UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra farmakologie a toxikologie

Screening nových látek chelatujících železo/měď – *in vivo* a *in vitro* studie

Screening of new iron- and copper-chelating substances – *in vivo* and *in vitro* studies

DISERTAČNÍ PRÁCE

Vedoucí disertační práce: doc. PharmDr. Přemysl Mladěnka, Ph.D.

Konzultant: prof. MUDr. Radomír Hrdina, CSc.

Hradec Králové, 2015 Mgr. Michal Říha

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně, pod vedením svého školitele doc. PharmDr. Přemysla Mladěnky, Ph.D. a konzultanta prof. MUDr. Radomíra Hrdiny, CSc. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

Disertační práce vznikla za podpory Specifického vysokoškolského výzkumu SVV 260 185.

V Hradci Králové …… ……

Mgr. Michal Říha

Poděkování

Na tomto místě bych rád poděkoval svému školiteli doc. PharmDr. Přemyslu Mladěnkovi, Ph.D. a konzultantovi prof. MUDr. Radomíru Hrdinovi, CSc. za jejich cenné rady při vypracování této práce a odborný i přátelský přístup během postgraduálního studia. Mé poděkování za spolupráci a vstřícnost patří i ostatním kolegům skupiny Kardiovaskulární a respirační farmakologie a toxikologie a také celému kolektivu Katedry farmakologie a toxikologie.

Děkuji za finanční podporu Univerzitě Karlově v Praze (GAUK 605712C a 1220314B, UNCE 204019/304019/2012, program PRVOUK P40, SVV 265 003, 267 003, 260 064 a 260 185) a Grantové agentuře České republiky (projekt P303/12/G163).

Srdečné poděkování bych chtěl vyjádřit své rodině, zejména manželce Ivě Říhové a rodičům, za podporu a pochopení během studia.

Abstrakt

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové
Katedra farmakologie a toxikologie

Kandidát:	Mgr. Michal Říha
Školitel:	doc. PharmDr. Přemysl Mladěnka, Ph.D.
Konzultant:	prof. MUDr. Radomír Hrdina, CSc.
Název disertační práce:	Screening nových látek chelatujících železo/měď – *in vivo* a *in vitro* studie

Železo a měď jsou esenciální stopové prvky, jež hrají roli v řadě fyziologických procesů v lidském organismu. Homeostáza těchto přechodných kovů je pečlivě regulována, neboť volné či labilně vázané železo nebo měď katalyzují tvorbu volných radikálů. Hereditární hemochromatóza, transfuzní hemosideróza a Wilsonova choroba představují stavy spojené s absolutním nadbytkem železa a mědi v organismu. Pro jejich léčbu mají zásadní význam chelátory těchto kovů. Existuje však několik dalších onemocnění, u nichž je dokumentováno zapojení železa a/nebo mědi do patofyziologie. Jedná se především o neurodegenerativní onemocnění, kardiovaskulární choroby, nádorová onemocnění či diabetes mellitus. V těchto potenciálních indikacích jsou zkoumány nejrůznější látky s chelatačními schopnostmi.

Cílem této disertační práce bylo provést screening látek chelatujících železo a měď a detailněji studovat vlastnosti těchto látek v *in vitro* a *in vivo* experimentech.

V rámci *in vitro* studií byly zkoumány chelatační vlastnosti syntetických i přírodních látek vůči mědi, pomocí vyvinuté spektrofotometrické metodiky využívající bathokuproindisulfonát nebo hematoxylin. Ukázalo se, že klinicky používaný chelátor

mědi D-penicilamin je oproti jiným látkám poměrně slabým chelátorem mědi s výraznými redukčními vlastnostmi. Řada flavonoidů vykázala schopnost vázat měď, silnými chelátory však byly především 3-hydroxyflavon, kempferol a baikalein. V návaznosti na přechozí výzkum skupiny byly také detailněji sledovány schopnosti redukovat železo a vliv na železem katalyzovanou Fentonovu reakci u flavonoidů. Tyto látky byly schopné redukovat ionty železa pouze v kyselém prostředí, přičemž prooxidační aktivita byla zjištěna zejména ve skupině flavonolů. Pro plnou charakterizaci interakcí nových nebo známých chelátorů byl vyvinut nový přístup ke stanovení stechiometrie komplexu přechodný kov-chelátor využívající matematických výpočtů. V porovnání se standardní Jobovou metodou se jeví jako výhodný zejména v případech slabších chelátorů.

V rámci *in vivo* studií byla publikována práce s perorální sedmidenní premedikací kvercetinem u potkanů. Tento přístup nebyl schopen ochránit kardiovaskulární systém před akutním poškozením katecholaminy. Kvercetin však ovlivnil některé hemodynamické parametry a v kontrolní skupině snížil odpověď aorty na vazokonstrikční podnět. Další práce s D-penicilaminem na stejné téma je v současné době ve fázi přípravy publikace a analogická studie s rutinem v recenzním řízení.

Závěrem lze shrnout, že celá paleta látek byla testována pro chelatační účinky mědi. Studovány byly rovněž další důležité vlastnosti chelátorů, jako je redukční a prooxidační aktivita či stechiometrie komplexu. Vyvinuté *in vitro* metody mohou navíc podpořit další výzkum v této oblasti. Účinné chelátory byly nalezeny ve skupině flavonoidů, ty však vykazují složité interakce s oběma kovy. Takovým příkladem je i kvercetin, jenž nebyl *in vivo* schopen zabránit komplexnímu kardiotoxickému působení katecholaminů a v *in vitro* studii působil v některých koncentracích prooxidačně.

Abstract

Charles University in Prague, Faculty of Pharmacy in Hradec Králové
Department of Pharmacology and Toxicology

Candidate:	Michal Říha, MSc.
First Supervisor:	Assoc. Prof. Přemysl Mladěnka, Ph.D.
Second Supervisor:	Prof. Radomír Hrdina, M.D., Ph.D.
Title of Doctoral Thesis:	Screening of new iron- and copper-chelating substances – *in vivo* and *in vitro* studies

Iron and copper are essential trace elements which play a role in a series of physiological processes in human organism. Homeostasis of these transition metals is meticulously regulated since free or loosely bound iron and copper can catalyse the production of free radicals. Hereditary hemochromatosis, transfusion hemosiderosis and Wilson´s disease are associated with absolute iron and copper overload in the organism. Transition metal chelators have crucial significance for the treatment of these states. There are several other diseases with documented involvement of iron and/or copper in their pathophysiology. Examples are primarily neurodegenerative diseases, cardio-vascular diseases, tumours and diabetes mellitus. Various chelating compounds are examined in these possible indications.

The aim of this doctoral thesis was to perform a screening of iron- and copper-chelating substances and to study their properties in detail using *in vitro* and *in vivo* experiments.

Copper-chelating properties of synthetic as well as natural substances were investigated *in vitro*, by a developed spectrophotometric method using

bathocuproinedisulphonate or hematoxylin. In contrast to other compounds, the clinically used copper chelator D-penicillamine was shown as a relatively weak copper-chelator, which possesses marked reducing properties. Many flavonoids were able to bind copper; however, potent chelators were especially 3-hydroxyflavone, kaempferol and baicalein. Iron-reducing properties and influence on the iron-catalysed Fenton reaction were also investigated in a series of flavonoids. These compounds reduced ferric ions only under acidic conditions and pro-oxidant activity was found particularly in flavonols. Novel approach aimed at the determination of transition metal-chelator complex stoichiometry based on mathematical calculations was developed. In comparison with the standard Job´s method, the approach appeared to be advantageous especially in the cases of moderately active chelators.

An *in vivo* study concerning oral administration of quercetin for seven days in rats has been published. This approach was unable to protect cardiovascular system against acute catecholamine cardiotoxicity. However, quercetin affected some haemodynamic parameters and decreased the responsiveness of aorta to vasoconstriction in control group. Other data concerning the effect of D-penicillamine are currently being prepared for publication and a similar experiment with rutin is in a peer-review process.

In conclusion, a spectrum of substances was tested for copper-chelating effects. Other important properties of chelators were investigated, such as reducing and pro-oxidant activities or the complex stoichiometry. Moreover, the developed *in vitro* methods may promote research in this field. Potent chelators were observed among flavonoids which showed numerous interactions with both metals. Quercetin represents such an example. It was unable to protect against complex catecholamine cardiotoxic effects and it was even pro-oxidant in some concentrations in an *in vitro* study.

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1. Úvod

Přechodné kovy železo a měď jsou stopové prvky s nezastupitelným významem pro lidský organismus, neboť jsou součástí celé řady enzymů a dalších proteinů. Homeostáza železa a mědi je v lidském těle pečlivě regulována důmyslnými mechanismy. Její narušení jak genetickými faktory, tak vlivy vnějšího prostředí vede k nežádoucímu působení těchto kovů. Volné či labilně vázané železo a měď jsou totiž účinnými katalyzátory reakcí vedoucích k tvorbě vysoce toxických volných radikálů.

Kromě stavů chronického přetížení organismu železem a Wilsonovy choroby, spojených s rozvratem homeostázy železa, respektive mědi, existuje několik dalších onemocnění či poruch, u nichž je dokumentována souvislost s přechodnými kovy. Jedná se o neurodegenerativní onemocnění, zejména Alzheimerovu a Parkinsonovu chorobu, kardiovaskulární onemocnění, nádorová onemocnění nebo diabetes mellitus.

Chelátory jsou organické vícevazné sloučeniny tvořící komplexy s kovy. Základním rysem jejich účinku je schopnost vytvořit komplex, v němž je přechodný kov redoxně neaktivní a může být vyloučen z organismu. Mimo uplatnění v terapii otrav těžkými kovy jsou chelátory klinicky používány především pro léčbu chronického přetížení železem vyvolaným opakovanými krevními transfuzemi a Wilsonovy choroby.

Celá paleta dalších chelátorů železa a/nebo mědi je experimentálně nebo dokonce v klinických zkouškách studována pro léčbu stavů spojených s nerovnováhou těchto kovů. Vzhledem k poměrně úzkému klinickému využití v současné praxi a na druhou stranu velkému terapeutickému potenciálu u různých onemocnění je žádoucí nalézat nové látky schopné příznivě ovlivnit dysbalanci přechodných kovů v lidském těle. Aby bylo možné takové látky uplatnit v terapii chorob, není dostačující pouze znalost vlastní chelatační aktivity, ale je nezbytné objasnit řadu jejich dalších vlastností.

2. Teoretická část

2.1. Úloha a kinetika železa a mědi v lidském organismu

Přechodné kovy železo (lat. *ferrum*, Fe) a měď (lat. *cuprum*, Cu) hrají důležitou roli v široké paletě biologických procesů v živých systémech. Společně s I, Co, Zn, Se, F a dalšími stopovými prvky tvoří přibližně pouze 0,01 % organismu. Nejsou nositeli energie, ale jsou nezbytnou složkou potravy (Trojan a Langmeier 2003). Homeostáza těchto prvků, pečlivě regulovaná mechanismy příjmu, transportu, skladování a vylučování, je udržována v přísných hranicích (Bertini a Cavallaro 2008, Tisato *et al.* 2010), neboť její narušení vede k některým významným patologickým stavům. Vzhledem k tématu disertační práce bude podrobněji zpracována zejména problematika absorpce přechodných kovů.

2.1.1. Železo

Železo je jeden z nejhojnějších prvků v zemské kůře (Frey a Reed 2012). V biologických systémech existuje v oxidačních stavech +II a +III, tedy jako ionty železnaté a železité. Zatímco železnaté ionty jsou rozpustné v biologických tekutinách, oxidovaná forma železa je v podstatě nerozpustná ve vodě při neutrálním pH a rychle precipituje za vzniku hydroxidu železitého (Jones-Lee a Lee 2005). Železnaté ionty jsou navíc nestabilní ve vodném prostředí a jejich tendence reagovat s molekulárním kyslíkem za vzniku Fe^{3+} a radikálu superoxidového aniontu je podkladem toxicity tohoto kovu (Jones-Lee a Lee 2005). Navzdory určité nedostupnosti obou iontů železa je tento kov paradoxně klíčovým katalytickým místem enzymů a proteinů transportujících kyslík (Jomova a Valko 2011).

Železo je nezbytné pro živé organismy, představuje totiž výkonný katalyzátor reakcí zahrnujících přenos elektronů (Geissler a Singh 2011). Vzhledem k vysoké reaktivitě a toxicitě volného železa musí být jeho chemická reaktivita v biologickém prostředí omezena vazbou s různými ligandy. V buněčných kompartmentech je téměř veškeré železo (> 95 %) vázáno na proteiny (Crichton 2001). I přesto zůstává malá část železa také ve formě označované jako labilní železo, které je redoxně aktivní a má význam pro metabolismus a homeostázu kovu (Cabantchik 2014). Fyziologicky nepředstavuje zřejmě žádné riziko, ale vzestup této frakce při různých patologických stavech, např. ischemii, je podkladem oxidativního poškození.

Proteiny obsahují železo ve formě prostetické skupiny jako Fe/S klastry nebo hem, kov však může být přímo koordinován řetězci aminokyselin. Proteiny obsahující železo přenášejí nebo uchovávají kyslík, katalyzují metabolické, signalizační a anti-mikrobiální redoxní reakce a transportují či skladují kov (Ganz 2013). Jejich přehled je uveden v **Tabulce 1** a dokládá význam železa v jednotlivých biologických procesech.

Tabulka 1. Přehled proteinů obsahujících železo

Proteiny obsahující železo	Funkce
Hemové proteiny	
hemoglobin myoglobin	transport kyslíku (erytrocyty) uskladnění kyslíku (svaly)
Hemové enzymy	
cytochromy a, b, c	přenos elektronů přenos elektronů na molekulární kyslík na konci dýchacího řetězce (vyžadují též měď)
cytochrom-c-oxidasa cytochrom P450 + b_5 duodenální cytochrom-b-reduktasa 1	mikrosomální oxidasy, tzv. oxidasy se smíšenou funkcí fáze 1 biotransformace xenobiotik redukce železitých iontů (duodenální enterocyty)
katalasa peroxidasy myeloperoxidasa	rozklad peroxidu vodíku rozklad peroxidů tvorba baktericidního chloristanu
sulfitoxidasa	přeměna siřičitanu na síran
tryptofan 2,3-dioxygenasa iodasa (iodoperoxidasa)	metabolismus pyridinu přeměna jodidu na jodičnan
Nehemové enzymy obsahující železo	
ribonukleotidreduktasa	přeměna ribonukleotidů na 2´-deoxyribonukleotidy syntetická fáze buněčného dělení
(Fe/S proteiny)	
akonitasa isocitrátdehydrogenasa sukcinátdehydrogenasa NADH dehydrogenasa	cyklus kyseliny citronové a počáteční kroky oxidativní fosforylace
aldehydoxidasa	přeměna aldehydů na karboxylové kyseliny
xantinoxidasa	přeměna rozkladných produktů purinových bází na kyselinu močovou
fenylalaninhydroxylasa tyrosinhydroxylasa tryptofanhydroxylasa	syntéza katecholaminů, neurotransmiterů a melaninu
prolylhydroxylasa lysylhydroxylasa	syntéza kolagenu

Převzato z: Geissler a Singh (2011).

Celkový obsah železa v lidském těle je zhruba 3,5 g u žen a 4,0 g u mužů (Geissler a Singh 2011). U dospělých je většina železa přítomna v hemoglobinu uvnitř cirkulujících erytrocytů (60-70 %), kde plní esenciální funkci pro přenos kyslíku, a ve svalech v myoglobinu (10 %). Zbývající část (20-30 %) představuje železo zásobní a nachází se v játrech a retikuloendoteliálním systému jako feritin a hemosiderin (Muñoz *et al.* 2009, Geissler a Singh 2011). Pouze zhruba 1 % tělesného železa je inkorporováno v enzymech a méně než 0,2 % se vyskytuje v plasmě navázáno na transferin (Geissler a Singh 2011). Většina plasmatického železa pochází ze starých erytrocytů, recyklovaných makrofágy ve slezině a dalších orgánech (Ganz 2013).

Vzhledem k významu železa pro lidský organismus a rizikům vyplývajícím z nedostatku, ale i nadbytku tohoto kovu musí existovat mechanismy, které za normálních podmínek udržují hladiny železa ve fyziologických mezích. Prvním a nejdůležitějším místem regulace je gastrointestinální trakt.

Železo je vstřebáváno v proximální části tenkého střeva, zejména duodenu. Fyziologicky se absorbuje 1-2 mg za den (Donovan a Andrews 2004). Protože lidský organismus postrádá aktivní exkreční mechanismus pro železo, musí být ztráty železa z těla v rovnováze s příjmem. Ztráty jsou převážně způsobené deskvamací epiteliálních povrchů (Green *et al.* 1968). Absorpce je tedy zásadním mechanismem regulujícím obsah železa v těle. V potravě se železo nachází v hemové a nehemové formě a ve formě feritinu, přičemž hlavní zdroj kovu představuje nehemové železo. Transport anorganické formy byl studován po desetiletí a je znám poměrně detailně. Nejdůležitější apikální přenašeč anorganického železa do buněk je transportér pro dvojmocné kovy (DMT1, z angl. divalent metal transporter), což je integrální transmembránový protein spřažený s elektrochemickým H^+ gradientem z venku dovnitř buňky (Gunshin *et al.* 1997). DMT1 přenáší železnaté ionty a několik dalších dvojmocných kovů, ale nikoliv

trojmocné železo (Illing *et al.* 2012), proto je pro vstřebání anorganického železa nezbytná konverze železitých iontů na železnaté. Na přeměně oxidačního stavu se pravděpodobně podílejí enzymy reduktasy, zejména duodenální cytochrom-b-reduktasa 1 (CYBRD1) (McKie *et al.* 2001), jejíž přesný význam však není dořešen, dále enzym, který je v anglické literatuře označován jako „cytochrome b_{558} ferric/cupric reductase“ (Knöpfel a Solioz 2002), a Steap2 (Steap z angl. six-transmembrane epithelial antigen of the prostate) (Ohgami *et al.* 2006). Co se týká absorpce hemového železa (obsaženého především v masných zdrojích), byl objeven apikálně exprimovaný intestinální hemový transportér (HCP1, z angl. heme carrier protein 1) (Shayeghi *et al.* 2005), ale dle současných poznatků mu je přisuzována role hlavně v intestinálním přenosu folátů (odtud druhé označení PCFT, z angl. proton-coupled folate transporter) (Qiu *et al.* 2006).

Pokud železo proniknuvší do buňky nemá být okamžitě využito nebo exportováno, musí být skladováno v cytoplasmě. Feritin je sférický heteropolymerní protein, který uvnitř své molekuly může uschovat velké množství železa, a to až 4500 atomů (Mladěnka *et al.* 2005). Představuje tak zásobní formu železa, a co je důležité, udržuje nadbytečný kov v redoxně inaktivní formě. Železo je dopravováno k feritinu pomocí cytoplasmatických chaperonů (Shi *et al.* 2008). Rozpustná, na železo relativně chudá forma feritinu se nachází i v krevní plasmě (Cohen *et al.* 2010). Druhou sloučeninou skladující železo je hemosiderin (Iancu 1992).

Jediný známý savčí přenašeč pro transport železa z buňky přes bazolaterální membránu je ferroportin (Slc40a1) (Le a Richardson 2002, Donovan *et al.* 2005). Jde o transmembránový protein exprimovaný na všech místech, kde železo přechází do plasmy, tedy na duodenálních enterocytech, makrofázích, hepatocytech a na bazálním povrchu placentárního syncytiotrofoblastu (Donovan *et al.* 2000). Ferroportin

transportuje Fe^{2+} ionty (a také Zn^{2+}), přičemž buněčný export je závislý na členech rodiny ferroxidas obsahujících měď: hephaestinu (důležitém pro přenos železa z enterocytů do plasmy) (Vulpe *et al.* 1999, Eisenstein 2000) nebo ceruloplasminu (vysoce exprimovaném v játrech a sítnici) (Osaki *et al.* 1966, Eisenstein 2000) a zřejmě též zyklopenu (exprimovaném převážně v placentě) (Chen *et al.* 2010). Tyto proteiny oxidují železnaté ionty, vytvářejí tak jejich gradient, čímž usnadňují ferroportinem mediovaný eflux železa a umožňují vazbu železa na apotransferin (Osaki *et al.* 1966).

Právě železité ionty mohou být vázány na plasmatický nosič železa, glykoprotein transferin. Ten může vázat až dva železité ionty. U lidí je plasmatický transferin fyziologicky saturovaný železem zhruba z 30 % (Brissot *et al.* 2012). Transferin představuje hlavní a nejdůležitější přenašeč železa, který dodává kov cílovým tkáním (Meyron-Holtz *et al.* 2011). Na druhé straně je ale známo, že savci bez transferinu mohou žít, jak vyplývá ze vzácných případů tzv. atransferinemie (Beutler *et al.* 2000). Transferin s navázaným železem (Fe-Tf) je z krve vychytáván specifickou vazbou na buněčné membránové receptory, transferinový receptor 1 (TfR1) a kubilin (Kozyraki *et al.* 2001). Funkce TfR2 je pravděpodobně odlišná, regulační (Camaschella *et al.* 2000, Mladěnka *et al.* 2005). Po endocytóze komplexu Fe-Tf/TfR dojde k acidifikaci vezikuly, uvolnění železa, jeho redukci na železnatou formu a konečně transportu do cytosolu prostřednictvím DMT1 (Graham *et al.* 2007). Obdobnou funkci jako transferin vykonává na sliznicích glykoprotein laktoferin, jenž má také antimikrobiální a protizánětlivé vlastnosti (Mladěnka *et al.* 2005). Železo se v plasmě za jistých podmínek může vyskytovat jako tzv. železo nevázané na transferin (v angl. non-transferrin bound iron) (Brissot *et al.* 2012). Jedná se o železo vázané na ostatní proteiny (např. feritin) a také neproteinové železo (např. ve formě citrátu či acetátu)

(Grootveld *et al.* 1989). Hladiny této formy železa v plasmě jsou extrémně nízké a za normálních okolností nepřekračují 1 μmol.l^{-1} (Anderson 1999).

Zásadní pochopení homeostázy železa a nové potenciální terapeutické možnosti přineslo relativně nedávné objevení hormonu hepcidinu, který má ústřední roli v systémové homeostáze železa. Název odráží jednak místo původu tohoto hormonu, jednak jeho antimikrobiální vlastnosti (Park *et al.* 2001). Hepcidin je peptid syntetizovaný a secernovaný játry, který posttranslačně ovlivňuje membránovou koncentraci svého receptoru ferroportinu, zmíněného efluxního transportéru železa (Ganz 2013). Navázání hepcidinu indukuje endocytózu ferroportinu a jeho následnou proteolýzu v lysosomu (Nemeth *et al.* 2004). Tento hormon tedy kontroluje tok železa do plasmy. Samotný hepcidin je kromě tkáňových zásob a plasmatických koncentrací železa regulován řadou dalších faktorů, například erytropoezou, zánětem či jaterním poškozením. Dosud jediná známá úroveň této regulace je transkripční, zahrnující celou řadu působků se složitými vztahy (Ganz 2013). Mutace v genech kódujících hepcidin, jeho rozličné regulátory nebo jeho molekulární cíl ferroportin se mohou manifestovat jako poruchy regulace železa (viz kapitola 2.2.1.).

Osud železa v buňkách je řízen posttranskripčně prostřednictvím specifických RNA vázajících proteinů IRP1 a IRP2 (IRP z angl. iron-regulatory protein). Tyto proteiny interagují s příslušnými responzivními elementy (IRE, z angl. iron-responsive element) na cílové mRNA (Hentze *et al.* 2010), přičemž vázání regulačních proteinů odpovídá na buněčné hladiny železa. IRE, zhruba třicet nukleotidů dlouhé sekvence, byly nalezeny v nekódujících oblastech mRNA u různých proteinů zapojených do kinetiky železa a energetického metabolismu (Mladěnka *et al.* 2005).

Po absorpci v tenkém střevě je železo v portální krvi vázáno na apotransferin a přenášeno do jater, kde je použito pro syntézu proteinů, skladováno nebo exportováno.

Transferin v cirkulaci pak přenáší kov k cílovým tkáním. Největšími konzumenty železa v lidském těle jsou erytroidní buňky v kostní dřeni. Téměř veškeré železo v těchto vyvíjejících se buňkách je směřováno do mitochondrií, kde je upotřebeno pro biosyntézu hemu a tvorbu Fe/S proteinů (Ponka *et al.* 2002). Na tomto místě nelze nezmínit protein frataxin, chaperon, který pravděpodobně dopravuje železo na místo utváření Fe/S klastrů a syntézy hemu a jehož snížená syntéza se projevuje jako Friedreichova ataxie (Bencze *et al.* 2007). Dalšími buňkami, které mají zásadní význam pro celotělovou homeostázu železa, jsou retikuloendoteliální makrofágy recyklující železo ze stárnoucích erytrocytů a ostatních buněk. Pozřený erytrocyt a jeho hemoglobin jsou degradovány ve fagolysosomu makrofágu. Enzym hemoxygenasa následně rozštěpí protoporfyrinový kruh hemu a dojde k uvolnění kovu. Retikuloendoteliální makrofágy slouží jako rezervoár železa, normálně skladující zhruba polovinu celkových tělesných zásob (Knutson a Wessling-Resnick 2003). Hlavním regulačním krokem u všech tkání transportujících železo je přenos kovu z buněk tkání do plasmy. Jak již bylo zmíněno, tento krok je zásadním způsobem ovlivněn hormonem hepcidinem (Ganz 2013).

2.1.2. Měď

Měď je přechodný kov, který se vyskytuje především ve dvou formách, jako ionty měďné (Cu^{+}) a měďnaté (Cu^{2+}). Měďné ionty tvoří početné komplexy jak s organickými, tak anorganickými ligandy, přičemž silně preferují ligandy mající jako donorové atomy síru (např. v cysteinu nebo methioninu), aromatický dusík a fosfor (Tisato *et al.* 2010). V biologických systémech se měď nachází převážně ve stavu Cu^{2+}, protože v přítomnosti kyslíku nebo jiných akceptorů elektronů je ochotně oxidována, pokud nejsou měďné ionty stabilizovány tvorbou komplexu. Měďnaté ionty jsou dosti rozpustné, zatímco rozpustnost měďných iontů je v rozsahu nižším než mikromolárním (Arredondo a Núñez 2005).

