bili su najbolji kelatori u sklopu ovog istraživanja. Slične zaključke navode Castangia i sur., kao i Kotian i sur. u svojim istraživanjima određivanja antiradikalne aktivnosti *G. glabra* (62,63).

U testu inhibicije oksidacijske razgradnje nezasićenih masnih kiselina, ekstrakti *S. marianum* i *E. purpurea* pokazali su izrazito visoku sposobnost inhibicije koja je bila jednaka ili viša od aktivnosti otopine standarda. Kod *S. marianum* izraženiju aktivnost imao je SYL optimiziran ekstrakt, dok je kod *E. purpurea* to bio RSA optimiziran ekstrakt. Ovo je posebno važno jer većina kozmetičkih proizvoda sadrži prirodna ulja bogata linolenskom kiselinom, kao i mnoštvom drugih polinezasićenih masnih kiselina. Takvi se proizvodi nerijetko koriste u terapiji atopičnih ili seboreičnih dermatitisa, kao i ostalih kožnih oboljenja, a ekstrakti koji pokazuju visoku moć inhibicije nezasićenih masnih kiselina povoljno djeluju na aktivnost, kao i trajnost kozmetičkih proizvoda koji ih sadrže (115). S druge strane, aktivnost inhibicije nezasićenih masnih kiselina u ekstraktima *B. vulgaris* i *G. glabra* bila je niža od aktivnosti standarda.

3.4.2. Utjecaj na enzime koji djeluju na pigmentaciju i strukturu kože

Pored hidratacijskog učinka i antiradikalnog potencijala, od modernih se dermatofarmaceutskih proizvoda očekuju dodatna povoljna djelovanja na kožu. Prejaka enzimatska aktivnost kože, do koje dolazi zbog brojnih ranije opisanih promjena uzrokovanih prirodnim starenjem, ali i vanjskim utjecajima, dovodi do preuranjene ili pretjerane razgradnje važnih strukturnih proteina u koži poput elastaze ili kolagenaze. Ove promjene također dovode do razgradnje važnih strukturnih polisaharida u koži poput hijaluronske kiseline (116).

Rezultati ovog istraživanja pokazuju kako je ekstrakt *E. purpurea* dobar inhibitor kolagenaze i elastaze. Ipak, te su aktivnosti i dalje niže od aktivnosti otopina standarda, galne i ursolične kiseline. Kod inhibicije oba enzima jača aktivnost opažena je u RSA optimiziranom ekstraktu što daje naslutiti kako i drugi spojevi osim fenolnih kiselina igraju važnu ulogu u ovome procesu. Uz to, ekstrakt *E. purpurea* snažan je inhibitor hijaluronidaze, što je u skladu s prijašnjim istraživanjima koja opisuju derivate kavene kiseline snažnim antihijaluronidaznim učinkom (117,118). Obje vrste ekstrakta jednako snažno inhibiraju ovaj enzim čime se nameće zaključak kako su upravo derivati kavene kiseline zaslužni za isti. Valja napomenuti kako je taj učinak bio izraženiji od otopine standarda, taninske kiseline. Ovo je posebice važno obzirom na sposobnost hijaluronske kiseline da zadržava vlagu čime njezino očuvanje u koži doprinosi sveukupnoj hidrataciji.

Slični rezultati vidljivi su i kod ekstrakta *S. marianum* obzirom na aktivnost inhibicije elastaze. S druge strane, svi ekstrakti *G. glabra* pokazali su izvrsne rezultate inhibicije elastaze, s posebnim naglaskom na GLA i ISO optimiziran ekstrakt koji je premašio aktivnost otopine standarda. Također je važno napomenuti da je u sklopu ovog istraživanja proveden eksperiment kako bi se utvrdilo utječe li otapalo (glicerol) na aktivnosti elastaze i dobiveni rezultati ukazuju da to nije slučaj što dodatno potvrđuje povoljno djelovanje sastavnica s ciljem primjene u dermatofarmaceutskim proizvodima.

Učinak UV zraka iz sunčevog svjetla ranije je opisan, kao i njegove negativne posljedice na kožu, prvenstveno zbog stvaranja melazmi i hiperpigmentacijskih pjega. To je posljedica aktivnosti tirozinaze, enzima koji katalizira sintezu proteina melanina. Ovim istraživanjem pokazano ja kako su svi ekstrakti dobri inhibitori tirozinaze, s posebnim naglaskom na ekstrakte *E. purpurea* i *G. glabra*, čiji su učinci bili usporedni ili bolji učincima kojične kiseline, korištene kao otopina standarda. Kod ovih biljnih droga aktivnijim su se pokazali TPA optimizirani ekstrakti, kao i GLA i ISO optimizirani ekstrakti. Važno je napomenuti kako niti u ovim eksperimentima glicerol nije pokazao značajan učinak na inhibiciju tirozinaze, čime sva inhibitorna aktivnost pripada aktivnim sastavnicama biljaka. Honisch i sur. pokazali su kako je CAF snažan kompetitivni inhibitor tirozinaze (53). Stoga je i očekivano kako je u ovom istraživanju ekstrakt optimiziran na TPA bio mnogo izraženiji inhibitor ovoga enzima. Imajući na umu ovo djelovanje derivata kavene kiseline, kao i GLA i ISO, nameće se zaključak kako bi ekstrakti biljnih droga bogatim ovim sastavnicama bili od znatne važnosti u proizvodima za izbjeljivanje kože.

