Sveučilište u Zagrebu

Farmaceutsko-biokemijski fakultet

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ANTIMIKROBNI I PROTUUPALNI UČINCI VRSTE *ERODIUM CICUTARIUM* (L.) L'HÉR. EX AITON U UVJETIMA *IN VITRO*

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DOKTORSKI RAD

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IN VITRO ANTIMICROBIAL AND ANTI-INFLAMMATORY ACTIVITY OF *ERODIUM CICUTARIUM* (L.) L'HÉR. EX AITON

DOCTORAL DISSERTATION

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SAŽETAK

U okviru doktorskog rada provedena su ispitivanja antimikrobnog, protuupalnog te antioksidacijskog djelovanja ekstrakata vrste *Erodium cicutrium* (L.) L'Hér. ex Aiton (Geraniaceae) u uvjetima *in vitro.* Ispitivanja navedenih aktivnosti praćena su opsežnom fitokemijskom analizom ektrakata i eteričnog ulja. Antimikrobna aktivnost iscrpina poglavito je izražena na sojeve vrste *Staphylococcus aureus*, uključujući i kliničke MRSA sojeve. Uz pokazanu kinetiku bakteriostatskog i baktericidnog učinka u vremenu te anti-biofilm aktivnost, bioautografijom je uz semipreparativnu izolaciju i UHPLC-LTQ OrbiTrap MS^4 analizu, kao vrhunac ovog doktorskog rada, provedena izolacija i karakterizacija antimikrobno aktivnih frakcija. Dobiveni rezultati ukazuju na derivate galne kiseline kao spojeve poglavito odgovorne za opaženi antimikrobni učinak. Među njima je najzastupljeniji spoj galoil šikiminska kiselina. Poseban je naglasak i na 7 spojeva iz antimikrobno aktivnih frakcija, koji su po prvi puta unutar ovog doktorskog rada identificirani za vrstu *E. cicutarium*, rod *Erodium*, te porodicu Geraniaceae. To su narcisin, galoil pentozid izomer 1 i izomer 2, metilgaloil-kafeoil heksozid, metilgaloil-kumaroil heksozid, te izomer 1 i izomer 2 trimetilelagične kiseline. Protuupalni potencijal vrste *E. cicutarium* u uvjetima *in vitro* ponajprije je pokazan kroz rezultate izravne inhibicije enzima COX1 i COX2 i inhibiciju citokina IL-6, te inhibiciju lučenja dušik (II) oksida na modelu THP-1 stanica. Antioksidacijsku aktivnost u uvjetima *in vitro,* ispitanu metodama DPPH, ABTS, FRAP, CUPRAC te metodom izbjeljivanja *β*-karotena, pokazali su svi ispitani uzorci u svim provedenim pokusnim uvjetima. Detaljan polifenolni profil vrste *E. cicutarium* utvrđen je metodom UHPLC-LTQ OrbiTrap MS^4. Analizirane su metanolne i vodene iscrpine ove vrste s četiri lokaliteta na području Republike Hrvatske (Podvinje, Plitvice, Trešnjevka, Buzin), a identificirano je ukupno 85 sastavnica. Najzastupljeniji su derivati galne kiseline (24 sastavnice), derivati elagične kiseline – uključujući elagitanine (22 sastavnice), 19 flavonolnih glikozida, 8 derivata fenolnih kiselina, 7 derivata drugih hidroksibenzojevih kiselina, 3 flavonolna aglikona te 2 procijanidina. Nagasak je na 25 identificiranih sastavnica, koje do sada nisu pronađene unutar roda *Erodium*, te porodice Geraniaceae. Među ukupno 17 kvantificiranih sastavnica metodom UHPLC-QqQ-MS/MS, najzastupljeniji spoj je galna kiselina. GC-MS analizom eteričnog ulja ukupno je identificirano 50 spojeva, s ugljikovodicima kao glavnom skupinom spojeva (najzastupljeniji spoj je heksadekanoična kiselina).

