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Mentor: dr. sc. Mirza Bojić, izv. prof.

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FACULTY OF PHARMACY AND BIOCHEMISTRY

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MECHANISM OF CYTOCHROME P450 3A4 INHIBITION MEDIATED BY FLAVONOIDS

DOCTORAL DISSERTATION

Supervisor: Associate Professor Mirza Bojić, PhD

Zagreb, 2021

Zahvale

Duboku zahvalnost želim iskazati svom mentoru, izv. prof. dr. sc. Mirzi Bojiću, koji je za moje nedoumice uvijek imao razumijevanja, vremena i strpljenja, bez obzira na to na kojem kraju svijeta se nalazio. Najviše sam mu zahvalan što je ujedno bio i moj najveći kritičar i tjerao me da sam krčim svoj put.

Zahvalnost dugujem i dekanici Farmaceutskog fakulteta Sveučilišta u Mostaru, prof. dr. sc. Moniki Tomić, bez čijeg predanog rada i svesrdne potpore ovaj moj životni korak ne bi bio moguć.

Uvelike sam zahvalan dekanici Farmaceutsko-biokemijskog fakulteta Sveučilišta u Zagrebu, izv. prof. dr. sc. Jasmini Lovrić te bivšem dekanu prof. dr. sc. Željanu Malešu, na otvorenim vratima, pomoći i povjerenju koje su mi ukazali.

Veliku zahvalnost moram iskazati i prof. dr. sc. Marici Medić-Šarić, koja me je gurnula u svijet znanosti, iz kojeg mi je se sada teško istrgnuti.

Hvala dr. sc. Hrvoju Rimcu na savjetima, podučavanju i pomoći pri eksperimentalnom radu, kao i tehničkoj suradnici Ljiljani Jurkin, koja je u svakom trenutku bila spremna pomoći.

Veliku zahvalu zaslužili su Mirjana, Anela, Franjo, Josipa, Igor i Ivona, moja ekipa s fakulteta koja je uvijek bila tu da podari malo smijeha kad je najviše trebalo.

Isto vrijedi i za sve članove Zavoda za farmaceutsku kemiju Farmaceutsko-biokemijskog fakulteta, od mlađih do iskusnijih; hvala na svim dobrim danima koje smo skupa provodili.

Na stalnoj potpori hvala mojoj braći, Marku i Jakovu, cijeloj mojoj užoj i široj obitelji, posebice prijateljima koji su našli razumijevanja za moj životni put.

Hvala mojem *ćaći* Viciju. Hrabrost je koračati kroz život s neizbrisivim osmijehom na licu, unatoč nedaćama koje život daje. Hvala mu što mi to svakodnevno strpljivo pokazuje.

Hvala mojoj *materi* Filomeni. Njezina urođena vedrina i dobrota oplemenjuju svako živo biće. Hvala joj što je razumjela ono što nisam ni izgovorio.

Najveća hvala mojoj Maji. Hvala joj što je neprestano tu, što je strpljivo vjerovala, što se ni u jednom trenutku nije umorila vjerovati, čak i onda kada ja jesam.

homo sum

humani nihil a me alienum puto

SAŽETAK

Uvod: Flavonoidi su spojevi koji se u većim količinama nalaze u biljkama. Čovjek prehranom svakodnevno konzumira flavonoide. Enzimi citokrom P450 su najvažniji enzimi koji sudjeluju u metabolizmu lijekova i lipofilnih ksenobiotika. Najveći broj lijekova na tržištu metabolizira se putem CYP3A4 enzima. Poznato je da flavonoidi mogu stupati u reakcije s CYP3A4 enzimom, pri čemu ga mogu inhibirati. Važno je razjasniti dosad nepoznat mehanizam inhibicijskog učinka flavonoida na CYP3A4 enzim.

Materijali i metode: Ispitana je vrsta inhibicije kojom akacetin, apigenin, krizin i pinocembrin inhibiraju aktivnost CYP3A4 enzima. Inaktivacijska kinetika flavonoida određena je uz testosteron i nifedipin kao marker supstrate. Određeni su osnovni parametri enzimske inaktivacije (konstanta inhibicije, konstanta brzine inaktivacije, učinkovitost inaktivacije i polovica maksimalne inhibitorne koncentracije). Ispitana je pseudoireverzibilna inhibicija enzima uz pomoć hemokrom-piridin testa. Primjenom glutationa, hvatača slobodnih radikala, nastojala se utvrditi struktura reaktivnih intermedijera odgovornih za inaktivaciju enzima.

Rezultati: Apigenin je uzrokovao o metabolizmu ovisnu inhibiciju CYP3A4 enzima (ostatna aktivnost enzima 10,6 ± 1,3%). Za krizin je utvrđena IC_{50} vrijednost od 0,6 ± 0,5 μM, konstanta inhibicije K_i = 0,6 ± 0,3 μM te učinkovitost inhibicije 0,108 min^{-1} μM^{-1}. Za apigenin je utvrđena konstanta brzine inaktivacije, k_{inact} = 0,11 ± 0,01 μM^{-1}. Svi flavonoidi su smanjili koncentraciju hema, a krizin najviše (ostatna koncentracija hema 5,5%, odnosno 2,9%). Nije uočena statistički značajna razlika između ostatne aktivnosti enzima nakon dijalize s dodatkom kalijevog heksacijanoferata i bez dodatka oksidansa. Reaktivni međuprodukti flavonoida nisu uočeni na spektrometru masa.

Zaključci: Svi flavonoidi pokazuju o metabolizmu ovisnu inhibiciju CYP3A4 enzima, pri čemu se krizin pokazao kao najsnažniji inhibitor. Flavonoidi se kovalentno vežu za hem te na taj način dovode do inaktivacije enzima. Niti jedan flavonoid nije pokazao inhibiciju pseudoireverzibilnog karaktera na CYP3A4 enzim. Reaktivni međuprodukti flavonoida nisu uočeni zbog niskih koncentracija ili nestabilnosti produkata.

Ključne riječi: flavonoidi, akacetin, apigenin, krizin, pinocembrin, CYP3A4, inhibicija

SUMMARY

Introduction: Flavonoids are compounds found in larger amounts in plants. People consume flavonoids in their diet on a daily basis. Cytochrome P450 enzymes are the most important enzymes involved in the metabolism of drugs and lipophilic xenobiotics. Most drugs on the market are metabolized by the CYP3A4 enzyme. It is known that flavonoids can react with the CYP3A4 enzyme, whereby they can inhibit it. It is important to elucidate the hitherto unknown mechanism of the inhibitory effect of flavonoids on the CYP3A4 enzyme.

Material and Methods: The type of inhibition by which acacetin, apigenin, chrysin and pinocembrin inhibit CYP3A4 enzyme activity was investigated. The inactivation kinetics of flavonoids were determined with testosterone and nifedipine as substrates. The basic parameters of enzyme inactivation (K_i, k_{inact}, inactivation efficiency and IC_{50} concentration) were determined. Pseudoireversible inhibition of the enzyme was examined using the hemochromopyridine test. The use of glutathione sought to determine the structure of reactive intermediates responsible for enzyme inactivation.

Results: Apigenin caused metabolism based inhibition of the CYP3A4 enzyme (residual enzyme activity 10.6 ± 1.3%). For chrysin, an IC_{50} value of 0.6 ± 0.5 µM, K_i = 0.6 ± 0.3 µM, and an inhibition efficiency of 0.108 min^{-1} $µM^{-1}$ were recorded. An inactivation rate constant, k_{inact} = 0.11 ± 0.01 $µM^{-1}$, was recorded for apigenin. All flavonoids reduced the concentration of heme, chrysin being the most potent (residual concentration of heme was 5.5% and 2.9%, respectively). No statistically significant difference was observed between the residual enzyme activity after dialysis with and without the addition of potassium hexacyanoferrate. Reactive intermediates of flavonoids were not observed.

Conclusions: All flavonoids show metabolism-dependent inhibition of the CYP3A4 enzyme, with chrysine proving to be the most potent inhibitor. Flavonoids bind covalently to heme, thus leading to enzyme inactivation. None of the flavonoids showed pseudo-irreversible inhibition of the CYP3A4 enzyme. Reactive intermediates of flavonoids were not observed due to low concentrations or product instability.

Keywords: flavonoids, acacetin, apigenin, chrysin, pinocembrin, CYP3A4, inhibition

EXTENDED SUMMARY

Flavonoids are secondary metabolites of plants and a large family of polyphenolic plant components. They are key components of higher plant cells. Their name comes from the Latin word *flavus*, which means yellow, and which is related to their appearance in nature and the color they give to parts of plants. They were first isolated from lemons in 1930 and were known as vitamin P. To date, more than 8,000 such compounds are known and described, and it is believed that their roles are to attract insects, protect against UV radiation, various microbes and the like. roles. Today, flavonoids are considered indispensable components in various food, pharmaceutical, medical and cosmetic preparations. The reason lies in the fact that flavonoids in isolated laboratory *in vitro* and *in vivo* studies show different effects, such as: antioxidant, anti-inflammatory, antimutagenic, antitumor, antiallergic, antiviral, antiplatelet, antipyretic, antibacterial, hepatoprotective and other effects. With regard to the substituents on the rings of the basic structure or degree of oxidation, groups and subgroups of flavonoids are distinguished, from which it is necessary to single out: anthocyanidins, flavanes, flavanones, flavanonols, flavones, flavonols and isoflavonoids.

Flavonoids occur in nature bound to sugar in conjugated form (glycosides), and less frequently without bound sugar (aglycones). Studies of human exposure to flavonoids state that isoflavones are best absorbed in humans, followed by catechins, flavanones and quercetin glycosides. The bioavailability of flavonoids is very diverse when viewed by groups. People are exposed to flavonoids daily through diet. There are data that the average person, who adheres to a standardized diet, ingests about 1-2 g of flavonoids per day. According to some studies, the average intake of flavonoids in American adults is 189.7 mg/day, of which flavan-3-oli is 83.5%, flavanones 7.6%, flavanols 6.8%, anthocyanidins 1.6%, flavones 0.8% and isoflavones 0.6%. However, exposure to flavonoids today needs to be viewed in a cultural and sociological context, as it can sometimes vary from region to region. It is estimated that in Poland the intake of flavonoids is around 989 mg/day, which has been shown to be higher than for example in Greece (744 mg/day for men and 584 mg/day for women), but lower than in France (1193 mg/day).

Acacetin has various beneficial effects such as heart disease prevention, antioxidant, anti-inflammatory and antiplasmodial properties. Also, research indicates antiproliferative properties on different types of tumor cells present in the liver, prostate, lungs, in *in vitro* studies. Apigenin is also associated with the prevention of heart disease. *In vitro* studies indicate a significant role of apigenin in the prevention of malignancies and stimulation of the immune system, therefore the consumption of apigenin, as well as foods rich in apigenin, is highly recommended. Chrysin is known to have anti-inflammatory and antioxidant effects, however, the chemoprotective effects of chrysin are increasingly being investigated. Chrysin is thought to exert its effect by inducing apoptosis. Studies show that pinocembrin has the potential to be a drug for the treatment of ischemic stroke and similar clinical conditions, as it modulates inflammatory responses. The mechanism of action of pinocembrin in ischemic stroke probably lies in the fact that pinocembrin provides protection of mitochondria, inhibition of autophagy, antioxidant effect and other biological activities.

Cytochrome P450 enzymes (CYP enzymes) are members of a large superfamily of enzymes containing heme as a prosthetic group. They are present in all kingdoms; animals, plants, fungi, protoctists, bacteria, archaea and viruses. By 2018, more than 300,000 different CYP proteins have been described Of all CYP enzymes involved in drug metabolism, most drugs are metabolized by cytochrome P450 3A4 enzymes (CYP3A4) (33%), followed by CYP2D6 (13%), CYP2C9 (10%), CYP2C19 (9%), and CYP1A2 (9%). Such a large amount of drugs metabolized by CYP3A4 can be partly explained by the relatively large active site of this enzyme. CYP3A4 is found on the membranes of the endoplasmic reticulum. It is known that various compounds, including flavonoids, can inhibit CYP3A4 enzyme activity. Probably one of the best known inhibitions that flavonoids achieve with CYP enzymes is the inhibition of CYP3A4 mediated by flavonoids present in grapefruit juice. The inhibitory potential of grapefruit juice, but also red wine, as beverages rich in flavonoids, was also investigated. Grapefruit juice and red wine were found to show strong inhibition of the CYP3A4 enzyme by as much as 90% and 84%, respectively. The flavonoids genistein and biocanin A, baicalein and 2', 5, 6', 7-tetrahydroxyflavone have also been shown to inhibit enzyme activity by inhibiting 6β-hydroxylation of testosterone mediated by CYP3A4. Flavonoid compounds I3 and II8-biapigenin have been shown to act as potent enzyme inhibitors. I3 and II8-

biapigenin showed competitive inhibition of the CYP3A4 enzyme. Flavonoid compounds in plant species of St. John's wort (*Hypericum perforatum*), ginkgo (*Ginkgo biloba*) and many other plant species have been shown to be potent enzyme inhibitors.

The research hypothesis is that acacetin, apigenin, chrysin and pinocembrin show inhibition of irreversible character by covalent binding to the enzyme cytochrome P450 3A4. Reactive intermediates from the cytochrome P450 catalytic cycle resulting from the biotransformation of acacetin, apigenin, chrysin, and pinocembrin are responsible for the inhibition. Determining the type of irreversible inhibition by which the flavonoids acacetin, apigenin, chrysin, and pinocembrin metabolically inactivate CYP3A4 will contribute to understanding the interactions with other xenobiotics that they exert via the cytochrome P450 3A4 enzyme. The binding site of the reactive intermediate (iron, heme or apoprotein) will be determined, based on incubations with glutathione and the structure of the reactive intermediate if it is sufficiently stable. This work will contribute to the understanding of the interaction of foods and dietary products rich in flavonoids with drugs.

Different methods were used in this study: incubation and determination of enzymatic activity, determination of inhibition type, high performance liquid chromatography, inactivation kinetics assay, reactive intermediate binding assay, pseudoirreversible inhibition assay, determination of reactive intermediate structure, liquid mass chromatography analysis data.

The highest inhibition of CYP3A4 enzyme activity was observed with acacetin, with residual enzyme activity being 10.6 ± 1.3% in metabolism-dependent inhibition. In the study of direct and time-dependent inhibition, the residual enzyme activity was 31.2 ± 1.8% and 37.2 ± 4.4%, respectively. Apigenin also showed the highest inhibition of the CYP3A4 enzyme in metabolism-dependent inhibition, with residual enzyme activity being 24.5 ± 4.3%. In a time-dependent and direct inhibition study, an inhibitory effect of apigenin was observed that did not differ too much between the two types of inhibition. The residual enzyme activity was 37.2 ± 4.4% for time-dependent inhibition and 31.2 ± 1.8% for direct inhibition. Chrysin showed the greatest inhibitory effect in metabolism-dependent inhibition (residual enzyme activity was 17.6 ± 2.0%). After metabolism-dependent inhibition the largest inhibitory effect of chrysin was observed in time-dependent inhibition (residual enzyme activity was 49.9 ± 2.9%), while in direct

inhibition it showed the lowest inhibitory effect (residual enzyme activity was 58.4 ± 2.6%). Pinocembrin did not show an inhibitory effect on CYP3A4 enzyme activity in time-dependent inhibition (residual enzyme activity was 84.2 ± 1.3%). In direct inhibition, an inhibitory effect can be observed (residual enzyme activity was 77.5 ± 0.8%). Pinocembrin showed the greatest inhibitory effect in metabolism-dependent inhibition (residual enzyme activity was 51.8 ± 2.8%). Chrysin was shown to be the inhibitor with the lowest IC_{50} value (0.6 ± 0.5 μM), pinocembrin showed the IC_{50} value that was 10 times higher (5.0 ± 0.6 μM), while acacetin and apigenin showed about 20 times higher IC_{50} values (10.9 ± 0.3 μM and 11.4 ± 0.4 μM, respectively). All flavonoids reduced the heme concentration in the assay with and without the addition of SOD and CAT. The residual heme concentration after incubation with acacetin was 48.88% and on retesting with the addition of SOD and CAT 63.33%. The residual heme concentration after incubation with apigenin was 45.05% and on repeated testing with the addition of SOD and CAT 55.11%. The residual heme concentration after incubation with chrisin was 5.5% and on retesting with the addition of SOD and CAT 2.99%. The residual heme concentration after incubation with pinocembrin was 25.27% and on repeated testing with the addition of SOD and CAT 35.33%. The residual activity of the enzyme after incubation and dialysis with acacetin was 1.54%, and after incubation and dialysis with prior treatment with potassium hexacyanoferrate 2.23%. The residual activity of the enzyme after incubation and dialysis with apigenin was 4.42%, and after incubation and dialysis with prior treatment with potassium hexacyanoferrate 4.48%. The residual activity of the enzyme after incubation and dialysis with chrysin was 0.57%, and after incubation and dialysis with prior treatment with potassium hexacyanoferrate 1.31%. The residual activity of the enzyme after incubation and dialysis with pinocembrin was 3.27%, and after incubation and dialysis with prior treatment with potassium hexacyanoferrate 6.35%. The reactive intermediates of flavonoids are epoxides formed by the biotransformation of acacetin due to the catalytic cycle of the CYP3A4 enzyme. The proposed molecular weights of the reactive intermediates acacetin, apigenin, chrysin and pinocembrin were 312.269, 310.297, 270.234 and 273.257 g/mol. Fragmented samples have been proposed for $[M + H]^+$ flavonoid epoxy ions: $1.3A^+$, $0.2B^+$ and $1.3B^+$, which show characteristic *m/z* values. Examination of the chromatogram resulted in the values of the proposed flavonoid epoxy fragments, apigenin epoxide conjugate with glutathione

$[M + H\text{-}H_2O]^+$, *m/z* = 639, apigenin epoxide conjugate with heme without iron $[M + H]^+$, *m/z* = 905, as well as glutathione itself $[M + H\text{-}H_2O]^+$, *m/z* = 362 and iron-free heme itself $[M + H]^+$, *m/z* = 562. *m/z* values for glutathione and one molecule of water, the shown fragmentation of glutathione fragmentation, as well as iron - free heme were observed on other signals with different retention times. In a flavonoid inhibition kinetics study, acacetin showed an IC_{50} value of 10.9 ± 0.3 μM in the testosterone assay and 7.5 ± 2.7 μM in the nifedipine assay. Like this study, there are studies that examined IC_{50} values for acacetin. There is research on the main components of *Lygodium japonicum* root in terms of their inhibitory effect on the CYP3A4 enzyme *in vitro* and *in vivo*. *In vitro* studies were performed on rat liver microsomes using midazolam as a substrate. *In vivo* studies were performed on adult Sprague Dawley rats injected with acacetin for seven days at a dose of 5 mg/kg. They found that of all the components, only acacetin and apigenin inhibited the activity of the CYP3A4 enzyme. Acacetin showed an IC_{50} value of 58.46 μM. In an *in vivo* study, acacetin was found to inhibit midazolam metabolism in rats. The higher IC_{50} value obtained by the researchers in this study can be explained using a different substrate compared to the results obtained in this doctoral dissertation. It should be noted that testosterone and nifedipine are marker substrates used in the study of CYP3A4 enzyme activity, whereas the above researchers used midazolam as a substrate. According to the data available in the literature, the rate of inactivation constant and the inactivation efficiency of the CYP3A4 enzyme have not yet been determined for apigenin. Testosterone values were determined in this study (k_{inact} = 0.11 ± 0.01 min^{-1} and k_{inact} / K_i = 0.073 $min^{-1}\mu M^{-1}$). Based on the obtained inhibition kinetics parameters, it can be assumed that they may be clinically significant. Namely, this is confirmed by *in vivo* studies performed on apigenin and its ability to inactivate the CYP3A4 enzyme, which were performed on rats treated with etoposide. Etoposide is metabolized by CYP3A4. Elevated etoposide levels occurred in rats in the presence of apigenin. However, this does not always have to be understood as a negative effect, as this approach represents a potential process by which the oral bioavailability of etoposide can be improved. Also, in addition to etoposide, an increase in the concentration of imatinib antineoplastics was observed with apigenin. Chrysin showed a strong inhibitory effect on the CYP3A4 enzyme in a pseudo-irreversible inhibition assay. Chrysin almost completely reversed enzyme activity (more than 99% and approximately 99%, respectively, in SOD and CAT

assays). However, there was no statistically significant difference between the residual enzyme activity after dialysis and dialysis with previous oxidant addition. Therefore, it can be concluded that chrysin does not act as a pseudo-reversible inhibitor. So far, the IC_{50} parameters of pinocembrin on the CYP3A4 enzyme have not been reported, and in this study, they were determined (5.0 ± 0.6 μM for testosterone and 4.3 ± 1.1 μM for nifedipine, respectively). In addition, values of inhibition kinetics (1.2 ± 0.3 min^{-1} and 5.1 ± 1.6 min^{-1}) are described. The inhibition kinetics values described here are important for further understanding the clinical interactions of food ingredients with drugs. The importance of knowing the kinetics of inhibition is also illustrated by the example of the drug mibefradil. Mibefradil shows an inactivation kinetics of 0.174 min^{-1}. This drug was used as an antihypertensive but was withdrawn from the market as it caused clinically significant interactions with more than 30 drugs registered on the market. Examination of the chromatograms obtained by analysis on a mass spectrometer revealed the proposed fragments of apigenin epoxide with corresponding *m/z* values of 137 and 153, as well as other described fragmentations of glutathione and heme in the absence of iron. This evidence is consistent with other studies that have described the characterization of flavonoid fragments, glutathione adducts, and heme. In addition to the sites suggested here, epoxides may form on other parts of flavonoids, but the most likely sites of epoxidation have been described in this study. However, changing the place of flavonoid epoxidation does not change the mass of the formed reactive intermediate, so a search was made based on the calculated molecular weights. No *m/z* values corresponding to conjugates of acacetin epoxide with glutathione or heme were observed. This is probably due to the low concentration or instability of the resulting reactive intermediates. The structure of the reactive intermediate of apigenin as a glutathione-conjugated epoxide was proposed in a glutathione incubation assay. A product weighing 578.565 g/mol is proposed. Examination of the chromatograms obtained by analysis on a mass spectrometer revealed the proposed fragments of apigenin epoxide with corresponding *m/z* values of 137 and 153, as well as other described fragmentations of glutathione and heme in the absence of iron. Other authors also describe similar *m/z* values of apigenin fragments, which are adapted to epoxidation sites in this study. No *m/z* values corresponding to apigenin epoxide conjugates with glutathione or heme were observed, as they are likely to form at the nanomolar level. Like acacetin and apigenin, *m/z* values

of the required adducts were not observed when examining the chromatograms of the chrysin and pinocembrin samples, although characteristic *m/z* values of flavonoid fragments were observed.

Apigenin proved to be the most potent inhibitor in the time-dependent inhibition assay, with residual enzyme activity being 37.7 ± 4.4%. After apigenin, chrysin (residual enzyme activity 49.9 ± 2.9%) and akacetin (residual enzyme activity 55.6 ± 6.4%) were shown to be inhibitors. The weakest inhibitor in the time-dependent inhibition assay was pinocembrin (84.2 ± 1.3% of residual enzyme activity). Apigenin proved to be both the most potent inhibitor in the direct inhibition assay, with residual enzyme activity being 31.2 ± 1.8%. After apigenin, acacetin caused the highest enzyme inhibition in the direct inhibition assay (residual enzyme activity 48.5 ± 6.5%), followed by chrysin (58.4 ± 2.6%) and finally pinocembrin (77.5 ± 0.8%). In a study on the metabolism of dependent inhibition, the strongest inhibitor was acacetin, with residual enzyme activity being 10.6 ± 1.3%. The residual enzyme activity during incubation with chrysin was 17.6 ± 2.0%, with apigenin 24.5 ± 4.3% and with pinocembrin 51.8 ± 2.8%, the dominant form of inhibition by which acacetin, apigenin, chrysin and pinocembrin inhibit CYP3A4 enzyme activity is a metabolism-dependent inhibition. Chrysin was shown to be the flavonoid with the lowest IC50 value (0.6 ± 0.5 μM) with testosterone and 2.5 ± 0.6 μM with nifedipine as a marker substrate. After chrysin, the lowest IC_{50} values were observed for pinocembrin (5.0 ± 0.6 μM and 4.3 ± 1.1 μM, respectively), acacetin (10.9 ± 0.3 μM and 7.5 ± 2.7 μM, respectively) and apigenin (11.4 ± 0.4 μM and 8.4 ± 1.1 μM, respectively). In addition to IC_{50} values, chrysin was shown to be the flavonoid with the highest binding affinity, K_i = 0.6 ± 0.3 μM with testosterone, and 2.5 ± 1.0 μM with nifedipine as substrate. After chrysin, the lowest values of the inhibition constant were found in pinocembrin (1.2 ± 0.3 μM, and 5.1 ± 1.6 μM), apigenin (1.5 ± 0.8 μM, and 20.2 ± 12, 7 μM) and akacetin (6.0 ± 3.0 μM and 12.1 ± 5.6 μM, respectively). Apigenin showed the highest constant inactivation rate, k_{inact} = 0.11 ± 0.1 min^{-1} with testosterone and 0.11 ± 0.04 min^{-1}, with nifedipine. The determined inactivation rate constants in the tested flavonoids were as follows; for chrysin 0.065 ± 0.005 min^{-1} and 0.07 ± 0.01 min^{-1}, respectively, akacetin 0.036 ± 0.006 min^{-1} and 0.10 ± 0.02 min^{-1}, respectively and pinocembrin 0.018 ± 0.001 min^{-1} and 0.04 ± 0.01 min^{-1}, respectively. Chrysin was shown to be the flavonoid with the highest inactivation efficiency, k_{inact} / K_i = 0.108 min^{-1} μM^{-1}

for testosterone and 0.03 min^{-1} μM^{-1} for nifedipine. The determined inactivation efficiency values were as follows; for apigenin 0.073 min^{-1} μM^{-1} and 0.01 min^{-1} μM^{-1}, respectively, for pinocembrin 0.015 min^{-1} μM^{-1} and 0.01 min^{-1} μM^{-1}, respectively and for akacetin 0.06 min^{-1} μM^{-1} and 0.01 min^{-1} μM^{-1}, respectively. All flavonoids tested; acacetin, apigenin, chrysin and pinocembrin led to a statistically significant decrease in heme concentration both in the study without the addition of SOD and CAT and in the study with the addition of these enzymes, which aim to prevent the destruction of heme by reactive oxygen species. Chrysin caused the largest decrease in heme concentration, with the remaining heme concentration being 5.5% and 2.9%, respectively (with the addition of SOD and CAT). After chrysin, the next largest decrease in heme concentration was observed with pinocembrin (heme concentration 25.3% and 35.3% with SOD and CAT, respectively), apigenin (45.1% and 55.1% with SOD and CAT), and acacetin (48.8% and 63.3% with SOD and CAT). None of these flavonoids showed pseudoireversible inhibition on the CYP3A4 enzyme. No statistically significant difference was observed between the residual enzyme activity after dialysis with and without oxidant addition, therefore no complexation of acacetin, apigenin, chrysin and pinocembrin with ferrous iron in the active site of the enzyme occurs. It has been suggested that acacetin, apigenin, chrysin, and pinocembrin be metabolized to epoxides due to the catalytic cycle of the CYP enzyme. The resulting epoxides should react with heme or glutathione as a free radical scavenger. Samples were analyzed by high performance liquid chromatography, but the expected adducts were not observed. It is possible that the adducts form in a small amount that is not noticeable or the adducts are not stable enough. Acacetin, apigenin, chrysin and pinocembrin show inhibition of irreversible character by covalent binding to the enzyme cytochrome P450 3A4. Reactive intermediates from the cytochrome P450 catalytic cycle resulting from the biotransformation of acacetin, apigenin, chrysin, and pinocembrin are responsible for inhibition.

SADRŽAJ

POPIS KRATICA I SIMBOLA

AUC – površina ispod krivulje

CAT – katalaza

CoA – koenzim A

CYP1A1 – citokrom P450 1A1 enzim

CYP1A2 - citokrom P450 1A2 enzim

CYP1B1 – citokrom P450 1B1 enzim

CYP2C19 - citokrom P450 2C19 enzim

CYP2C9 - citokrom P450 2C9 enzim

CYP2D6 - citokrom P450 2D6 enzim

CYP3A4 – citokrom P450 3A4 enzim

CYP3A5 - citokrom P450 3A5 enzim

DI – izravna inhibicija

EROD - *O*-deetilacija 7-etoksirezorufina

g – ubrzanje Zemljine sile teže

G6P – Glukoza-6-fosfat

G6PDH – glukoza-6-fosfat dehidrogenaza

GABA – gama-aminomaslačna kiselina

HIV – virus humane imunodeficijencije

HPLC – tekućinska kromatografija visoke djelotvornosti

***IC_{50}* –** polovica maksimalne inhibitorne koncentracije

IU – međunarodna jedinica

K_i – konstanta inhibicije

k_{inact} – konstanta brzine inaktivacije

MDI – o metabolizmu ovisna inhibicija

Mr – molekularna masa

NADP - β-ninkotinamid-dinukleotid fosfat

p.a. – *pro analysi*

rpm – okretaja po minuti

SIM – promatranje pojedinačnog iona

SOD – superoksid dismutaza

TDI – o vremenu ovisna inhibicija

UV – ultraljubičasto

Vis – vidljivi dio spektra

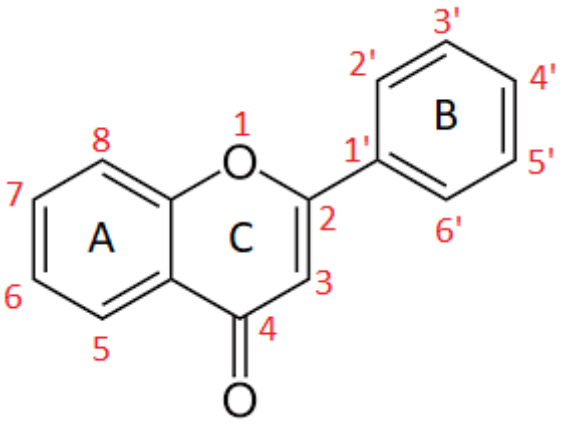
1.UVOD

1.1. Flavonoidi

Moglo bi se reći da su ljudi od svojih samih početaka bili izloženi flavonoidima, bilo kroz prehranu ili koristeći ih u ljekovite svrhe. Od tada pa sve do danas oni predstavljaju bitnu sastavnicu ljudske prehrane. Flavonoidi predstavljaju sekundarne metabolite biljaka, odnosno veliku porodicu polifenolnih biljnih sastavnica. Ključne su sastavnice stanica viših biljaka. Njihov naziv dolazi od latinske riječi *flavus,* što znači žut, a što je povezano s njihovom pojavom u prirodi i bojom koju daju dijelovima biljaka (Mukohata i sur., 1978). Prvi put su izolirani iz limuna 1930. godine te su bili poznati pod nazivom vitamin P (Scarborough, 1945). Do danas je poznato i opisano više od 8000 ovakvih spojeva, pri čemu se vjeruje da su njihove uloge privlačenje insekata, zaštita od UV zračenja, različitih mikroba i slično. Danas se flavonoidi smatraju neizostavnim sastavnicama u različitim prehrambenim, farmaceutskim, medicinskim i kozmetičkim pripravcima. Razlog leži u tome što flavonoidi pokazuju različite učinke na ljudski organizam, poput: atnioksidativnog, protuupalnog, antimutagenskog ili antitumorskog učinka (Panche i sur., 2016), antialergijskog i antivirusnog (Xia i sur., 2017), antiagregacijskog (Bojić i sur., 2015) te kardioprotektivnog, antibakterijskog, hepatoprotektivnog, antimalarijskog, neuroprotektivnog, kao antilišmanijskog, antiamebijalnog i antitripanosomskog učinka (Tapas i sur., 2008; Ferreyra i sur., 2012). Osim toga, flavonoidi su danas zanimljivi i istraživačkoj zajednici, budući da je poznato da mogu stupati u reakcije s citokrom P450 enzimima, pri čemu mogu djelovati kao inhibitori metaboličke aktivnosti enzima (Bojić, 2015).

1.1.1. Skupine flavonoida

Pregledom strukture flavonoida (slika 1), uočava se osnovni kostur flavonoida, kojeg čini 15 ugljikovih atoma koji su međusobno povezani u obliku C6-C3-C6, odnosno tri prstena (A, B i C), od kojih su A i B aromatski (Mierziak i sur., 2014).



Slika 1. Osnovna struktura većine flavonoida

S obzirom na supstituente na prstenima osnovne strukture ili stupnju oksidacije, razlikuju se skupine i podskupine flavonoida, od kojih valja izdvojiti:

- Antocijanidini
- Flavani
- Flavanoni
- Flavanonoli
- Flavoni
- Flavonoli
- Izoflavanoidi

Antocijanidini u odnosu na osnovnu strukturu većine flavonoida ne posjeduju keto skupinu na položaju 4 prstena C. Mogu biti supstituirani u položajima 3 na C prstenu, 4, 6 i 7 na A prstenu i 3', 4' i 5' na B prstenu. Za razliku od antocijanidina, flavani posjeduju jednostruku vezu na položaju C2/3 na prstenu C. S obzirom na položaj supstitucije s hidroksilnom skupinom dijele se na flavan-3-ole, flavan-4-ole i flavan-3,4-diole. Flavanoni imaju sličnu strukturu osnovnoj strukturi flavonoida, s tim da, isto kao flavani, imaju jednostruku vezu na položaju C2/3, a za razliku od flavana posjeduju keto skupinu na položaju 4 prstena C. Flavanonoli u odnosu na flavanone imaju barem jednu hidroksilnu skupinu u položaju 3 prstena C. S obzirom na osnovnu strukturu flavonoida, flavoni mogu biti supstituirani u položajima 3, 5, 6, 7 i 8 te 2', 3', 4', 5' i 6'. Flavonoli s obzirom na osnovnu strukturu flavonoida imaju jednu hidroksilnu skupinu u položaju 3 na prstenu C. Izoflavonoidi se od ostalih flavonoida razlikuju po izmijenjenom prstenu

B. Naime, kod izoflavonoida je prsten B vezan za prsten C u položaju 3, a ne u položaju 2 kao kod ostalih flavonoida (Alzand i Mohamed, 2012).

Bez obzira na to o kojoj se skupini flavonoida radi, sinteza većine njih počinje kondenzacijom *p*-kumaroil-CoA s tri molekule malonil-CoA, kako bi nastao kalhon (2′, 4′, 6′, 4-tetrahidroksikalhon). Ovu reakciju katalizira enzim kalhon sintaza, a reakciju pretvorbe kalhona u flavanon katalizira enzim kalhon-flavanon izomeraza (Alzand i Mohamed, 2012; Koes i sur., 1994).

1.1.2. Izloženost čovjeka flavonoidima

Kao što je već navedeno, flavonoidi su široko zastupljeni u prirodi te su im ljudi izloženi od pamtivijeka. Međutim, flavonoidi nisu podjednako zastupljeni u svim sastavnicama hrane. Od svih skupina flavonoida, u hrani su najzastupljeniji izoflavoni, flavonoli i flavoni (Kumar i Pandey, 2013).

Flavonoidi se u prirodi pojavljuju vezani sa šećerom u konjugiranom obliku (glikozidi), a rjeđe bez vezanog šećera (aglikoni). Flavoni su široko prisutni u listovima, cvjetovima biljaka, kao i voću u obliku glikozida. Najviše ih se može pronaći u celeru, peršinu, crvenim paprikama, kamilici, menti i dvorežnjastom ginkgu (luteolin, apigenin, tangeretin). Flavonoli su flavonoidi koji imaju ketonsku skupinu, a pojavljuju se značajno u voću i povrću. Kemferol, kvercetin, miricetin i fisetin česti su u luku, kelju, zelenoj salati, rajčicama, jabukama, grožđu. Flavanoni se mogu pronaći u svom citrusnom voću, poput limuna i naranči. Hesperetin i naringenin su česti predstavnici ove skupine. Za razliku od njih izoflavonoidi (kao što su genistein i daidzein) se ne javljaju tako često u svim biljnim vrstama, već je njihovo prisustvo ograničeno na soju i druge mahunarke. Antocijanini su pretežito pigmenti u biljnim vrstama (borovnice, jagode, maline) (Panche i sur., 2016).

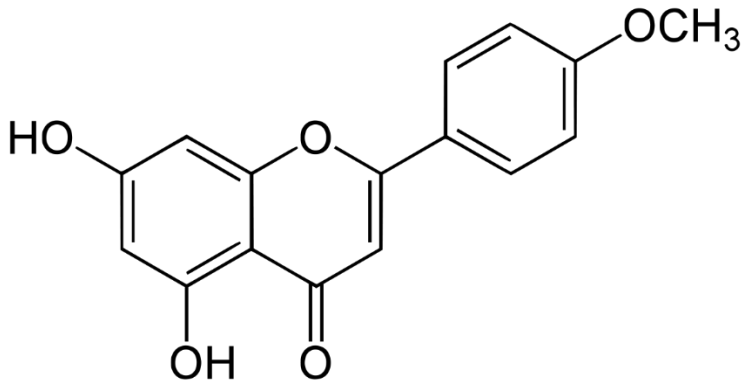
Manach i sur. (2006) navode da se kod ljudi najbolje apsorbiraju izoflavoni, a zatim katehini, flavanoni i glikozidi kvercetina. Bioraspoloživost flavonoida vrlo je raznolika ako se promatra po skupinama (Manach i sur., 2005). Čovjek je flavonoidima izložen svakodnevno putem prehrane. Postoje podaci o tome da prosječan čovjek, koji se drži standardizirane prehrane, dnevno unese oko 1-2 g flavonoida (De Vrijes i sur., 1997). Chun i sur. su 2007. godine izračunali prosječni unos flavonoida kod odraslih

Amerikanaca. Unos iznosi 189,7 mg/dan, od toga flavan-3-oli čine 83,5%, flavanoni 7,6%, flavanoli 6,8%, antocijanidini 1,6%, flavoni 0,8% i izoflavoni 0,6%. Međutim, izloženost flavonoidima danas treba promatrati u kulturološkom i sociološkom kontekstu, budući da se nekada zna razlikovati od regije do regije. Tako Zujko i sur. (2015) procjenjuju da je u Poljskoj unos flavonoida oko 989 mg/dan, pri čemu se pokazalo da je on veći u odnosu na primjerice Grčku (744 mg/dan za muškarce i 584 mg/dan za žene), ali manji od Francuske (1193 mg/dan).

Akacetin, apigenin krizin i pinocembrin strukturno su slični flavonoidi. Međusobno se razlikuju po supstituentima (hidroksilne i metoksi skupine) ili po načinu na koji su ugljikovi atomi međusobno povezani u osnovnom kosturu (jednostruke ili dvostruke veze). Utvrđeno je da ovi flavonoidi mogu inhibirati aktivnosti CYP3A4 enzima u *in vitro* uvjetima (Šarić Mustapić i sur., 2018).

1.1.3. Akacetin

Akacetin je *O*-metilirani flavon (slika 2) koji je u prirodi prisutan obično u biljkama iz porodice Asteraceae, a posebice u sjemenkama šafranike (Ha i sur., 2012).



Slika 2. Strukturna formula akacetina

Osim toga, akacetin je od glavnih polifenola prisutnih u medu, za koje se vjeruje da su povezani s prevencijom srčanih oboljenja (Khalil i Sulaiman, 2010). Akacetin je prisutan u biljkama kao što su: *Carthamus tinctorius* (Roh i sur., 2004), *Tanacetum sinaicum* (Marzouk i sur., 2016a), *Chrozophora tinctoria* (Marzouk i sur., 2016b), *Wissadula periplocifolia* (Teles i sur., 2015), *Agastache mexicana* (Flores-Flores i sur.,

2016) i *Turnera diffusa* (Ha i sur., 2012). Akacetin je sintetskim putem dobiven već 1926. godine (Robinson i Ventakataraman, 1926).

Akacetin se danas može pronaći na slobodnom tržištu kao sastavnica dijetetskih proizvoda, pri čemu se najčešće ističe njegova mogućnost inhibicije aktivnosti aromataze te se akacetin promovira kao pojačivač aktivnosti testosterona (Semwal i sur., 2019).

Različita ispitivanja ukazuju na blagotvorna djelovanja koja pokazuje ovaj flavonoid. Naime, u dosadašnjoj literaturi, osim prevencije srčanih oboljenja, prikazana su njegova antioksidativna (Wu i sur., 2018), protuupalna (Liou i sur., 2017; Pinzon i sur., 2011) i antiplazmodijalna svojstva (Kraft i sur., 2003; Pan i sur., 2006). Također, istraživanja ukazuju i na antiproliferativna svojstva na različite vrste tumorskih stanica prisutnih u jetri, prostati, plućima, u *in vitro* ispitivanjima (Singh i sur., 2005; Hsu i sur., 2004).

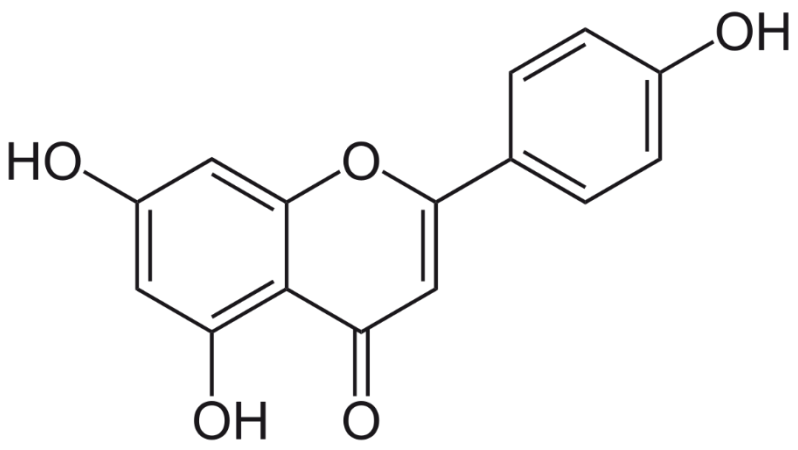
Kim i sur. (2012) su utvrdili i neuroprotektivni učinak kojeg ostvaruje akacetin, pri čemu se spominje kao učinkovito terapijsko rješenje za neurološke poremećaje povezane s pretjeranom stimulacijom neurotransmiterima. Različita istraživanja govore u prilog tome da akacetin inhibira aktivnost acetilkolinesteraze, enzima uključenog u razvoj Alzheimerove bolesti. Prema tome, postoji niz istraživanja koji akacetin spominju kao potencijalnog kandidata u liječenju Alzheimerove bolesti (Lim i sur., 2007; Dragičević i sur., 2011). Yang i sur. (2014) su uspjeli uz pomoć akacetina smanjiti koncentraciju malondialdehida kod neonatalnih štakorskih kardiomiocita s ozljedom uslijed hipoksije. Stoga je opravdano tvrditi da akacetin ima svoje mjesto i kao potencijalni kardioprotektivni agens. Istraživanje koje su proveli Asthana, Mishra i Pandey (2016) govori o produženju životnog vijeka vrste crva *Caenorhabditis elegans*. U tom istraživanju pokazano je da je izloženost akacetinu u dozi od 25 μM povisila životni vijek ove životinjske vrste za 27,31% u odnosu na one koji nisu bili izloženi akacetinu. Iako je nezahvalno donositi zaključke na osnovi jednog izoliranog istraživanja, važno je ukazati na mogućnosti koje ima akacetin u produženju životnog vijeka. U novije vrijeme spominje se i kao kandidat u liječenju infekcija uzrokovanih novim SARS-CoV-2 virusom (Adhikari i sur., 2020).

Istraživanja odnosa strukture i aktivnosti ukazuju na to da su hidroksilne skupine na položajima C5 i C7, dvostruka veza na C2/C3 i metoksi skupina na C4' ključne funkcionalne skupine zaslužne za biološku aktivnost akacetina. Primjerice,

Androutsopoulos i sur. (2009) tvrde da je za inhibiciju etoksirezorfurin-*O*-deetilaze odgovorna 5,7-dihidroksidni dio prstena A, kao i metoksi skupina na prstenu B. Kawaii i sur. (1999) su utvrdili da je dvostruka veza na C2/C3 ključna u indukciji diferencijacije HL-60 stanica.

1.1.4. Apigenin

Apigenin je po svojoj strukturi vrlo sličan akacetinu, a za razliku od njega na B prstenu ne posjeduje metoksi, već hidroksilnu skupinu (slika 3). Apigenin je jedan od najprisutnijih flavonoida u hrani, posebice u peršinu (Ross i Kasum, 2002). Štoviše, obilno je prisutan i u špinatu, celeru, kineskom celeru i suhom origanu. U manjim količinama prisutan je u narančama, luku, kumkvatu, sirku, čaju i korijanderu (Bhagwat i sur., 2011).



Slika 3. Strukturna formula apigenina

Apigenin je povezan s prevencijom srčanih oboljenja (Hertog i sur., 1993; Dong i sur., 2018; Hu i sur., 2015). *In vitro* ispitivanja ukazuju na značajnu ulogu apigenina u prevenciji malignih oboljenja (Czyz i sur., 2005; Patel, Shukla i Gupta, 2007) i stimulaciji imunološkog sustava (Cardenas i sur., 2016; Yano i sur., 2006; Lefort i Blay, 2013), stoga se konzumacija apigenina, kao i hrane bogate apigeninom, uvelike preporučuje (Shukla i

Gupta, 2010). Apigenin se u *in vitro* uvjetima pokazao kao spoj koji djeluje protektivno kod raka dojke (Lee i sur., 2008), debelog crijeva (Ruela-DeSousa i sur., 2010), prostate (Skuhla i Gupta, 2010) i drugih vrsta raka. Navedena antitumorska svojstva apigenina očituju se u inhibiciji rasta staničnih kultura melanoma, stoga se razmišlja i o njegovoj primjeni u različitim terapijskim kombinacijama protiv metastatskog melanoma (Caltagirone i sur., 2000). Osim navedenih, brojna istraživanja upućuju na druga biološka djelovanja, poput: protuupalnog (Gutierrez-Venegas i Gonzales-Rosas, 2017) antioksidativnog, antimutagenskog i antiproliferativnog (Patel, Shukla i Gupta, 2007).