Zacjeljivanje rana je proces dinamičkih staničnih i molekularnih mehanizama, podijeljenih u nekoliko faza, koje se mogu preklapati tijekom vremena: hemostaza, upala, proliferacijacija/migracija i sazrijevanje ili preoblikovanje, karakterizirano stvaranjem novog tkiva. U fazi proliferacije migracija keratinocita i fibroblasta obnavlja mrežu krvnih žila i sudjeluje u procesu granulacije. Ova karakteristika koristi se za *in vitro* metodu ispitivanja cijeljenja rana "grebanjem". U ovom postupku, ogrebotina koja ostavlja prazan prostor (“rana”) na dnu jažice stvara se u HaCaT staničnom monosloju. Ako su uvjeti zadovoljavajući, dolazi do kretanja stanica i proliferacije, nakon čega slijedi postupno zatvaranje staničnog modela rane (119).

U ovom su istraživanju stanice tretirane različitim razrjeđenjima ekstrakata *E. purpurea* i glicerol. Hankova otopina (engl. *Hank's balanced salt solution*, HBSS) je korištena kao negativna kontrola. Pratio se proces zatvaranja rane preko 48 h. Oba ekstrakta (optimizirana na TPA i RSA) su ubrzala zatvaranje rana u stanica. Posebno je aktivan bio RSA optimiziran ekstrakt. Nakon 48 h, površina ogrebotine u staničnom monosloju tretiranom tim ekstraktom bila je jedva vidljiva, što ukazuje na izvrsnu aktivnost cijeljenja rana. S druge strane, smanjenje površine rane u stanicama tretiranih negativnom kontrolom bila je jedva primjetna. Također je ispitana aktivnost različitih koncentracija glicerola, čije je aktivnost bila jednaka ili niža od aktivnosti HBSS kontrole. Nedostatak aktivnosti otapala ukazuje kako su upravo aktivne sastavnice *E.purpurea* bile odgovorne za poticanje proliferacije HaCaT stanica tijekom ispitivanog vremena inkubacije. Zanimljivo, djelovanje RSA optimiziranog nije ovisilo o dozi. Na primjer, RSA optimizirani ekstrakt u koncentraciji od 2,5 µL ekstrakta/mL pokazao je bolju aktivnost cijeljenja rana nego u koncentraciji od 12,5 µL ekstrakta/mL. Sposobnost biljnih ekstrakata na cijeljenje rana neovisno o koncentraciji istih nije neobična pojava. Razlog može biti složeno međudjelovanje pojedinih aktivnih sastavnica u ekstraktu (120). Sukladno tomu, jedan od budućih istraživačkih pravaca može se usmjeriti na pronalaženje komponenti koje su prvenstveno odgovorne za uočenu aktivnost cijeljenja rana ekstrakata *E. purpurea*, kao kao i optimalni raspon doza za primjenu istih. Slično je ponašanje biljnih pripravaka također yabilježeno u *in vivo* ispitivanjimana, primjerice s mašću koja sadrži ekstrakt lišća *Ocimum gratissimum* (121).

Ovaj eksperiment potvrđuje ranije navedenu tvrdnju Europske agencije za lijekove (EMA) u kojoj se navodi kako se E. purpurea i njezini pripravci mogu koristiti u biljnim lijekovima za ublažavanje kožnih oboljenja i pospješenje zacijeljivanja manjih rana.

3.4.3. Protuupalno djelovanje

Denaturacija tkivnih proteina jedan je od uzroka upalnih procesa u koži (122). Stoga bi supresija denaturacije proteina kože mogla usporiti daljnji razvoj upalnih promjena kože što je od posebne važnosti prilikom razvitka dermofarmaceutskih proizvoda (123). U ovom su istraživanju svi ekstrakti pokazali dobra svojstva inhibicije toplinom inducirane koagulacije ovalbumina. Kako je u literaturi opisana uloga glicerola na denaturaciju proteina poput kolagena (124), u ovom je istraživanju proveden eksperiment uloge otopina glicerola na denaturaciju ovalbumina jajeta. Očekivano, rezultati su pokazali kako glicerol značajno inhibira denaturaciju ovalbumina te je većina aktivnosti inhibicije ekstrakata upravo zbog uloge otapala. Ova sposobnost glicerola dodatno potvrđuje važnost uporabe glicerolnih ekstrakata u dermatofarmaceutskim proizvodima budući da nadilazi osnovne prednosti glicerola kao zelenog otapala.

Nadalje, lipoksigenaza (LOX) je enzim koji je uključen u metabolizam arahidonske kiseline i otpuštanje raznih proupalnih eikozanoida poput leukotriena i lipoksina. LOX igra važnu ulogu u aktivaciji upale kože i posreduje u upalnom odgovoru kože na razne okolišne čimbenike poput UV zračenja i alergena (125). Iako je u literaturi BER iz *Mahonia aquifolium* pokazao vrlo nisku anti-LOX aktivnost, aktivnost ekstrakta *B. vulgaris* pripremljenog u ovome istraživanju bila je znatno jača. Točnije, RSA-optimiziran ekstrakt svojom je anti-LOX aktivnošću bio relativno usporedan s aktivnosti 1 mg/mL otopine nordihidroguajaretične kiseline (NDGA), korištene kao standard.

Zaključno, opisana aktivnost ekstrakata u upalnim promjenama uzrokovanim aktivnosti LOX-a, kao i koagulacijom proteina čini ih dobrim kandidatima za uporabu u kozmetici.