Ključne riječi: *Erodium cicutarium*, antimikrobni učinak, MRSA, bioautografija, galoil šikiminska kiselina, protuupalno djelovanje, COX, IL-6, NO, polifenolni profil, galna kiselina, antioksidacijsko djelovanje.

SUMMARY

Introduction. *Erodium cicutarium* (L.) L'Hér. ex Aiton, Geraniaceae is known by the common names stork's bill, alfilaria, pin-clover, and redstem filaree. The genus *Erodium* is widespread and contains 74 species. There are 34 species of *Erodium* in the flora of Europe and, depending on references, 4 to 6 *Erodium* species can be found in Croatia. It is a native species to the Mediterranean and has fern-like, pinnate leaves, 10-30 cm long with a "green" grassy aroma, arising from a rosette. It is an annual or biennial with small, pink flowers with 5 petals. After flowering, the fruits, which consist of 5 mericarps joined together, grow in a large spine-like style. The *Erodium* dispersal mechanism contributed to the global distribution of the genus. Many *Erodium* species are known for their ethnopharmacological use in traditional medicine, and ethnopharmacological data suggest the use of *E. cicutarium* in dermatological diseases, hepatitis, nephritis and bleeding from wounds, stomach pain, constipation, dysentery, heart problems, diabetes, influenza, sores and rashes, and even as an abortifacient. *E. cicutarium* also has been used internally and externally for the treatment of dysentery, fever, wounds, and worm infections as a traditional medicine. It was employed as an antihemorrhagic drug in gynecology to stop uterine bleeding and as a general haemostipticum. In combination with other plants, it was used for preparing astringent and antiseptic teas used for stomatitis. Wide traditional use of *E. cicutarium* can be related to the so far known phytochemical composition. A literature search reveals that only few data regarding phytochemical analysis of different types of extracts exist, and they indicate the presence of hydroxycinnamic acids, flavonoids, tannins, saponins, sugars, amino acids and fatty acids. Brevifolin, gallic acid methyl ester, gallic acid, ellagic acid, protocatechuic acid, ellagitannin geraniin, as well as flavonol glycosides rutin and isoquercitrin, erodiol, gallotannins ((–) 3-*O*-galloylshikimic acid, methyl gallate 3-*O*-*β*-D-glucopyranoside), ellagitannins (didehydrogeraniin, corilagin, geraniin), flavonoid glycosides (quercetin 3-*O*-(6-*O*-galloyl)-*β*-D-galactopyranoside, rutin, hyperoside and isoquercitrin) are shown to be present as well. Despite broad literature based on essential oil composition of other species of the family Geraniaceae, only few investigations have focused on the chemical composition of *E. cicutarium* essential oil. Previous experimental activity studies showed data supporting antioxidant, antimicrobial, spasmogenic, interferonogenic, antiviral and antiproliferative activity.

Antimicrobial activity of naturally occurring compounds is constantly being investigated and a need for new approaches is higher than ever, particularly due to a global increase of antimicrobial resistance. The *in vitro* antimicrobial activity testing in this work was designed with the starting phase of whole and complex, but extensively profiled plant extracts and concluded with isolated and, again, extensively phytochemically characterized antimicrobial fractions. In literature seach so far, relatively scarce literature data describe *E. cicutarium* antimicrobial activity and only with classical screenings methods, but no detailed or more in depth studies were found. To the best of our knowledge, no data on time-dependence of the *E. cicutarium* activity, the anti-biofilm activity, or overall analysis of separated active segments of the complex *E. cicutarium* extracts are available in the literature. Next to exploring the first highly detailed polyhenolic profile of *E. cicutarium* extracts, this research was designed including further investigations of elemental composition of the herbal material. The determination of essential and non-essential elements in plants is significant for estimation of their phyto-nutritional value in general, and in consideration of possible adverse effects in case of intake. Since *E. cicutarium* is still a poorly investigated traditional medicinal plant, incorporating a study on the contents of macroelements and microelements could provide a new perspective in the deliberation of potential synergistic effect with phytochemical compounds present in the investigated plant material. Further steps were made to evaluate *in vitro* antioxidant, antimicrobila and anti-infammatory activity, along with cytotoxic effects.

