Isolation, solubilization and biological activity of isoflavonoids from Fabaceae family

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DOCTORAL DISSERTATION

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Farmaceutsko-biokemijski fakultet

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Izolacija, solubilizacija i biološka aktivnost izoflavona iz porodice Fabaceae

DOKTORSKI RAD

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“Dreams are great.

In fact, dreams are necessary in life or no one would ever go anywhere!

But a dream without a goal, and without action, has no opportunity to come true.”

Denzel Washington

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SUMMARY

Isoflavones are naturally occurring phenols distributed in many plants belonging to Fabaceaee family. They show a number of positive effects on various aspects of human health by displaying antioxidant, anti-inflammatory, estrogenic, chemopreventive and many other activities. Recently, it was found that isoflavones show significant potential in treatment of mucopolysaccharidoses (MPSs), inherited metabolic diseases characterized by accumulation of undegraded glycosaminoglycans (GAGs) in lysosomes. However, their therapeutic potential is significantly limited by relatively low solubility in water, poor bioavailability, as well as by rapid metabolic degradation of isoflavone aglycones. The aim of this doctoral thesis was to optimize the extraction of isoflavones and other phenolic constituents from *Medicago sativa* L. and *Lotus corniculatus* L. (Fabaceaee) by using hydroxypropyl-β-cyclodextrin and ultrasound-assisted extraction. Various phenolic classes (total phenolic compounds, total flavonoids and total phenolic acids) and antiradical activity were determined by using UV/Vis spectroscopy, while individual isoflavone aglycons were determined by HPLC-DAD. The influence of extraction parameters on concentration of target molecules was determined using response surface methodology. The procedures were performed according to the Box–Behnken design with three independent varaiables and the extraction efficiency compared to extraction using water/ethanol mixtures. The ability of the extracts to modulate accumulation of GAGs was evaluated on the fibroblasts obtained from patients suffering from MPS type III. In addition, the activity of cyclodextin-encapsulated isoflavones genistein and daidzein to inhibit GAGs synthesis in fibroblasts originating from patients with MPS type II and III was also evaluated. The results have shown that the developed technology using hydroxypropyl-*β*-cyclodextrin led to more selective flavonoid and isoflavone extraction from the selected plants than obtained either by the use of water or ethanol. The extracts reduced the levels of GAGs in fibroblasts of MPS III patients in a dose-dependent manner. Neither the extracts nor the cycledextrin-encapsulated isoflavones exerted any significant cytotoxic effect. Taken together, the results highlight the promising role of the cyclodextrin-mediated extraction of isoflavones and other phenols from *M. sativa* and *L. corniculatus*. Furthermore, the prepared extracts showed a potential to be considered as a part of the substrate reduction therapy of MPS III and other types of MPS diseases.

Keywords: isoflavones, cyclodextrines, mucopolysaccharidoses, *Medicago sativa*, *Lotus corniculatus*, ultrasound-assisted extraction

SAŽETAK

Uvod

Izoflavoni (IF) su biljni polifenoli rasprostranjeni u brojnim biljnim vrstama iz porodice Fabaceae. IF pokazuju brojne pozitivne učinke na ljudsko zdravlje poput antioksidativnog, protuupalnog, estrogenog, kempreventivnog te brojnih drugih učinaka. Novija istraživanja pokazuju da IF imaju značajan potencijal u liječenju mukopolisaharidoza (MPS), naslijednih metaboličkih bolesti kod kojih dolazi do akumulacije nerazgrđenih glikozaminoglikana (GAG) u lizosomima. Ipak, terapijski potencijal IF značajno je ograničen brzom metaboličkom razgradnjom te relativno niskom topljivošću njihovih aglikona. To značajno smanjuje njihovu oralnu bioraspoloživost te koncentraciju u ciljnim tkivima. Budući se stvaranjem inkluzijskih kompleksa brojnih lijekova s ciklodekstrinima (CD) značajno poboljšava njihova topljivost i bioraspoloživost, ovaj rad je usmjeren na optimizaciju ciklodekstrinske ekstrakcije izoflavona iz zeleni biljnih vrsta *Medicago sativa* L. i *Lotus corniculatus* L. (Fabaceae), njezinu usporedbu s ekstrakcijom pomoću vode i etanola te istraživanje utjecaja biljnih ekstrakata, izoliranih IF, te inkluzijskih kompleksa odabranih IF s različitim CD na oksidativni stres i sintezu glikozaminoglikana u staničnim linijama kultiviranih kožnih fibroblasta bolesnika s mukopolisaharidozom tipa II i III.

Metode

Prethodno osušen biljni materijal samljeven je u prah i prosijan kroz sito veličine 850 μm. Kako bi se učinkovito ekstrahirao sadržaj zeleni vrsta *M. sativa* i *L. corniculatus* korištena je ultrazvučna ekstrakcija. Istraživanje je provedeno korištenjem metodologije površine odgovora primjenom Box-Benkhen dizajna. Podaci su analizirani višestrukom regresijskom analizom i analizom varijance za odabrane modele. Vrijednosti $P < 0.05$ smatrane su statistički značajnima. Koncentracija etanola, temperatura i pH otapala bili su neovisne varijable kod pripreme vodeno-etanolnih ekstrakata dok su ciklodekstrinski ekstrakti pripremljeni su variranjem količine CD, temperature i snage ultrazvuka. Fitokemijska karakterizacija provedena je spektrofotometrijskim određivanjem sadržaja fenolnih sastavnica (ukupnih fenola, ukupnih flavonoida i ukupnih fenolnih kiselina) ekstrakta te analizom IF aglikona pomoću tekućinske kromatografije visoke djelotvornosti (HPLC). Antiradikalna aktivnost ekstrakata ispitana je UV/VIS spektrofotometrijom. Binarni ciklodekstrinski kompleksi daidzeina i genisteina s hidroksipropil-β-ciklodekstrinom (HPβCD) ili sulfobutileter-β-ciklodekstrinom (SBEβCD) u čvrstom stanju pripremljeni su mljevenjem ekvimolarnih

količina tvari u visokoenergetskim vibracijskim mikromlinovima te karakterizirani primjenom termogravimetrije (TGA), diferencijalne pretražne kalorimetrije (DSC) i difrakcijom rentgenskih zraka na prašku (XRPD). Njihove morfološke značajke ispitane su primjenom pretražnog elektronskog mikroskopa (SEM). Budući su izoflavoni slabo topljivi u vodi, ispitana je i topljivost dobivenih kompleksa. Inhibicija sinteze GAG ispitana je kulturi kožnih fibroblasta pacijanata koji pate od MPS III te MPS II. Nakon tretiranja stanica ispitivanim ekstraktima, IF aglikonima ili njihovim CD kompleksima mjerena je koncentracija glikozaminoglikana i DNA u supernatantu. Koncentracija glikozaminoglikana u stanicama određena je korištenjem Blyscan metode. Za mjerenje koncentracije DNA u supernatantu iz postupka izolacije GAG iz fibroblasta korištena je fluorimetrijska metoda s bojom Hoechst 33258. Procjena preživljavanja stanica određena pomoću 3-(4,5-dimetiltiazol-2-il)-2,5-difheniltetrazolium bromida (MTT) te mjerenjem aktivnosti laktat dehidrogenaze (LD) u mediju.