4. ZAKLJUČAK

Glicerol se pokazao kao optimalno zeleno otapalo za ekstrakciju aktivnih sastavnica biljnih vrsta *G. glabra, E. purpurea, B. vulgaris* i *S. marianum* zbog svojih brojnih prednosti nad konvencionalnim otapalima od kojih se poglavito ističu njegova netoksičnost, kao i opisana humektantna svojstva zbog kojih doprinosi hidratacijskom učinku na koži čime se ukida potreba za uklanjanjem otapala iz ekstrakta. Zbog svoje učinkovitosti i kratkog vremena ekstrakcije UAE su se pokazala ekstrakcijskom metodom izbora. Optimizirani glicerolni ekstrakti pokazali su dobru biološku aktivnost na poljima antiradikalnog i protuupalnog učinka, kao i učinkom na odabrane enzime važne za zdravlje i izgled kože. Zaključno, priređeni ekstrakti pokazuju obećavajuću ulogu u proizvodima namijenjenim liječenju i zaštiti kože, s ciljem smanjenja kožnih promjena starenjem kože. Dodatna istraživanja su potrebna kako bi se utvrdio način ugradnje spomenutih ekstrakata u dermatofarmaceutske proizvode, kao i njihova točna doza.

5. LITERATURA

1. Walters K, Roberts M. The Structure and Function of Skin. U: Dermatological and Transdermal Formulations. Boca Raton: CRC Press; 2002. str. 1–11.

2. Rijken F, Kiekens RCM, van den Worm E, Lee PL, van Weelden H, Bruijnzeel PLB. Pathophysiology of photoaging of human skin: focus on neutrophils. Photochem Photobiol Sci. veljača 2006.;5(2):184–9.

3. Zhang S, Duan E. Fighting against Skin Aging: The Way from Bench to Bedside. Cell Transplant. svibanj 2018.;27(5):729–38.

4. Callaghan TM, Wilhelm KP. A review of ageing and an examination of clinical methods in the assessment of ageing skin. Part I: Cellular and molecular perspectives of skin ageing. Int J Cosmet Sci. listopad 2008.;30(5):313–22.

5. Zillich OV, Schweiggert-Weisz U, Eisner P, Kerscher M. Polyphenols as active ingredients for cosmetic products. International Journal of Cosmetic Science. 2015.;37(5):455–64.

6. Kumari S, Tien Guan Thng S, Kumar Verma N, Gautam HK. Melanogenesis Inhibitors. Acta Derm Venereol. 05. studeni 2018.;98(10):924–31.

7. Kerscher M, Buntrock H. Update on cosmeceuticals. J Dtsch Dermatol Ges. travanj 2011.;9(4):314–26; quiz 327.

8. Mitjans M, Del Campo J, Abajo C, Martínez V, Selga A, Lozano C, i ostali. Immunomodulatory Activity of a New Family of Antioxidants Obtained from Grape Polyphenols. J Agric Food Chem. 01. prosinac 2004.;52(24):7297–9.

9. Parish LC, Crissey JT. Cosmetics: A historical review. Clinics in Dermatology. 01. srpanj 1988.;6(3):1–4.

10. Hu X, He H. A review of cosmetic skin delivery. J of Cosmetic Dermatology. srpanj 2021.;20(7):2020–30.

11. Bozza A, Campi C, Garelli S, Ugazio E, Battaglia L. Current regulatory and market frameworks in green cosmetics: The role of certification. Sustainable Chemistry and Pharmacy. 01. prosinac 2022.;30:100851.

12. Costa R, Santos L. Delivery systems for cosmetics - From manufacturing to the skin of natural antioxidants. Powder Technology. prosinac 2017.;322:402–16.

13. De Lima Cherubim DJ, Buzanello Martins CV, Oliveira Fariña L, Da Silva De Lucca RA. Polyphenols as natural antioxidants in cosmetics applications. J of Cosmetic Dermatology. siječanj 2020.;19(1):33–7.

14. Borowska S, Brzóska MM. Metals in cosmetics: implications for human health. J Appl Toxicol. lipanj 2015.;35(6):551–72.

15. Rice-evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The Relative Antioxidant Activities of Plant-Derived Polyphenolic Flavonoids. Free Radical Research. siječanj 1995.;22(4):375–83.

16. F G, Pr E, Cm S, U W. Luteolin as a modulator of skin aging and inflammation. BioFactors (Oxford, England) [Internet]. ožujak 2021. [citirano 09. rujan 2024.];47(2). Dostupno na: https://pubmed.ncbi.nlm.nih.gov/33368702/

17. Neelam null, Khatkar A, Sharma KK. Phenylpropanoids and its derivatives: biological activities and its role in food, pharmaceutical and cosmetic industries. Crit Rev Food Sci Nutr. 2020.;60(16):2655–75.

18. Stępniowska A, Cieplińska P, Fac W, Górska J. Selected Alkaloids Used in the Cosmetics Industry. J Cosmet Sci. 2021.;72(2):229–45.

19. Franca C, Ueno H. Green cosmetics: perspectives and challenges in the context of green chemistry. Desenvolvimento e Meio Ambiente. 27. travanj 2020.;53.

20. Ciganović P, Jakupović L, Momchev P, Nižić Nodilo L, Hafner A, Zovko Končić M. Extraction Optimization, Antioxidant, Cosmeceutical and Wound Healing Potential of Echinacea purpurea Glycerolic Extracts. Molecules. siječanj 2023.;28(3):1177.

21. Yahya NA, Attan N, Wahab RA. An overview of cosmeceutically relevant plant extracts and strategies for extraction of plant-based bioactive compounds. Food and Bioproducts Processing. 01. studeni 2018.;112:69–85.

22. Abubakar AR, Haque M. Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. J Pharm Bioallied Sci. 2020.;12(1):1–10.

23. Azmir J, Zaidul ISM, Rahman MM, Sharif KM, Mohamed A, Sahena F, i ostali. Techniques for extraction of bioactive compounds from plant materials: A review. Journal of Food Engineering. kolovoz 2013.;117(4):426–36.