Thus, the aim of the present work is to take a step towards improving the scientific knowledge on *E. cicutarium* and its bioactivity potential. Elucidating the main compounds associated with observed antibacterial activity is of particular interest. This especially retains to the obtained activity data on *Staphylococcus aureus* strains, including the methicillin resistant strains (MRSA), and potentially opens new doors for further in-depth antimicrobial activity research in the future.

Methods. Plant material was collected from 4 naturally occurring localities in Croatia during the blooming period (Trešnjevka, Buzin, Mukinje, and Podvinje). Phytochemical composition analysis was performed with several methods and strongly underlines the performed bioactivity testing, which respresents the core of this thesis.

In vitro activity testing. Antimicrobial experiments were performed with standard laboratory strains: a gram-positive strain – Staphylococcus aureus ATCC (American Type Culture Collection) 6538, a gram-negative strain – Pseudomonas aeruginosa ATCC 27853, and a yeast model – Candida albicans ATCC 90028, all from Collection of Microorganisms stock-cultures of the Department of Microbiology, Faculty of Pharmacy and Biochemistry University of Zagreb (MFBF). Additionally, clinical *S. aureus* strains were included in the experiments – a methicillin sensitive (MSSA MFBF 10663) and a methicillin resistant strain (MRSA MFBF 10679). The agar well diffusion assay was performed according to European Pharmacopoeia, and the serial microdilution broth assay was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, both with some modifications. The time-kill assay was performed on two *S. aureus* strains, MSSA MFBF 10663 and MRSA MFBF 10679, in order to invetigate the aspect of time-dependency of the antibacterial activity and distincion between bacteriostatic and bactericidal activity. Evaluation of anti–biofilm activity of investigated plant extracts was undertaken on *S. aureus* ATCC 6538 and MRSA MFBF 10679 biofilm formation with a crystal violet assay. In order to examine if antimicrobial activity occurs due to modulation of cell wall integrity, the *S. aureus* model was tested for protein and nucleic acid leackage under the affect from the investigated extracts. Anti-hemolytic activity testing of extracts was performed with sub-inhibitory concentrations (MIC/2 and MIC/4), again on the *S. aureus* model. An TLC-bioautography assay was performed with *E. cicutarium* extracts on the *S. aureus* model, in order to determine active fractions from the complex extracts, responsible for the observed antimicrobial activity. Inhibition zones were seen as clear spots around the active chromatogram zones with antibacterial activity against red background. After detecting active antimicrobial zones on the developed TLC chromatograms, their R_F values were determined. Active bands were marked, scraped and semipreparatively isolated. In the following step, in order to remove the liquid phase, drying in a stream of nitrogen was applied. Composition of the obtained sample *i.e.*, the bioactive bands, was analyzed using UHPLC-LTQ OrbiTrap MS^4 analysis. Anti-inflamatory activity of *E. cicutarium* extracts was investigated with *in vitro* methodology, including investigation of the effect on the enzyms lipoxygenase (LOX) and cyclooxinegase (COX1 and COX2) with comercially awailable kits (Lipoxygenase inhibitor screening assay kit and COX colorimetric inhibitor screening assay kit). For the investigation on potential anti-inflammatory activity of *E. cicutarium* extracts in regards to cytikine production,

the cell line THP-1 ATCC® TIB-202™ was stimulated with LPS, and cytikines were screened with the Multi-analyte elisarray kit (IL 1α, IL 1*β*, IL 2, IL 4, IL 10, IL 12, IL 17α, IFN-γ, GM-CSF), and selected cytokines were andditionally quantified with the Human IL-6, IL-8 and TNF alpha uncoated ELISA kits. Arginase activity and the production of nitric oxide (NO) were evaluated with the Arginase activity assay kit and Griess Reagent System DeadEnd™ colorimetric TUNEL system on the same cell line. Investigation on pottential inhibition of TNF-α induced intercellular adhesion molecule 1(ICAM-1) was performed on a human microvascular endothelial cell line (HMEC-1) with fluorescence-activated cell sorting. Antioxidant activity testing included five *in vitro* methods, namely Ferric reducing/antioxidant power assay (FRAP assay), DPPH radical scavenging assay, ABTS•+ radical scavenging assay, cupric ion reducing antioxidant capacity assay (CUPRAC) and *β*-carotene bleaching assay. In order to give an overview and simplify comparison, an overall antioxidant potency composite index (ACI) was calculated as well. To assess the cytotoxicity of investigated extracts, two experiments were performed – (1) testing against human erythrocytes, where hemolytic activity was evaluated by the percentage of hemolysis of a 4% suspension of human red blood cells *ex situ*, and (2) against human lymphocytes *ex situ*, evaluating their cell viability, apoptosis and necrosis with the dye exlusion method using DNA-binding dyes.