Apigenin je umjereni antioksidativni spoj, vjerojatno zbog dvostruke veze na C2/C3, unatoč odsustvu hidroksilne skupine na poziciji 3 i kateholne strukture u prstenu B (Sichel i sur., 1991; Tripoli i sur., 2007). Iako postoje proturječna istraživanja glede antibakterijskog učinka apigenina (Liu i sur., 2013a), postoje istraživanja koja ukazuju na njegovu učinkovitost u suzbijanju virusa, i to: enterovirusa 71, herpes-simplex virusa 1 i 2, virusa hepatitisa C, virusa gripe te virusa afričke svinjske kuge (Wang i sur., 2019). Važno je napomenuti kako postoje istraživanja u kojima je liječenje apigeninom tijekom 24 sata pokazalo inhibiciju stanične proliferacije *Leishmania amazonesis* (Fonseca-Silva, 2015) parazita koji uzrokuje lišmaniozu, bolest koja zahvaća oko 12 milijuna ljudi na svijetu (Alvar i sur., 2015). Još jedna antimikrobna aktivnost koju pokazuje apigenin je i učinak na gljivice, odnosno na gljivice vrste *Candida albicans* i *Candida parapsilosis*, pri čemu inhibira njihov rast i razvoj pri koncentracijama od 8, odnosno 16 μg/mL (Singh, Joshi i Kumar, 2014).

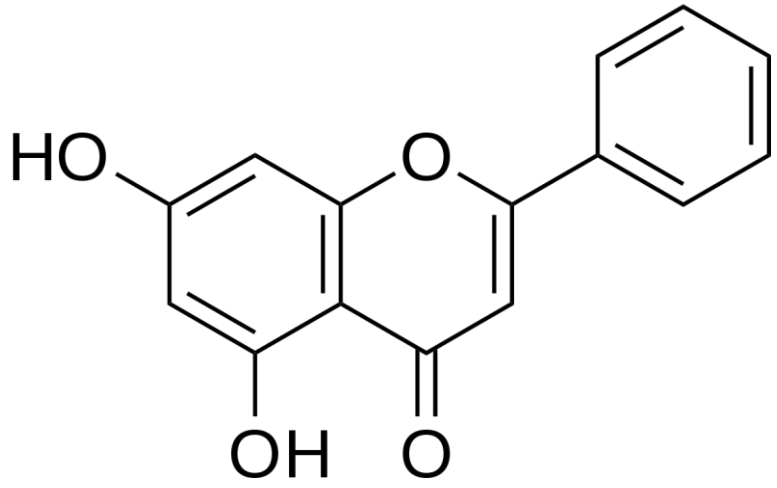
Apsorpcija apigenina nije sasvim do kraja objašnjena, jer postoje proturječna istraživanja o brzini apsorpcije. Gradolatto (2004) navodi apsorpciju apigenina 24 sata nakon oralne primjene, a Chen i sur. (2007) navode mnogo bržu apsorpciju od čak 3,9 sati nakon oralne primjene. Prilikom oralne primjene apigenina vršna koncentracija (C_{max}) kod štakora je iznosila 1,33 ± 0,24 μg/mL, odnosno površina ispod krivulje (engl. *area under the curve,* AUC) iznosila je 11,76 ± 1,52 μg sat/mL, što ukazuje na vrlo niske vrijednosti apigenina u krvi (Ding i sur., 2014).

Na osnovu dosadašnjih istraživanja moguće je zaključiti da se apigenin vjerojatno nakuplja u tijelu, budući da mu je i izlučivanje vrlo sporo. Nakon pojedinačne oralne primjene radioaktivno označenog apigenina kod štakora, uočeno je da je 51,0%

radioaktivnog oblika akacetina pronađeno u mokraći, a 12,0% u fecesu tijekom 10 dana. Pri tome, utjecaj na brzinu izlučivanja imali su spol i dob laboratorijskih životinja (Gradolatto, 2004).

1.1.5. Krizin

Krizin je analog apigenina, 5,7-dihidroksi-flavon (slika 4). Krizin je prirodno prisutan u medu, ali i različitim biljkama i propolisu (Mani i Natesan, 2018). Osim toga, prisutan je i u ekstraktima biljke *Passiflora caerulea*. U novije vrijeme, pokazuje se da je krizin potentan inhibitor aromataze (Sanderson i sur., 2004) te da inhibira aktivaciji virusa humane imunodeficijencije (HIV) (Critchfield i sur., 1996).



Slika 4. Strukturna formula krizina

Već su poznati njegovi protuupalni (Yeo i sur., 2020; Farkhondeh, Abedi i Smarghandian, 2019; Shin i sur., 2019; Zhang i sur., 2004) i antioksidativni učinci (Sim i sur., 2007; Woo i sur., 2005; Fan, Shen i Tang, 2004). Međutim, sve više se ispituju kemoprotektivni učinci krizina. Smatra se da krizin svoj učinak ostvaruje inducirajući apoptozu. Neke od vrsta tumorskih stanica u *in vitro* ispitivanjima, u kojima je krizin pokazao pozitivne učinke, su primjerice rak prostate i dojke (Joshee i sur., 2009), rak

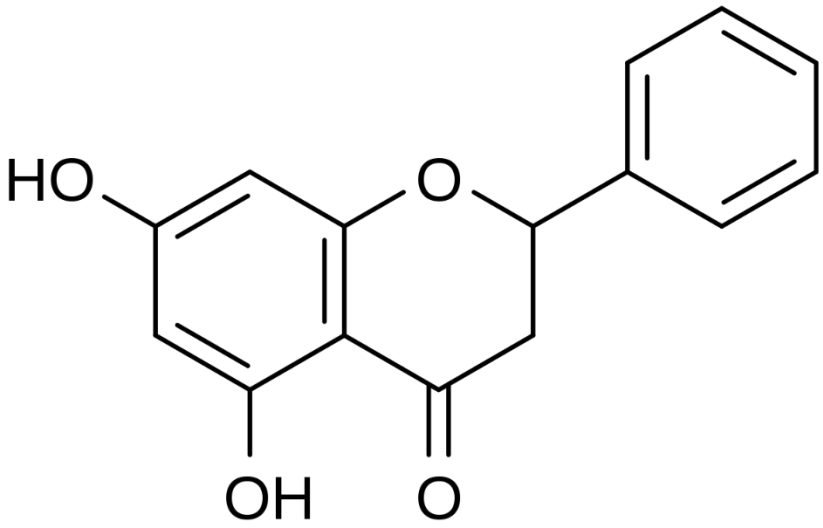
debelog crijeva (Wang i sur., 2004). U nekim istraživanjima se utvrdilo da krizin djeluje odgađajući razvoj tumorskih stanica, a ne kao inhibitor rasta već nastalih stanica, kao što je to slučaj u raku usne šupljine kod štakora, pri dozi od 250 mg/kg tjelesne mase (Karthikeyan i sur., 2013). Krizin se u određenim vrstama raka pokazuje kao učinkovita tvar, kako u ispitivanjima *in vitro*, tako i u ispitivanjima *in vivo* (Liu i sur., 2013b). Tako je dokazana njegova antitumorska učinkovitost kod raka štitnjače (Yu i sur., 2013) te u raku kože (Liu i sur., 2013). Smanjenje proliferacije stanica, kao i induciranje apoptoze zaslužni su i za antitumorski učinak kojeg krizin ostvaruje na raku jetre uzrokovanom *N*-nitrodietilaminom kod štakora (Khan i sur., 2011). Rehman i sur. (2013) su utvrdili da krizin djeluje protektivno kod razvoja tumora bubrega, također ublažavanjem stanične hiperproliferacije. Dodatna istraživanja potvrđuju ovu tezu, pri čemu se krizin promatra kao nefroprotektivna tvar, ublažavajući oksidativni stres izazvan toksičnom dozom cisplatina na štakorima (Sultana i sur., 2012), kao i kolonprotektivna tvar, gdje je također pokazao učinke na cisplatinom uzrokovanu ozljedu kolona putem apoptoze i ublažavanja oksidativnog stresa (Khan i sur., 2012).

Krizin posjeduje i antiastmatični potencijal. U istraživanju kojeg su proveli Wadibhasme i sur. (2011), krizin je pokazao antiasmatične učinke na bronho-alveolarnu hiperaktivnost izazvanu ovalbuminom kod štakora. U navedenom istraživanju krizin smanjuje alergijsku upalu dišnih putova degranulacijom određenih vrsta stanica (Filho i sur., 2015). Zanoli, Avallone i Baraldi (2000) su utvrdili da krizin aktivacijom GABA receptora pokazuje i antidepresivni učinak. Slično kao i njegov analog, apigenin, pokazao se i kao učinkovita antivirusna tvar, pri čemu posjeduje snažan inhibicijski učinak na enterovirus 71 (Wang i sur., 2014).

1.1.6. Pinocembrin

Pinocembrin je po svojoj strukturi pripadnik skupine flavanona, a od krizina se razlikuje samo po jednostrukoj vezi na položaju C2/C3 (slika 5). U prirodi je značajno prisutan u propolisu, pri čemu je njegova koncentracija od 606-701 mg/g propolisa (Escriche i Juan-Borras, 2018). Prisutan je i u medu te u vrsti đumbira *Boesenbergia rotunda* (Punvittayagul i sur., 2011). Značajno je prisutan u određenim biljnim vrstama,

poput: *Turnera diffusa*, *Peperomia obtusifolia* i *Piper genera*, kao i u biljnim vrstama porodice Asteraceae i Passifloraceae (Zhao i sur., 2008; Lopez i sur., 2002; Danelutte i sur., 2003; Feng i sur., 2012).



Slika 5. Strukturna formula pinocembrina

Istraživanja pokazuju da pinocembrin ima potencijala biti lijek za liječenje ishemijskog udara i sličnih kliničkih stanja, budući da modulira upalne odgovore (Soromou i sur., 2012). Mehanizam djelovanja pinocembrina kod ishemijskog udara vjerojatno leži u tome da pinocembrin osigurava zaštitu mitohondrija, inhibiciju autofagije (Tao i sur., 2018; Arias i sur., 2019), antioksidativni učinak, ali taj mehanizam ima svoju podlogu i u drugim biološkim aktivnostima (Ying, 2011).

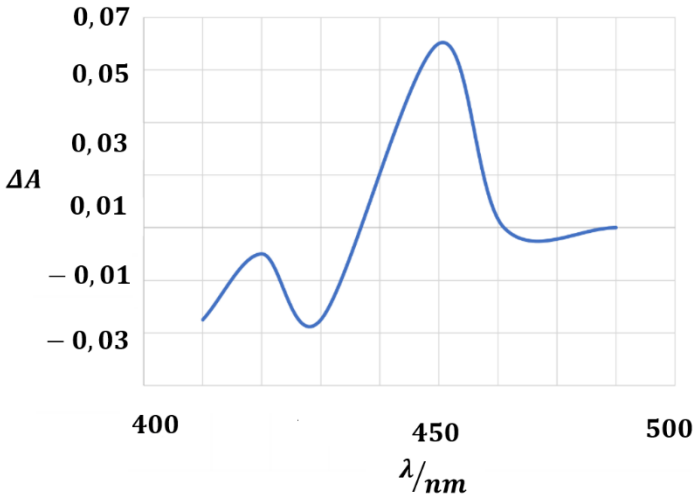
Pinocembrin suzbija ekspresiju upalnih markera, poput čimbenika nekroze tumora – α, interleukina-1-β, unutarstanične adhezijske molekule-1, inducibilne dušik-oksid sintetaze te akvaporina-4. Također, vrlo je važno naglasiti da pinocembrin inhibira aktivaciju mikroglija-stanica i astrocita te ekspresiju metaloproteinaza matriksa u ishemijskom dijelu mozga. Zbog toga pinocembrin pokazuje neuroprotektivni učinak na neurovaskularne stanice (Saad i sur., 2018; Gao i sur., 2010).

Za pinocembrin je dokazano da utječe na kognitivnu sposobnost i štiti živčane stanice od β-amiloidnih plakova, odnosno toksičnosti koju oni uzrokuju. Takve nakupine

su povezane s pojavom Alzheimerovom bolesti, a pinocembrin je uspio očuvati živčana vlakna štakora, pospješujući njihovu kognitivnu sposobnost (Liu i sur., 2012; Liu i sur., 2014a; Liu i sur., 2014b). Osim toga, pinocembrin značajno smanjuje stvaranje unutarstaničnih reaktivnih kisikovih spojeva, apoptozu stanica te drugih značajnih markera, koji su uključeni u razvoj Parkinsonove bolesti (Shen i sur., 2019; Jin i sur., 2015). Stoga je opravdano tvrditi da pinocembrin pokazuje velik potencijal u daljnjem istraživanju terapijskih rješenja u liječenju Alzheimerove i Parkinsonove bolesti. Nadalje, temeljito je opisano i njegovo antifungalno (Shain i Miller., 1982; Peng i sur., 2012), vazodilatacijsko (Li i sur., 2013) i hepatoprotektivno (Zhu i sur., 2007) antivirusno djelovanje (Lee, 2019; Schnitzler i sur., 2010), kao i poticanje apoptoze kod stanica raka debelog crijeva (Kumar i sur., 2007).

1.2. Citokrom P450 enzimi

1955. godine izvješćem o enzimskoj aktivnosti prisutnoj u endoplazmatskom retikulumu stanica jetre zeca, znanstveni svijet je postao svjestan jedne do tada neopisane vrste enzima, koja će se kasnije nazvati sustav citokroma P450, enzimi citokrom P450 ili jednostavno CYP enzimi (Axelrod, 1955). Kasnije se otkrilo kako su CYP enzimi uključeni u metabolizam ksenobiotika (grč. *xenos* – stran, *bios* – život), spojeva koji su nepoznati organizmu. Naziv citokrom P450 dolazi od toga što ovi enzimi imaju prisutan crveni pigment (P) koji apsorbira u vidljivom dijelu spektra na valnoj duljini $\lambda = 450$ nm (slika 6) kada je u reduciranom obliku i kompleksiran s ugljikovim monoksidom (Omura i Sato, 1964a). Do danas su CYP enzimi ostali jedina skupina enzima koja prikazuje apsorpcijski maksimum na 450 nm (Silverman, 2002), dok većina drugih hemoproteina ne pokazuje slična spektralna svojstva. Citokrom P450 sustav kasnije je postao predmet istraživanja kojim su se bavili Omura i Sato te su utvrdili njegovu hemoproteinsku strukturu i povezanost s citokromom b_5 (1964b).



$$[P450]/\mu M = \frac{\Delta A_{450} - \Delta A_{490}}{0{,}091}$$

Slika 6. Diferencijalni spektar citokroma P450 (prema Bojić, 2015)

Danas se zna da su citokromi pripadnici velike superporodice enzima koja sadrži hem kao prostetsku skupinu. Prisutni su u svim carstvima; životinjama, biljkama, gljivama, protoktistima, bakterijama, arhejama i virusima (Lamb i sur., 2009). Do 2018. godine opisano je više od 300 000 različitih CYP proteina (Nelson, 2018).

Citokromi dobivaju svoj naziv sukladno ustanovljenoj nomenklaturi (Nelson i sur., 1996.) pri čemu se u literaturi *kurziv* načinom pisanja označuju geni koji kodiraju određene enzime. Primjerice, *CYP1A2* je gen koji kodira CYP1A2 enzim. Bilo da se radi o genima ili enzimima, svima naziv počinje oznakom „CYP“ što je oznaka za superporodicu, a kratica izvedena iz engleskog izraza: *cytochrome P450*. Zatim se arapskim brojem označava vrsta porodice, nakon čega slijedi oznaka slovom latinske abecede koja predstavlja potporodicu te u konačnici oznaka arapskim brojem koja predstavlja određeni enzim. Enzimi se klasificiraju na osnovi sličnosti aminokiselinskog slijeda u njihovoj strukturi (tablica 1).

Tablica 1. Nomenklatura CYP enzima na osnovu sličnosti primarne strukture (Medić-Šarić i Rendić, 2013)

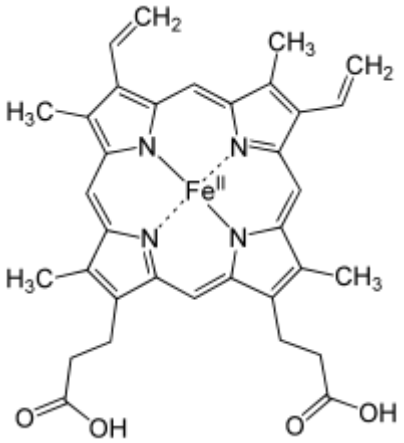
klasifikacija	oznaka	sličnost primarne strukture
superporodica	CYP	-
porodica	CYP3	≥ 40%
potporodica	CYP3A	≥ 55%
enzim	CYP3A4	≥ 98%

Iako se funkcija CYP enzima može svesti na dvije najvažnije zadaće; metabolizam ksenobiotika i biosinteza signalnih molekula (Guengerich, 1991), raznolike uloge CYP enzima uzrok su rasprostranjenosti u mnogim vrstama organizama i tkiva, pri čemu metaboliziraju širok raspon spojeva. Pri tome sudjeluju u različitim reakcijama, poput oksidativne i reduktivne dehalognacije, *N*-oksidacije i hidroksilacije, alifatske i aromatske hidroksilacije i slično (Sono i sur., 1996).

CYP enzimi su monooksigenze, odnosno vanjske monooksigenaze. CYP enzimi trebaju vanjskog donatora elektrona (nikotinamid dinukleotid fosfat – NAD(P)H) potrebnog za aktivaciju kisika, odnosno hidroksilaciju supstrata. Njihova opća formula monooksigenacije može biti predočena na sljedeći način (Bernhardt, 2006):

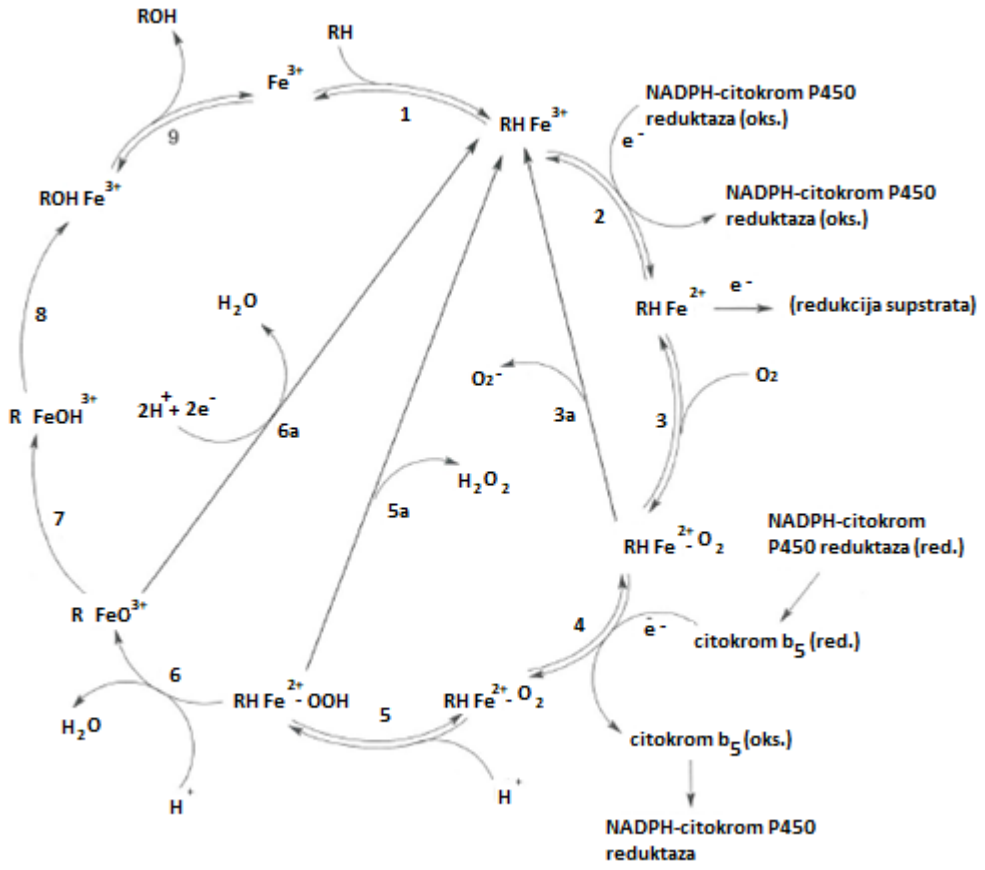
$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$$

CYP enzimi sadrže između 400 do 500 aminokiselinskih ostataka i jednu hem prostetsku skupinu u aktivnom mjestu, željezo u protoporfirinu IX (Medić-Šarić i Rendić, 2013). U toj strukturi četiri pirolna prstena (I-IV) su međusobno povezana metilnim mostovima *α, β, γ* i *δ*. Željezo u trovalentnom (feri, Fe^{3+}) obliku nalazi se u središtu protoporfirinskog prstena (slika 7) te je koordinirano pirolnim dušicima. Osim toga, u nativnoj strukturi na željezo je vezana molekula vode. Hemsko željezo je vezano na apoprotein preko tiolne skupine cisteinskog ostatka.



Slika 7. Strukturna formula hema s dvovalentnim željezom u središtu

Prema Medić-Šarić i Rendić (2013.) reakcijski ciklus enzima citokroma P450 kreće vezanjem supstrata na enzim (Fe^{3+}), pri čemu dolazi do konformacijskih promjena aktivnog mjesta enzima. Uklanja se molekula vode iz položaja šestog liganda. Zatim dolazi do prijenosa prvog elektrona s NAD(P)H na enzim. Dolazi do redukcije hemskog željeza iz Fe^{3+} u Fe^{2+}. Molekula kisika se veže za fero-ion, pri čemu nastaje nestabilan kompleks (Fe^{2+}-O_2), koji može prijeći u stabilniji feri-oblik (Fe^{3+}-O_2^-). Prijenos drugog elektrona dolazi s NADPH-P450-reduktaze, citokroma b_5 ili feredoksina, pri čemu nastaje perokso-skupina. Brzina ovog koraka određuje brzinu cijele reakcije. Nastala perokso-skupina se protonira (koriste se protoni iz vode ili aminokiselinskih ostataka proteinskog lanca). Jedan atom kisika s dva protona tvori vodu, a drugi atom ulazi u sastav $[FeO]^{3+}$ kompleksa. Svojstva supstrata i reakcije koju enzim katalizira odredit će hoće li cijepanje kovalentne *O-O* veze biti heterolitičko ili homolitičko. Potpuni katalitički ciklus citokroma P450 prikazan je na slici 8.



Slika 8. Shematski prikaz katalitičkog ciklusa citokroma P450; 1 – vezanje supstrata, 2 – prva redukcija, 3 – vezanje kisika, 4 – druga redukcija, 5 – kidanje kovalentne veze molekule kisika, 6 – odvajanje molekule vode, 7 – nastanak radikala, 8 – nastanak hidroksiliranog supstrata, 9 – oslobađanje produkta; RH – supstrat, ROH – produkt, Fe – atom željeza u hemskoj skupini na aktivnom središtu enzima, FeO^{3+} - pretpostavljeni aktivni kompleks enzim citokrom P450-kisik (prema Medić-Šarić i Rendić, 2013)

CYP enzimi su kod ljudi većinom vezani za membrane, nalaze se ili na unutrašnjim membranama mitohondrija ili u endoplazmatskom retikulumu stanica. Kod ljudi su zaslužni za metabolizam više tisuća endogenih i egzogenih spojeva, pri čemu se može dogoditi da pojedinačni CYP enzimi sudjeluju u metabolizmu samo jednog ili par supstrata, dok neki mogu sudjelovati u metabolizmu velikog broja supstrata. Citokromi P450 su itekako bitni za metabolizam estrogena i testosterona, vitamina D i sintezi kolesterola (Hakkola i sur., 2020). Osim toga, zaslužni su velikim dijelom za metabolizam ksenobiotika i toksičnih spojeva (Berka i sur., 2011). Do sada je poznato 57 ljudskih gena koji kodiraju različite CYP enzime (Lozić, Rimac i Bojić, 2016).

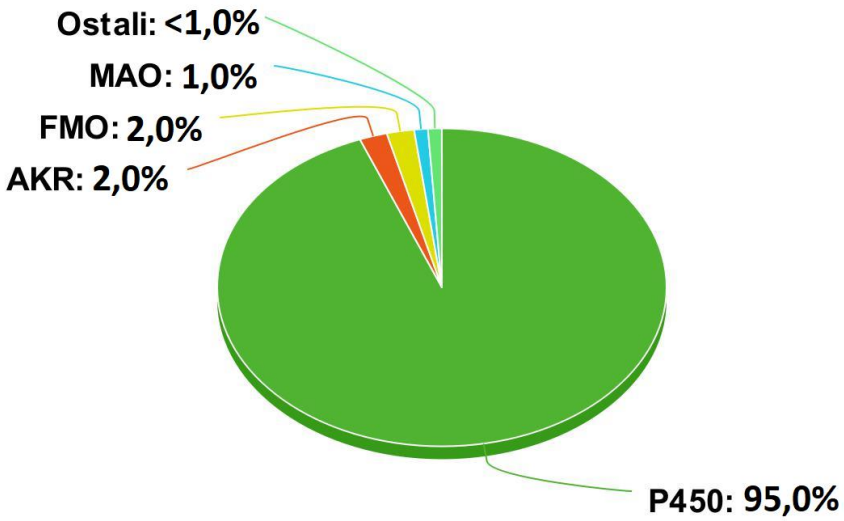
CYP enzimi mogu pokazivati i genetsku varijabilnost, koja se može očitovati u vidu procesa metabolizacije određenih spojeva. Genetski polimorfizam se javlja kada se neka osobina, u istoj populaciji određene vrste pojavljuje u dva ili više jasno različitih fenotipa (Ford, 1977). Budući da točno određeni gen kodira svaki CYP enzim, a svaka osoba naslijedi jedan alel od svakog roditelja, polimorfizam može nastati kada varijabilni alel zamijeni jedan ili oba alela divljeg tipa. Pri tome, varijabilni aleli obično kodiraju CYP enzim koji ima smanjenu ili gotovo nikakvu aktivnost. Tako se može dogoditi da osobe koje prime dva alela varijabilnog tipa budu tzv. spori metabolizatori, osobe koje prime jedan alel varijabilnog i jedan divljeg tipa mogu imati smanjenu enzimatsku aktivnost. S druge strane, osobe koje naslijede višestruke kopije divljeg alela mogu biti ultrabrzi metabolizatori, pri čemu imaju višu aktivnost enzima (Medić-Šarić i Rendić, 2013).

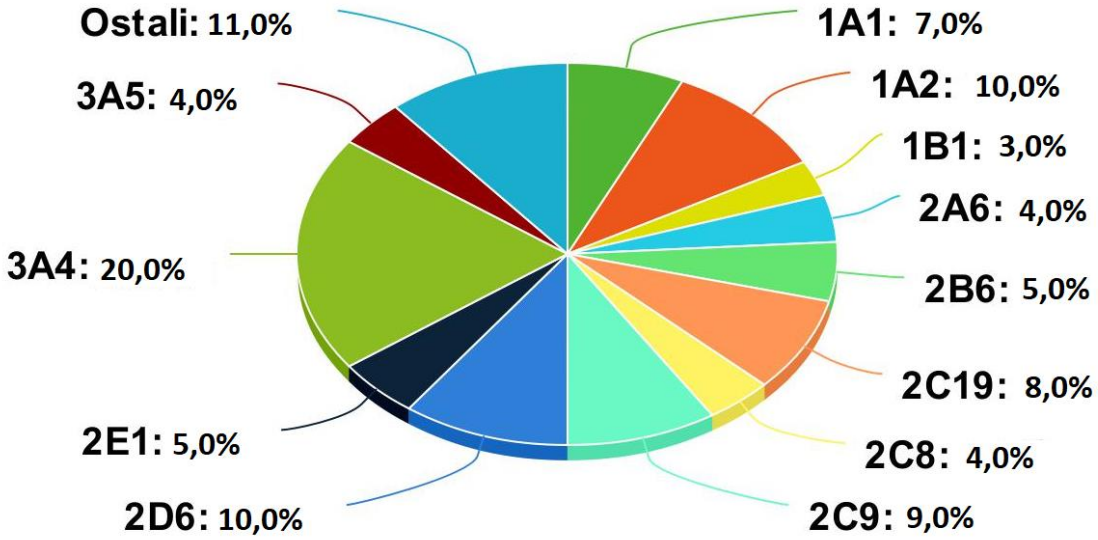
Poznavati ovaj polimorfizam važno je i zbog toga što su upravo CYP enzimi ti koji sudjeluju u metabolizmu većine lijekova, točnije u metabolizmu otprilike 96% poznatih lijekova (Rendić i Guengerich, 2015).

1.2.1. Citokrom P450 3A4 enzim

Od svih CYP enzima uključenih u metabolizam lijekova, najveći broj lijekova metabolizira se preko citokrom P450 3A4 enzima (CYP3A4) (33%). Zatim slijede, CYP2D6 (13%), CYP2C9 (10%), CYP2C19 (9%) i CYP1A2 (9%) (slika 9) (Rendić i Guengerich, 2015). Takva velika količina lijekova koje metabolizira CYP3A4 može se donekle objasniti relativno velikim aktivnim mjestom kojeg ima ovaj enzim. CYP3A4 se nalazi na membranama endoplazmatskog retikuluma. Osim što metabolizira spojeve u njihove polarnije metabolite, CYP3A4 može sudjelovati i u metabolizmu spojeva stvarajući njihove aktivne oblike. Neki od supstrata CYP3A4 enzima su: klaritromicin, eritromicin, telitromicin, kinidin, alprazolam, diazepam, midazolam, triazolam, ciklosporin, takrolimus, indinavir, ritonavir, sakvinavir, cisaprid, astemizol, klorfeniramin, amlodipin, diltiazem, felodipin, nifedipin, nisoldipin, nitrendipin, verapramil, atorvastatin, lovastatin, simvastatin, boceprevir, buspiron, imatinib,

haloperidol, metadon, pimozid, kinin, sildenafil, tamoksifen, telaprevir, trazodon, vinkristin (Lozić, Rimac i Bojić, 2016).





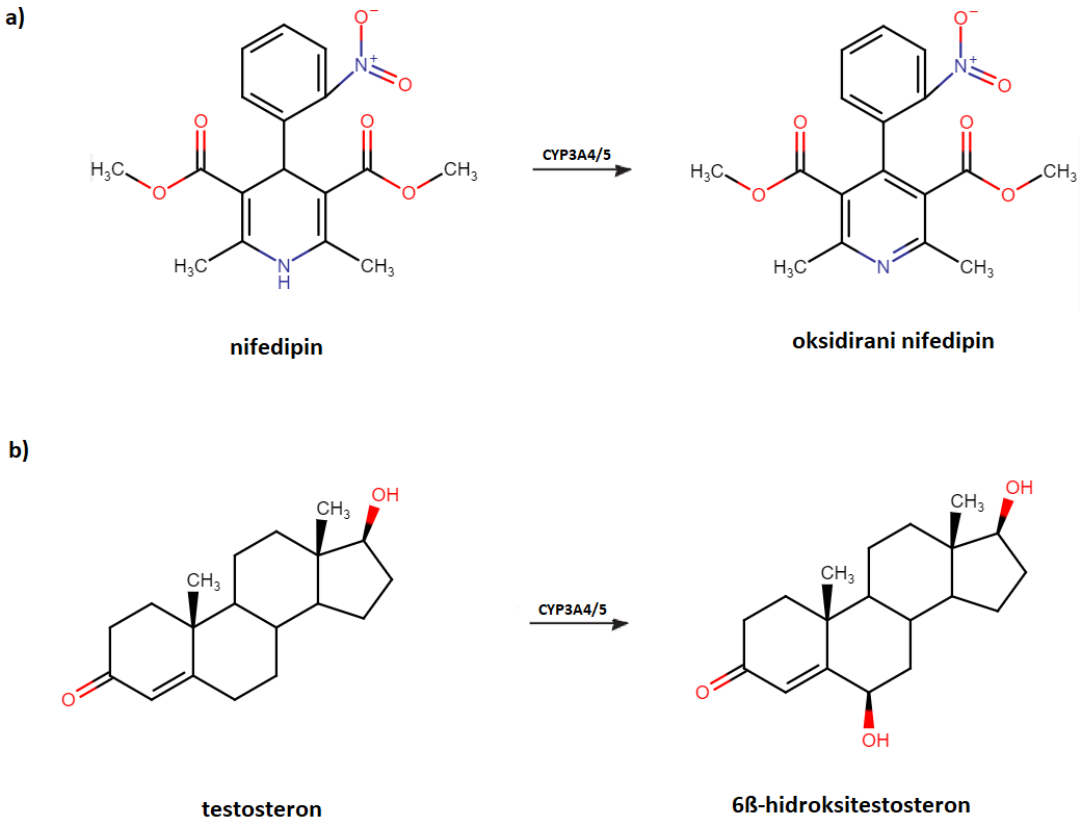
Slika 9. Udio citokroma P450 u reakcijama oksidacija i redukcija; P450 – citokrom P450, AKR – aldo-keto reduktaze, FMO – flavinmonooksigenaze, MAO – monoaminoksidaze (prema Lozić, Rimac i Bojić, 2016; Rendić i Guengerich, 2015)

Osim što posjeduje relativno veliko aktivno mjesto, CYP3A4 enzim posjeduje mogućnost vezanja više supstrata odjednom te metabolizira niz endogenih i egzogenih spojeva. Reakcije biotranformacije uključuju: hidroksilaciju, epoksidaciju, aromatsku

oksidaciju, oksidaciju heteroatoma, *N*- i *O*- dealkilaciju, oksidaciju aldehida, reakciju dehidrogenacije i slično. Hidroksilacija sp^3 C-H veze jedan je od načina na koji CYP3A4 enzim utječe na ligand, a nakon hidroksilacije ponekad slijedi i dehidrogenacija, što dovodi do stvaranja složenijih metabolita (Schmiedlin-Ren, 1997).

Budući da CYP3A4 sudjeluje u metabolizmu velikog broja spojeva, različiti spojevi mogu djelovati kao inhibitori ili kao induktori njegove aktivnosti. Pri tome može doći i do interakcija s drugim lijekovima koji se koriste u terapiji, pa čak do klinički značajnih interakcija (Bojić, 2015). Neki od spojeva koji mogu dovesti do inhibicije CYP3A4 enzima i do navedenih interakcija su: indinavir, nelfinavir, ritonavir, klaritromicin, itrakonazol, ketokonazol, nefazodon, eritromicin, verapramil, subokson, diltiazem, cimetidin, amiodaron, fluvoksamin, mibefradil, troleandomicin (Lozić, Rimac i Bojić, 2016). S druge strane, kao induktori enzimatske aktivnosti ovog enzima mogu djelovati lijekovi poput: karbamazepin, fenobarbiton, fenitoin, pioglitazon, rifabutin, rifampin, gospina trava, troglitazon. Primjerice, jedna od značajnih mogućih interakcija je primjena gospine trave i oralnih kontraceptiva, pri čemu dolazi do smanjene djelotvornosti oralnih kontraceptiva i posljedičnih trudnoća (Henderson i sur., 2002), stoga je vrlo važno poznavati spojeve koji inhibiraju ili induciraju aktivnost CYP3A4 enzima.

Kada se spominje djelovanje CYP3A4 enzim, onda se i u istom kontekstu spominje i CYP3A5 enzim, budući da se ova dva enzima preklapaju u pogledu supstrata, pa tako nije rijetko pronaći da se ti enzimi spominju kao CYP3A4/5, pri čemu treba naglasiti da CYP3A4 ima desetak puta veću katalitičku učinkovitost (Lozić, Rimac i Bojić, 2016). Dakle, CYP3A5 nema toliko značajan udio u metabolizmu lijekova u odnosu na CYP3A4, ali je važno poznavati osobine enzima, budući da je podložan genetskom polimorfizmu. CYP3A5 enzim odgovoran je za katalizu O^6-demetilacije tebaina čak deset puta više u odnosu na CYP3A4 (Kramlinger i sur., 2015). Najznačajnija, odnosno marker reakcija za ispitivanje aktivnosti CYP3A4 enzima je reakcija hidroksilacije testosterona u 6β-hidroksitestosteron. Osim navedene, koristi se i reakcija oksidacije nifedipina (slika 10).



Slika 10. a - oksidacija nifedipina; b - 6β-hidroksilacija testosterona

1.2.2. Inhibicija CYP3A4 enzima

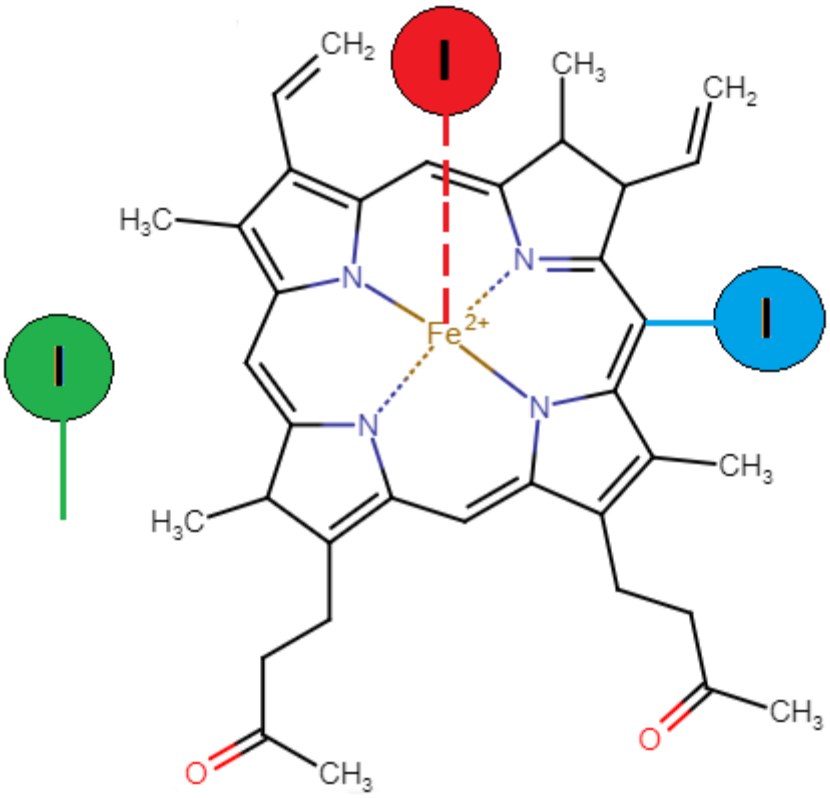
Inhibitori enzima su spojevi koji se vežu za enzim i rezultiraju smanjenjem aktivnosti enzima. Inhibitor se može vezati za enzim te na taj način spriječiti vezanje supstrata za aktivno mjesto i/ili spriječiti enzim u kataliziranju određene kemijske reakcije. Poznajemo dvije osnovne vrste inhibicije – reverzibilnu i ireverzibilnu inhibiciju. Jačina inhibitora određuje se njegovom IC_{50} vrijednošću. IC_{50} (engl. *inhibitory concentration of 50%*) oznaka je za polovicu maksimalne inhibitorne koncentracije. To je mjera za jačinu kojom određeni spoj može inhibirati određenu biološku ili biokemijsku funkciju. IC_{50} vrijednost je kvantitativni prikaz, koji nam govori o tome koliko je potrebno određenog spoja ili tvari (primjerice flavonoida) da inhibira određeni biološki proces ili tvar za 50%, u *in vitro* uvjetima (Enting i Hoetelmans, 1998).

Pri reverzibilnoj inhibiciji inhibitor se veže za enzim nekovalentnim vezama (vodikovim, ionskim vezama ili hidrofobnim interakcijama). Reverzibilni inhibitori mogu biti kompetitivni, akompetitivni, nekompetitivni te se može javiti miješana vrsta

inhibicije (Berg, Tymoczko i Stryer, 2002). Kod kompetitivne inhibicije supstrat i inhibitor se u isto vrijeme natječu za vezno mjesto, dakle ne može doći do vezanja i jednog i drugog. U slučaju kompetitivne inhibicije, vrlo često su i supstrat i inhibitor slične molekularne strukture. Ova vrsta inhibicije može se poništiti dodavanjem dovoljno velikih koncentracija supstrata (V_{max} ostaje nepromijenjen) (Berg, Tymoczko i Stryer, 2002). Međutim, uočena K_m vrijednost će se povisiti, budući da je potrebna veća koncentracija supstrata. Kod nekompetitivne inhibicije, vezanje inhibitora na enzim smanjuje aktivnost enzima, ali ne utječe na vezanje supstrata. U tom slučaju jačina inhibicije zavisi samo od koncentracije inhibitora (Srinivasan, 2020a). U slučaju akompetitivne inhibicije događa se nešto sasvim drugo; dolazi do vezanja inhibitora na enzim-supstrat kompleks. U ovoj vrsti inhibicije V_{max} se smanjuje, ali se smanjuje i K_m (Srinivasan, 2020b). Kod inhibicije miješanog tipa, inhibitor se može vezati za enzim u isto vrijeme kad i supstrat enzima. Međutim, vezanje inhibitora utječe na vezanje supstrata i obrnuto. Iako se ova vrsta inhibicije može smanjiti, ona se ne može u potpunosti premostiti povećanjem koncentracije supstrata. Iako to ne mora biti slučaj, kod ove vrste inhibicije vrlo često dolazi do toga da se inhibitor veže na drugačije mjesto od veznog mjesta na supstratu, pri čemu se mijenja konformacija enzima (Segel, 1993).

Za razliku od reverzibilne inhibicije, kod ireverzibilne inhibicije obično dolazi do toga da supstrat kovalentno modificira enzim, stoga se aktivnost ne može povratiti. Ireverzibilni inhibitori obično imaju neke reaktivne funkcionalne skupine poput dušikovog iperita, aldehide, haloalkane, alkene ili slično. Ove nukleofilne skupine reagiraju s aminokiselinskim postraničnim lancima kako bi stvorili kovalentne adukte. Ireverzibilni inhibitori su općenito specifični za jednu vrstu enzima i ne inaktiviraju sve proteine. Glavno je obilježje ireverzibilnih inhibitora to da je potrebno vrijeme, odnosno da su ovisni o vremenu te se njihova snaga ne može karakterizirati IC_{50} vrijednošću (Srinivasan, 2020b; Srinivasan i sur., 2020). Prema Bojiću (2015) ireverzibilna inhibicija predstavlja puno veći problem u dizajnu novih lijekova i interakcijama koje se ostvaruju na relaciji lijek – lijek ili lijek – drugi ksenobiotik, u odnosu na reverzibilnu inhibiciju, jer se enzimska aktivnost ne uspostavlja samim prestankom primjene inhibitora. U tom slučaju potrebno je vrijeme za sintezu novog enzima, od nekoliko dana do nekoliko tjedana. O vremenu ovisna inhibicija, nije karakteristična za citokrom P450 enzime (Bojić i sur., 2014), već je najčešće uočena inhibicija izravna inhibicija citokroma P450 (Bojić,

2015). Međutim, najznačajniji oblik inhibicije je o metabolizmu ovisna inhibicija, koja je posljedica nastanka reaktivnih metabolita. Takvi nastali reaktivni metaboliti mogu se kovalentno vezati za apoprotein ili hem te na taj način dovesti do inaktivacije enzima (slika 11).



Slika 11. Potencijalna mjesta inaktivacije citokroma P450 kovalentnom modifikacijom hema (plavo), apoproteina (zeleno) ili stvaranjem pseudoireverzibilnog kompleksa sa željezom (crveno označeni inhibitor – I) (prema Bojić, 2015)

Poseban slučaj inhibicije citokrom P450 enzima je tzv. pseudo (kvazi) ireverzibilna inhibicija enzima. Ona se događa u slučaju da vezanjem inhibitora na fero oblik hemskog željeza nastaje stabilni kompleks, pri čemu se inhibicija očituje kao ireverzibilna. Međutim, ukoliko se enzim u *in vitro* uvjetima prevede ponovno u aktivni oblik onda govorimo o pseudoireverzibilnoj inhibiciji. Enzim se u aktivni oblik može prevesti dijalizom ili ultracentrifugiranjem uz dodatak oksidansa poput kalijevog heksacijanoferata (Bojić, 2015). Uobičajen primjer pseudoireverzibilne inhibicije je na

CYP3A4 enzimu je diltiazem koji stvara kompleks s fero oblikom željeza (Lee i sur., 2012).

1.3. Inhibicija CYP3A4 enzima posredovana flavonoidima

Vjerojatno jedna od najpoznatijih inhibicija koju flavonoidi ostvaruju s CYP enzimima je inhibicija CYP3A4 posredovana flavonoidima prisutnima u soku od grejpa. Kao najsnažniji inhibitori prisutni u soku od grejpa pokazali su se flavonoidi s prisutnom hidroksilnom skupinom (Ho, Saville i Wanwimolruk, 2011). Chan i sur. (1998) također su ispitivali inhibicijski potencijal soka od grejpa, ali i crnog vina, kao napitaka koji obiluju flavonoidima. Utvrdili su da sok od grejpa i crno vino pokazuju snažnu inhibiciju CYP3A4 enzima od čak 90%, odnosno 84%. Sok od grejpa u ovom ispitivanju pokazuje o vremenu i o metabolizmu ovisnu inhibiciju.