24. Fu X, Belwal T, Cravotto G, Luo Z. Sono-physical and sono-chemical effects of ultrasound: Primary applications in extraction and freezing operations and influence on food components. Ultrasonics Sonochemistry. 01. siječanj 2020.;60:104726.

25. Huang H, Xu Q, Belwal T, Li L, Aalim H, Wu Q, i ostali. Ultrasonic impact on viscosity and extraction efficiency of polyethylene glycol: A greener approach for anthocyanins recovery from purple sweet potato. Food Chemistry. 15. lipanj 2019.;283:59–67.

26. Chemat F, Vian MA, Cravotto G. Green Extraction of Natural Products: Concept and Principles. Int J Mol Sci. 11. srpanj 2012.;13(7):8615–27.

27. Chemat F, Abert Vian M, Ravi HK, Khadhraoui B, Hilali S, Perino S, i ostali. Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects. Molecules. siječanj 2019.;24(16):3007.

28. Berlan J, Mason TJ. Sonochemistry: from research laboratories to industrial plants. Ultrasonics. 01. siječanj 1992.;30(4):203–12.

29. Chemat F, Abert-Vian M, Fabiano-Tixier AS, Strube J, Uhlenbrock L, Gunjevic V, i ostali. Green extraction of natural products. Origins, current status, and future challenges. TrAC Trends in Analytical Chemistry. 01. rujan 2019.;118:248–63.

30. Herrera MC, de Castro MDL. Ultrasound-assisted extraction of phenolic compounds from strawberries prior to liquid chromatographic separation and photodiode array ultraviolet detection. J Chromatogr A. 23. prosinac 2005.;1100(1):1–7.

31. Mason TJ, Paniwnyk L, Lorimer JP. The uses of ultrasound in food technology. Ultrasonics Sonochemistry. 01. studeni 1996.;3(3):S253–60.

32. Rosarina D, Narawangsa DR, Chandra NSR, Sari E, Hermansyah H. Optimization of Ultrasonic-Assisted Extraction (UAE) Method Using Natural Deep Eutectic Solvent (NADES) to Increase Curcuminoid Yield from Curcuma longa L., Curcuma xanthorrhiza, and Curcuma mangga Val. Molecules. 18. rujan 2022.;27(18):6080.

33. Rodríguez-Fernández R, Fernández-Gómez Á, Mejuto JC, Astray G. Modelling Polyphenol Extraction through Ultrasound-Assisted Extraction by Machine Learning in Olea europaea Leaves. Foods. 14. prosinac 2023.;12(24):4483.

34. Wang W, Liu X, Wang L, Song G, Jiang W, Mu L, i ostali. Ficus carica polysaccharide extraction via ultrasound-assisted technique: Structure characterization, antioxidant, hypoglycemic and immunomodulatory activities. Ultrason Sonochem. prosinac 2023.;101:106680.

35. Wolfson A, Dlugy C, Shotland Y. Glycerol as a green solvent for high product yields and selectivities. Environ Chem Lett. 01. svibanj 2007.;5(2):67–71.

36. Becker LC, Bergfeld WF, Belsito DV, Hill RA, Klaassen CD, Liebler DC, i ostali. Safety Assessment of Glycerin as Used in Cosmetics. Int J Toxicol. 2019.;38(3_suppl):6S-22S.

37. Aalim H, Belwal T, Jiang L, Huang H, Meng X, Luo Z. Extraction optimization, antidiabetic and antiglycation potentials of aqueous glycerol extract from rice (*Oryza sativa L.*) bran. LWT. 01. travanj 2019.;103:147–54.

38. Vieira V, Calhelha RC, Barros L, Coutinho JAP, Ferreira ICFR, Ferreira O. Insights on the Extraction Performance of Alkanediols and Glycerol: Using Juglans regia L. Leaves as a Source of Bioactive Compounds. Molecules. 27. svibanj 2020.;25(11):2497.

39. Thong-on W, Pathomwichaiwat T, Boonsith S, Koo-amornpattana W, Prathanturarug S. Green extraction optimization of triterpenoid glycoside-enriched extract from Centella asiatica (L.) Urban using response surface methodology (RSM). Sci Rep. 11. studeni 2021.;11(1):22026.

40. Anderson-Cook CM, Borror CM, Montgomery DC. Response surface design evaluation and comparison. Journal of Statistical Planning and Inference. 01. veljača 2009.;139(2):629–41.

41. Alberti A, Zielinski AAF, Zardo DM, Demiate IM, Nogueira A, Mafra LI. Optimisation of the extraction of phenolic compounds from apples using response surface methodology. Food Chemistry. 15. travanj 2014.;149:151–8.

42. Karvela E, Makris DP, Kalogeropoulos N, Karathanos VT. Deployment of response surface methodology to optimise recovery of grape (*Vitis vinifera*) stem polyphenols. Talanta. 15. listopad 2009.;79(5):1311–21.

43. Zhang Y lao, Kong L chun, Yin C ping, Jiang D hua, Jiang J quan, He J, i ostali. Extraction optimization by response surface methodology, purification and principal antioxidant metabolites of red pigments extracted from bayberry (*Myrica rubra*) pomace. LWT - Food Science and Technology. 01. travanj 2013.;51(1):343–7.

44. 5.3.3.6.2. Box-Behnken designs [Internet]. [citirano 16. rujan 2024.]. Dostupno na: https://www.itl.nist.gov/div898/handbook/pri/section3/pri3362.htm

45. Ahmad A, Rehman MU, Wali AF, El-Serehy HA, Al-Misned FA, Maodaa SN, i ostali. Box-Behnken Response Surface Design of Polysaccharide Extraction from Rhododendron arboreum and the Evaluation of Its Antioxidant Potential. Molecules. 24. kolovoz 2020.;25(17):3835.