Phytochemical composition. Phytochemical analysis was performed with TLC and by analysing the content of total polyphenols (TP), total flavonoids (TF), tannins (T) and total phenolic acids (TPA). Analysis of phytochemical composition of essential oils was perfomed *via* GC-MS. Detailed phenolic profiling of water and methanolic extracts was undertaken with UHPLC-LTQ OrbiTrap MS^4 for identification of polyphenolic compounds, and polyphenolics quantification was performed using UHPLC-QqQ-MS/MS. The elemental composition of *E. cicutarium* from four native localities was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). The elemental profiling of dried pulverized herb material included the determination of concentrations of eighteen elements (Al,Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sr, and Zn).

Results. Hence the variety and volume of the performed research, obtained results can be devided in two domains – *in vitro* activity testing and phytochemical composition.

In vitro activity testing. The initial antimicrobial activity screening showed highest activity on the *S. aureus* model, which led to further tests including clinical isolates of methicillin sensitive (MSSA) and methicillin resistant (MRSA) strains. The sample from locality Podvinje stands out as the most active sample and was therefore chosen as experimental model for further in-depth antimicrobial examination of *E. cicutarium* extracts. The activity time frame for activity onset for the water extract is t_9-t_{24} hours, being bacteriostatic for MSSA MFBF 10663 and bactericidal for MRSA MFBF 10679 (t_{24} hours). The methanolic extract has faster and bactericidal activity (t_3 hours) for both bacterial strains. Due to a relatively fast manifestation of bactericidal activity against *S. aureus* in the case of methanolic extracts, the question of possible mechanism of action was directed towards the cell wall and/or membrane integrity disruption, but no protein leakage was detected from treated bacterial cells. Biofilm formation of *S. aureus* (MSSA) ATCC 6538 and MRSA MFBF 10679 was inhibited by both types od extracts. The lowest extract concentrations at which bacterial biofilm mass was inhibited during formation are lower for the MSSA than for MRSA strain. Also, the methanolic extract showed higher antibiofilm activity, than the water extract. Subinhibitory concentrations (c_1 = MIC/2; c_2 = MIC/4) of both extracts from locality Podvinje reduce hemolytic activity of the tested *S. aureus* strains (MSSA and MRSA), with higher concentrations exhibiting higher anti-hemolytic activity (with a maximum of close to 50%). After performing TLC chromatogram development of water and methanolic extracts, their bioautography with *S. aureus* ATCC 6538 and semi-preparative isolation of the observed active fractions, as well as identification with UHPLC-LTQ OrbiTrap MS^4 was performed. Galloyl-shikimic acid was identified as the most abundant compound of the active antimicrobial zones of both extracts. A total of 27 compounds were identified in the methanolic sample (the extract that showed bactericidal activity in the time-kill assay) and a total of 24 compounds were found in the active fraction of the water sample (the sample that showed bacteriostatic activity in the time-kill assay). The identified compounds can be classified into gallic acid derivatives and flavonol glycosides. Seven of the identified compounds are compounds detected for the first time not only for *E. cicutarium*, but in the Geraniaceae family as well. Among them, there is one flavonol glycoside, isorhamnetin 3-*O*-(6"-rhamnosyl)glucoside (narcissin), and several gallic acid derivatives: galloyl pentoside isomer 1 and isomer 2, methylgalloyl-caffeoyl hexoside, methylgalloyl-coumaroyl hexoside, trimethylellagic acid isomer 1 and isomer 2. When comparing the two samples, the three missing