Rezultati

Etanolno-vodena ekstrakcija biljnog materijala vrsta *M. sativa* i *L. corniculatus* provedena je primjenom ultrazvuka prema Box-Behnkenovom dizajnu. Statistička analiza je pokazala da se kod obe zeleni odnos između neovisnih (koncentracija etanola, temperatura i pH otapala) i ovisnih varijabli (koncentracija različitih vrsta polifenola te antiradikalna aktivnost) mogao zadovoljavajuće opisati kvadratnim jednadžbama te da su dobiveni modeli bili statistički signifikantni ($P < 0,05$). Učinkovitost ekstrakcije fenolnih sastavnica iz vrste *M. sativa* ovisila je u prvom redu o koncentraciji etanola (ukupni fenoli i flavonoidi) i temperaturi (ukupne fenolne kiseline) dok je pH bio značajan faktor u interakciji s koncentracijom glicerola (ukupni fenoli i flavonoidi) te sa snagom ultrazvuka (ukupni flavonoidi i fenolne kiseline). Pri tome se 96%-tni etanol pokazao najboljim ekstrakcijskim otapalom. Ukupni fenoli su se najbolje ekstrahirali pri višoj (60°C), a flavonoidi i fenolne kiseline pri nižoj (23°C) temperaturi. Niži pH (5,5) je bio pogodniji za ekstrakciju ukupnih flavonoida dok je za ekstrakciju fenolnih kiselina bio bolji nešto viši pH (8,5). Korištenjem optimalnih uvjeta koncentracija ukupnih polifenola dosegnula je 6,63 mg/mL, ukupnih flavonoida 0,292 mg/mL dok je koncentracija ukupnih fenolnih kiselina iznosila 0.275 mg/mL. Najzastupljeniji izoflavoni pronađeni u vrsti *M. sativa* bili su glikozidi formononetina i genisteina. Temperatura je bila jedina neovisna varijabla koja je značajno utjecala na antiradikalnu aktivnost ekstrakata *M. sativa*, a činjenica da je najbolju antiradikalnu aktivnost pokazao ekstrakt priređen pri 60°C govori u prilog termostabilnosti sastavnica odgovornih za taj učinak. Najpovoljniji uvjeti za ekstrakciju

fenolnih sastavnica u vrsti *L. corniculatus* su se značajno razlikovali od onih zabilježenih za ekstrakciju *M. sativa*. Najvažnija varijabla za ekstrakciju odabranih vrsta fenola u *L. corniculatus* bila je koncenracija etanola dok utjecaj preostalih varijabli nije bio statistički značajan. Za ekstrakciju ukupnih polifenola i fenolnih kiselina iz vrste *L. corniculatus* najučinkovitije ekstrakcijsko sredstvo bio je 45% etanol, dok je viši udio etanola (95%) bio potreban za ekstrakciju ukupnih flavonoida. Od izoflavona je u zeleni *L. corniculatus* pronađen samo genistein (2.34-15.22 μg/mL) koji je u ovoj biljnoj vrsti zabilježen po prvi put. Najvažniji flavonoidi u vrsti *L. corniculatus* bili su derivati kvercetina i kemferola čija se koncentracija kretala u rasponu od 6,07 to 65,10 mg/mL odnosno od 6,69 do 92,75 mg/mL što znači da je primjena optimalnih ekstrakcijskih uvijeta dovela do desetorostrukog povećanja koncentracije ciljnih komponeti. Najpovoljnija koncentracija etanola za ekstrakciju navedenh flavonoida iznosila je 50%. Za razliku od koncentracije fenolnih sastavnica, na koje je značajno utjecala samo koncentracija etanola u ekstrakcijskom mediju, na antiradikalnu su aktivnost utjecale sve neovisne varijable. Najbolju sposobnost hvatanja slobodnih radikala pokazali su vodeni ekstrakti priređeni pri 60°C i neutralnom pH.

Kako bi se povećala topljivost izoflavona i ostalih fenolnih sastavnica biljnih vrsta *M. sativa* i *L. corniculatus* u vodi, a količina organskog otapala u ekstrakciji svela na minimum, priređeni su ekstrakti primjenom ultrazvučne ekstrakcije uz vodene otopine HPβCD kao ekstrakcijskog otapala. Nezavisne varijable uključene u ekstrakciju bile su koncentracija HPβCD, snaga ultrazvuka i duljina ekstrakcije. Iz zeleni vrste *M. sativa* dobiveno je, ovisno o uvjetima ekstrakcije, 1.98-2.47 mg/mL ukupnih polifenola te 0.41-0.75 mg/mL ukupnih flavonoida. Svi ekstrakti pokazali su značajnu sposobnost hvatanja slobodnih radikala. Korištenjem 20 mM otopine HPβCD, snage ultrazvuka od 432 W te ekstrakcijskim vremenom od 45 min, dobiven je ekstrakt s najvećim udjelom ukupnih polifenola te najboljom antiradikalnom aktivnosti, koji je korišten u testiranju na fibroblastima. Količine ukupnih polifenola, flavonoida i fenolnih kiselina u ekstraktima vrste *L. corniculatus* bile su 2,66 - 2,88 mg/mL, 0,80 - 1,46 mg/mL odnosno 0,033 - 0,04 mg/mL. Svi ekstrakti pokazali su značajnu antiradikalnu aktivnost. Optimirani ekstrakt korišten za tretiranje fibroblasta pripremljen je korištenjem 20 mM otopine HPβCD, te snage ultrazvuka od 648 W tijekom 45 minuta. Ekstrakti *M. sativa* i *L. corniculatus* priređeni korištenjem optimalnih uvjeta pokazali su značajno smanjenje količine GAG u fibroblastima pacijenata oboljelih od MPS III. Tako je ekstrakt vrste *M. sativa* u koncentracijama od 3 i 6 μg/mL pokazao smanjenje količine GAG 41.2% odnosno 51.1%.

Nadalje, količina GAG u stanicama tretiranim *L. corniculatus* esktraktom u koncentracijama 3 i 6 µg/mL bila je 33.97% tj. 50.08% niža nego u netretiranim fibroblastima.

Priređeni su kompleksi daidzeina i genisteina s HPβCD i SBEβCD te ispitana njihova sposobnost da inhibiraju sintezu GAG u fibroblastima koji potječu od bolesnika s MPS tipa II i III. Primjenom metoda termičke (TGA i DSC), spektralne (XRPD), i SEM analize potvrđeno je stvaranje kompleksa s povećanom topljivošću u odnosu na polazne spojeve. Oba izoflavona, kao i njihovi ciklodekstrinski kompleksi, smanjili su razinu GAG u fibroblastima bolesnika MPS II i MPS III sa 54,8 – 77,5%, na način ovisan o dozi, bez ikakvog značajnog citotoksičnog učinka. Stvaranje kompleksa s ciklodekstrinima nije promijenila intrinzično visok učinak daidzeina i genisteina na smanjenje razine GAG u tretiranim stanicama, što ukazuje na značajan potencijal priređenih kompleksa u terapiji MPS-a.

Zaključak

Metodologija površine odgovora temeljena na Box-Behnken dizajnu pokazala se dobrim pristupom za optimizaciju ekstrakcije izoflavona i drugih fenolnih sastavnica biljnih vrsta *M. sativa* i *L. corniculatus*. Velike razlike među koncentracijama fenolnih sastavnica u pojedinim ekstraktima ukazuju na važnost pravilnog odabira ekstrakcijskih uvjeta kako bi se, uz smanjenu količinu organskog otapala i biljnog materijala, postigao jednak ili bolji prinos ekstrakcije. Ultratzvučna ekstrakcija izoflavona i drugih fenolnih sastavnica ciklodekstrinima pokazala se kao učinkovita alternativa konvencionalnoj ekstrakciji pomoću etanola. Priređeni ekstrakti bili su bogati izoflavonima i drugim fenolnim sastavnicama, te su pokazali značajnu antiradikalnu aktivnost. Osim toga zabilježen je i značajan učinak ekstrakata vrsta *M. sativa i L. corniculatus* te izoliranih izoflavona i njihovih ciklodekstrinskih kompleksa na akumulaciju GAG u lizosomoma pacijenata oboljelih od MPS. Daljnja ispitivanja će pokazati koji je sastav ekstrakata izoflavona i u kojoj dozi optimalan za najučinkovitije smanjivanje razine GAG u fibroblastima. Stoga nije isključeno da bi priređeni ekstrakti i kompleksi mogli poslužiti kao osnova za razvoj lijekova i dodataka prehrani koji bi mogli poboljšati terapijske ishode kod pacjenata oboljelih od različitih tipova MPS.

Ključne riječi: izoflavoni, ciklodekstrini, mukopolisaharidoze, *Medicago sativa*, *Lotus corniculatus*, ultrazvučna ekstrakcija

1. INTRODUCTION

Isoflavones are naturally occurring dietary phytoestrogens distributed in leaves, seeds, bark, and flowers of some plants. They have shown a number of positive effects on various aspects of human health (Panche et al., 2016). However, although isoflavones display a broad spectrum of beneficial biological effects, their therapeutic potential is significantly limited by rapid metabolic degradation and relatively low solubility of isoflavone aglycones. This considerably reduces their oral bioavailability and concentration in target tissues (Stancanelli et al., 2007). Isoflavones are suitable guest molecules for inclusion complex formation with Cyclodextrins (CDs). The molecular encapsulation with CDs results in a solid, molecularly dispersed product and in a significantly improved aqueous solubility of isoflavones. In this form, they are protected from degradation and have better bioavailability so that they can be used for therapeutic purposes (Stancanelli et al, 2007). Progress in understanding the pathophysiology of more than 1100 inborn errors of metabolism (IEM) has led to the discovery of several new therapies that have made it possible to attenuate the severity of the clinical manifestations associated with some IEMs (Alfadhel et al., 2013).