Od bakterií k rostlinám a savcům, všechny živé buňky potřebují ionty mědi (Delangle a Mintz 2012). Nepostradatelnost mědi vychází z jejích redoxních vlastností a specifické inkorporace do rozličných enzymatických a strukturálních proteinů (Harris 2000), v nichž je vázána koordinačně-kovalentní vazbou. Mnoho těchto enzymů (tzv. kuproenzymů) a proteinů má dvojí nebo víceré role (Linder a Hazegh-Azam 1996). Měď funguje jako intermediát elektronového přenosu v reakcích zahrnujících buněčnou respiraci, ochranu před volnými radikály, je důležitá pro metabolismus železa, syntézu pojivové tkáně, pigmentaci, srážení krve, angiogenezi, produkci peptidových hormonů a biosyntézu neurotransmiterů, je nezbytná pro normální buněčný růst a vývoj (Tapiero *et al.* 2003, Lalioti *et al.* 2009, Tümer a Møller 2010). Přehled kuproenzymů a proteinů transportujících měď je uveden v **Tabulce 2**. Rychle přibývající, i když dosud relativně opomíjené důkazy dokládají, že měď má také esenciální regulační funkci pro buněčnou signalizaci, od modulace vazby proteinů na cílové receptory po ovlivnění nukleárních transkripčních faktorů (Grubman a White 2014). Může tak být považována za třetí klíčový modulátor vedle vápníku a zinku (Grubman a White 2014).

Tabulka 2. Přehled kuproenzymů a proteinů transportujících měď

Kuproenzym/protein transportující měď	Funkce
cytochrom-c-oxidasa	buněčné dýchání
Cu/Zn-superoxiddismutasa	zhášení volných radikálů
metallothionein	detoxikace mědi a dalších kovů
dopamin-β-hydroxylasa	syntéza katecholaminů
tyrosinasa (katecholoxidasa)	syntéza melaninu
fenylalaninhydroxylasa	přeměna fenylalaninu na tyrosin
peptidylglycin-α-amidující monooxygenasa	zrání peptidových hormonů
ceruloplasmin (ferroxidasa I)	transport mědi, ferroxidasa, protein akutní fáze
lysyloxidasa	tvorba pojivové tkáně, důležitá pro příčné zesíťování kolagenu a elastinu
albumin	mj. transport mědi
transkuprein	transport mědi
měď-dependentní aminoxidasy	metabolismus aminů
krevní srážecí faktory V (proakcelerin) a VIII (antihemofilický faktor)	hemokoagulace
hephaestin	metabolismus železa
sulfhydryloxidasa	příčné zesíťování keratinu

Údaje použité v tabulce byly čerpány z těchto zdrojů: Linder a Hazegh-Azam (1996), Uauy *et al.* (1998), Tapiero *et al.* (2003), Tümer a Møller (2010).

Ačkoliv od 90. let 20. století mnoho molekulárních mechanismů kontrolujících homeostázu mědi bylo objeveno (Lutsenko 2010), oproti železu jsou mnohé aspekty osudu mědi v lidském těle stále neobjasněny. Hladiny a distribuce mědi v lidském organismu však taktéž musejí být přísně regulovány, neboť jak nadbytek, tak nedostatek mědi mohou mít patofyziologické následky. To potvrzuje i zjištění, že za normálních podmínek na jednu buňku připadá méně než jeden volný iont mědi (Rae *et al.* 1999). V lidské plasmě je odhadována koncentrace volných měďnatých iontů řádově 10^{-13} mol.l^{-1} (Linder a Hazegh-Azam 1996). Měď je třetím nejhojněji se vyskytujícím

přechodným prvkem v lidském těle, je ale obsažena v mnohem menším množství v porovnání se železem nebo zinkem, je to pouze zhruba 0,10-0,15 g (Trojan a Langmeier 2003).

Měď je absorbována z potravy v proximální části tenkého střeva, průměrný příjem u lidí je denně okolo 1 mg (Tümer a Møller 2010). Přesná kontrola transportu mědi ve střevě není známa, ale absorpce je modulována ve vztahu k přijímaným hladinám kovu. Typicky je totiž vyšší podíl mědi z potravy absorbován při nižším příjmu (Turnlund *et al.* 1998). Pro transport přes apikální membránu enterocytu, obdobně jako u železa, zřejmě musí být redukována. Kandidátů pro tuto úlohu je v literatuře popsáno několik: „cytochrome b_{558} ferric/cupric reductase“ (Knöpfel a Solioz 2002), Steap 2 (Ohgami *et al.* 2006) a CYBRD1 (Wyman *et al.* 2008). Klíčová úloha v získávání mědi z potravy může být pravděpodobně připsána lidskému transportéru mědi (hCTR1, z angl. human copper transporter) (Nose *et al.* 2006a), vysokoafinitnímu transportéru mědi, který vytváří kanál přenášející redukovanou formu kovu (Lee *et al.* 2002). Přesný mechanismus transportu dosud není znám. Ačkoliv je hCTR1 esenciální pro biodostupnost mědi (Nose *et al.* 2006a), jeho funkce v iniciálním apikálním příjmu kovu se zdá být spíše nepřímá (Zimnicka *et al.* 2007), protože import mědi se zjevně může odehrávat i v jeho nepřítomnosti (Nose *et al.* 2006a). Dokonce i lokalizace hCTR1 v enterocytech je nedořešená, uvádí se jak intracelulární, tak apikální, tak bazolaterální (Zimnicka *et al.* 2007, Gupta a Lutsenko 2009, van den Berghe a Klomp 2009, Nose *et al.* 2010). Pravděpodobný model předpokládá, že hCTR1 získává zpět z krve měď, která je určena pro intracelulární potřebu enterocytu, zatímco měď z intestinálního lumen je transportována odlišným mechanismem, např. prostřednictvím jiného transportního proteinu nebo pinocytózou/endocytózou (Zimnicka *et al.* 2007, van den Berghe a Klomp 2009). Vliv jiných možných importérů

mědi kromě hCTR1 není jasný. Protichůdné názory jsou i na DMT1. Některými autory je považován za Cu^{2+} transportér (Gunshin *et al.* 1997), jinými dokonce i za Cu^{+} přenašeč (Arredondo *et al.* 2003). Illing *et al*. (2012) naopak vylučují roli DMT1 v transportu mědi. Další mechanismy intestinální absorpce mědi mohou být: hCTR2 (van den Berghe a Klomp 2009), ATP-dependentní vysokoafinitní Cu^{2+} transport (Knöpfel *et al.* 2005) nebo aniontový transportní systém přenášející Cu^{+} i Cu^{2+} způsobem závislým na chloridových iontech (Zimnicka *et al.* 2011). Role DMT1 je zdůrazňována zejména při dietním nedostatku železa, kdy mRNA pro DMT1 a hladiny vlastního proteinu jsou indukovány (Collins *et al.* 2005, Shah *et al.* 2009). V buňce je měď vázána jedním z několika chaperonových proteinů, které ji doručí jednotlivým buněčným cílům: mitochondrii (COX17, chaperon pro cytochrom-c-oxidasu) (Palumaa *et al.* 2004), Golgiho aparátu (ATOX1, chaperon pro ATP7A) (Larin *et al.* 1999) nebo cytosolické Cu/Zn-superoxiddismutase (CCS, chaperon pro Cu/Zn-superoxiddismutasu) (Casareno *et al.* 1998). Nadbytečný kov v buňce je okamžitě vázán glutathionem a následně metallothioneiny, nízkomolekulárními proteiny bohatými na cystein (Tapiero *et al.* 2003). Měď je dále transportována z enterocytu do portální cirkulace, kde je vázána na α_2-makroglobulin transkuprein, albumin a aminokyseliny (Sarkar 1999, Liu *et al.* 2007) pro transport do jater. Export mědi z enterocytu závisí na činnosti ATP7A (tzv. Menkesovy ATPasy, Menkesova proteinu). Jde o transmembránový protein s duální funkcí, exprimovaný ve většině tkání kromě jater. Normálně zprostředkovává transport mědi do Golgiho aparátu, při zvýšení hladin mědi se však tato ATPasa přesune do plasmatické membrány, čímž umožní export kovu z buňky (Tapiero *et al.* 2003). Stejně jako většina intracelulárních proteinů vážících měď, i tento transportér ji váže v redukované formě (Kodama *et al.* 2012). ATP7A je zásadní pro příjem mědi do lidského organismu.

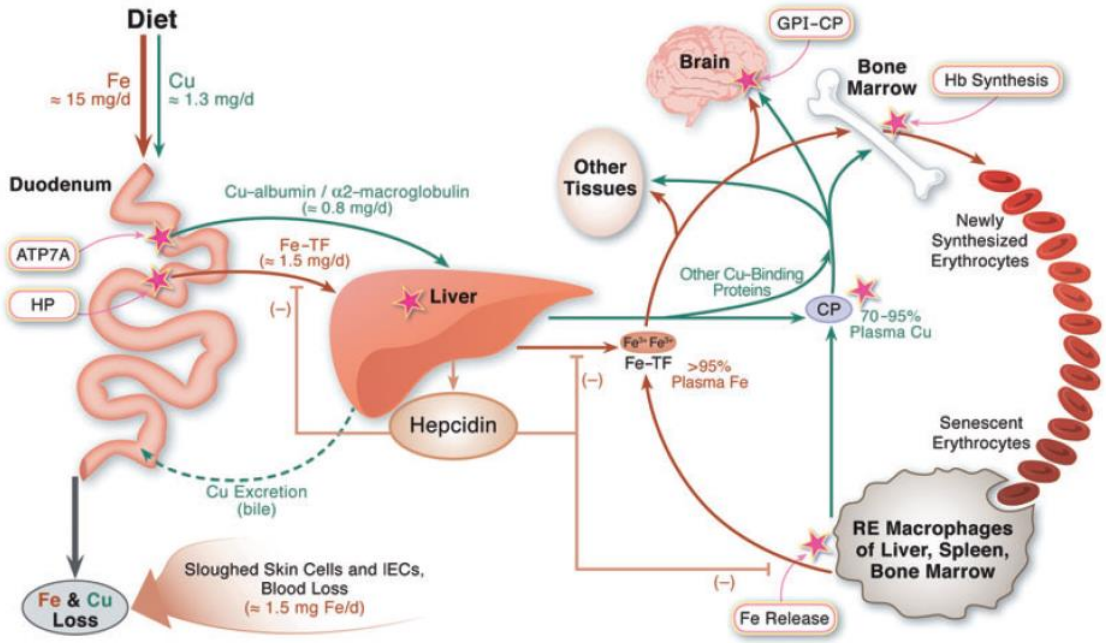
Játra jsou ústředním orgánem skladování a homeostázy mědi (Tümer a Møller 2010). Pro její příjem v játrech má zásadní význam hCTR1 (Kim *et al.* 2009). Zde je vyžadován redukční krok, protože hCTR1 propouští pouze měďné ionty (Nose *et al.* 2006b). Bylo zjištěno, že α_2-makroglobulin dodává měď primárně hCTR1, zatímco albumin předává kov jinému cíli (Moriya *et al.* 2008). V jaterní buňce je měď, jako v enterocytu, vázána chaperony a jimi distribuována. ATP7B (tzv. Wilsonova ATPasa, Wilsonův protein) je protein homologní s ATP7A, avšak exprimovaný primárně v játrech. Transportuje měď do Golgiho aparátu, kde je využita pro syntézu kupro-proteinů (Terada *et al.* 1998). Funkce ATP7B v nejaterních tkáních je nejasná (Kodama *et al.* 2012). Většina mědi opouští játra navázána na ceruloplasmin a je roznášena ke všem orgánům, tkáním a buňkám v těle. Na ceruloplasmin je pevně vázáno zhruba 85-90 % sérové mědi. Zbývajících 10-15 % je vázáno na albumin a aminokyseliny, zejména histidin, přičemž tato měď je vázaná volněji a je dostupná buňkám (Sarkar 1999, Brewer 2009, Kodama *et al.* 2012). Za zmínku také stojí, že ceruloplasmin není nepostradatelný pro účinnou distribuci mědi (Hellman a Gitlin 2002), při tzv. aceruloplasminemii nejsou pozorovány změny tkáňových koncentrací kovu (Harris *et al.* 1998). Prvořadou roli v příjmu mědi do těchto buněk zastává již zmiňovaný hCTR1 (Gupta a Lutsenko 2009). Nadbytek mědi stimuluje translokaci ATP7B z Golgiho aparátu do kanalikulární membrány hepatocytu, tím umožňuje její sekreci do žluče (Roelofsen *et al.* 2000) a odchod z těla stolicí. Na rozdíl od železa má tedy lidské tělo aktivní exkreční mechanismus pro měď.

Homeostáza přechodných kovů v mozku je pečlivě regulována řadou transportérů (Collins *et al.* 2010), neboť její narušení vede k neuropatologickým stavům (Rivera-Mancía *et al.* 2010). Regulace hladin mědi v mozku však zatím není dobře prostudována (Tümer a Møller 2010).

2.1.3. Interakce železa a mědi

Interakce mezi esenciálními kovy železem a mědí v lidském organismu jsou známy mnoho let, přičemž prokázání významu mědi pro syntézu hemoglobinu v roce 1928 je považováno za počátek této problematiky (Fox 2003). Základní místa interakce na úrovni celého organismu jsou znázorněna na **Obrázku 1**.

Obr. 1. Přehled kinetiky železa a mědi na úrovni organismu, včetně základních míst jejich interakce



Místa interakce jsou vyznačená hvězdami. Hephaestin (HP) je regulován hladinami mědi, zatímco exprese Menkesovy ATPasy (ATP7A) je ovlivňována hladinami železa. Nepřítomnost ceruloplasminu (CP) vede k akumulaci železa v pankreatu, retině a mozku, ferroxidasová aktivita cirkulujícího ceruloplasminu je totiž rozhodující pro normální homeostázu železa (Xu *et al.* 2004). Jaterní hladiny mědi se mění nepřímo úměrně ke stavu železa (Collins *et al.* 2010). Aktivita ceruloplasminu vázaného na glykosylfosfatidylinositol (GPI-CP) je nezbytná pro uvolnění železa z mozku a retikuloendoteliálních (RE) makrofágů, tento děj je tedy závislý na hladinách mědi. Neznámý aspekt využití železa v kostní dřeni také závisí na mědi, neboť při jejím nedostatku je tvorba hemoglobinu neefektivní, navzdory normálním sérovým hladinám železa (Pyatskowit a Prohaska 2008). Převzato z: Collins *et al*. (2010).

Ferroxidasy hephaestin a ceruloplasmin hrají důležitou roli v transportu železa ve střevě. Aktivita hephaestinu je závislá na normálních hladinách mědi (Chen *et al.* 2006). Při jejím nedostatku byla pozorována snížená absorpce železa (Reeves a DeMars 2004), zřejmě právě na podkladě snížené aktivity duodenálního hephaestinu (Reeves *et al.* 2005). Analogické výsledky byly získány také v *in vitro* experimentech s Caco-2 buňkami (Chen *et al.* 2009). Bylo zjištěno, že ceruloplasmin může také participovat na intestinálním transportu železa, zejména při stavech hematopoetického stresu (Cherukuri *et al.* 2005). Ke změnám v absorpci jednoho z těchto stopových prvků může docházet odchylkami v homeostáze toho druhého. Vstřebávání mědi tak může být zvýšené při nedostatku železa (Collins *et al.* 2005, Ravia *et al.* 2005, Collins *et al.* 2009), což pravděpodobně souvisí s pozorovanou zvýšenou expresí mRNA ATP7A a vlastního proteinu při deficitu železa (Collins *et al.* 2005, Ravia *et al.* 2005). Bylo zjištěno, že geny pro transportér železnatých iontů DMT1 a enzym redukující železité ionty CYBRD1, tedy proteiny významné pro absorpci železa, jsou regulovány hypoxií indukovaným faktorem HIF-2α (HIF z angl. hypoxia inducible factor) (Shah *et al.* 2009). HIF faktory jsou stabilizovány mědí, čímž dochází ke zvýšení exprese HIF-responzivních genů (Martin *et al.* 2005, Feng *et al.* 2009). Zvýšená hladina mědi v enterocytech, játrech a tělesných tekutinách tak může hrát roli ve zvýšení aktivity HIF, což ovlivňuje geny související s intestinální a následně tělesnou homeostázou železa (Collins *et al.* 2010).

Metabolismus železa a mědi v hepatocytech je propojen ferroxidasou ceruloplasminem (Harris *et al.* 1999). Z neznámých příčin se navíc jaterní koncentrace mědi mění inverzně ke stavu železa (Collins *et al.* 2010).

Metabolismus mědi a železa v retikuloendoteliálních makrofázích je opět propojen přes ceruloplasmin. Nedostatek této ferroxidasy vyplývající z dietního strádání

mědí nebo genové ablace má za následek akumulaci železa v retikuloendoteliálních buňkách sleziny a jater (Harris *et al.* 1999). Buněčná forma ceruloplasminu vázaná na glykosylfosfatidylinositol je rozhodující pro uvolnění železa z uvedených buněk více než cirkulující forma (Collins *et al.* 2010).

Důležité je zmínit, že interakce mědi s železem jsou ovlivněny věkem (homeostatické mechanismy se liší mezi dětmi a dospělými) a živočišným druhem (Collins *et al.* 2010). Kupříkladu příjem železa ve střevě kojících savců, včetně lidí, může záviset spíše na laktoferrinu než na DMT1 (Lopez *et al.* 2006). Dále je zajímavé, že u potkanů a myší trpících nedostatkem mědi se vyvíjí těžká anemie, ale pouze u potkanů dochází ke snížení plasmatických hladin železa (Pyatskowit a Prohaska 2008).

2.2. Stavy spojené s poruchou homeostázy železa a mědi

Schopnost přeměny oxidačního stavu, která činí železo a měď esenciální pro mnohé biologické procesy, je za jistých okolností paradoxně podkladem jejich toxicity. Porušení homeostázy přechodných kovů je spojeno s celou řadou patologických stavů. V některých případech dochází k absolutní ztrátě rovnováhy kovu a jeho nadměrnému hromadění v organismu (např. hereditární hemochromatóza, Wilsonova choroba), nebo naopak jeho nedostatku (např. Menkesova choroba). V dalších případech dochází k určité dysbalanci kovu, jejíž příčinný vztah k rozvoji onemocnění často není zcela znám. Základem škodlivého působení redoxně aktivního železa a mědi je navození oxidačního stresu. Důvodem je schopnost přechodných kovů podporovat reakce vedoucí k tvorbě reaktivních forem kyslíku (Delangle a Mintz 2012).

Za přítomnosti aniontu superoxidového radikálu nebo redukčních činidel, jako glutathion či kyselina askorbová, mohou být železité a měďnaté ionty redukovány (Gaetke a Chow 2003, Jomova a Valko 2011):

$$Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2$$

$$Cu^{2+} + O_2^{-\bullet} \rightarrow Cu^{+} + O_2$$

Oba kovy jsou v redukovaném stavu schopny katalýzy Fentonovy reakce, jejímž produktem je mj. vysoce toxický hydroxylový radikál (Gaetke a Chow 2003, Prousek 2007, Jomova a Valko 2011):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-}$$

Kombinaci Fentonovy reakce a redukce kovů superoxidovým radikálem vyjadřuje Haberova-Weissova reakce, jejímž katalyzátorem je přechodný kov (M):

$$O_2^{-\bullet} + H_2O_2 \xrightarrow{M} O_2 + OH^{\bullet} + OH^{-}$$

Hydroxylový radikál, jehož biologický poločas ve vodném roztoku je kratší než 1 ns (Pastor *et al.* 2000), může reagovat v podstatě s jakýmikoli biologickými molekulami, včetně nukleových kyselin, lipidů a proteinů (Jomova a Valko 2011). Měď může být dokonce 10-60x účinnější než železo v katalýze Fentonovy reakce (Shinar *et al.* 1989).

S ohledem na zaměření disertační práce budou v následujícím textu podrobněji zpracovány patologické stavy spojené se zvýšenou akumulací přechodných kovů nebo jejich dysbalancí.

2.2.1. Chronické přetížení železem

Několik milionů lidí na celém světě trpí chronickým přetížením železem se závažnými klinickými dopady (Flaten *et al.* 2012). Tento stav je důsledkem vrozeného onemocnění a/nebo je navozený opakovanými krevními transfuzemi.

Hereditární hemochromatóza je vrozená porucha homeostázy železa, klasifikovaná do čtyř typů na základě příčinné mutace (**Tabulka 3**). Podkladem většiny hemochromatóz (typy 1-3) je snížená produkce hepcidinu s následnou hyperabsorpcí železa z potravy, typ 4 je spojen s neschopností ferroportinu vázat hepcidin nebo jeho rezistencí k hepcidinu, protože ferroportin je mutován (Kanwar a Kowdley 2013). Dochází k ukládání železa v různých orgánech – játrech, pankreatu, srdci, hypofýze, kůži a kloubech – a jejich poškození s rozvojem fibrózy, cirhózy jater a hepatocelulárního karcinomu, diabetu, kardiomyopatii a dalším postižením (Kanwar a Kowdley 2013). Labilní plasmatické železo, tj. železo nevázané na transferin, je považováno za hlavního činitele způsobujícího oxidační poškození (Cabantchik 2014). V diagnostice onemocnění se používají stanovení sérového feritinu, saturace transferinu a jaterní biopsie. Velký pokrok znamenalo zavedení genetického testování a zobrazovací techniky magnetické rezonance (Kanwar a Kowdley 2013). Léčba spočívá v odstranění železa z těla terapeutickou flebotomií (venesekcí), což je metoda levná, účinná a jednoduchá (Kontoghiorghes *et al.* 2010). Použití chelátorů železa je vyhrazeno pro pacienty, kteří nejsou schopni nebo ochotni podstoupit flebotomii (Nielsen *et al.* 2003). Nové terapeutické možnosti potenciálně představují hepcidinová mimetika nebo stimulátory produkce hepcidinu. Tyto látky jsou v současné době intenzivně studované (Ruchala a Nemeth 2014).

Tabulka 3. Typy hemochromatózy

Typ	Název	Mutovaný gen	Typ dědičnosti	Patogeneze
1	klasická hemochromatóza	*HFE*	recesivní	snížený hepcidin
2A	juvenilní hemochromatóza	*HJV* (gen pro hemojuvelin)	recesivní	inhibice exprese hepcidinu
2B	juvenilní hemochromatóza	*HAMP* (gen pro hepcidin)	recesivní	snížený hepcidin
3	hemochromatóza	*TfR2* (gen pro transferinový receptor 2)	recesivní	porušení kontroly hladiny železa, snížený hepcidin
4	„ferroportinová nemoc“	*SLC40A1* (gen pro ferroportin)	dominantní	snížený export železa z makrofágů a enterocytů, vzácně rezistence k hepcidinu

Převzato z: Kanwar a Kowdley (2013).

Hlavní příčinou získaného přetížení železem jsou posttransfuzní stavy – tzv. transfuzní sideróza. Nadměrná potravní konzumace železa je velmi vzácná (Brissot *et al.* 2008). Pacienti se závažnými hematologickými onemocněními, především anemiemi s neefektivní erytropoezou (ß-talasemií a srpkovitou anemií), jsou léčeni opakovanými krevními transfuzemi (Flaten *et al.* 2012). Tato terapie však má dvě strany: každá jednotka krevní transfuze obsahuje velké množství železa, které se v organismu hromadí, neboť lidské tělo postrádá aktivní způsob vyloučení přebytečného kovu. Dva základní mechanismy se podílejí na rozvoji přetížení: vlastní nálož železa opakovanými transfuzemi a nedostatek hepcidinu při neefektivní erytropoeze. Zvýšená aktivita kostní dřeně v tomto ohledu totiž převyšuje reakci na přetížení železem (Brissot *et al.* 2008). Na rozdíl od hemochromatózy, u které dochází primárně k poškození jater, je srdeční poškození nejčastější příčinou smrti u talasemických pacientů podstupujících opakované krevní transfuze (Borgna-Pignatti *et al.* 1998). Při expozici vysokým

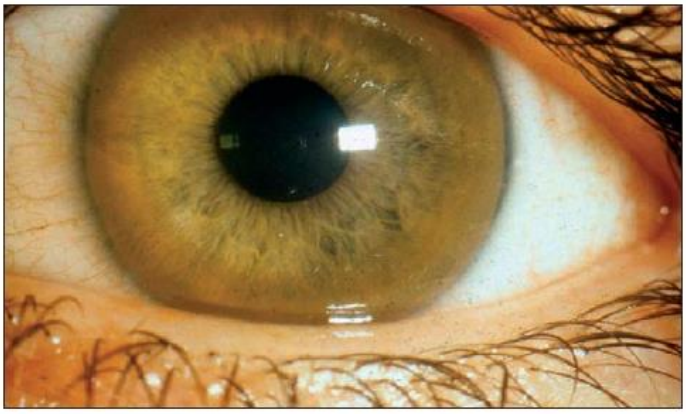
hladinám železa se významné známky orgánového selhání projeví obecně zhruba za deset let (Coates 2014). Hlavním pilířem léčby transfuzní siderózy je chelatační terapie, jež byla rozsáhle použita zvláště u pacientů s *thalassaemia major*. Flebotomie v těchto případech nemůže být použita (Flaten *et al.* 2012). Chelátory mají odstranit nadbytečné železo z organismu, ihned také váží volný kov a poskytují tak ochranu před oxidačním poškozením (Coates 2014). Délka terapie koresponduje s transfuzní léčbou, u většiny pacientů je nutné doživotní podávání (Brittenham 2011). Kromě kontroly účinnosti chelatační léčby musí být také pečlivě monitorovány nežádoucí účinky použitých léčiv (Brittenham 2011). V současné době jsou klinicky používány tři látky: deferoxamin, deferipron a deferasirox. Deferoxamin během svého více než čtyřicetiletého použití znamenal záchranu života tisíců pacientů (Aaseth *et al.* 2014), poslední dvě jmenovaná léčiva však znamenají pokrok v léčbě, i vzhledem k možnosti perorální aplikace. Kombinační terapie různými chelátory železa přináší benefit v podobě zvýšené exkrece kovu a snížení vedlejších účinků (Sheth 2014).