Za trajnu zeljastu biljku ponosni protivak (*Lysimachia clethroides*) dokazano je da posjeduje hepatoprotektivni učinak. Zhang i sur. (2016) predlažu da su za hepatoprotektivni učinak ove biljke odgovorni flavonoidi kempferol i kvercetin. Nadalje, predlažu da hepatoprotektivni učinak ostvaruju inhibirajući aktivnost CYP3A4 enzima. Pri tom ispitivanju za kvercetin i kempferol utvrđene su IC_{50} vrijednosti od 18,77 ± 1,69 µM, odnosno 32,65 ± 1,32 µM.

Kao inhibitori enzimske aktivnosti pokazali su se i flavonoidi genistein i biokanin A. Istraživanje koje su proveli Kopečná-Zapletalová i sur. (2016) pokazalo je inhibicijski učinak na hidroksilaciju testosterona posredovanu CYP3A4 enzimom. Utvrđene IC_{50} vrijednosti u ovom ispitivanju ustanovljene su 24,42 ± 4,04 µM za genistein te 65,11 ± 3,97 µM za biokanin A. Utvrđene K_i vrijednosti iznosile su 23,25 ± 5,85 mmol/L za genistein i 57,69 ± 2,36 mmol/L za biokanin A. Iako autori istraživanja navode da je mogućnost interakcije ovih flavonoida s lijekovima kod ljudi rijetka, ona se ipak ne može isključiti.

Baikalein i 2',5,6',7-tetrahidroksiflavon inhibiraju 6β-hidroksilaciju testosterona posredovanu CYP3A4 enzimom. Kim i sur. (2002) utvrdili su IC_{50} za ova dva flavonoida od 17,4, odnosno 7,8 µM.

Obach (2000) je istraživao inhibicijski učinak na citokrom P450 enzime kojeg ostvaruju spojevi gospine trave (*Hypericum perforatum*). Dokazao je da su se kao snažni

inhibitori enzima ponašali flavonoidni spojevi I3 i II8-biapigenin. I3 i II8-biapigenin pokazali su kompetitivnu inhibiciju CYP3A4 enzima. Obach je utvrdio da je konstanta inhibicije za ove flavonoide iznosila 0,038 i 0,32 μM. Budući da se gospina trava upotrebljava kao biljni preparat u liječenju depresije, potrebno je dodatno ispitati istovremenu primjenu ovog preparata s lijekovima koji se metaboliziraju putem CYP3A4 enzima.

Lou i sur. (2019) ispitivali su učinak flavonoida miricetina na aktivnost CYP3A4 enzima. Miricetin je značajno zastupljen u čaju, crnom vinu, borovnicama i povrću. U provedenom istraživanju Lou i sur. su dokazali da je miricetin nekompetitivni inhibitor CYP3A4 enzima. Utvrdili su i konstantu inhibicije od K_i = 143,1 μM.

Gaudineau i sur. (2004) ispitivali su učinak spoja EGb761, ekstrakta biljke *Ginkgo biloba* na aktivnost CYP enzima. Spoj EGb761 uzrokovao je tek neznatnu inhibiciju aktivnosti CYP3A4 enzima (K_i = 155 ± 43 μg/mL). Međutim, autori su zatim promatrali dvije zasebne frakcije ovog spoja – terpenoidnu i flavonoidnu. Terpenoidna frakcija nije uzrokovala inhibiciju CYP3A4 enzima (K_i >900 μg/mL). S druge strane, flavonoidna frakcija uzrokovala je značajnu inhibiciju aktivnosti enzima (K_i = 43 ± 9 μg/mL).

Flavonoidi se ne ponašaju uvijek kao inhibitori enzimske aktivnosti. U istraživanju kojeg su proveli Han i sur. (2014) ispitivan je utjecaj skutelarina na šest ljudskih i štakorskih CYP enzima u *in vitro* uvjetima. Ustanovilo se da skutelarin nije pokazao značajan inhibicijski učinak niti na jedan ljudski CYP enzim. Za CYP2C19 ustanovljena je IC_{50} vrijednost od 63,8 μM, dok je za sve ostale, pa tako i za CYP3A4 iznosila više od 100 μM.

Osim pojedinačnih flavonoida koji mogu inhibirati aktivnost CYP3A4 enzima, često se događa da više prisutnih flavonoida može dovesti do sinergističkog učinka inhibicije. Takav slučaj opisali su Weiss i sur. (2020). U njihovom istraživanju ispitivan je tangeretin, flavonoid prisutan u soku od klementine, na aktivnost CYP3A4 enzima. U tom ispitivanju tangeretin se pokazao kao induktor enzimske aktivnosti. Međutim, sinergistički učinak svih flavonoida prisutnih u soku od klementine uzrokovao je inhibiciju CYP3A4 enzima.

2. CILJEVI I HIPOTEZA ISTRAŽIVANJA

Opći cilj ovog istraživanja je ispitati mehanizam inhibicije enzima citokrom P450 3A4 posredovanu flavonoidima.

Ispitat će se mehanizam vezanja akacetina, apigenina, krizina i pinocembrina za enzim, vrstu vezanja, kinetiku inhibicije i strukturni oblik flavonoida koji inhibira enzim. Mehanizam inhibicijskog učinka u ovom slučaju predstavlja određivanje zahvatnih točaka u katalitičkom ciklusu citokroma P450. U svrhu ostvarivanja ciljeva istraživanja odredit će se ostatna aktivnost enzima, inaktivacijska kinetika flavonoida, ispitat će se vrsta inhibicije (reverzibilna, pseudoireverzibilna) i odrediti specifičnost vezanja međuprodukta na enzim.

Hipoteza istraživanja je da akacetin, apigenin, krizin i pinocembrin pokazuju inhibiciju ireverzibilnog karaktera kovalentnim vezanjem na enzim citokrom P450 3A4. Za inhibiciju su odgovorni reaktivni međuprodukti iz katalitičkog ciklusa citokroma P450 koji nastaju biotransformacijom akacetina, apigenina, krizina i pinocembrina.

Određivanje vrste ireverzibilne inhibicije kojom flavonoidi akacetin, apigenin, krizin i pinocembrin metabolički inaktiviraju CYP3A4, doprinijet će razumijevanju interakcija s drugim ksenobioticima koje ostvaruju preko enzima citokrom P450 3A4. Odredit će se vezno mjesto reaktivnog intermedijera (željezo, hem ili apoprotein), a temeljem inkubacija s glutationom i struktura reaktivnog intermedijera ukoliko je isti dovoljno stabilan. Ovim radom doprinijet će se razumijevanju interakcije hrane i dijetetskih proizvoda bogatih flavonoidima s lijekovima.

3. MATERIJALI I METODE

3.1. Materijali

U istraživanju su korišteni flavonoidi: akacetin, apigenin, krizin i pinocembrin (Sigma-Aldrich, St. Louis, Montana, Sjedinjene Američke Države). Rekombinantni citokromi P450 3A4 zajedno s NADPH reduktazom i citokromom b_5 u bakulosomima nabavljeni su od Thermo Fisher Scientific (Waltham, Massachusetts, Sjedinjene Američke Države). Glukoza-6-fosfat (G6P), glukoza-6-fosfat dehidrogenaza (G6PDH) i β-ninkotinamid-dinukleotid fosfat dinatrijeva sol (NADP) nabavljeni su od Sigma-Aldrich. Kalijev fosfat (p.a.) i diklormetan (p.a.) nabavljeni su od Kemika d.d. (Zagreb, Hrvatska), mravlja kiselina (85%, p.a.) od Semikem d.o.o. (Sarajevo, Bosna i Hercegovina), a metanol korišten za otapanje reagensa i kromatografiju od Merck KGaA (Darmstadt, Njemačka). U inkubacijskim smjesama i kromatografiji korištena je ultračista voda. Za pripremu kalijevog fosfatnog pufera (pH=7,4 i pH=7,85) korišten je kalijev dihidrogen fosfat (Kemika d.d.), a pH je prilagođen uz pomoć natrijevog hidroksida nabavljenog od Semikem d.o.o. Pri ispitivanju ostatne aktivnosti enzima, kao i ispitivanju inaktivacijske kinetike korišteni su testosteron (Sigma-Aldrich), 6β-hidroksitestosteron Cayman Europe (Tallinn, Estonija), nifedipin (Sigma-Aldrich) i oksidirani nifedipin (Europsko ravnateljstvo za kakvoću lijekova, standard prema Europskoj farmakopeji, 10. izdanje, Strasbourg, Francuska). U hemokrom-piridin ispitivanju korišten je piridin (p.a.), Semikem d.o.o., goveđi hemin (Sigma-Aldrich) te dimetilsulfoksid (Semikem d.o.o.). Pri ispitivanju reverzibilne i pseudoireverzibilne inhibicije korišteni je kalijev heksacijanoferat (Siegfried AG, Zofingen, Švicarska), a pri ispitivanju specifičnosti vezanja glutation (Fluka, Buchs, Švicarska), superoksid dismutaza (Sigma-Aldrich), katalaza (Sigma-Aldrich), klorovodična kiselina (36%, p.a.) (Semikem d.o.o.) te acetonitril (Lach-Ner, s.r.o, Neratovice, Češka).

Inkubacije enzima provedene su u vodenoj kupelji. Ispitivanja ostatne aktivnosti enzima i inaktivacijske kinetike flavonoida provedena su koristeći uređaj za obrnuto faznu tekućinsku kromatografiju visoke djelotvornosti spregnutu s UV-Vis detekcijom (engl. *high performance liquid chromatography coupled with UV-Vis*, HPLC UV-Vis). Ispitivanje mase konjugata s glutationom provedeno je na spektrometru masa. Izračuni ostatne aktivnosti i parametri inhibicijske kinetike napravljeni su uz pomoć Michaelis-Mentenine jednadžbe.

3.2. Metode

3.2.1. Inkubacije i određivanje enzimske aktivnosti

Inkubacije enzima provedene su u triplikatu, uz mehaničko miješanje u vodenoj kupelji (Inkolab, Zagreb, Hrvatska) na 37 °C. Alikvoti flavonoida konačne koncentracije 1 µM otopljeni su u metanolu, preneseni u staklene epruvete te upareni do suha, osim u kontrolnim uzorcima koji ne sadržavaju inhibitor (flavonoid). Nakon isparavanja otapala pripremljena je inkubacijska smjesa na volumen od 100 µL sastavljena je od 5 pmol CYP3A4 enzima, 50 mM kalijevog fosfatnog pufera (pH=7,4) te ultračiste vode (pripravljena koristeći uređaj Arium comfort combined water production system od tvrtke Sartorius AG, Goettingen, Njemačka). Neposredno prije upotrebe pripravljen je i generirajući sustav sastavljen u omjeru 0,1 M G6P : 10 mg/mL $NADP^+$: 1000 IU/mL G6PDH = 50 : 25 : 1 (*v/v/v*). Generirajući sustav poslužio je kao izvor koenzima (15% volumena u konačnoj inkubaciji, *v/v*) te se dodatkom generirajućeg sustava započinje reakcija. Supstrati (testosteron i nifedipin) su se koristili za ispitivanje ostatne aktivnosti enzima u konačnoj koncentraciji od 200 µM.

3.2.2. Određivanje tipa inhibicije

Kako bi se utvrdila vrsta inhibicijskog učinka flavonoida na CYP3A4 enzim, provedene su tri vrste ispitivanja: ispitivanje izravne, o vremenu ovisne i metaboličke inhibicije enzima. Kod ispitivanja izravne inhibicije ne provodi se predinkubacija flavonoida s enzimom. Generirajući sustav dodaje se izravno u inkubacijsku smjesu u kojoj se nalazi i supstrat te se inkubacija provodi 15 minuta. Kod ispitivanja o vremenu ovisne inhibicije prvo se provede predinkubacija flavonoida s enzimom tijekom 30 min na vodenoj kupelji, a zatim se započinje reakcija dodatkom generirajućeg sustava i supstrata. Uzorci se zatim inkubiraju tijekom dodatnih 15 min. U slučaju ispitivanja o metabolizmu ovisne inhibicije prvo se provede predinkubacija flavonoida, enzima i generirajućeg sustava tijekom 30 min, a reakcija se započinje dodatkom supstrata. Uzorci se inkubiraju tijekom dodatnih 15 min.

Reakcija se zaustavlja dodatkom ledeno hladne 1 mL otopine 1% mravlje kiseline u diklormetanu. Nakon toga se uzorci mućkaju te se centrifugiraju tijekom 10 min na 1900 g (3000 rpm) (Rotofix 32, Westphalia, Njemačka). Po završetku centrifugiranja u uzorku se odvajaju dva sloja; gornji vodeni i donji organski sloj. 850 µL donjeg organskog sloja prenosi se u staklene epruvete, pri čemu se otapalo upari do suha. Nakon uparavanja, analit se otopi u 30 µL metanola te se prenosi u vijale za HPLC analizu (Agilent 1100 instrument, Agilent Technologies, Santa Clara, Kalifornija, Sjedinjene Američke Države).

3.2.3. Tekućinska kromatografija visoke djelotvornosti spregnuta s UV detekcijom

Ispitivanja inkubacije provedena su u triplikatu, a uzorci otopljeni u 30 µL metanola. Pri analizi uzoraka tekućinskom kromatografijom visoke djelotvornosti spregnute s UV detekcijom korištena je Agilent Zorbax SB C18 kolona (4,6 x 250 mm, 3 µm). Mobilna faza bila je sastavljena od metanola i vode u omjeru 64 : 36. Analiza je izokratska. Protok je postavljen na 1,0 $mLmin^{-1}$. Volumen injektiranja postavljen je na 10 µL. Kao marker supstrati korišteni su testosteron i nifedipin. Provedbom analize s testosteronom, pratila se 6β-hidroksilacija testosterona. Kromatogrami su snimani na 240 nm. Trajanje analize postavljeno je na 35 min. Vrijeme retencije testosterona je 19,2 min, a 6β-hidroksitestosterona 5,8 min. Nakon provedbe analize s testosteronom, uzorci su analizirani i s nifedipinom kao marker supstratom. Promatrana reakcija bila je oksidacija nifedipina. Kromatogrami su snimani na 254 nm. Trajanje analize postavljeno je na 25 min. Vrijeme retencije nifedipina je 7,1 min, a oksidiranog nifedipina 10,8 min (Sohl, Cheng i Guengerich, 2009). U oba slučaja količina dobivenog produkta promatrana je kao količina AUC u odnosu na kontrolni uzorak.

3.2.4. Ispitivanje inaktivacijske kinetike flavonoida

Za ispitivanje inaktivacijske kinetike flavonoida, pripremljene su koncentracije u rasponu od 0,1 do 20 mM. Otopine su prenesene u staklene epruvete te je otapalo upareno do suha. Inkubacijska smjesa i generirajući sustav su pripravljeni po gore opisanoj metodi, a predinkubacija je trajala 30 min na vodenoj kupelji pri 37 °C uz mehaničko

miješanje. Reakcija je započeta dodatkom supstrata (testosterona ili nifedipina) u različitim vremenskim razdobljima (0, 5, 10, 15, 20 i 25 min). Reakcije su prekinute dodatkom ledene otopine mravlje kiseline u diklormetanu. Uzorci su se analizirali na HPLC-u prema prethodno opisanoj metodi, zavisno od korištenog supstrata za mjerenje ostatne aktivnosti (testosteron ili nifedipin).

3.2.5. Ispitivanje vezanja reaktivnog međuprodukta na hem

Ispitivanje promjene koncentracije hema uslijed vezanja na reaktivnog međuprodukta na hem provedeno je po metodi koju su opisali Flink i Watson (1942), odnosno Paul i sur. (1953) uz određene preinake. Za provođenje testa prvo je pripravljen baždarni pravac otopine hemina u dimetilsulfoksidu (od 0,6 do 0,1 µM) te je na spektrofotometru snimljen spektar na valnoj duljini od 500 do 600 nm. Inkubacijska smjesa pripravljena je na volumen od 1,5 mL, a sastojala se od ultračiste vode, odgovarajuće otopine hemina, 0,83 M piridina (konačna koncentracija) i 0,06 M natrijevog hidroksida (konačna koncentracija). Pripravljeni baždarni pravac korišten je za izračun koncentracije hema u uzorcima.

Inkubacijska smjesa pripravljena je po već opisanoj metodi, pri čemu je volumen inkubacijske smjese povećan na 200 µL, a koncentracija inhibitora iznosila je 25 µM. Ispitivanja su provedena u triplikatu. Reakcija se započinje dodatkom generirajućeg sustava, pri čemu se provodi inkubacija tijekom 30 min. Nakon toga se u smjesu dodaje ultračista voda. Smjesa se prenosi u kivete za spektrofotometar te se dodaju piridin i natrijev hidroksid. Uzorci se snimaju na spektrofotometru (UV-1280, Shimadzu Coorporation, Kyoto, Japan) unutar 1 min od dodatka lužnate otopine, zbog nestabilnosti piridin hemokromogena pri bazičnim uvjetima (Bojić i sur., 2014). Apsorbancija se mjeri na valnoj duljini od 500 do 600 nm. Rezultati su se potvrdili ponovnom inkubacijom, pri čemu je dodano po 5 IU katalaze i superoksid dismutaze.

3.2.6. Ispitivanje reverzibilne i pseudoireverzibilne inhibicije

Cilj ovog ispitivanja je provjeriti nastajanje kompleksa sa Fe^{2+}, odnosno dolazi li do povrata aktivnosti enzima nakon dijalize s ili bez dodatka oksidansa. Ispitivanja su

provedena u triplikatu. Provedene su tri vrste ispitivanja za svaki flavonoid; inkubacijska smjesa bez flavonoida (kontrola), inkubacijska smjesa s flavonoidom te inkubacijska smjesa s flavonoidom i dodatkom oksidansa. Uzorci se predinkubiraju tijekom 30 min bez supstrata, s istim, gore navedenim, postavkama. Nakon inkubacije uzorci se prenose u kazete za dijalizu. U uzorke u kojima ispitujemo flavonoid s oksidansom dodamo i 20 mM otopinu kalijevog heksacijanoferata prije provođenja dijalize (konačna koncentracija) (Bojić i sur., 2014). Kazete (Slide-A-Lyzer Dialysis Cassettes, Thermo Fisher Scientific, Waltham, Massachusetts, Sjedinjene Američke Države) se urone u 50 mM otopinu kalijevog fosfatnog pufera (pH=7,4) tijekom 30 min. Nakon dijalize uzorak se iz kazete ponovno prenosi u staklenu epruvetu, pri čemu se za ispitivanje ostatne aktivnosti enzima koristi 200 μM testosteron (konačna koncentracija) te se ponovno dodaje generirajući sustav. Uzorci se inkubiraju 30 min s istim postavkama (mehaničko miješanje, temperatura od 37 °C). Reakcija se završava dodatkom ledene otopine mravlje kiseline u diklormetanu. Uzorci se analiziraju na HPLC-u, pri čemu je testosteron korišten kao marker supstrat.

3.2.7. Određivanje strukture reaktivnog produkta flavonoida

Za potrebe određivanja strukture reaktivnog međuprodukta odgovornog za ireverzibilnu inhibiciju provedeno je ispitivanje s glutationom (hvatač slobodnih radikala) uz dodatak superoksid dismutaze i katalaze kako bi se spriječilo moguće uništenje enzima uslijed neproduktivnih ciklusa citokroma P450. Uvjeti provođenja inkubacije ostali su isti, ali je volumen inkubacije povećan na 500 μL te je trajanje inkubacije povećano na 4 h. U uzorcima je upareno 10 μL flavonoida. Osim CYP3A4, kalijevog fosfatnog pufera i ultračiste vode, inkubacijska smjesa sadržavala je 50 IU superoksid dismutaze, 50 IU katalaze te 1 mM glutationa (konačne koncentracije), na način koji su opisali Yu i sur. (2005). Glutation se kao nukleofil dodaje u suvišku, kako bi se uhvatili bilo kakvi nespecifični elektrofilni spojevi, koji se mogu vezati za proteine u inkubacijskoj smjesi (Sharma i sur., 1997). Reakcija se završava dodatkom 1 mL ledene 25% otopine koncentrirane klorovodične kiseline u acetonitrilu (*v/v*). Uzorci se mućkaju, zatim centrifugiraju. Nakon centrifugiranja, gornji neproteinski sloj se prenosi u vijale, koje se

analiziraju tekućinskom kromatografijom spregnutom sa spektrometrijom masa (HPLC-Triple Quad Mass Spectrometer Agilent 6420, Agilent Technologies).

3.2.8. Tekućinska kromatografija spregnuta sa spektrometrijom masa

Uzorci su snimani na uređaju za tekućinsku kromatografiju visoke djelotvornosti spregnutim sa spektrometrijom masa opremljenim izvorom elektroionizacijskog raspršenja (ESI). Kao mobilna faza korišteni su A (0,1% trifluoroctena kiselina u vodi) i B (0,05% trifluoroctena kiselina u acetonitrilu). Gradijentno eluiranje postavljeno je na sljedeći način: na 0, 30, 35, 40 min postotak B faze iznosio je 30, 80, 30 i 30%. Protok mobilne faze postavljen je na 0,5 mL/min. Volumen injektiranja postavljen je na 5 µL. Korištena je kolona Jupiter C4 (250 x 4,6 mm, 5 µm). Temperatura plina za sušenje (dušik) postavljena je na 300 °C. Protok plina za sušenje postavljen je na 8 L/min. Tlak nebulizatora postavljen je na 15 psi. Napon i jakost struje na kapilari postavljeni su na 3,5 kV (+), 20 nA. Kromatogrami su snimani na $\lambda = 400$ nm.

Za metodu kromatograma ukupne ionske struje (TIC metoda), korišten je raspon 10-2250 amu, pozitivna i negativna ionizacija, *step size* 0,1 amu, vrijeme snimanja 500 ms, fragmentor 135 V. Metodom promatranja pojedinačnog iona (SIM metoda) svaki je uzorak testiran na moguće protonirane forme $[M+H]^+$ analita uzoraka koji se mogu iščitati iz kromatograma. ESI (+), fragmentor 135 V.

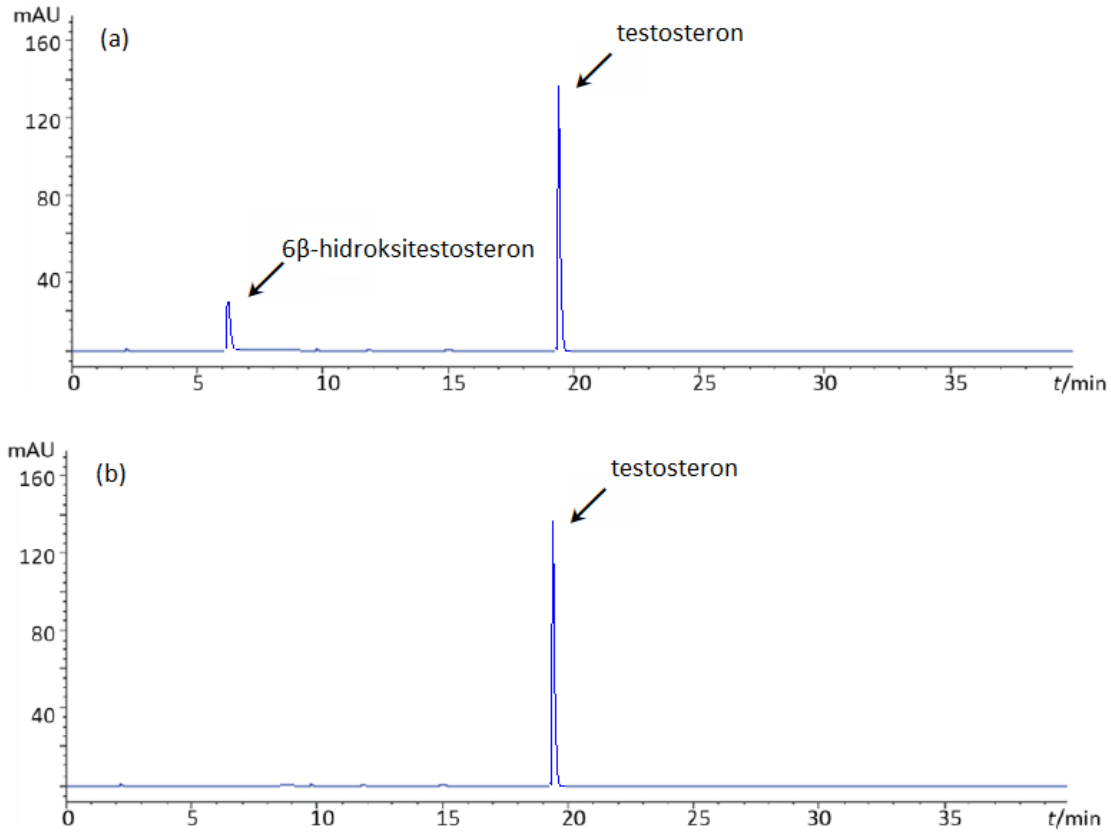
3.2.9. Statistička analiza

Nakon obavljenih ispitivanja provedena je analiza podataka. Ispitana je normalna raspodjela podataka. Ukoliko bi se ustanovilo da podaci slijede normalnu raspodjelu, koristio bi se jednostrani *t*-test za procjenu statističke značajnosti u razlikama između uzoraka i kontrole, na osnovu mjerenja ostatne aktivnosti. Za izračun IC_{50} vrijednosti koristila se nelinearna jednadžba. Za određivanje inaktivacijske konstante (*Ki*) i inaktivacijske stope inhibitora (I) koristila se Michaelis-Mentenina jednadžba. Za kinetičke je parametre izračunata i standardna devijacija. Statistička obrada je napravljena koristeći Program R (The R Project for Statistical Computing, Beč, Austrija) i Microsoft Excel (Microsoft, Redmond, Washington, Sjedinjene Američke Države).

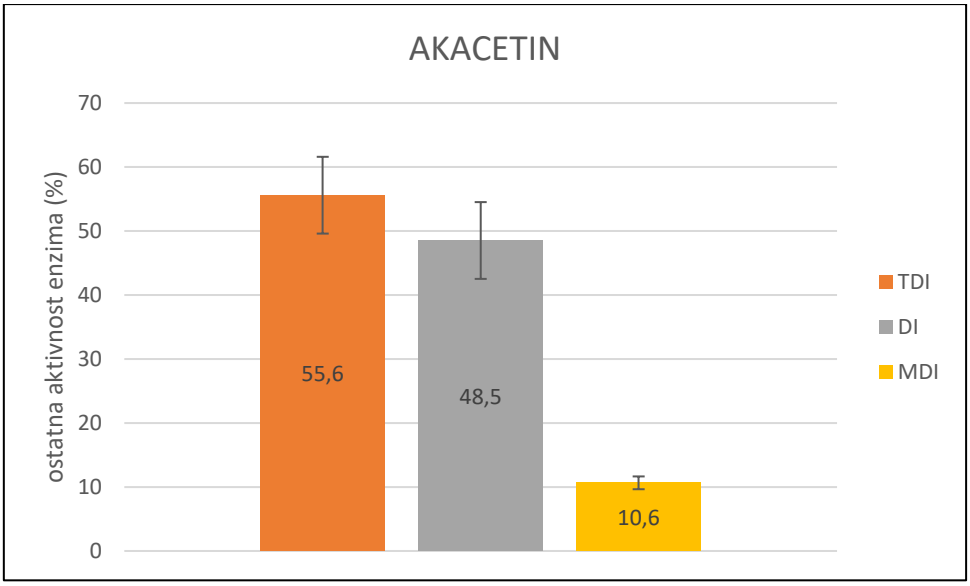
4. REZULTATI

4.1. Određivanje vrste inhibicije CYP3A4 enzima

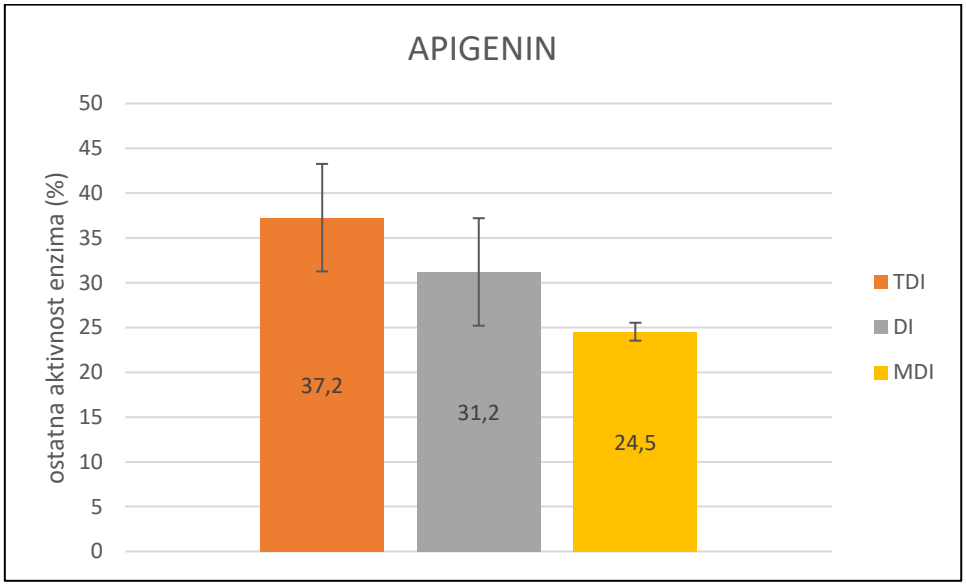
Kako bi se odredila vrsta inhibicije kojom flavonoidi inaktiviraju CYP3A4 enzim provedene su tri vrste ispitivanja; ispitivanje izravne, o vremenu i o metabolizmu ovisne inhibicije. Ispitivanja ostatne aktivnosti enzima su provedena praćenjem hidroksilacije testosterona (slika 12). Iako je većina flavonoida pri koncentraciji od 1µM pokazala inhibicijski učinak u sve tri vrste ispitivanja, značajan inhibicijski učinak uočen je kod metaboličke inhibicije (slike 13-16). Najveća inhibicija aktivnosti CYP3A4 enzima uočena je kod akacetina, pri čemu je ostatna aktivnost enzima iznosila 10,6 ± 1,3% u metabolizmu ovisnoj inhibiciji. Pri ispitivanju izravne i o vremenu ovisne inhibicije ostatna aktivnost enzima iznosila je (31,2 ± 1,8%, odnosno 37,2 ± 4,4%) (tablica 2).



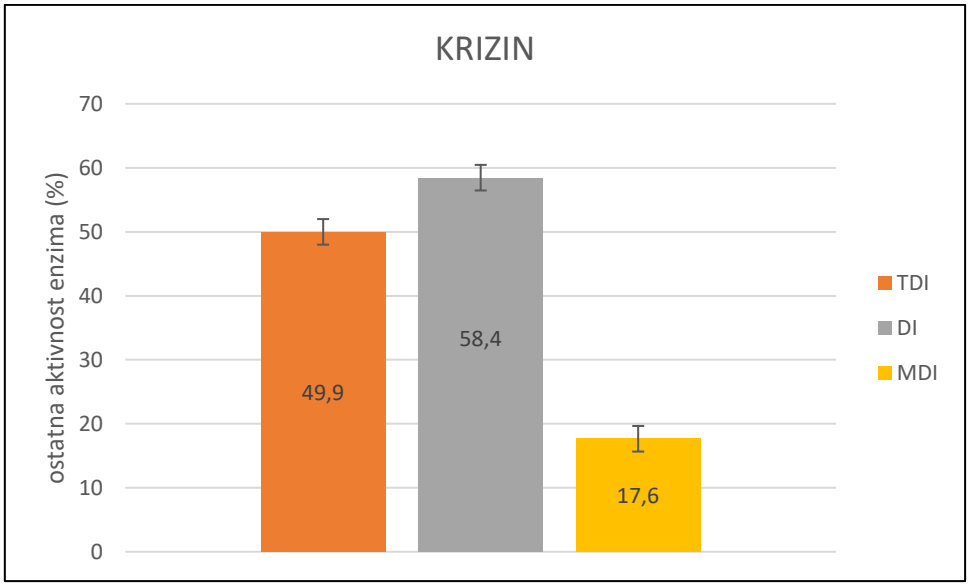
Slika 12. Primjer kromatograma s HPLC analize; a – 6β-hidroksilacija testosterona bez prisustva flavonoida, uočava se signal testosterona i 6β-hidroksitestosterona; b - 6β-hidroksilacija testosterona uz flavonoid kao inhibitor, dolazi do smanjenja ili potpunog nestanka 6β-hidroksitestosterona, uslijed nemogućnosti enzima da katalizira reakciju



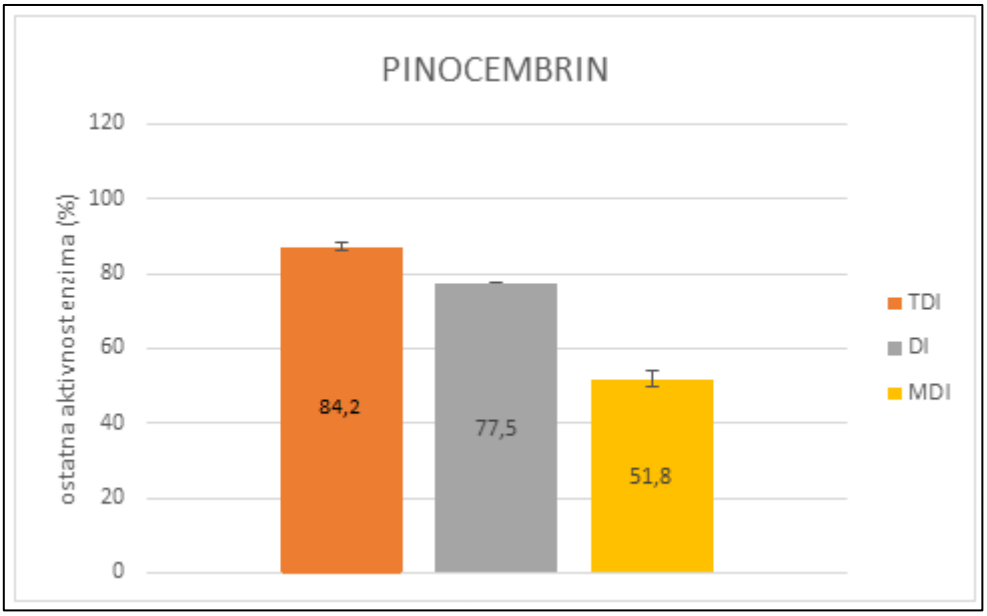
Slika 13. Inhibicijski učinak akacetina (1 µM) na CYP3A4 enzimu; KON – kontrola, TDI – o vremenu ovisna inhibicija, DI – izravna inhibicija, MDI – o metabolizmu ovisna inhibicija



Slika 14. Inhibicijski učinak apigenina (1 µM) na CYP3A4 enzimu; KON – kontrola, TDI – o vremenu ovisna inhibicija, DI – izravna inhibicija, MDI – o metabolizmu ovisna inhibicija



Slika 15. Inhibicijski učinak krizina (1 µM) na CYP3A4 enzimu; KON – kontrola, TDI – o vremenu ovisna inhibicija, DI – izravna inhibicija, MDI – o metabolizmu ovisna inhibicija



Slika 16. Inhibicijski učinak pinocembrin (1 µM) na CYP3A4 enzimu; KON – kontrola, TDI – o vremenu ovisna inhibicija, DI – izravna inhibicija, MDI – o metabolizmu ovisna inhibicija

Tablica 2. Ostatna aktivnost nakon inkubacije flavonoida s CYP3A4 (%)

Vrsta inhibicije	AKACETIN	APIGENIN	KRIZIN	PINOCEMBRIN
TDI	55,6 ± 6,4	37,2 ± 4,4	49,9 ± 2,9	84,2 ± 1,3
DI	48,5 ± 6,5	31,2 ± 1,8	58,4 ± 2,6	77,5 ± 0,8
MDI	10,6 ± 1,3	24,5 ± 4,3	17,6 ± 2,0	51,8 ± 2,8

TDI – o vremenu ovisna inhibicija, DI – izravna inhibicija, MDI – o metabolizmu ovisna inhibicija

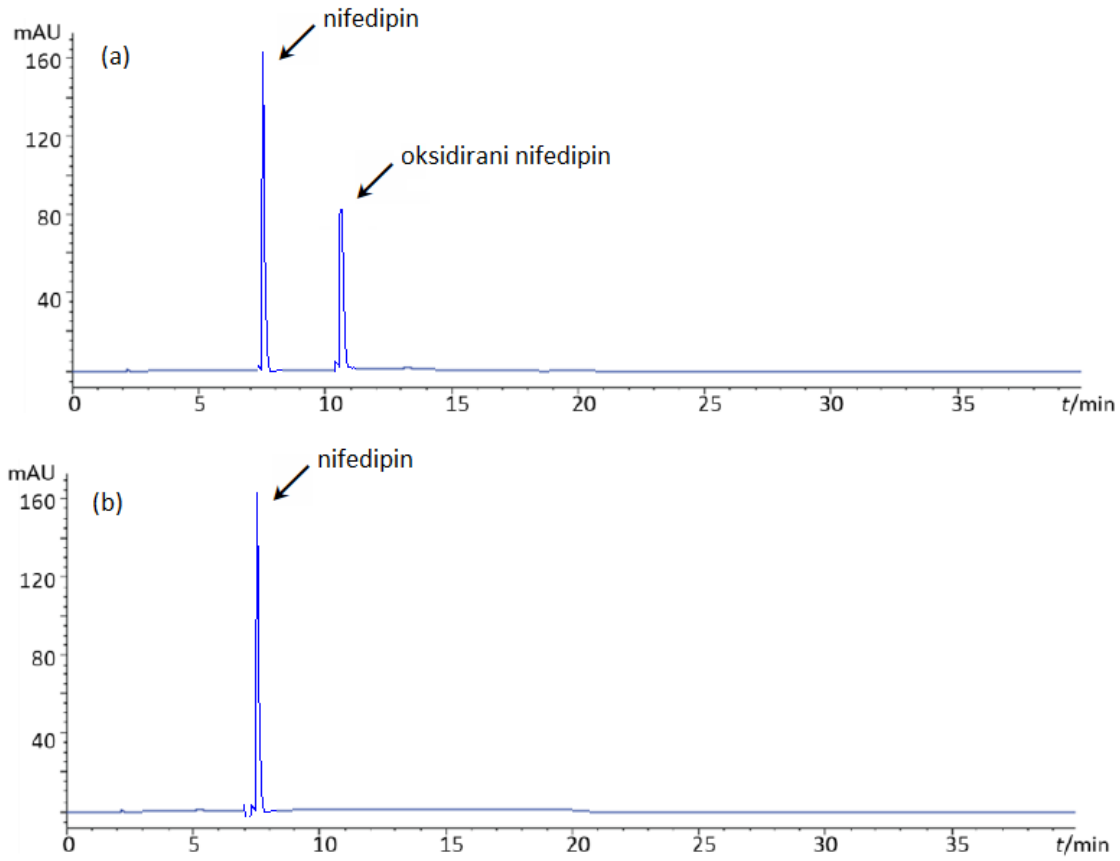
Apigenin je također pokazao najveću inhibiciju CYP3A4 enzima u metabolizmu ovisnoj inhibiciji, pri čemu je ostatna aktivnost enzima iznosila 24,5 ± 4,3%. Pri ispitivanju o vremenu ovisne i izravne inhibicije uočen je inhibicijski učinak apigenina koji se nije previše razlikovao između jedne i druge vrste inhibicije. Ostatna aktivnost enzima iznosila je 37,2 ± 4,4% za o vremenu ovisnu inhibiciju, odnosno 31,2 ± 1,8% za izravnu inhibiciju. Krizin je najveći inhibicijski učinak pokazao u metabolizmu ovisnoj inhibiciji (ostatna aktivnost enzima iznosila je 17,6 ± 2,0%. Zatim je najveći inhibicijski učinak krizina uočen u o vremenu ovisnoj inhibiciji (ostatna aktivnost enzima iznosila je 49,9 ± 2,9%), dok je u izravnoj inhibiciji pokazao najmanji inhibicijski učinak (ostatna aktivnost enzima iznosila je 58,4 ± 2,6%). Pinocembrin nije pokazao inhibicijski učinak na aktivnost CYP3A4 enzima u o vremenu ovisnoj inhibiciji (ostatna aktivnost enzima iznosila je 84,2 ± 1,3%). U izravnoj inhibiciji može se uočiti inhibicijski učinak (ostatna aktivnost enzima iznosila je 77,5 ± 0,8%). Najveći inhibicijski učinak pinocembrin je pokazao u o metabolizmu ovisnoj inhibiciji (ostatna aktivnost enzima iznosila je 51,8 ± 2,8%).

4.2. Kinetika inhibicije CYP3A4 flavonoidima

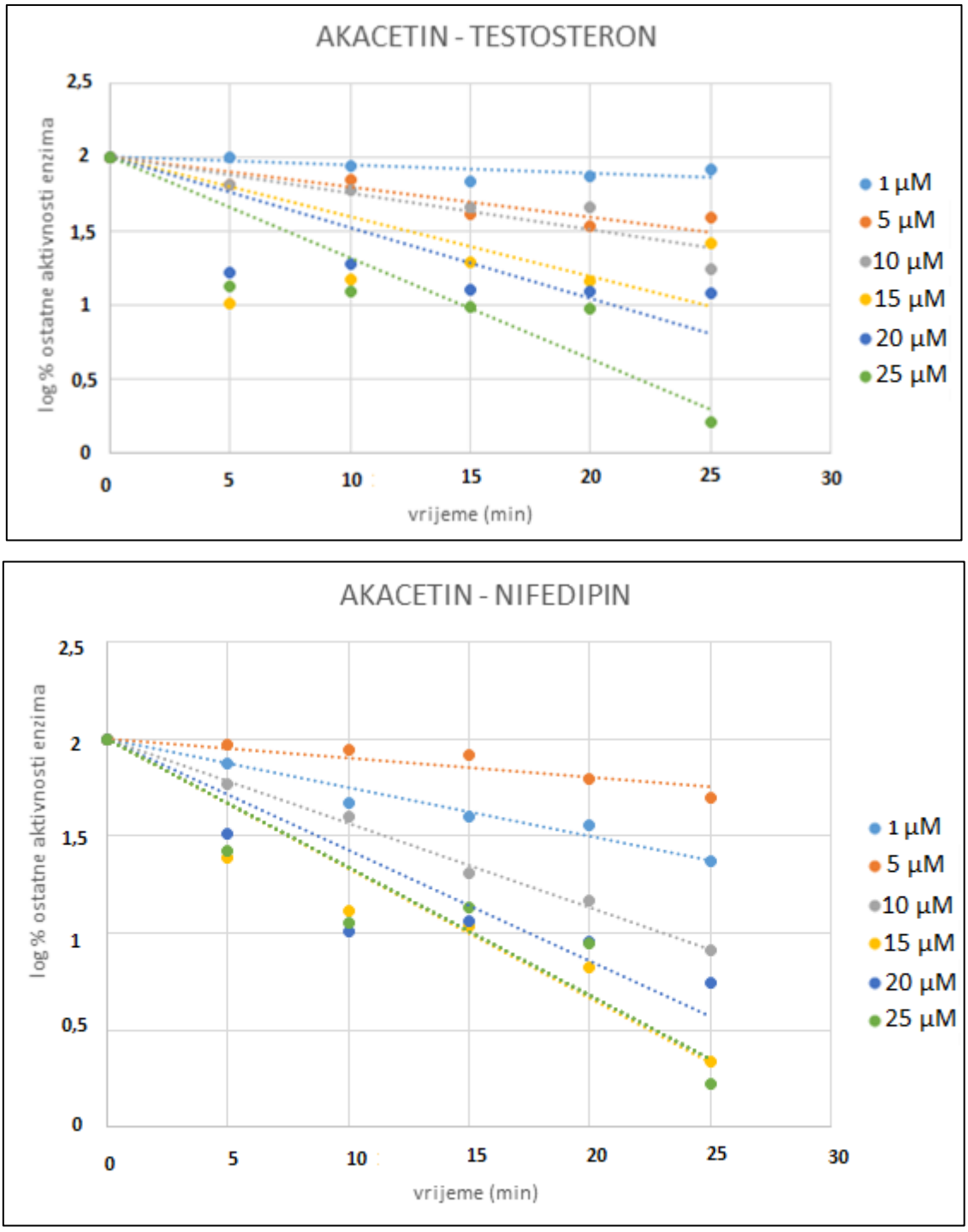
Kinetika inhibicije CYP3A4 flavonoidima ispitana je dodatkom različitih koncentracija inhibitora u različitim vremenskim razmacima, pri čemu je mjerenje ostatne aktivnosti enzima provedeno na ranije prikazani način. Određivanje inaktivacijske kinetike ispitano je uz pomoć marker supstrata testosterona i nifedipina (slika 17). Ispitivanja su provedena u duplikatu, a primjenjivali su se flavonoidi u koncentraciji od 1 μM do 25 μM, osim u slučaju krizina gdje su primijenjene i koncentracije od 0,01 μM

i 0,1 µM (slike 18-20). Na osnovu Michaelis-Mentenine jednadžbe izračunati su osnovni parametri inhibicijske kinetike enzima; konstanta inhibicije, konstanta brzine inhibicije, učinkovitost inaktivacije (omjer konstante brzine inaktivacije i konstante inhibicije) te IC_{50} vrijednosti na programu R.