Therefore the main aim of our research was to investigate effects of plant extracts, pure isoflavones and inclusion complexes of selected isoflavonoids with different CDs on oxidative stress and correction of glycosaminoglycan (GAGs) cell storage.

1.1. Polyphenols

Polyphenols are aromatic compounds with several hydroxyl substituents and widely distributed in nature. They are seldom found in free form but rather in esterified or conjugated form. Polyphenols may be classified into different groups according to the number of benzene rings and the structural links between them. Regarding the structural characteristics, we can distinguish among phenolic acids, flavonoids, stilbenes and lignans (Manach et al., 2004).

Phenolic acids are aromatic secondary plant metabolites widespread throughout the entire plant kingdom. The term generally describes phenols that have one carboxyl functional group (e.g. salicylic acid). Naturally occurring phenolic acids contain two different constitutive carbon skeletons: hydroxycinnamic and hydroxybenzoic structure. C6-C1 unit represents the basic structure of hydroxybenzoic acids, and C6-C3 of hydroxycinnamic acids. Although the basic skeleton remains the same, the difference lays in the number and position of hydroxyl groups on the aromatic ring. In many cases, aldehyde analogs are also classified into the group of

phenolic acids (e.g. vanillin). Caffeic, *p*-coumaric, vanillic, ferulic and protocatechuic acid are present in almost all plants (Ghasemzadeh and Ghasemzadeh, 2011).

Flavonoids are a group of polyphenols with the C6-C3-C6 structure (two benzene nuclei linked by a propane chain), and they are known to be synthesized by plants in response to microbial infection (Dixon et al., 1990). In most flavonoids, the central fragment is joined to oxygen into a heterocyclic chain. The variety of their structures depends on degree of oxidation of the heterocyclic ring and the number and position of hydroxyl groups on benzene nuclei. Flavonoids may be divided into different categories like flavones, flavonols, flavanols, isoflavones, anthocyanins. Structural variety of flavonoids is the result of numerous modifications of the basic skeletal structure. This group of polyphenols represents one of the most studied classes of phyto compounds. The studies carried out so far show their role in the prevention of degenerative diseases including cancers, cardiovascular and neurodegenerative diseases (Tsao, 2010).

Isoflavones are a subgroup of flavonoids, derivatives of 3-phenyl chromone that occur in a plant by biosynthesis of calconaringenin and isoliquiritigenin. Most isoflavone aglycones are derivatives of genistein (5,7,4-trihydroxyisoflavone, GEN) and daidzein (7,4-dihydroxyisoflavone, DAD), and are differentiated mainly according to the number and position of hydroxyl groups on aromatic rings, and according to the number and type of bonded sugars. Isoflavone concentration in individual plants depends on the climate where they grow, on the stress to which a plant is exposed and, to a large extent, also on the post-harvest processing (Križova, 2019). Most isoflavones have been isolated from species of the Fabaceae family, one of the largest and economically most important plant families (Veitch, 2013).

Phenolic compounds in plants display a variety of biological activities, including antioxidant, antiinflammatory, antimutagenic and antimicrobial effects (Martins et al., 2011). There is a large number of epidemiological studies that refer to the protective effect of phenolic compounds on chronic diseases such as cancer, inflammatory diseases, and bacterial disorders, as well as on reducing diabetes, cardiovascular and neurodegenerative diseases (Messina and Wood, 2008; Andres-Lacueva et al., 2011). These effects of phenolic compounds result from the activity of functional hydroxyl groups that capture free radicals and/or chelate metal ions. The chelation of metals could be crucial in preventing the generation of free radicals which damage target biomolecules. Isoflavones are of particular interest among phenolic compounds as they engage in a wide range of hormonal and nonhormonal activities related to human health. They show a number of positive effects on various aspects of human health by displaying

antioxidant, anti-inflammatory, estrogenic, chemopreventive and many other activities. Epidemiological data suggest that regular intake of isoflavones from food reduces the incidence of estrogen-dependent and aging-associated disorders, such as menopausal symptoms, osteoporosis, cardiovascular diseases and cancer (Mayo et al., 2019). Recently, it was found that isoflavones show significant potential in treatment of mucopolysaccharidoses (MPSs), inherited metabolic diseases characterized by accumulation of undegraded GAGs in lysosomes (Jakóbkiewicz-Banecka et al., 2009).

1.2. Selected species of the Fabaceae family

Fabaceae is a family from the class of dicotyledonous plants with more than 300 genera and approximately 10000 species distributed worldwide. Important species within the Fabaceae family are broad beans (*Vicia faba* L.), peas (*Pisum sativum* L.), soybeans (*Glycine max* L., *Soja hispida* L.), liquorice (*Glycyrrhiza glabra* L., bird's-foot trefoil (*Lotus corniculatus* L.), lucerne (*Medicago sativa* L.) and many others. It is precisely due to their wide distribution that the species from the genus Fabaceae are so much used in both food and pharmaceutical industry.

1.2.1. *Medicago sativa*

M. sativa (alfalfa or lucerne) is a perennial herbaceous plant species, cultivated throughout the world as a fodder plant. It has long been used as traditional herbal medicine in Europe, Asia and America for treatment of a variety of ailments. In Croatia, about 4% of the arable land is covered with lucerne (Štos, 2011).

The plant is upright, widely spread, and has a shrubby growth up to 100 cm in height. The root is well developed and very deep, often about five meters, and there are data confirming that it can be even deeper (up to 15 meters). The leaves are alternate, tripartite with elongated egg-like, serrated and weakly hairy blades which have small prickles on the top. The flowers are bisexual, irregular, have short stems, and are borne in rather long racemes. They bloom 10-14 days from June to September. The perianth is double, consisting of the calyx and the corolla. The calyx is bell-shaped with five teeth, the corolla resembles a butterfly. The pistil has an ovary and carries a number of ovules; there are ten stamens, nine of which are fused into a tube, and one is free. The fruit is a spirally bent pod containing 5-10 kidney-shaped, small yellow-brown seeds (Lesins & Lesins, 1979).

Due to the presence of a wide range of chemical compounds, i.e. saponins, flavonoids, phytoestrogens, coumarins, alkaloids, amino acids, phytosterols, vitamins, digestive enzymes and terpenes, the plant is valuable source of novel bioactive agents. So far, its significant hypocholesterolemic, antiatherosclerotic and neuroprotective activity has been clinically confirmed (Khaleel et al, 2005). It has been suggested that *M. sativa* may have a slight central antidopaminergic action without any side effects (De Leo, 1998). The plant has been shown to affect gram-positive bacteria and has some antifungal activity (Avato et al., 2006). In addition, several pharmacological reports have been published on the use of *M. sativa* in treatment of heart disease, stroke, cancer, diabetes and menopausal symptoms in women (Bora and Sharma, 2011).

1.2.2. *Lotus corniculatus*

L. corniculatus L., (bird's foot trefoil) is an herbaceous perennial from the Fabaceae family. It grows as a shrub with upright, or often slanted, weakly branched stalks up to 30 cm in height. The stalk is woody at the base. The root is spindle-shaped. Leaves are alternate and situated on short petioles, composed of five blades, and the two lower blades are close to the stalk. Flowers are on the stems about two centimeters long, they are bisexual, yellow, with bell-shaped calyx, and 4-6 of them are gathered in an umbellate inflorescence. They have intense fragrance and bloom from April to October. The fruit is a somewhat bent, reddish-brown pod, 2-3 cm long. It contains 6-12 radiant dark seeds that are released by sudden breaking of the pod along the two seams after one or two weeks of maturation when they change from green into brown color (Frame et al., 1998).

L. corniculatus L., Fabaceae (birdsfoot trefoil), is a perennial plant species with a wide distribution throughout the world. The plant is used in traditional medicine as febrifuge, dermatic, (Nikolić, 2008) anti-inflammatory, antispasmodic, cardiotonic, carminative, hypoglycemic, restorative, sedative, tonic and vermifuge agent (Hedqvist et al., 2000). *In vitro* studies have demonstrated good anti-inflammatory (Koelzer, et al., 2009) and antimicrobial activity (Dalmarco et. al. 2010) of *L. corniculatus*, as well as its lectin-induced apoptotic activity in various human tumor cell lines (Rafiq, et. al. 2013). Bioactive constituents in *L. corniculatus* include lipid antioxidants such as α-tocopherol, β-carotene, lutein (Elgersma et. al. 2013) and oleanolic acid (Dalmarco et. al. 2010). However, the most notable among numerous constituents of *L. corniculatus* are various phenolic compounds such as flavonoids, predominantly derivatives of kaempferol and quercetin (Reynaud et al. 1982, Dalmarco et. al.