2.2.2. Wilsonova choroba

Wilsonova choroba, označovaná též jako hepatolentikulární degenerace, je vzácná dědičná porucha metabolismu mědi, poprvé definovaná jako syndrom v roce 1912 (Wilson 1912). Prevalence tohoto autozomálně recesivního onemocnění je zhruba 1/30 000, ačkoliv se různí napříč populacemi (Mak a Lam 2008). Nemoc je klinicky velmi variabilní, stejně jako různé mutace, které ji způsobují (Bandmann *et al.* 2015). Projevuje se zejména jaterním a/nebo neurologickým postižením (Bandmann *et al.* 2015), a pokud není léčena, způsobuje těžkou invaliditu a smrt (Ala *et al.* 2007). Příčinný gen na chromozómu 13, *ATP7B*, kóduje stejnojmennou ATPasu transportující měď (Bandmann *et al.* 2015). Při Wilsonově chorobě je *ATP7B* mutantní a jeho expresí vzniká protein, který není schopný transportovat měď, je narušena sekrece holo-ceruloplasminu do plasmy a exkrece mědi do žluče. Hromaděná měď je následně uvolňována do plasmy ve formě nevázané na ceruloplasmin. Tato měď je vázána volněji na jiné komponenty plasmy a je zvýšeně exkretována ledvinami, ale i ukládána do různých tkání (Brewer 2009, Kodama *et al.* 2012). Snížená koncentrace ceruloplasminu navíc narušuje i homeostázu železa (Merle *et al.* 2010). Kromě obecně uznávaného patofyziologického mechanismu na podkladě oxidačního poškození z důvodu přetížení mědí (Ferenci 2005) spouští nerovnováha tohoto kovu řadu specifičtějších metabolických odpovědí, které pravděpodobně významně ovlivňují rozvoj nemoci (Burkhead *et al.* 2011). Neurologické symptomy se vyskytují u 40-50 % nemocných (Walshe 1962) a obvykle začínají ve druhé a třetí dekádě života (Machado *et al.* 2006). Příznaky choroby však mohou nastoupit i v podstatně pozdějším věku (Ala *et al.* 2005). Neurologické příznaky mohou být dystonické, ataktické nebo parkinsonické, mohou se rovněž kombinovat (Machado *et al.* 2006). Jaterní postižení se může manifestovat jako akutní jaterní selhání i chronické jaterní poškození. Nemocní

mají vyšší riziko rozvoje hepatocelulárního karcinomu, navzdory terapii (Kodama *et al.* 2012). Pro diagnózu Wilsonovy choroby se používají následující vyšetření: přítomnost Kayserových-Fleischerových prstenců (**Obrázek 2**), stanovení sérové koncentrace ceruloplasminu, analýza 24h exkrece mědi močí, penicilaminový test, stanovení jaterní mědi a inkorporace ^{64}Cu do sérového ceruloplasminu. Dále se uplatňují zobrazovací metody, např. magnetická rezonance, a genetické testování (Ala *et al.* 2007).

Obr. 2. Kayserovy-Fleischerovy prstence



Hromadění mědi vytváří zlatě hnědé zabarvení vnějšího okraje rohovky. Převzato z: Ala *et al*. (2007).

Wilsonova choroba byla postupně progredující a smrtelná nemoc až do roku 1951, kdy byl použit intramuskulárně dimerkaprol (BAL, z angl. British anti-Lewisit) jako první chelatující činidlo. Revoluci v léčbě představovalo zavedení perorálně účinného chelátoru penicilaminu v roce 1956 (Walshe 1956), v roce 1969 následoval trientin jako alternativa pro pacienty netolerující penicilamin (Walshe 1969). Farmakologická léčba, jež musí být doživotní, má dvě fáze: iniciální fázi, která je agresivnější a akutně snižuje množství mědi v organismu, a následnou fázi udržovací. Nastavení udržovací léčby pomáhá předcházet předávkování léčivy, které může vést k deficienci kovu (Weiss a Stremmel 2012). V současné době jsou k terapii Wilsonovy

choroby používány chelátory mědi (penicilamin, trientin a tetrathiomolybdenan), soli zinku, nebo se kombinuje chelátor se zinkem. Chelatační látky váží měď přímo v krvi nebo tkáních a usnadňují její exkreci, zatímco zinek interferuje s intestinálním příjmem kovu. Tetrathiomolybdenan může působit oběma mechanismy. Dostupná data neumožňují definitivní doporučení jedné chelatační látky před ostatními (Ala *et al.* 2007, Bandmann *et al.* 2015), obecně je ale léčba Wilsonovy choroby účinná (Ala *et al.* 2007). Problémem jsou však nežádoucí účinky léčiv a paradoxní zhoršení neurologických příznaků až u 20 % pacientů po iniciální terapii. Mechanismus této reakce není zcela pochopen, ale pravděpodobně dochází k nadměrné mobilizaci mědi vedoucí ke zvýšení její volné frakce (Bandmann *et al.* 2015).

Zinek, jehož začátek použití spadá do 60. let 20. století, interferuje s příjmem mědi ve střevě. Jeho působením dochází k indukci syntézy metallothioneinu ve střevních epiteliálních buňkách. To vede k přednostnímu vázání dietní mědi v těchto buňkách, které jsou následně fyziologicky odloučeny. Léčba solemi zinku je méně často spojena se zhoršením neurologických projevů po iniciální terapii, vykazuje však nižší účinnost a navození negativní bilance mědi trvá déle než při použití chelátorů. Terapie zinkem je často spojena s gastrointestinálním diskomfortem (Ala *et al.* 2007, Bandmann *et al.* 2015).

Krajním řešením u pacientů trpících Wilsonovou chorobou je transplantace jater. Koriguje jaterní genotyp, obnovuje schopnost exkrece mědi a měla by být uvážena u pacientů vykazujících akutní jaterní selhání nebo dekompenzovanou cirhózu (Bandmann *et al.* 2015).

2.2.3. Neurodegenerativní onemocnění

Navzdory řadě známých patofyziologických mechanismů není vyvolávající příčina těchto chorob dosud přesně objasněna. Železo a měď vykonávají esenciální funkce v mozkové tkáni. Mozek přirozeně koncentruje vysoké hladiny kovů, avšak výsledky mnohých studií naznačují souvislost přechodných kovů s rozvojem neurodegenerativních onemocnění (Delangle a Mintz 2012). Jedná se zejména o Alzheimerovu, Parkinsonovu a Huntingtonovu chorobu, amyotrofickou laterální sklerózu a dále aceruloplasminemii a Friedreichovu ataxii, kdy jsou železo a měď zapojeny do neurodegenerativních mechanismů jako je agregace proteinů, tvorba volných radikálů a oxidační stres (Rivera-Mancía *et al.* 2010, Ward *et al.* 2014). Nebylo však dosud objasněno, jestli oxidační stres je příčinou, nebo následkem základních patologických procesů (Eskici a Axelsen 2012).

Nejvíce pozornosti v souvislosti s Alzheimerovou chorobou bylo věnováno mědi, protože amyloidní prekurzorový protein a amyloidní beta peptidy mají významné interakce s tímto kovem (Eskici a Axelsen 2012). U pacientů s Alzheimerovou chorobou byly nalezeny zvýšené koncentrace mědi nevázané na ceruloplasmin v séru a cerebrospinální tekutině (Bandmann *et al.* 2015). Naopak intracelulární měď v mozkové tkáni se zdá být nedostatečná (Maynard *et al.* 2005), což může přispívat k akumulaci amyloidního beta peptidu vně buněk (White *et al.* 2006). Nedávné studie podporují koncept nerovnováhy mědi v mozku spíše než její samotnou akumulaci či deficienci, proto látky schopné vyvážit hladiny tohoto kovu, spíše než klasické chelátory, jsou nyní rozsáhle studovány (Manso *et al.* 2011, Faller 2012). V současnosti je klinicky testována jediná skupina látek vázajících kov, 8-hydroxychinoliny (Ward *et al.* 2014). Bylo prokázáno, že modelová látka kliochinol transportuje kov dovnitř buňky a kromě chelatační schopnosti aktivuje signální dráhy vedoucí k upregulaci

metaloproteas v matrix a degradaci amyloidních beta peptidů (White *et al.* 2006). Příbuzná látka PBT2 prokázala slibné výsledky v řadě *in vivo* modelů Alzheimerovy choroby a také ve fázi 2 klinických studií (Manso *et al.* 2011).

U Parkinsonovy choroby se naopak více dokladů soustředí na neurotoxický potenciál železa (Rivera-Mancía *et al.* 2010), jehož koncentrace jsou zvýšené v mozkové tkáni u těchto nemocných (Jellinger 2013). K depozici železa v *substantia nigra* mohou vést snížené hladiny ceruloplasminu (Jin *et al.* 2011).

Aceruloplasminemie je autozomálně recesivní onemocnění způsobené mutací genu pro ceruloplasmin, což vede k poruše homeostázy železa, nikoliv mědi, v souvislosti s ferroxidasovou aktivitou tohoto proteinu. Redoxně aktivní kov se hromadí v mozku, játrech a pankreatu (Bandmann *et al.* 2015). Pro velmi nízké nebo nulové koncentrace sérového ceruloplasminu může být aceruloplasminemie zaměněna s Wilsonovou chorobou (Kerkhof a Honkoop 2014).

Friedreichova ataxie je také dědičné neurodegenerativní onemocnění, jehož podkladem je defektní exprese mitochondriálního proteinu frataxinu. Přestože přesná funkce frataxinu není známa, jeho absence vede k narušení metabolismu železa, funkci proteinů obsahujících Fe/S klastry a deregulaci redoxního stavu buňky (Richardson *et al.* 2013). Vzhledem k dokladům o porušené homeostáze železa byl v *in vitro* modelech i u pacientů trpících Friedreichovou ataxií (Santos *et al.* 2010) testován vliv chelátorů tohoto kovu. Klinické studie s deferoxaminem a deferipronem však přinesly rozporuplné výsledky (Richardson *et al.* 2013).

2.2.4. Kardiovaskulární onemocnění

Kardiovaskulární onemocnění, mezi kterými zaujímá první místo ischemická choroba srdeční, představují celosvětově nejčastější příčinu úmrtí (Mendis *et al*. 2011). Volné kyslíkové radikály hrají ústřední roli v patogenezi postischemického myokardiálního reperfuzního poškození (Powell a Tortolani 1992). Při znovuobnovení průtoku ischemickým ložiskem jsou železo a měď mobilizovány z myokardiálních buněk do koronární cirkulace. Vyplavené množství závisí na trvání ischemie a koreluje se stupněm poškození srdce (Chevion *et al.* 1993, Berenshtein *et al.* 1997, Altekin *et al.* 2005). Zapojení přechodných kovů do katalýzy tvorby velmi reaktivního hydroxylového radikálu vedlo k hypotéze, že použití specifického chelatačního agens by mohlo udržet kov v inaktivní formě a zabránit tak nežádoucí reakci (Chevion 1988). Význam železa v ischemicko-reperfuzním poškození byl podpořen *in vitro* studiemi ukazujícími, že chelatace kovu ochránila srdeční tkáň (DeBoer a Clark 1992), zatímco přidání železa nebo mědi zvýšilo míru poškození (Zeltcer *et al.* 1997). Různé chelatační látky v experimentálních podmínkách prokázaly slibné účinky. Na modelu izolovaného potkaního srdce vykázal neokuproin, vysoce účinný chelátor železa i mědi (de Mello Filho a Meneghini 1985), protektivní působení jak při non-ischemickém, tak při ischemicko-reperfuzním poškození (Applebaum *et al.* 1990). *In vivo* deferoxamin i bathokuproin snížily tvorbu volných radikálů během reperfuze (Spencer *et al.* 1998). Avšak v klinické studii deferoxamin podaný pacientům s akutním infarktem myokardu s elevací ST-segmentu, podstupujícím perkutánní koronární intervenci, přes zmírnění oxidačního stresu neomezil velikost infarktu (Chan *et al.* 2012).

Třebaže velké množství prací týkající se patofyziologických mechanismů spojuje stav tělesného železa s kardiovaskulárními nemocemi, epidemiologické studie přinášejí rozporuplné výsledky. Nadbytek železa v těle, stejně jako deficience kovu,

jsou ve většině studií asociovány se zvýšeným kardiovaskulárním rizikem. Na základě observačních studií však nelze stanovit kauzální vztah (Lapice *et al.* 2013).

Měď má esenciální roli v kardiovaskulárním systému, zejména na podkladě podpory angiogeneze a je také zapojena do regulace antioxidačních aktivit (Kang 2011, He a Kang 2013). Nedostatek mědi má nepříznivé kardiovaskulární následky, avšak vysoké koncentrace kovu v krvi jsou považovány za nezávislý rizikový faktor pro aterosklerózu, ischemickou chorobu srdeční i její nejzávažnější formu, akutní infarkt myokardu (Kang 2011). Zvýšené koncentrace mědi a ceruloplasminu jsou nezávisle asociovány se zvýšeným rizikem mortality ze všech příčin i příčin kardiovaskulárních (Grammer *et al.* 2014). Z dostupných dat však není možno vyvodit, zdali tyto zvýšené koncentrace mají aktivní a kauzální roli v rozvoji aterosklerózy nebo jejích klinických následků. Není bez zajímavosti, že v mechanismu rozvoje kardiovaskulárních nemocí byla dokumentována role interakce mědi s neesenciální aminokyselinou homocysteinem (Kang 2011).

Na tomto místě nelze alespoň nezmínit problematiku anthracyklinové kardiotoxicity. Anthracyklinová antibiotika jsou protinádorová léčiva, hojně využívaná v klinické praxi, jejichž závažný nežádoucí účinek představuje kardiotoxicita, vedoucí až k srdečnímu selhání. Mechanismus této toxicity stále není dořešen, ačkoliv se dlouho předpokládalo, že v něm má zásadní postavení železo participující na tvorbě volných radikálů. K této hypotéze vedla i skutečnost, že známé kardioprotektivum dexrazoxan, respektive jeho metabolit ADR-925, působí jako chelátor železa. Výzkum analogických chelátorů však nevedl k uspokojivým výsledkům a dosavadní studie naznačují, že mechanismus působení dexrazoxanu pravděpodobně spočívá spíše v katalytické inhibici topoisomerasy II než v chelataci železa (Šimůnek *et al.* 2009, Vavrova *et al.* 2013).

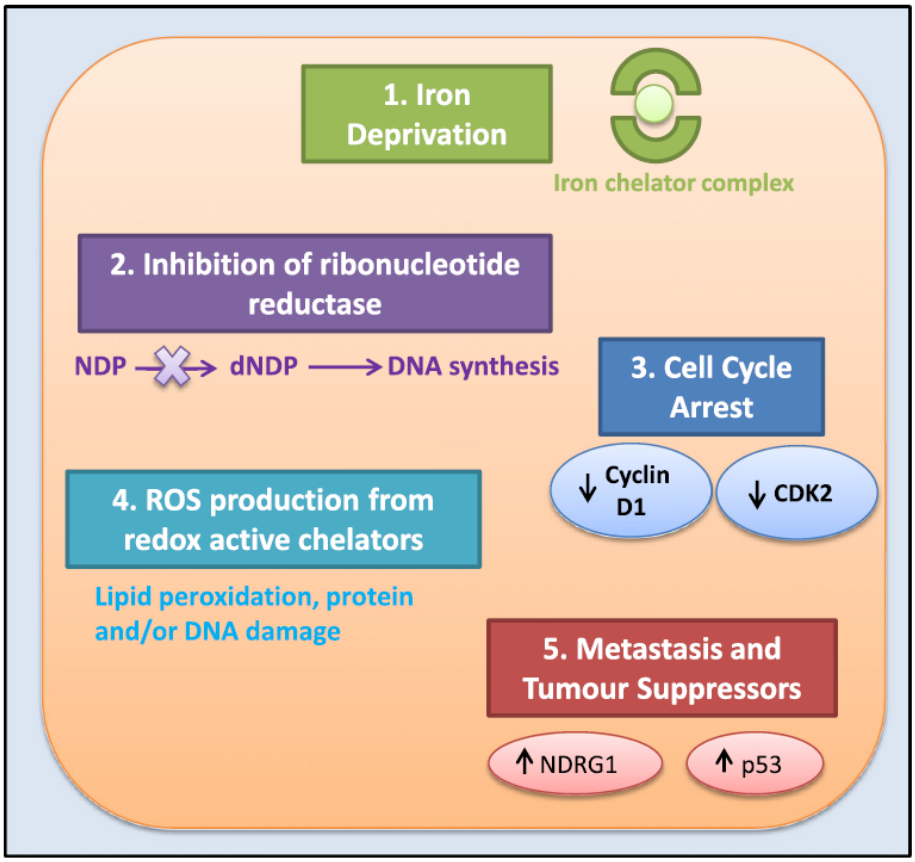
2.2.5. Nádorová onemocnění

Nádorová onemocnění představují významnou příčinu úmrtí ve vyspělých zemích a celosvětově závažný zdravotní a sociální problém (Jemal *et al.* 2011). Vzhledem k přibývajícím důkazům o souvislosti přechodných kovů železa a mědi s nádorovými onemocněními jsou studovány chelátory kovů jako potenciální protinádorová léčiva.

U nádorových buněk se obecně popisuje zvýšený příjem železa, který vede k jeho vyšším intracelulárním hladinám (Torti a Torti 2013). Železo má totiž zásadní význam pro buněčnou proliferaci. Jedná se o kofaktor enzymu ribonukleotidreduktasy, je rovněž zapojeno do regulace řady molekul důležitých pro buněčný cyklus, včetně cyklinů, cyklin-dependentních kinas (CDK) a jejich inhibitorů (Lane *et al.* 2014). Je známo, že oxidační stres indukovaný železem může hrát roli v tumorigenezi navozením mutace DNA a aktivace onkogenů (Valko *et al.* 2006). Příčinný vztah je však obtížné stanovit, neboť se jedná o velmi komplexní proces (Merlot *et al.* 2013). Zvýšené hladiny či příjem železa jsou spojeny se zvýšeným rizikem některých nádorů, zejména kolorektálního karcinomu (Nelson 2001). Naopak u dárců krve bylo pozorováno nižší riziko rozvoje nehematologických malignit (Merk *et al.* 1990).

Působení chelátorů železa jako potenciálních chemoterapeutik nádorových onemocnění lze rozdělit na dva základní principy: snížení hladiny kovu a podporu tvorby reaktivních forem kyslíku (ROS, z angl. reactive oxygen species) (Torti a Torti 2013). Jednotlivé mechanismy, které jsou zodpovědné za protinádorovou aktivitu těchto látek, jsou znázorněny na **Obrázku 3**. Se záměrem zvýšit selektivitu k nádorovým buňkám jsou studovány různé přístupy, například konjugace chelátoru s tumor-specifickým ligandem (Torti a Torti 2013).

Obr. 3. Mechanismy protinádorového účinku chelátorů železa



Na protinádorovém účinku chelátorů železa se podílejí následující mechanismy: 1) snížení hladin železa v buňce, 2) inhibice ribonukleotidreduktasy, 3) navození zástavy buněčného cyklu, 4) podpora lokalizované a cytotoxické produkce ROS, 5) indukce supresorů metastáz a tumorových supresorů (NDRG1, z angl. N-myc downstream regulated gene 1, respektive proteinu p53). NDP, ribonukleosid-5´-difosfát; dNDP, 2´-deoxyribonukleosid-5´-difosfát. Převzato z: Lane *et al*. (2014).

Řada látek vykázala slibné účinky v *in vitro* a *in vivo* experimentech (Merlot *et al.* 2013), informace z klinických studií jsou však zatím nedostatečné. Zajímavou skupinou syntetických chelátorů specificky navržených pro terapii nádorů jsou thiosemikarbazony, z nichž 3-aminopyridin-2-karbaldehyd-thiosemikarbazon (3-AP, Triapine®) dokonce vstoupil do několika klinických studií fáze 1 a 2 (Lane *et al.* 2014).

Zvýšené sérové i tkáňové hladiny mědi byly nalezeny u několika typů nádorů a korelují se stádiem nádoru a/nebo jeho progresí (Gupte a Mumper 2009). Měď hraje

ústřední roli v angiogenezi, procesu klíčovém pro růst nádoru (Finney *et al.* 2009, Gupte a Mumper 2009, Antoniades *et al.* 2013). Kromě samotných chelátorů mědi, které vykazují antiangiogenní potenciál a/nebo cytotoxický potenciál k nádorovým buňkám produkcí ROS, jsou studovány také účinky komplexů rozličných ligandů s mědí (Gupte a Mumper 2007, Gupte a Mumper 2009, Brewer 2014). Nejvíce pozornosti bylo věnováno schopnosti těchto komplexů interagovat s DNA, zjištěny však byly i další molekulární cíle, topoisomerasy nebo proteasom (Santini *et al.* 2014).

Není bez zajímavosti, že metabolismus mědi má souvislost s rezistencí nádorových buněk k platinovým cytostatikům: zvýšená exprese hCTR1, který představuje významný transportér pro cisplatinu (Ishida *et al.* 2002), je spojena s příznivými výsledky léčby těmito chemoterapeutiky (Kuo *et al.* 2012). Protože exprese hCTR1 s následným zvýšením příjmu cisplatiny do buňky může být indukována deplecí buněčných hladin mědi (Kuo *et al.* 2012), logickým vyústěním je kombinační léčba chelátorem mědi trientinem s karboplatinou, testovaná ve fázi 1 klinického zkoušení (Fu *et al.* 2012).

2.2.6. Diabetes mellitus

Významně je diskutováno zapojení železa i mědi do rozvoje diabetu mellitu a jeho chronických komplikací, chelatace těchto kovů tak může představovat terapeutický přístup zmírňující závažnost stavu a mortalitu diabetických pacientů (Cooper 2012, Hansen *et al.* 2014).

Železo je vyžadováno pro správnou funkci ß-buněk Langerhansových ostrůvků pankreatu a tím homeostázu glukózy (Hansen *et al.* 2014), hraje však přímou roli v patogenezi onemocnění prostřednictvím inzulinové rezistence i selhání ß-buněk (Simcox a McClain 2013). Základní mechanismy tohoto vlivu zahrnují oxidační stres a modulaci adipokinů a intracelulárních signálních drah (Simcox a McClain 2013). Ačkoliv řada experimentálních studií na zvířecích modelech a průkazných epidemiologických studií dokládá zvýšené riziko rozvoje diabetu v souvislosti se železem, na základě omezených intervenčních studií nelze zatím potvrdit ani vyvrátit hypotézu, že snížení hladin železa v těle zlepšuje stav nemoci (Kunutsor *et al.* 2013, Simcox a McClain 2013, Hansen *et al.* 2014, Orban *et al.* 2014).

Zatímco některé experimentální práce spojují měď s patogenezí diabetu mellitu (Tanaka *et al.* 2009), předmětem zájmu je v případě mědi především její souvislost s rozvojem chronických diabetických komplikací a vliv chelátorů tohoto kovu (Keegan *et al.* 1999, Eaton a Qian 2002, Hamada *et al.* 2005, Gong *et al.* 2008, Cooper *et al.* 2009, Cooper 2012). Hyperglykemie a související metabolické poruchy totiž narušují homeostázu mědi, dochází k poškození citlivých tkání oxidačním stresem a rozvoji závažných komplikací (Cooper 2012).

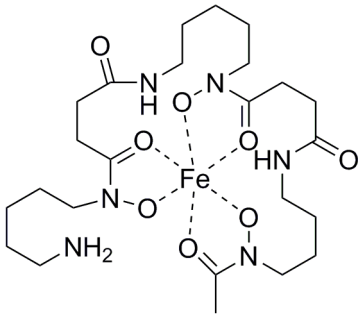
2.2.7. Revmatoidní artritida

U revmatoidní artritidy byly ve většině studií zjištěny zvýšené plasmatické koncentrace mědi, a ačkoliv patofyziologie homeostázy mědi není u tohoto onemocnění zcela objasněna, pravděpodobně dochází ke zvýšené syntéze a sekreci ceruloplasminu vlivem zánětlivých cytokinů (Honkanen *et al.* 1991, Zoli *et al.* 1998, Yazar *et al.* 2005, Önal *et al.* 2011, Strecker *et al.* 2013). Přestože je D-penicilamin užíván mnoho let v terapii revmatoidní artritidy, mechanismus jeho účinku zřejmě nesouvisí se schopností chelatovat měď (Omoto *et al.* 2005, Wood *et al.* 2008).

2.3. Chelátory železa a mědi

Chelatace znamená tvorbu či přítomnost dvou nebo více samostatných koordinačních vazeb mezi vícevazným ligandem a jedním centrálním atomem (Muller 1994). Obvykle jsou těmito ligandy organické sloučeniny označované jako chelátory nebo chelatační činidla, které tímto způsobem váží kationt kovu. Uvedené termíny pocházejí z řeckého slova znamenajícího klepeto, a tak velmi výstižně vyjadřují strukturu vzniklého komplexu (chelátu, **Obrázek 4**) (Morgan a Drew 1920).

Obr. 4. Komplex železa s deferoxaminem



Kalinowski a Richardson (2005), upraveno.

Terapie chelátory představuje přímý přístup k řešení patologických stavů spojených s nadbytkem nebo akumulací kovu v organismu (Ding *et al.* 2011). Typicky je tento přístup v klinické praxi uplatňován u transfuzního přetížení železem a Wilsonovy choroby (viz kapitoly 2.2.1. a 2.2.2.). V těchto případech je chelatační léčba zcela zásadní, prodlužující život pacientů (Ala *et al.* 2007, Flaten *et al.* 2012). Přehled v současnosti klinicky používaných chelátorů, včetně jejich významných charakteristik, je uveden v **Tabulkách 4** a **5**.

Chelatační terapie však neznamená pouze vyvázání nadbytečného kovu a jeho odstranění z organismu – chelátory mohou regulovat redoxní cyklus přechodných kovů nebo měnit distribuci kovu v organismu, působit jako chaperony. Dále tyto látky

představují nástroj pro studium buněčných procesů souvisejících s transportem, uchováním nebo využitím přechodných kovů, na buněčné úrovni i úrovni celého organismu (Ding *et al.* 2011). Mimo uvedeného je třeba vzpomenout také využití chelatačních látek v případě intoxikací kovy (Chang a Rangan 2011, Aaseth *et al.* 2014).

V kontrastu s relativně omezeným počtem látek v klinické praxi je široká paleta sloučenin s chelatačními vlastnostmi zkoušena v řadě potenciálních terapeutických aplikací v experimentálních modelech nebo klinickém testování. Významné skupiny látek, které jsou předmětem současného výzkumu, uvádí **Tabulka 6**.

Účinek chelátorů je ve své podstatě nespecifický, na rozdíl od naprosté většiny používaných léčiv, které farmakodynamicky působí na konkrétní cílovou strukturu. Pro pochopení účinku těchto látek je však třeba vycházet z patofyziologických poznatků o konkrétních onemocněních či poruchách, u nichž dochází buď k absolutnímu přetížení železem/mědí v organismu, nebo k dysbalanci těchto kovů, často s dokumentovaným zvýšením volněji vázané, tzv. labilní, chelatovatelné frakce kovu (Cabantchik 2014, Bandmann *et al.* 2015). Selektivní účinek chelátorů se v některých případech vysvětluje hypotézou, že snížení sérových koncentrací kovu inaktivuje v první řadě proteiny závislé na jeho vyšších hladinách, než které jsou zapotřebí pro základní buněčné procesy (Sen *et al.* 2002). U některých stavů navíc dochází k odlišným změnám v metabolismu kovů v různých kompartmentech (Squitti a Zito 2009, Kang 2011) a místo prosté chelatace je za výhodnější přístup považováno obnovení rovnováhy přechodného kovu (Ding *et al.* 2011, Delangle a Mintz 2012). Toxicita chelátorů vyplývá z několika faktorů, zejména inhibice enzymů závislých na železe/mědi, nízké selektivitě ke kovům a možnosti vázaného kovu podstoupit redoxní cyklus (Liu a Hider 2002).