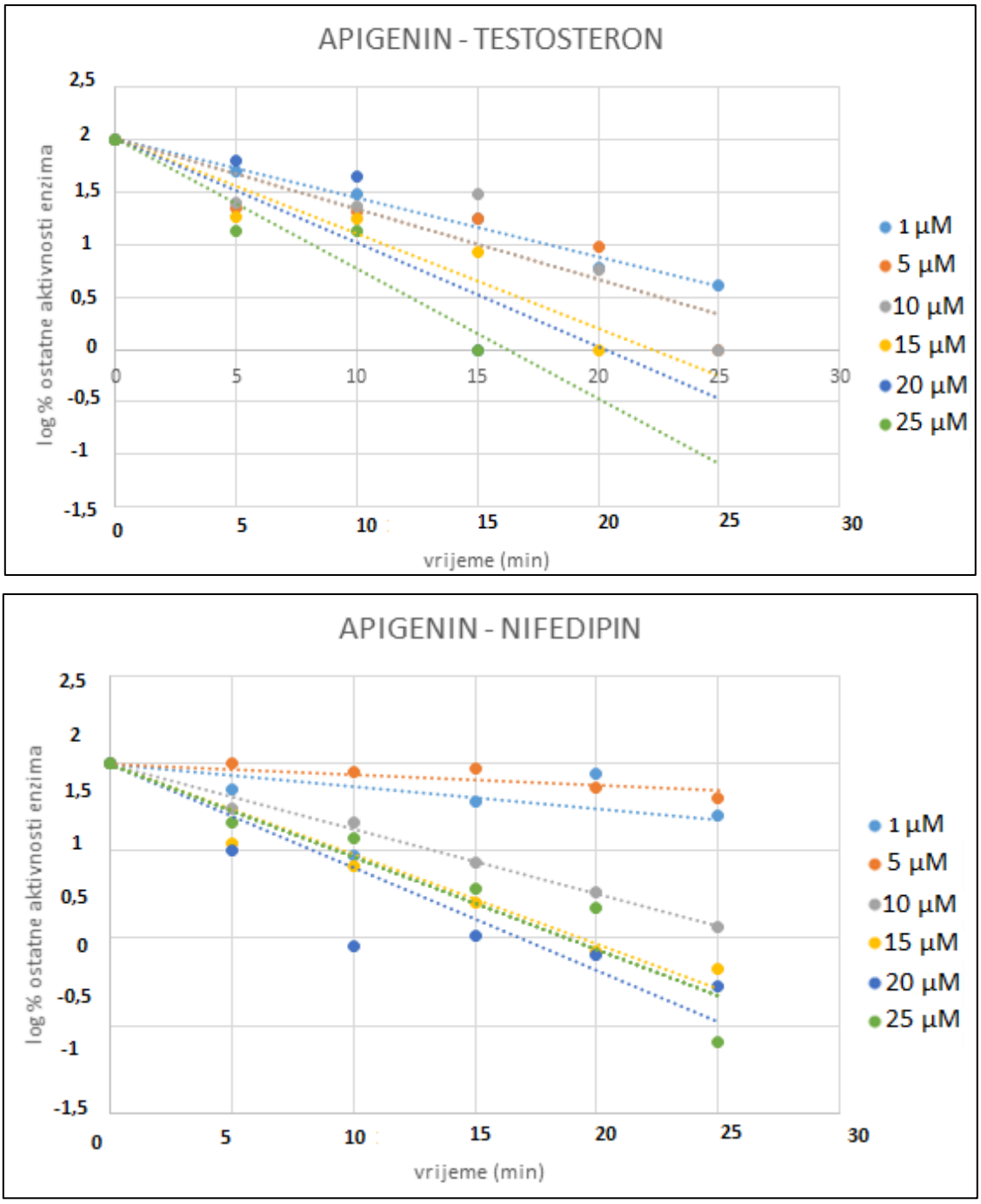
Krizin se pokazao kao inhibitor s najnižom IC_{50} vrijednošću (0,6 ± 0,5 µM), pinocembrin je pokazao 10 puta veću IC_{50} vrijednost (5,0 ± 0,6 µM), dok su akacetin i apigenin pokazali oko 20 puta veću IC_{50} vrijednost (10,9 ± 0,3 µM, odnosno 11,4 ± 0,4 µM), do 0,6 ± 0,5 µM) (slike 21-26; tablica 3).



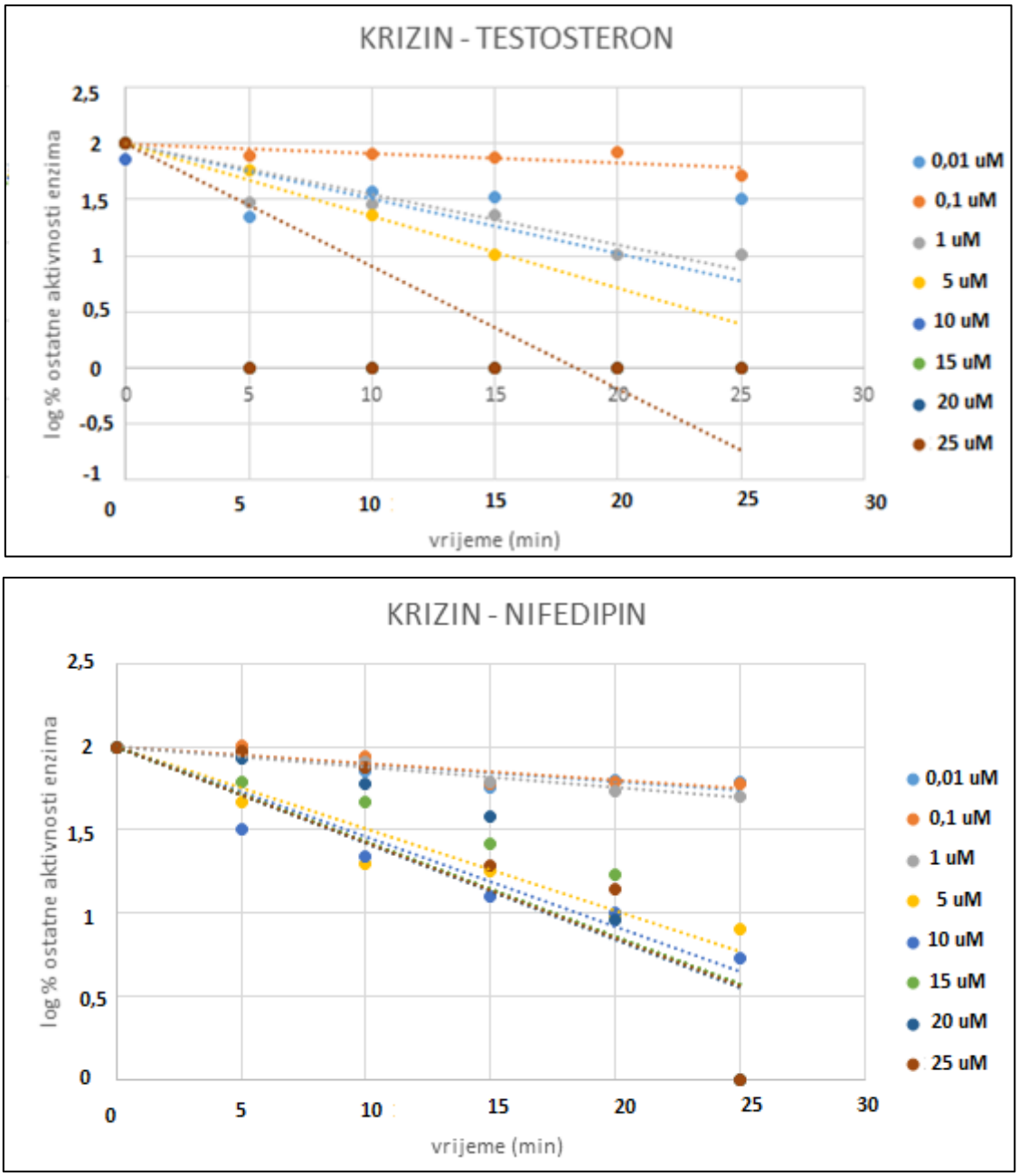
Slika 17. Primjer kromatograma s HPLC analize; a – oksidacija nifedipina bez prisustva flavonoida, uočava se signal nifedipina i oksidiranog nifedipina; b - oksidacija nifedipina uz flavonoid kao inhibitor, dolazi do smanjenja ili potpunog nestanka oksidiranog nifedipina, uslijed nemogućnosti enzima da katalizira reakciju



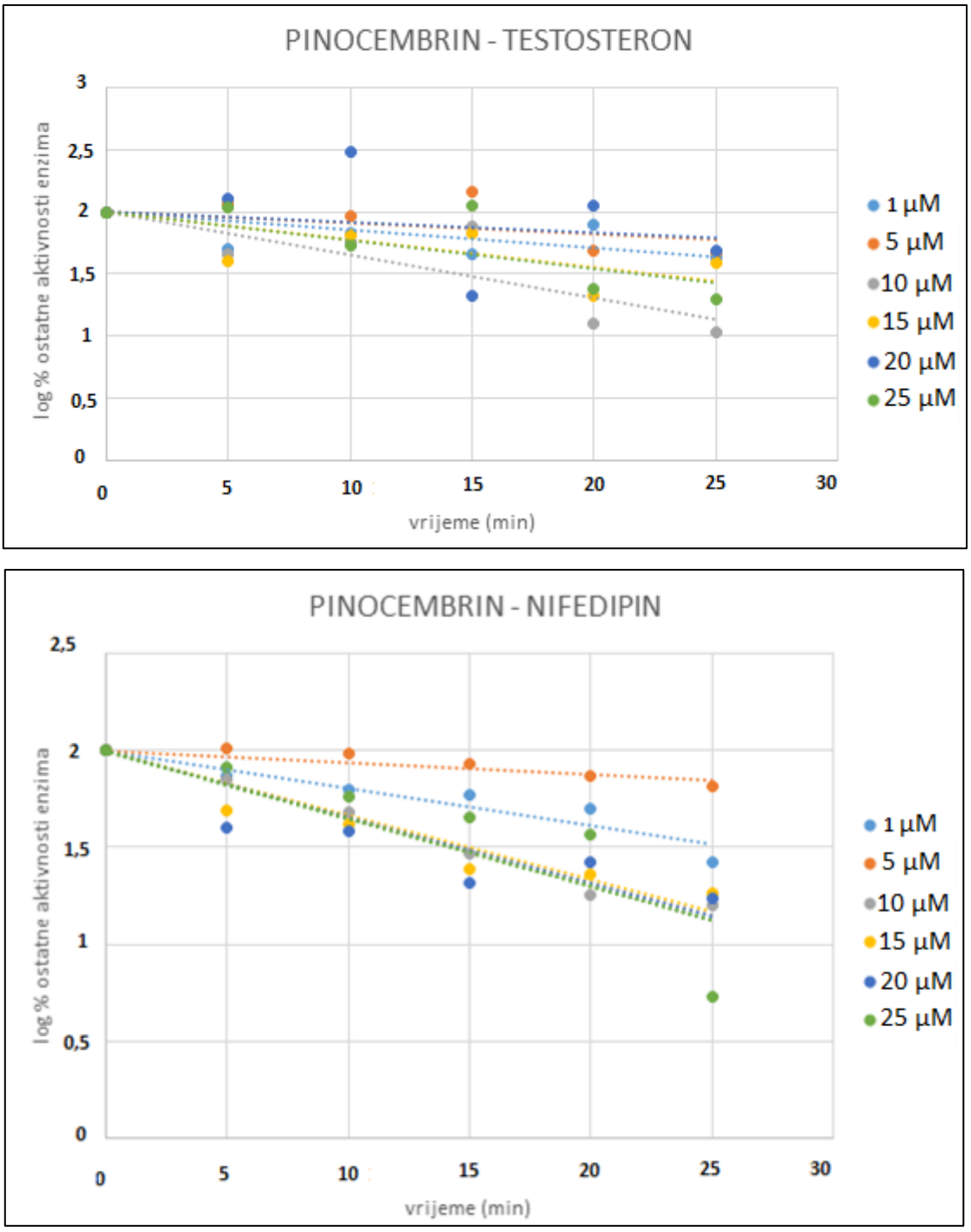
Slika 18. Zbirni prikaz inaktivacijske kinetike akacetina (1µM – 25 µM) na CYP3A4 enzimu, kao marker supstrat korišten testosteron (gore) i nifedipin (dolje)



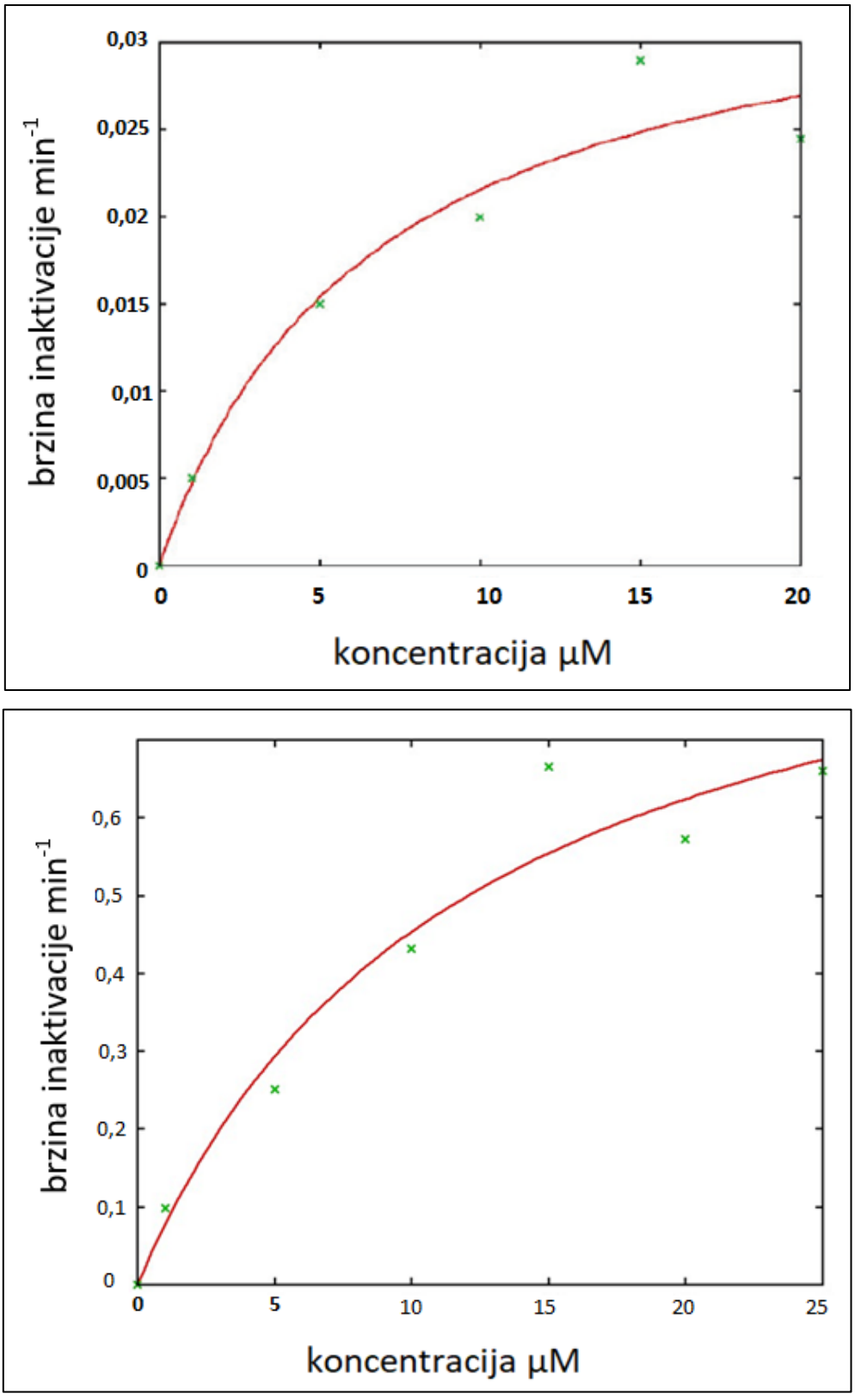
Slika 19. Zbirni prikaz inaktivacijske kinetike apigenina (1µM – 25 µM) na CYP3A4 enzimu, kao marker supstrat korišten testosteron (gore) i nifedipin (dolje)



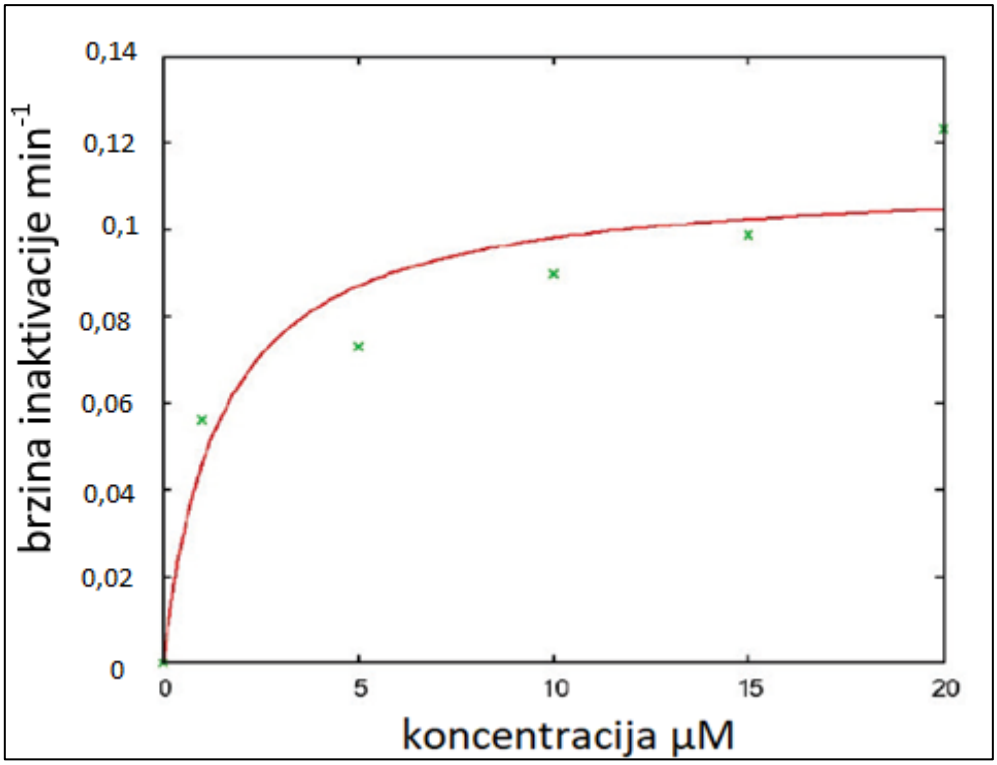
Slika 20. Zbirni prikaz inaktivacijske kinetike krizina (0,01 µM – 25 µM) na CYP3A4 enzimu, kao marker supstrat korišten testosteron (gore) i nifedipin (dolje)

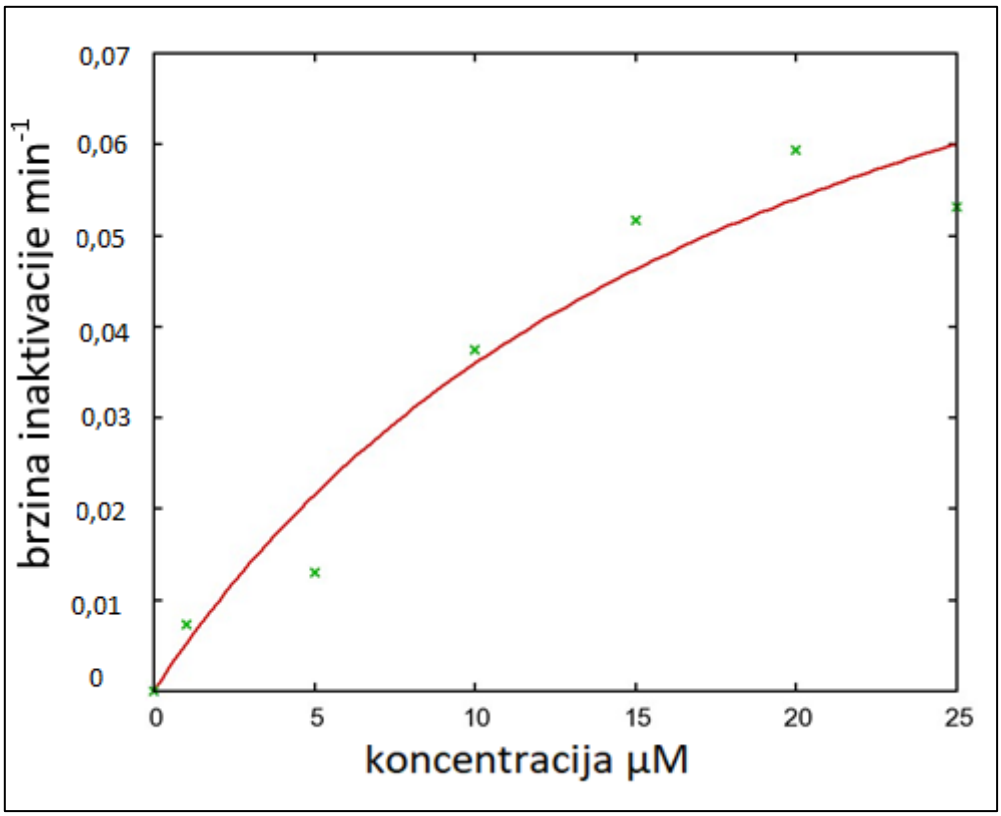


Slika 21. Zbirni prikaz inaktivacijske kinetike pinocembrina (1 µM – 25 µM) na CYP3A4 enzimu, kao marker supstrat korišten testosteron (gore) i nifedipin (dolje)

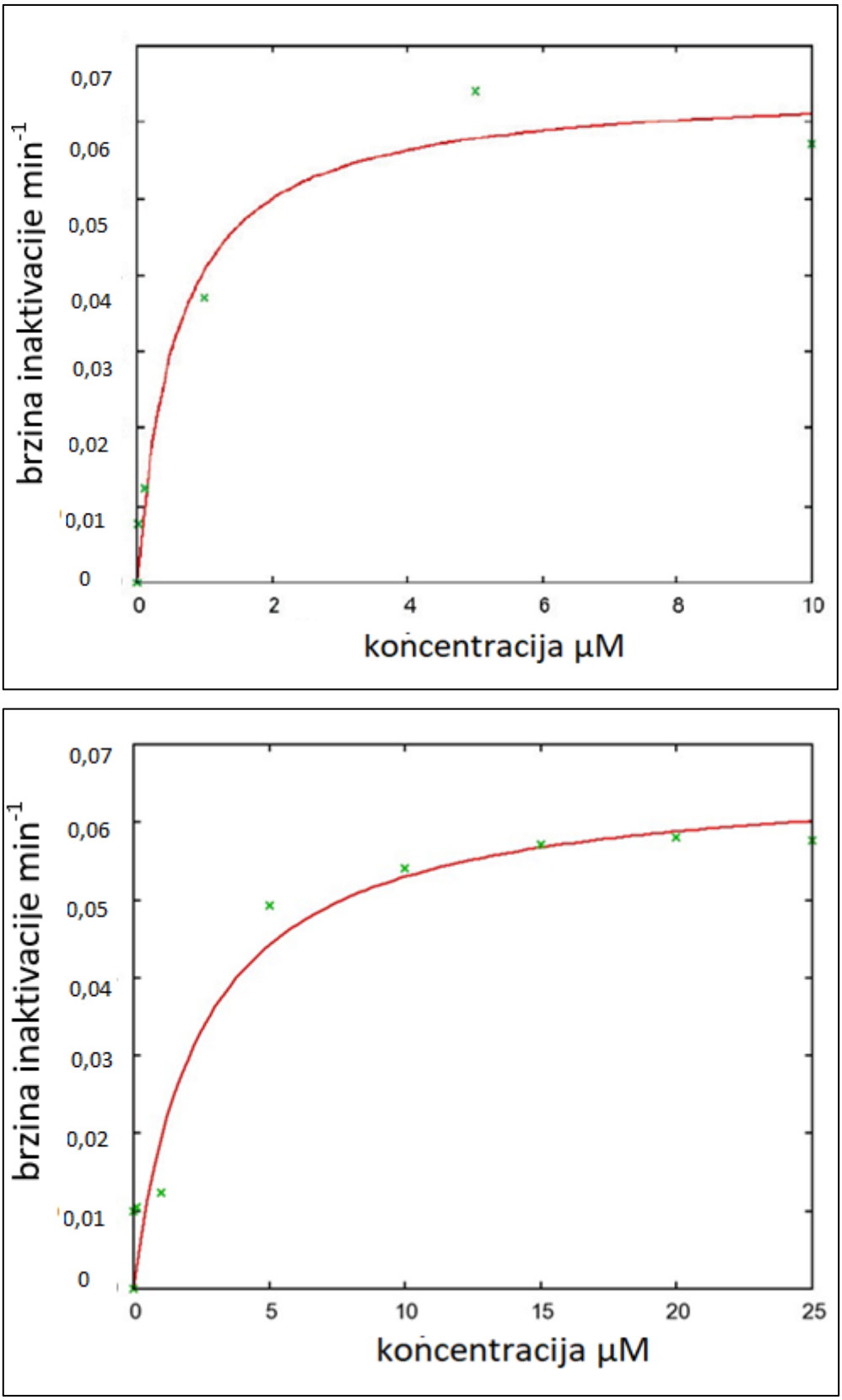


Slika 22. Kinetika inaktivacije CYP3A4 akacetinom; marker supstrati testosteron (gore) i nifedipin (dolje)

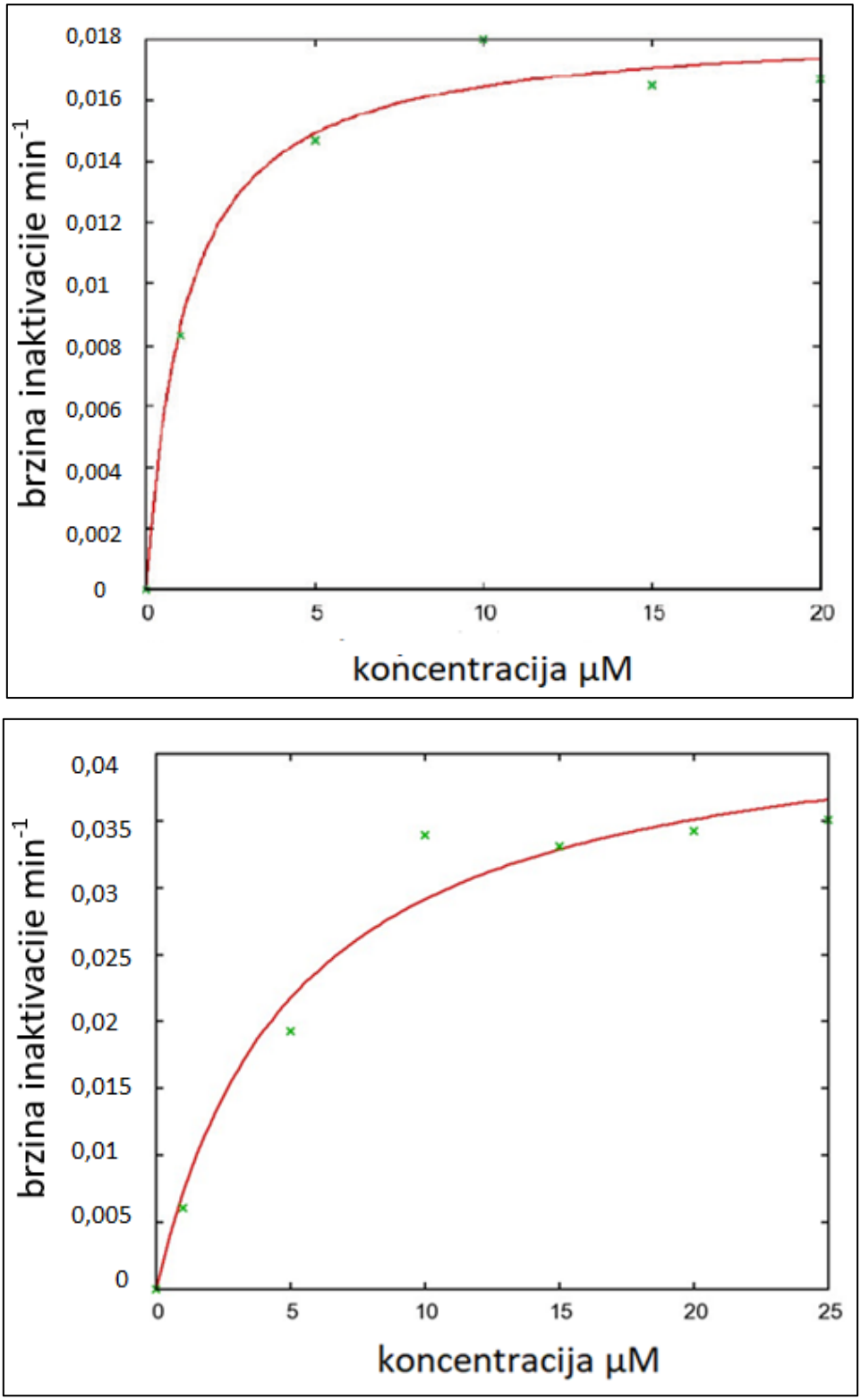




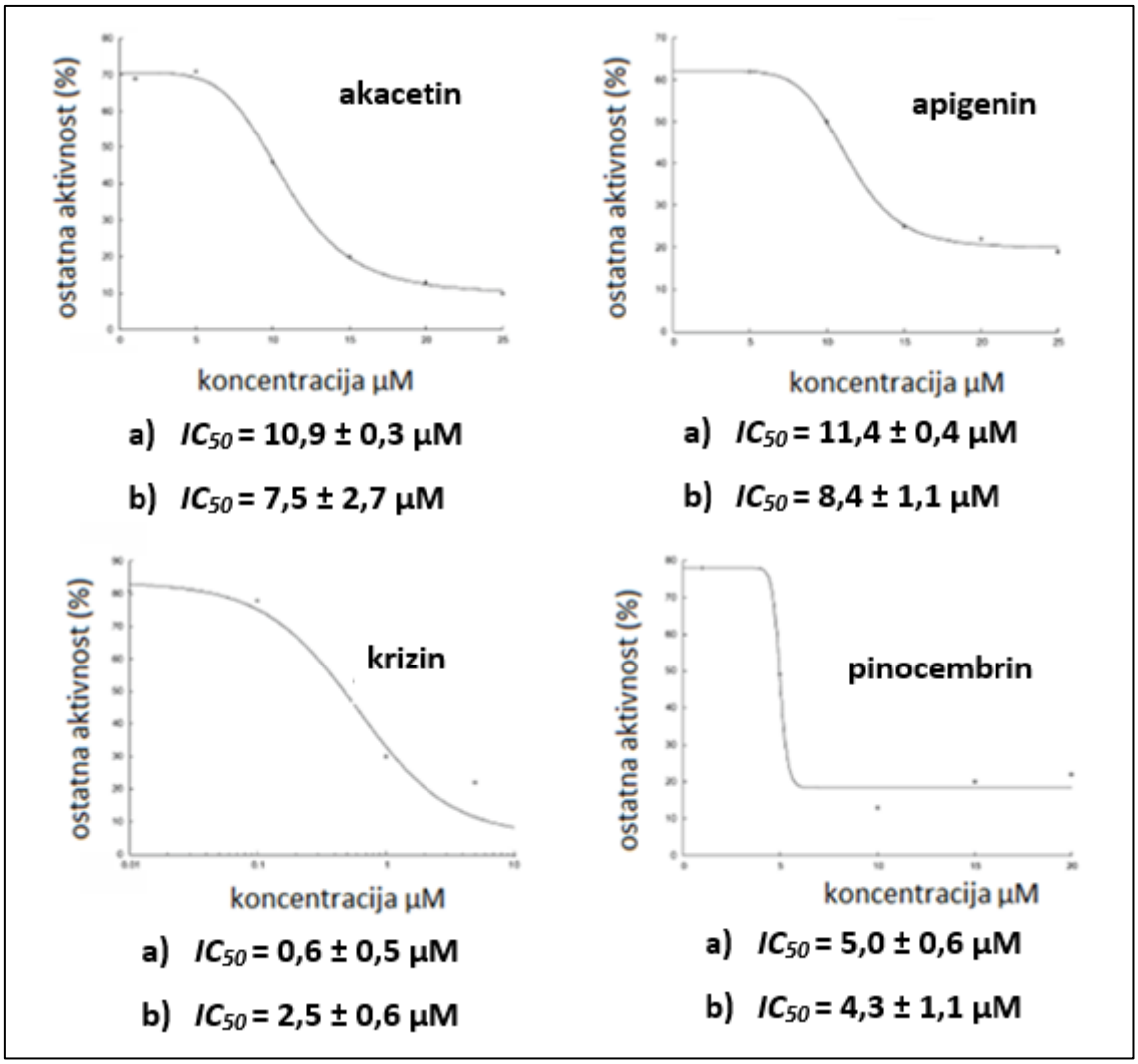
Slika 23. Kinetika inaktivacije CYP3A4 apigeninom; marker supstrati testosteron (gore) i nifedipin (dolje)



Slika 24. Kinetika inaktivacije CYP3A4 krizinom; marker supstrati testosteron (gore) i nifedipin (dolje)



Slika 25. Kinetika inaktivacije CYP3A4 pinocembrinom; marker supstrati testosteron (gore) i nifedipin (dolje)



Slika 26. Vrijednosti IC_{50} koncentracija; marker supstrat testosteron (a) i nifedipin (b)

Ponovljeno ispitivanje IC_{50} flavonoida s nifedipinom kao marker supstratom dalo je ponešto drugačije rezultate. Najmanja IC_{50} vrijednost utvrđena je za krizin (2,5 ± 0,6 µM). Za pinocembrin je IC_{50} vrijednost iznosila 4,3 ± 1,1 µM, a za apigenin i akacetin 8,4 ± 1,1 µM i 7,5 ± 2,7 µM. Prema ranije opisanoj metodi utvrđene su i konstante inhibicije za pojedine flavonoide. Na ispitivanju provedenom s testosteronom kao marker supstratom za akacetin, apigenin, krizin i pinocembrin utvrđene su sljedeće K_i vrijednosti: 6,0 ± 3,0 µM, 1,5 ± 0,8 µM, 0,6 ± 0,3 µM i 1,2 ± 0,3 µM. Na ponovljenom ispitivanju konstanti inhibicije pojedinih flavonoida koristeći nifedipin kao marker supstrat, za akacetin, apigenin, krizin i pinocembrin utvrđene su sljedeće K_i vrijednosti: 12,1 ± 5,6 µM, 20,2 ± 12,7 µM, 2,5 ± 1,0 µM i 5,1 ± 1,6 µM. Za flavonoide je određena i konstanta brzine inaktivacije (k_{inact}). Uz testosteron kao marker supstrat određene su konstante

brzine inaktivacije za akacetin, apigenin, krizin i pinocembrin 0,036 ± 0,006 min^{-1}, 0,11 ± 0,01 min^{-1}, 0,065 ± 0,005 min^{-1} i 0,018 ± 0,001 min^{-1}. Uz ponovljeno ispitivanje s nifedipinom kao marker supstratom određene su sljedeće vrijednosti konstante brzine inaktivacije za akacetin, apigenin krizin i pinocembrin: 0,10 ± 0,02 min^{-1}, 0,11 ± 0,04 min^{-1}, 0,07 ± 0,01 min^{-1} i 0,04 ± 0,01 min^{-1}. Učinkovitost inaktivacije određena je za svaki flavonoid kao omjer konstante brzine inaktivacije i konstante inhibicije. Pri ispitivanju s testosteronom kao marker supstratom uočene su sljedeće vrijednosti učinkovitosti inaktivacije za akacetin, apigenin, krizin i pinocembrin: 0,006 min^{-1} μM^{-1}, 0,073 min^{-1} μM^{-1}, 0,108 min^{-1} μM^{-1} i 0,015 min^{-1} μM^{-1}. U ponovljenom ispitivanju s nifedipinom kao marker supstratom uočene su sljedeće vrijednosti učinkovitosti inaktivacije za akacetin, apigenin, krizin i pinocembrin: 0,01 min^{-1} μM^{-1}, 0,01 min^{-1} μM^{-1}, 0,03 min^{-1} μM^{-1} i 0,01 min^{-1} μM^{-1} (tablica 3).

Tablica 3. Osnovni parametri inhibicije enzima CYP3A4 pojedinim flavonoidima

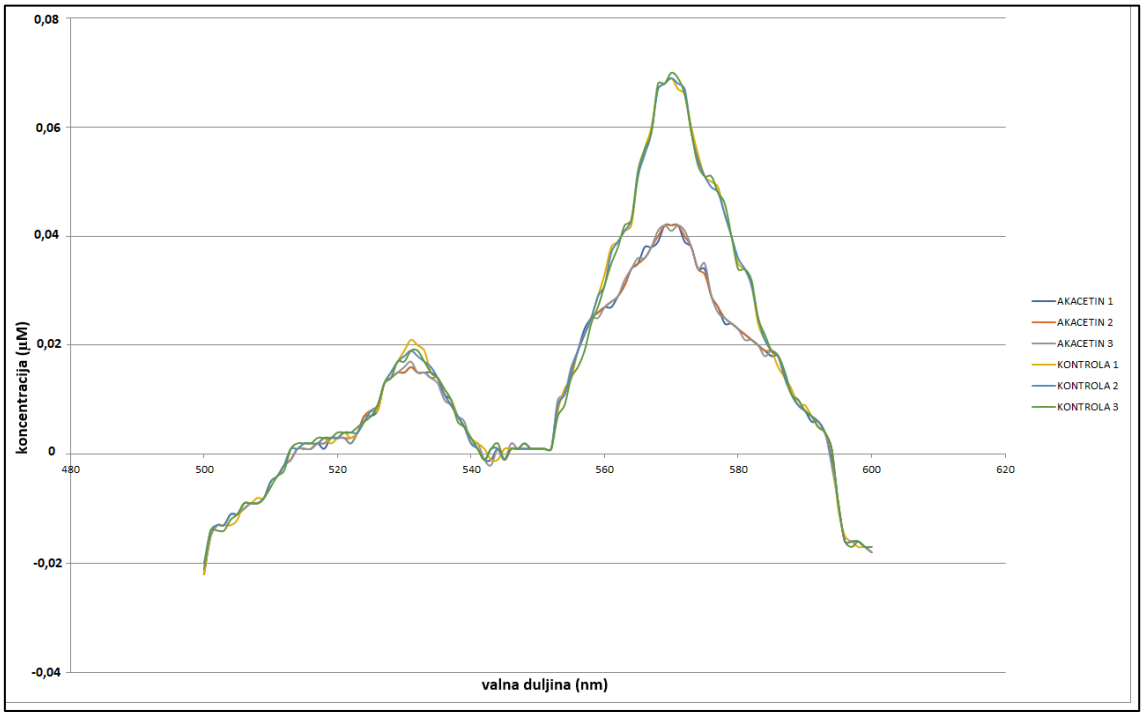
	akacetin	**apigenin**	**krizin**	**pinocembrin**
T-IC_{50} (μM)	10,9 ± 0,3	11,4 ± 0,4	0,6 ± 0,5	5,0 ± 0,6
T-K_i (μM)	6,0 ± 3,0	1,5 ± 0,8	0,6 ± 0,3	1,2 ± 0,3
T-k_{inact} (min^{-1})	0,036 ± 0,006	0,11 ± 0,01	0,065 ± 0,005	0,018 ± 0,001
T-k_{inact}/K_i (min^{-1} μM^{-1})	0,006	0,073	0,108	0,015
N-IC_{50} (μM)	7,5 ± 2,7	8,4 ± 1,1	2,5 ± 0,6	4,3 ± 1,1
N-K_i (μM)	12,1 ± 5,6	20,2 ± 12,7	2,4 ± 1,0	5,1 ± 1,6
N-k_{inact} (min^{-1})	0,10 ± 0,02	0,11 ± 0,04	0,07 ± 0,01	0,04 ± 0,01
N-k_{inact}/K_i (min^{-1} μM^{-1})	0,01	0,01	0,03	0,01

T- ili N-IC_{50} – polovica maksimalne inhibitorne koncentracije određena uz supstrat testosteron, odnosno nifedipin, T- ili N-K_i – konstanta inhibicije uz supstrat testosteron, odnosno nifedipin, T- ili N-k_{inact} – konstanta brzine inaktivacije uz supstrat testosteron, odnosno nifedipin, T- ili N-k_{inact}/K_i – učinkovitost inaktivacije uz supstrat testosteron, odnosno nifedipin

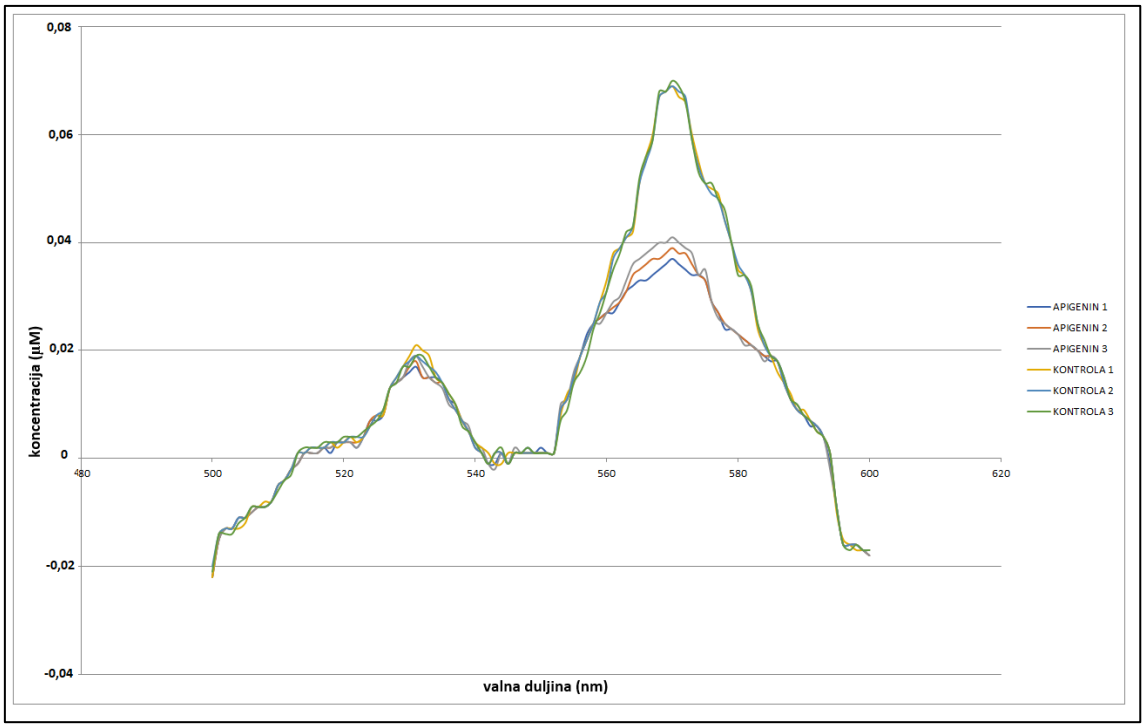
4.3. Hemokrom-piridin test

Hemokrom-piridin test služi za određivanje kovalentnog vezanja reaktivnih međuprodukata s protoporfirinskim dijelom hema. Pri ispitivanju su uočeni maksimumi

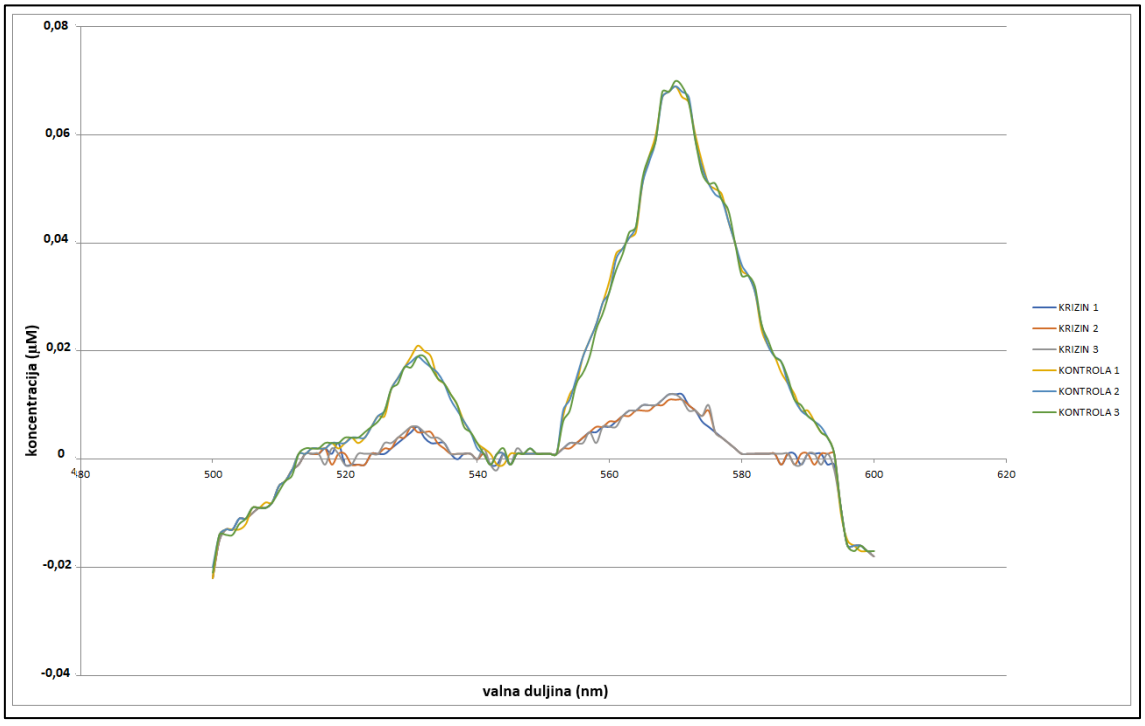
apsorpcije na 531 nm i 570 nm. Korištenjem svih flavonoida kao inhibitora (akacetin, apigenin, krizin i pinocembrin) uočeno je smanjenje količine hema (slike 27-31), koje je izračunato koristeći postavljeni baždarni pravac. Ispitivanja su zatim potvrđena dodatnom inkubacijom s katalazom i superoksid dismutazom, pri čemu je potvrđeno smanjenje količine hema (slike 32-36). Inkubacijom s akacetinom došlo je do smanjenja količine hema za 51,12%, apigeninom za 54,95%, krizinom za 94,5% i pinocembrinom za 74,73% (tablica 4).