2010) and condensed tannins (Ramírez-Restrepo et. al. 2006). Furthermore, the leaves are rich in vitamin C and proteins. The leaves of this plant are also used in human diet (Nikolić, 2008). Even though Fresh bird's-foot trefoil contains small amounts of cyanogenic glycosides (Scriber, 1978) the toxicity of this plant has not been reported due to a relatively low dose and fast metabolism of cyanides in normal concentrations, as well as the impairment of cyanide formation during at high temperatures. Thus, infusion of *L. corniculatus* has been used as a sedative in traditional medicine. It is also used in agriculture as a forage plant, grown for pasture, hay, and silage. It may be used as an alternative to alfalfa in poor soils (Barry et al. 1999).

1.3. Cyclodextrins

CDs are a group of structurally related oligosaccharides able to improve solubility, chemical stability, safety and bioavailability of different compounds through inclusion complex formation. CDs have an internal non-polar hole and hydroxyl groups placed on the molecule surface. They can form inclusion complexes with poorly water-soluble molecules (such as polyphenols), improving their solubility by hydrophobic interactions between guest molecules and the walls of CD cavity (Buschmann & Schollmeyer, 2002). However, other forces, such as van der Walls and dipole–dipole interactions, may be involved in the binding of the guest. CDs can also protect bioactive molecules from harmful environmental conditions (temperature, pH, light) thereby enhancing their shelf-life, bioavailability and antioxidant activity (Fang & Bhandari, 2010). In addition, CDs can modify the properties of the encapsulated molecule reducing its toxic side-effects or modulating its transport to the target site (Aqil et al., 2013). Due to the above-mentioned properties, CD complexation of drugs and other bioactive compounds have been increasingly applied in pharmaceutical industry.

Despite the number of factors and different forces involved in complexation with CDs, the production of complexes is a rather simple process. There are different methods to prepare CD complexes. The first is the method of preparation in a solution where the molecule to be encapsulated and CDs are in water or a mixture of water and organic solvent, and conditions for complexation are created by adjusting pH and the temperature of the solution. After that, the solvent is removed by a suitable technique, like freze-drying, spray-drying or evaporation under reduced preassure. Another method involves a semi-solid state where the mixture of solid CDs and the molecule to be encapsulated is moistened with a small amount of water or a mixture of water and organic solvent until a homogeneous paste is obtained from which solvents are

removed. The drawbacks of the solvent-based complexation methods are related to possible hydrolysis of sensitive molecules and complex removal of the organic solvent from the final product. Namely, the organic solvent residuals in the final product can cause toxic effects. The third method of molecular encapsulation in CDs is in solid state by applying different technologies. In some of them, as in microwave irradiation or "sealed heating" method, the physical mixture of the molecule to be encapsulated and CD is subjected to irradiation or high temperature which may cause its degradation. In addition to that, the grinding method has been increasingly used recently as a simple, fast and highly efficient method for preparation of CD inclusion complexes without the use of organic solvents and the risk of molecule degradation under harsh conditions (Mura, 2015).

Chemical modification of parent CDs consisting of 6, 7, or 8 glucopyranose units (αCD, βCD, and γCD) resulted in numerous derivatives with improved solubility and complexation efficiency. In general, CDs are biocompatible and nontoxic when applied orally, while some derivatives, such as hydroxypropyl-β-CD (HPβCD), sulfobuthyl-β-CD (SBEβCD), and γCD are also suitable for parenteral application (Duchêne and Bochot, 2016).

Past studies of the interaction between CD and isoflavonoids have mainly been focused on investigating the structure and stability of individual isoflavones with natural and chemically modified CDs. Thus, complexes were described of DAD, GEN and puerarin with βCD and hydroxypropyl derivatives of βCD (Xiao et al., 2012; Stancanelli et al., 2007; Yatsu et al., 2013), and their molecular mechanics was studied (Zhang et al., 2013). In addition, βCD complexation increases the oral bioavailability of the soy isoflavones by 126% (Lee et al., 2007), and formation of inclusion complexes was confirmed to significantly enhance antioxidative potential of various natural antixodants, like hydroxytyrosol (Lopez-Garcia et al., 2010).

CDs are known encapsulators of hydrophobic molecules widely used in extraction without organic solvents (Diamanti et al., 2017). A definition of ‘‘green” chemistry is the invention, design and application of chemical products and processes to reduce or to eliminate the use and generation of hazardous substances (Chemat et al., 2012). For example, the selective inclusion properties of CDs toward phenolic stilbenes isolated from the roots of *P. cuspidatum* L. yielded a much cleaner analytical extract profile if compared with that obtained with methanol. Thanks to polyphenol encapsulation, this extract showed very good water dispersibility, higher stability and antioxidant power (Mantegna et al., 2012).

Recent publications have indicated that CDs could also exhibit some pharmacological effects, such as antitumor effect against primary effusion lymphoma *in vitro* and *in vivo* (Gotoh et al., 2014). Studies on animal models have shown that βCD are good candidates for atherosclerosis treatment. Actually, they reduce the concentration of cholesterol, low-density lipoprotein, very low-density lipoprotein and triglycerides in blood and, in interaction with macrophages and lymphocytes, act on inflammatory processes which are triggers for formation of atherosclerotic plaques (Zimmer et al., 2016). Furthermore, 2-hydroxyalkylated β-CD showed an attenuating potential against Niemann-Pick Type C disease (NPC). This is a rare autosomal recessive lysosomal storage disease characterized by accumulation of esterified cholesterol and other lipids in lysosomes, predominantly in liver and central nervous systems. The clinical spectrum of NPC disease ranges from a neonatal rapidly progressive fatal disorder to an adult-onset chronic neurodegenerative disease. In disease animal models, HPβCD appears to reduce the cholesterol and lipid accumulation and prolongs survival; it has been assumed to be beneficial probably due to the ability to modify the internal environment of the endosomal/lysosomal compartment (Kondo et al., 2016). This ability has encouraged studies of potential utilization of HPβCD differing degrees of hydroxypropylation of the CD ring for treatment of NPC patients (VTS-270 and Trappsol Cyclo are HPβCD products under investigation). Results of these studies should reveal the most effective, concentrations of CD forms showing acceptable risk to benefit ratio in treated patients (Matsuo et al., 2014). In addition, interaction of CDs with plasma membranes and extraction of different lipids are highly relevant at the level of the blood brain barrier since central nervous system (CNS) is affected in most neurodegenerative disorders

GM1 gangliosidosis is another incurable lysosomal storage disease where a limited *in vitro* study suggested that CDs may have the potential as a therapeutic drug (Motoyama et al., 2015). In affected patients, GM1 gangliosides accumulate in many tissues and organs, particularly in neurons. This type of gangliosidosis is clinically characterized by a wide range of variable neurovisceral, ophthalmological and dysmorphic features. Although CDs can very effectively extract cholesterol and various phospholipid compounds from cell membranes, it appears that CDs impair the GM1-ganglioside levels in a different, still unknown manner (Maeda et al., 2015).

Neurodegenerative Alzheimer's disease is characterized by β-amyloid aggregation/ deposition in neurons. It has been shown that HPβCD could significantly improve memory deficits in mouse models and reduce β-amyloid peptide neuronal deposition. Pathophysiological

mechanism has not been elucidated yet, but authors presume that HPβCD acts on β-amyloid peptide and reduces its accumulation (Ren et al., 2016).

Apart from this potential therapeutic application, CDs may be part of innovative pharmacological approaches and find their application also in treatment of other neurodegenerative disorders closely linked to alterations of cholesterol metabolism in the brain, including Parkinson's disease and Huntington's disease (Vecsernyes et. al., 2014).

Results of the CD application for therapeutic purposes have so far pointed to their great potential, particularly in treatment of degenerative brain diseases, but there is still need for a extensive research to confirm their safety in clinical use.