Různé chelátory mají odlišné vlastnosti, které vedou k jejich specifickému užití za určitých okolností (Ding *et al.* 2011). Mezi významné aspekty patří především:

1) Selektivita k centrálnímu kovu

Ze struktury, konkrétně povahy jednotlivých donorových atomů, vyplývá nejen selektivita chelátoru k různým kovům, ale také k určitému oxidačnímu stupni daného kovu (Ding *et al.* 2011, Delangle a Mintz 2012, Aaseth *et al.* 2014). Jako příklady lze uvést velmi selektivní trientin a ethylendiamintetraoctovou kyselinu (EDTA), resp. její soli, mající vysokou afinitu k řadě dvojmocných a trojmocných iontů (Cooper 2012, Aaseth *et al.* 2014). Nedostatečná selektivita může vést k depleci některých fyziologicky důležitých kovů (Liu a Hider 2002).

2) Stechiometrie a stabilita komplexu

Komplexy, v nichž nejsou koordinační místa plně obsazená, jsou náchylnější k produkci toxického hydroxylového radikálu (Filipský *et al.* 2013, Merlot *et al.* 2013). Šestivazný chelátor deferoxamin tvoří stabilní komplex se železem v poměru 1:1 a zabraňuje přímému přístupu peroxidu vodíku nebo kyslíku ke kovu, jenž se stává redoxně neaktivní (Kalinowski a Richardson 2005). Komplex deferipronu v mikromolárních koncentracích při pH 7 má stechiometrii 3:1 (deferipron:Fe) (Motekaitis a Martell 1991). Ve velmi zředěných roztocích však byly pozorovány cheláty s poměrem 1:1 nebo 2:1 (deferipron:Fe) a nekompletní koordinací železa, která dovoluje tvorbu ROS (Merlot *et al.* 2013). Tato skutečnost může přispívat k toxicitě deferipronu (Merlot *et al.* 2013). Na druhou stranu potenciace redoxního cyklu přechodného kovu může být příznivá při terapii nádorových onemocnění (Kalinowski a Richardson 2005, Lane *et al.* 2014). Znalost stechiometrie komplexu má tedy klinický význam.

3) Lipofilně-hydrofilní charakter

Tato fyzikálně-chemická vlastnost určuje farmakokinetiku látky. Dostatečná lipofilita je tak zásadní například u chelatačních látek studovaných pro léčbu neurodegenerativních onemocnění (Budimir 2011). Vysoce polární povaha deferoxaminu znemožňuje jeho aplikaci perorální cestou (Callender a Weatherall 1980). Lipofilně-hydrofilní charakter chelátoru také může oběma směry ovlivnit absorpci přechodného kovu v gastrointestinálním traktu (Kontoghiorghes 1990).

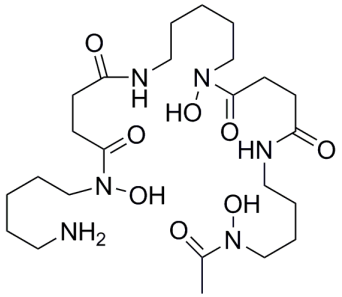
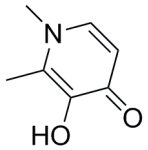
4) Vliv pH prostředí

Kyselost či zásaditost prostředí může mít velký význam nejen pro farmakokinetické vlastnosti chelátoru, ale i vlastní účinnost vázání kovu. Je třeba uvažovat jak fyziologické rozdíly pH v různých orgánech či tkáních, tak poměry dané patologickými stavy, např. ischemií a nádory (Mladěnka *et al.* 2011).

5) Metabolismus látky

Některé látky podléhají intenzivní metabolické přeměně, ať již v játrech, gastrointestinálním traktu či jiných tkáních, což může mít vliv na chelatační vlastnosti a výslednou účinnost i toxicitu (Liu a Hider 2002).

Tabulka 4. Klinicky používané chelátory železa a jejich základní charakteristika

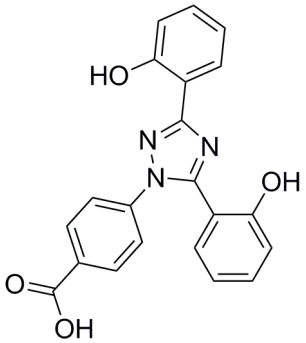
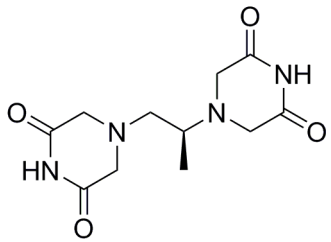
Chelátor	Přípravky registrované v ČR, indikace	Způsob podání, farmakokinetické vlastnosti	Mechanismus účinku, způsob chelatace	Hlavní nežádoucí účinky
deferoxamin (desferrioxamin B)[1] 	Desferal® (deferoxamin mesylát) - transfuzní hemosideróza - primární hemochromatóza se souběžným onemocněním vylučujícím flebotomii - akutní intoxikace železem	kontinuální s.c. nebo i.v. infuze, podávání 8-12 h 5-7 dní v týdnu, exkrece žlučí i močí, $t_{1/2}$ 20-30 min	šestivazný chelátor, komplex 1:1, především extracelulární chelatace	alergické a kožní reakce, infekce, krevní změny, zrakové a sluchové obtíže, syndrom dechové tísně, hypotenze při rychlé infuzi
deferipron (L1) 	Ferriprox® - zvýšená zátěž železem u pacientů s *thalassaemia major*, u kterých je léčba deferoxaminem kontraindikovaná nebo nedostatečná	p.o., 3x denně, především renální exkrece, $t_{1/2}$ 1,5-2,5 h	dvojvazný chelátor, komplex 3:1, především intracelulární chelatace[2]	gastrointestinální diskomfort, agranulocytóza a neutropenie, deplece zinku, bolest svalů a kloubů

Poznámky:

[1] přírodní látka izolovaná z bakterie *Streptomyces pilosus* (Brittenham 2011)

[2] vyšší kardioprotektivní efekt ve srovnání s deferoxaminem u chronického přetížení železem (Pepe *et al*. 2011)

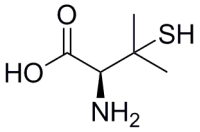
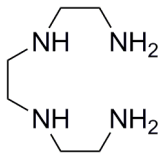
Pokračování tabulky 4

Chelátor	Přípravky registrované v ČR, indikace	Způsob podání, farmakokinetické vlastnosti	Mechanismus účinku, způsob chelatace	Hlavní nežádoucí účinky
deferasirox (ICL670) 	Exjade® - transfuzní hemosideróza - chronické přetížení železem u pacientů s talasemií nezávislou na podávání krevních transfuzí v případech, kdy je léčba deferoxaminem kontraindikovaná nebo nevhodná	p.o., 1x denně, exkrece přednostně žlučí, $t_{1/2}$ 8-16 h	trojvazný chelátor, komplex 2:1	gastrointestinální diskomfort, zvýšení hladin kreatininu, ledvinné a jaterní poškození, vyrážka
dexrazoxan (ICRF-187) 	Cardioxane®, Cyrdanax®, Savene® (dexrazoxan hydrochlorid) - prevence chronické kumulativní kardiotoxicity způsobené anthracyklinovými antibiotiky - léčba anthracyklinové extravazace (přípravek Savene®)	i.v. podání, především renální exkrece, $t_{1/2}$ 2-2,7 h	chelatace železa/inhibice topoisomerasy II,[3] možný vliv metabolitu ADR-925	hematologické poruchy, zejména leukopenie, gastrointestinální diskomfort, alopecie

Poznámky:

[3] mechanismus kardioprotektivního účinku přehodnocován (viz kapitola 2.2.4.)

Tabulka 5. Používané chelátory mědi a jejich základní charakteristika

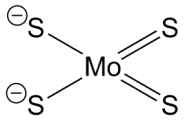
Chelátor	Přípravky registrované v ČR, indikace	Způsob podání, farmakokinetické vlastnosti	Mechanismus účinku, způsob chelatace	Hlavní nežádoucí účinky
D-penicilamin[1] 	Metalcaptase® - chronická revmatická polyartritida - Wilsonova choroba - otrava těžkými kovy - sklerodermie - cystinurie s prokázanou tvorbou močových kaménků nebo při pokročilém stádiu onemocnění	p.o., 2-4x denně, především renální exkrece, bifázická eliminace	reduktivní chelatace mědi, výrazné zvýšení její exkrece močí, indukce metallothioneinu	četné a závažné; hypersenzitivní reakce, horečka, vyrážka, proteinurie, nefrotoxicita, suprese kostní dřeně, kožní obtíže, poruchy imunitního systému a pojivové tkáně; vysoké riziko počátečního zhoršení neurologických příznaků
trientin (triethylentetramin, trien) 	není v ČR registrován, v zahraničí používán[2]	p.o., 2-4x denně	chelatace především Cu^{2+}	suprese kostní dřeně, proteinurie, jaterní sideróza, riziko počátečního zhoršení neurologických příznaků

Poznámky:

[1] rozkladný produkt penicilinu (Abraham *et al*. 1943)

[2] intenzivně studován z hlediska prevence a léčby chronických diabetických komplikací (Cooper 2012)

Pokračování tabulky 5

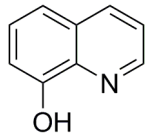
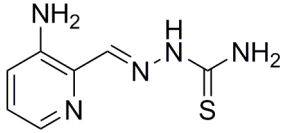
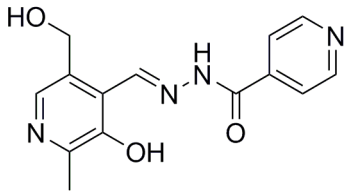
Chelátor	Přípravky registrované v ČR, indikace	Způsob podání, farmakokinetické vlastnosti	Mechanismus účinku, způsob chelatace	Hlavní nežádoucí účinky
tetrathiomolybdenan 	není registrován[3]	p.o., 3x denně, exkrece žlučí	tvoří komplex s mědí a proteinem; užíván s jídlem brání absorpci mědi, při užití mezi jídly je absorbován a chelatuje kov v krvi	anemie a leukopenie, zvýšení hladin transaminas

Poznámky:

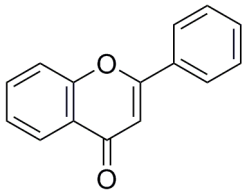
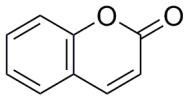
[3] intenzivně zkoumán pro terapii nádorových onemocnění (Brewer 2014)

Údaje použité v Tabulkách 4 a 5 byly čerpány z těchto zdrojů: Muijsers *et al.* (1984), Liu a Hider (2002), Cvetković a Scott (2005), Ala *et al.* (2007), Brewer (2009), Šimůnek *et al.* (2009), Brittenham (2011), Ding *et al.* (2011), Chang a Rangan (2011), Cooper (2012), Flaten *et al.* (2012), Kodama *et al.* (2012), Vavrova *et al.* (2013), Sheth (2014), Mikro-verze AISLP - 2015.2.

Tabulka 6. Vybrané významné skupiny látek s chelatačním potenciálem v experimentálním nebo klinickém zkoušení

Skupina látek, příklad	Zástupci	Chelatovaný kov	Potenciální indikace, účinky
8-hydroxychinoliny  8-hydroxychinolin	kliochinol, PBT2, kloroxin	měď, ale i zinek a železo	neurodegenerativní a nádorová onemocnění, antimikrobiální účinky (kliochinol a kloroxin klinicky používány v této indikaci)
thiosemikarbazony  3-aminopyridin-2-karbaldehyd-thiosemikarbazon (3-AP)	Dp44mT, DpC, 3-AP	železo, ale i měď a zinek	nádorová onemocnění
aroylhydrazony  PIH (pyridoxal-isonikotinoylhydrazon)	PIH, SIH (salicylaldehyd-isonikotinoylhydrazon), BSIH (boronylester salicylaldehyd-isonikotinoylhydrazonu)	železo	nádorová onemocnění, chronické přetížení organismu železem

Pokračování tabulky 6

Skupina látek, příklad	Zástupci	Chelatovaný kov	Potenciální indikace, účinky
flavonoidy  flavon	3-hydroxyflavon, kempferol, baikalein, kvercetin, myricetin	železo a měď	přes celou řadu studií nejsou jejich možné kardiovaskulární účinky jednoznačně dokumentovány, s výjimkou účinků isoflavonoidů a podávání některých látek u chronické žilní nedostatečnosti
kumariny  jednoduchý kumarin	6,7-dihydroxykumarin (eskuletin), 7,8-dihydroxykumarin (dafnetin)	železo a měď	kardiovaskulární účinky, kumarinová antikoagulancia v klinickém použití

Údaje použité v tabulce byly čerpány z těchto zdrojů: de Kleijn *et al.* (2002), Kalinowski a Richardson (2005), Kokubo *et al.* (2007), Nicolaides *et al.* (2008), Mladěnka *et al.* (2010b), Ding *et al.* (2011), Schimmer (2011), Bareggi a Cornelli (2012), Eskici a Axelsen (2012), Merlot *et al.* (2013), Prachayasittikul *et al.* (2013), Lane *et al.* (2014), Říha *et al.* (2014), Filipský *et al.* (2015), Najmanová *et al.* (2015).

3. Cíl práce

Cílem disertační práce bylo provést screening látek chelatujících přechodné kovy železo a měď a současně hlouběji studovat vlastnosti těchto látek v *in vitro* a *in vivo* experimentech. Jelikož se jedná o velmi široké téma, byly vytyčeny následující dílčí cíle:

- Provést rešerši literatury týkající se antioxidačních účinků kumarinů.
- Vyvinout *in vitro* metodu pro screening chelatace a redukce iontů mědi.
- Testovat chelatační, případně i redukční účinky vybraných látek přírodního a syntetického původu.
- Pokračovat v práci skupiny ve studiu interakcí flavonoidů s ionty železa, tj. dokončit studium vztahu redukce iontů železa flavonoidy a jejich antioxidačních nebo prooxidačních účinků.
- Vyvinout *in vitro* metodu pro stanovení stechiometrie komplexu chelátoru s ionty železa.
- Na *in vivo* modelu isoprenalinové kardiotoxicity studovat účinky premedikace látkami s různě vyjádřeným potenciálem chelatovat či redukovat měď a/nebo železo.

4. Recenzované odborné články publikované v časopisech s impaktním faktorem související s tématem disertační práce

4.1. Antioxidant effects of coumarins include direct radical scavenging, metal chelation and inhibition of ROS-producing enzymes

4.2. Novel method for rapid copper chelation assessment confirmed low affinity of D-penicillamine for copper in comparison with trientine and 8-hydroxyquinolines

4.3. *In vitro* evaluation of copper-chelating properties of flavonoids

4.4. Iron reduction potentiates hydroxyl radical formation only in flavonols

4.5. Mathematical calculations of iron complex stoichiometry by direct UV-Vis spectrophotometry

4.6. Oral administration of quercetin is unable to protect against isoproterenol cardiotoxicity

4.7. Is a highly linear relationship between the dose of quercetin and the pharmacological effect possible? – A comment on Liu, *et al*. Evaluation of antioxidant and immunity activities of quercetin in isoproterenol-treated rats. *Molecules* 2012, *17*, 4281-4291

Kompletní bibliografické údaje jsou uvedeny u jednotlivých článků a v kapitole 11.1.

4.1. Antioxidant effects of coumarins include direct radical scavenging, metal chelation and inhibition of ROS-producing enzymes

FILIPSKÝ, T.; ŘÍHA, M.; MACÁKOVÁ, K.; ANZENBACHEROVÁ, E.; KARLÍČKOVÁ, J.; MLADĚNKA, P. Antioxidant effects of coumarins include direct radical scavenging, metal chelation and inhibition of ROS-producing enzymes. *Current Topics in Medicinal Chemistry*. 2015, **15**(5), 415-431.

(IF 2013: 3,453)

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Current Topics in Medicinal Chemistry, 2015, *15*, 415-431

Antioxidant Effects of Coumarins Include Direct Radical Scavenging, Metal Chelation and Inhibition of ROS-producing Enzymes

Tomáš Filipský[1,a], Michal Říha[1,a], Kateřina Macáková[1,b], Eva Anzenbacherová[2], Jana Karlíčková[1,b] and Přemysl Mladěnka[1,a,*]

[1]*Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Czech Republic:* [a]*Department of Pharmacology and Toxicology and* [b]*Department of Pharmaceutical Botany and Ecology;* [2]*Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University Olomouc, Czech Republic*

Abstract: Coumarins represent a large group of 1,2-benzopyrone derivatives which have been identified in many natural sources and synthetized as well. Several studies have shown that their antioxidant capacity is not based only on direct scavenging of reactive oxygen and nitrogen species (RONS) but other mechanisms are also involved. These include: a) the chelation of transient metals iron and copper, which are known to catalyse the Fenton reaction; and b) the inhibition of RONS-producing enzymes (*e.g.* xanthine oxidase, myeloperoxidase and lipoxygenase), suggesting that mechanism(s) involved on cellular level are complex and synergistic. Moreover, many factors must be taken into account when analysing structure-antioxidant capacity relationships of coumarins due to different *in vitro/in vivo* methodological approaches. The structural features necessary for the direct RONS scavenging and metal chelation are apparently similar and the ideal structures are 6,7-dihydroxy- or 7,8-dihydroxycoumarins. However, the clinical outcome is unknown, because these coumarins are able to reduce copper and iron, and may thus paradoxically potentiate the Fenton chemistry. The similar structural features appear to be associated with inhibition of lipoxygenase, probably due to interference with iron in its active site. Contrarily, 6,7-dihydroxycoumarin seems to be the most active coumarin in the inhibition of xanthine oxidase while its derivative bearing the 4-methyl group or 7,8-dihydroxycoumarin are less active or inactive. In addition, coumarins may hinder the induction of inducible NO-synthase and cyclooxygenase-2. Sparse data on inhibition of myeloperoxidase do not enable any clear conclusion, but some coumarins may block it.

Keywords: Antioxidant, Copper, Coumarins, Iron, Lipoxygenase, Myeloperoxidase, Xanthine oxidase.

CLASSIFICATION AND OCCURRENCE OF NATURAL COUMARINS

Natural coumarins are abundant derivatives of 2*H*-1-benzopyran-2-one. This basic compound was the first coumarin isolated initially from tonka bean (*Dipteryx odorata* Willd., family Fabaceae), which vernacular name "coumarou" gave the name not only to the compound itself but to the whole class of these substances too. Since 1820, when coumarin itself was isolated, over one thousand derivatives have been identified [1-4].

Wide range of coumarins is produced by Angiosperm dicots belonging mainly to families Apiaceae and Rutaceae but also to Asteraceae and Fabaceae and by some species of monocots from families Poaceae and Orchideaceae [1-3, 5]. *E.g.* 7-hydroxycoumarin (umbelliferone, hydrangine, skimmetine), one of the most common simple coumarins, is found as aglycone in families Apiaceae, Rutaceae and Asteraceae [6-8], or as glycoside skimmin in branches and stems of *Hydrangea paniculata* (Hydrangeaceae) or in leaves and anthodia of *Chamomilla recutita* (Asteraceae) [9, 10]. Although coumarins are distributed throughout all parts of a plant, the highest content of coumarins is usually in fruits or seeds followed by roots, leaves and latex of some species [1]. Simple coumarins, commonly found in plants, arise from the metabolism of phenylalanine *via* cinnamic acid and *p*-coumaric acid. The specificity of this process lies in the 2-hydroxylation, izomerization and lactonization in particular (Fig. **1**) [2, 3]. Moreover, some members have been discovered in microorganisms as well, *e.g.* novobiocin, coumermycin A1 and clorobiocin from bacteria *Streptomyces*, and aflatoxins from *Aspergillus* species of fungi [11].

According to the chemical structure, coumarins can be divided into the following groups: simple coumarins (including biscoumarins and triscoumarins), pyranocoumarins, furanocoumarins (Fig. **2**) and miscellaneous coumarins (*e.g.* coumarinolignans) [2, 4, 12].

POTENTIAL TOXICITY OF COUMARINS

Coumarins are metabolized in human and animals by cytochromes P450 (CYP 450). Two pathways are considered to be the most common (Fig. **4**) [13]:

*Address correspondence to this author at the Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic; Tel: +420-495-067-295; Fax: +420-495-067-170;
E-mail: mladenkap@faf.cuni.cz

1568-0266/15 $58.00+.00

- epoxidation in the positions C-3 and C-4, with coumarin-3,4-epoxide as the product;
- 7-hydroxylation.

The former is considered to be a potential risk because of the toxicity [14]. However, its clinical outcome is not well defined. Firstly, coumarin-3,4-epoxide can be conjugated with glutathione and thus detoxified or could be further metabolized into *ortho*-hydroxyphenyl acetaldehyde which is considered to be a hepatotoxic intermediate [13-19]. Secondly, this pathway is minor in humans and even in humans with complete deficiency of 7-hydroxylation the chances on formation of the hepatotoxic *ortho*-hydroxyphenyl acetaldehyde is lower than in rat [13, 19]. The reason is higher susceptibility of rats, because their major pathway of the coumarin metabolism leads to the mentioned epoxide while hydroxylation is the minor pathway [16]. In addition, there have been attempts to relate pharmacogenetic profiles of CYP enzymes and coumarin toxicity. At first, the mentioned absence of 7-hydroxylation of coumarin in subjects expressing CYP2A6 form with Leu160His amino-acid substitution (arising from the CYP2A6*2 allele) has been blamed for increased production of hepatotoxic *ortho*-hydroxyphenylacetic acid [20]. On the other hand, it has been found that the protein resulting from the presence of this variant allele does not contain heme and is devoid of any enzyme activity and hence unable to take part in formation of hepatotoxic intermediate. Recently, another form of human microsomal CYP of the CYP2A subfamily, CYP2A13, present mainly in the lungs, has been characterized [21] and found to be ten times less efficient in the formation of 7-hydroxycoumarin; however, no conclusion regarding the coumarin toxicity has been drawn out so far [22].

There have been also attempts to investigate the possible carcinogenicity of coumarin in the literature as studies on animals (rats, mice) indicated that coumarin may induce tumours in some cases (liver in rats, liver and kidney in mice). However, the purported carcinogen, *ortho*-hydroxyphenyl acetaldehyde was found to not be genotoxic. The results of these studies were then interpreted that coumarin in these cases induced the tumor formation by a non-genotoxic mode of action (reviewed in [14, 15, 19]). The Occupational Health and Safety Administration concluded that due to The International Agency for Research on Cancer classification in Group 3, coumarin is not currently classifiable as a carcinogen in human [23].

Coumarin hepatotoxicity is still, however, an open question. It became clear that a subgroup of human population reacts sensitively to coumarin with hepatotoxic effects. Detailed studies on genetic polymorphisms in the CYP2A6 gene have shown that CYP2A6 gene polymorphism alone is not the cause of the susceptibility to hepatotoxicity in this human subgroup [15, 24]. A study with coumarin-troxerutin combination realized under standard conditions indicated that possibly previous hepatitis and elevated baseline level of gamma glutamyl transferase may be the risk factors [25] but the evidence for a metabolic cause of the high susceptibility to hepatotoxicity is still missing. It has been also speculated that a yet unknown immune mechanism may be involved and coumarin hepatotoxicity hence appears to be an idiosyncratic response potentially influenced by multiple outside and inherent factors [15, 18].

The finding of hepatotoxicity as well as formation of tumors in rats in long-term toxicity experiments led to ban of synthetic coumarin in foods in the U.S. which has been followed also by European Union in 1988 setting a 2 mg/kg limit for food in general. In 2005, it was found that certain cookies (mostly German traditional Christmas cookies), cereals and teas may exhibit higher amounts of coumarin from cinnamon and the limit of daily intake (TDI, tolerable daily intake refers to the amount of a compound assessed safe for human being on long-term basis) has been set to 0.1 mg/kg body weight [15, 26].

The other important problem is that above mentioned data are related mostly to the non-substituted coumarin, so data on its derivatives are missing. Because of the mentioned risk of epoxide formation and possible hematotoxicity and carcinogenesis, most researchers used rather 4-methylderivatives of coumarins because of the 4-methyl group block the possible epoxide formation [27].

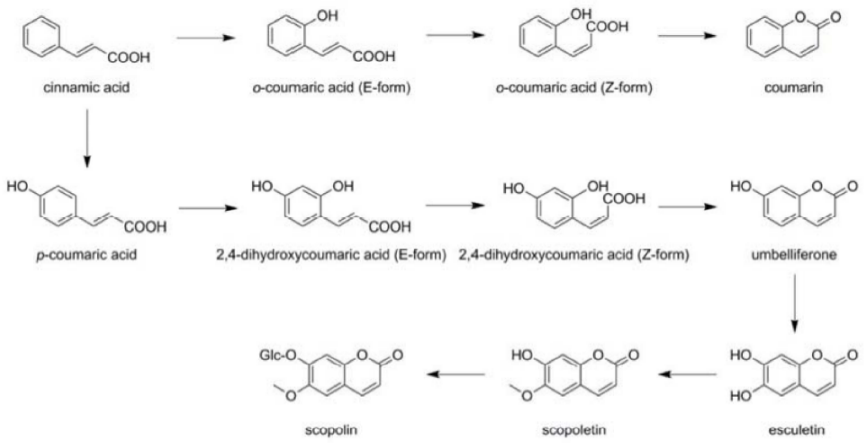


Fig. (1). Synthesis of coumarins in the plants according to Dewick [3].

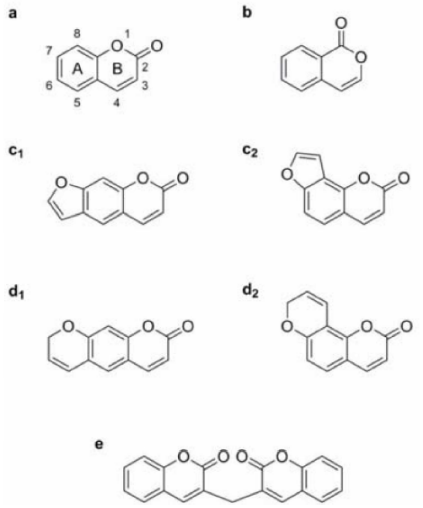


Fig. (2). Classification of coumarins. (**a**) simple coumarin, (**b**) isocoumarin, (**c**) furanocoumarin (c1 - linear furanocoumarin - psoralen, c2 - angular furanocoumarin - angelicin), (**d**) pyranocoumarin (d1 - linear pyranocoumarin, d2 - angular pyranocoumarin, for example see Fig. 3) (**e**) an example of biscoumarins (dicoumarol).