46. Della Posta S, Gallo V, Dugo L, De Gara L, Fanali C. Development and Box-Behnken design optimization of a green extraction method natural deep eutectic solvent-based for phenolic compounds from barley malt rootlets. Electrophoresis. listopad 2022.;43(18–19):1832–40.

47. Polo-Castellano C, Álvarez JÁ, Palma M, Barbero GF, Ayuso J, Ferreiro-González M. Optimization through a Box-Behnken Experimental Design of the Microwave-Assisted Extraction of the Psychoactive Compounds in Hallucinogenic Fungi (Psylocibe cubensis). J Fungi (Basel). 02. lipanj 2022.;8(6):598.

48. Karsch-Völk M, Barrett B, Kiefer D, Bauer R, Ardjomand-Woelkart K, Linde K. Echinacea for preventing and treating the common cold. Cochrane Database of Systematic Reviews [Internet]. 2014. [citirano 15. kolovoz 2024.];(2). Dostupno na: https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD000530.pub3/full

49. Echinaceae purpureae herba - herbal medicinal product | European Medicines Agency (EMA) [Internet]. 2015 [citirano 15. kolovoz 2024.]. Dostupno na: https://www.ema.europa.eu/en/medicines/herbal/echinaceae-purpureae-herba

50. European Directorate for the Quality of Medicines & HealthCare [Internet]. [citirano 15. kolovoz 2024.]. European Pharmacopoeia (Ph. Eur.) - European Directorate for the Quality of Medicines & HealthCare - EDQM. Dostupno na: https://www.edqm.eu/en/european-pharmacopoeia

51. Peng Y, Sun Q, Park Y. The Bioactive Effects of Chicoric Acid As a Functional Food Ingredient. J Med Food. srpanj 2019.;22(7):645–52.

52. Caftaric acid: an overview on its structure, daily consumption, bioavailability and pharmacological effects. Biointerface Res Appl Chem. 29. ožujak 2020.;10(3):5616–23.

53. Honisch C, Osto A, Dupas de Matos A, Vincenzi S, Ruzza P. Isolation of a tyrosinase inhibitor from unripe grapes juice: A spectrophotometric study. Food Chemistry. 01. veljača 2020.;305:125506.

54. Yotsawimonwat S, Rattanadechsakul J, Rattanadechsakul P, Okonogi S. Skin improvement and stability of Echinacea purpurea dermatological formulations. International Journal of Cosmetic Science. 2010.;32(5):340–6.

55. Kondo K, Shiba M, Nakamura R, Morota T, Shoyama Y. Constituent properties of licorices derived from Glycyrrhiza uralensis, G. glabra, or G. inflata identified by genetic information. Biol Pharm Bull. srpanj 2007.;30(7):1271–7.

56. El-Saber Batiha G, Magdy Beshbishy A, El-Mleeh A, Abdel-Daim MM, Prasad Devkota H. Traditional Uses, Bioactive Chemical Constituents, and Pharmacological and Toxicological Activities of Glycyrrhiza glabra L. (Fabaceae). Biomolecules. 25. veljača 2020.;10(3):352.

57. Encyclopedia of Food Sciences and Nutrition [Internet]. 2003 [citirano 15. kolovoz 2024.]. Dostupno na: https://shop.elsevier.com/books/encyclopedia-of-food-sciences-and-nutrition/caballero/978-0-08-091791-7

58. F P, Q D, C P, N W, H T, X X, i ostali. A Review: The Pharmacology of Isoliquiritigenin. Phytotherapy research : PTR [Internet]. srpanj 2015. [citirano 09. rujan 2024.];29(7). Dostupno na: https://pubmed.ncbi.nlm.nih.gov/25907962/

59. Simmler C, Pauli GF, Chen SN. Phytochemistry and biological properties of glabridin. Fitoterapia. listopad 2013.;90:160–84.

60. Pastorino G, Cornara L, Soares S, Rodrigues F, Oliveira MBPP. Liquorice (Glycyrrhiza glabra): A phytochemical and pharmacological review. Phytother Res. prosinac 2018.;32(12):2323–39.

61. Ferreira MS, Magalhães MC, Oliveira R, Sousa-Lobo JM, Almeida IF. Trends in the Use of Botanicals in Anti-Aging Cosmetics. Molecules. 11. lipanj 2021.;26(12):3584.

62. Castangia I, Caddeo C, Manca ML, Casu L, Latorre AC, Díez-Sales O, i ostali. Delivery of liquorice extract by liposomes and hyalurosomes to protect the skin against oxidative stress injuries. Carbohydrate Polymers. 10. prosinac 2015.;134:657–63.

63. Mostafa DM, Ammar NM, Abd El-Alim SH, El-anssary AA. Transdermal microemulsions of Glycyrrhiza glabra L.: characterization, stability and evaluation of antioxidant potential. Drug Deliv. ožujak 2014.;21(2):130–9.

64. Kotian S, Bhat K, Pai S, Nayak J, Souza A, Gourisheti K, i ostali. The Role of Natural Medicines on Wound Healing: A Biomechanical, Histological, Biochemical and Molecular Study. Ethiop J Health Sci. studeni 2018.;28(6):759–70.

65. Yu H, Li H, Li Y, Li M, Chen G. Effect of isoliquiritigenin for the treatment of atopic dermatitis-like skin lesions in mice. Arch Dermatol Res. prosinac 2017.;309(10):805–13.