1.4. Response surface methodology

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for modeling and optimization of complex processes. It enables creation of an empirical model by development of an adequate functional relationship between a response(s) of interest and a number of associated input variables. The use of RSM effectively reduces the number of experimental trials needed to evaluate multiple parameters and their interactions. Therefore, it is widely used in optimizing the extraction of polyphenols (Bouras et al., 2015; Liu et al., 2013), alkaloids (Wu et al., 2015) and other biologically active compounds from herbal material. Box–Behnken is one of the most commonly used RSM designs which requires fewer runs in a 3-factor experimental design than all other RSM designs, thereby sharply reducing the number of experimental sets without decreasing the accuracy of the optimization compared with traditional factorial design methods.

1.5. Lysosomal storage diseases

Lysosomes, described for the first time by De Duve in 1955 (De Duve et al., 1955), are ubiquitous intracellular organelles whose primary function is the degradation and recycling of macromolecules from secretory, endocytic, autophagic and phagocytic membrane-trafficking pathways (Luzio et al., 2007). The lysosome is the cell's main digestive compartment for the degradation of a wide variety of structurally diverse substances, such as proteins, GAGs, nucleic acids, oligosaccharides, and complex lipids. They are part of the highly dynamic endosome/lysosome system which aids the internalization, recycling, transport and breakdown of cellular and extracellular components (Lüllmann-Rauch, 2005).

As described above, numerous cellular functions depend on normal lysosomal function. Impaired lysosomal function can lead to lysosomal storage disorders (LSDs), a diverse group of devastating inherited metabolic disorders (Ferreira and Gahl, 2017). Each LSD results from a mutation in a protein critical for lysosomal function, most commonly mutations in genes encoding for lysosomal hydrolases, but also in genes encoding for lysosomal membrane proteins, proteins involved in post-translational modification and trafficking of lysosomal enzymes or non-lysosomal proteins involved in lysosomal biogenesis (Schultz et al., 2011). All these diseases share as a common biochemical characteristic the progressive accumulation of undegraded substrates within the lysosome which ultimately leads to cellular dysfunction and death. Recently there has been increasing evidence of the correlation of LSDs with some of the most frequent, complex disorders such as cancer and Parkinson's disease (Coutinho and Alves, 2016).

1.5.1. Mucopolysaccharidoses

Mucopolysaccharidoses (MPS) are a group of LSDs caused by the deficiency of enzymes necessary for the degradation of GAGs that leads to intralysosomal accumulation of GAGs (Suarez-Guerrero et al., 2016). GAGs (formerly called mucopolysaccharides) are long, unbranched polysaccharides consisting of a repeating disaccharide unit. GAG chains covalently bound to proteins form proteoglycans which are ubiquitous both in the cell membrane and in the extracellular matrix and are an important component of connective tissues due to their water-binding capacity (Valstar et al., 2008).

Depending on the function they fulfil, GAGs form a chain consisting of several to a few hundreds of disaccharide units. The first step in GAG synthesis is covalent addition of a tetrasaccharide chain onto a serine residue of the core protein. Each further step is catalyzed by specific enzymes whose composition diverges towards the formation of specific GAGs: heparan sulfate, dermatan sulfate, chondroitin sulfate, etc. They are divided in two groups: nonsulfated GAGs (hyaluronic acid or hyaluronate) and sulfated GAGs (chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin) (Kircher et al., 2018). Degradation of GAGs that form sulfated polysaccharide chains occurs in lysosomes via lysosomal enzymes. Four different pathways of lysosomal degradation of GAGs are distinguished depending on the molecule to be degraded: dermatan sulfate, heparan sulfate, keratan sulfate, and chondroitin sulfate. Mutations in each gene of the involved enzymes have been reported and result in different MPS types. Faulty degradation of any GAG causes its

accumulation over time and consequently tissue and organ damage –actually, the clinical picture of MPS (Lacombe D, Germain DP, 2014).

1.5.2. Treatment of lysosomal storage diseases

Over the past decades, the number of treatments available for patients with LSDs has rapidly increased. Currently, many different therapies are available besides supportive care: hematopoietic stem cell transplantation, enzyme replacement therapy (ERT), substrate reduction therapy (SRT), chaperone, gene therapy and others (Platt, 2018). The choice of therapy depends on the type of MPS, and on age and general status of a patient.

ERT has so far been commercially available for MPS type I, II, IVA, VI and VII. The main problems associated with ERT are the short half-life of enzyme, low efficacy in some tissues, and cellular and humoral responses to enzymes. LSDs affecting the brain present formidable difficulties in terms of treatment because existing approaches are only poorly efficient with regard to CNS manifestations. An important obstacle comes from the blood brain barrier that restricts entry of therapeutic molecules to the CNS (Tsai, 2005).

1.5.3. Isoflavones and mucopolysaccharidoses

In addition to numerous biological effects of isoflavones stated in section 1.2.2, recent studies have indicated their use in treating MPS. GEN effectively reduces GAG synthesis in cells of patients affected by MPS (Piotrowska et al., 2006; Wegrzyn et al., 2010; Banecka-Majkutewicz et al., 2012). It is already known that the addition of epidermal growth factor (EGF) to the cell culture leads to increased synthesis of GAGs (Pisano & Greene, 1987). GEN activity is directed to inhibiting the EGF receptor (Akiyama et al., 1987), which reduces the synthesis and consequent GAG accumulation in lysosomes; it is the so-called „gene expression-targeted isoflavone therapy“(GET IT). Besides the actual GAG accumulation in cells, secondary effects significantly contribute to the disease pathophysiology. The accumulated GAGs, particularly heparan sulfate, stimulate inflammatory processes in cells and increase cytokine and chemokine secretion (Taylor & Gallo, 2006). GEN may also play a significant role in reducing the secondary factors of MPS: accumulation of gangliosides (Malinowska et al., 2010), protection of neurons against neurotoxicity (Bang et al., 2004), prevention of neuroinflammatory conditions (Valles et al., 2010), and inhibition of apoptosis in neuronal cells (Kajta et al., 2007). However, it is not only GEN that is efficient in reducing GAG synthesis, it was shown that DAD also has a considerable potential to inhibit GAG synthesis (Kloska et al., 2011).

Secondary accumulation of particular classes of compounds may explain some features of the disease pathology and some clinical manifestations, such as activation of inflammation.

Besides impairment of GAG synthesis through EGF pathway, GEN could contribute to activation of lysosomal biogenesis through increasing the levels of transcription factor EB (TFEB) and stimulation of its translocation to the nucleus. TFEB is a master regulator for transcription of genes involved not only in lysosome biogenesis but also in autophagy and mitochondrial biogenesis (Rao et al., 2017). It seems that GEN affects the GAG biosynthesis in a cell type- and heparan sulfate/chondroitin sulfate-dependent manner (Lan et al., 2018). This is consistent with the reported beneficial effects of GEN in treating MPS type III by inhibiting heparan sulfate biosynthesis and enhancing lysosomal biogenesis (Wegrzyn et al., 2010, Kloska et al., 2011, Piotrowska et al., 2008 , Banecka-Majkutewicz et al., 2012, Malinowska et al., 2009, Arfi et al., 2010). Beneficial effect of GEN on heparan sulfate reduction has been shown also on fibroblast cell lines of patients with mucolipidosis type II. In this metabolic disorder, cholesterol, phospholipids, saccharides and proteins accumulate in lysosomes in addition to GAGs (Takanobu et al., 2012).

Along with all of the above, it is important to state that isoflavones, unlike enzyme replacement therapy, may still pass through the blood brain barrier and thus affect also the symptoms of MPS in the CNS (Malinowska et al., 2010).

1.6. Oxidative stress in lysosomal storage diseases

An unavoidable consequence of aerobic metabolism is production of reactive oxygen species ROS. They include free radicals like the superoxide anion ($O_2\bullet^-$), hydroxyl radical (•OH), and non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). When the level of ROS overwhelms defense mechanisms, a cell is said to be in the condition of 'oxidative stress'. Oxidative stress is caused by impaired balance between production and elimination of ROS which then become precursors for the development of a number of neurodegenerative, inflammatory, vascular and neoplastic disorders (Halliwell, 1994; Reznick & Packer, 1993; Ben-Menachem et al., 2000). The consequence of such imbalance is even more pronounced in lysosomal storage diseases due to overloading of lysosomes which are particularly sensitive to oxidative stress. Increased ROS production may cause lipid peroxidation that results in impaired lysosomal membrane and consequent release of the content of lysosomes into the cytoplasm (Terman et al., 2006). A study carried out on mice with MPS type 1 showed changes in oxidative markers and antioxidative enzymes in the cerebellum and peripheral organs

(Reolon et al., 2009). Another study has shown a high level of lipid peroxidation in patients with MPS type 1, and also the effect of enzyme replacement therapy on the activity of antioxidative enzymes. All this points to the significant role that oxidative stress plays in the pathophysiology of MPS type 1 (Pereira et al., 2007). Oxidative imbalance was also found in animal models of MPS IVA (Donida, 2015), MPS IIIA (Arifi, 2011) and MPS IIIB (Villani, 2007). It is interesting to note that plants have an ability to biosynthesize a wide range of non-enzymatic antioxidants capable of attenuating oxidative damage induced by ROS making plants and products derived thereof potentially valuable agents for different therapeutic applications including MPS (Kasote, 2015).