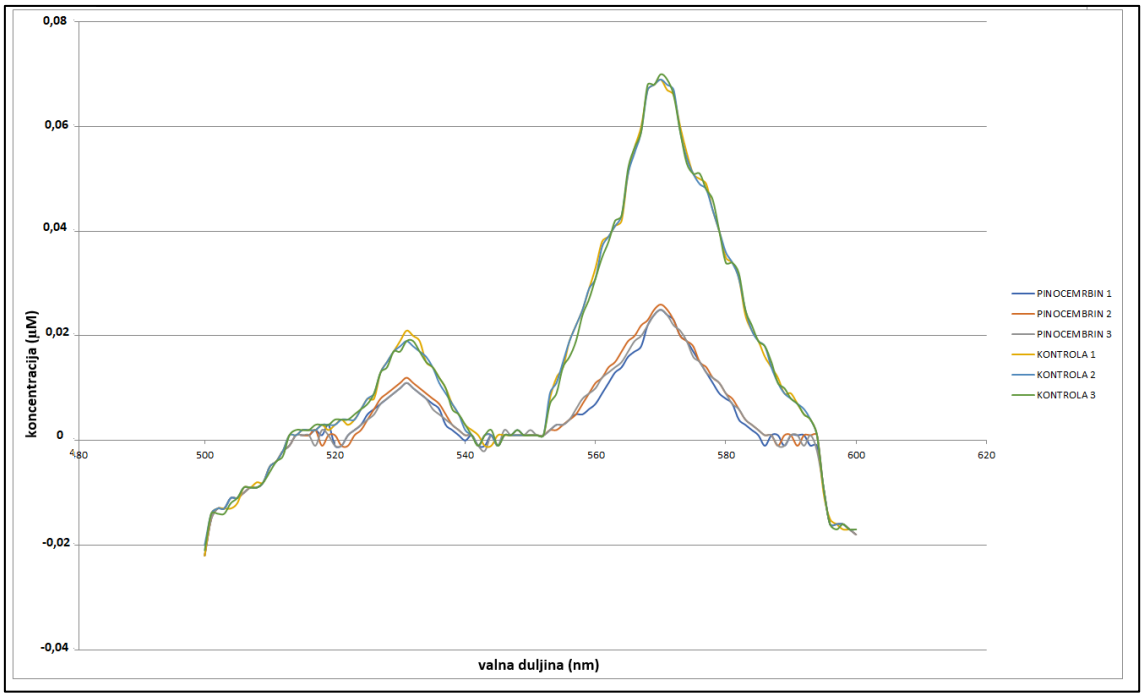
Slika 27. Smanjenje koncentracije hema uslijed inkubacije s akacetinom (25 µM)



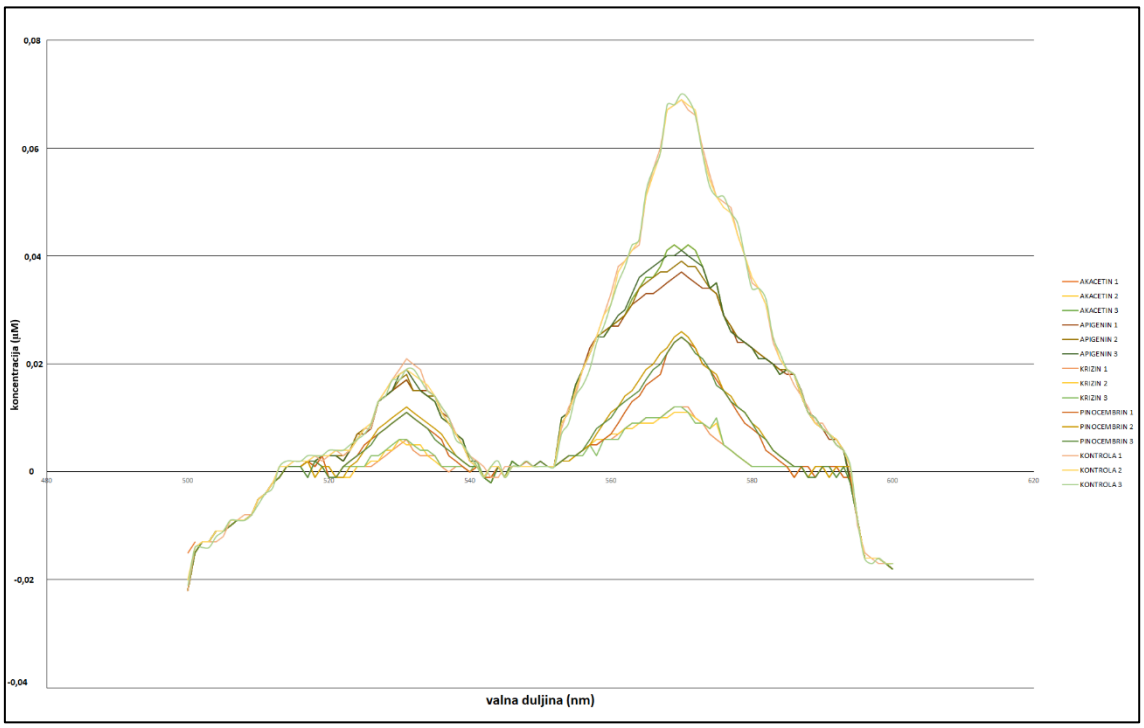
Slika 28. Smanjenje koncentracije hema uslijed inkubacije s apigeninom (25 µM)



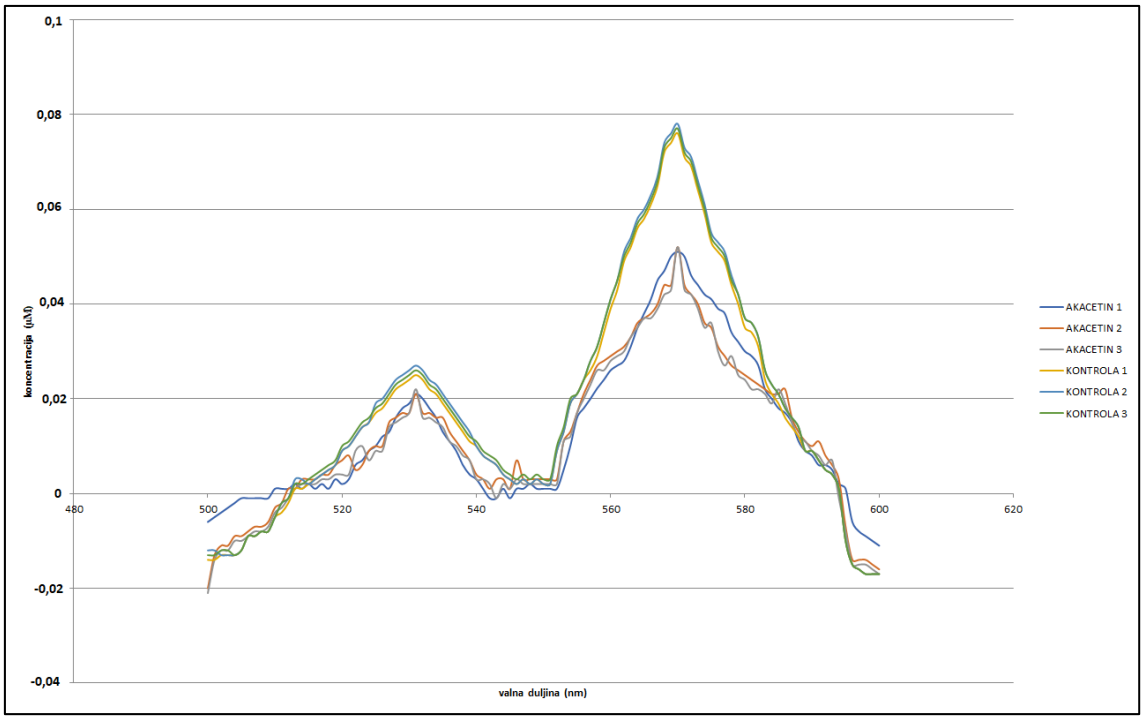
Slika 29. Smanjenje koncentracije hema uslijed inkubacije s krizinom (25 µM)



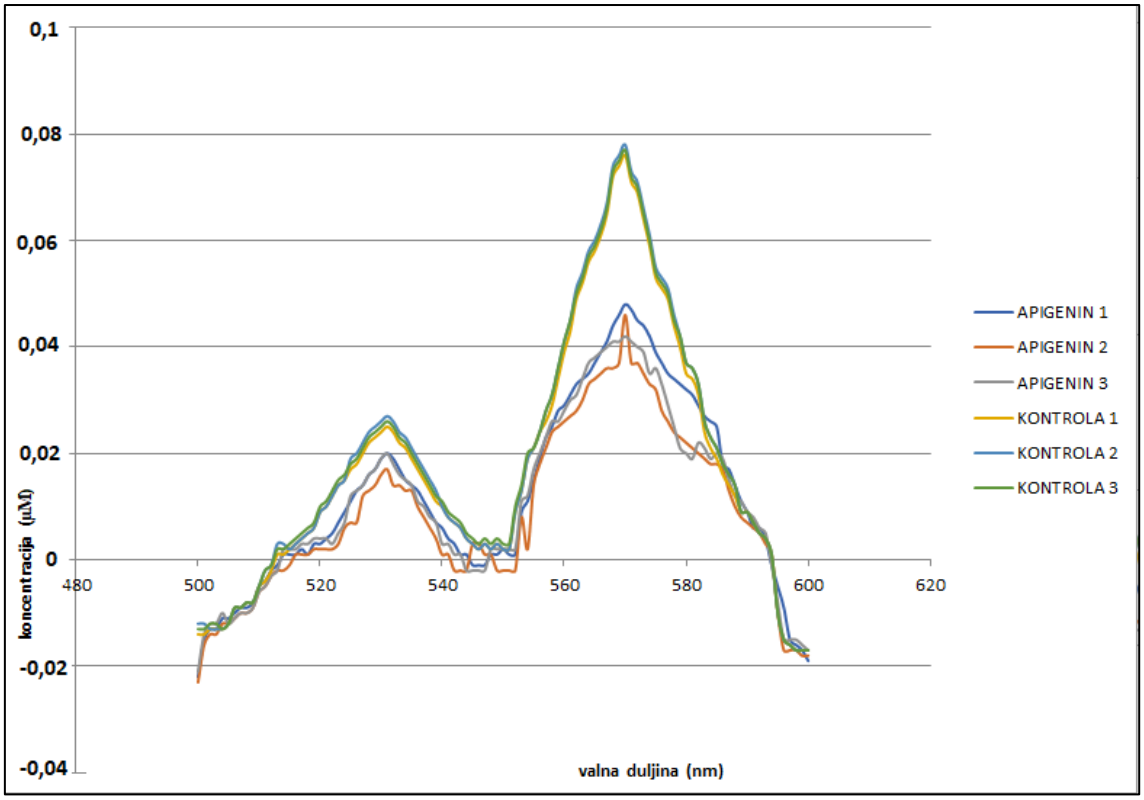
Slika 30. Smanjenje koncentracije hema uslijed inkubacije s pinocembrinom (25 µM)



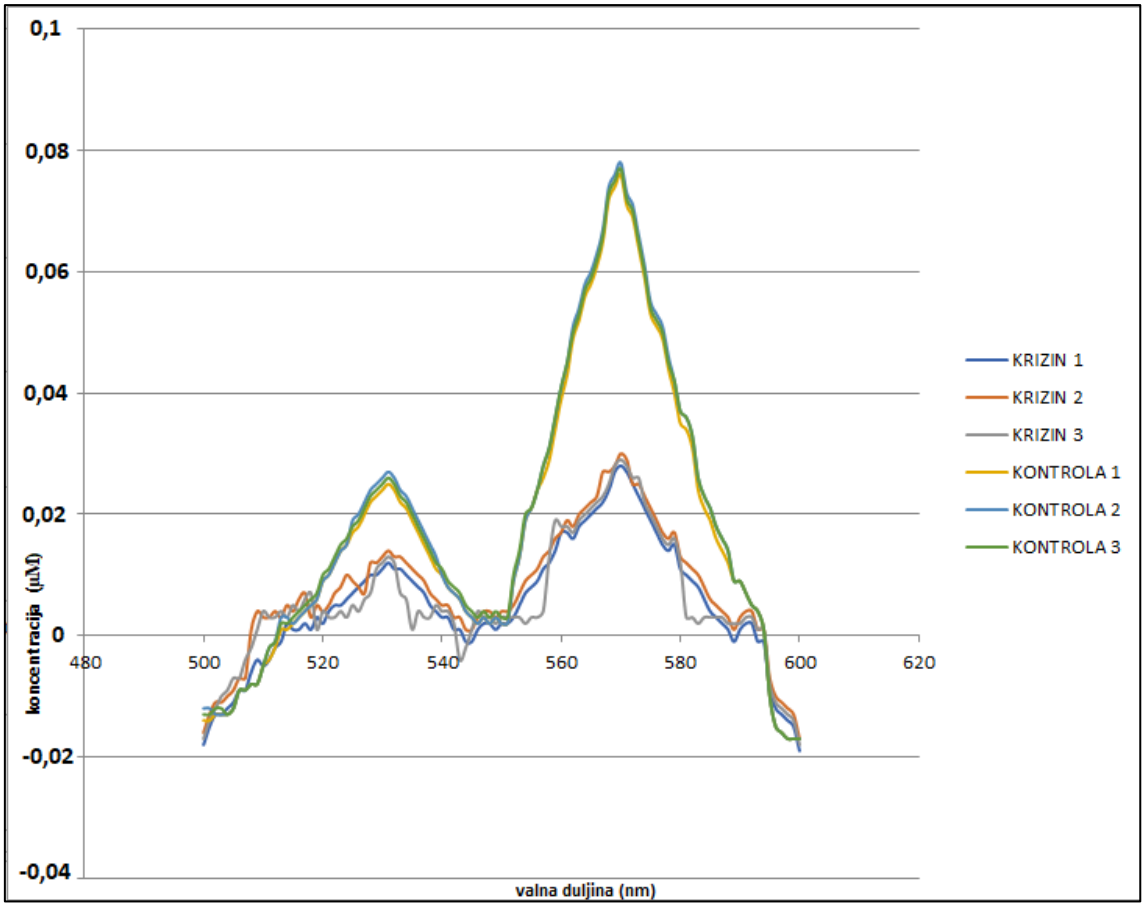
Slika 31. Zbirni prikaz smanjenja koncentracije hema uslijed inkubacije s flavonoidima (25 µM)



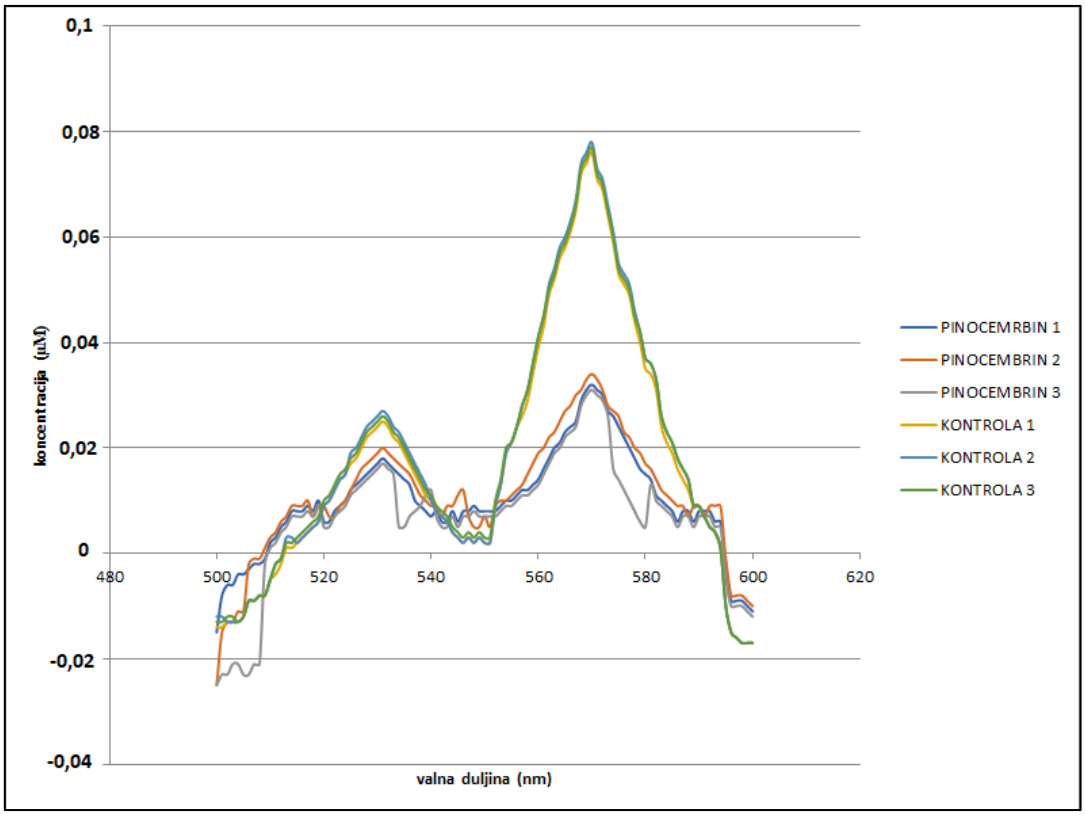
Slika 32. Smanjenje koncentracije hema uslijed inkubacije s akacetinom (25 µM), ponovljeno ispitivanje s dodatkom SOD i CAT



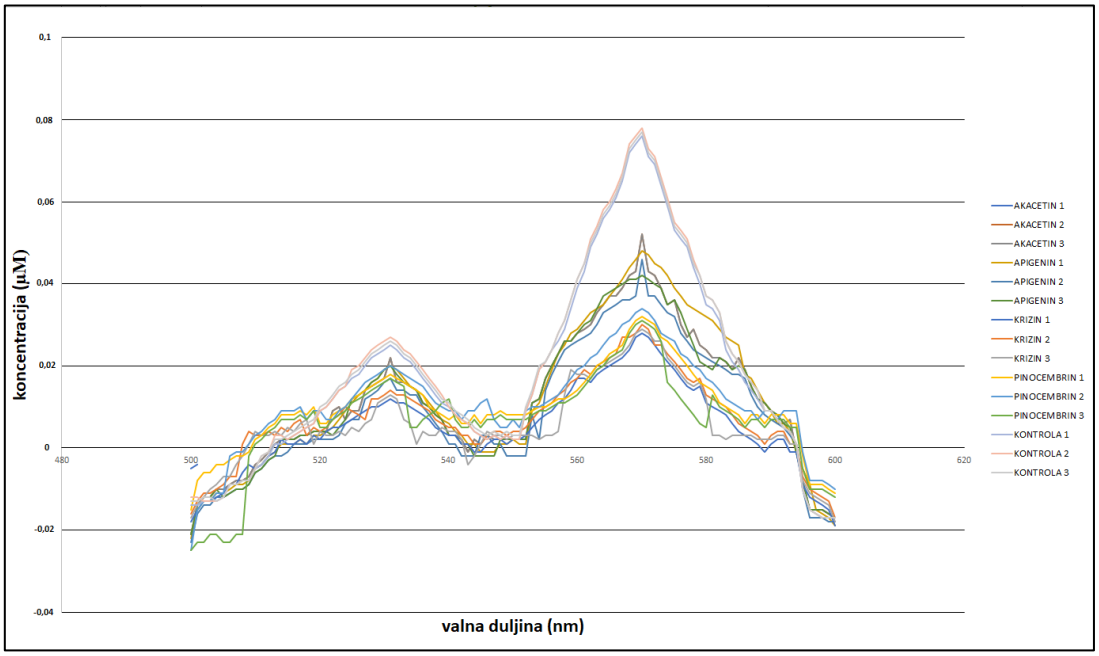
Slika 33. Smanjenje koncentracije hema uslijed inkubacije s apigeninom (25 µM), ponovljeno ispitivanje s dodatkom SOD i CAT



Slika 34. Smanjenje koncentracije hema uslijed inkubacije s krizinom (25 µM), ponovljeno ispitivanje s dodatkom SOD i CAT



Slika 35. Smanjenje koncentracije hema uslijed inkubacije s pinocembrinom (25 µM), ponovljeno ispitivanje s dodatkom SOD i CAT



Slika 36. Zbirni prikaz smanjenja koncentracije hema uslijed inkubacije s flavonoidima (25 µM), ponovljeno ispitivanje s dodatkom SOD i CAT

Svi su flavonoidi smanjili koncentraciju hema u ispitivanju s i bez dodatka SOD i CAT. Ostatna koncentracija hema nakon inkubacije s akacetinom bila je 48,88% te na ponovljenom ispitivanju uz dodatak SOD i CAT 63,33%. Ostatna koncentracija hema nakon inkubacije s apigeninom iznosila je 45,05% te na ponovljenom ispitivanju uz dodatak SOD i CAT 55,11%. Ostatna koncentracija hema nakon inkubacije s krizinom bila je 5,5% te na ponovljenom ispitivanju uz dodatak SOD i CAT 2,99%. Ostatna koncentracija hema nakon inkubacije s pinocembrinom bila je 25,27% te na ponovljenom ispitivanju uz dodatak SOD i CAT 35,33% (tablica 4).

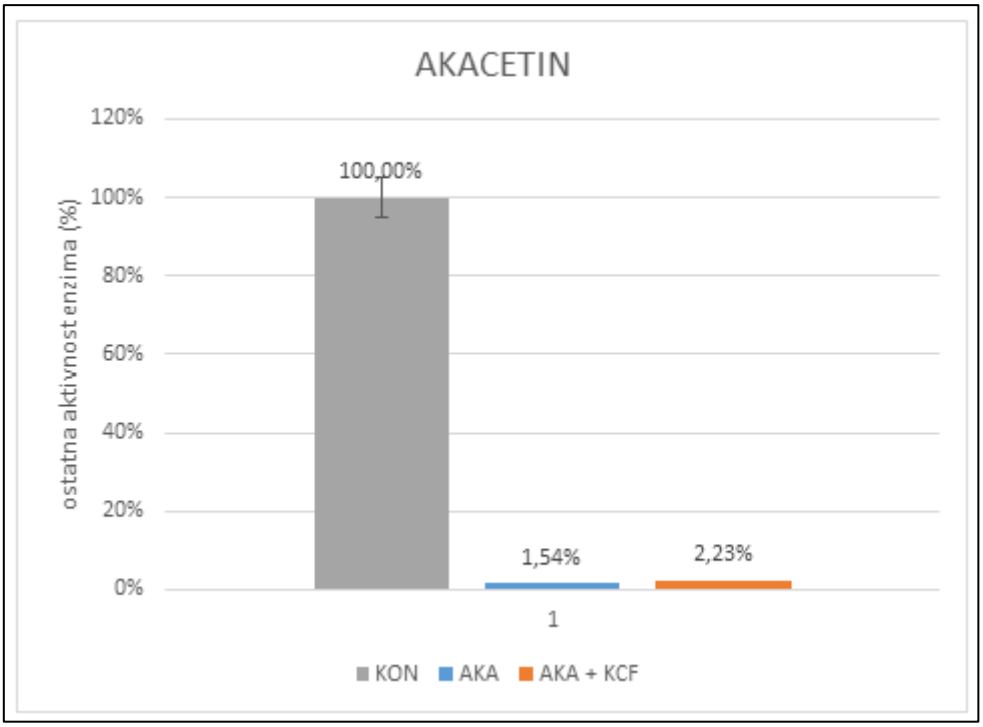
Tablica 4. Koncentracije hema nakon inkubacije s flavonoidom

Flavonoid	Koncentracija hema (%)	Srednja vrijednost	Koncentracija hema uz dodatak SOD i CAT (%)	Srednja vrijednost
Akacetin 1	49,3%		62,3%	
Akacetin 2	49,3%	48,8%	63,8%	63,3%
Akacetin 3	48,0%		63,8%	
Apigenin 1	42,1%		58,0%	
Apigenin 2	45,0%	45,1%	58,0%	55,1%
Apigenin 3	48,0%		49,3%	
Krizin 1	6,0%		2,8%	
Krizin 2	4,5%	5,5%	3,2%	2,9%
Krizin 3	6,0%		3,0%	
Pinocembrin 1	24,8%		34,8%	
Pinocembrin 2	26,1%	25,3%	37,6%	35,3%
Pinocembrin 3	24,8%		33,5%	

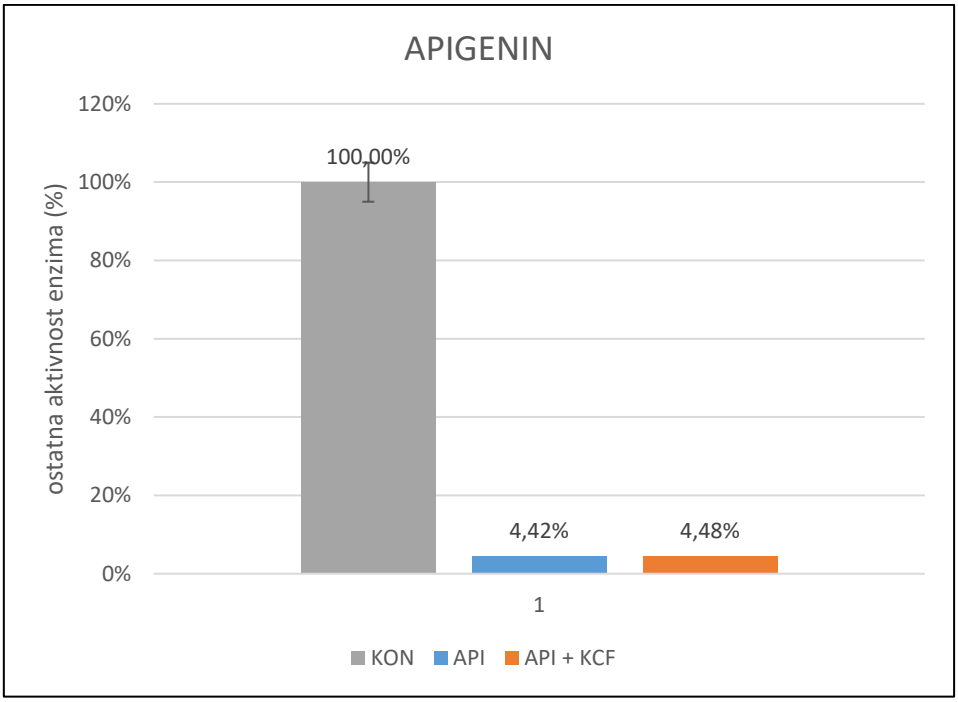
4.4. Ispitivanja pseudoireverzibilne inhibicije

Pri ispitivanju pseudoireverzibilne inhibicije, uzorci su inkubirani s flavonoidima te po potrebi tretirani s oksidansom, nakon čega su podvrgnuti dijalizi. Ukoliko je u pitanju pseudoireverzibilna inhibicija, enzim bi se trebao vratiti u aktivni oblik, što bi se dokazalo njegovom kasnijom katalizom hidroksilacije testosterona u 6β-hidroksitestosteron. U svim slučajevima došlo je do značajne inhibicije enzimske aktivnosti (slike 37-40). Niti u jednom slučaju razlika ostatne aktivnosti enzima između

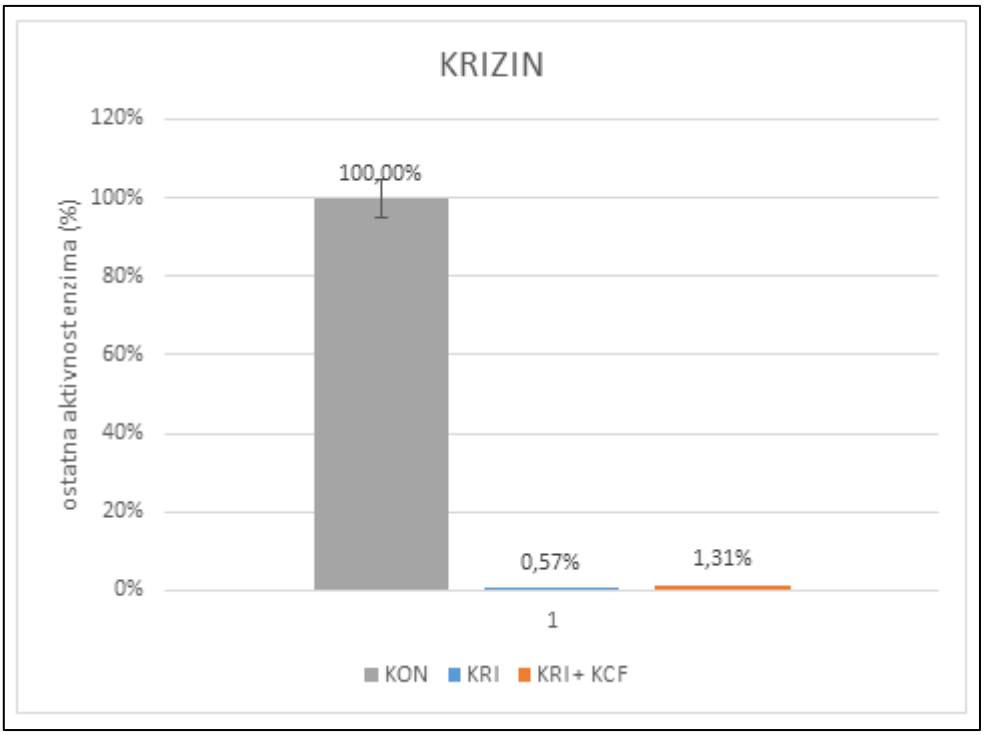
uzorka s flavonoidom i uzorka s kalijevim heksacijanoferatom nije bila statistički značajna ($p \leq 0,05$).



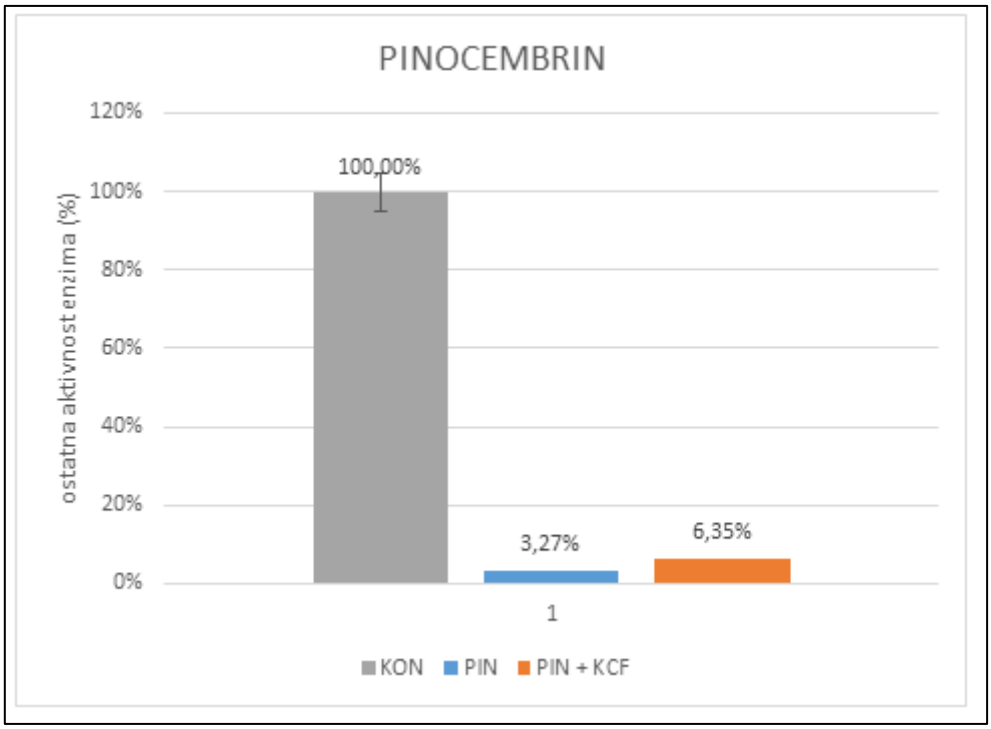
Slika 37. Ostatna aktivnost CYP3A4 enzima nakon inkubacije i dijalize s akacetinom (AKA) te inkubacije i dijalize s akacetinom uz prethodni tretman kalijevim heksacijanoferatom (AKA + KCF), u odnosu na kontrolu (KON)



Slika 38. Ostatna aktivnost CYP3A4 enzima nakon inkubacije i dijalize s apigeninom (AKA) te inkubacije i dijalize s apigeninom uz prethodni tretman kalijevim heksacijanoferatom (AKA + KCF), u odnosu na kontrolu (KON)



Slika 39. Ostatna aktivnost CYP3A4 enzima nakon inkubacije i dijalize s krizinom (AKA) te inkubacije i dijalize s krizinom uz prethodni tretman kalijevim heksacijanoferatom (AKA + KCF), u odnosu na kontrolu (KON)



Slika 40. Ostatna aktivnost CYP3A4 enzima nakon inkubacije i dijalize s pinocembrinom (AKA) te inkubacije i dijalize s pinocembrinom uz prethodni tretman kalijevim heksacijanoferatom (AKA + KCF), u odnosu na kontrolu (KON)

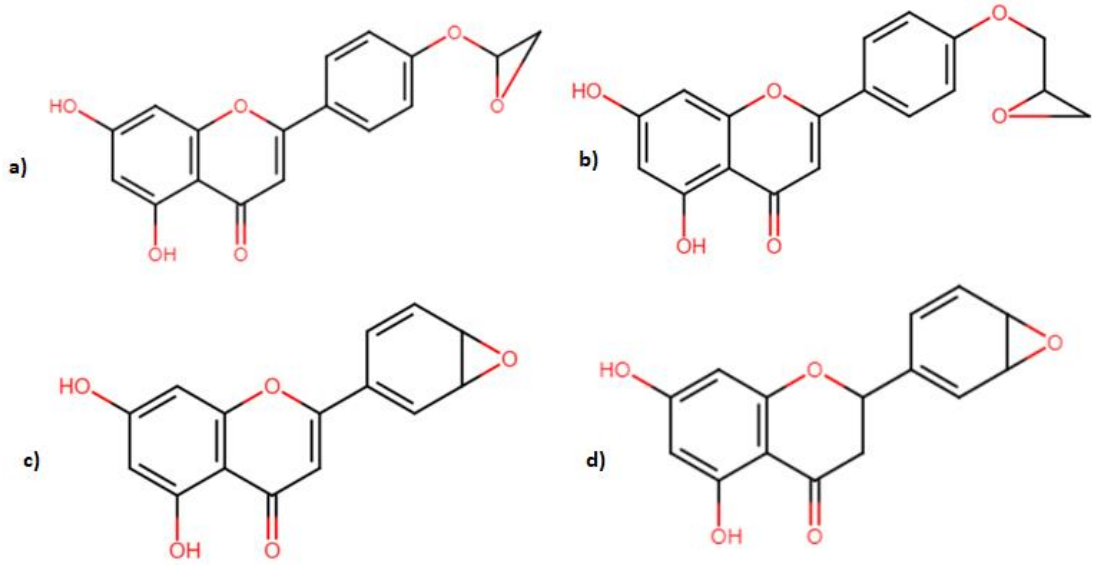
Ostatna aktivnost enzima nakon inkubacije i dijalize s akacetinom iznosila je 1,54%, a nakon inkubacije i dijalize uz prethodni tretman kalijevim heksacijanoferatom 2,23%. Ostatna aktivnost enzima nakon inkubacije i dijalize s apigeninom iznosila je 4,42%, a nakon inkubacije i dijalize uz prethodni tretman kalijevim heksacijanoferatom 4,48%. Ostatna aktivnost enzima nakon inkubacije i dijalize s krizinom iznosila je 0,57%, a nakon inkubacije i dijalize uz prethodni tretman kalijevim heksacijanoferatom 1,31%. Ostatna aktivnost enzima nakon inkubacije i dijalize s pinocembrinom iznosila je 3,27%, a nakon inkubacije i dijalize uz prethodni tretman kalijevim heksacijanoferatom 6,35% (tablica 5).

Tablica 5. Ostatna aktivnost CYP3A4 enzima (%) nakon inkubacije i dijalize s flavonoidima s i bez tretmana kalijevim heksacijanoferatom (KCF)

	akacetin	apigenin	krizin	pinocembrin
bez KCF	1,54	4,42	0,57	3,27
s KCF	2,23	4,48	1,31	6,35

4.5. Određivanje strukture reaktivnog intermedijera

Prilikom ispitivanja specifičnosti vezanja provedena su ispitivanja neproteinskog dijela uzorka, inkubiranog s glutationom, SOD i CAT. Za svaki uzorak predložena je struktura reaktivnog međuprodukta (epoksida) (slika 41) koja će se nastojati uhvatiti kao konjugat s glutationom ili uočiti kao konjugat s hemom.

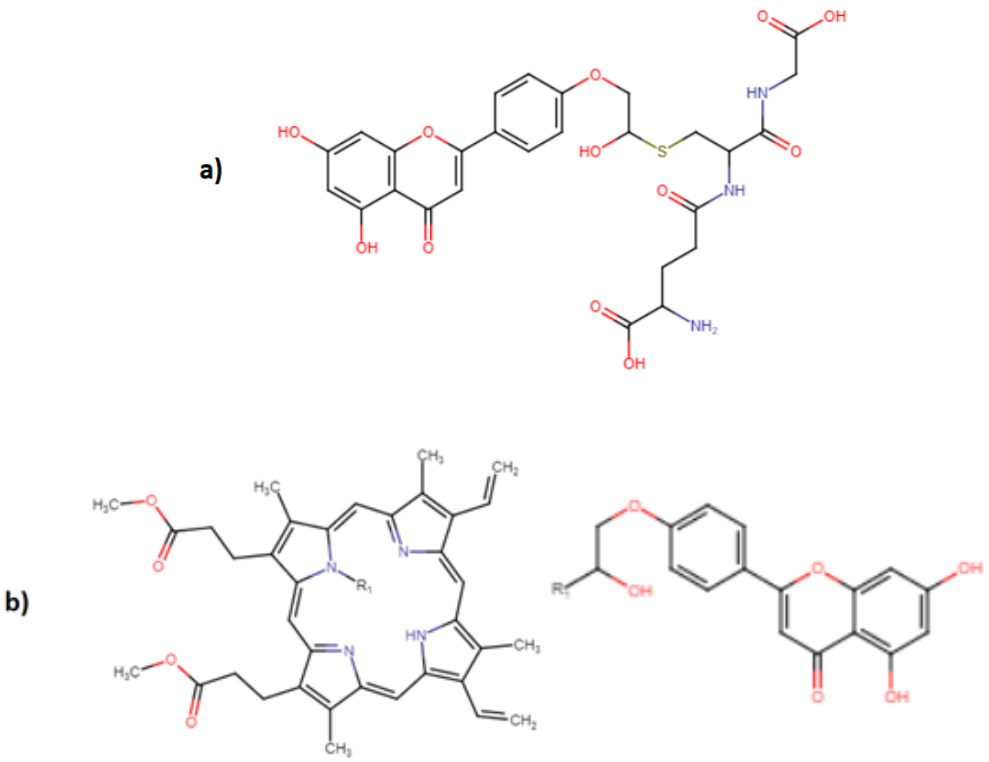


Slika 41. Predložena molekularna struktura reaktivnih međuprodukata akacetina (a), apigenina (b), krizina (c) i pinocembrina (d)

4.5.1. Akacetin

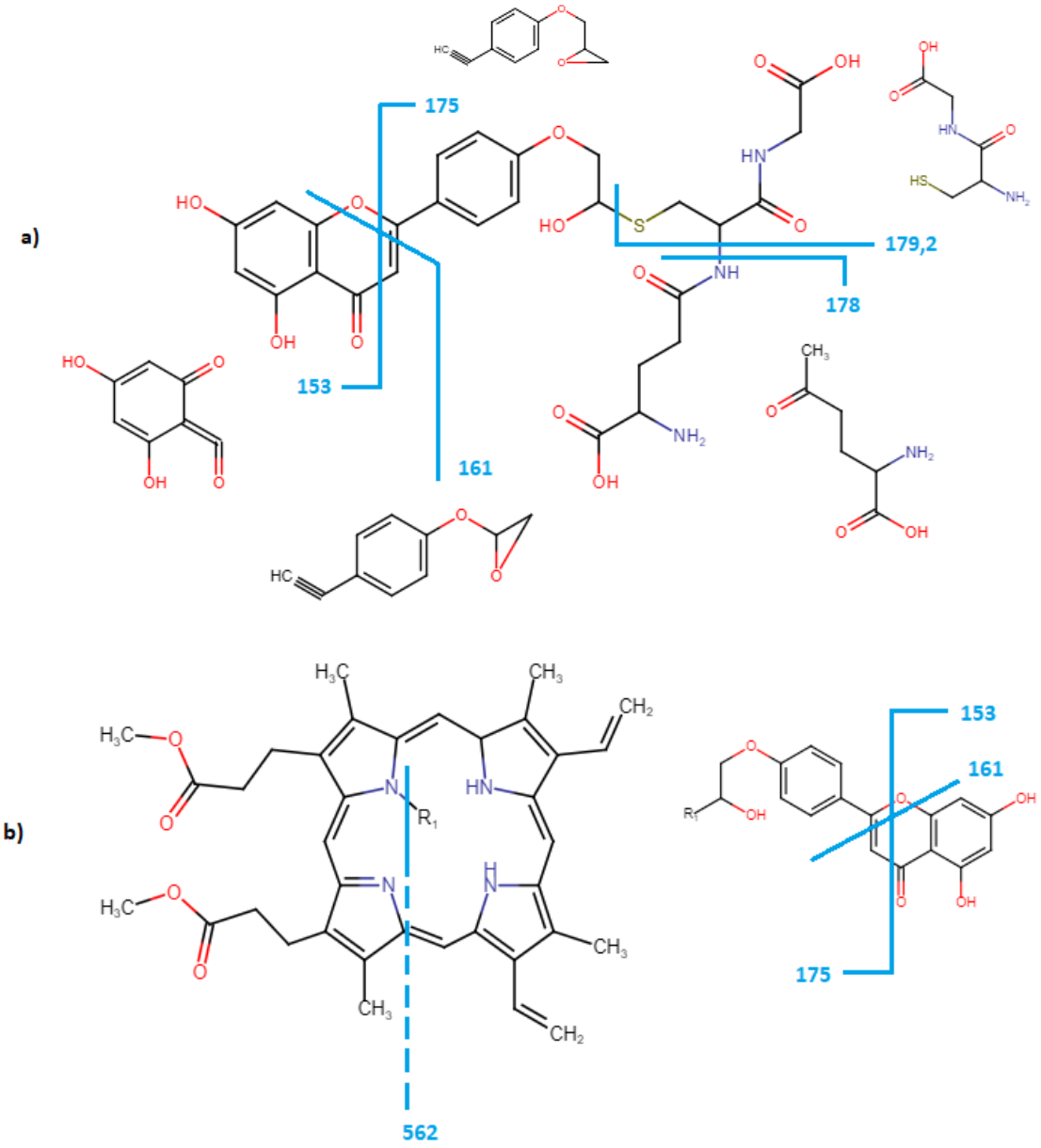
Za reaktivni međuprodukt akacetina predložena je struktura prikazana na slici 41. Reaktivni međuprodukt je epoksid koji nastaje biotransformacijom akacetina uslijed katalitičkog ciklusa CYP3A4 enzima. Predložena molekularna masa reaktivnog međuprodukta iznosi 312,269 g/mol. Predložena struktura konjugata epoksida akacetina

s glutationom (M_r = 620,602 g/mol) i epoksida akacetina s hemom (M_r = 904,278 g/mol) prikazana je na slici 42.



Slika 42. Prijedlog molekularnih struktura konjugata epoksida akacetina s glutationom (a) i hemom (b)

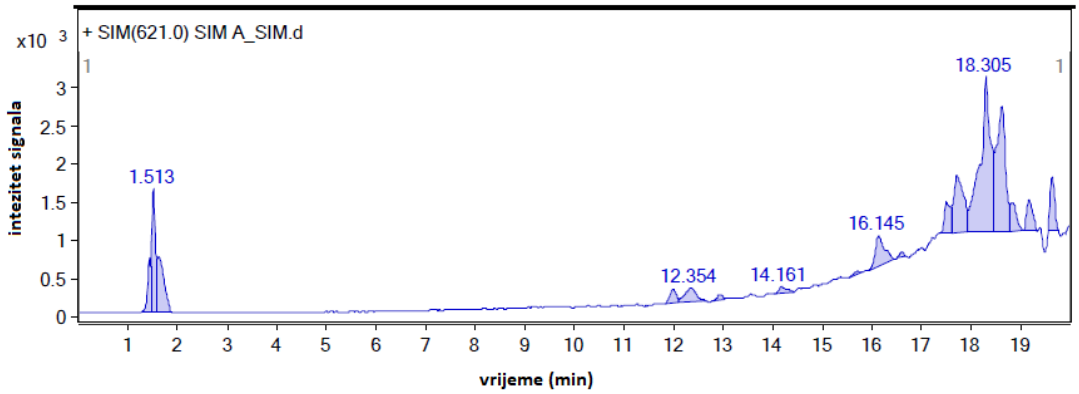
Za konjugat epoksida akacetina s hemom i glutationom predložene su karakteristične fragmentacije prikazane na slici 43.