66. Silybi mariani fructus - herbal medicinal product | European Medicines Agency (EMA) [Internet]. 2019 [citirano 15. kolovoz 2024.]. Dostupno na: https://www.ema.europa.eu/en/medicines/herbal/silybi-mariani-fructus

67. Singh RP, Agarwal R. Cosmeceuticals and silibinin. Clinics in Dermatology. 01. rujan 2009.;27(5):479–84.

68. Drouet S, Leclerc EA, Garros L, Tungmunnithum D, Kabra A, Abbasi BH, i ostali. A Green Ultrasound-Assisted Extraction Optimization of the Natural Antioxidant and Anti-

Aging Flavonolignans from Milk Thistle Silybum marianum (L.) Gaertn. Fruits for Cosmetic Applications. Antioxidants. kolovoz 2019.;8(8):304.

69. Neag MA, Mocan A, Echeverría J, Pop RM, Bocsan CI, Crişan G, i ostali. Berberine: Botanical Occurrence, Traditional Uses, Extraction Methods, and Relevance in Cardiovascular, Metabolic, Hepatic, and Renal Disorders. Front Pharmacol. 2018.;9:557.

70. Kosalec I, Gregurek B, Kremer D, Zovko M, Sanković K, Karlović K. Croatian barberry (Berberis croatica Horvat): a new source of berberine—analysis and antimicrobial activity. World J Microbiol Biotechnol. 01. siječanj 2009.;25(1):145–50.

71. da Silva AR, de Andrade Neto JB, da Silva CR, Campos R de S, Costa Silva RA, Freitas DD, i ostali. Berberine Antifungal Activity in Fluconazole-Resistant Pathogenic Yeasts: Action Mechanism Evaluated by Flow Cytometry and Biofilm Growth Inhibition in Candida spp. Antimicrob Agents Chemother. lipanj 2016.;60(6):3551–7.

72. Yang P, Ju Y, Liu X, Li Z, Liu H, Yang M, i ostali. Natural self-healing injectable hydrogels loaded with exosomes and berberine for infected wound healing. Mater Today Bio. 19. studeni 2023.;23:100875.

73. Kim S, Chung JH. Berberine prevents UV-induced MMP-1 and reduction of type I procollagen expression in human dermal fibroblasts. Phytomedicine. rujan 2008.;15(9):749–53.

74. Kim S, Kim Y, Kim JE, Cho KH, Chung JH. Berberine inhibits TPA-induced MMP-9 and IL-6 expression in normal human keratinocytes. Phytomedicine. svibanj 2008.;15(5):340–7.

75. Chen J, Jiang Z, Liu X, Wang K, Fan W, Chen T, i ostali. Berberine promotes the viability of random skin flaps via the PI3K/Akt/eNOS signaling pathway. Phytother Res. veljača 2023.;37(2):424–37.

76. Namba T, Sekiya K, Toshinal A, Kadota S, Hatanaka T, Katayama K, i ostali. [Study on baths with crude drug. II.: the effects of coptidis rhizoma extracts as skin permeation enhancer]. Yakugaku Zasshi. kolovoz 1995.;115(8):618–25.

77. Imenshahidi M, Hosseinzadeh H. Berberine and barberry (Berberis vulgaris): A clinical review. Phytother Res. ožujak 2019.;33(3):504–23.

78. Wu Y, Li W, Vovers J, Thuan Lu H, Stevens GW, Mumford KA. Investigation of green solvents for the extraction of phenol and natural alkaloids: Solvent and extractant selection. Chemical Engineering Journal. 15. kolovoz 2022.;442:136054.

79. Wallace SN, Carrier DJ, Clausen EC. Batch solvent extraction of flavanolignans from milk thistle (Silybum marianum L. Gaertner). Phytochem Anal. 2005.;16(1):7–16.

80. Senica M, Mlinsek G, Veberic R, Mikulic-Petkovsek M. Which Plant Part of Purple Coneflower (Echinacea purpurea (L.) Moench) Should be Used for Tea and Which for Tincture? J Med Food. siječanj 2019.;22(1):102–8.

81. Turrini F, Donno D, Beccaro GL, Zunin P, Pittaluga A, Boggia R. Pulsed Ultrasound-Assisted Extraction as an Alternative Method to Conventional Maceration for the

Extraction of the Polyphenolic Fraction of Ribes nigrum Buds: A New Category of Food Supplements Proposed by The FINNOVER Project. Foods. 10. listopad 2019.;8(10):466.

82. Izadiyan P, Hemmateenejad B. Multi-response optimization of factors affecting ultrasonic assisted extraction from Iranian basil using central composite design. Food Chemistry. 01. siječanj 2016.;190:864–70.

83. Michail A, Sigala P, Grigorakis S, Makris DP. Kinetics of Ultrasound-Assisted Polyphenol Extraction from Spent Filter Coffee Using Aqueous Glycerol. Chemical Engineering Communications. 03. ožujak 2016.;203(3):407–13.

84. Eyiz V, Tontul I, Turker S. Optimization of green extraction of phytochemicals from red grape pomace by homogenizer assisted extraction. Food Measure. 01. veljača 2020.;14(1):39–47.

85. Chemat F, Vian MA, urednici. Alternative Solvents for Natural Products Extraction [Internet]. Berlin, Heidelberg: Springer; 2014 [citirano 25. listopad 2024.]. (Green Chemistry and Sustainable Technology). Dostupno na: https://link.springer.com/10.1007/978-3-662-43628-8

86. Perrucci M, Dezio M, Saleem H, Ruggieri F, Locatelli M. Green extraction, chemical profile and biological activity of waste products from the olive oil industry: From waste to wealth. Journal of Chromatography Open. 01. studeni 2024.;6:100161.

87. Dai J, Mumper RJ. Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. Molecules. listopad 2010.;15(10):7313–52.

88. Vatai T, Škerget M, Knez Ž. Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide. Journal of Food Engineering. 01. siječanj 2009.;90(2):246–54.