compounds in the water sample are ellagic acid, galloyl hexoside isomer 2, and methylgalloyl hexoside isomer 2, all of them gallic acid derivatives. *In vitro* anti-inflammatory activity testing of *E. cicutarium* extracts showed inhibitroy activity on COX-1 and COX-2, cytikine IL-6 production, as well as production of NO, indicating their anti-inflammatory activity. TNF-α production was induced, indicating that *E. cicutarium* extracts do not have only anti-infammatory activity, but also show potential for immunomodulation. The inhibirtory affect on LOX activity was evident only partially for the water sample, while the affect on the activity of the enzyme arginase, production of IL-8 and TNF-α induced ICAM-1 production were not significantly affected by *E. cicutarium* extracts in this study. According to the overall antioxidant potency composite index (ACI), localities Podvinje and Plitvice show similar and higher antioxidant activity than other samples. Investigation of cytotxic activity showed cytotoxic effect on human lymphocytes *ex situ* was up to 20%, while no statistically significant cytotoxicity against human erythrocytes *ex situ* was observed.

Phytochemical composition. The contents of TP, TF, T, TPA in aerial parts of *E. cicutarium* significantly varied depending on the collection site, and were in the range of 4.78%-12.85% (TP), 3.23%-5.80% (T), 0.42%-1.09% (TF), and 1.08%-2.59% (TPA) of the dry weight of plant material. The sample from Plitvice stands out with the highest phytochemical content in comparison with other samples. Within this study, to our best knowledge, comprehensive detailed phenolic identification of *E. cicutarium* extracts was performed generally for the first time. In total, 85 phenolic compounds, mainly gallic acid derivatives (24 compounds), ellagic acid derivatives including ellagitannins (22 compounds), flavonol glycosides (19 compounds), hydroxycinnamic acid derivatives (8 compounds), other hydroxybenzoic acid derivatives (7 compounds), flavonol aglycones (3 compounds), and procyanidins (2 compounds) were identified. The presence of phenolics is similar among localities and only the methanolic extract from Trešnjevka showes somewhat less presence of phenolics. To stress out, very important results from this study show the presence of newly identified compounds – 25 new compounds were found, that have not been reported for the *Erodium* genera or the Geraniaceae family before (11 hydroxybenzoic acid derivatives – 8 of them are gallic acid derivatives, 7 ellagic acid derivatives, and 7 derivatives of flavonol glycosides). The quantitative analysis of phenolic compounds yielded a total of 17 phenolic constituents quantified in the methanolic and water extracts of *E. cicutarium* herb. Gallic acid stands out as the phenolic constituent with highest

quantified content among all samples (0.679 – 2.310 mg/g). Protocatechuic acid (0.224 – 0.463 mg/g), rutin (0.116 – 0.667 mg/g) and narcissin (0.104 – 0.449 mg/g) follow as the next three constituents with relatively high presence in investigated samples. The analysis of essential oils from 4 locations showed a total yield from 0.03% to 0.09%. In total, 50 compounds were identified (90.4% – 96.7% of the total oil) and classified on the basis of their chemical structures into 7 classes. Hydrocarbons (59.8% – 65.7%) were the main class of constituents, with hexadecanoic acid (41.5% – 49.6%), followed by octadecanoic acid (3.8% – 8.1%), octacosane (2.1% – 4.8%), and heptadecanoic acid (2.3% – 4.6%) as the major component. Carbonylic compounds were found in concentrations under 6% in all our *Erodium* oils, except the E-caryophyllene content of the Podvinje population with 14.5%. Obtained data showed similarity in composition to the major compounds of all investigated essential oils. The concentrations of eighteen elements were determined and the concentrations of the most abundant elements decreased as follows: Mg > Ca > K > S > P > Na. Concentrations of Al, B, Ba, Cd, Co, Cr, Cu, Fe, Li, Mn, Ni, Pb, Sr, Zn were below method detection limits. *In vitro* antioxidant activity testing showed that alls samples exhibited antioxidant activity in all performed assays (FRAP, DPPH, ABTS, CUPRAC and *β*-carotene bleaching assay).