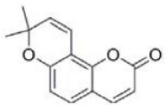


Fig. (3). Angular pyranocoumarin seselin.

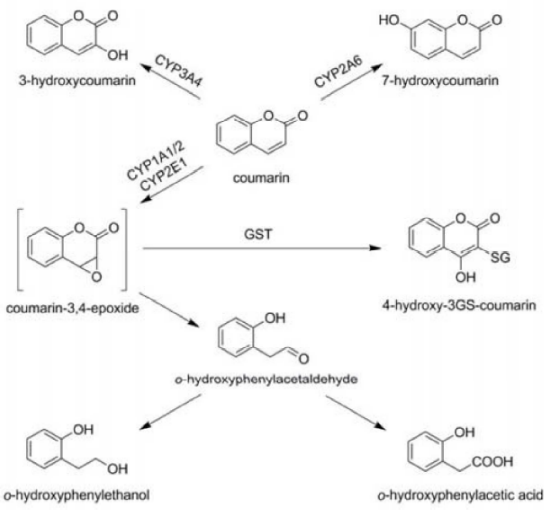


Fig. (4). Major pathways of coumarin metabolism. Cytochromes P450 (CYP) contributing mainly to formation of the respective metabolites are indicated. GST, glutathione S-transferase.

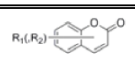
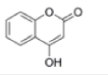
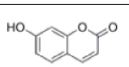
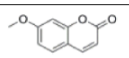
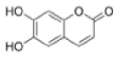
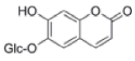
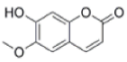
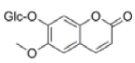
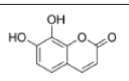
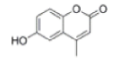
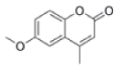
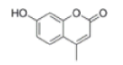
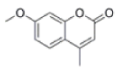
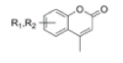
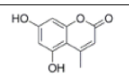
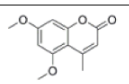
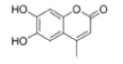
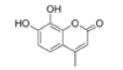
DIRECT RONS SCAVENGING POTENTIAL

A considerable amount of literature has been published on experimental and theoretical elucidation of direct scavenging effects of coumarins against reactive oxygen and nitrogen species (RONS). However, due to different methodological approaches, many factors must be taken into account when analysing scavenging effects in relation to a coumarin structure. These factors include particularly a nature and concentration of the involved reactive species or oxidative stress inducers, lipophilicity of a reaction microenvironment (*i.e.* aqueous or alcoholic *vs* lipid peroxidation models), and last but not least, the involvement of transient metals and RONS-producing enzymes. An overview of the studies focussing on the direct scavenging effects is summarized in this section and Table **1**, in which it can be seen that a scavenging potential (shown as SC_{50}) of commonly tested simple coumarins strongly depends not only on the chemical structure of a coumarin but on performed assays, resp. oxidative stress inducers, as well.

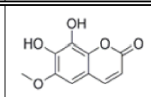
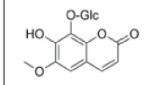
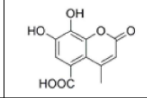
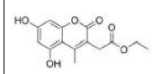
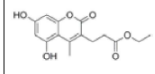
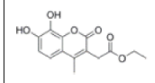
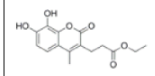
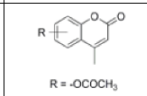
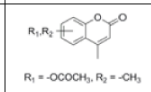
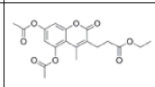
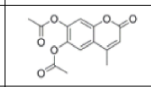
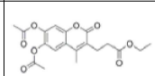
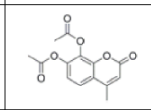
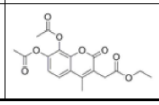
In coumarins, the most crucial factor to reach the maximal scavenging effect towards various RONS is the presence of two hydroxyl groups in the *ortho*-position in ring A because the catechol moiety may easily undergo a bi-electronic oxidation leading to the formation of a highly stable quinone structure [27-54]. Summarizing the studies, the 7,8-dihydroxyl group and the similarly efficient 6,7-dihydroxyl group are significantly superior to the 5,7-dihydroxyl one in simple coumarins [27, 29-32, 36, 39, 41, 42, 45, 50, 55]. This priority has been observed in both hydrophilic and hydrophobic reaction environments *via* different methodologies, *e.g.* DPPH (1,1-diphenyl-2-picrylhydrazyl), $ABST^{\cdot+}$ [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] or peroxyl radical assays [APPH (2,2'-azobis(2-amidinopropane)dihydrochloride, ORAC-FL values (oxygen radical antioxidant capacity-fluorescein)]. The exception seems to be the scavenging of the superoxide anion in which 7,8-dihydroxycoumarins (7,8-DHCs) were more active than those of 6,7-dihydroxycoumarins (6,7-DHCs). Moreover, 7,8-dihydroxy-6-methoxycoumarin (fraxetin) containing the electro-donating methoxyl group appeared to be slightly more efficient than 7,8-DHCs [28, 31, 35, 41-45, 47]. Even that it was also reported that 5,7-dihydroxy-4-methylcoumarin scavenged hypochlorous acid more effectively than *ortho*-dihydroxy-4-methylcoumarins, the *ortho*-dihydroxyl site appears to be the most important [35, 49]. In line with this finding, coumarins containing 4,5-dihydropyrazole with an *ortho*-dihydroxyphenyl substitution are promising scavengers of different RONS [56, 57]. On the other hand, scavenging of RONS does not equal to a cytoprotection at all conditions since 4-(chloromethyl)-5,7-dihydroxycoumarin bearing the resorcinol group has been found to scavenge the hydroxyl radical but simultaneously to be cytotoxic to the bovine aortic endothelial cell line (BAEC) [53].

It has been theoretically suggested that the marginal difference between these 6,7 and 7,8 *ortho*-positions could be ascribed to the higher capacity of 7,8-dihydroxyl to form intramolecular hydrogen bonds which could prevent a formation of intermolecular hydrogen bond with a hydrogen-accepting solvent [37]. Moreover, introduction of a carboxyl in the position C-5 may slightly enhance the scavenging effect of 7,8-dihydroxy-4-methylcoumarin (7,8-DHMC) in both DPPH and peroxyl [ABAP assay; 2,2'-azobis(2-methylpropionamidine) dihydrochloride] assays [55].

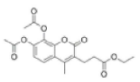
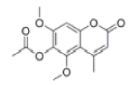
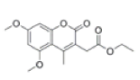
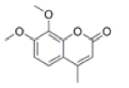
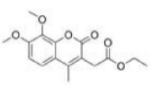
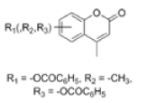
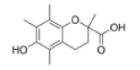
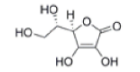
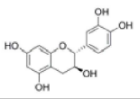
Table 1. Scavenging effects of several simple coumarins towards various RONS and oxidative stress inducers.

Substance	Chemical formula	SC_{50}																	
		DPPH						$O_2^{\cdot-}$					NO	HOCl		$ABTS^{+}$		peroxyl AAPH assay	
		[30][b]	[33][c]	[31][b]	[36][b]	[42][a]	[55][b]	[31][b]	[45][b]	[41][b]	[42][a]	[47][e]	[31][b]	[31][b]	[42][a]	[31][b]	[51][c]	[31][b]	[42][a]
various (di)methylcoumarins	 $R_1 = R_2 = -CH_3$		>77,300														>4,470		
4-hydroxycoumarin		>750							nd.						>2,000				
7-hydroxycoumarin (umbelliferon)		>750				>200			nd.		26.7 ± 2.8				>2,000				187.2 ± 13.1
7-methoxycoumarin									nd.										
6,7-dihydroxycoumarin (esculetin)				379.5		17.2 ± 1.2		nd.	nd.		2.3 ± 0.4	33.9	53.1	182.1	>2,000	22.0		10.35	15.8 ± 1.9
7-hydroxy-6-O-glucosylcoumarin (esculin)						>200	nd.		nd.		>200				>2,000				
7-hydroxy-6-methoxycoumarin (scopoletin)		>750				>200			nd.		>200				>2,000				93.5 ± 6.9
7-O-glucosyl-6-methoxycoumarin (scopolin)		>750					25.18												
7,8-dihydroxycoumarin (daphnetin)						20.6 ± 2.3					3.2 ± 0.4	10.6			>2,000				17.8 ± 2.2
6-hydroxy-4-methylcoumarin			5,860 ± 190														320 ± 0.0		
6-methoxy-4-methylcoumarin			13,800 ± 110														890 ± 20		
7-hydroxy-4-methylcoumarin (hymecromon)		>750	7,450 ± 160			nd.		nd.	nd.								630 ± 30		
7-methoxy-4-methylcoumarin			14,700 ± 150						nd.								880 ± 50		
various monohydroxydimethylcoumarins	 $R_1 = -OH, R_2 = CH_3$		>2,400														>10		
5,7-dihydroxy-4-methylcoumarin		>750		nd.				nd.		8,800.0			396.2	2,389.0		nd.		nd.	
5,7-dimethoxy-4-methylcoumarin		>750																	
6,7-dihydroxy-4-methylcoumarin		74.7		431.2				nd.	nd.	62.0		82.0	126.8	415.9		53.0		3.67	
7,8-dihydroxy-4-methylcoumarin		24.9	10 ± 10	286.0	14.0			39.32		34.0		8.5	121.61	164.5		22.8	3 ± 1	2.98	

(Table 1) contd….

Substance	Chemical formula	SC_{50}																		
		DPPH						$O_2^{\cdot-}$					NO	HOCl		$ABTS^{\cdot+}$		peroxyl AAPH assay	
		[30][b]	[33][c]	[31][b]	[36][b]	[42][a]	[55][b]	[31][b]	[45][b]	[41][b]	[42][a]	[47][cl]	[31][b]	[31][b]	[42][a]	[31][b]	[51][c]	[31][b]	[42][a]
7,8-dihydroxy-6-methoxycoumarin (fraxetin)						39.9 ± 2.8			17.3		1.1 ± 0.2	2.3 5.8			1,858 ± 198				26.3 ± 2.1
7-hydroxy-6-methoxy-8-O-glucosylcoumarin (fraxin)						>200			nd.		>200				>2,000				
5-carboxy-7,8-dihydroxy-4-methylcoumarin							33.46												
5,7-dihydroxy-3-ethoxycarbonylmethyl-4-methylcoumarin		>750																	
5,7-dihydroxy-3-ethoxycarbonylethyl-4-methylcoumarin		>750																	
7,8-dihydroxy-3-ethoxycarbonylmethyl-4-methylcoumarin		24.0		423.0			17.49	nd.					27.3	174.3		92.8		7.53	
7,8-dihydroxy-3-ethoxycarbonylethyl-4-methylcoumarin		27.0		257.6				46.54					59.6	177.2		139.6		1.14	
various monoacetoxy-methylcoumarins	 R = -OCOCH$_3$		>2,170														>160		
various monoacetoxy-dimethylcoumarins	 R_1 = -OCOCH$_3$, R_2 = -CH$_3$		>15,300														>890		
5,7-diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin		>750																	
6,7-diacetoxy-4-methylcoumarin					31.0														
6,7-diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin					172.0														
7,8-diacetoxy-4-methylcoumarin		492.8	1,140 ± 100	2,070.0	31.0			nd.					78.5	517.6		350.5	60 ± 0.0	nd.	
7,8-diacetoxy-3-ethoxycarbonylmethyl-4-methylcoumarin		517.2		4,611.0				nd.					188.9	930.8		489.2		nd.	

(Table 1) contd….

Substance	Chemical formula	SC_{50}																		
		DPPH						$O_2^{\cdot-}$					NO	HOCl		$ABTS^{\cdot+}$		peroxyl AAPH assay	
		[30][b]	[33][c]	[31][b]	[36][b]	[42][a]	[55][b]	[31][b]	[45][b]	[41][b]	[42][a]	[47][c1]	[31][b]	[31][b]	[42][a]	[31][b]	[51][c]	[31][b]	[42][a]
7,8-diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin		415.4		1,022.0	85.0			nd.					95.9	445.3		508.5		nd.	
6-acetoxy-5,7-dimethoxy-4-methylcoumarin		>750																	
5,7-dimethoxy-3-ethoxycarbonylmethyl-4-methylcoumarin		>750																	
7,8-dimethoxy-4-methylcoumarin		>750																	
7,8-dimethoxy-3-ethoxycarbonylmethyl-4-methylcoumarin (trigocoumarin)		>750																	
various (di)benzoate-(di)methylcoumarins			>21,300														>1,280		
trolox		27.8	160 ± 30														10 ± 0.0		
ascorbic acid		42.4			35.0														34.7 ± 3.5
(+)-catechin		49.2				22.6 ± 2.1					3.8 ± 0.6				862 ± 117				16.8 ± 2.7

Data express representative scavenging concentration SC_{50} [μM]; [a] mean ± SD; [b] mean; [c] deviation not specified; [1] two different assays. AAPH – 2,2'azobis(2-amidinopropane)dihydrochloride; $ABST^{\cdot+}$ – 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); DPPH – 1,1-diphenyl-2-picrylhydrazyl; Glc – glucose; nd. – not determined.

Several synthetic 3-phenylcoumarins bearing the catechol ring exerted noticeable scavenging effects towards various ROS or DPPH, and surprisingly, those bearing methoxyl groups in the phenyl ring instead of hydroxyls and an electron-withdrawing halogen (chlorine or bromine) at C-6 of ring A inhibited AAPH-induced lipid peroxidation [(2,2'-azobis(2-amidinopropane)dihydrochloride] [58, 59]. Moreover, a series of differently hydroxylated 3-phenylcoumarins, in particular 3-(4'-hydroxyphenyl)-8-hyd- roxycoumarin and 3-(4'-hydroxyphenyl)-6-chlorocoumarin, possessing no theoretical violations of Lipinski's rule, showed scavenging capacity towards different types of RONS – peroxyl (AAPH, ORAC-FL values), hydroxyl, superoxide and DPPH radicals [60, 61]. Similarly, several coumarin-chalcone hybrids fulfilling the theoretical predictions of Lipinski's rule as well, *e.g.* the most active 6-bromo-3-(3',4'-dihydroxybenzoyl)-8-hydroxycoumarin, resp. 6-hydroxy-3-(4′-hydroxybenzoyl) coumarin, were found to scavenge the peroxyl (AAPH, ORAC-FL values) and the hydroxyl radical confirming that the presence, the number and the position of hydroxyl group(s), resp. the catechol moiety, and an electron-withdrawing group (chlorine or bromine) at C-6 of the coumarin skeleton are important structural features [62, 63].

In contrast to the above mentioned active coumarins, those possessing only one hydroxyl group in the aromatic ring have shown only moderate or negligible effects in general [27, 30, 32, 33, 35, 37-40, 42, 46, 47, 51, 54, 55, 57, 64-66]. The isolated 4-hydroxyl group seems to be slightly more active than the isolated 6- or 7-hydroxyl group in simple coumarins [38, 40, 67]. In hydroxycoumarins, especially in 4-hydroxycoumarins, an additional electron-donating group, particularly at C-3 or C-7, may increase the scavenging effect, on the other hand, an electron-withdrawing group may decrease it, as was demonstrated in various assays including DPPH, peroxyl (AAPH, ORAC-FL values), hydroxyl radical as well as hypochlorous one [36, 53, 67-70]. Interestingly,

the scavenging capacity of 6-hydroxydimethylcoumarin against $ABST^{\cdot+}$ was comparable to that of trolox. This suggests that the coumarin bearing the isolated hydroxyl group at C-6 may show stronger scavenging effect than that bearing the isolated hydroxyl group at C-7 confirming that the resonance structure of the radical derived from 6-hydroxycoumarin is more stable than that of 7-hydroxyl one due to the formation of *ortho*-quinone form [51]. Some Mannich bases containing the 7-hydroxycoumarin core with piperazine or morpholine at C-8 were found active towards various free radicals [71]. A few 7-hydroxycoumarin, resp. 7-amino- derivatives, with disubstituted ethylenediamine moiety and pyrrolcoumarins have been described as promising scavengers of ROS or DPPH and inhibitors of AAPH-induced lipid peroxidation as well [72, 73].

Coumarins possessing either methyl, methoxyl, benzoxyl group(s) or ester side chain without the aromatic hydroxyl group(s) are associated either with very weak or none scavenging effects [27, 30, 32, 33, 35, 40, 42, 45, 50, 51, 64, 74]. Similarly, a sugar moiety markedly reduces the activities of coumarin glycosides although possessing a free hydroxyl group [30, 35, 42, 45, 46, 52, 66]. Notwithstanding the absence of a free hydroxyl group, simple coumarins containing a heterocyclic substituent have possessed some scavenging effects [75, 76]. In this case, two possible mechanisms which may contribute to a promising scavenging effects towards ROS or DPPH radical have been postulated: 1) resonance and inductive effects, or 2) keto-enol tautomerism [75].

In view of the fact that ethoxycarbonylmethyl or ethoxycarbonylethyl at C-3 may interact with the oxypyran oxygen above the planar coumarin core, the influence of these medium-length ester side chains has been evaluated predominantly in the most active *ortho*-dihydroxylated simple coumarins [27, 29-31, 39, 77]. The introduction of the ethoxycarbonylmethyl chain did not influence the effect against most of oxidative stress inducers and RONS, however, it significantly enhanced that effect against NO [27, 29-31, 39]. The findings describing influences of the ethoxycarbonylethyl chain can be summarized as follows [27, 29-31]:

- it improved the effect against both peroxyl and galvinoxyl radical and AAPH-induced peroxidation on lipidic liposomes;
- it did not induce any changes in prevention of AAPH-induced LDL oxidation;
- it did not abolish the excellent effect against the superoxide anion in 7,8-DHMCs (see above);
- it decreased the effect against $ABST^{\cdot+}$;
- and moreover, its theoretical lipophilicity was consistent with its antioxidant capacity on phospholipidic membranes.

Surprisingly, *ortho*-diacetoxylated simple coumarins have good scavenging effects, as well against the superoxide anion, in spite of the absence of the catechol ring or esterases in the reaction microenvironment. However, their effects are less pronounced than those of the corresponding dihydroxylated derivatives [27, 29-31, 33, 34, 36, 39, 51, 78, 79]. Their activity may be explained by a possibility of the formation of an extensively resonance-stabilised coumarin-phenoxyl radical in particular and ketene in the presence of an initiating free radicals, including the superoxide radical as well [34, 36, 78]. Similarly, as described above, the ethoxycarbonylmethyl ester side chain had either any or slightly negative influence with the exception of AAPH-induced LDL oxidation, in which it increased the effect [29, 31, 39]. There was rather an increase in the effect in the case of ethoxycarbonylethyl ester side chain. This has been reported to be significant in a scavenging of peroxyl radical and prevention of AAPH-induced LDL oxidation [27, 29, 34]. In LDL system, the enhancing effect of the 7,8-diacetoxylated esters in comparison to the 7,8-dihydroxylated esters could be due to their more pronounced hydrophobic nature, and therefore due to better distribution of a coumarin in LDL surface [29].

It is important to note that a presence of the methyl group at C-4 is strongly desirable from a therapeutic point of view because it prevents the formation of toxic 3,4-coumarin epoxides (see the above chapter), although it may decrease very slightly the scavenging effect due to its weak electron-donor property [14, 27, 31, 32, 35, 37, 80, 81]. However, its weak positive effect has been published as well [39].

One of the principal structural feature of a coumarin core is a presence of the unique ring B, *i.e.* the 1,2-pyrone core [37, 42, 52]. In theory, the 1,2-pyrone core is a weaker electron-withdrawing group than the 1,4-pyrone one, a group ubiquitous in isoflavones and many flavonoids, and conclusively, it has a lower influence on the effect [37, 42, 52]. A different subclass of coumarins represents thionocoumarins, with a sulphur atom in the core structure, however, their scavenging activity seems to be comparable to that of coumarins [27, 34]. Few studies have analysed the scavenging effects in simple isocoumarins. Those isolated from the endophytic fungus *Colletotrichum* sp., monocerin derivate and particularly fusarentin derivative both bearing 6,7-dihydroxyl group have been proposed to scavenge free radicals as well [82] in line with similar findings in simple coumarins. Isocoumarins isolated from *Paepalanthus bromelioides*, paepalantine (Fig. **5a**) and its dimer (Fig. **5b**) both bearing a catechol-like arrangement and a 3,4-double bond, had some scavenging effect against DPPH radical and ROS [83].

Some of 4-hydroxy-bis-coumarins bearing the catechol structure [such as 3,3'-[(3,4-dihydroxyphenyl)methylene]bis (4-hydroxycoumarin), Fig. **5c**] may be of interest because their scavenging effect seems to be superior to that of 4-hydroxycoumarin [74].

Although studies analysing structure-activity relationships are limited in linear or angular coumarins, i.e. furanocoumarins and pyranocoumarins, particularly due to their complicated structures, the basic characteristics involving the presence of a phenol group seems to be valid as well; coumarins without a hydroxyl group or bearing another group, such as a methoxyl one, have either any or very marginal scavenging effects [42, 84, 85]. Two linear furanocoumarins isolated from *Angelica dahurica*, 9-hydroxy-4-methoxypsoralen (Fig. **5d**) and alloisoimperatorin (Fig. **5e**) bearing an isolated hydroxyl group, have been effective towards the DPPH radical and AAPH-induced lipid peroxidation [85]. Notwithstanding the absence of a free hydroxyl group, some of linear or angular furanocoumarins, pyranocoumarins or heterocyclic derivatives have been found as

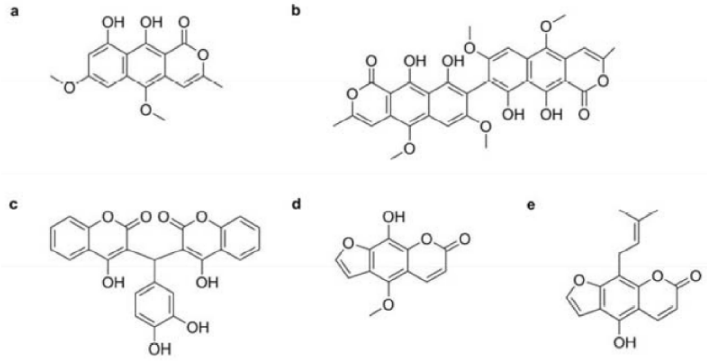


Fig. (5). Selected coumarins scavenging RONS. (**a**) paepalantine, (**b**) 8,8′-paepalantine dimer, (**c**) 3,3'-[(3,4-dihydroxyphenyl)methylene]bis(4-hydroxycoumarin), (**d**) 9-hydroxy-4-methoxypsoralen, (**e**) alloisoimperatorin.

promising scavengers as well [86]. Noticeable effects have been observed in 6-hydroxybenzo[h]coumarin and 5-hydroxybenzo[f]coumarin towards DPPH radical, the superoxide anion and particularly AAPH-induced lipid peroxidation [65].

INTERACTIONS INVOLVING METAL (IRON/COPPER) CHELATION AND REDUCTION

Interactions of coumarins with iron and/or copper ions have been documented in a series of studies, usually indirectly by affecting oxidative stress induced by a metal. Mostly they include the iron-catalysed lipid peroxidation or the iron-based Fenton reaction. 4-Methylcoumarins have been investigated in a majority of studies especially due to their mentioned inability to form toxic epoxides. In general, structural requirements for iron chelation seem to be very similar to structural features for antioxidants because, at least partially, antioxidant properties are based on chelation of a transient metal as well [79, 87, 88].

Coumarins possessing *ortho*-dihydroxyl or *ortho*-diacetoxygroups have been shown to chelate iron while coumarins with one hydroxyl group, two methoxyl groups, or hydroxyl or acetoxyl groups in *meta*-positions have been practically inactive which is in accordance with indirect assessment of iron chelation [44, 87]. *Ortho*-dihydroxy-4-methylcoumarins (*ortho*-DHMCs) and *ortho*-diacetoxy-4-methylcoumarins have showed pronounced activity against initiation and propagation of iron-induced lipid peroxidation. The order of the decreasing effects based on a dihydroxyl group in ring A was following: 7,8- > 6,7- > 5,7- [36]. This is in a good agreement with other iron chelation assays or iron-based production of ROS in which 5,7-dihydroxycoumarins (5,7-DHCs) were inactive or slightly active while 7,8-DHCs were slightly more or similarly active in comparison to 6,7-DHCs [41, 87]. 7,8-DHMC has been shown to be similarly active as the well-known iron chelator deferoxamine at pH 7.5, however, it seemed that coumarins lost their ability under the acidic conditions [87], similarly to other polyphenols [89]. The effect of substitution of an amino group instead of one hydroxyl group appears ambiguous because in one study the substitution did not affect the potency while in another 7-amino-8-hydroxy-4-methylcoumarin was absolutely inactive [86, 90]. The catechol moiety represents the key factor in protection against iron-induced lipid peroxidation in natural coumarins [42], similarly, 6,7-DHCs, esculetin and 4-methylesculetin, as well as cortex *Fraxini* extract rich in *ortho*-dihydroxyl simple coumarins, effectively inhibited iron-induced Fenton reaction [38, 52]. Interestingly, the 6-glycoside of esculetin, esculin, supressed Fe^{2+}- but not Fe^{2+}-EDTA-induced lipid peroxidation [91]. Herein, authors have suggested, in line with previous findings [92], that iron chelation was responsible for this effect but this does not seem probable because the iron-chelating moiety was blocked by the sugar. However, the possible deglycosylation at those reaction conditions (trichloroacetic acid, 100 °C) or the direct scavenging potential cannot be fully excluded. The latter can be relevant in coumarins: 1) compounds lacking iron-chelating functionalities like linear furanocoumarin feroniellamin (Fig. **6a**) blocked iron-induced lipid peroxidation [93], and 2) even in isocoumarins possessing some iron-chelating activity, *i.e.* paepalantine (Fig. **5a**), 8,8′-paepalantine dimer (Fig. **5b**) and vioxanthin (Fig. **6b**), the scavenging potential seems to be responsible for the major part of the antioxidant effect [83].

There is a discussion about the precise mechanism of inhibition of iron-induced ROS production. Raj *et al.* proposed that Fe-DHMC complex is unstable and therefore has no impact on the generation of ROS while a stable ternary mixed ligand complex ADP-Fe-DHMC has very efficiently inhibited lipid peroxidation process *via* the prevention of the ADP-perferryl radical production [79]. This mechanism is likely common to *ortho*-amino-hydroxycoumarins [90]. Moreover, the scavenging potential of DHMC has increased significantly after forming the ternary complex with Fe^{3+} and ADP [41].