89. Wang L, Weller CL. Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology. 01. lipanj 2006.;17(6):300–12.

90. Toma M, Vinatoru M, Paniwnyk L, Mason TJ. Investigation of the effects of ultrasound on vegetal tissues during solvent extraction. Ultrasonics Sonochemistry. 01. travanj 2001.;8(2):137–42.

91. Antony A, Farid M. Effect of Temperatures on Polyphenols during Extraction. Applied Sciences. siječanj 2022.;12(4):2107.

92. Mocan A, Babotă M, Pop A, Fizeșan I, Diuzheva A, Locatelli M, i ostali. Chemical Constituents and Biologic Activities of Sage Species: A Comparison between Salvia officinalis L., S. glutinosa L. and S. transsylvanica (Schur ex Griseb. & Schenk) Schur. Antioxidants. lipanj 2020.;9(6):480.

93. Chen Y, Luo H, Gao A, Zhu M. Ultrasound-assisted extraction of polysaccharides from litchi (*Litchi chinensis* Sonn.) seed by response surface methodology and their structural characteristics. Innovative Food Science & Emerging Technologies. 01. srpanj 2011.;12(3):305–9.

94. Leopoldini M, Chiodo SG, Russo N, Toscano M. Detailed Investigation of the OH Radical Quenching by Natural Antioxidant Caffeic Acid Studied by Quantum Mechanical Models. J Chem Theory Comput. 13. prosinac 2011.;7(12):4218–33.

95. Bergeron C, Gafner S, Batcha LL, Angerhofer CK. Stabilization of caffeic acid derivatives in Echinacea purpurea L. glycerin extract. J Agric Food Chem. 03. srpanj 2002.;50(14):3967–70.

96. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. Clarendon Press; 1989. 568 str.

97. Cisneros-Yupanqui M, Chalova VI, Kalaydzhiev HR, Mihaylova D, Krastanov AI, Lante A. Ultrasound-assisted extraction of antioxidant bioactive compounds from wastes of rapeseed industry and their application in delaying rapeseed oil oxidation. Environmental Technology & Innovation. 01. svibanj 2023.;30:103081.

98. Mousavi SA, Nateghi L, Javanmard Dakheli M, Ramezan Y, Piravi-Vanak Z. Maceration and ultrasound-assisted methods used for extraction of phenolic compounds and antioxidant activity from Ferulago angulata. Journal of Food Processing and Preservation. 2022.;46(3):e16356.

99. Tsai CC, Chou CH, Liu YC, Hsieh CW. Ultrasound-assisted extraction of phenolic compounds from hyllanthus emblica L. and evaluation of antioxidant activities. International Journal of Cosmetic Science. 2014.;36(5):471–6.

100. Le HV, Le VVM. Comparison of enzyme-assisted and ultrasound-assisted extraction of vitamin C and phenolic compounds from acerola (Malpighia emarginata DC.) fruit. International Journal of Food Science & Technology. 2012.;47(6):1206–14.

101. Riciputi Y, Diaz-de-Cerio E, Akyol H, Capanoglu E, Cerretani L, Caboni MF, i ostali. Establishment of ultrasound-assisted extraction of phenolic compounds from industrial potato by-products using response surface methodology. Food Chemistry. 15. prosinac 2018.;269:258–63.

102. Fumić B, Jug M, Zovko Končić M. Optimization of Ultrasound-Assisted Extraction of Phenolic Antioxidants from Lotus corniculatus. Croat chem acta (Online). 2019.;92(3):369–77.

103. Pandey A, Belwal T, Sekar KC, Bhatt ID, Rawal RS. Optimization of ultrasonic-assisted extraction (UAE) of phenolics and antioxidant compounds from rhizomes of *Rheum moorcroftianum* using response surface methodology (RSM). Industrial Crops and Products. 01. rujan 2018.;119:218–25.

104. Philippi K, Tsamandouras N, Grigorakis S, Makris DP. Ultrasound-Assisted Green Extraction of Eggplant Peel (Solanum melongena) Polyphenols Using Aqueous Mixtures of Glycerol and Ethanol: Optimisation and Kinetics. Environ Process. 01. lipanj 2016.;3(2):369–86.

105. El Kantar S, Rajha HN, Boussetta N, Vorobiev E, Maroun RG, Louka N. Green extraction of polyphenols from grapefruit peels using high voltage electrical discharges, deep eutectic solvents and aqueous glycerol. Food Chem. 15. listopad 2019.;295:165–71.

106. Limwachiranon J, Jiang L, Huang H, Sun J, Luo Z. Improvement of phenolic compounds extraction from high-starch lotus (Nelumbo nucifera G.) seed kernels using glycerol: New insights to amylose/amylopectin - Phenolic relationships. Food Chem. 15. veljača 2019.;274:933–41.

107. Herrera MC, Luque de Castro MD. Ultrasound-assisted extraction for the analysis of phenolic compounds in strawberries. Anal Bioanal Chem. 01. kolovoz 2004.;379(7):1106–12.

108. Lê T. Barel, Paye, Maibach - Handbook of Cosmetic Science and Technology. [citirano 17. kolovoz 2024.]; Dostupno na: https://www.academia.edu/28676594/Barel_Paye_Maibach_Handbook_of_Cosmetic_Science_and_Technology

109. Mlakar A, Batna A, Dudda A, Spiteller G. Iron (II) Ions Induced Oxidation of Ascorbic Acid and Glucose. Free Radical Research. 01. siječanj 1996.;25(6):525–39.