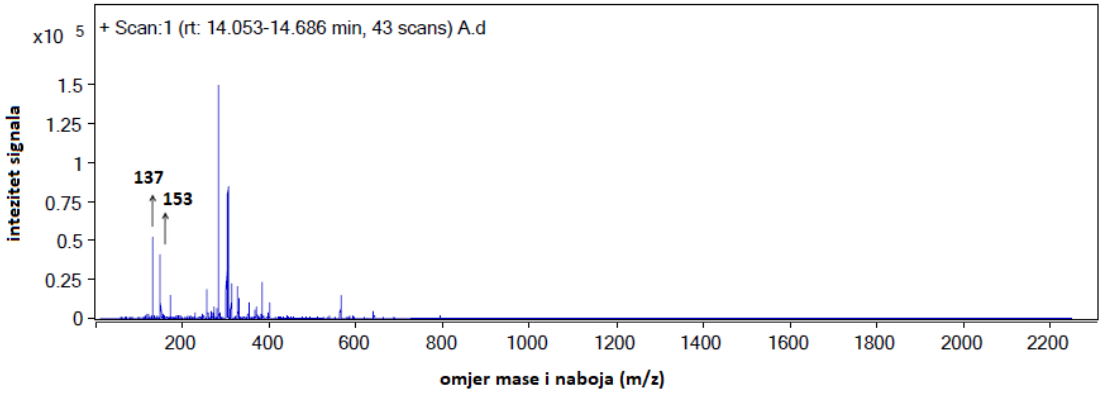
Slika 43. Fragmentacija konjugata epoksida akacetina s glutationom (a) i hemom (b)

Za $[M+H]^+$ ione epoksida akacetina predloženi su fragmentirani uzorci: $^{1,3}A^+$, $^{0,2}B^+$ i $^{1,3}B^+$, koji pokazuju *m/z* vrijednosti od 153, 137 i 161. Pregledom kromatograma tražene su vrijednosti predloženih fragmenata epoksida flavonoida (*m/z* = 137, 153 i 161), konjugata epoksida apigenina s glutationom $[M+H-H_2O]^+$, *m/z* = 639, konjugata epoksida apigenina s hemom bez željeza $[M+H]^+$, *m/z* = 905, kao i samog glutationa $[M+H-H_2O]^+$, *m/z* = 362 i samog hema bez željeza $[M+H]^+$, *m/z* = 562. Pregledom kromatograma dobivenih analizom na spektrometru masa uočen je signal na 14,053 min (slika 44), a na

kojem su uočeni predloženi fragmenti epoksida apigenina s pripadajućim *m/z* vrijednostima od 137 i 153. Fragment epoksida apigenina s pripadajućim *m/z* od 161, kao i konjugati s hemom i glutationom nisu uočeni (slika 45). Vrijednosti *m/z* za glutation i jednu molekulu vode, prikazane fragmentacije glutationa, kao i hem bez željeza uočeni su na drugim signalima s različitim vremenom zadržavanja.



Slika 44. SIM uzorka s akacetinom

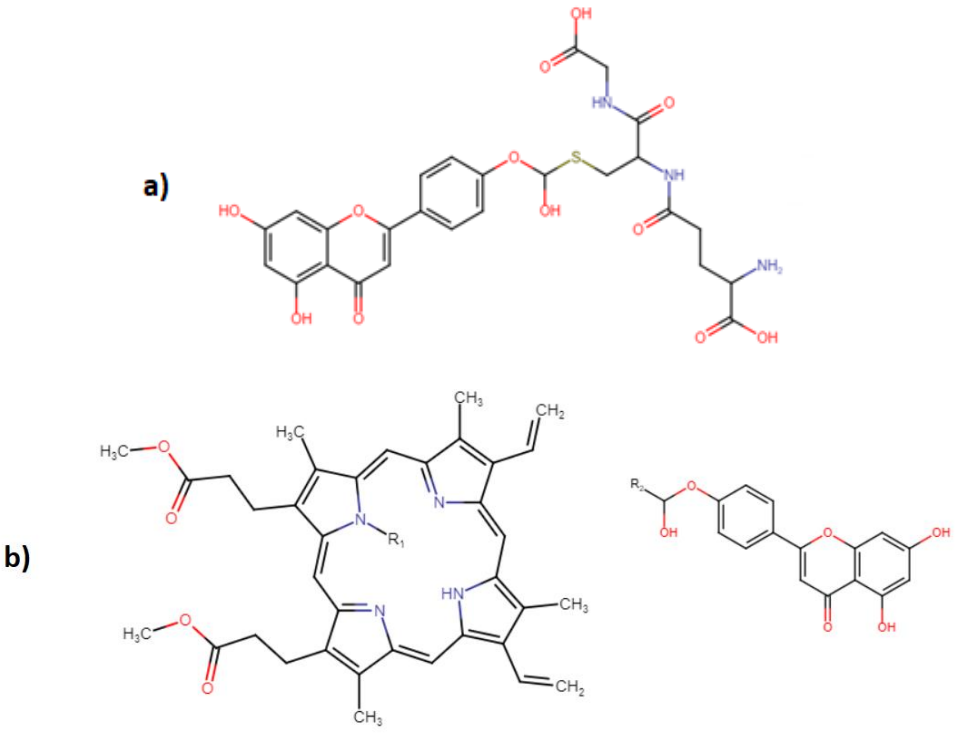


Slika 45. Raščlamba signala na 14,053 min; uočavaju se fragmenti epoksida apigenina s *m/z* = 137 i 153

4.5.2. Apigenin

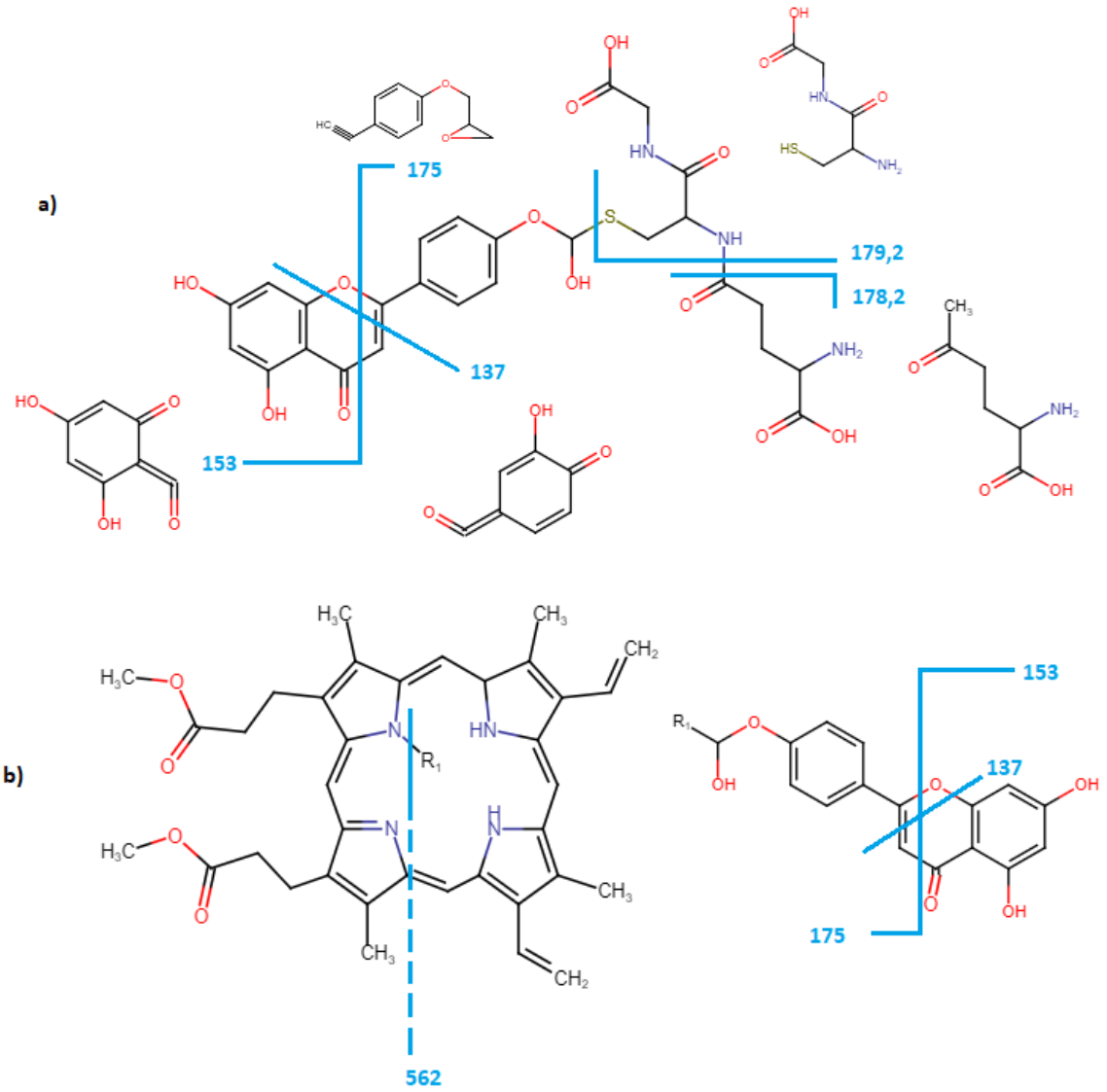
Za reaktivni međuprodukt apigenina predložena je struktura prikazana na slici 41. Reaktivni međuprodukt je epoksid koji nastaje biotransformacijom apigenina uslijed katalitičkog ciklusa CYP3A4 enzima. Predložena molekularna masa reaktivnog međuprodukta iznosi 310,297 g/mol. Predložena struktura konjugata epoksida apigenina

s glutationom (M_r = 578,565 g/mol) i epoksida apigenina s hemom (M_r = 891,259 g/mol) prikazana je na slici 46.



Slika 46. Prijedlog molekularnih struktura konjugata epoksida apigenina s glutationom (a) i hemom (b)

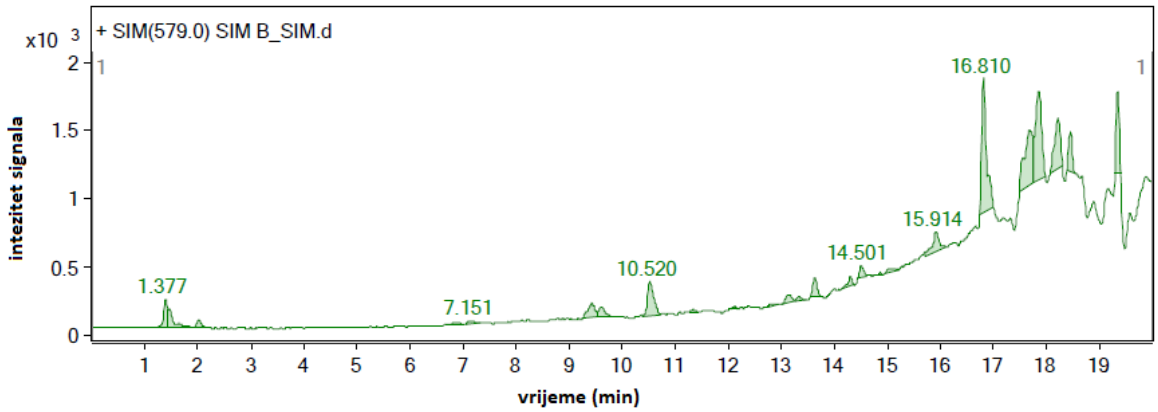
Za konjugat epoksida apigenina s hemom i glutationom predložene su karakteristične fragmentacije prikazane na slici 47.



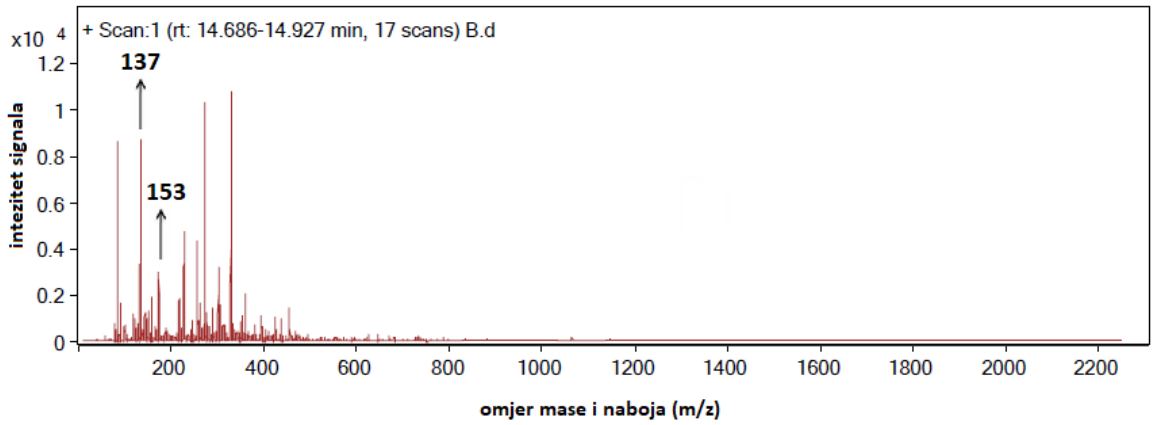
Slika 47. Fragmentacija konjugata epoksida apigenina s glutationom (a) i hemom (b)

Pregledom kromatograma tražene su vrijednosti predloženih fragmenata epoksida flavonoida (*m/z* = 137, 153 i 175), konjugata epoksida apigenina s glutationom $[M+H-H_2O]^+$, *m/z* = 597, konjugata epoksida apigenina s hemom bez željeza $[M+H]^+$, *m/z* = 892, kao i samog glutationa $[M+H-H_2O]^+$, *m/z* = 362 i samog hema bez željeza $[M+H]^+$, *m/z* = 562. Pregledom kromatograma dobivenih analizom na spektrometru masa uočen je signal na 14,686 min (slika 48), a na kojem su uočeni predloženi fragmenti epoksida apigenina s pripadajućim *m/z* vrijednostima od 137 i 153. Fragment epoksida apigenina s pripadajućim *m/z* od 175, kao i konjugati s hemom i glutationom nisu uočeni (slika 49).

Vrijednosti *m/z* za glutation i jednu molekulu vode, prikazane fragmentacije glutationa, kao i hem bez željeza uočeni su na drugim signalima s različitim vremenom zadržavanja.



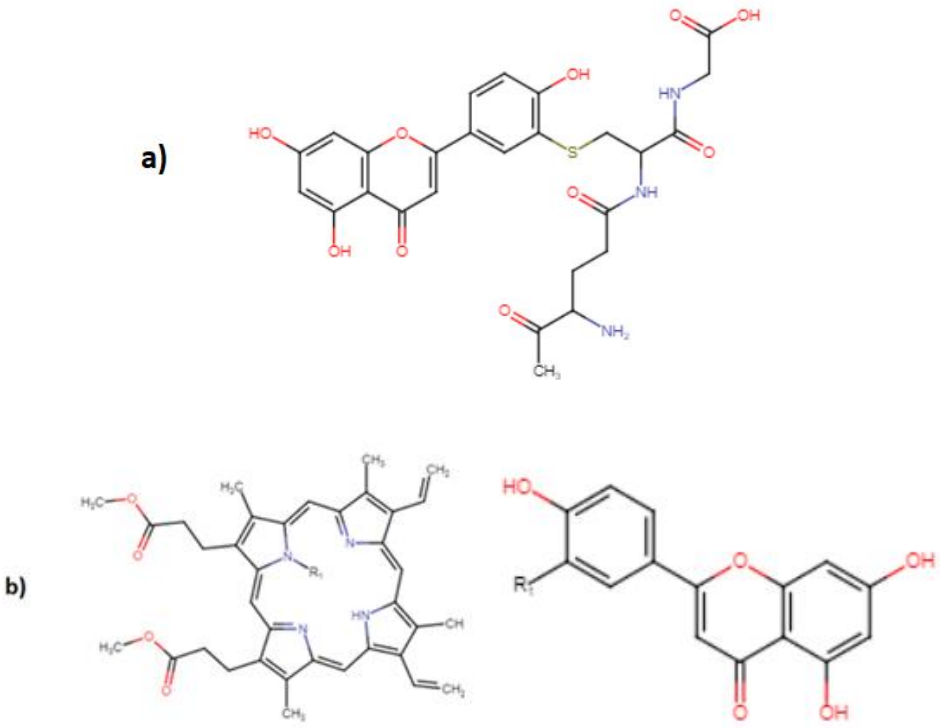
Slika 48. SIM uzorka s apigeninom



Slika 49. Raščlamba signala na 14,686 min; uočavaju se fragmenti epoksida apigenina s *m/z* = 137 i 153

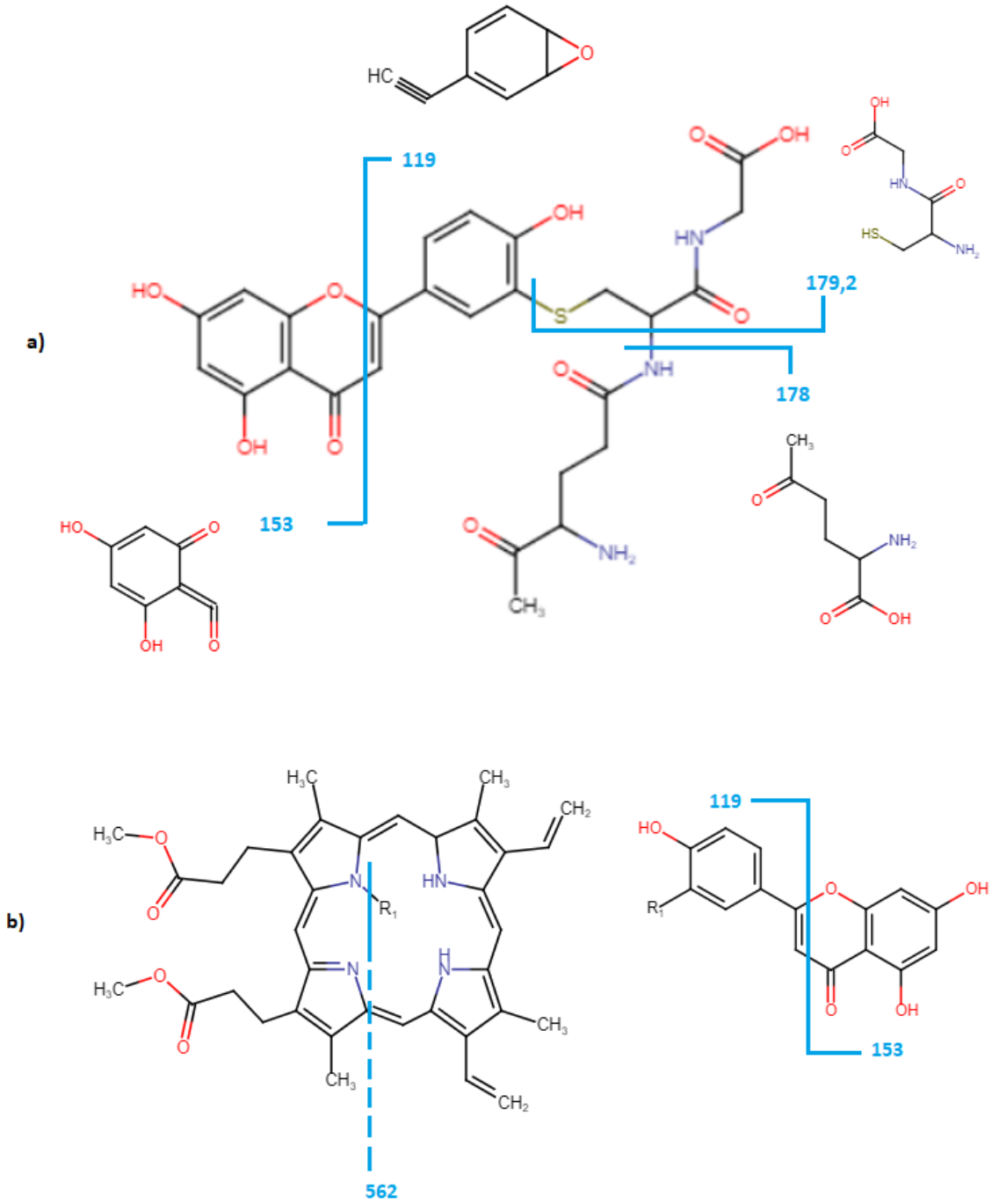
4.5.3. Krizin

Za reaktivni međuprodukt krizina predložena je struktura prikazana na slici 41. Reaktivni međuprodukt je epoksid koji nastaje biotransformacijom krizina uslijed katalitičkog ciklusa CYP3A4 enzima. Predložena molekularna masa reaktivnog međuprodukta iznosi 270,234 g/mol. Predložena struktura konjugata epoksida krizina s glutationom (M_r = 576,549 g/mol) i epoksida krizina s hemom (M_r = 866,268 g/mol) prikazana je na slici 50.



Slika 50. Prijedlog molekularnih struktura konjugata epoksida krizina s glutationom (a) i hemom (b)

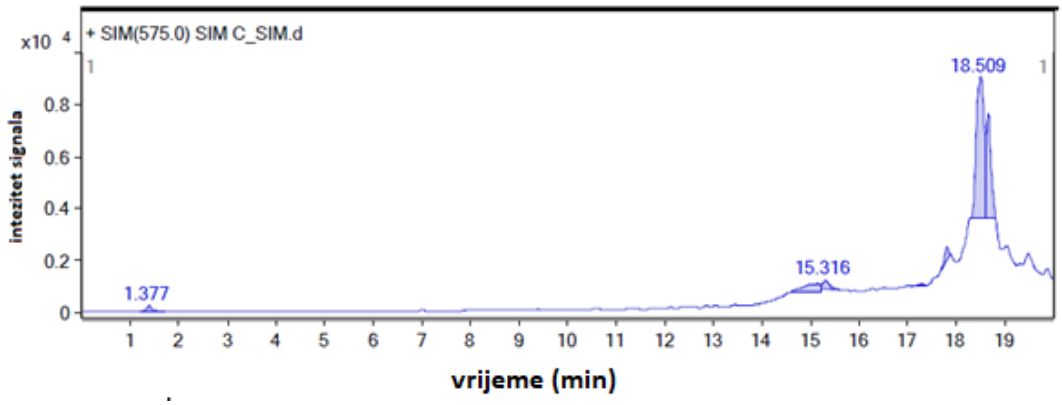
Za konjugat epoksida krizina s hemom i glutationom predložene su karakteristične fragmentacije prikazane na slici 51.



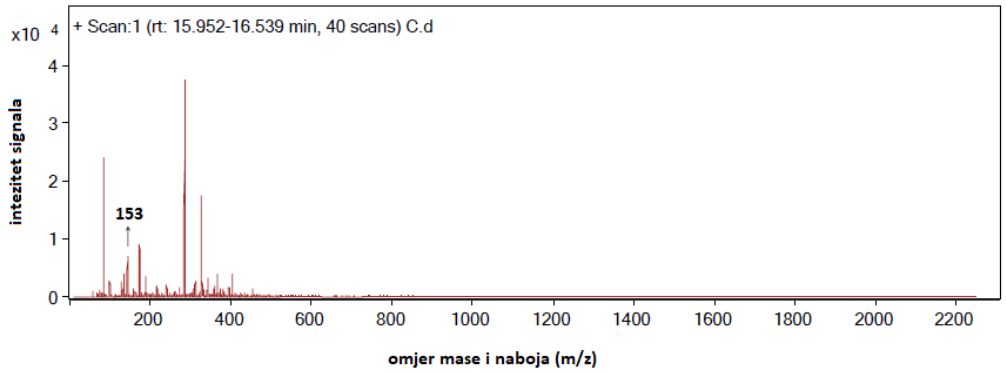
Slika 51. Fragmentacija konjugata epoksida krizina s glutationom (a) i hemom (b)

Pregledom kromatograma tražene su vrijednosti predloženih fragmenata epoksida flavonoida (*m/z* = 153 i 119), konjugata epoksida krizina s glutationom $[M+H-H_2O]^+$, *m/z* = 595, konjugata epoksida krizina s hemom bez željeza $[M+H]^+$, *m/z* = 867, kao i samog glutationa $[M+H-H_2O]^+$, *m/z* = 362 i samog hema bez željeza $[M+H]^+$, *m/z* = 562. Pregledom kromatograma dobivenih analizom na spektrometru masa uočen je signal na

15,952 min (slika 52), a na kojem je uočen predloženi fragment epoksida krizina s pripadajućom *m/z* vrijednošću od 153. Fragment epoksida krizina s pripadajućom *m/z* od 119, kao i konjugati s hemom i glutationom nisu uočeni (slika 53). Vrijednosti *m/z* za glutation i jednu molekulu vode, prikazane fragmentacije glutationa, kao i hem bez željeza uočeni su na drugim signalima s različitim vremenom zadržavanja.



Slika 52. SIM uzorka s krizinom

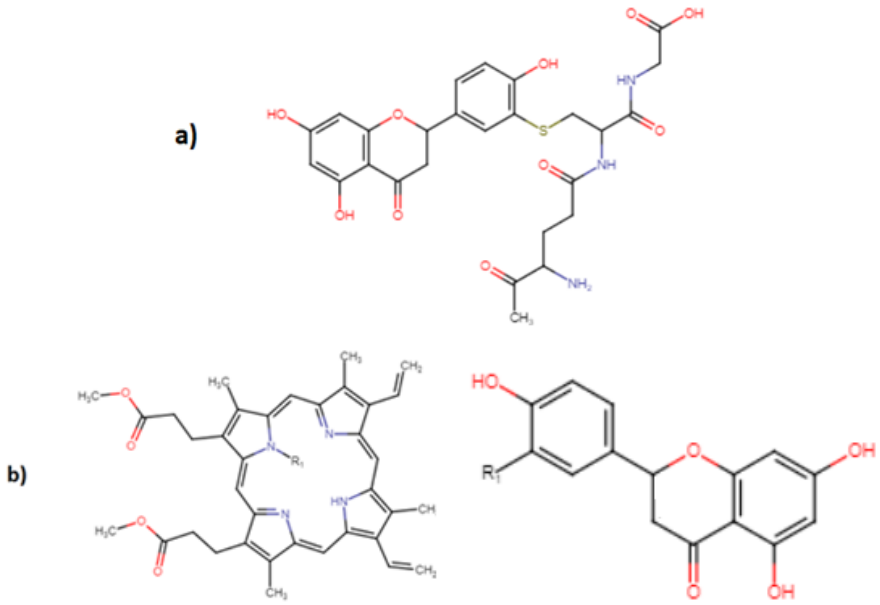


Slika 53. Raščlamba signala na 15,952 min; uočava se fragment epoksida krizina (*m/z* = 153)

4.5.4. Pinocembrin

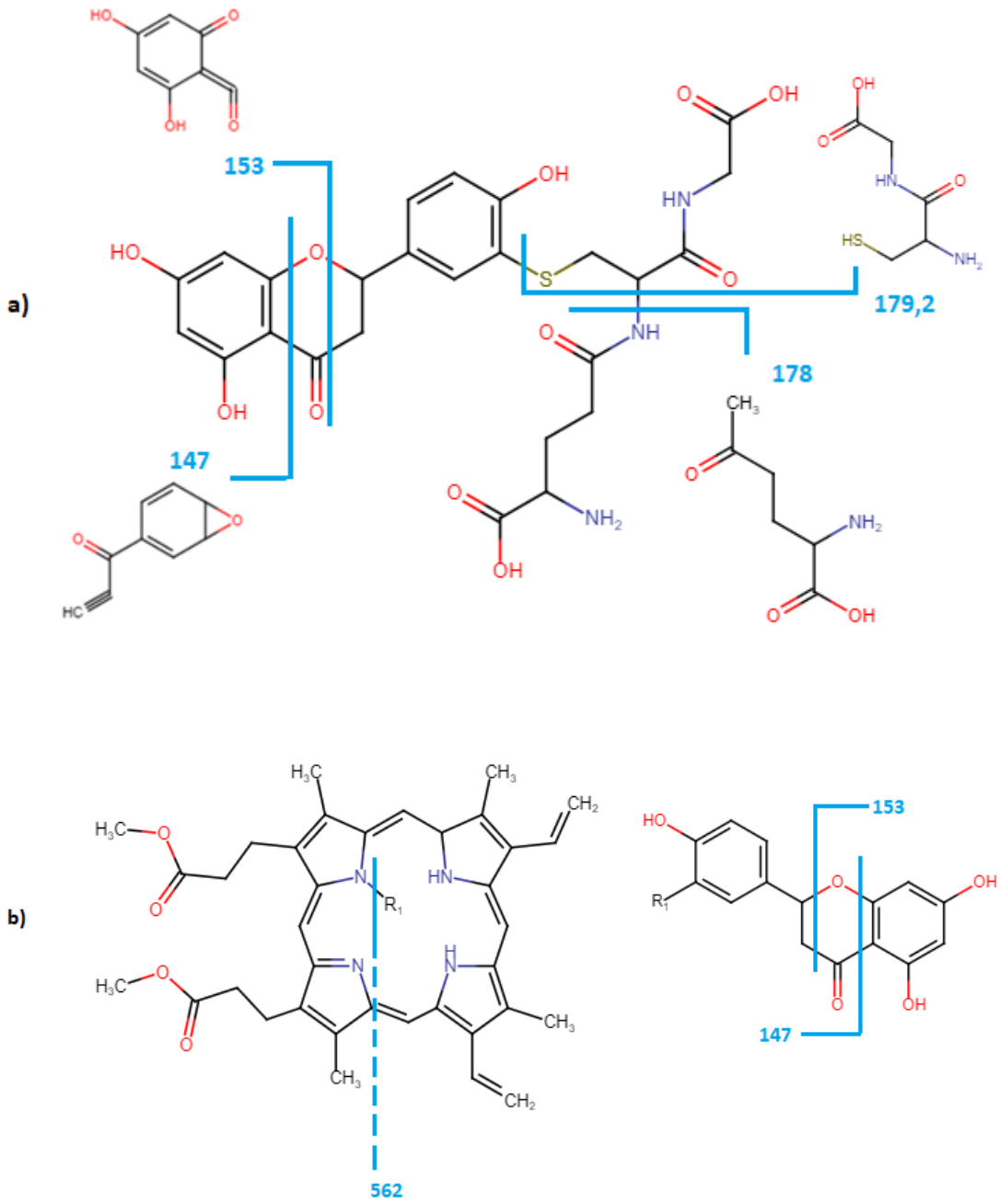
Za reaktivni međuprodukt pinocembrina predložena je struktura prikazana na slici 41. Reaktivni međuprodukt je epoksid koji nastaje biotransformacijom pinocembrina uslijed katalitičkog ciklusa CYP3A4 enzima. Predložena molekularna masa reaktivnog međuprodukta iznosi 273,257 g/mol. Predložena struktura konjugata epoksida

pinocembrina s glutationom (M_r = 578,565 g/mol) i epoksida pinocembrina s hemom (M_r = 862,242 g/mol) prikazana je na slici 54.



Slika 54. Prijedlog molekularnih struktura konjugata epoksida pinocembrina s glutationom (a) i hemom (b)

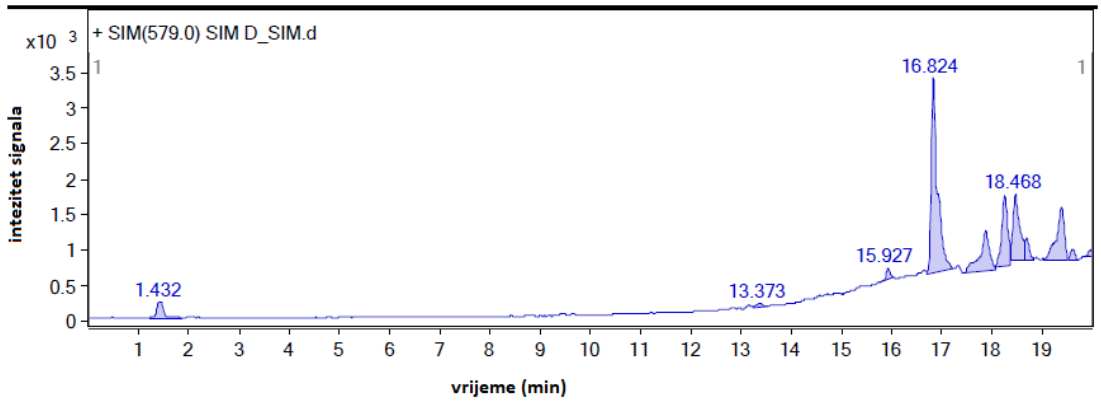
Za konjugat epoksida pinocembrina s hemom i glutationom predložene su karakteristične fragmentacije prikazane na slici 55.



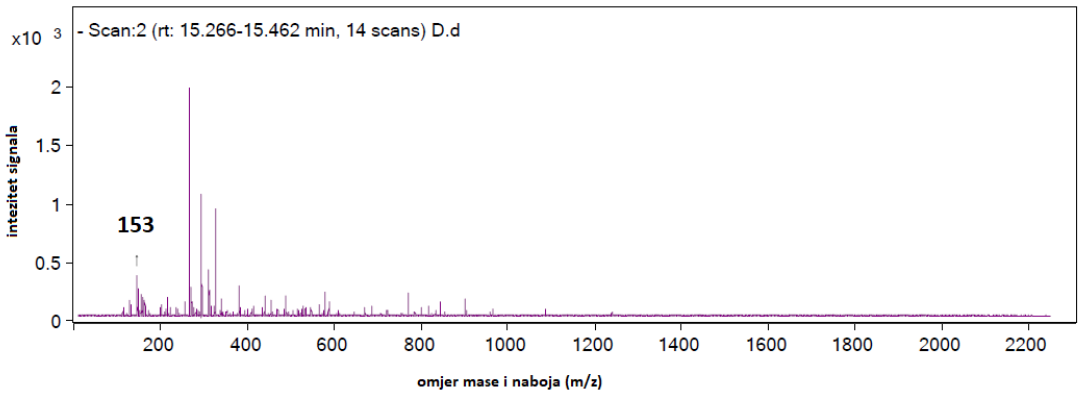
Slika 55. Fragmentacija konjugata epoksida pinocembrina s glutationom (a) i hemom (b)

Pregledom kromatograma tražene su vrijednosti predloženih fragmenata epoksida flavonoida (*m/z* = 153 i 147), konjugata epoksida pinocembrina s glutationom $[M+H-H_2O]^+$, *m/z* = 597, konjugata epoksida pinocembrina s hemom bez željeza $[M+H]^+$, *m/z* = 863, kao i samog glutationa $[M+H-H_2O]^+$, *m/z* = 362 i samog hema bez željeza $[M+H]^+$, *m/z* = 562. Pregledom kromatograma dobivenih analizom na spektrometru masa uočen je signal na 15,266 min (slika 56), a na kojem je uočen predloženi fragment epoksida

pinocembrina s pripadajućom *m/z* vrijednošću od 153. Fragment epoksida pinocembrina s pripadajućom *m/z* od 147, kao i konjugati s hemom i glutationom nisu uočeni (slika 57). Vrijednosti *m/z* za glutation i jednu molekulu vode, prikazane fragmentacije glutationa, kao i hem bez željeza uočeni su na drugim signalima s različitim vremenom zadržavanja.



Slika 56. SIM uzorka s pinocembrinom



Slika 57. Raščlamba signala na 15,266 min; uočava se fragment epoksida pinocembrina (*m/z* = 153)

5. RASPRAVA

5.1. Akacetin

Akacetin (*O*-metilirani flavon) je uzrokovao o vremenu ovisnu inhibiciju CYP3A4 enzima, pri čemu je utvrđena ostatna aktivnost enzima od 55,6 ± 6,4%. Osim toga, njegov inhibicijski učinak utvrđen je i prilikom ispitivanja izravne inhibicije (ostatna aktivnost enzima iznosila je 48,5 ± 6,5%). Prilikom ispitivanja o metabolizmu ovisne inhibicije akacetin se pokazao kao najsnažniji inhibitor. Inhibirao je aktivnost enzima za više od 90%, pri čemu je ostatna aktivnost enzima iznosila 10,6 ± 1,3%. Ovo je u skladu s istraživanjem kojeg su obavili Šarić Mustapić i sur. (2018), utvrdivši da od 30 ispitivanih flavonoida, njih sedam pokazuje inhibicijski učinak na CYP3A4 enzim. Također, kao i u ovom ispitivanju, najsnažniji inhibitor bio je akacetin, koji je uzrokovao 95%-tnu inhibiciju.

Inhibicijski učinak ovog flavonoida na CYP enzime nije dovoljno ispitan. Međutim, u dostupnoj znanstvenoj literaturi postoje određeni podaci o inhibicijskom učinku akacetina na druge CYP enzime. Tako su Beijer i sur. (2010) ustanovili inhibicijski učinak akacetina na CYP1A potporodici enzima u škrgama ribe koljuške (*Gasterosteus aculeatus*). Akacetin je pri koncentraciji od 1 µM u tom ispitivanju smanjio aktivnost enzima za 50%. Promatrana reakcija bila je *O*-deetilacija 7-etoksirezorufina (EROD).

Prilikom ispitivanja kinetike inhibicije flavonoida, akacetin je pokazao IC_{50} vrijednost od 10,9 ± 0,3 µM prilikom ispitivanja s testosteronom te 7,5 ± 2,7 µM prilikom ispitivanja s nifedipinom. Slično kao u ovom ispitivanju, postoje ispitivanja koja su ispitivala IC_{50} vrijednosti za akacetin. Zhou i sur. (2020) su ispitivali glavne sastavnice korijena biljke *Lygodium japonicum* u smislu njihovog inhibicijskog učinka na CYP3A4 enzim *in vitro* i *in vivo*. *In vitro* ispitivanja provedena su na štakorskim jetrenim mikrosomima, pri čemu je kao supstrat korišten midazolam. *In vivo* ispitivanja provedena su na odraslim Sprague Dawley štakorima kojima je ubrizgavan akacetin tijekom sedam dana u dozi od 5 mg/kg. Ustanovili su da su od svih sastavnica jedino akacetin i apigenin inhibirali aktivnost CYP3A4 enzima. Akacetin je pokazao IC_{50} vrijednost od 58,46 µM. U *in vivo* ispitivanju ustanovljeno je da akacetin može inhibirati metabolizam midazolama kod štakora. Viša IC_{50} vrijednost koju su dobili Zhou i sur. može se objasniti korištenjem drugačijeg supstrata u odnosu na rezultate dobivene u ovom ispitivanju.

Treba naglasiti kako su testosteron i nifedipin marker supstrati koji se koriste pri ispitivanju aktivnosti CYP3A4 enzima, dok su Zhou i sur. koristili midazolam kao supstrat.

Osim toga, akacetin se pokazao kao snažan inhibitor i drugih ljudskih enzima, poput monoaminooksidaza. Monoaminooksidaze s CYP enzimima dijele određen sličnosti. Zajedno s CYP enzimima monoaminoksidaze mogu sudjelovati u metaboličkim putovima određenih lijekova i drugih ksenobiotika (Theobald i Maurer, 2007). U istraživanju kojeg su proveli Lee i sur. (2017) pokazano je da akacetin snažno inhibira rekombinantne ljudske monoaminooksidaze A i B. Utvrđena je IC_{50} vrijednost od 0,19 µM za monoaminooksidazu A, odnosno 0,17µM za monoaminooksidazu B. Osim toga, utvrđena je i konstanta inhibicije za akacetin koja je za monoaminooksidazu A i B iznosila 0,045 µM i 0,037 µM. Za usporedbu, u ovom ispitivanju određena je K_i vrijednost od 6,0 ± 3,0. Takvi rezultati navode na zaključak da akacetin čak i snažnije inhibira monoaminoksidaze nego CYP enzime.

Važno je naglasiti da je u ovom istraživanju opisana i konstanta brzine inaktivacije koju pokazuje akacetin na aktivnost CYP3A4 enzima (k_{inact} = 0,036 ± 0,006 min^{-1} za testosteron, odnosno 0,10 ± 0,02 min^{-1} za nifedipin), kao i učinkovitost inaktivacije enzima (k_{inact}/K_i = 0,006 $min^{-1}\mu M^{-1}$ za testosteron i 0,01 za nifedipin $min^{-1}\mu M^{-1}$). Pregledom literature utvrđeno je da se radi o podacima koji do sada nisu bili opisani u dostupnoj znanstvenoj literaturi.

Osim toga, radi se i o prvom opisu vezanja akacetina za hem, odnosno dokaza da se reaktivni oblici flavonoida kovalentno vežu za hem. Istraživanje koje su proveli Wilsher i sur. (2017) obrađivalo je vezanje apigenina i luteolina (flavonoida strukturno sličnih akacetinu) na hem. Pri tom istraživanju predložili su da se navedeni flavonoidi za hem vežu preko prstena A, ali nisu proveli eksperimentalno *in vitro* ispitivanje. U ovom ispitivanju koncentracija hema primjenom akacetina smanjena je za otprilike 52%. Ispitivanje je ponovljeno primjenom SOD i CAT, kojima se nastojalo spriječiti uništenje hema reaktivnim kisikovim spojevima. Međutim, pri ponovljenom ispitivanju također je došlo do smanjenja koncentracije hema za otprilike 37%. Takvi rezultati navode na zaključak da se reaktivni međuprodukti akacetina kovalentno vežu za protoporfirinski dio hema. Kako navode Bojić i sur. (2019), postoji mogućnost da je razlika u afinitetu vezanja rezultat razlike u strukturi flavonoida. Naime, smatra se da su za stvaranje reaktivnih

međuprodukata odgovorne metoksi i hidroksilna funkcionalna skupina na akacetinu i apigeninu, u položaju 4' na B prstenu. Time bi akacetin i apigenin doveli do inaktivacije enzima.

Kako bi se ispitalo je li akacetin pokazuje inhibiciju pseudoireverzibilnog karaktera na CYP3A4 provedeno je ispitivanje dodatkom kalijevog heksacijanoferata i dijalizom inkubacijske smjese. Akacetin je nakon dijalize, a bez dodatka kalijevog heksacijanoferata, doveo do značajne inhibicije CYP3A4 enzima. Utvrđena je ostatna aktivnost enzima od 1,54%. U ispitivanju provedenom s dodatkom oksidansa utvrđena je ostatna aktivnost enzima od 2,23%. Nije utvrđena statistički značajna razlika između ispitivanja provedenog s i bez dodatka kalijevog heksacijanoferata ($p \leq 0,05$). Budući da se aktivnost enzima nije mogla povratiti dodatkom oksidansa, ovakvi dokazi upućuju na to da vezanje akacetina na hem nije pseudoireverzibilnog karaktera.

Za strukturnu formulu reaktivnih međuprodukata koji se kovalentno vežu na hem predložena je epoksidna struktura. Moguće je da se akacetin tijekom oksidativnog ciklusa CYP enzima oksidira u području metoksi skupine B prstena. Istraživanje koje su proveli He i sur. (1996) ispitivalo je inhibiciju citokrom P450 2B1 enzima sa sedativom sekobarbitalom. Ustanovili su da se na sekobarbitalu uslijed katalitičkog ciklusa CYP enzima formira epoksid. Takav epoksid može se vezati na protoporfirin IX te na taj način dovesti do inhibicije enzimske aktivnosti. Nadalje, Han i sur. (2005) su ispitivali djelovanje bifenil dioksigenaze iz soja bakterije *Pseudomonas pseudoalcaligenes* KF707. Ustanovili su da djelovanjem bifenil dioksigenaze dolazi do uspostavljanja epoksida na B prstenu flavanona, što je u skladu sa strukturama epoksida flavonoida koje su predložene u ovom istraživanju. U ovom istraživanju predloženo je da se epoksid akacetina u inkubaciji s glutationom veže preko molekule sumpora za glutation, pri čemu nastaje konjugat glutationa i epoksida akacetina. Ovaj prijedlog strukture u skladu je s istraživanjem kojeg su proveli Fjellstedt i sur. (1973), a koji su ispitivali konjugacija epoksida s glutationom. Utvrdili su da konjugacijom nastaje spoj $RCHCH_2$, koji je supstituiran jednom hidroksilnom skupinom na CH dijelu te vezan za glutation preko molekule sumpora u dijelu CH_2. Molekularna masa ovdje opisanog konjugata epoksida akacetina s glutationom određena je na 620,602 g/mol. Pregledom kromatograma dobivenih analizom na spektrometru masa uočeni su predloženi fragmenti epoksida apigenina s pripadajućim *m/z* vrijednostima od 137 i 153, kao i ostale opisane

fragmentacije glutationa i hema u odsustvu željeza. Ovi dokazi su u skladu s drugim istraživanjima koja su opisivala karakterizaciju fragmenata flavonoida, adukata glutationa i hema (Tsimogiannis i sur., 2007; Schebb i sur., 2009; von Weymarn, Blobaum i Hollenberg, 2004). Osim na ovdje predloženim mjestima, epoksidi mogu nastati i na drugim dijelovima flavonoida, ali su u ovom istraživanju opisana najvjerojatnija mjesta epoksidacije. Međutim, promjenom mjesta epoksidacije flavonoida ne dolazi do promjene mase nastalog reaktivnog međuprodukta, tako da je napravljena pretraga na osnovu izračunatih molekularnih masa. Nisu uočene *m/z* vrijednosti koje bi odgovarale konjugatima epoksida akacetina s glutationom, odnosno hemom. Uzrok tomu vjerojatno je mala koncentracija ili nestabilnost nastalih reaktivnih međuprodukata.

5.2. Apigenin

Apigenin je trihidroksiflavon. Za razliku od akacetina, apigenin u položaju C4' B prstena posjeduje hidroksilnu umjesto metoksi skupine. Apigenin je u ovom istraživanju uzrokovao inhibiciju CYP3A4 u ispitivanju o vremenu ovisne i izravne inhibicije te u ispitivanju o metabolizmu ovisne inhibicije. Najveću inhibiciju enzima uzrokovao je pri ispitivanju o metabolizmu ovisne inhibicije, pri čemu je ostatna aktivnost enzima iznosila 24,5 ± 4,3%. Prilikom ispitivanja o vremenu ovisne i izravne inhibicije, aktivnost CYP3A4 enzima smanjila se za otprilike 63%, odnosno 69%. Ovo ispitivanje u skladu je s istraživanjem kojeg su proveli Šarić Mustapić i sur. (2018). Budući da između ostatne aktivnosti enzima u o vremenu ovisnoj i izravnoj inhibiciji ne možemo govoriti o značajnoj razlici, možemo zaključiti da apigenin nije spori inhibitor CYP3A4 enzima. Istraživanje inhibicijskog potencijala apigenina kojeg su proveli Kampschulte i sur. (2020) pokazalo je da je apigenin slab inhibitorom (postotak inhibicije <15%). Treba uzeti u obzir da ispitivanje koje su proveli Kampschulte i sur. nije provedeno koristeći testosteron i nifedipin kao supstrate, već su pratili hidroksilaciju arahidonske kiseline.