Some studies have categorized coumarins as powerful antioxidants due to their ability to reduce ferric iron (FRAP test, ferric reducing antioxidant power). Such interpretation is, however, inaccurate because iron reduction may be associated with potentiation of the Fenton reaction and thus to be pro-oxidative [94]. Although such studies are available for other polyphenols, only few data have been published in relation to coumarins. A study using hydroxyl radical generation in the Fe^{3+}-EDTA/ascorbate-H_2O_2 deoxyribose sys-

tem demonstrated that *ortho*-DHCs can both chelate iron ions and also readily donate electrons for redox cycling of Fe^{3+}, thus promoting the Fenton-type reaction [44]. Such a pro-oxidative effect may also underline their cytotoxic activity [47]. In contrast, non-reducing 5,7-DHMC was not associated with pro-oxidation [44, 87]. The strong antioxidant activity of *ortho*-DHMCs due to iron reduction has been observed with stable radicals [31, 95, 96]. Therefore the clinical significance is unknown, but interestingly, 7,8-DHMC was able to decrease ROS production generated by DOX treatment in MCF7 cells [95]. On the other hand, iron participation in DOX cardiotoxicity is currently largely discussed [97, 98].

In general, the outcome of metal-coumarin anti- or pro-oxidation is not easily predictable because the concentration ratio of transient metal and a coumarin is an extremely important factor. FRAP methodology generally used one fixed ratio, however, the relationship between transient metal reduction and chelation is largely dependent on a concentration ratio between a coumarin and metal. Thus, to have a complex view, the measurement should be conducted at different transient metal:compound ratios. As follows, the "bell-shaped" curves of the iron reduction were found for potent iron chelators (DHMCs) because at low ratios of the compound to iron, the coumarin is not able to chelate all iron, while in the excess, the reduction is decreased due to formation of stable complexes. Contrarily, low-potent chelators like diacetoxy-4-methylcoumarins were reducing iron progressively in relation to their concentration [87]. This fact could explain some discrepancies between studies [87, 96]. In particular, we have shown that *ortho*-DHMCs and 7,8-diacetoxy-4-methylcoumarins significantly reduced ferric ions especially under the acidic conditions, with the reduction potential of 7,8-DHMC three times more expressed in comparison to 6,7-DHMC [87], while in FRAP methodology, 6,7-DHMC was the most efficient reducing agent [96].

Studies focusing on copper chelation by coumarins are rather sparse in contrast to iron and to our knowledge, only one study has explored direct interactions of coumarins with copper: esculetin has been directly demonstrated as a ligand presenting a significant chelating power towards Cu^{2+} ion which is bound on the doubly deprotonated catechol, leading to a 1:1 complex [99]. *Ortho*-DHMCs highly effectively inhibited the oxidative modification of human LDL catalysed by copper and copper chelation was, at least partly, responsible for this effect [29]. The structural features seem to be similar to those of iron chelation: 7,8-DHMC showed higher effect than 6,7-DHMC, on the other hand, 5,7-DHMC acted rather as pro-oxidant (non-significant effect) *via* promotion of redox cycling of copper [29] – this effect is related with easier copper reduction which could be explained by the lower standard reduction potential of Cu^{2+}/Cu^{+} (+0.15 V) in comparison with Fe^{3+}/Fe^{2+} (+0.77 V). Moreover, a natural linear furanocoumarin, bergapten (Fig. **6c**), with a low direct scavenging potential and lacking the appropriate substituents for metal chelation, has showed almost any protection against copper-initiated lipid radical-mediated oxidation [84]. Analogously to iron, an *ortho*-dihydroxylated coumarin, 7,8-dihydroxy-4-phenylcoumarin, has notably reduced cupric ions (CUPRAC assay, cupric ions reducing power assay) in contrast to those of 4-phenylcoumarins containing a single hydroxyl group or 5,7-dihydroxyl substitution [50]. However, it should be taken into account that the generation of the reduced cuprous ions can promote oxidative stress under certain conditions. Accordingly, 7,8-DHMC showed pro-oxidant behaviour in the assay using copper-induced hydroxyl radical (ORAC) [51], despite its potent antioxidant capacity in various assays as described above.

Coumarin-metal interactions may have a practical impact. For example, various coumarin backbone-containing compounds, mostly Schiff base-derived, and especially their metal (including copper) complexes have been tested for diverse antimicrobial activities [100-102] which were generally enhanced after formation of a metal complex. Additionally, coumarins can serve as chromogenic and fluorogenic dyes with high selectivity towards copper and/or iron [103-105].

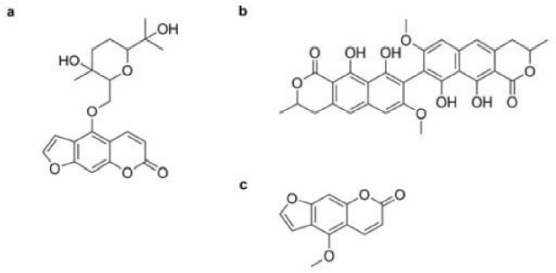


Fig. (6). Structures of (**a**) furanocoumarin feroniellamin, (**b**) isocoumarin vioxanthin and (**c**) furanocoumarin bergapten.

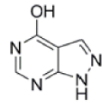
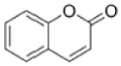
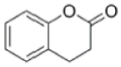
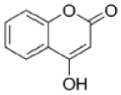
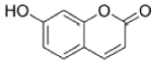
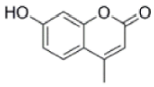
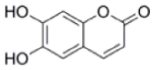
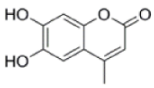
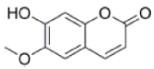
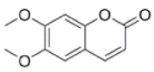
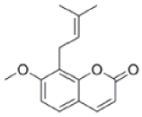
INTERACTION WITH ROS-PRODUCING ENZYMES

Xanthine Oxidase

Xanthine oxidoreductase involving two interconvertable forms, *i.e.* xanthine oxidase (XO) and xanthine dehydrogenase, is a member of the molybdoenzyme family and is the best known for its catalytic role in physiological purine degradation, metabolizing hypoxanthine and xanthine to uric acid [106-108]. On the other hand, there is a growing awareness that the activity of xanthine oxidase deteriorates pathological processes, *e.g.* ischemia, hypertension or heart failure involving endothelial dysfunction and inflammation, through the generation of ROS such as the superoxide anion and hydrogen peroxide [106, 108-110]. Several coumarins have been proposed to be active in comparison to the known XO inhibitor allopurinol (see Table **2**).

Coumarins represent a group of competitive inhibitors of XO like allopurinol, but paradoxically, few coumarins may act as uncompetitive or a mixed type inhibitors of this enzyme [38, 111-116]. Among the tested simples coumarins, 6,7-dihydroxycoumarin (esculetin) has the highest affinity toward the binding molybdopterin site of the enzyme, and this may be mainly due to the interaction of the 6-hydroxyl group with the Glu802 residue through a formation of a hydrogen bond [38, 40, 113, 116]. Moreover, possible interactions of carbonyl toward the Arg880 and the oxygen atom of the 1,2-pyrone ring with Thr1010 may play the role at the active site [38]. The 3,4-double bond also plays an important role in the activity [113]. Although its 7-hydroxyl group may

Table 2. Inhibition of xanthine oxidase by simple coumarins.

Substance	Chemical formula	IC_{50}				
		[38][a]	[113][a]	[114][b]	[115][b]	[116][c]
allopurinol		1.07 ± 0.01	9.64 ± 0.11	13.70 ± 0.15	213,06 ± 2.06	10.66
coumarin		>100				
dihydrocoumarin		>100				
4-hydroxycoumarin		78.13 ± 3.11				
7-hydroxycoumarin (umbelliferone)		>100	43.65 ± 0.55	>500	215,85 ± 5.67	
7-hydroxy-4-methylcoumarin		>100	96.70 ± 5.10		157,18 ± 3.60	
6,7-dihydroxycoumarin (esculetin)		10.84 ± 0.14	20.91 ± 0.55			28.4
6,7-dihydroxy-4-methylcoumarin		75.79 ± 1.98				
7-hydroxy-6-methoxycoumarin (scopoletin)		>100	123.21 ± 5.02	475 ± 3.7		149.40
6,7-dimethoxycoumarin (scoparone)						143.82
7-methoxy-8-isopentenylcoumarin (osthole)						138.17

Data are expressed as IC_{50} [μM]; [a] mean ± SD; [b] mean ± SEM; [c] mean.

interact with the residue S876, its contribution seems to be variable [38, 113, 115]. An additional derivatization of the hydroxyl group(s), *i.e.* methoxylation or glycosylation, hampers the interaction with the Glu802 residue resulting in negligible inhibitory activity of a coumarin [38, 113, 116, 117]. However, *i.p.* administration of scopoletin, the 6-methoxylated esculetin, resulted in dose-dependent inhibition of the enzyme in liver homogenates of hyperuricemic mice [117]. 4-Hydroxycoumarin, 7-hydroxycoumarin and their 4-methylated analogues are proposed to be XO inhibitors as well, however, their activity seems to be significantly lower in comparison to esculetin [38, 113-115]. The methyl group has been speculated to generate a repulsive force with a water molecule inside the enzyme leading to an additional reduction in that effect [38]. Studies analysing a superiority of isolated 7- or 4-hydroxyl group are not in concordance [38, 113]. In the case of the dihydroxylated 4-methylcoumarins (7,8-; 6,7- and 5,7-) and the 7,8-diacetoxylated congeners as well, it appears that there is any XO inhibition, however, 6,7-DHMC has been found to slightly inhibit the enzyme [38, 41, 78]. An additional substituent at C-8, *e.g.* the hydroxyl group or glucose, may significantly decrease the inhibitory activity [113].

NADPH Oxidase

NADPH oxidase comprises seven isoforms of multi-subunit enzyme complex that catalyses the generation of the superoxide anion from oxygen and NADPH. They were originally discovered in neutrophils but are localized in vascular smooth muscle cells, endothelial cells and fibroblasts as well [118-120]. The activity of NADPH oxidase is considered as a factor contributing to the endothelial dysfunction [121]. Therefore, a development of new drugs for inhibition of NADPH oxidase in cardiovascular diseases with endothelial dysfunction is highly desirable [119, 122].

Data on the inhibition of NADPH oxidase by coumarins are limited, almost no structure-activity relationship study is available [45, 49]. Inhibition of NADPH oxidase by several *ortho*-DHCs including 7,8-dihydroxy-6-methoxycoumarin (fraxetin) and those bearing an isolated 7-hydroxyl group may be relevant since the formation of the superoxide anion has been decreased in leucocytes [45, 81]. 6,7-DHC (esculetin) has been demonstrated to inhibit NADPH oxidase by blocking translocation of the cytosolic subunits in cultured human endothelial cells and in neutrophils [123, 124]. On the other hand, no activity of 6,7-DHCs in leukocytes analysed by cytochrome c assay has been reported as well [45]. The blockade of active hydroxyl groups by methylation or glycosylation appears to decrease the activity [45]. In human neutrophils, possible mechanisms of *ortho*-dihydroxy-4-methylcoumarins and 7-hydroxy-3-(4'-hydroxyphenyl) coumarin on the inhibition of NADPH oxidase have been proposed. These include decreases in PKC activity, phosphorylation of PKCα and PKCβII isoforms, respectively, but no effects on phosphorylation of both PKCδ isoform and the NADPH oxidase subunit $p40^{phox}$ [49, 125, 126]. On the other hand, 7-hydroxy-3-(4'-hydroxyphenyl)dihydrocoumarin lacking the double bond between C-3 and C-4 in ring B has been found as ineffective [125]. Another coumarin, auraptene, may inhibit the multicomponent NADPH oxidase system as well [127].

Inducible NO Synthase

Nitric oxide ($NO^{\cdot}$) is exclusively synthesized by a family of NO-synthases (NOS), which exist in three major isoforms. Two isoforms are constitutively expressed, one mostly in endothelial cells (eNOS), the other in the nervous system and several other tissues (neuronal or nNOS) [128]. The expression of the third major isoform is inducible (iNOS). When its activity is triggered, *e.g.* during inflammation and immune reactions, the consequent reaction of $NO^{\cdot}$ and the superoxide anion may lead to vascular and cellular inflammatory reactions due to formation of peroxynitrite [128, 129]. Therefore, the inhibition of iNOS appears to be a promising strategy for the treatment of inflammatory diseases in particular. However, data on the inhibiton of iNOS by coumarins are rather limited.

Several sesquiterpene coumarins isolated from *Ferula fukanensis* and some of 6-alkoxycoumarin and 7-alkoxycoumarin derivatives have inhibited NO production and iNOS protein and mRNA expression in a mouse macrophage-like cell line (RAW264.7) after induction with lipopolysaccharide and INF-γ [130-133]. In sesquiterpene coumarins, the α,β-unsaturated ketone moiety, a position of double-bond in the sesquiterpene unit may be those structural features of interest, moreover, a methoxyl group at C-6, Z-configuration and the dimethyldihydrofuran moiety enhanced the effect [130-132]. Some simple coumarins isolated from *Mammea siamensis* including kayeassamin G, mammea A/AD and that geranylated one, mammeasin A, all bearing 7-hydroxyl group, a linear pyranocoumarin decursin isolated from *Angelica gigas,* a linear furanocoumarin imperatorin isolated from *Glehnia littoralis* and 6-hydroxy-7-methoxycoumarin derivative from *Ruta graveolens* have been found to inhibit both the protein and mRNA expression in RAW264.7 cells as well [134-137]. In lipopolysaccharide-stimulated RAW264.7 or human monocyte cell line (THP-1), a suppression of nuclear protein levels of phosphorylated signal transducer and activator of transcription-1 (STAT1) and NF-κB, which may be through inhibition of phosphorylation and subsequent degradation of inhibitor-kB (IkB), respectively, have been proposed to be involved in the inhibitory effects on iNOS expression [134, 135, 137]. 3-Triazolyl substituent of the coumarin core may decrease NO production, by reducing iNOS gene and protein expressions and iNOS activity in neutrophils without affecting the eNOS activity [138]. Herein, it has been shown that π-π interactions with the heme group of the enzyme seemed to be important for the iNOS inhibition [138].

Lipoxygenase

Lipoxygenases (LOX) are a family of monomeric non-heme, non-sulfur, iron dioxygenases, which catalyse the addition of oxygen to arachidonic acid and polyunsaturated fatty acids, yielding hydroperoxyl derivatives including hydroperoxyeicosatetraenoic acids [139, 140]. Upon reduction, the corresponding hydroxyl derivatives hydroxyeicosatetraenoic acid, leukotriene and lipoxins are generated [139]. Human LOX are classified as 5-, 8-, 12-, or 15-LOX, according to the site of oxygen insertion within arachidonic acid [139]. At least three types of 12-LOX have been characterized, *i.e.* platelet-type, leukocyte-type and epidermal 12-LOX [139]. LOX have been implicated in the pathogenesis of several inflammatory conditions involving cardiovascular diseases as well [140, 141]. Although, LOX metabolites contribute to ROS generation, there are few structure-activity studies analysing the inhibitory activity of coumarins.

In general, studies have suggested that a mechanism of the action of coumarins likely depends upon both iron-chelating and iron-reducing properties of coumarins because catalytically active LOX contain a non-heme ferric iron at its active site [140, 142, 143]. However, a competitive non-redox mechanism may play a role in a synthetic fluorophenyl-substituted coumarin containing bis(trifluoromethyl) carbinol substituent which has been reported to inhibit the formation of leukotrienes *via* recombinant human 5-LOX *in vitro* and in rats when administered as a pro-drug [144].

In simple coumarins, the most important is the *ortho*-dihydroxyl substitution. 6,7-DHCs (esculetin and 4-methylesculetin) and 7,8-DHCs (daphnetin) containing the catechol ring have been reported to extensively inhibit 5-LOX [142, 145, 146]. The effect of 6-methoxyl group seems to be variable [142, 145]. Although a presence/absence of the 4-methyl group did not play a role in 6,7-DHCs, it was re-

sponsible for blocking of inhibitory effect of 7,8-DHC (daphnetin) [142, 145]. On the other hand, unequivocally weak 5-LOX inhibitors were 5,7-DHC, coumarins possessing one hydroxyl group, glycosides and coumarin or dihydrocoumarin themselves [142, 145]. Moreover, esculetin showed inhibition of LOXs in neutrophils, platelets, cloned mastocytoma cells and in a model of bile salt stimulation of rat colonic epithelial proliferation [124, 147-149]. As expected, a consequent increase in prostaglandin synthesis has been observed especially at higher doses of esculetin following the inhibition of leukotriene synthesis [148].

In a soybean LOX assay, the basic structural features regarding simple coumarins have been confirmed because the active compounds were those bearing a hydroxyl group, *i.e.* 6-hydroxycoumarin, 7-hydroxycoumarin and 6-hydroxybenzo[h]coumarin [65]. Similarly, in 6-hydroxycoumarin, the 4-methyl group decreased the effect significantly [65]. Coumarin itself is a weak inhibitor of soybean LOX [72, 86]. Mannich bases containing the 7-hydroxycoumarin core, in particular that bearing piperazine at C-8, inhibited the enzyme [71]. Some of 7-amino- or 7-hydroxycoumarin derivatives with disubstituted ethylenediamine moiety, simple coumarins bearing a pyrrol ring, one of the synthetic 3-phenylcoumarin bearing both *ortho*-methoxyl groups and bromine were found as perspective structures [58, 72, 73]. On the other hand, linear or angular furanocoumarins and pyranocoumarins were found to be almost inactive [86].

Cyclooxygenase

An elevated formation of prostanoids in assistance of the constitutive (COX-1) or inducible (COX-2) cyclooxygenases has been involved in cardiovascular diseases such as hypertension [150]. It is important to note that ROS can activate COXs, and hereafter, COX-derived products can induce ROS production through effects on different ROS generating enzymes [150]. Therefore, an additional inhibition of COXs by coumarins may be of interest in prevention of oxidative stress.

In polymorphonuclear leukocyte, a structure-activity relationship study has revealed that the inhibition of COX pathway occurred in 5,7-DHMC in particular, which did not inhibit 5-LOX, implying the fact that COX inhibitory mechanism appears to be different than that of LOX [35, 142, 146]. *Ortho*-DHCs (such as esculetin, daphnetin and fraxetin) were found as COX pathway inhibitors as well, however their IC_{50} were significantly higher than those necessary for LOX inhibition [142, 145, 146, 149]. 7-Methoxycoumarins have exerted some activity as well [142]. Interestingly, esculetin was found to act as the inhibitor in a model of bile salt stimulation of rat colonic epithelial proliferation, suggesting that coumarins may be beneficial in the pathological inflammatory processes [147].

Imperatorin, a linear furanocoumarin isolated from *Glehnia littoralis* inhibited COX-2 protein expression in lipopolysaccharide-stimulated RAW264.7 cell line [136]. The inhibition of COX-2 may be probably mediated by the inhibition of the NF-κB pathway in linear pyranocoumarin decursin [135]. Mannich bases with both 7-hydroxycoumarin core and 8-piperazine inhibiting the soybean LOX did not significantly decrease the activity of COX-1 [71].

Myeloperoxidase

Myeloperoxidase (MPO) is an essential heme peroxidase enzyme produced by activated leukocytes and characterised by powerful pro-oxidative and proinflammatory properties [151, 152]. This enzyme catalyses the reaction of hydrogen peroxide with chloride ion to form hypochlorous acid. Hypochlorous acid is a chemical oxidant, possessing antibacterial, antiviral, and antifungal properties, which play key roles in the human immune system [152]. However, many studies have suggested a possible role of MPO-derived oxidants in cardiovascular diseases in which it may serve as a non-specific biomarker [151-154]. In coumarins, data on evaluation of structure-MPO inhibition relationship are very limited according to our knowledge.

Nevertheless, simple 4-methylcoumarins bearing *ortho*-dihydroxyl moiety decreased MPO activity in general [49, 54]. Some synthetic 3-phenylcoumarins bearing 6,7-dihydroxyl group and the catechol structure in the phenyl ring have inhibited MPO activity in human neutrophils as well [59]. However, oxidation of 7-hydroxylated simple coumarins by the neutrophil MPO may produce highly reactive coumarin-radical intermediates, which may contrarily act as pro-oxidants [81].

In an experimental model of rat colitis induced by trinitrobenzenesulphonic acid, synthetic 6,7-DHMC (4-methylesculetin) significantly inhibited MPO activity at doses of 5 and 10 mg/kg, but at the higher dose (25 mg/kg) a loss of efficacy occurred, contrarily no effect on MPO inhibition was found in the case of 6,7-DHC (esculetin) [155]. Interestingly, esculin (6,7-dihydroxy-6-*O*-glucosylcoumarin), which is promptly hydrolysed to its aglycone esculetin, in the highest tested dose of 25 mg/kg [156], and 7,8-DHC in a dose of 5 mg/kg inhibited MPO as well [54]. On the other hand, scopoletine, scoparone, fraxetin and 4-methylumbelliferone were found as almost inactive [54]. Similarly, coumarin at the lowest tested dose of 5 mg/kg, 4-hydroxycoumarin (25 mg/kg) and isocoumarin paepalantine (5 and 10 mg/kg; Fig. **5a**), isolated from *Paepalanthus bromelioides*, were found to be effective in that model [157, 158]. Moreover, cloricromen has been found to decrease levels of MPO in different *in vivo* ischemic models including transient ischemic cerebral injury in the Mongolian gerbil [159-162].

CONCLUSIONS

This review has shown that many coumarins possess a broad *in vitro* antioxidant capacity. Indeed, the synergism involving the direct RONS-scavenging effects and the chelation of transient metals and/or the inhibition of RONS-producing enzymes have been suggested in cellular as well as *in vivo* models [27, 53, 54, 63].

Moreover, many factors must be taken into account when analysing chemical structure-antioxidant capacity relationships of coumarins due to different *in vitro/in vivo* methodological approaches, e.g. a nature and a concentration of RONS or oxidative stress inducers, lipophilicity of a reaction microenvironment, and last but not least, the involvement of transient metals and RONS-producing enzymes. In fact, comparing aqueous assays and lipophilic systems, coumarins

which are able to interact with lipidic biomembranes and penetrate into cells may be of particular interest.

A substantial amount of literature enables the elucidation of the structure-activity relationship for direct RONS-scavenging and iron chelation. Data on other antioxidant mechanisms are more modest but they suggest that coumarins are able to block the activity of xanthine oxidase, lipoxygenase and myeloperoxidase, and likely to decrease the expression of inflammatory mediators iNOS and COX-2.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This review was supported by grant of The Czech Science Foundation (P303/12/G163). M.Ř. thanks to Charles University in Prague (SVV 260 064).

ABBREVIATIONS

AAPH	=	(2,2'-azobis(2-amidinopropane) dihydrochloride
ABAP	=	2,2'-azobis(2-methylpropionamidine) dihydrochloride)
$ABST^{\cdot+}$	=	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BAEC	=	Bovine aortic endothelial cell line
COX	=	Cyclooxygenase
DHC	=	Dihydroxycoumarin
DHMC	=	Dihydroxy-4-methylcoumarin
DPPH	=	1,1-diphenyl-2-picrylhydrazyl
LOX	=	Lipoxygenase
MPO	=	Myeloperoxidase
ORAC-FL	=	Oxygen radical antioxidant capacity-fluorescein
ROS	=	Reactive oxygen species
RONS	=	Reactive oxygen and nitrogen species
XO	=	Xanthine oxidase

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Received: June 05, 2014 **Revised: December 04, 2014** **Accepted: December 09, 2014**

4.2. Novel method for rapid copper chelation assessment confirmed low affinity of D-penicillamine for copper in comparison with trientine and 8-hydroxyquinolines

ŘÍHA, M.; KARLÍČKOVÁ, J.; FILIPSKÝ, T.; MACÁKOVÁ, K.; HRDINA, R.; MLADĚNKA, P. Novel method for rapid copper chelation assessment confirmed low affinity of D-penicillamine for copper in comparison with trientine and 8-hydroxyquinolines. *Journal of Inorganic Biochemistry*. 2013, **123**, 80-87.

(IF 2013: 3,274)

Journal of Inorganic Biochemistry 123 (2013) 80–87

Contents lists available at SciVerse ScienceDirect

Journal of Inorganic Biochemistry

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

Novel method for rapid copper chelation assessment confirmed low affinity of D-penicillamine for copper in comparison with trientine and 8-hydroxyquinolines

Michal Říha [a], Jana Karlíčková [b], Tomáš Filipský [a], Kateřina Macáková [b], Radomír Hrdina [a], Přemysl Mladěnka [a,*]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 15 November 2012
Received in revised form 6 February 2013
Accepted 28 February 2013
Available online 19 March 2013

Keywords:
8-Hydroxyquinolines
Bathocuproinedisulfonic acid
Copper
Chelators
Penicillamine
Triethylenetetramine

ABSTRACT

Copper is an essential trace element involved in many physiological processes. Since disorder of copper homeostasis is observed in various pathologies, copper chelators may represent a promising therapeutic tool. This study was aimed at: 1) formation of an *in vitro* methodology for screening of copper chelators, and 2) detailed analysis of the interaction of copper with clinically used D-penicillamine (D-PEN), triethylenetetramine (trientine), experimentally tested 8-hydroxyquinolines, and the disodium salt of EDTA as a standard chelator. Methodology based on bathocuproinedisulfonic acid disodium salt (BCS), usable at (patho)physiologically relevant pHs (4.5–7.5), enabled assessment of both cuprous and cupric ions chelation and comparison of the relative affinities of the tested compounds for copper. In the case of potent chelators, the stoichiometry could be estimated too. Clioquinol, chloroxine and EDTA formed very stable complexes with Cu^{+}/Cu^{2+} at all tested pHs, while copper complexes with trientine were stable only under neutral or slightly acidic conditions. Non-substituted 8-hydroxyquinoline was a less efficient copper chelator, but still unequivocally more potent than D-PEN. Both 8-hydroxyquinoline and D-PEN chelation potencies, similarly to that of trientine, were pH-dependent and decreased with pH. Moreover, only D-PEN was able to reduce cupric ions. Conclusively, BCS assay represents a rapid, simple and precise method for copper chelation measurement. In addition, lower binding affinity of D-PEN compared with 8-hydroxyquinolines and trientine was demonstrated.

1. Introduction

Copper plays an essential role in the human organism. With its association with enzymes, copper is involved in many vital metabolic processes, *e.g.*, decomposition of superoxide radical, formation of ATP, synthesis of important mediators, and regulation of transcription [1–3].