110. Kitazawa M, Iwasaki K, Sakamoto K. Iron chelators may help prevent photoaging. Journal of Cosmetic Dermatology. 2006.;5(3):210–7.

111. Zovko Koncić M, Kremer D, Karlović K, Kosalec I. Evaluation of antioxidant activities and phenolic content of Berberis vulgaris L. and Berberis croatica Horvat. Food Chem Toxicol. 2010.;48(8–9):2176–80.

112. Tomosaka H, Chin YW, Salim AA, Keller WJ, Chai H, Kinghorn AD. Antioxidant and cytoprotective compounds from Berberis vulgaris (barberry). Phytotherapy Research. 2008.;22(7):979–81.

113. Han L, Suo Y, Yang Y, Meng J, Hu N. Optimization, characterization, and biological activity of polysaccharides from *Berberis dasystachya* Maxim. International Journal of Biological Macromolecules. 01. travanj 2016.;85:655–66.

114. Spagnol CM, Assis RP, Brunetti IL, Isaac VLB, Salgado HRN, Corrêa MA. *In vitro* methods to determine the antioxidant activity of caffeic acid. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 05. kolovoz 2019.;219:358–66.

115. Lin TK, Zhong L, Santiago JL. Anti-Inflammatory and Skin Barrier Repair Effects of Topical Application of Some Plant Oils. International Journal of Molecular Sciences. siječanj 2018.;19(1):70.

116. Ganceviciene R, Liakou AI, Theodoridis A, Makrantonaki E, Zouboulis CC. Skin anti-aging strategies. Dermato-Endocrinology. 01. srpanj 2012.;4(3):308–19.

117. Facino RM, Carini M, Aldini G, Marinello C, Arlandini E, Franzoi L, i ostali. Direct characterization of caffeoyl esters with antihyaluronidase activity in crude extracts from Echinacea angustifolia roots by fast atom bombardment tandem mass spectrometry. Farmaco. listopad 1993.;48(10):1447–61.

118. Lengers I, Herrmann F, Le Borgne M, Jose J. Improved Surface Display of Human Hyal1 and Identification of Testosterone Propionate and Chicoric Acid as New Inhibitors. Pharmaceuticals. travanj 2020.;13(4):54.

119. Kawano Y, Patrulea V, Sublet E, Borchard G, Iyoda T, Kageyama R, i ostali. Wound Healing Promotion by Hyaluronic Acid: Effect of Molecular Weight on Gene Expression and In Vivo Wound Closure. Pharmaceuticals (Basel). 28. ožujak 2021.;14(4):301.

120. Ghuman S, Ncube B, Finnie JF, McGaw LJ, Mfotie Njoya E, Coopoosamy RM, i ostali. Antioxidant, anti-inflammatory and wound healing properties of medicinal plant extracts used to treat wounds and dermatological disorders. South African Journal of Botany. 01. studeni 2019.;126:232–40.

121. Ibezim EC, Momoh MA, Onyishi VI, Nwabunike I, Odimegwu DC, Ibezim NE, i ostali. Evaluating the ethyl-acetate fraction of crude methanol leaf extract of Ocimum gratissimum formulated as ointments for wound healing properties using the excision wound model. Journal of Pharmaceutical and Allied Sciences [Internet]. 22. ožujak 2018. [citirano 27. listopad 2024.];15(1). Dostupno na: https://www.ajol.info/index.php/jophas/article/view/168599

122. Bessada SMF, C. Alves R, P. P. Oliveira MB. Coffee Silverskin: A Review on Potential Cosmetic Applications. Cosmetics. ožujak 2018.;5(1):5.

123. Chandra S, Chatterjee P, Dey P, Bhattacharya S. Evaluation of *in vitro* anti-inflammatory activity of coffee against the denaturation of protein. Asian Pacific Journal of Tropical Biomedicine. 01. siječanj 2012.;2(1, Supplement):S178–80.

124. Penkova R, Goshev I, Gorinstein S, Nedkov P. Stability of Collagen During Denaturation. J Protein Chem. 01. svibanj 1999.;18(4):397–401.

125. Aruna V, Amruthavalli GV, Gayathri R. Use of cosmetic products for treating certain diseases—Know the science. Journal of Cosmetic Dermatology. 2019.;18(1):221–5.

6. ŽIVOTOPIS AUTORA

Petar Ciganović rođen je 1989. godine u Zagrebu, Hrvatska. Završetkom Privatne klasične gimnazije u Zagrebu, upisuje integrirani preddiplomski i diplomski studij farmacije na Farmaceutsko-biokemijskom fakultetu Sveučilišta u Zagrebu (FBF SuZ) kojeg završava 2015. godine. Pripravnički staž obavlja 2016. godine u javnoj ljekarni te polaže stručni ispit za magistre farmacije pri Ministarstvu zdravstva čime stječe licencu magistra farmacije za samostalan rad. Poslijediplomski doktorski studij „Farmaceutsko-biokemijske znanosti" upisuje 2016. godine. Od 2016. godine zaposlen je u tvrtki Providens d.o.o. gdje obnaša dužnost voditelja ključnih kupaca divizije bolničkih lijekova.

U razdoblju 2018. – 2021. bio je suradnik je na projektu Hrvatske zaklade za znanost „Ekstrakcija bioaktivnih biljnih sastavnica pomoću zelenih otapala – korak prema zelenim kozmeceuticima" akronima „CosmoGreen" (IP-2018-01-6504, voditeljica: prof.dr.sc. Marijana Zovko Končić).

Kao autor ili koautor objavio je pet znanstvenih radova i dva kongresna priopćenja. Član je Hrvatske ljekarničke komore, kao i Hrvatskog ljekarničkog društva. Oženjen je i otac je dvoje maloljetne djece.