In vitro ispitivanja inhibicijskog učinka apigenina na aktivnost CYP3A4 potvrđena su i *in vivo* ispitivanjima provedenim na štakorima (Choi i Choi, 2010). Apigenin je u dozi od 50 μM povisio apsorpciju paklitaksela u odnosu na kontrolu. (Kumar i Pryanka, 2015). Budući da citokrom P450 3A4 ima veliko aktivno mjesto, preporučuje se ispitivanje inhibicijskih učinaka na ovom enzimu provoditi uz pomoć dva

različita supstrata (Bojić, 2015). Zbog toga se mogu objasniti različito dobiveni rezultati kada pogledamo rezultate kinetike inhibicije s testosteronom ili nifedipinom. Primjerice, tako je za apigenin utvrđena različita IC_{50} vrijednost, od 1,8 μM i od 31 ± 8 μM (Choi i Choi, 2010; Brahmi i sur., 2011). Naime, u jednom istraživanju korišten je supstrat 7-benziloksi-4-trifluorometilkumarin, a u drugom istraživanju 7-benziloksimetiloksi-3-cijanokumarin. S druge strane, Kimura i sur. (2010) utvrdili su da je apigenin u dozi od 1μM u potpunosti inhibirao aktivnost CYP3A4 enzima (0% ostatne aktivnosti enzima), prateći hidroksilaciju testosterona. U ovom istraživanju utvrđena je IC_{50} vrijednost apigenina od 11,4 ± 0,4 μM te 8,4 ± 1,1 μM koristeći testosteron i nifedipin kao supstrate.

Iako ne postoje dostupna istraživanja koja opisuju K_i vrijednost apigenina prilikom inhibicije CYP3A4 enzima, dostupna su istraživanja koja govore o inhibiciji drugih CYP enzima. Tako je u istraživanju kojeg su proveli Kim i sur. (2005) utvrđena K_i vrijednost apigenina pri inhibiciji CYP1A1, CYP1A2, CYP1B1 enzima od 2,0 μM, 2,45 μM i 0,5 μM. Ispitivanja su provedena koristeći EROD kao marker reakciju. Autori inhibicijski učinak na CYP1A potporodicu pripisuju broju hidroksilnih supina B prstena.

Poznato je da apigenin posjeduje antivirusna svojstva (Wang i sur., 2019). Roy i sur. (2017) otkrili su djelovanje apigenina na protein ključan u replikaciji Zika virusa, koji se smatra ključnom metom za otkrivanje novih antivirusnih lijekova. U tom ispitivanju apigenin je pokazao K_i vrijednost od 34,02 μM. Konstanta inhibicije apigenina u ovom istraživanju iznosila je 1,5 ± 0,8 μM za testosteron, odnosno 20,2 ± 12,7 μM za nifedipin. Takve vrijednosti konstante inhibicije govore u prilog snažnom inhibitornom djelovanju apigenina, kao i o potencijalima za kliničku primjenu.

Prema podacima dostupnima u literaturi za apigenin još nije utvrđena konstanta brzine inaktivacije, kao ni učinkovitost inaktivacije CYP3A4 enzima. U ovom istraživanju utvrđene su vrijednosti na testosteronu (k_{inact} = 0,11 ± 0,01 min^{-1} i k_{inact}/K_i = 0,073 $min^{-1}\mu M^{-1}$). Na osnovu dobivenih parametara kinetike inhibicije, može se pretpostaviti da one mogu biti klinički značajne. Naime, to potvrđuju *in vivo* ispitivanja provedena na apigeninu i njegovoj mogućnosti inaktiviranja CYP3A4 enzima, a koja su provedena na štakorima kod kojih je bio primijenjen etopozid. Etopozid se metabolizira posredovanjem CYP3A4. U prisustvu apigenina došlo je do povišene razine etopozida kod štakora. Međutim, to se ne mora uvijek shvaćati kao negativan učinak, jer ovakav pristup predstavlja potencijalni proces kojim se može popraviti oralna bioraspoloživost

etopozida (Lim i sur., 2011). Također, osim kod etopozida, prilikom primjene apigenina uočeno je i povećanje koncentracije antineoplastika imatiniba (Liu i sur., 2013).

Inhibitorni učinak apigenina očituje se i u smanjenju koncentracije hema. Apigenin je smanjio koncentraciju hema za otprilike 55%, a u ponovljenom ispitivanju s dodatkom SOD i CAT također je utvrđeno smanjenje hema za otprilike 45%. Ovaj podatak u skladu je s istraživanjem kojeg su proveli Wilsher i sur. (2017) o vezanju apigenina i luteolina na hem.

Ovdje provedeno istraživanje ukazuje na to da se apigenin ne ponaša kao pseudoireverzibilni inhibitor. Apigenin je uzrokovao inhibiciju CYP3A4 enzima, koja se nije mogla povratiti nakon dijalize i nakon dijalize uz prethodni dodatak kalijevog heksacijanoferata. Ostatna aktivnost enzima i u jednom i u drugom slučaju iznosila je oko 4%.

Struktura reaktivnog međuprodukta apigenina kao epoksida koji se konjugira s glutationom predložena je prilikom ispitivanja u inkubaciji s glutationom. Predložen je produkt mase 578,565 g/mol. Pregledom kromatograma dobivenih analizom na spektrometru masa uočeni su predloženi fragmenti epoksida apigenina s pripadajućim *m/z* vrijednostima od 137 i 153, kao i ostale opisane fragmentacije glutationa i hema u odsustvu željeza. Tsimogiannis i sur. (2007) opisuju slične *m/z* vrijednosti fragmenata apigenina, koje su u ovom istraživanju prilagođene mjestima epoksidacije. Nisu uočene *m/z* vrijednosti koje bi odgovarale konjugatima epoksida apigenina s glutationom, odnosno hemom, zbog toga što se vjerojatno formiraju na nanomolarnoj razini.

5.3. Krizin

Krizin se u ovom istraživanju pokazao kao drugi najsnažniji inhibitor, uzrokujući inhibicijski učinak na CYP3A4 enzim, pri čemu je ostatna aktivnost enzima u metaboličkoj inhibiciji iznosila 17,6 ± 2,0%. Metabolička inhibicija bila je najsnažnija inhibicija koju je uzrokovao krizin, dok je u izravnoj i o vremenu ovisnoj inhibiciji ostatna aktivnost enzima iznosila 58,4 ± 2,6%, odnosno 49,9 ± 2,9%. Ovakvo ispitivanje u skladu je s rezultatima koje su dobili Šarić Mustapić i sur. (2018). Međutim, u ovom istraživanju nije uočena statistički značajna razlika između o vremenu ovisne i izravne inhibicije koju je uzrokovao krizin.

Budući da je krizin u ovom istraživanju pokazao dosta nisku IC_{50} vrijednost, za njega je provedeno ispitivanje i na dodatnim koncentracijama (0,01 i 0,1 µM), za razliku od ostalih ispitivanih flavonoida, za koje je ispitivanje provedeno u koncentracijama od 1, 5, 10, 15 i 20 µM, kako bi se odredili parametri kinetike inaktivacije enzima. Inhibicijski potencijal krizina istraživali su i Maharao, Venitz i Gerk (2019). Pregledavali su učinak spojeva koji se općenito smatraju sigurnima za primjenu kod ljudi, a među kojima je bio i krizin. Istraživači su promatrali pretvorbu buprenorfina u norbuprenorfin koju kataliziraju CYP enzimi na ljudskim jetrenim i crijevnim mikrosomima. Ustanovili su da krizin uzrokuje 70% inhibiciju aktivnosti CYP enzima. Ustanovljena IC_{50} vrijednost za krizin bila je 38,9 ± 5,8 µM na ljudskim jetrenim mikrosomima. Utvrđena IC_{50} vrijednost predstavlja čak 64, odnosno 15 puta veće vrijednosti od onih koje su uočene u ovom istraživanju (0,6 ± 0,5 µM za testosteron i 2,5 ± 0,6 za nifedipin). Osim toga, krizin u ovom istraživanju predstavlja flavonoid s najnižom IC_{50} vrijednošću od svih promatranih inhibitora. Ovakve rezultate treba staviti u kontekst provođenja eksperimenata, kao i supstrata na kojima je provedeno ispitivanje. Mahao i sur. nisu provodili ispitivanje ne specifičnom enzimu niti su koristili marker supstrate CYP3A4 enzima, testosteron i nifedipin. Kimura i sur. (2010) su uočili IC_{50} vrijednost od 0,9 µM. U tom istraživanju također se koristio testosteron kao marker supstrat. Međutim, kada se koristio marker supstrat 7-benziloksimetiloksi-3-cijanokumarin, Brahmi i sur. (2011) su dobili IC_{50} vrijednost od 95 ± 31 µM.

Babangida i sur. (2018) su pregledavali utjecaj krizina na oštećenje tkiva štakora uslijed korištenja natrijevog arsenita. U tom istraživanju krizin je potvrđen kao antioksidativni spoj s blagotvornim učincima na oštećeno tkivo. Predlaže se nastavak istraživanja krizina kao ljekovite tvari te je utvrđena K_i vrijednost od 0,959 µM. To je u skladu s ispitivanjima konstante inhibicije u ovom istraživanju gdje se utvrđena K_i vrijednost za krizin kreće od 0,6 ± 0,3 µM do 2,5 ± 1,0 µM zavisno od korištenog supstrata. Konstanta inhibicije krizina bila je najmanja od svih ispitivanih flavonoida. Osim toga, u ovom je istraživanju opisana i brzina kinetike inaktivacije krizina od 0,065 ± 0,005 min^{-1} (testosteron) i 0,07 ± 0,01 min^{-1} (nifedipin) te učinkovitost inaktivacije krizina 0,108 min^{-1} μM^{-1} (testosteron) i 0,03 min^{-1} μM^{-1} (nifedipin).

Azami-Movahed i sur. (2013) su utvrdili da krizin može utjecati na hem vezno mjesto apomioglobina. Ispitan je učinak krizina i drugih malih molekula na vezanje s

hemom te je zaključeno da krizin ostvaruje svoj učinak vezanjem za hem. Osim toga, Chen i sur. (2012) su utvrdili snažan inhibitorni učinak kojeg pokazuje krizin na aktivnost enzima indolamin deoksigenze. Slično kao CYP3A4, indolamin deoksigenaza sadrži hem u aktivnom mjestu. Takvi rezultati su u skladu s ovim istraživanjem, gdje je primjenom krizina utvrđeno smanjenje koncentracije hema za otprilike 95%, odnosno 97% (ponovljeno ispitivanje sa SOD i CAT). Iako se radi o različitim enzimima, moguće je da je princip inhibicije isti – kovalentno vezanje intermedijera krizina za hem. Ostatna koncentracija hema bila je najmanja nakon primjene krizina u odnosu na druge promatrane flavonoide. Krizin kao neutralna molekula izlaže svoj B prsten aktivnom mjestu enzima te ima veći afinitet vezanja za CYP3A4 (Šarić Mustapić i sur., 2018; Bojić i sur., 2019). Izlaganje prstena B hemskom željezu vjerojatno rezultira nastajanjem reaktivnih međuprodukata, koji se vežu za enzim i na taj način ga inaktiviraju.

Slično je bilo i u ispitivanju pseudoireverzibilne inhibicije. Krizin je gotovo u potpunosti poništio aktivnost enzima (više od 99%, odnosno otprilike 99% prilikom ispitivanja sa SOD i CAT). Međutim, nije bilo statistički značajne razlike između ostatne aktivnosti enzima nakon dijalize i dijalize uz prethodni dodatak oksidansa. Stoga se može zaključiti da krizin ne djeluje kao pseudoireverzibilni inhibitor.

Reaktivni međuprodukt koji nastaje uslijed katalitičkog ciklusa CYP enzima nastojao se uhvatiti s glutationom, hvatačem slobodnih radikala. Predložena je struktura konjugata epoksida krizina s glutationom. Određena je molekularna masa za opisani spoj, 576,549 g/mol. Pregledavanjem kromatograma spektrometra masa uočen je fragment krizina *m/z* vrijednosti od 153. Međutim, pregledom kromatograma spektrometra masa nije pronađen traženi spoj, kao ni konjugat epoksida krizina s hemom. Pregledom konstante brzine inaktivacije i učinkovitosti inaktivacije može se doći do zaključka da se konjugat reaktivnog međuprodukta formira u koncentracijama na nanomolarnoj razini. Tako niske koncentracije reaktivnog međuprodukta teško je potvrditi analizom te slični pokušaji utvrđivanja reaktivnih međuprodukata također nisu bili uspješni (Bojić, 2015). Osim toga, kao i kod ranije opisanih reaktivnih međuprodukata, moguće je da su reaktivni međuprodukti nestabilni. Time je njihovo opisivanje i uočavanje na spektrometru masa dodatno otežano.

5.4. Pinocembrin

Pinocembrin (dihidroksiflavanon) posjeduje zasićeni C prsten (tetrahidro-4*H*-piran-4-on). Pinocembrin se od krizina razlikuje dvostrukom vezom u C prstenu na poziciji C2/C3. Ta veza čini strukturu pinocembrina rigidnijom te prilikom vezanja za aktivno mjesto ne dolazi do gubitka energije (Kondža i sur., 2020). Pri ispitivanju o vremenu ovisne inhibicije pinocembrin je pokazao najslabiji inhibicijski učinak u odnosu na sve ispitivane flavonoide (84,2 ± 1,3% ostatne aktivnosti enzima). Pri ispitivanju izravne i o metabolizmu ovisne inhibicije utvrđen je inhibicijski učinak na CYP3A4, pri čemu je o metabolizmu ovisna inhibicija bila najsnažnija; 51,8 ± 2,8% ostatne aktivnosti enzima u odnosu na 77,5 ± 0,8% u izravnoj inhibiciji. Šarić Mustapić i sur. (2018) su dobili slične rezultate te također nisu ustanovili pinocembrinom uzrokovanu o vremenu ovisnu inhibiciju.

Lee i sur. (2019) opisuju antivirusno djelovanje pinocembrina na Zika virus. U navedenom istraživanju utvrđena je IC_{50} vrijednost pinocembrina od 17,4 μM. Autori navode važnost daljnjeg istraživanja antivirusnih i drugih potencijala koje posjeduje pinocembrin. Pri ispitivanju kinetike inhibicije u ovom istraživanju, koristeći se testosteronom kao marker supstratom, uočena je oko osam puta veća IC_{50} vrijednost pinocembrina u odnosu na krizin, dok je kod marker supstrata nifedipina ona bila oko dva puta veća. Do sada nisu bili iskazani parametri IC_{50} vrijednosti pinocembrina na CYP3A4 enzimu, a u ovom istraživanju su određeni (5,0 ± 0,6 μM za testosteron, odnosno 4,3 ± 1,1 μM za nifedipin). Osim toga, opisane su i vrijednosti kinetike inhibicije (1,2 ± 0,3 min^{-1} i 5,1 ± 1,6 min^{-1}). Ovdje opisane vrijednosti kinetike inhibicije od važnosti su za daljnje razumijevanje kliničkih interakcija sastavnica hrane s lijekovima. O važnosti poznavanja kinetike inhibicije govori i primjer lijeka mibefradila. Mibefradil pokazuje inaktivacijsku kinetiku od 0,174 min^{-1} (Prueksaritanont i sur., 1999). Ovaj lijek koristio se kao antihipertenziv, ali je povučen s tržišta budući da je uzrokovao klinički značajne interakcije s više od 30 lijekova registriranih na tržištu (Po i Zhang, 1998).

Citokrom P450 1A2 i 2D6 dovodi do *O*-demetilacije akacetina i aromatskoj hidroksilaciji, pri čemu nastaju dva produkta, apigenin i luteolin. Posredovanjem citokroma P450 1A2 dolazi do aromatske hidroksilacije apigenina u luteolin, a za aromatsku monohidroksilaciju krizina u luteolin jednim dijelom je odgovoran CYP1A2.

Međutim, za pinocembrin nije utvrđen metabolizam posredovan CYP enzimima (Kondža i sur., 2020; Benković i sur., 2019a; Benković i sur., 2019b).

Konstanta brzine inaktivacije za pinocembrin iznosi 0,018 ± 0,001 min^{-1} (testosteron), odnosno 0,04 ± 0,01 min^{-1} (nifedipin), a opisana je i učinkovitost inaktivacije kao omjer konstante brzine inaktivacije i konstante inhibicije; 0,015 $min^{-1}\mu M^{-1}$ (testosteron) i 0,01 $min^{-1}\mu M^{-1}$ (nifedipin).

Pinocembrin je kao i svi ostali flavonoidi uzrokovao smanjenje koncentracije hema. Uslijed inkubacije s pinocembrinom koncentracija hema smanjila se za otprilike 75% te u ponovljenoj inkubaciji s dodatkom SOD i CAT za otprilike 65%. Za pinocembrin također nije utvrđena pseudoireverzibilna inhibicija, koja nije specifična za CYP enzime. Nije uočena statistički značajna razlika između ostatne aktivnosti enzima nakon dijalize (3,27%) i enzima nakon dijalize uz prethodni dodatak kalijevog heksacijanoferata (6,35%).

Za reaktivni međuprodukt pinocembrina koji nastaje katalitičkim ciklusom CYP enzima predložen je epoksid koji se nastojao uhvatiti inkubacijom tijekom 4 h s glutationom. Konjugat glutationa i epoksida pinocembrina opisan je strukturnom formulom te mu je određena masa od 578,565 g/mol. Fragmentne *m/z* vrijednosti, koje opisuju i drugi autori (Mari i sur., 2013), uočene su i ovdje za pinocembrin (153). Međutim, kao i kod drugih ovdje opisanih međuprodukata, zbog izrazito malih vrijednosti nastalog međuprodukta ili nestabilnosti, isti nije utvrđen analizom na spektrometru masa.

6. ZAKLJUČCI

Provedena su ispitivanja inhibicijskog učinka akacetina, apigenina, krizina i pinocembrina na CYP3A4 enzimu u tri različite vrste inhibicija; ispitivanje o vremenu ovisne inhibicije, ispitivanje izravne inhibicije i ispitivanje o metabolizmu ovisne inhibicije. Apigenin se pokazao kao najsnažniji inhibitor u ispitivanju o vremenu ovisne inhibicije, pri čemu je ostatna aktivnost enzima iznosila 37,7 ± 4,4%. Nakon apigenina kao inhibitori su se pokazali krizin (ostatna aktivnost enzima 49,9 ± 2,9%) i akacetin (ostatna aktivnost enzima 55,6 ± 6,4%). Najslabiji inhibitor u ispitivanju o vremenu ovisne inhibicije bio je pinocembrin (84,2 ± 1,3% ostatne aktivnosti enzima).

Apigenin se pokazao i kao najsnažniji inhibitor i u ispitivanju izravne inhibicije, pri čemu je ostatna aktivnost enzima iznosila 31,2 ± 1,8%. Iza apigenina, akacetin je uzrokovao najveću inhibiciju enzima u ispitivanju izravne inhibicije (ostatna aktivnost enzima 48,5 ± 6,5%), zatim krizin (58,4 ± 2,6%) te u konačnici pinocembrin (77,5 ± 0,8%). U ispitivanju o metabolizmu ovisne inhibicije najsnažniji inhibitor bio je akacetin, pri čemu je ostatna aktivnost enzima iznosila 10,6 ± 1,3%. Ostatna aktivnost enzima pri inkubaciji s krizinom iznosila je 17,6 ± 2,0%, s apigeninom 24,5 ± 4,3% te s pinocembrinom 51,8 ± 2,8%, Dominantan oblik inhibicije kojim akacetin, apigenin, krizin i pinocembrin inhibiraju aktivnost CYP3A4 enzima je o metabolizmu ovisna inhibicija.

Korištenjem dvaju marker supstrata (testosterona i nifedipina) ispitani su osnovni parametri kinetike inhibicije: IC_{50} vrijednost, konstanta inhibicije (K_i), konstanta brzine inaktivacije (k_{inact}) i učinkovitost inaktivacije (k_{inact}/ K_i). Krizin se pokazao kao flavonoid s najmanjom IC_{50} vrijednošću, IC_{50} = 0,6 ± 0,5 µM s testosteronom, odnosno 2,5 ± 0,6 µM s nifedipinom kao marker supstratom. Nakon krizina najmanje IC_{50} vrijednosti zabilježene su kod pinocembrina (5,0 ± 0,6 µM, odnosno 4,3 ± 1,1 µM), akacetina (10,9 ± 0,3 µM, odnosno 7,5 ± 2,7 µM) te apigenina (11,4 ± 0,4 µM, odnosno 8,4 ± 1,1 µM). Osim IC_{50} vrijednosti, krizin se pokazao kao flavonoid s najvećim afinitetom vezanja, K_i = 0,6 ± 0,3 µM s testosteronom, odnosno 2,5 ± 1,0 µM s nifedipinom kao supstratom. Nakon krizina, najmanje vrijednosti konstante inhibicije utvrđene su kod pinocembrina (1,2 ± 0,3 µM, odnosno 5,1 ± 1,6 µM), apigenina (1,5 ± 0,8 µM, odnosno 20,2 ± 12,7 µM) te akacetina (6,0 ± 3,0 µM, odnosno 12,1 ± 5,6 µM).

Apigenin je pokazao najveću konstantu brzinu inaktivacije, k_{inact} = 0,11 ± 0,1 min^{-1} s testosteronom, odnosno 0,11 ± 0,04 min^{-1}. Sljedeće utvrđene konstante brzine inaktivacije kod ispitivanih flavonoida bile su za krizin (0,065 ± 0,005 min^{-1}, odnosno 0,07 ± 0,01 min^{-1}), akacetin (0,036 ± 0,006 min^{-1}, odnosno 0,10 ± 0,02 min^{-1}) te pinocembrin (0,018 ± 0,001 min^{-1}, odnosno 0,04 ± 0,01 min^{-1}). Krizin se pokazao kao flavonoid s najvećom učinkovitošću inaktivacije, k_{inact}/K_i = 0,108 min^{-1} μM^{-1} za testosteron, odnosno 0,03 min^{-1} μM^{-1} za nifedipin. Sljedeće utvrđene vrijednosti učinkovitosti inaktivacije bile su za apigenin (0,073 min^{-1} μM^{-1}, odnosno 0,01 min^{-1} μM^{-1}), za pinocembrin (0,015 min^{-1} μM^{-1}, odnosno 0,01 min^{-1} μM^{-1}) te za akacetin (0,06 min^{-1} μM^{-1}, odnosno 0,01 min^{-1} μM^{-1}).

Kako bi se ispitalo kovalentno vezanje reaktivnih međuprodukata s protoporfirinskim dijelom hema proveden je hemokrom-piridin test. Svi ispitivani flavonoidi; akacetin, apigenin, krizin i pinocembrin doveli su do statistički značajnog smanjenja koncentracije hema kako u ispitivanju bez dodatka SOD i CAT, tako i u ispitivanju uz dodatak navedenih enzima, koji za cilj imaju sprječavanje uništenja hema reaktivnim kisikovim spojevima. Krizin je uzrokovao najveće smanjenje koncentracije hema, pri čemu je ostatna koncentracija hema iznosila 5,5%, odnosno 2,9% (uz dodatak SOD i CAT). Nakon krizina, sljedeće najveće smanjenje koncentracije hema uočeno je prilikom primjene pinocembrina (koncentracija hema 25,3%, odnosno 35,3% uz primjenu SOD i CAT), apigenina (45,1% te 55,1% uz SOD i CAT) te akacetina (48,8% te 63,3% uz SOD i CAT).

Kako bi se ispitalo vezanje akacetina, apigenina, krizina i pinocembrina na fero oblik željeza provedeno je ispitivanje pseudoireverzibilne inhibicije dijalizom enzima, odnosno dodatkom kalijevog heksacijanoferata kao oksidansa. Nijedan od navedenih flavonoida nije pokazao pseudoireverzibilnu inhibiciju na CYP3A4 enzim. Između ostatne aktivnosti enzima nakon dijalize s i bez dodatka oksidansa nije uočena statistički značajna razlika, prema tome ne dolazi do nastanka kompleksa akacetina, apigenina, krizina i pinocembrina s fero oblikom željeza u aktivnom mjestu enzima.

Predloženo je da se akacetin, apigenin, krizin i pinocembrin uslijed katalitičkog ciklusa CYP enzima metaboliziraju u epokside. Nastali epoksidi bi trebali reagirati s hemom ili glutationom kao hvatačem slobodnih radikala. Uzorci su analizirani

tekućinskom kromatografijom visoke djelotvornosti, ali očekivani adukti nisu uočeni. Moguće je da se adukti formiraju u maloj količini koja nije uočljiva ili nisu dovoljno stabilni.

Akacetin, apigenin, krizin i pinocembrin pokazuju inhibiciju ireverzibilnog karaktera kovalentnim vezanjem na enzim citokrom P450 3A4. Za inhibiciju su odgovorni reaktivni međuprodukti iz katalitičkog ciklusa citokroma P450 koji nastaju biotransformacijom akacetina, apigenina, krizina i pinocembrina.

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8. PRILOZI

Ovaj dodatak sadrži dva znanstvena rada, koji su objavljeni u časopisima zastupljenim u bazama *Web of Science (WOS), SCOPUS, Public Medline (PUBMED)*, a obrađuju teme vezane za sadržaj ovog doktorskog rada.

8.1. Prilog 1

Kondža M, Rimac H, Maleš Ž, Turčić P, Ćavar I, Bojić M. (2020) Inhibitory Effect of Acacetin, Apigenin, Chrysin and Pinocembrin on Human Cytochrome P450 3A4. Croatica Chemica Acta. 93:1-7.

ORIGINAL SCIENTIFIC PAPER

CROATICA CHEMICA ACTA

Croat. Chem. Acta **2020**, *93*(1)
Published online: August 03, 2020
DOI: 10.5562/cca3652

HKD 1926

Inhibitory Effect of Acacetin, Apigenin, Chrysin and Pinocembrin on Human Cytochrome P450 3A4

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RECEIVED: June 26, 2020 ★ REVISED: July 28, 2020 ★ ACCEPTED: July 30, 2020

Abstract: Cytochrome P450 3A4 is the most significant enzyme in metabolism of medications. Flavonoids are common secondary plant metabolites found in fruits and vegetables. Some flavonoids can interact with other drugs by inhibiting cytochrome P450 enzymes. Thus, the objective of this study was to determine inhibition kinetics of cytochrome P450 3A4 by flavonoids: acacetin, apigenin, chrysin and pinocembrin. For this purpose, testosterone was used as marker substrate, and generation of the 6β-hydroxy metabolite was monitored by high performance liquid chromatography coupled with diode array detector. IC_{50} values, inhibition constants, and rates of inhibition were determined. IC_{50} values ranged between 0.6 and 11.4 μM. The strongest inhibitor was chrysin (IC_{50} 0.6 μM, inhibition constant 0.6 μM, inhibition rate constant 0.065 min^{-1}, inhibition efficacy 0.108 min^{-1} μM^{-1}). Compared to other flavonoids analyzed, chrysin's inhibitory effect can be attributed to the hydrophobic nonsubstituted B ring, as well as rigidity of the structure. When foods rich in chrysin are consumed, e.g. honey and propolis, chrysin can cause food-drug interactions. Further *in vitro* studies are needed to determine the reactive intermediate responsible for inactivation of cytochrome P450 3A4 enzyme, as well as *in vivo* studies to determine possible clinical significance of this inhibition.

Keywords: flavonoids, inhibition, cytochromes P450.

INTRODUCTION

CYTOCHROMES P450 are the most important metabolic enzymes responsible for over 94 % of metabolic reactions of drugs and other xenobiotics.[1] These enzymes do not fit to a lock (enzyme) and key (substrate) enzyme model. Rather, every cytochrome P450 can have numerous substrates and each xenobiotic can be a substrate to multiple cytochrome P450 enzymes.[2] Consequently, interactions between different xenobiotics can occur, e.g. drug-drug interactions that can have repercussions on the outcome of the pharmacotherapy as well as possible side-effects.

Most common reason for metabolic drug-drug interactions are inhibitions of cytochromes P450.[3] These inhibitions can be reversable, most commonly competitive, and are regarded as less severe, as an adjustment of the medication dose will usually resolve possible unwanted effects of the interaction.[3] However, in the case of irreversible inhibition, a simple adjustment of the dose is not possible, and a drug, the perpetrator of the inhibition, needs to be discontinued and preferably replaced. If an irreversible inhibition is observed, enzyme activity diminishes, and new copies of the enzyme have to be expressed for the enzyme activity to be recovered completely. This process can take from a few days to a few weeks, depending of the metabolic enzyme.[3]

While metabolic drug-drug interactions are of significant interest, food-drug interactions have just come into focus.[4,5] Flavonoids, as the most common secondary metabolites found in higher plants, are constituents of daily foods.[6] These compounds have been extensively studied

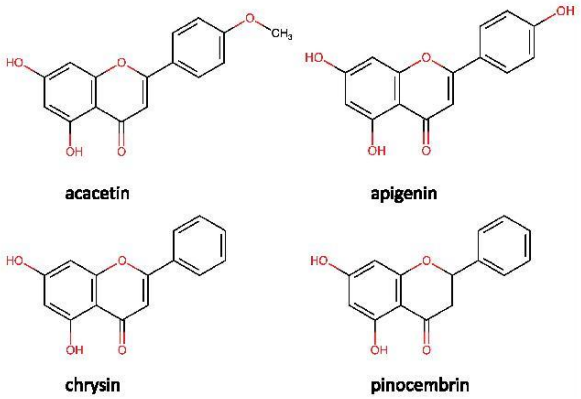

Figure 1. Structural characteristics of studied flavonoids: flavones acacetin, apigenin and chrysin, and flavanone pinocembrin.

for over a century when they were discovered as vitamin P.[7] It has been shown that they have antioxidant, anti-inflammatory, hepatoprotective, antimicrobial, cardio-protective, and other pharmacological properties.[6,8] The major issue of their application *in vivo* is their bioavailability. However, it has been shown that some flavonoids, e.g. chrysin, can achieve submicromolar concentrations in plasma and some flavonoids, as soya isoflavones, hesperetin and diosmetin, have been in commercial use as dietary supplements.[9]

Cytochrome P450 3A4 is the most significant cytochrome P450 enzyme as it metabolizes about one third of the drugs.[1,10] In the previously published screening study, it has been shown that acacetin, apigenin, chrysin and pinocembrin are the most prominent inhibitors of human cytochrome P450 3A4 at 1 μM concentration.[11] Flavanone pinocembrin (Figure 1.) reduces the enzyme activity by 50 %, while flavones acacetin, apigenin and chrysin reduce the enzyme activity to 5 %, 24 % and 17 %, respectively.[11]

The inhibition of P450 3A4 by acacetin, apigenin, chrysin and pinocembrin is not well characterized, and, if available, is reported as IC_{50} value. The IC_{50} values are dependent of the type of inhibition (direct, time and metabolism dependent), as well as experimental setup. Thus, the objective of this study was to determine metabolism dependent inhibition kinetics, inhibition constants and rates of inhibition of P450 3A4 by the aforementioned flavonoids. As these flavonoids have different structural features (Figure 1.): presence or absence of double bond at the position C2-C3, hydroxylation of B ring and methylation of hydroxyl groups, conclusions about structure-activity relationship can be made.

EXPERIMENTAL SECTION

Materials

Flavonoids used in this study (acacetin, apigenin, chrysin, and pinocembrin) were acquired commercially from Sigma-Aldrich (St. Louis, MO, USA).

Recombinant cytochromes P450 3A4 coexpressed with NADPH cytochrome P450 reductase and cytochrome b_5 in baculosomes were obtained from Thermo Fisher Scientific, Waltham, MA, USA.

Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD) and NADP disodium salt were purchased from Sigma Aldrich. Potassium phosphate (p.a.) and dichloromethane (p.a.) were purchased from Kemika d.d. (Zagreb, Croatia). Methanol for chromatography was purchased from Merck KGaA (Darmstadt, Germany). Ultra-pure water was produced using Arium comfort combined water production system from Sartorius AG (Goettingen, Germany).

Incubations for Determination of Inhibition Kinetics

To achieve the objective of this study, testosterone was used as the marker substrate. Generation of the 6β-hydroxy metabolite, that reflected residual enzyme activity, was monitored by high performance liquid chromatography coupled with diode array detector (HPLC-DAD).[12] For different concentrations of flavonoid, residual

enzyme activity was determined after different periods of incubation. This data was used to construct Michaelis-Menten curve, and inhibition constants and rate of inhibition were determined. The results were assessed in the light of current guidelines on inhibition studies.[13]

Evaluation of enzyme kinetics was conducted using baculosomes of recombinant cytochrome P450 3A4 coexpressed with NADPH cytochrome P450 reductase and cytochrome b_5. A range of flavonoid aglycons concentrations from 0.01 to 20 μM was prepared; appropriate aliquots of 20 mM flavonoid solutions were transferred to glass tubes and evaporated until dry on a water bath equipped with mechanical shaking. After solvent evaporation, a 100 μL incubation mixture was prepared in each of the tubes by adding cytochrome P450 baculosomes (5 pmol), 50 mM potassium phosphate buffer pH 7.4, and ultra-pure water. Generating system containing glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase in a ratio 100:50:2 (*V*/*V*/*V*) was used as a source of the coenzyme (15 % volume in final incubation, *V*/*V*), and its addition marked the beginning of the reaction. Pre-incubations were conducted in duplicate for zero, 5, 10, 15, 20 and 25 minutes.[14]

After the appropriate time period, the residual enzyme activity was tested by adding 1 μL of testosterone solution (final concentration 200 μM). For determination of IC_{50} values, preincubation was set to 15 minutes. Testosterone served as the marker substrate of cytochrome P450 activity. The reaction was quenched by adding 1 mL of cold dichloromethane. Reaction tubes were centrifuged at 1900 *g* (3000 rpm) for 10 minutes. The dichloromethane layer was transferred into a HPLC vial, and the organic solvent was evaporated under a stream of nitrogen. Dry residues were dissolved in methanol (30 μL) and analyzed by HPLC-DAD.[12]

HPLC-DAD Analysis

High performance liquid chromatography coupled with diode array detection (Agilent 1100 instrument, Santa Clara, CA, USA) was used for the analysis of testosterone and the 6β-hydroxy metabolite. A C_{18} analytical column (Agilent Zorbax SB C18 column 4.6 × 250 mm, 3 μm) was used for isocratic analysis with a mixture of 64 % CH_3OH/ 36 % H_2O (*V*/*V*) at a flow rate of 1.0 mL/min. Analytes were detected at 240 nm, and the amount of generated 6β-hydroxy testosterone was determined as the area under the curve based on the calibration curve of the standard.[12]

Determination of Enzyme Inhibition Parameters

All incubations were conducted in duplicate. The results were expressed as the amount of generated metabolite based on HPLC-DAD analysis (*vide supra*). Based on these results, inhibition rates were determined and used for calculation of the major parameters of enzyme inhibition kinetics (inhibition constant and inhibition rate constant) based on the Michaelis-Menten equation. Non-linear three parameters sigmoidal-logistic equation was used for IC_{50} calculations. Program R (The R Project for Statistical Computing, Vienna, Austria) was used for calculations.

RESULTS AND DISCUSION

In the previously published screening study, it was shown that acacetin, apigenin, chrysin, and pinocembrin inhibit human cytochrome P450 3A4 at 1 μM concentration.[11] Herein, we have characterized the inhibition kinetics and determined IC_{50} values inhibition constants (K_I), and rates of inhibition (k_{inact}) for each of the aforementioned flavonoids. The strongest inhibitor was chrysin (IC_{50} 0.6 μM, inhibition constant 0.6 μM, inhibition rate constant 0.065 min^{-1}, inhibition efficacy 0.108 min^{-1} μM^{-1}).

Since the inhibition was characterized as metabolism-dependent, the first objective herein was to determine the inhibitory concentrations IC_{50} (Figure 2.). For this purpose, flavonoids were first preincubated with the cytochrome P450 3A4 enzyme for 15 minutes, after which the residual activity was determined using testosterone as the marker substrate.

Chrysin had the lowest IC_{50} value. This is in agreement with the fact that a 1 μM concentration of chrysin reduced the enzyme activity by 95 %, indicating that the IC_{50} value is in a submicromolar range.[11] Herein, we obtained a value of 0.6 ± 0.5 μM. Acacetin and apigenin had around twentyfold higher IC_{50} values, 10.9 ± 0.3 μM and 11.4 ± 0.4 μM, respectively. In the previously conducted molecular docking study, it was shown that chrysin has a higher binding affinity to cytochrome P450 3A4 as a neutral molecule, exposing the B ring to the iron in the active center of the enzyme.[11,15] This confirms that cytochromes P450 tend to metabolize more lipophilic species.[3] By exposing the B ring to the cytochrome P450 active site, a reactive intermediate responsible for inactivation can be generated.

Acacetin and apigenin differ from chrysin as they have a methoxy and a hydroxy group, respectively, at the 4' position of the B ring. Oxygen at the 4' position probably interacts with the ferric ion in the active site of the enzyme and thus tends to inhibit enzyme activity reversibly as a ligand, which has also been reported.[15] Consequently, the observed IC_{50} values for acacetin and apigenin were higher when compared to chrysin.

Interestingly, pinocembrin had around tenfold higher IC_{50} value of 5.0 ± 0.6 μM when compared to chrysin (0.6 ± 0.5 μM). Pinocembrin belongs to the flavanone class while chrysin is a flavone, and the only difference in their structures is a single bond at the C2-C3 position in

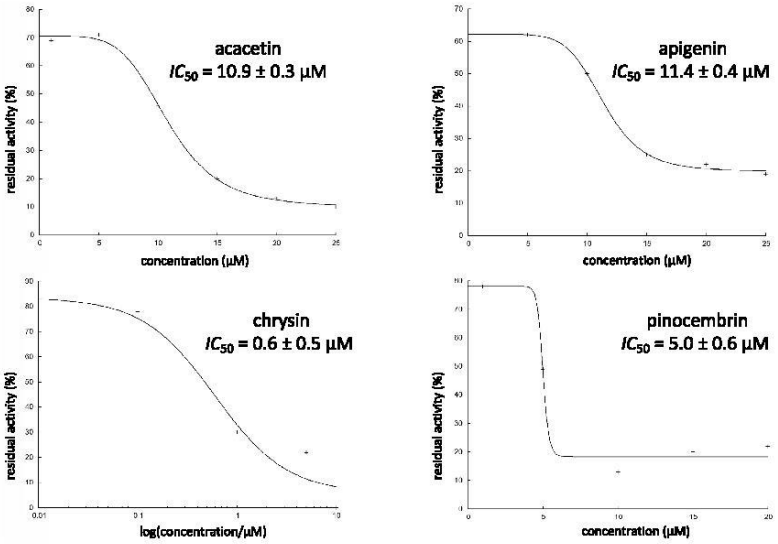

Figure 2. Values of inhibitory concentration that reduces enzyme activity to 50 % (IC_{50}) for each of the analysed flavonoids.

pinocembrin *vs.* a double C2=C3 bond in chrysin. A double bond in chrysin makes the structure rigid, and no energy loss is observed while binding to the active site.

Acacetin is one of the major polyphenols present in honey, which is believed to be associated with the prevention of heart disease.[16] Different studies indicate beneficial effects this flavonoid shows. In addition to the prevention of heart disease, its antioxidant, anti-inflammatory and antiplasmodial properties have also been shown.[17,18] Additionally, studies indicate its antiproliferative properties on different types of tumor cells present in the liver, prostate, and lungs.[19] On a rat cytochrome P450 3A subfamily it was determined that acacetin inhibits the enzyme with a IC_{50} value of 8.2 µM, using midazolam as the marker substrate.[20] This is similar to the IC_{50} value of 10.9 ± 0.3 µM reported herein.

Apigenin is one of the most present flavonoids in food, especially in parsley.[21] As reported in the review by Ross and Kasum[21], *in vitro* studies indicate a significant role of apigenin in the prevention of malignancies and cardiovascular diseases as well as the stimulation of the immune system. The stated antitumor properties of apigenin are evident in inhibiting the growth of melanoma cell cultures, therefore its use in various therapeutic combinations against metastatic melanoma has been studied.[22] Apigenin has been shown to inhibit P450 3A4 in an assay using 7-benzyloxy-4-trifluoromethylcoumarin as the marker substrate with an IC_{50} value of 1.8 µM.[23] Another group obtained an IC_{50} value of 31 ± 8 µM for apigenin using 7-benzyloxymethyloxy-3-cyanocoumarin as the marker substrate of cytochrome P450 3A4.[24] Use of different substrates for determining cytochrome P450 3A4 enzyme activity can explain observed differences in IC_{50} values between studies. As the cytochrome P450 3A4 has a large active site when compared to other human liver cytochromes P450, use of at least two marker substrates is advisable for the assessment of inhibition kinetics.[3,13]

Chrysin is naturally present in honey, but also in various plants and propolis.[25] Its anti-inflammatory and antioxidant effects are well documented.[26,27] Its chemoprotective effects are increasingly being investigated, and chrysin is believed to exert its effect by inducing apoptosis. Chrysin has shown positive *in vitro* effects on cervical cancer, leukemia, prostate and breast cancer, as well as colon cancer.[28,29] Chrysin has been shown to inhibit cytochrome P450 3A4 in an enzyme activity assay using 7-benzyloxymethyloxy-3-cyano-coumarin as a the marker substrate, and the obtained IC_{50} value was 95 ± 31 µM.[24] In contrast, when testosterone was used as the marker substrate, IC_{50} value was determined to be 0.9 µM, similar to this study (0.6 ± 0.5 µM).[30]

Pinocembrin modulates inflammatory responses, and there is a potential for its use in the treatment of ischemic stroke and similar clinical conditions.[31] In addition, its antifungal activity has been thoroughly described, as well as the induction of apoptosis in colon cancer cells.[32] While its *in vitro* pharmacological effects have been extensively studied, this is not the case with inhibition of cytochrome P450 3A4 enzyme. Herein, we have reported an IC_{50} value of 5.0 ± 0.6 μM.

Aforementioned results of inhibition assays have been expressed as IC_{50} values. As IC_{50} values are depend on the experimental setup further characterization of inhibition is needed. Thus, we have analyzed inhibition kinetics for each flavonoid, results of which are presented in Figure 3.

While for the most flavonoids the rate of inactivation was assessed at 0, 1, 5, 10, 15 and 20 μM final concentration in flavonoid incubation, due to its low IC_{50} value, concentrations for chrysin were adjusted to 0.01, 0.1, 1, 5 and 10 μM.

The lowest inhibition constant was found for chrysin, with a value of 0.6 ± 0.3 μM. A flavanone analogue of chrysin – pinocembrin had a higher value of 1.2 ± 0.3 μM, with a much lower value of inhibition rate of 0.018 ± 0.001 min^{-1} compared to chrysin (0.065 ± 0.005 min^{-1}). Hydroxylated and methoxylated chrysin derivatives, i.e. apigenin and acacetin had higher values of inhibition constant: 1.5 ± 0.8 μM and 6 ± 3 μM, respectively, while their inhibition rate constants were 0.11 ± 0.01 min^{-1} and 0.036 ± 0.006 min^{-1}.

Consequently, inactivation efficacy was the highest for chrysin, 0.108 min^{-1} μM^{-1}. This was followed by apigenin (0.073 min^{-1} μM^{-1}), pinocembrin (0.015 min^{-1} μM^{-1}) and acacetin (0.006 min^{-1} μM^{-1}). In comparison, one of the well-known time dependent cytochrome P450 3A4 inhibitors, mibefradil, shows an inactivation efficacy of 0.174 min^{-1} μM^{-1}.[33] This antihypertensive drug has been retracted from market due to clinically significant interactions it caused with over 30 drugs in the market.