The fate of copper in the human body is complex and not fully understood. Briefly, dietary copper is absorbed after reduction into cuprous ions in the upper part of the small intestine. The transport of copper within the enterocytes, as well as in other cells, is ensured by several chaperons (ATOX1, Cox17 and Ccs1). Copper in the enterocytes can be incorporated in copper-containing proteins, detoxified when presented in excess by binding with metallothionein or transported into the circulation by the specific copper-transporting ATPase — ATP7A. In the portal vein, copper is transported in the oxidized cupric form bound to albumin, α_2-macroglobulin or amino acids. This copper is generally designated as exchangeable copper. Before liver uptake *via* a copper transporter, hCTR1, copper should be reduced again. In the liver, it can be incorporated into ceruloplasmin or excreted into the bile. Both processes are controlled by another ATPase transporter — ATP7B [4–6]. Ceruloplasmin binds 6 atoms of copper and one of its roles is to supply copper for peripheral tissues. Copper in ceruloplasmin is generally considered tightly bound, but some pathological factors such as oxidative stress may release it [7,8].

Copper levels have to be meticulously regulated since disruption of copper homeostasis at systemic or local levels has various pathophysiological consequences. Systemic copper homeostasis disruption is well known due to human genetic disorders, in particular Wilson's and Menkes diseases [4]. Moreover, relative (local) disorder of copper homeostasis is pathologically relevant and is associated with a lot of pathologies, especially neurodegenerative diseases, tumors, inflammatory diseases and acute myocardial infarction [9–13]. Especially, excess of copper is highly toxic due to the capability of free copper to potentiate the formation of reactive oxygen species (ROS) and thus promote oxidative stress [3,14].

Notwithstanding these facts and the intense research in this field, clinical use of copper chelators is restricted mainly to Wilson's disease. This autosomal recessive disorder based on the mutation of the ATP7B gene is associated with copper accumulation, especially in the liver,

* Corresponding author. Tel.: +420 495067295; fax: +420 495067170.
E-mail address: mladenkap@faf.cuni.cz (P. Mladěnka).

0162-0134/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jinorgbio.2013.02.011

brain and kidney [4]. Life-long pharmacological therapy with copper chelators D-penicillamine (D-PEN), triethylenetetramine (trientine) or tetrathiomolybdate potentially combined with zinc represents the key current approach to the treatment of this disease [15,16].

D-PEN, a standard copper chelator, was first introduced in the treatment of Wilson's disease in 1956 [17] and represents the "gold standard" of the therapy [15]. However, D-PEN has significant side effects concerning particularly the immune system, connective tissue and the kidney. Moreover, the risk of initial worsening of neurological symptoms in patients with neurological impairment is relatively high [18,19]. Trientine, another copper chelating agent, was introduced in 1982 as an alternative for D-PEN-intolerant patients with a better safety profile [16,20]. 8-Hydroxyquinolines, in particular clioquinol, are non-selective chelators, which were shown to possess some positive impact on Alzheimer's disease patients and on *in vivo* tumors as well [21,22]. Clioquinol, a halogenated derivative of 8-hydroxyquinoline, was originally used due to its antiseptic and amebicide properties. Although it was withdrawn from the market as an oral drug in the early 1970s due to its probable association with a specific kind of neuropathy, subacute myelo-optic neuropathy, it remains an important prototype substance. Its derivative, PBT2, without the risk of the mentioned neuropathy is currently under clinical investigation [23–25]. EDTA, a well-known complexing agent, was included in this study for comparison as a standard chelator.

Considering different pathophysiologies, the research of novel copper chelators should emphasize various characteristics, namely: 1) affinity to cuprous or cupric ions, as well as the possible reduction of cupric ions; 2) stability of the complex with possible participation on ROS production; 3) chelation under various pH conditions; 4) specificity to chelate other ions (especially iron and zinc), and 5) lipophilicity, which affects penetration into the cells and central nervous system.

This *in vitro* study was aimed at: 1) formation of a rapid, simple and precise methodology enabling the testing of novel copper chelators, and 2) a detailed analysis of the interaction of clinically used hydrophilic D-PEN, trientine and experimentally tested lipophilic clioquinol and its related 8-hydroxyquinolines with copper under (patho)physiological relevant pHs.

2. Materials and methods

2.1. Reagents

A stock solution of cuprous ions (5 mM) was prepared by dissolving cuprous chloride (CuCl) in an aqueous solution of 0.1 M HCl and 1 M NaCl. Working solutions were prepared by use of distilled water. Cupric ions (cupric sulfate pentahydrate, $CuSO_4.5H_2O$) were dissolved directly in distilled water. Hydroxylamine hydrochloride (hydroxylamine), bathocuproinedisulfonic acid disodium salt (BCS), D-PEN, disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA) and trientine were dissolved in distilled water; hematoxylin, 8-hydroxyquinoline, chloroxine (5,7-dichloroquinolin-8-ol) and clioquinol (5-chloro-7-iodoquinolin-8-ol) were dissolved in DMSO (see chemical structures in Supplementary data — Fig. S1).

Measurements were conducted in 15 mM buffers, acetate for pH 4.5 and 5.5 and HEPES for pH 6.8 and 7.5, at room temperature. All chemicals were purchased from Sigma-Aldrich (Germany).

2.2. BCS assay

Copper solutions (50 μl) were mixed for 2 min with a solution of chelator (50 μl) in a buffer (100 μl). In the case of cuprous ions, the mixture contained 50 μl of hydroxylamine (final concentration according to pH, see below). In the case of cupric ions, hydroxylamine was added after 2 min in order to reduce non-chelated cupric ions into cuprous ones. Finally, 50 μl of BCS or water (blank) was added and the absorbance at 484 nm was measured by spectrophotometry using a Synergy HT Multi-Detection Microplate Reader (BioTec Instruments, Inc., Winooski, Vermont, U.S.A.) immediately and at 5 min.

A similar approach was used for determination of reducing properties, *i.e.*, cupric ions were mixed with a tested substance in a buffer without hydroxylamine. BCS was added after 2 min of mixing. Hydroxylamine was used only as a positive control — it caused 100% reduction.

2.3. Hematoxylin assay

A solution of chelator (50 μl) was mixed with cupric ions solutions (50 μl) in a buffer (150 μl). After 2 min of mixing, 50 μl of either hematoxylin or DMSO (blank) was added and mixed for an additional 3 min. The absorbance was measured immediately and after 4 min. The wavelength differed according to pH (see results below).

2.4. Statistical analysis

The amount of remaining copper was calculated from the difference of absorbance between the tested sample (with the indicator) and its corresponding blank (without indicator) divided by the difference of the control sample (the known amount of copper without the tested substance) and its control blank.

The normalized dose-dependent curves with 95% confidence intervals were constructed by GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, California, U.S.A.). Each curve of an efficient substance was composed from at least five points (first point 0% chelation, last point 100% chelation).

Data are expressed as means ± SD. Differences among tested substances were carried out by comparing 95% confidence intervals.

3. Results

3.1. Development of BCS methodology

A previous study has shown that bathocuproine forms a complex with cuprous ions with maximum absorbance at 484 nm [26]. Indeed, such a complex at this wavelength maximum was found when BCS was mixed with cuprous ions in the presence of the reducing agent hydroxylamine at all tested pH values (shown for pH 6.8 in Fig. 1A). The spectra were identical in the measured range of 400 to 800 nm at all tested pH values (Supplementary data — Fig. S2A). Cuprous or cupric ions without hydroxylamine did not form such a complex with BCS at any of the tested pHs (Fig. 1A). Moreover, neither cuprous/cupric ions nor hydroxylamine nor BCS showed significant absorbance in the measured wavelengths suggesting the specificity of the reaction (Supplementary data — Fig. S2B). Although cupric ions cannot be directly assessed by BCS, the methodology can be extended for these ions by administration of a reductant, *e.g.*, hydroxylamine (Supplementary data — Fig. S2B). With both cuprous and cupric ions, the complex formation was linearly dependent on the copper concentration (Fig. 1B).

To optimize the methodology, we tested the effects of different concentrations of hydroxylamine and BCS together with the time influence (Figs. 2, 3 and Supplementary data — Fig. S3). The complex BCS–Cu^+ was formed gradually and the rate of formation was dependent on the acidity of the environment. At 5 min, the complex formation seemed to be finished under all conditions, as little or no change was observed in the next measurements. In the case of pH 7.5 and 6.8, the addition of 1 mM hydroxylamine (final concentration of 0.167 mM) was sufficient for rapid complex formation and thus enabling immediate measurement. At pH 5.5 and especially 4.5, however, a higher concentration was necessary (Fig. 2). The addition of 10 mM hydroxylamine (final concentration of 1.67 mM) was set for these pH conditions. At lower pH (4.5–6.8), the complex BCS–Cu^+ was formed rapidly even in low concentrations of BCS. However, the complex formation was slower up to

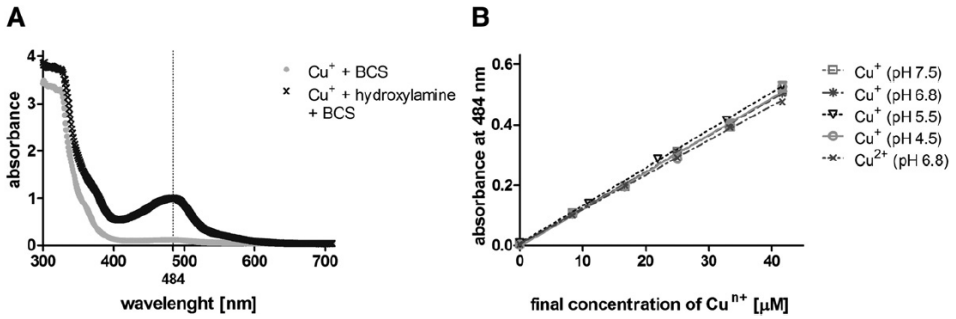

Fig. 1. Absorption spectra (A) and calibration of copper ions (B). Absorption spectra (A) at pH 6.8 show cuprous ions with BCS (grey) and cuprous ions with hydroxylamine and BCS (black). Final concentrations were: 100 μM /Cu^{+}/, 1 mM /BCS/ and 20 mM /hydroxylamine/. Graph (B) shows calibration of cuprous ions at different pHs, as well as cupric ions for comparison. Coefficients of linear regression were 0.9988 (pH 7.5), 0.9996 (pH 6.8), 0.9993 (pH 5.5), 0.9945 (pH 4.5) and 0.9989 (cupric ions, pH 6.8).

the BCS:Cu^{+} concentration ratio 10:1 at neutral pH (Fig. 3). Thus, in order to maintain the same competitive environment for copper and to ascertain rapid complex formation immediately after BCS addition, we set the final ratio at 20:1, *i.e.*, addition of 50 μL of 5 mM BCS. No significant differences were found between cuprous and cupric ions in these experiments for methodology optimization.

3.2. Copper chelation and reduction assessment

Six clinically or experimentally known copper chelators were first tested by BCS for both cupric and cuprous chelation properties. All tested compounds were able to chelate copper under various pH conditions, although there were marked differences in chelation efficacy (Fig. 4 and Supplementary data — Figs. S4 and S5). There was no significant difference between chelation of cupric and cuprous ions in the 8-hydroxyquinoline group, while Na_2EDTA, trientine and D-PEN showed slightly lower affinity for cuprous ions (Fig. 4CD *vs* Supplementary data — Fig. S5). The 8-hydroxyquinoline derivatives clioquinol and chloroxine, and Na_2EDTA were potent copper chelators with chelation efficacy independent of the acidity of the environment (Fig. 4B and Supplementary data — Figs. S4 and S5AB). In addition, their complexes were stable, *i.e.*, there was no change in the chelation curve measured immediately and after a 5 min interval (*e.g.*, clioquinol in Fig. 5A). Moreover, the methodology enabled the assessment of the complex stoichiometry: clioquinol and chloroxine chelated about 50% of copper at the ratio 1:1 (Fig. 4B and Supplementary data — Fig. S4A), thus at the ratio 2:1, chelator to copper, 100% of copper was chelated implicating the stoichiometry 2:1. Analogously, Na_2EDTA formed likely complexes with the stoichiometry 1:1. Trientine showed as high an affinity as that of Na_2EDTA to copper ions (either cuprous or cupric ions) at neutral or close to neutral conditions. However, this ability significantly dropped under more acidic conditions (pH 5.5 and 4.5) (Fig. 4C). In line with this finding, complexes in those acidic conditions were not stable, in contrast to complexes in neutral or close to neutral pH (Fig. 5B). Complexes with cuprous ions were slightly, but significantly less stable than cupric complexes (data not shown). In contrast to its halogenated congeners, non-substituted 8-hydroxyquinoline was a considerably weaker copper chelator. Its copper chelation was even less expressed in more acidic conditions and its complexes were less stable (Fig. 4A *vs* B; Fig. 5A). Interestingly, D-PEN was only a poor chelator in neutral or slightly acidic conditions. At pH 7.5, it was able to chelate only 26 ± 1% of cupric ions and 15 ± 4% of cuprous ions at a ratio of 400:1, D-PEN to copper. To verify the reliability of these results, 1 mM BCS solution instead of 5 mM was tested. However, measured chelation reached only approximately twofold higher values (data not shown). At acidic pH, no copper chelation was observed at all (Fig. 4D and Supplementary data — Fig. S5C). Additionally, complexes of D-PEN were not stable and their decay followed an approximately linear character in relation to the concentration of the chelator (Fig. 5C).

Since D-PEN is a well-known copper chelator, the measured very low chelation efficacy using the BCS-chelation methodology was rather unexpected, and so the copper chelation activity was rechecked by other methodology based on hematoxylin. This dye forms complexes specifically with cupric ions [27]. However, it is useless in more acidic conditions (pH 4.5). In contrast to BCS, addition of Cu^{2+} to hematoxylin led to different absorption maxima in relation to the pH used (Fig. 6A). Despite the fact that the maximum absorbance was situated at 750 nm at pH 7.5 and 6.8, a linear relationship between copper concentration and absorbance at this wavelength was not found. Therefore, the relationship between copper concentration and absorbance was analyzed at all wavelengths in order to find an appropriate wavelength for precise copper assessment. This wavelength was found around 600 nm at all tested pHs, where hematoxylin alone had negligible absorbance (Fig. 6B). There were slight differences between particular pH conditions

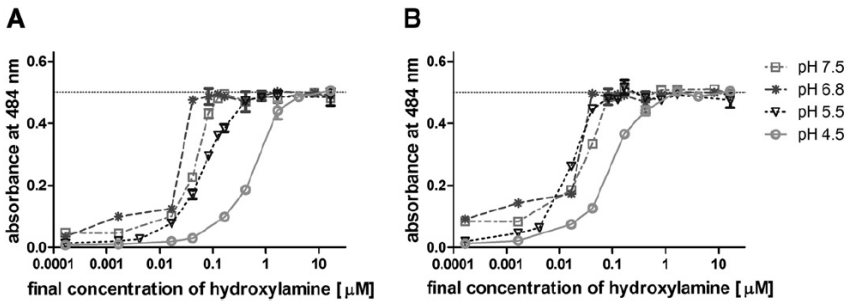

Fig. 2. Influence of hydroxylamine on BCS–Cu^{+} complex formation at 0 min (A) and 5 min (B).

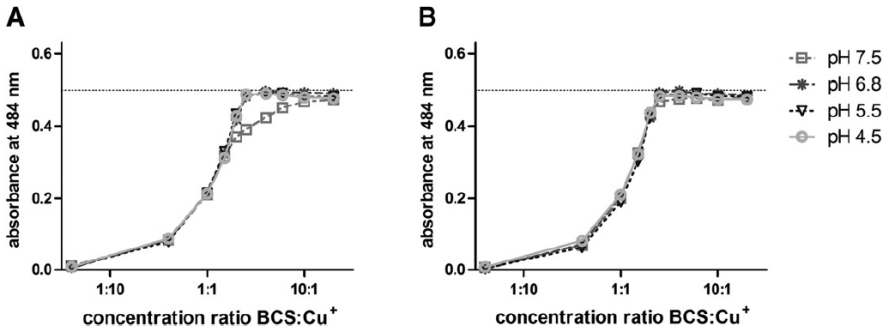

Fig. 3. Effect of BCS on complex formation with cuprous ions at 0 min (A) and 5 min (B).

and, based on more detailed calibration curves (data not shown), the wavelength 610 nm was selected for pH 7.5, 590 nm for pH 6.8 and 595 nm for pH 5.5. Additionally, different concentrations of the indicator were tested to optimize the methodology. Hematoxylin in equal concentration to copper was sufficient for sensitive measurement while further increases in the concentration did not substantially influence the absorbance at the wavelengths around 600 nm (Fig. 7). Since the absorbance of the complex was changing gradually, the time aspect was also included in all measurements.

For the reason of comparison, all mentioned substances were analyzed in this assay too. As in previous methodology, similar results were found in the case of clioquinol, chloroxine and Na_2EDTA. The stable complexes with 2:1 or 1:1 stoichiometry were confirmed by this assay (Fig. 8B and Supplementary data — Figs. S4B and S6). Chelation of cupric ions by trientine was similar to that using BCS methodology, except that the curves were identical at all tested pHs (Fig. 8C). Complexes with the 1:1 stoichiometry were, in contrast to BCS methodology, stable even at pH 5.5. The activity of 8-hydroxyquinoline and D-PEN was, however, much higher when compared with the BCS assay (Figs. 8AD *vs* 4AD). 8-Hydroxyquinoline likely formed the complex with the stoichiometry 2:1, but the complex did not appear to be stable in neutral conditions (Fig. 8A). Complexes of D-PEN were very variable at different pHs and changed with time (Fig. 5D). The stoichiometry approximated, but did not equal, the ratio 1:1 at pH 5.5–6.8. This may correspond to the known fact that its complex has a rather complicated character depending on experimental conditions [28,29].

To fully characterize the copper-chelator interactions, cupric ion reducing experiments were performed. None of the tested substances except for D-PEN possessed copper reducing properties. Reducing ability of D-PEN was observed at all tested pH values, with the maximum and complete reduction at a concentration ratio of 4:1 (Supplementary data — Fig. S7). Although the reducing potential dropped slightly in higher ratios of D-PEN to copper at pH 6.8 and 7.5, it remained substantial.

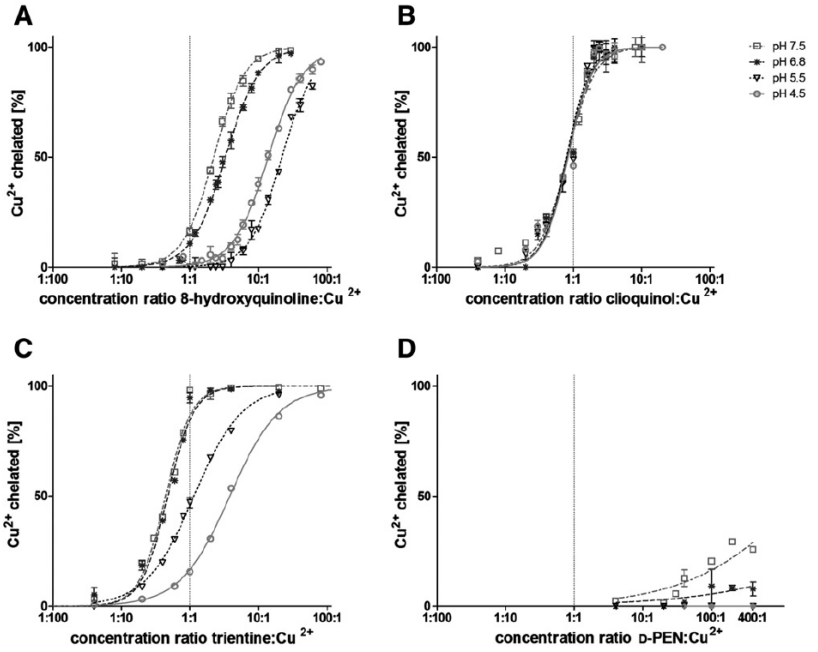

Fig. 4. Chelation of cupric ions under different pH conditions by 8-hydroxyquinoline (A), clioquinol (B), trientine (C), and D-PEN (D). Chelation properties of chloroxine were not different from those of clioquinol (Supplementary data — Fig. S4A).

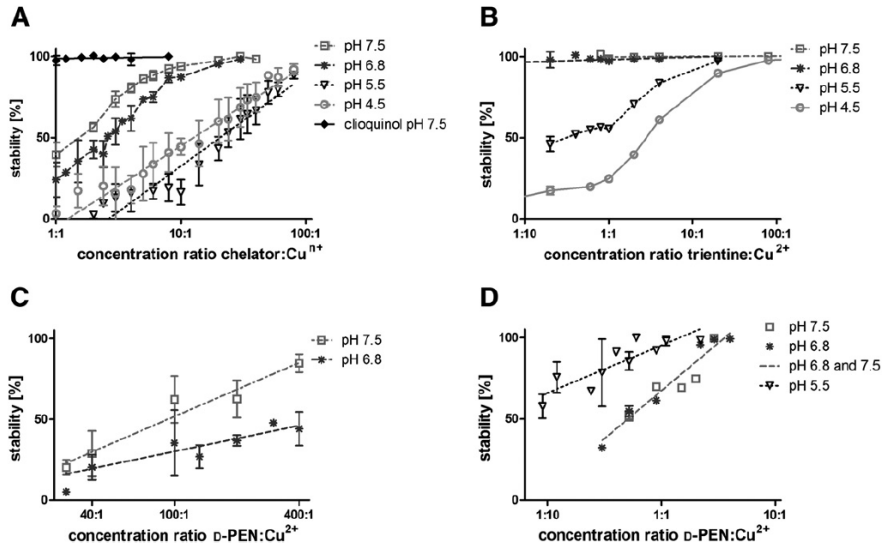

Fig. 5. Stability of copper complexes. (A) Comparison of 8-hydroxyquinoline- and clioquinol-copper complex stabilities measured by BCS methodology. Clioquinol formed stable complexes at all tested pH (shown only for pH 7.5), while complexes of 8-hydroxyquinoline were unstable. (B) Stability of trientine-cupric ion complexes. The complexes were stable at neutral or slightly acidic conditions. (C–D) Stability of D-PEN complexes with copper as measured by BCS methodology (C) and hematoxylin method (D). In both experimental methods, the complexes of D-PEN were unstable.

4. Discussion

There are various principles for the determination of copper ions *in vitro*. Since spectrophotometric measurements are rapid and inexpensive, they are often used [30]. However, several spectrophotometric methods reported in the literature have only limited use in pharmacological research; in particular, some of them have low sensitivity (*e.g.*, low molar absorption coefficient in the case of bis(acetylacetone)ethylenediimine) or can be used only in a limited pH range (*e.g.*, β-benzoyl-α-pyridyl thiourea) [31,32]. In light of these facts, BCS represents a suitable indicator: 1) it avidly chelates cuprous ions with high affinity; 2) the complex is formed in a wide range of pHs and its absorbance is stable; 3) this method can be used for cupric chelation assessment too, when coupled with a suitable reducing agent; 4) the methodology can reveal the relative affinity of the tested compound for copper as well, because of the high affinity of BCS for copper, and 5) in the case of strong copper chelators, *i.e.*, those which have high affinity for copper, the complex stoichiometry can be determined. Although BCS has some affinity for cupric ions too [33], this appears to be low to influence the assay significantly (Supplementary data — Fig. 2SB). Moreover, BCS is specific for cuprous ions compared with other ions [34]. The accuracy of BCS measurement can be confirmed by available data, which are in apparent agreement with the results from this study: EDTA forms 1:1 complexes with heavy metals, and complexes of 8-hydroxyquinolines with copper of 2:1 stoichiometry were reported in the literature [35–37].

In contrast, hematoxylin is rather a cumbersome indicator. It and its complex with copper are not stable and the assay is not usable at lower pH. It can, however, be used at pH 5.5, but it has to be taken into consideration that the affinity of hematoxylin for cupric ions is low in this condition. Thus, this approach may be used at pH 5.5 only for the determination of chelation stoichiometry, but not for the comparison of relative affinities of the tested substances for copper. Because of the mentioned instability, the lowest possible concentration of hematoxylin was used. This concentration was equal to the concentration of copper (Fig. 7). This is in agreement with the published data showing that the stoichiometry of Cu^{2+}-hematoxylin, or more precisely Cu^{2+}-hematein (active form of hematoxylin), complex is 1:1 [38].

The second aim of the study was to analyze the copper chelation activity in harmony with the known and anticipated biological aspects of copper (patho)physiology. Firstly, pH influences chelation and appears to be an important factor for the fate of transient metals (iron and copper) in the organism: 1) when cells are in contact with excess of copper, lysosomes (pH 4–5) appear to accumulate this; 2) copper absorption, similar to that of iron, is likely taking place in the upper part of the intestine where the pH is still rather acidic; 3) pathological conditions like tumors and ischemia are associated with lower pH, as well [5,39–42]. Secondly, although not very much is known about the pathophysiological changes of the copper oxidative state, presumably both cupric and cuprous ions have important roles in the organism [4–6]. A detailed investigation of copper interaction with potential chelators in relation to pH and oxidative status has not been published yet. We are of the opinion that such type of study may facilitate further research on copper chelators. In particular, weak copper chelation associated with copper reduction could potentially increase copper absorption and increase production of ROS, while potent chelators may block copper from redox cycling.

The most potent cupric/cuprous ion chelators in this study were chloroxine, clioquinol and Na_2EDTA. These chelators differed only in the complex stoichiometry. Both of the halogenated 8-hydroxyquinolines formed 2:1 complexes, while EDTA, a known hexadentate ligand, formed the complex with a stoichiometry of 1:1. This was confirmed by both assays used in this study. Trientine chelated copper in the ratio 1:1, as suggested by both methodological approaches. Previous papers reported cupric complexes with clioquinol of the 2:1 stoichiometry only at neutral pH and with trientine of the stoichiometry 1:1 over a large pH range [35,43,44]. It ensues from the summarized data that trientine forms complexes of 1:1 at all tested pHs, but its complexes at pH 5.5 and 4.5 were not stable. The affinity of EDTA for cuprous ions was slightly lower than that for cupric ones, which is in agreement with the known fact that EDTA forms stable complexes especially with divalent or multivalent metal cations [45].