2. Multi-response optimization of ultrasound-assisted extraction of bioactive components from *Medicago sativa* L.

3. Therapeutic potential of hydroxypropyl-β-cyclodextrin-based extract of *Medicago sativa* in the treatment of mucopolysaccharidoses

4. Cyclodextrin encapsulation of daidzein and genistein by grinding: implication on the glycosaminoglycan accumulation in mucopolysaccharidosis type II and III fibroblasts

5. Development of cyclodextrin-based extract of *Lotus corniculatus* as a potential substrate reduction therapy in mucopolysaccharidoses type III

6. Optimization of Ultrasound-Assisted Extraction of Phenolic Antioxidants from *Lotus corniculatus*

7. GENERAL DISCUSSION

Advances in modern medicine have allowed detection of an increasing number of metabolic disorders associated with various diseases. This detection was during past years further facilitated particularly by different methods of new generation sequencing. Adequate diagnostics and treatment of these diseases remains a great challenge for all who take part in the processes of patient care. Unfortunately, metabolic disorders often involve rare diseases with no accompanying commercial interest in developing adequate specific therapy. This makes the life of those affected by metabolic disorders even more difficult, along with all other social problems of such patients and their families. Available drugs often have limited activity due to highly complex clinical data and insufficient knowledge of the pathophysiological background of a disorder. They are directed toward treatment of complications and are not specific for the underlying abnormality. They improve the quality of life for patients, but they cannot prevent the inevitable decline in function. The same is true for some types of MPS where specific therapies are not available, such as ERT or hematopoietic cell transplantation which may alter the natural history of these disorders. In such patients, it is of utmost importance to provide supportive and symptomatic management that also includes reduced concentration of GAGs as basic pathological substrate in lysosomes. This is one form of SRT that may significantly contribute to success of therapy.

Isoflavones belong to natural products for which new biological activities are being detected almost on a daily basis, including also their efficacy in reducing GAG synthesis (Kloska et al., 2011). Like other polyphenols, GEN and DAD also display antioxidative activity and may reduce the rate of lipid peroxidation in cells by reacting with metabolically produced free radicals (Patel et al., 2001).

One of the main reasons for this study was the fact that these compounds can modify the expression of genes encoding for enzymes required for metabolism of sphingolipids and MPS in human dermal fibroblasts. Therefore, natural flavonoids, such as DAD, GEN and kaempferol, can be considered as potential drug candidates for treatment of MPS and other LSD disorders that involve an error in sphingolipid metabolism (Moskot et al., 2015)

Complementary medicine offers many opportunities to a wide range of pathologies. Natural phenols are important functional compounds distributed in plants, with their potential beneficial effects on human health that may be utilized in various aspects of therapy (Grosso et al., 2017). This fact has encouraged an ever-growing scientific interest in all aspects of their optimal extraction and pharmacological application of extracts from plant species, and also in in vitro and in vivo verification of efficacy of such preparations (Ko, 2014).

If isoflavones –and other biologically active substances from plants– are to be prepared in a form suitable for production of pharmaceutical formulations, it is necessary to first extract them from herbal material. Extraction is the most important step in isolating different types of bioactive compounds from fruits and vegetables. Different compounds may be present in different products depending mostly on extraction method applied. As most phenolic compounds and other pharmacologically interesting compounds are almost insoluble in water, extraction of the *M. sativa* species is mainly associated with organic solvents (Ranu et al, 2010; Caunii et al., 2012).

On the other hand, *L. corniculatus* is a plant with high agronomic value in some parts of the world that has not gained importance in pharmaceutical industry yet, despite its rich flavonoid content (Reynaud and Lussignol, 2005). Available studies of extracts of the species *L. corniculatus* also involve nonaqueous media (Girardi et al., 2014; Gorski et al., 1975).

Ultrasound was used as a method of extraction in this study, as it has great potential in the extraction of bioactive compounds from plant material and it is a key technology in achieving the objective of sustainable ‘‘green” chemistry and extraction due to its high extraction efficiency as well as low energy and solvent consumption resulting in increasingy pure products. The main mechanism behind highly efficient ultrasound-assisted extraction has been attributed to mechanical, cavitation, and thermal effects which can result in disruption of cell walls, particle size reduction, and enhanced mass transfer across cell membranes.

Because of their different composition and forms in which they occur in each plant, the final composition of bioactive compounds in an extract largely depends on selected extraction conditions. According to available literature data, the type of extraction medium, pH and temperature appear to be the most significant parameters (Sannino et al., 2017).

Ultrasound-assisted extraction of the species *M. sativa* has shown that a high ethanol proportion and low pH are optimal for extraction of flavonoids, polyphenols and phenolic acids. Such findings were also revealed in a study by Caunii et al., 2012, where the proportion of phenolic components in the extract decreased with solvent polarity, whereas lignan and terpenoid concentrations increased.

Reduction in ethanol quantity in extraction medium and increase in pH result in increased proportion of isoflavones in the extract, with the highest quantities of the formononetin glycoside and GEN. In a study by Martin et al., 2006, significant amounts of formononetin glycoside and GEN were also found in the extract of the *M. sativa* species, but DAD and

biochanin were found as well. As the form in which isoflavones are found in a plant depends on its origin and the time when it was gathered, this fact may explain why the above-mentioned isoflavones were not detected in this study.

Increased temperature of extraction is beneficial for extraction of a number of polyphenolic compounds which are promoters of antiradical activity (Liyana-Pathirana and Shahidi, 2005). The results obtained in this study are consistent with such statement and show that the best antiradical activity was registered precisely in extracts prepared at high temperature.

In the species *L. corniculatus*, a high proportion of total flavonoids –but not of total polyphenols and phenolic acids– was observed when a high proportion of ethanol was present in extraction medium, which suggests that a large proportion of total polyphenols of the *L. corniculatus* species are not flavonoids but rather some other polyphenolic compounds. It is interesting to note that in contrast to other papers (Sarelli, 2003) isoflavone Genistein was also found. The extract prepared at high extraction temperature showed, similarly to the species *M. sativa*, better antiradical effect.

Due to its composition and weak polar nature, flavonoids are substances hardly soluble in water; on the other hand, extracts with a high proportion of organic solvents are not applicable for clinical purposes.

In order to obtain extracts with increased proportion of flavonoids in a water medium, CDs have been used. When extracts prepared by using water and ethanol as extraction medium have been compared to extracts prepared by applying water solution of CDs, it was evident that the use of CDs resulted in better selective extraction of flavonoids irrespective of whether the species *M. sativa* or *L. corniculatus* was in question.

Extraction of other polyphenols by using CDs was not as effective, probably due to the lower affinity of those substances for encapsulation in the lipophilic CD cavity. Among many factors that may affect the encapsulation of flavonoids and polyphenols into CD cavities is their concentration in extraction medium. If the ratio between the amount of CD and plant material in the extraction medium is not sufficient, only a certain portion of polyphenols will be complexed, i.e. extracted and utilization of extraction will not be complete. Theoretically, it is difficult to predict the optimal amount of CDs necessary for encapsulation because complexation depends on other factors as well. One of them is also the intensity of the ultrasound used during extraction.

Solvent extraction from dry plant material includes soaking of the plant material into the solvent to encourage swelling and hydration of the material, as well as the transfer of the mass of soluble components by diffusion and osmosis from the plant into extraction medium. Ultrasound facilitate swelling and hydration and consequently causes an increase in the cell wall pores, which improves diffusion of bioactive components from the plant. This very fact may be used to explain why a higher ultrasound intensity resulted in better flavonoid extraction from the both investigated species (Chemat et al., 2017).

Results of extraction of total phenolic content (TP) from *M. sativa* by conventional methods showed higher extraction yield than extraction by simultaneous use of ultrasound extraction and HPßCD complexation. However, application of HPßCD enabled more selective extraction of the total flavonoid content (TF) whose levels were more than three times higher. The extracted amount of the total flavonoid content from 1 g of the herbal material from *L. corniculatus* when using HPßCD was twice higher than the extracted total flavonoid content from *M. sativa*.