Extrapolation of the inactivation kinetic data obtained in the herein study is not straightforward. The setup of the experiment enables us to characterize observed metabolism/time dependent inhibition. Other parameters can also influence the results, e.g. some drugs can act as substrates and inhibitors at the same time.[3] However, based on previous reports by Benković *et al.*,[34,35] metabolism of acacetin, apigenin, chrysin and pinocembrin mediated by cytochrome P450 3A4 can be disregarded. Acacetin is susceptible to *O*-demethylation and aromatic hydroxylation, generating two products, apigenin and luteolin, which is attributed to cytochromes P450 1A2 and 2D6; apigenin undergoes aromatic hydroxylation to luteolin and this is mediated by cytochrome P450 1A2; chrysin is aromatically monohydroxylated to baicalein or norvogonin or dihydroxylated to luteolin, and this is mediated to some extent by cytochrome P450 1A2; while pinocembrin was not found to be susceptible to the metabolism mediated by hepatic cytochromes P450.[34,35]

Usually, observed time-dependent inhibition is at the same time metabolism-dependent, meaning that cytochrome P450 generates a reactive intermediate that inactivates the enzyme (suicide substrates).[3] It can be speculated that this could be due to generation of a reactive epoxide on an aromatic ring and further research

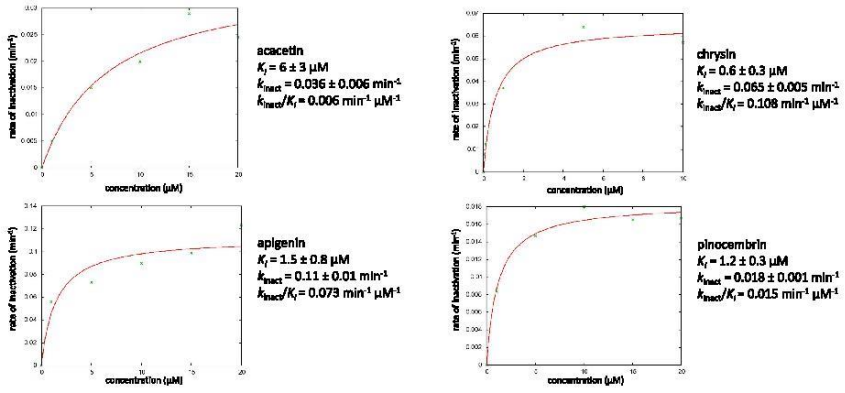

Figure 3. Inhibition kinetic parameters for each of the analysed flavonoids: K_I – inhibition constant, k_{inact} – inhibition rate constant, and k_{inact}/K_I – inactivation efficacy.

should focus on determining the structure of the reactive intermediate. However, this is not an easy task, as the inhibition rate constant of chrysin is 0.065 ± 0.005 min^{-1}. Incubations are usually limited to 30 minutes, as longer incubations can result in hydrogen peroxide formation due to futile catalytic cycles. Hydrogen peroxide is known to destroy the heme moiety in the active site of the enzyme. Consequently, in half an hour, under maximal inhibition rate, in an incubation with 5 pmol of enzyme only 9.75 pmol of the reactive intermediate would be generated. That means that in an incubation in which 10 μM chrysin is present (concentration that enables maximal inhibition rate), around 100 nM of the reactive intermediate would be generated (1 ‰). Not surprisingly, even for the aforementioned mibefradil, the reactive intermediate has not been trapped nor was its structure described.[3]

To put the obtained results into perspective, an assessment parameter *R* can be calculated as per Food and Drug Administration guidelines.[13] For time-dependent inhibition $R = (k_{obs} + k_{deg}) / k_{deg}$ where $k_{obs} = (k_{inact} \times 50 \times I_{max}) / (K_I + 50 \times I_{max})$; k_{obs} is the observed inhibition rate constant of the affected enzyme, k_{deg} is the apparent degradation rate constant of the affected enzyme (0.0138 h^{-1} for cytochrome P450 3A4)[36], K_I is inhibition constant – the inhibitor concentration causing half-maximal inactivation (0.6 μM), k_{inact} is the inhibition rate constant (0.065 min^{-1}), and I_{max} is the maximal unbound plasma concentration of the interacting drug at steady state (12 nM for chrysin)[9]. Thus, assessment parameter *R* has a value of 142, well above the threshold of 1.25, and consequently further assessment and pharmacokinetic modeling is needed to evaluate if this inhibition is clinically significant.

Based on the inhibition kinetic data, it can be assumed that interactions with herein studied flavonoids will be clinically significant at the level of metabolic enzyme cytochrome P450 3A4. *In vivo* data on apigenin in animal models confirm this observation.[37,38] The inhibition effect of apigenin against cytochrome P450 3A4 mediated metabolism was confirmed in rats when combined with etoposide. Etoposide is metabolized primarily by cytochrome P450 3A4 and, in the presence of apigenin, bioavailability of oral etoposide in rats was increased. This interaction does not necessarily need to be unwanted, as combined use of apigenin might be helpful to improve etoposide bioavailability in chemotherapeutic applications.[37] Imatinib, another chemotherapeutic agent, is metabolized by cytochrome P450 3A4 and it has been shown that in the short term apigenin can increase imatinib concentration *in vivo* in rats as an animal model.[38]

The major contribution of this study is characterization of inhibition kinetics of cytochrome P450 3A4 by selected flavonoids that show metabolism dependent inhibition i.e. inactivation of the enzyme. Further *in vitro* studies can be conducted on different enzyme sources (e.g. tissue, liver microsomes) or using other marker substrates of cytochrome P450 3A4 (e.g. midazolam, nifedipine). The data from this study can be used to assess flavonoid-drug or food-drug interactions. While the data suggest that clinically significant interaction can exist, further assessment is needed through pharmacokinetic modeling or *in vivo* models to confirm the relevance of the results.[13]

CONCLUSION

Acacetin, apigenin, chrysin and pinocembrin cause time-dependent inhibition of cytochrome P450 3A4. IC_{50} value is the lowest for chrysin, indicating that hydrophobicity of the nonsubstituted B ring, as well as rigidity of the structure (absence of a single bond between C2 and C3 atoms), plays an important role in the inhibition. Based on the data for inactivation kinetics, it can be concluded that chrysin has the highest potential to cause food-drug interactions when used with foods rich in this flavonoid, e.g. honey and propolis. Further *in vitro* studies are needed to determine the reactive intermediate responsible for inactivation of cytochrome P450 3A4 enzyme, as well as *in vivo* studies to determine possible clinical significance of this inhibition.

Acknowledgment. This research was supported by the Croatian Science Foundation under the project UIP-2014-09-5704 (M. B.) and Federal Ministry of Education and Science of the Federation of Bosnia and Herzegovina (I. Ć.).

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Bojić M, Kondža M, Rimac H, Benković G, Maleš Ž. (2019) The Effect of Flavonoid Aglycones on the CYP1A2, CYP2A6, CYP2C8 and CYP2D6 Enzymes Activity. Molecules. 24: 3174.

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The Effect of Flavonoid Aglycones on the CYP1A2, CYP2A6, CYP2C8 and CYP2D6 Enzymes Activity

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Received: 11 August 2019; Accepted: 31 August 2019; Published: 1 September 2019

Abstract: Cytochromes P450 are major metabolic enzymes involved in the biotransformation of xenobiotics. The majority of xenobiotics are metabolized in the liver, in which the highest levels of cytochromes P450 are expressed. Flavonoids are natural compounds to which humans are exposed through everyday diet. In the previous study, selected flavonoid aglycones showed inhibition of CYP3A4 enzyme. Thus, the objective of this study was to determine if these flavonoids inhibit metabolic activity of CYP1A2, CYP2A6, CYP2C8, and CYP2D6 enzymes. For this purpose, the *O*-deethylation reaction of phenacetin was used for monitoring CYP1A2 enzyme activity, coumarin 7-hydroxylation for CYP2A6 enzyme activity, 6-α-hydroxylation of paclitaxel for CYP2C8 enzyme activity, and dextromethorphan *O*-demethylation for CYP2D6 enzyme activity. The generated metabolites were monitored by high-performance liquid chromatography coupled with diode array detection. Hesperetin, pinocembrin, chrysin, isorhamnetin, and morin inhibited CYP1A2 activity; apigenin, tangeretin, galangin, and isorhamnetin inhibited CYP2A6 activity; and chrysin, chrysin-dimethylether, and galangin inhibited CYP2C8. None of the analyzed flavonoids showed inhibition of CYP2D6. The flavonoids in this study were mainly reversible inhibitors of CYP1A2 and CYP2A6, while the inhibition of CYP2C8 was of mixed type (reversible and irreversible). The most prominent reversible inhibitor of CYP1A2 was chrysin, and this was confirmed by the docking study.

Keywords: flavonoids; CYP1A2; CYP2A6; CYP2C8; CYP2D6; inhibition

1. Introduction

Cytochrome P450 (CYP) enzymes are the most significant enzymes for the metabolism of substances foreign to the human body, including drugs [1]. These enzymes are hemoproteins containing heme, which is bound to the apoprotein part. The iron ion is linked to the heme by four coordinative covalent bonds, while the fifth coordinative covalent bond links iron to the cysteine residue of the apoprotein. Functionally cytochromes P450 are monooxygenases that incorporate one atom of oxygen from molecular oxygen into the substrate [1].

The reactions catalyzed by cytochromes P450 are not limited to one type of reaction or one substrate. Instead, each cytochrome P450 enzyme has numerous substrates and catalyzes chemically diverse reactions. Beside oxidation reactions (e.g., hydroxylations, dealkylations), cytochromes catalyze reduction, desaturation, ester cleavage, ring expansion, ring formation, aldehyde scission, dehydration, ipso attack, one-electron oxidation, coupling reactions, rearrangement of fatty acid, and prostaglandin hydroperoxides [2].

The major site of cytochromes P450 activity within the human organism is the liver. The liver cytochromes P450 involved in the metabolism of most drugs are: CYP3A4/5 (30%), CYP2D6 (20%), CYP2C9 (13%), CYP1A2 (9%), and others [3]. As nearly 94% of oxidation reactions of medicinal drugs are catalyzed by cytochromes P450, the involvement of these enzymes in the pharmacokinetic of the drugs is significant.

Drugs and other xenobiotics can be substrates of cytochromes P450, but they can also act as inhibitors. Two substrates competing for the same active site of the cytochrome P450 enzyme present the most common observed interaction, e.g., ketoconazole is a competitive CYP3A4 inhibitor that can reduce terfenadine metabolism, which is almost exclusively metabolized by CYP3A4 [4]. The aforementioned interaction is reversible, i.e., discontinuation of the ketoconazole application normalizes terfenadine metabolism and pharmacokinetic.

Inhibitions that can have more significant consequences are irreversible. These are usually caused by inactivation of cytochromes P450 by a reactive intermediar that covalently binds to the apoprotein or the heme part of cytochrome P450. A good example is the antihypertensive drug mibefradil, which irreversibly inhibits CYP3A4. Mibefradil is converted to a reactive intermediar in the catalytic cycle of CYP3A4. The generated intermediar covalently binds to the heme part of CYP3A4, causing the inactivation of the enzyme [5]. The enzyme activity is lost and can only be regained by the expression of an additional protein (enzyme), which takes days to weeks [6].

Except drugs, other xenobiotics like flavonoids can interact with cytochrome P450. Flavonoids are natural polyphenols abundantly present in higher plants, including fruits, vegetables, and plant-derived products, such as wine and propolis [7–9]. Flavonoids are regarded as vitamins that are important in the regulation of oxidative stress and act as antioxidants. Consequently, flavonoids have many beneficial health effects such as antiallergic, anti-inflammatory, antioxidative, antimicrobial, antitumorigenic, and antimutagenic effects, thus preventing cancer, heart disease, bone loss, and a number of other diseases [8,10–13].

Flavonoids, in nature, mainly come bound to a sugar moiety as glycosides. Glycosides are susceptible to hydrolysis, and aglycones are liberated and absorbed in the gut [6]. In the liver, aglycones are metabolized by the cytochromes P450. The major metabolic reactions to which flavonoids are susceptible are aromatic hydroxylations and *O*-dealkylations, which are catalyzed by cytochromes P450 [14,15]. The major cytochromes P450 involved in the metabolism of flavonoid aglycones are CYP1A2, CYP3A4, and CYP2D6 [14,15]. Flavonoids can also act as inhibitors of metabolic enzymes, causing clinically significant interactions [16–21].

In the previous study [22], it was shown that some flavonoid aglycones can cause the inhibition of CYP3A4 metabolic activity. These flavonoids were: Hesperetin, pinocembrin, acacetin, chrysin, chrysin-dimethylether, flavone, tangeretin, galangin, isorhamnetin, morin, and tamarixetin (statistical significance $p < 0.1$) (Figure 1). Thus, the aim of this study was to assess the inhibitory effect of the aforementioned flavonoids on the CYP1A2, CYP2A6, CYP2C8, and CYP2D6 enzymes activity.

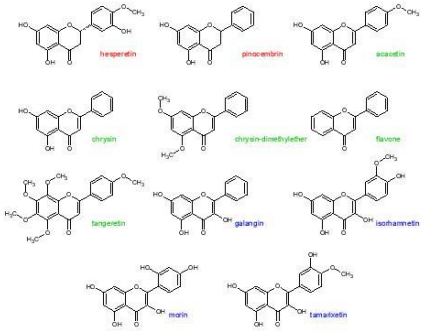

Figure 1. Structural characteristics of flavonoids used in this study. Flavanones are marked in red, flavones in green, and flavanols in blue.

2. Results and Discussion

To test the enzyme activity of each individual cytochrome P450, marker substrates/reactions were used, namely phenacetin *O*-deethylation for monitoring CYP1A2 enzyme activity, coumarin 7-hydroxylation for monitoring CYP2A6 enzyme activity, paclitaxel 6-α-hydroxylation for monitoring CYP2C8 enzyme activity, and dextromethorphan *O*-demethylation for monitoring CYP2D6 enzyme activity (Figure 2). All generated products were determined using high-performance liquid chromatography coupled with a diode-array detector (HPLC-DAD) with the appropriate method of analysis for each individual reaction (*vide infra*).

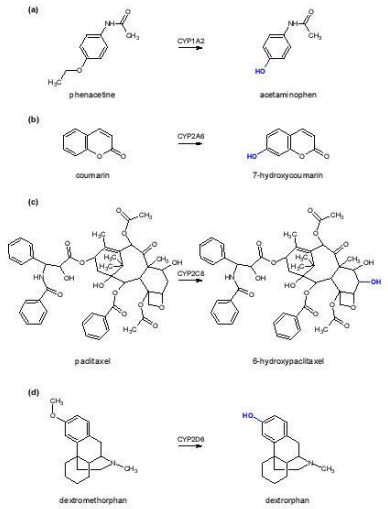

Figure 2. Marker substrates and reactions used for monitoring enzyme activity: (**a**) Phenacetin *O*-deethylation for the CYP1A2 enzyme, (**b**) coumarin 7-hydroxylation for the CYP2A6 enzyme, (**c**) paclitaxel 6-α-hydroxylation for the CYP2C8 enzyme, and (**d**) dextromethorphan *O*-demethylation for the CYP2D6 enzyme. The sites of reaction are marked in blue.

CYP1A2 activity was inhibited by five of the tested flavonoids, namely chrysin, hesperetin, isorhamnetin, morin, and pinocembrin (Figure 3).

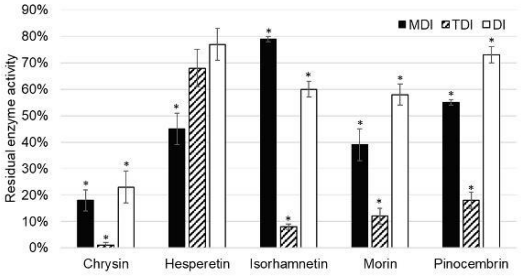

Figure 3. Residual CYP1A2 enzyme activity after incubation with 1 μM flavonoid in the experiments for the determination of metabolism dependent inhibition (MDI), time dependent inhibition (TDI), and direct inhibition (DI). * denotes that observed inhibition is statistically significant ($p < 0.05$) when compared to the control (without addition of flavonoid).

Chrysin showed inhibition of CYP1A2 in all inhibition assays: Metabolism (MDI) and time dependent (TDI), as well as in the direct inhibition (DI) assay. As direct inhibition and metabolism inhibition assay are of comparable residual activities (23 ± 6% (p = 0.001) and 18 ± 4% (p = 0.004), respectively), it can be concluded that inhibition is reversible. This is in accordance with previously published data. Lee et al. [23] tested 21 flavonoids, out of which chrysin was shown to be the strongest inhibitor of CYP1A2. The enzyme activity was tested with N^3-demethylation of caffeine as a marker reaction, and chrysin showed reversible, competitive inhibition. On rat liver microsomes, Siess et al. [24] showed that chrysin inhibited ethoxy- and pentoxy-resorufin dealkylation and characterized the inhibition as mix type reversible. Phenacetin *O*-deethylation and caffeine N^3-demethylation are marker reactions of CYP1A2, ethoxy- and pentoxy-resorufin, while dealkylation was used to characterize the overall contribution of all cytochromes. The inhibition observed in the study performed by Siess et al. [24] can be consequence of the inhibition of other cytochromes P450, rather than CYP1A2. However, Kim et al. [25] confirmed the reversible mix inhibition of CYP1A2 using the ethoxyresorufin *O*-deethylation reaction on a recombinant cytochrome P450 system. Mixed inhibition is in accordance with observed results of the TDI assay that showed complete inhibition of CYP1A2 (Figure 3). In the TDI assay, sufficient time is given by preincubating chrysin with the enzyme before starting the catalytic marker reactions.

Hesperetin showed a less extensive inhibition of CYP1A2 under the same conditions compared to other flavonoid inhibitors of CYP1A2. As residual activity increased in the order of DI, TDI, and MDI assays, it can be concluded that hesperetin showed reversible, as well as irreversible, inhibition of CYP1A2, although the later one was not statistically significant (Figure 3). Doostdar et al. [26] showed that hesperetin is an inhibitor of CYP1B1, while residual CYP1A2 enzyme activity was not statistically different to control in the analyzed range of concentrations, including the one used in the present study (1 μM). This difference could be attributed to different marker reaction used by Doostdar et al. [26], i.e., ethoxyresorufin *O*-dealkylation. The significance of hesperetin CYP1A2 inhibition was relevant for the pharmacokinetic and metabolism of rasagiline mesylate in Wistar rats, explaining the observed drug-hesperetin interaction [27].

Chang et al. [28] showed competitive inhibition of CYP1A2-mediated 7-ethoxyresorufin *O*-dealkylation with an inhibition constant of 0.14 μM. This is in accordance with results of our study, in which isorhamnetin was shown to be a reversible inhibitor of CYP1A2. Interestingly, if isorhamnetin is preincubated with CYP1A2, the residual activity of CYP1A2 decreases to 8 ± 2% (p = 0.012).

On rat liver microsomes, Siess et al. [24] showed that morin inhibited ethoxy- and pentoxy-resorufin dealkylation, and characterized the inhibition as mix type reversible similarly to chrysin. However, the inhibition constant for morin was 16-fold greater (5 μM). This is in accordance with the results of our study, as patterns observed in MDI, TDI, and DI assays were the same for morin and chrysin, and chrysin was a more potent inhibitor than morin. Sahu et al. [29] showed that the inhibition of CYP1A2 could influence the febuxostat metabolism (substrate of CYP1A2), causing drug-flavonoid interactions, while Li et al. [30] confirmed interactions of etoposide and morin, although later one was attributed to the other cytochromes P450 as well.

Pinocembrin inhibited CYP1A2 enzyme activity. The observation that pinocembrin acts as a metabolism dependent, time dependent, and direct inhibitor of CYP1A2 has not been previously reported. As in the case of the most of aforementioned flavonoid inhibitors of CYP1A2, time dependent inhibition is the most prominent compared to other types. This indicates that these flavonoids require time to interact with CYP1A2 for the inhibition to be observed, and could be attributed to allosteric, nonspecific binding to the enzyme.

Out of 11 analyzed flavonoids, apigenin, galangin, isorhamnetin, and tangeretin inhibited CYP2A6 enzyme activity (Figure 4). Although TDI was an important type of CYP1A2 enzyme, this was not the case with the CYP2A6 enzyme, as it was shown only to be significant in the case of isorhamnetin.

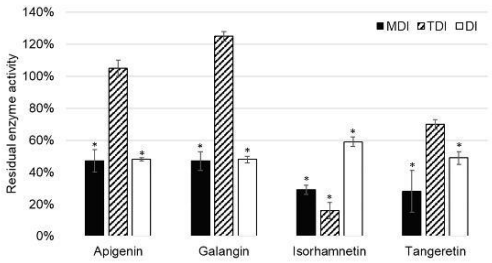

Figure 4. Residual CYP2A6 enzyme activity after incubation with 1 μM flavonoid in the experiments for the determination of metabolism dependent inhibition (MDI), time dependent inhibition (TDI), and direct inhibition (DI). * denotes that observed inhibition is statistically significant ($p < 0.05$) when compared to the control (without addition of flavonoid).

Apigenin was reported to inhibit CYP2A6 activity in the metabolism dependent inhibition assay, as well as the direct inhibition assay of CYP2A6. Boonruanga et al. [31] reported similar IC_{50} values in metabolism dependent and direct inhibition assays of 0.77 ± 0.16 μM and 0.90 ± 0.07 μM, respectively. Likewise, under the similar experimental conditions and 1 μM concentration of apigenin, we observed comparable values of residual CYP2A6 enzyme activity: 47 ± 7% ($p = 0.037$) and 48 ± 1% ($p = 0.018$). This indicates that apigenin is a direct, reversible inhibitor of the CYP1A2 enzyme.

Galangin showed similar values of residual CYP2A6 activity in MDI and DI assays, indicating that it is a reversible, direct inhibitor of CYP1A2. This is in accordance with the results of Tiong et al. [32], who conducted experiments using the recombinant enzyme and the same marker reaction as our study.

Isorhamnetin showed inhibition of CYP2A6 enzyme activity, which was most prominent in the TDI assay: 16 ± 5% ($p = 0.025$). (Figure 4). This indicates that isorhamnetin acts as a reversible, time dependent inhibitor of CYP1A2. This could explain the observed interactions of the *Ginkgo biloba* extract with valproic acid observed by Numa et al. [33].

Although it has been reported that tangeretin is metabolized by cytochromes P450, namely CYP1A2, CYP3A4, and CYP2D6 [14,15], it is interesting to note that tangeretin acts as a direct inhibitor of CYP2A6 enzyme activity.

Although the inhibition of CYP2C8 was reported for some flavonoids [34,35], this is the first report on the inhibition of CYP2C8 paclitaxel 6-α-hydroxylation by chrysin, chrysin-dimethylether,

and galangin. In the TDI assay, the reduction of enzyme activity was not observed, and values of residual CYP2C8 enzyme activity were lower in MDI compared to DI assay (Figure 5). Thus, it can be concluded that chrysin, chrysin-dimethylether, and galangin are mixed reversible and irreversible inhibitors of CYP2C8.

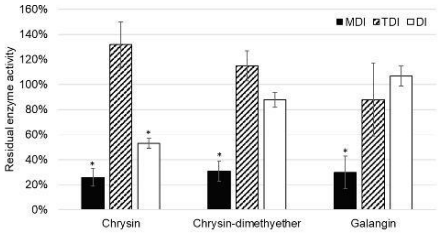

Figure 5. Residual CYP2C8 enzyme activity after incubation with 1 μM flavonoid in the experiments for determination of metabolism dependent inhibition (MDI), time dependent inhibition (TDI), and direct inhibition (DI). * denotes that observed inhibition is statistically significant ($p < 0.05$) when compared to the control (without addition of flavonoid).

Interestingly, no inhibition of CYP2D6 was observed on the set of analyzed flavonoids, neither reversible nor irreversible. Flavonoids are weak acids, having mainly hydroxyl groups, while typical CYP2D6 substrates contain a nitrogen atom, which can be protonated at physiological pH [36]. This could explain why not even direct competitive inhibition was observed when dextromethorphan was used as the marker substrate of CYP2D6 activity.

In our previous research, a significant number of irreversible flavonoid inhibitors of CYP3A4 was reported [22]. However, flavonoids in this study mainly served as reversible inhibitors of CYP1A2 and CYP2A6, while the inhibition of CYP2C8 was of mixed type (reversible and irreversible).

To assess the reversible binding of flavonoids to cytochromes P450, a docking study of the most potent reversible inhibitor, i.e., chrysin, to the CYP1A2 enzyme was conducted.

The redocking of alpha-naphthoflavone, both in presence and in absence of HOH 733, was performed and compared with crystallographic data (Figure 6). The docked positions of alpha-naphthoflavone with and without the water molecule were rotated by approximately 180° compared to each other. Alpha-naphthoflavone was docked with the water molecule having the virtually same coordinates as the crystallographic alpha-naphthoflavone. This was used as a confirmation that the docking of our chrysin species with the HOH 733 water molecule was a valid approach, as the water molecule can affect the ligand position in the active site of CYP1A2 [37,38].

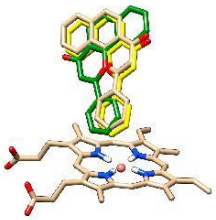

Figure 6. Comparison of the docked alpha-naphthoflavone with all water molecules removed (green), docked alpha-naphthoflavone in presence of HOH 733 (yellow), and crystallographic alpha-naphthoflavone (brown) in vicinity of cytochrome P450 1A2 heme. Oxygen atoms are depicted in red, nitrogen atoms in blue, hydrogen atoms in white, and heme iron in orange.

For both chrysin species, docking with and without the HOH 733 water molecule was done and their positions were compared (Figure 7, Table 1).

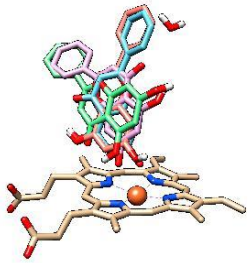

Figure 7. Comparison of docked chrysin species in presence and in absence of the HOH 733 water molecule (the results are identical in both cases). Anion species are depicted in blue (top-ranked docked species) and pink (second top-ranked docked species), while molecule species are depicted in green (top-ranked molecule species) and orange (third top-ranked molecule species in presence of HOH 733 and second top-ranked molecule species in absence of HOH 733). Oxygen atoms are depicted in red, nitrogen atoms in blue, hydrogen atoms in white, and heme iron in orange.

Table 1. Summary of Chrysin Species Docking.

Chrysin Anion at Position 7							
In Presence of HOH 733				In Absence of HOH 733			
Cluster Rank	Percentage of Runs	Binding Energy (kcal/mol)	Inhibitory Constant (μM)	Cluster Rank	Percentage of Runs	Binding Energy (kcal/mol)	Inhibitory Constant (μM)
1.	84	−6.91	8.66	1.	80	−6.94	8.23
2.	16	−6.70	12.22	2.	20	−6.75	11.26
Chrysin Molecule							
In Presence of HOH 733				In Absence of HOH 733			
Cluster Rank	Percentage of Runs	Binding Energy (kcal/mol)	Inhibitory Constant (μM)	Cluster Rank	Percentage of Runs	Binding Energy (kcal/mol)	Inhibitory Constant (μM)
1.	81	−8.23	0.93	1.	98	−8.26	0.89
2.	17	−8.13	1.10	2.	2	−8.09	1.18
3.	2	−8.07	1.21				

The anion species at position 7 binds in the same position both in presence and in absence of the HOH 733 water molecule, with the 7-O- group closest to the heme iron. For the docked molecule species, the HOH 733 water molecule does not play a significant role. The top-ranked docking pose in both cases was identical, with the 5-OH and 4-keto group being oriented toward the heme iron. Meanwhile, in the less populated clusters, the group closest to the heme iron was the 7-OH group. The second-ranked cluster of docked molecule species in presence of HOH 733 is not shown, but it was orientated with the B ring facing the heme iron. The binding energy for the molecule species (Table 1) was much lower than for the anion species, suggesting that the molecule species was responsible for most of the chrysin inhibitory effects. Even though the exact values of energy were probably overestimated due to incorrect energy calculations [39], their relative comparison is still possible since we are comparing different poses of the same molecule. The inhibitory constants obtained by computational studies are of similar range to those observed experimentally (micromolar).

In conclusion, out of 11 analyzed flavonoids, hesperetin, pinocembrin, chrysin, isorhamnetin, and morin inhibited CYP1A2 activity; apigenin, tangeretin, galangin, and isorhamnetin inhibited CYP2A6 activity; and chrysin, chrysin-dimethylether, and galangin inhibited CYP2C8. None of the analyzed flavonoids showed inhibition of CYP2D6. The flavonoids in this study were mainly reversible inhibitors of CYP1A2 and CYP2A6, while the inhibition of CYP2C8 was of mixed type (reversible and irreversible). The determined types of inhibition are important for the further assessment of

flavonoid-drug interactions. If flavonoid is a reversible inhibitor, flavonoid-rich foods or dietary supplements could inhibit drug metabolism mediated by cytochromes P450 and dose adjustment if needed. If flavonoid is an irreversible inhibitor of cytochrome P450 enzyme, combinations with drugs that it can interact with should be avoided.

3. Materials and Methods

3.1. Materials

Eleven flavonoids were used in this study. Acacetin, hesperetin, pinocembrin, chrysin, flavone, tangeretin, galangin, isorhamnetin, and morin were acquired commercially from TransMIT (Gießen, Hessen, Germany), while chrysin-dimethylether and tamarixetin were obtained from Extrasynthèse (Genay, Lyon, France). The marker substrates and their corresponding metabolites were purchased from Sigma-Aldrich (St. Louis, MO, USA), except 7-hydroxycoumarin, which was purchased from Extrasynthèse (Genay, Lyon, France).

The recombinant baculosomes with hyperexpressed cytochromes P450 (1A2, 2A6, 2C8, and 2D6) and coexpressed NADPH cytochrome P450 reductase and cytochrome b_5 were used as a source of enzyme. Baculosomes were commercially obtained from Thermo Fisher Scientific (Waltham, MA, USA), and all other chemicals from Sigma-Aldrich if not otherwise indicated. A 1.0 M solution phosphate buffer of pH 7.4 was prepared in house. NADPH generating system was prepared of 0.1 M glucose-6-phosphate, 10 mg mL^{-1} $NADP^+$, and 1000 IU mL^{-1} glucose-6-phosphate dehydrogenase in ratio 100:50:2 (*v*/*v*/*v*) [40].

3.2. Determination of CYP1A2 Enzyme Activity

Phenacetine O-deethylation was used as a marker reaction for monitoring CYP1A2 enzyme activity (Figure 2). Incubations of 100 µL were conducted at the temperature of 37 °C. The pH of the incubation mixture was set at 7.4 using a potassium phosphate buffer (final concentration 50 mM). The total amount of CYP1A2 enzyme was 5 pmol, and the final concentration of substrate was 150 µM. Residual activity was determined by incubating the flavonoid (1 µM) with phenacetine as a substrate for 15 min, with or without preincubation, depending of the type of inhibition assay. Reactions were started by the addition of 15 µL of NADPH generating system, while the reactions were stopped by the addition of 1 mL 1% formic acid in acetonitrile. After centrifugation (10 min, 1900× *g*), the clear solution was transferred to a vial for HPLC analysis [22].

HPLC analysis was performed on Agilent 1100 instrument (Santa Clara, CA, USA) coupled with a diode array detector (DAD) on Luna C18 column (4.6 × 150 mm, 3 µm). The gradient method was used for separation. Mobile phase A consisted of water, acetonitrile, and glacial acetic acid in the volume ratio of 90:10:0.1, while mobile phase B contained the same solvents in the ratio 10:90:0.1. The following gradient timetable was used (*t*/min, %B): (0, 0), (2, 0), (13, 55), (14, 0), (20, 0). The flow rate was 1 mL/min, and chromatograms were recorded at 254 nm. Volume of incubation injected to the column was 15 µL. Retention time of substrate was 12.2 min, while product was detected at 5.7 min. The amount of generated product (acetaminophen) was determined as the area under the curve based on the calibration curve of the standard.

3.3. Determination of CYP2A6 Enzyme Activity

The aromatic hydroxylation of coumarin at the position 7 was used as a marker reaction for CYP2A6. The incubation conditions were the same as for CYP1A2 enzyme (vide supra) unless otherwise stated. The final concentration of coumarin was 10 µM, while the incubation time was extended to 30 min.

HPLC-DAD analysis was conducted on the same system as the CYP1A2 enzyme with the following modifications: The gradient timetable (t/min, %B) was (0, 20), (1.5, 20), (10, 70), (11, 20), (17, 20), and the detection wavelength was 330 nm. The retention time of the substrate (coumarin) was 7.5 min,

while the product (7-hydroxycoumarin) was detected at 4.8 min. The amount of generated product was determined as the area under the curve based on the calibration curve of the standard [22].

3.4. Determination of CYP2C8 Enzyme Activity

Paclitaxel 6α-hydroxylation was used as a marker reaction for monitoring CYP2C8 enzyme activity (Figure 2). The incubation conditions were the same as for CYP1A2 enzyme (vide supra) unless otherwose stated. The final concentration of paclitaxel was 20 μM, while the incubation time was set to 30 min.

HPLC-DAD analysis was conducted on the same system as the CYP1A2 enzyme with the following modifications: The gradient timetable (t/min, %B) was (0, 50), (2, 50), (10, 90), (11, 50), (17, 50), and the detection wavelength was 227 nm. The retention time of the substrate (paclitaxel) was 6.1 min, while the product (6α-hydroxypaclitaxel) was detected at 3.4 min. The amount of generated product was determined as the area under the curve based on the calibration curve of the standard [22].

3.5. Determination of CYP2D6 Enzyme Activity

O-demethylation of dextromethorphan to dextrorphan was used as a marker reaction for monitoring CYP2D6 enzyme activity (Figure 2). The incubation conditions were the same as the CYP1A2 enzyme (vide supra) unless otherwise stated. The final concentration of the marker substrate in the incubation was 100 μM, while the incubation time was set to 30 min.

HPLC-DAD analysis was conducted on the same system as for CYP1A2 enzyme with the following modifications: The gradient timetable (t/min, %B) was (0, 18), (5, 18), (10, 40), (15, 18), and the detection wavelength was set to 224 nm. The retention time of the substrate (dextromethorphan) was 7.1 min, while the product (dextrorphan) was detected at 4.1 min. The amount of generated product was determined as the area under the curve based on the calibration curve of the standard [22].

3.6. Determination of the Inhibition Type

Three types of experiments were conducted to determine metabolism dependent inhibition, time dependent inhibition, and direct inhibition of CYP1A2, CYP2A6, CYP2C8, and CYP2D6 enzymes [40].

To determine metabolism dependent inhibition (MDI assay), the flavonoid was first preincubated with the enzyme with the addition of the generating system for 30 min, after which the marker substrate was added to determine residual enzyme activity (as described above for each enzyme).

If metabolism dependent inhibition was determined, time dependent inhibition and direct inhibition were tested. Time dependent inhibition (TDI assay) was assessed by preincubating the flavonoid and enzyme, without the generating system, after which residual activity was determined by adding the NADPH generating system along with the substrate (as described above for each enzyme).

The direct inhibition assay (DI assay) was conducted without preincubation, i.e., the NADPH generating system was added to the incubation mixture containing flavonoid and substrate following the experimental set-up described above for each individual cytochrome P450 enzyme [40].

If the flavonoid is an irreversible inhibitor of the cytochrome P450, it will decrease enzyme activity in the MDI assay, while no reduction of enzyme activity will be observed in TDI and DI assays. Pure time dependent inhibitors require time to interact with the enzyme. Thus, TDI inhibitors will show a reduction of enzyme activity in assays with preincubations, i.e., the MDI and TDI assays, but not in the DI assay (no preincubation). Pure direct inhibitors are reversible inhibitors that usually compete with the substrate binding to the active site and will show a decrease of enzyme activity with or without preincubation. DI inhibitors will reduce enzyme activity in all three assays (MDI, TDI, and DI).

3.7. Docking Studies

To examine the binding of chrysin to the active site of cytochrome P450 1A2, a docking study was performed using AutoDock 4.2.6. (The Scripps Research Institute, La Jolla, CA, USA) [39], which used dispersion, hydrogen bonds, and electrostatic and desolvatation energy components to determine the

conformation of the most probable complex. The three-dimensional coordinates of the cytochrome P450 1A2 molecule co-crystallized with alpha-naphthoflavone were obtained from the RCSB [41]. This crystal structure was chosen due to similarity of alpha-naphthoflavone with our ligand, and the crystal structure had a satisfactory resolution of 1.95 Å. The protein molecule was prepared for docking by adding the missing side-chain atoms and hydrogen atoms, all Lys, Arg, His, and Cys side-chains were protonated, all Asp and Glu side-chains were deprotonated, and the amino and carboxy termini were charged. Since there was a presence of a water molecule (HOH 733) in the vicinity of alpha-naphthoflavone molecule in the active site, the docking study was performed both with all water molecules removed, as well as with all water molecules removed except HOH 733. The three-dimensional forms of the ligands were drawn, and their initial geometries were minimized in HyperChem 8.0 (Hypercube, Inc., Gainesville, FL, USA). Their charge was set to represent the most abundant species at pH 7.4, calculated at chemicalize.com. At pH 7.4, chrysin-7-anion represents 65.49% and chrysin molecule represents 11.44% of all chrysin species. Both species were docked due to the fact that the percentage of the molecule species sharply increases with lowering of the pH, as happens in intrahepatic conditions [42]. Partial charges for flavonoid ligands were set according to Ionescu et al. [43]. In AutoDock grid maps of size 70 × 70 × 70 Å were generated with 0.375 Å spacing centered in the CYP1A2 active site cavity (4.0, 12.0, 23.0) by the AutoGrid program [39] and Lamarckian genetic algorithm (LGA) [44] was applied. The receptor molecule was regarded as rigid, while all ligand single bonds were allowed to rotate freely during the Monte Carlo simulated annealing procedure. Ligand flexible docking simulations were performed with 100 runs, population size of 150, 2.5 × 107 energy evaluations, 27,000 numbers of generations, rate of gene mutation of 0.02, and rate of crossover 0.08. A root-mean-square-deviation (RMSD) of 2.0 Å was used as a criterion for cluster analysis of the docking results (in order to determine if two docked conformations were similar enough to be included in the same cluster). First, the docking of alpha-naphthoflavone in presence and in absence of HOH 733 was conducted in order to assess the appropriateness of the system. Afterward, the docking of chrysin species was conducted, also in the presence and absence of HOH 733.

3.8. Statistical Analysis

All incubations were conducted in triplicate. The results are expressed as the residual activity of the enzyme, i.e., the percentage of product generated in incubation with the addition of flavonoid in ratio to the control without flavonoid. The statistical significance was tested with Student's t-test in the program R (The R Project for Statistical Computing, Vienna, Austria).

Author Contributions: M.B. designed the study; M.K., H.R. and G.B. performed incubation assays and HPLC-DAD analysis; H.R. conducted computational study; Ž.M. and M.B. analyzed the data and wrote the paper.

Funding: This research was funded by Croatian Science Foundation, grant number UIP-2014-09-5704.

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

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Sample Availability: Samples of the compounds are available from the authors and commercially.

9. ŽIVOTOPIS

Martin Kondža rođen je 18. srpnja 1992. godine u Grudama, Bosna i Hercegovina, gdje je završio osnovnu školu Ruđera Boškovića i opću gimnaziju u srednjoškolskom centru Antun Branko Šimić. Farmaceutski fakultet Sveučilišta u Mostaru upisuje 2011. godine, a završava 2016. godine obranom diplomskog rada i stjecanjem naziva magistar farmacije. Stručno osposobljavanje za magistre farmacije obavio je u Ljekarničkoj zdravstvenoj ustanovi „Ljekarne Čolak“ te 2016. godine položio stručni ispit pred povjerenstvom Federalnog ministarstva zdravstva, čime je stekao licenciju za samostalno obavljanje djelatnosti. Iste godine upisuje se na poslijediplomski doktorski studij farmaceutsko-biokemijske znanosti na Farmaceutsko-biokemijskom fakultetu Sveučilišta u Zagrebu. 2017. godine zapošljava se u Agenciji za lijekove i medicinska sredstva Bosne i Hercegovine, kao stručnjak za poslove farmakovigilancije. Zaslužan je za uspostavljanje prvog nacionalnog sustava za nuspojave u Bosni i Hercegovini, pri čemu se započinje sa sustavnim bilježenjem i analiziranjem nuspojava na lijekove, cjepiva i medicinska sredstva. Iste godine je na Sveučilištu u Mostaru izabran u znanstveno-nastavno zvanje asistent za područje biomedicine i zdravstva, polje farmacija, grana farmacija te započinje rad na Farmaceutskom fakultetu. Na fakultetu je uključen u znanstveno-nastavni proces za predmete Farmaceutska kemija 1 i 2, Biokemija lijekova i Analitika lijekova 1 i 2. Kao koordinator i suradnik angažiran je na 6 projekata (2 projekta uz potporu Europske komisije), kao predavač i sudionik sudjelovao je na 21 znanstvenom skupu u zemlji i svijetu, član je 12 znanstvenih odbora i stručnih povjerenstava. U nekoliko navrata bio je glavni urednik časopisa *Placebo,* a član je uredničkog odbora časopisa *Farmaceut.* Stručno se usavršavao na Sveučilištu u Gentu, Belgija (Fakultet farmaceutskih znanosti) i Sveučilištu u Zagrebu, Hrvatska (Medicinski i Farmaceutsko-biokemijski fakultet). Autor je i koautor 24 znanstvene i stručne publikacije, od kojih 3 citira *Current Contents.* Član je nekoliko stručnih i znanstvenih udruženja – Komora magistara farmacije Federacije Bosne i Hercegovine (član Upravnog odbora), Cochrane Bosna i Hercegovina, Europske federacije za medicinsku kemiju, Matice hrvatske, Hrvatskog farmaceutskog društva itd. Tečno govori engleski i njemački jezik, služi se francuskim i talijanskim jezikom.

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10. TEMELJNA DOKUMENTACIJSKA KARTICA

Temeljna dokumentacijska kartica

Sveučilište u Zagrebu **Doktorski rad**
Farmaceutsko-biokemijski fakultet
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A. Kovačića 1, 10000 Zagreb, Hrvatska

MEHANIZAM INHIBICIJSKOGA UČINKA FLAVONOIDA NA CITOKROM P450 3A4

Martin Kondža

SAŽETAK

Flavonoidi su spojevi koji se u većim količinama nalaze u biljkama. Čovjek prehranom svakodnevno konzumira flavonoide. Enzimi citokrom P450 su najvažniji enzimi koji sudjeluju u metabolizmu lijekova i lipofilnih ksenobiotika. Najveći broj lijekova na tržištu metabolizira se putem CYP3A4 enzima. Poznato je da flavonoidi mogu stupati u reakcije s CYP3A4 enzimom, pri čemu ga mogu inhibirati. Važno je razjasniti dosad nepoznat mehanizam inhibicijskog učinka flavonoida na CYP3A4 enzim. Ispitana je vrsta inhibicije kojom akacetin, apigenin, krizin i pinocembrin inhibiraju aktivnost CYP3A4 enzima. Inaktivacijska kinetika flavonoida određena je uz testosteron i nifedipin kao marker supstrate. Određeni su osnovni parametri enzimske inaktivacije (konstanta inhibicije, konstanta brzine inaktivacije, učinkovitost inaktivacije i polovica maksimalne inhibitorne koncentracije). Ispitana je pseudoireverzibilna inhibicija enzima uz pomoć hemokrom-piridin testa. Primjenom glutationa, hvatača slobodnih radikala, nastojala se utvrditi struktura reaktivnih intermedijera odgovornih za inaktivaciju enzima. Apigenin je uzrokovao o metabolizmu ovisnu inhibiciju CYP3A4 enzima (ostatna aktivnost enzima 10,6 ± 1,3%). Za krizin je utvrđena IC_{50} vrijednost od 0,6 ± 0,5 µM, konstanta inhibicije K_i = 0,6 ± 0,3 µM te učinkovitost inhibicije 0,108 min^{-1} $µM^{-1}$. Za apigenin je utvrđena konstanta brzine inaktivacije, k_{inact} = 0,11 ± 0,01 µM-1. Svi flavonoidi su smanjili koncentraciju hema, a krizin najviše (ostatna koncentracija hema 5,5%, odnosno 2,9%). Nije uočena statistički značajna razlika između ostatne aktivnosti enzima nakon dijalize s i bez dodatka kalijevog heksacijanoferata. Reaktivni međuprodukti flavonoida nisu uočeni na spektrometru masa. Svi flavonoidi pokazuju o metabolizmu ovisnu inhibiciju CYP3A4 enzima, pri čemu se krizin pokazao kao najsnažniji inhibitor. Flavonoidi se kovalentno vežu za hem te na taj način dovode do inaktivacije enzima. Niti jedan flavonoid nije pokazao inhibiciju pseudoireverzibilnog karaktera na CYP3A4 enzim. Reaktivni međuprodukti flavonoida nisu uočeni zbog niskih koncentracija ili nestabilnosti produkata.

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

Rad sadrži: 106 stranica, 57 grafičkih prikaza, 5 tablica i 180 literaturnih navoda. Izvornik je na hrvatskom jeziku.

Ključne riječi: flavonoidi, akacetin, apigenin, krizin, pinocembrin, CYP3A4, inhibicija

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Rad prihvaćen: ožujak 2021.

Basic documentation card

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Doctoral thesis

MECHANISM OF CYTOCHROME P450 3A4 INHIBITION MEDIATED BY FLAVONOIDS

Martin Kondža

SUMMARY

Introduction: Flavonoids are compounds found in larger amounts in plants. People consume flavonoids in their diet on a daily basis. Cytochrome P450 enzymes are the most important enzymes involved in the metabolism of drugs and lipophilic xenobiotics. Most drugs on the market are metabolized by the CYP3A4 enzyme. It is known that flavonoids can react with the CYP3A4 enzyme, whereby they can inhibit it. It is important to elucidate the hitherto unknown mechanism of the inhibitory effect of flavonoids on the CYP3A4 enzyme.The type of inhibition by which acacetin, apigenin, chrysin and pinocembrin inhibit CYP3A4 enzyme activity was investigated. The inactivation kinetics of flavonoids were determined with testosterone and nifedipine as substrates. The basic parameters of enzyme inactivation (K_i, k_{inact}, inactivation efficiency and IC_{50} concentration) were determined. Pseudoireversible inhibition of the enzyme was examined using the hemochromopyridine test. The use of glutathione sought to determine the structure of reactive intermediates responsible for enzyme inactivation.Apigenin caused metabolism based inhibition of the CYP3A4 enzyme (residual enzyme activity 10.6 ± 1.3%). For chrysin, an IC_{50} value of 0.6 ± 0.5 μM, K_i = 0.6 ± 0.3 μM, and an inhibition efficiency of 0.108 min^{-1} μM^{-1} were recorded. An inactivation rate constant, k_{inact} = 0.11 ± 0.01 μM^{-1}, was recorded for apigenin. All flavonoids reduced the concentration of heme, chrysin being the most potent (residual concentration of heme was 5.5% and 2.9%, respectively). No statistically significant difference was observed between the residual enzyme activity after dialysis with and without the addition of potassium hexacyanoferrate. Reactive intermediates of flavonoids were not observed.All flavonoids show metabolism-dependent inhibition of the CYP3A4 enzyme, with chrysine proving to be the most potent inhibitor. Flavonoids bind covalently to heme, thus leading to enzyme inactivation. None of the flavonoids showed pseudo-irreversible inhibition of the CYP3A4 enzyme. Reactive intermediates of flavonoids were not observed due to low concentrations or product instability.

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

Thesis includes: 106 pages, 57 figures, 5 tables and 180 references. Original is in Croatian language.

Keywords: flavonoids, acacetin, apigenin, chrysin, pinocembrin, CYP3A4, inhibition

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Thesis accepted: March 2021