Conclusions. The obtained results confirm that *E. cicutarium*, which has a profiled and rich phytochemical composition, is a plant species with both, ethnopharmacological value, and *in vitro* antimicrobial, antioxidative and anti-infammatory activity.

Antimicrobial activity was confirmed for *S. aureus* strains (MSSA and MRSA) for both types of extracts – water and methanol – in the agar well diffusion assay, the serial microdilution broth assay, and the "time-kill" assay (showing bactericidal and bacteriostatic activity). Biofilm formation inhibition and inhibition of bacterial hemolytic activity was confirmed for the *S. aureus* model as well. The obtained experimental data indicate that the observed antibacterial effects are not a result of bacterial cell wall disruption and, as such, open several new questions regarding the potential mechanism of antibacterial action. For the first time for *E. cicutarium*, in this work activity guided extracts' fractionation was performed using bioautography coupled with UHPLC-LTQ OrbiTrap MS^4, after which identification of gallic acid derivatives and flavonol glycosides were identified as the most important compounds for the observed *in vitro S. aureus* antimicrobial activity. Overall, galloyl-shikimic acid is the most abundant compound.

The galloyl-shikimic acid content in the active antimicrobial fractions in the methanolic sample (with bacericidal activity) was lower than in the water sample (with baceriostatic activity), which could potentially lead to a conclusion that the observed bactericidal activity of the methanolic sample is a result of synergistic activity of several compounds in the extract, as well as of their amount. It might be indicative to deliberate gallic acid derivatives as the most responsible compounds for the antimicrobial activity of *E. cicutarium* extracts. Gallic acid content was about 2.5 folds higher in the methanolic sample, than in the water sample, as well as protocatechuic acid and the methylgallate content were approximately 6 folds higher in the methanolic, than in the water sample. However, obtained results motivate further, more in-depth, investigation of antimicrobial activity and synergy with other phytochemical compounds. Considering that a few previous studies elucidated such direct relation between *E. cicutarium* (and *Erodium* species in general) with phytochemicals responsible for their *in vitro* antimicrobial activity, the obtained results (especially on MRSA), show high potential for further research. **T**his study, to our best knowledge, reveals results regarding antimicrobial activity of *E. cicutarium* from the phase of complex extracts to the phase of active fractions, generally for the first time. *E. cicutarium* extracts showed also *in vitro* inhibitroy activity directed towards COX1 and COX2 activity, as well as IL-6 inhibition and inhibition of production of NO (indicatig also possible wound healing potential). Since the TNF-α production was induced, *E. cicutarium* extracts show also potential for immunomodulation. The overall results of this study encourage further bioactivity testing regarding *E. cicutarium*, since it has been shown that this species is a valuable source of various phenolic compounds with substantial activity potential. Future investigation, especially *in vivo* studies, should also take potential cytotoxicity into further consideration.

Investigated extracts exhibited *in vitro* antioxidant activity according to all performed assays (FRAP, DPPH, ABTS, CUPRAC and *β*-carotene bleaching assay).

Extensive polyphenolic profiling yielded a total of 85 identified and 17 quantified phenolic constituents in methanolic and water extracts, representing the first highly detailed profile of such kind for EC. To stress out, 25 new compounds were found, that have not been reported for the *Erodium* genera or the Geraniaceae family before. Gallic acid stands out as the phenolic constituent with highest quantified content among all investigated extracts. Essential oil analysis identified a total of 50 compounds, with hexadecanoic acid as the main component. The

concentrations of eighteen elements were determined in plant material and the concentrations of the most abundant elements is decreasing as follows: Mg > Ca > K > S > P > Na.

Keywords: *Erodium cicutarium*, antimicrobial activity, MRSA, bioautography, galloyl-shikimic acid, anti-inflammatory activity, COX, IL-6, NO, plyphenolic proflie, gallic acid, antioxidative activity.