It is important to emphasize that isoflavones are naturally found in the form of glycosides (daidzin, genistin, glycitin), and in fermented food in the form of aglycones (DAD, GEN and glycitein). Glycoside conjugates are transformed into aglycones or "free isoflavones" during hydrolysis in the gastrointestinal tract by means of the enzyme β-glucosidase from bacteria in that tract. In adults, intestinal bacteria enzymatically hydrolyze glycosides into active aglycones, and aglycones are immediately absorbed from the stomach and the small intestine. Studies have shown that aglycones have better availability than glycosides because they are not dependent on the intestinal flora. Further transformation takes place by means of bacteria into specific metabolites: equol, O-desmethylagolen, dihydroGEN and p-ethyl phenol. Also, a proportion of isoflavones can directly pass to peripheral circulation (Yang et al., 2012).

The above was the reason to use aglycones only in testing the efficacy of decreasing GAG concentration in trials with cultivated fibroblasts.

Already mentioned papers describing potential use of CDs and isoflavones in tratment of rare metabolic diseases prompted us to investigate in depth the potential effect of CDs on intrinsic ability of DAD and GEN to inhibit GAG synthesis. In this study, focus was on 2-hydroxypropyl- (HPβCD) and sulfobuthylether-β-CD sodium salt (SBEβCD), both biocompatible and commercially available derivatives suitable even for parenteral administration (Jambhekar and Breen, 2016). Several papers have already been published about

CD complexation of both DAD and GEN, but their focus was mainly on determination of the stability constants and structure of the complexes formed with natural CDs (Crupi et al., 2007; Daruházi et al., 2008), as well as with hydrophilic (Borghetti et al., 2011; Danciu et al., 2014; Deng et al., 2016; Stancanelli et al., 2015) and amphiphilic CD derivatives (Cannavà et al., 2010; Crupi et al., 2007). In those papers, a kneading method was generally used for the preparation of DAD/GEN CD complexes in solid state.

In contrast to that, in this research neat grinding in the high energy vibrational mills was evaluated as a solvent free, sustainable green chemistry approach for the preparation of 2-HPβCD and SBEβCD complexes of DAD and GEN.

The obtained complexes were evaluated by differential scanning calorimetry (DSC) and thermogravimetric analysis (TG), as well as X-ray powder diffraction (XRPD) and scanning electron microscopy (SEM) analyses. Results of the analyses – which should indeed be observed as a whole (Mura, 2015) – indicated good complexation of both isoflavones, with somewhat higher affinity of GEN for complex formation. The formation of a complex does not alter the intrinsic ability of DAD and GEN to reduce the accumulation of GAG in cells and in this light our results are different from those obtained by Kamiski et al 2015, using cationic derivatives of gama CD.

Our results highlight the fact that HPßCD and SBEßCD do not enhance the isoflavone uptake in fibroblasts, but only act as carriers of the included molecules, increasing their solubility.

Our data show that both pure isoflavones GEN and DAD reduce GAG levels in the fibroblasts of MPS III patients in a dose-dependent manner. These results are consistent with literature data from other authors (Piotrowska et al., 2006; Malinowska et al., 2011) although they should always be interpreted taking into account the factors that cannot be standardized (passage times, growth rate, patient age at sample collection).

Besides *in vitro* biological testings, some pilot studies which included MPS III patients showed reduction in GAG excretion and improvements in some clinical parameters. In those studies GEN was orally administered at the relatively low doses of 5 and 10 mg/kg/day (Piotrowska, 2008; De Ruijter, 2012). Studies with higher doses of GEN (up to 150 mg/kg/day) have so far showed that such doses are also safe for patients, while further long-term evaluation is needed to reach conclusions about their clinical effects. Positive results have been observed on different tissues including improvement of hair morphology, range of joint motion, connective tissue elasticity, and behavior and cognitive abilities (Kim, 2013; Friso et al., 2010; Delgadillo et al.,

2011). For complete elucidation of the attained pharmacological effect on target tissues, past experience has indicated that, apart from the applied dose, it is highly significant that we understand the microflora involved in the metabolism of isoflavones because it can convert the parent molecules into metabolites with altered activity (Križova et al., 2019).

Due to all of the above, it is important to investigate the possibilities of safe application of high concentrations of GEN (and other flavonoids) to target tissues and evaluate their efficacy in reducing GAGs accumulation.

To the best of our knowledge, the impact of phytochemicals from isoflavone-rich plants, extracted by using novel eco-friendly methods, on GAG accumulation have not been published so far.

Although the cascade of events and cellular processes that lead to tissue pathology and clinical manifestations of the MPS is still not known in detail, what is currently certain is that several factors participate in those events, including oxidative stress. These factors are also subjected to mutual interactions, which contributes to the complexity of the pathological, functional, and phenotypic manifestations of the MPS. Previous studies, performed in animal models, suggested a role of oxidant/antioxidant imbalance in the pathophysiology of some manifestations of the MPS (Villani, 2012; Trudel, 2015). Moreover, excessive ROS production in the brain contributes to neurodegenerative processes (Broussalis et al., 2012). Neurological symptoms of the MPS include psychomotor retardation followed by cognitive and motor regression after the first decade, and spinal cord compression and hydrocephalus due to meningeal thickening and narrowing of the craniocervical junction. For such patients, it would be crucial to develop novel CD derivatives capable of enhancing delivery of isoflavones into fibroblasts and neuronal cells in a safe and controlled manner. This is important because past studies have also reported neuroprotective effect of DAD (Ahmad et al., 2016).

The mechanism of GEN action is different from action of other flavonoids (DAD, kaempferol, formononetin, glycitein, apigenin, naringenin), and their synergistic action could contribute to further GAG reduction (Koska, 2011).

As already stated, the prepared optimized extracts contain –in addition to a significant portion of GEN and other isoflavones– other polyphenols that may exhibit synergistic action. The presented results clearly demonstrated that such preparations lead to a more effective GAG decline in fibroblasts of MPS III patients than pure GEN and DAD. In comparison with pure GEN applied in the dose of 6 mg/mL, the optimised *M. sativa* extract showed 25% lower capacity to reduce GAG concentration. However, it should be emphasized that this extract

contained only 0.04% of GEN. Fibroblasts of the same MPS III patients treated with optimised extract from *L. corniculatus* showed the same ability to inhibit GAG synthesis as pure GEN at the dose of 6 mg/mL. At the same time, this extract was showed to be more effective than DAD.

On the other hand, previous studies indicated that flavonoids act differently on expression of a number of genes included in the metabolism of GAGs (some of the genes are downregulated, others are upregulated) (Moskot et al., 2015). Therefore, it is difficult to speculate on the extent in which the extracted isoflavone mixture may act on reduction in GAG concentration and the metabolism of other sphingolipids.

Furthermore, LDH and MTT assays showed that the used isoflavones (e.g. GEN and DAD) as well as optimised CD extracts in tested concentrations did not interfere with cellular membrane nor caused decrease in cellular viability. This is an important encouraging factor for further studies because potential SRT for MPS must be considered as a long-term therapy, with as few adverse effects as possible. Natural flavonoids are relatively weak inhibitors of GAG synthesis but have advantage over synthetic inhibitors of tyrosine kinases which were shown to possibly have unintended consequences (Sherman, 2009; Takeuchi and Ito, 2010). The fact that such extracts have moderate effect on inhibiting GAG synthesis is very important for their potential long-term clinical application, particularly in children. Actually, GAGs are compounds necessary for proper development and functions of many tissues and organs. On the other hand, it was shown that isoflavones may also have some negative influence on health (Woclawek-Potocka et al., 2013). The conflicting results in the available literature are strongly dependent on the experimental models used (different age and ethnicity of subjects, duration, dose and the manner of therapy application, etc.).

Although currently no ideal therapy is available for MPS III disease, there is an outstanding progress in both understanding the mechanism of the disease and in development of treatment methods (Gaffke et al., 2018). GET IT therapy, based on the use of different isoflavones (GEN, DAD or mixture of different isoflavones), is one option in slowing down pathological processes in MPS patients.

8. CONCLUSIONS

Ultrasound-assisted extraction of from *M. sativa* and *L. corniculatus* using cyclodextrins was viable alternative to ethanolic extraction, yielding the extracts with high phenolic content, rich in isoflavones, with high antioxidant potential. The inclusion complexes with cyclodextrins improved the biological availability of aglycone forms of the isoflavonoids isolated from selected species of the Fabaceae family and thus increased their therapeutic potential in treatment of several types of mucopolysaccharidoses. Taken together, our results highlight the promising role of the eco-friendly cyclodextrins extract of flavonoids from *M. sativa* and *L. corniculatus* in supportive treatment of various types of MPS diseases. Regrettably, the exact mechanism behind their influence on glycosaminoglycans metabolism is still uknown. Further studies might indicate what composition of flavonoids is optimal and in which dosage for the most efficient correction of the MPS phenotype.

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10. BIOGRAPHY

Barbara Fumić was born on 31st of January 1989 in Zagreb, Croatia. She finished elementary school and the XVth Gymnasium in Zagreb. She graduated from the Faculty of Pharmacy and Biochemistry, University of Zagreb, in 2012. During her studies she was awarded the Dean's Prize for scientific work „Influence of endothelin and endothelin A receptor on MDA-MB 231 cells in breast cancer ". She was also a Leader of Lectures and Practice team in eSTUDENT, student association which connects best students at the University of Zagreb and the best companies operating in Croatia. In 2013., she started working as a Research scientist at the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, on the project entitled „Solubilization of phenols in propolis using natural polymers"(2012-2013., project ID: 0006531 POC4 38)". In parallel, she enrolled in postgraduate doctoral study "Pharmaceutical-Biochemical Sciences" at the Faculty of Pharmacy and Biochemistry, University of Zagreb. During her postgraduate education she was representative of postgraduate students in the Student Union of Pharmacy and Biochemistry. From 2014. to 2016. she worked as a teaching assistant at the Department of Pharmaceutical Analysis and Department of Food Chemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb. Barbara co-authored 7 peer-reviewed papers, of which on 5 is the first author and have participated in different international scientific conferences. Since 2016 she has been working as Research scientist in Xellia Pharmaceuticals Ltd. (Formulation department, Research and Development of Finished Dosage Forms).

List of publications:

1. Bljajić, K., Šoštarić, N., Petlevski, R., Vujić, L., Brajković, A., **Fumić, B**., Saraiva de Carvalho, I., Zovko Končić, M. (2016). Effect of *Betula pendula* Leaf Extract on α-Glucosidase and Glutathione Level in Glucose-Induced Oxidative Stress. *Evidence-Based Complementary and Alternative Medicine*. 8429398-

2. **Fumić, B**., Jug, M., Zovko Končić, M. (2017), Multi-response Optimization of Ultrasound-assisted Extraction of Bioactive Components from *Medicago sativa* L. *Croatica Chemica Acta*. 90,481-491.

3. **Fumić, B**., Zovko Končić, M., Jug, M. (2017). Therapeutic potential of hydroxypropyl-β-cyclodextrin based extract of *Medicago sativa* in the treatment of mucopolysaccharidoses. *Planta Medica*. 83(1-2), 40-50.

4. **Fumić, B**., Jablan, J., Cinčić, D., Zovko Končić, M., Jug, M. (2018). Cyclodextrin encapsulation of daidzein and genistein by grinding: Implication on the glycosaminoglycan accumulation in mucopolysaccharidosys type II and III fibroblasts. *Journal of microencapsulation*. 35(1), 1-12.

5. **Fumić, B**., Zovko Končić, M., Jug, M. (2018). Development of cyclodextrin-based extract of *Lotus corniculatus* as a potential substrate reduction therapy in mucopolysaccharidoses type III. *Journal of inclusion phenomena and macrocyclic chemistry*. 92, 369-379.

6. Amidžić, M., Marić, P., **Fumić, B**., Petlevski, R., Bljajić, K, Zovko Končić, M. (2018). Oleuropein-Rich Olive Leaf Extracts May Ameliorate Consequences of Glucose-Induced Oxidative Stress in Hep G2 Cells. *Natural Product Communications*. 13, 657-660.

7. **Fumić, B.,** Jug, M., Zovko Končić, M. (2019). Optimization of Ultrasound-Assisted Extraction of Phenolic Antioxidants from *Lotus corniculatus*. *Croatica Chemica Acta*. 92, 1-9

Basic documentation card

University of Zagreb
Faculty of Pharmacy and Biochemistry
Doctoral study: Pharmaceutical-biochemical sciences
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Doctoral thesis

ISOLATION, SOLUBILIZATION AND BIOLOGICAL ACTIVITY OF ISOFLAVONOIDS FROM FABACEAE FAMILY

Barbara Fumić

SUMMARY

Mucopolysaccharidoses are hereditary metabolic diseases characterized by the accumulation of various glycosaminoglycans in the tissues of the diseased. Isoflavones are secondary plant metabolites that can slow down the synthesis of glycosaminoglycans, but their therapeutic potential is significantly limited by their poor oral bioavailability. In this thesis, the ethanol and cyclodextrin ultrasound-assisted extraction of aerial parts of two plant species from the family Fabaceae, *Medicago sativa* and *Lotus corniculatus*, was compared. The study was conducted using response surface methodology based on a Box-Benkhen design with three independent variables. The influence of isoflavones, the prepared extracts and inclusion complexes of selected isoflavones with different cyclodextrins on oxidative stress and glycosaminoglycan synthesis in fibroblast cell lines of patients with mucopolysaccharidosis was evaluated. Although ethanol was a more suitable solvent for the extraction of total polyphenols from *M. sativa*, the use of hydroxypropyl-β-cyclodextrin allowed for a more selective extraction of total flavonoids whose amount was as much as three times higher than in ethanol extracts. The amount of total flavonoids extracted using cyclodextrin from *L. corniculatus* was two times greater than the amount of total flavonoids of the species *M. sativa*. The cyclodextrin complexes of these extracts showed, depending on the dose, a significant decrease in glycosaminoglycan levels in fibroblast cells of patients with mucopolysaccharidoses, with no significant cytotoxic effect.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 109 pages, 17 figures, 18 tables and 352 references. Original is in English language.

Keywords: Cyclodextrines, isoflavones, *Lotus corniculatus*, *Medicago sativa*, mucopolysaccharidoses.

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IZOLACIJA, SOLUBILIZACIJA I BIOLOŠKA AKTIVNOST IZOFLAVONA IZ PORODICE FABACEAE

Barbara Fumić

SAŽETAK

Mukopolisaharidoze su nasljedne metaboličke bolesti koje karakterizira nakupljanje različitih glikozaminoglikana u tkivima oboljelih. Izoflavoni su sekundarni biljni metaboliti koji mogu usporiti sintezu glikozaminoglikana, ali je njihov terapijski potencijal značajno ograničen slabom oralnom bioraspoloživošću. U radu su uspoređene etanolna i ciklodekstrinska ultrazvučna ekstrakcija zeleni dvije biljne vrste iz porodice Fabaceae, *Medicago sativa* i *Lotus corniculatus*. Istraživanje je provedeno korištenjem metodologije površine odgovora temeljene na Box-Benkhen dizajnu s tri nezavisne varijable. Uspoređen je utjecaj izoflavona, priređenih ekstrakata te inkluzijskih kompleksa odabranih izoflavona s različitim ciklodekstrinima na oksidativni stres i sintezu glikozaminoglikana u staničnim linijama fibroblasta bolesnika oboljelih od mukopolisaharidoza. Iako je etanol bio prikladnije otapalo za ekstrakciju ukupnih polifenola iz *M. sativa*, uporaba hidroksipropil-β-ciklodekstrina omogućila je selektivniju ekstrakciju ukupnih flavonoida čija je količina bila čak tri puta veća nego u etanolnim ekstraktima. Količina ukupnih flavonoida ekstrahirana korištenjem ciklodekstrina iz biljnog materijala vrste *L. corniculatus* bila je još dva puta veća od količine ukupnih flavonoida iz vrste *M. sativa*. Ciklodekstrinski kompleksi navedenih ekstrakata pokazali su, ovisno o dozi, značajno sniženje razine glikozaminoglikana u stanicama fibroblasta bolesnika oboljelih od mukopolisaharidoza, bez značajnog citotoksičnog učinka.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Rad sadrži:	109 stranica, 17 grafičkih prikaza, 18 tablica i 352 literaturnih navoda. Izvornik je na engleskom jeziku.
Ključne riječi:	Ciklodekstrini, izoflavoni, *Lotus corniculatus* ,*Medicago sativa*, mukopolisaharidoze.
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