In our study, D-PEN and 8-hydroxyquinoline were copper chelators of lower potency than that of the other tested compounds. The hematoxylin assay showed that D-PEN was able to form complexes with

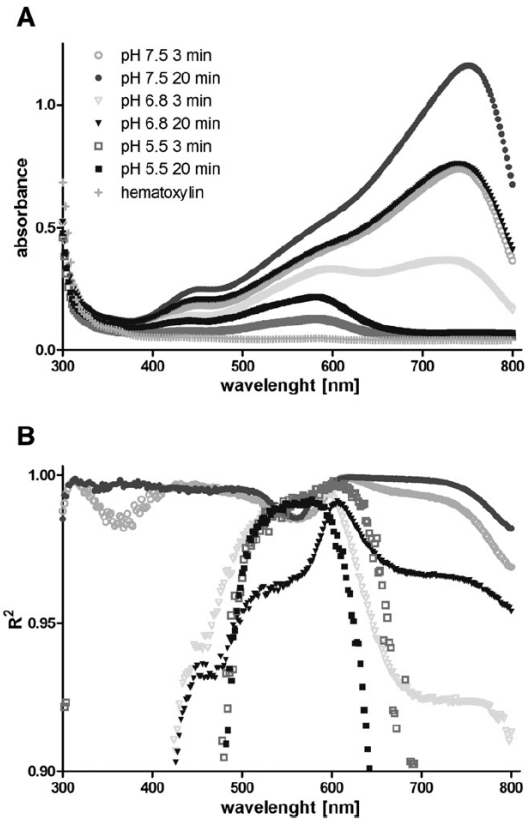

Fig. 6. Absorption spectra (A) and coefficients of linear regression of hematoxylin-Cu^{2+} complexes (B) at different times and pH conditions. Final concentrations were: 50 μM /Cu^{2+}/ and 100 μM /hematoxylin/. The spectrum of hematoxylin alone is also depicted in graph A. Graph B shows the linear regression coefficients (R^2) for the relationship between the concentration of cupric ions and absorbance at all measured wavelengths.

cupric ions. Their stoichiometric ratio varied according to pH and changed with time due to complex instability. 8-Hydroxyquinoline was able to chelate copper with the stoichiometry 2:1. Obviously, weaker activity and complex instability of non-substituted 8-hydroxyquinoline can be assigned to the absence of electronegative halogen(s) in the aromatic ring when compared with its congeners clioquinol and chloroxine. The latter compounds have identical chelation curves, probably due to the fact that they have similar pK_a values. The difference between halogenated congeners and non-substituted 8-hydroxyquinoline can be of particular importance, since 8-hydroxyquinoline has more potent antiproliferative activity than clioquinol [21]. The explanation may consist in the observed instability of the copper complex with 8-hydroxyquinoline. In addition, the effect of 8-hydroxyquinoline can be even intensified at the low pH of tumors. Considering the competitive BCS assay, D-PEN was even less efficient than 8-hydroxyquinoline. In general, the weaker chelators D-PEN and 8-hydroxyquinoline were losing their chelation efficacy in more acidic conditions since the acid moiety has probably a lower tendency to liberate a proton in a more acidic environment. This phenomenon was apparent, particularly in the BCS assay, because this indicator had balanced activity at all tested pHs (Supplementary data — Fig. S2A).

The confrontation of this *in vitro* data with known clinical and *in vivo* data is of interest. Although both D-PEN and trientine are known to potentiate urine copper excretion in similar doses, their effect on copper in the organism seems to be markedly different [44]. In agreement with our data, where trientine was shown to be a more potent copper chelator than D-PEN, other studies confirmed a relatively low chelation effect of D-PEN. Trientine was markedly more active in mobilization of copper from albumin than D-PEN [33,44,46]. In addition, D-PEN may rather increase or tend to increase copper in the circulation during the first weeks of the therapy [47,48]. Whether this is associated with a risk of worsening of the neurological impairment in patients with Wilson's disease is unknown, but this risk is the highest in the case of D-PEN when compared with either trientine or zinc therapy [18,19,49]. Moreover, the impairment generally starts early after initiation of the therapy with D-PEN, in contrast to the latter treatment modalities [19]. The potent reducing properties of D-PEN were previously reported and suggested as the mechanism of copper mobilization in the treatment of Wilson's disease [50]. However, copper reduction may lead to increased oxidative stress. Indeed, induction of oxidative stress was observed in the brain after D-PEN treatment in an animal model [48].

High complex stability in the case of clioquinol, chloroxine and Na_2EDTA emphasized the chelation potency of these substances, because they were able to preserve their chelation activity in competition with BCS. This finding should be compared with *in vivo* conditions. Recent reports suggested that clioquinol can deplete cells of zinc, but it acts as a copper shuttle, thus transporting copper inside the cells [51,52]. It is highly possible that copper specific intracellular chaperons, like Ccs1, with higher affinity for copper ions, may be responsible for this paradoxical effect [52].

In conclusion, we report here a simple, rapid, inexpensive and precise methodology for copper chelation screening. We confirmed that Na_2EDTA, clioquinol and chloroxine are potent copper chelators at all tested pH conditions, but trientine is only in neutral or slightly acidic conditions. D-PEN appears to be a quite weak copper chelator, which can even cause reduction of cupric ions.

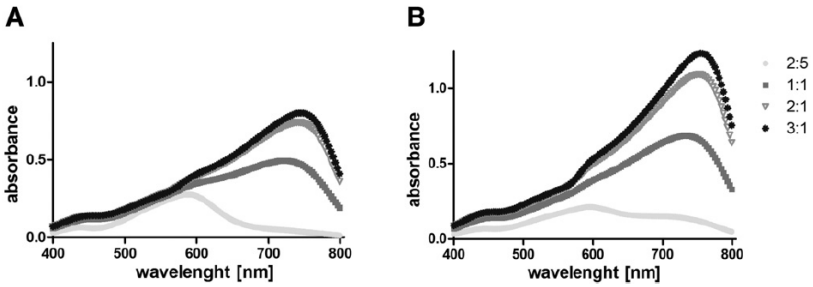

Fig. 7. Influence of hematoxylin on complex formation at 3 min (A) and 20 min (B). Concentration ratios, hematoxylin to copper, are shown in the legend. The absorbance markedly increased in higher hematoxylin to copper ratios (2:1 and 3:1) when 3-min and 20-min measurements were compared.

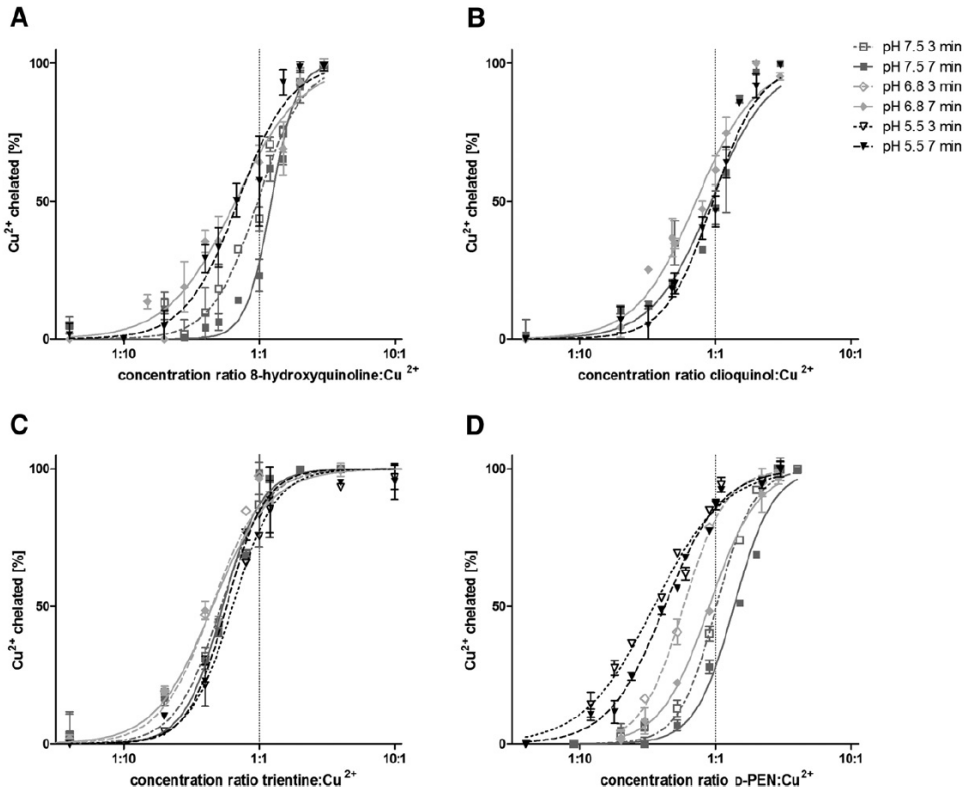

Fig. 8. Cupric ions chelation under different pH conditions, hematoxylin methodology. 8-Hydroxyquinoline (A), clioquinol (B), trientine (C), and D-PEN (D).

Abbreviations

BCS	bathocuproinedisulfonic acid disodium salt
D-PEN	D-penicillamine
ROS	reactive oxygen species
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Acknowledgments

This study was supported by the Charles University (GAUK 605712C and SVV 267 003). We would like to thank Prof. Gerald Blunden for the corrections.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2013.02.011.

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NOVEL METHOD FOR RAPID COPPER CHELATION ASSESSMENT CONFIRMED LOW AFFINITY OF D-PENICILLAMINE FOR COPPER IN COMPARISON WITH TRIENTINE AND 8-HYDROXYQUINOLINES

JOURNAL OF INORGANIC BIOCHEMISTRY

SUPPLEMENTARY DATA

5 pages

Michal Říha, Jana Karlíčková, Tomáš Filipský, Kateřina Macáková, Radomír Hrdina, Přemysl Mladěnka

Faculty of Pharmacy in Hradec Králové

Charles University in Prague

Heyrovského 1203

500 05 Hradec Králové

Czech Republic

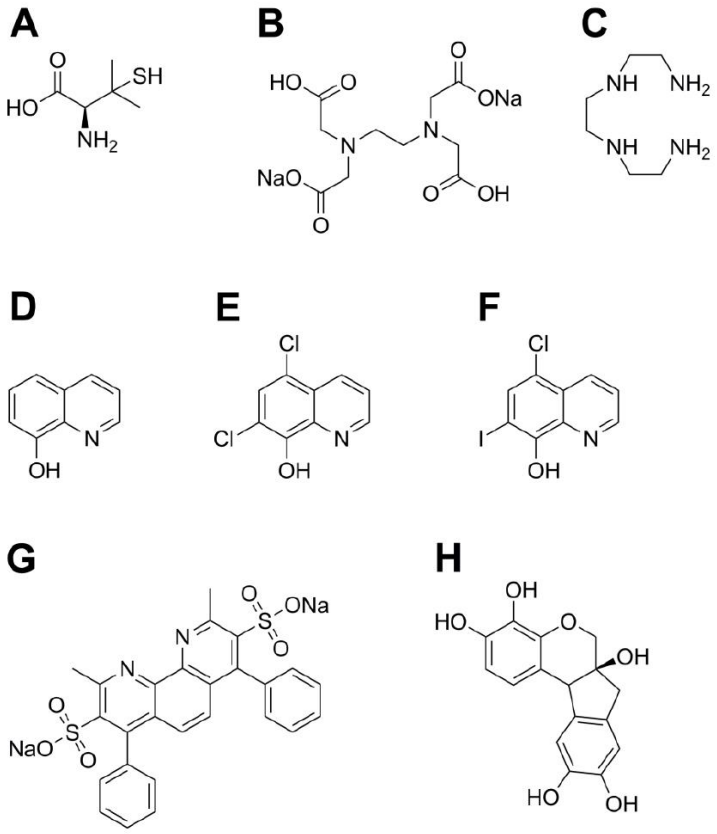

Fig. S1. Chemical structures of the tested substances (**A-F**) and the indicators used for the assays (**G** and **H**). D-PEN (**A**), Na_2EDTA (**B**), trientine (**C**), 8-hydroxyquinoline (**D**), chloroxine (**E**), clioquinol (**F**), BCS (**G**), and hematoxylin (**H**).

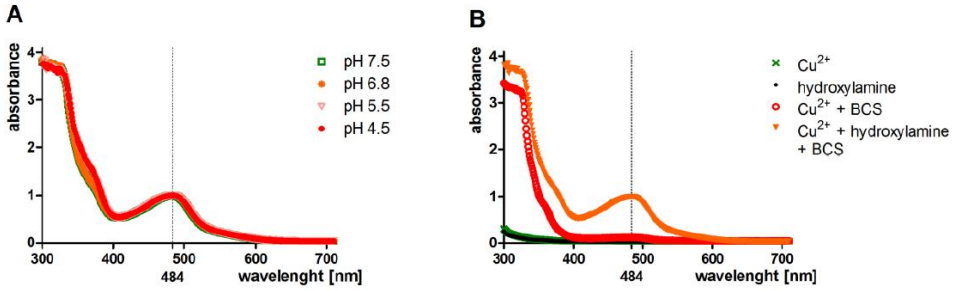

Fig. S2. Absorption spectra of BCS-Cu^{+} complex in the presence of hydroxylamine (**A**). Supplementary absorption spectra at pH 6.8 (**B**). Final concentrations were: 100 µM /Cu^{2+}/, 1 mM /BCS/, and 20 mM /hydroxylamine/. Data show results measured at 5 min interval.

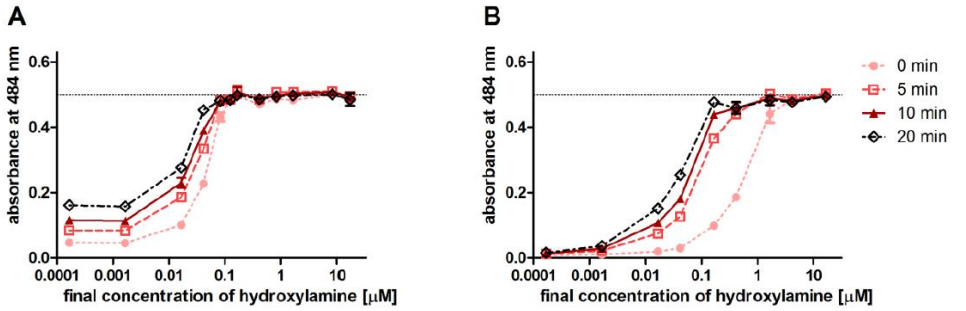

Fig. S3. Effect of different concentrations of hydroxylamine in time: cuprous ions, pH 7.5 (**A**), and cuprous ions, pH 4.5 (**B**).

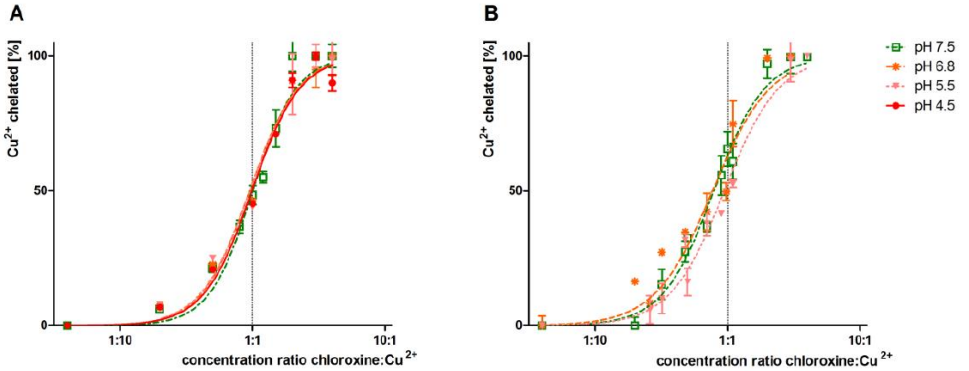

Fig. S4. Chelation of cupric ions by chloroxine: BCS (**A**) and hematoxylin (**B**) methodology.

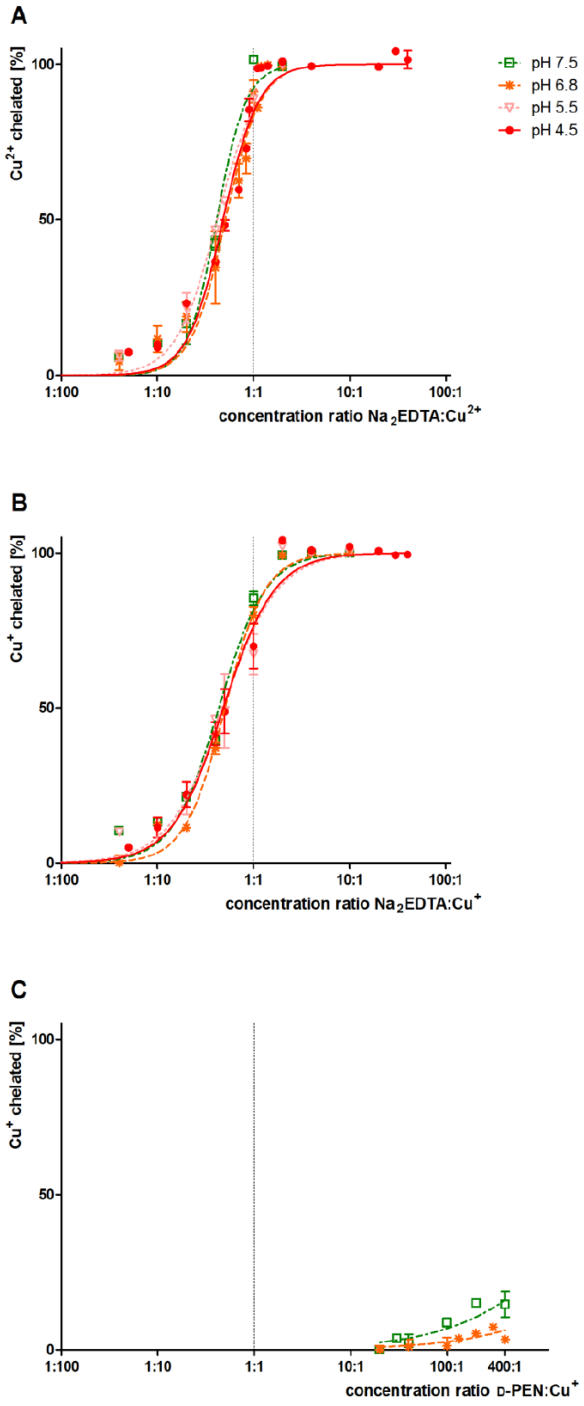

Fig. S5. Copper ions chelation by Na_2EDTA (**A-B**) compared with D-PEN (**C**).

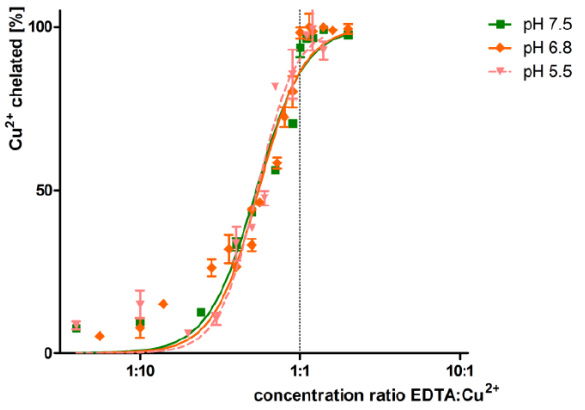

Fig. S6. Chelation of cupric ions by Na_2EDTA(hematoxylin methodology).

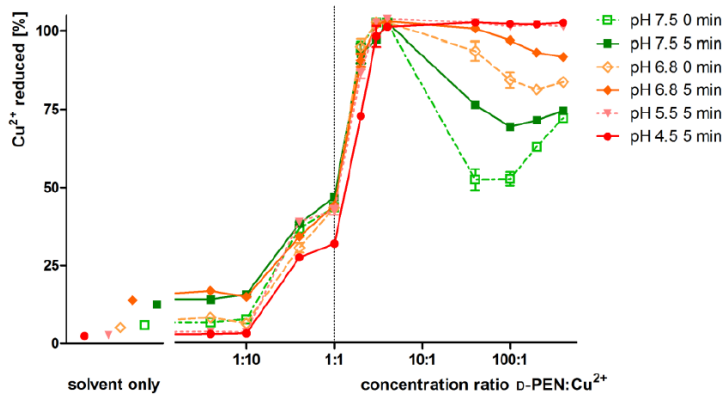

Fig. S7. Reducing properties of D-PEN. Data for pH 4.5 and 5.5 at 0 min were identical at 5 min and are not shown for better comprehensibility.

4.3. *In vitro* evaluation of copper-chelating properties of flavonoids

ŘÍHA, M.; KARLÍČKOVÁ, J.; FILIPSKÝ, T.; MACÁKOVÁ, K.; ROCHA, L.; BOVICELLI, P.; PROIETTI SILVESTRI, P.; SASO, L.; JAHODÁŘ, L.; HRDINA, R.; MLADĚNKA, P. *In vitro* evaluation of copper-chelating properties of flavonoids. *RSC Advances*. 2014, **4**(62), 32628-32638.

(IF 2013: 3,708)

RSC Advances

PAPER

Cite this: *RSC Adv.*, 2014, 4, 32628

In vitro evaluation of copper-chelating properties of flavonoids†

Michal Říha,[a] Jana Karlíčková,[b] Tomáš Filipský,[a] Kateřina Macáková,[b] Liliana Rocha,[ac] Paolo Bovicelli,[d] Ilaria Proietti Silvestri,[e] Luciano Saso,[f] Luděk Jahodář,[b] Radomír Hrdina[a] and Přemysl Mladěnka*[a]

Copper is an essential trace element involved in plenty of redox reactions in living systems, however, unbound copper ions cause damage to various biomolecules *via* excessive generation of reactive oxygen species. Flavonoids, ubiquitous plant secondary metabolites, possess complex effects on human health and chelation of transient metal ions is one of their proposed mechanisms of action. In this *in vitro* study, 26 flavonoids from various subclasses were screened for their interactions with both copper oxidation states at four (patho)physiologically relevant pH conditions (4.5, 5.5, 6.8 and 7.5) by two spectrophotometric approaches and compared with the clinically used copper chelator trientine. In a slightly competitive environment, the majority of flavonoids were able to chelate cupric ions, however, under more competitive conditions, only flavones and flavonols were able to chelate both cupric and cuprous ions. Apparently, the 2,3-double bond was essential for stable copper chelation. The most efficient copper chelation sites were the 3-hydroxy-4-keto group in flavonols and the 5,6,7-trihydroxyl group in flavones. On the other hand, the 3′,4′-dihydroxyl group was associated only with a weak activity. 3-Hydroxyflavone, kaempferol and partly baicalein were even more potent than trientine in the acidic environment, however, none of the tested flavonoids was able to surpass it at physiological pH or slightly acidic conditions. In conclusion, flavonoids possessing appropriate structural characteristics were efficient copper chelators and some of them were even more potent than trientine under acidic conditions.

Received 15th May 2014
Accepted 16th July 2014

DOI: 10.1039/c4ra04575k

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Introduction

Flavonoids represent a large group of polyphenolic compounds which belong to plant secondary metabolites. Their ubiquity in the plant kingdom and high content especially in fruits and vegetables make them a common component of the human diet. Flavonoids are 2-phenyl-1-benzopyran-4-one derivatives which can be classified into several subclasses according to their basic chemical structure: flavones, flavonols, flavanones, flavanonols, and flavanols. Plenty of *in vitro*, *in vivo* and several epidemiological studies have suggested their health-promoting effects. Miscellaneous properties including antioxidant, antimicrobial, anti-inflammatory, anticancer, vasoactive or hepatoprotective may be responsible for this.[1–3] The former theories explaining the effects only by direct scavenging of reactive oxygen species (ROS) seem to be overcome because flavonoids are able to chelate transient metals (particularly iron and/or copper), to block several enzymes producing ROS, and to specifically interact with other targets (direct antiaggregatory and vasodilatory potential) as well.[4] While the interactions of flavonoids with iron, enzymes and specific targets have been extensively studied (reviewed in ref. 4), data on interactions of flavonoids with copper are rather sparse. Quercetin and rutin were mostly examined and only a few other compounds have been tested.[5–10] As far as we know, no complex comparison of copper-chelating properties of flavonoids has been published yet.

Copper is an essential trace metal whose function in living systems is based predominantly on its ability to change between two oxidation states, a cuprous and a cupric one. Cuproenzymes are involved in many vital processes such as cell respiration,

[a]*Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic. E-mail: mladenkap@faf.cuni.cz; Fax: +420 495067170; Tel: +420 495067295*

[b]*Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic*

[c]*ERASMUS student at [a]; home institution: Faculty of Pharmacy, University of Porto, Praça Gomes Teixeira, 4099-002 Porto, Portugal*

[d]*Department of Chemistry, Institute of Biology, Molecular Medicine and NanoBiotechnologies (IBMN), Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy*

[e]*Department of Chemistry, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy*

[f]*Department of Physiology and Pharmacology "Vittorio Erspamer", Sapienza University of Rome, Italy*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4ra04575k

ROS elimination, biosynthesis of neurotransmitters and connective tissue.[11] The fate of copper in the human body is complex and has not been fully understood. However, a meticulous regulation of copper homeostasis is necessary because unbound copper ions participate in the formation of ROS due to the catalysis of the Fenton-like reaction which consequently causes damage to various biological structures.[12] A typical example of copper homeostasis disorder is Wilson's disease, which is caused by the chronic copper overload due to a specific genetic abnormality.[13] Despite some controversy, the disruption of copper homeostasis is involved in various pathological states including neurodegenerative diseases, tumours and acute myocardial infarction.[14–17]

The chelation approach is promising in pathological states associated with the excess or dysregulation of transition metals, however, the clinical use of copper chelators is currently restricted only for the treatment of Wilson's disease. On the other hand, the use of copper-chelating agents is absolutely essential in this case: until the effective pharmacological treatment based on D-penicillamine was available, Wilson's disease was fatal. The research of copper chelators has not been so successful up to now since currently only two other copper chelators, trientine and tetrathiomolybdate, are used in the treatment of Wilson's disease.[13] Interestingly, although D-penicillamine is considered as a standard copper chelator in the clinical practice, it is a much less potent copper chelator than trientine.[18] The research of novel copper chelators for the other above mentioned disorders, in particular that of the central nervous system, is ongoing but most compounds are still in the phase of experimental testing.

When screening metal chelators, several other factors should be considered, *e.g.* lipophilicity of the parent compound and the complex, affinity for the both cupric and cuprous ions, participation of the complex in redox chemistry, influence by pH because lower than physiological pH are characteristic for both physiological conditions (copper absorption in the duodenum) and several pathologies (tumours, acute myocardial infarction and inflammation), and the last but not the least the potential toxicity of the compound.

Therefore this study was aimed at a detailed *in vitro* evaluation of copper-chelating properties of various flavonoids which are generally considered as non-toxic. Their chelation efficiency was assessed at four pH conditions ranging from 4.5 to 7.5, towards both cupric and cuprous ions, and was compared with trientine, a clinically used copper chelator. The emphasis was given especially on the elucidation of the structure–activity relationship. Additionally, the relative stability of the formed complexes was assessed.

Materials and methods

Reagents and solutions

Stock solutions of cupric ions (cupric sulphate pentahydrate, $CuSO_4 \cdot 5H_2O$) and cuprous ions (cuprous chloride, CuCl) were prepared in water (Milli-Q RG, Merck Millipore, Massachusetts, U.S.A.) or in the aqueous solution of 0.1 M HCl and 1 M NaCl, respectively. The corresponding fresh working solutions (0.25 mM) were prepared by dilution in DMSO (BCS method) or distilled water (hematoxylin method). Hydroxylamine hydrochloride and bathocuproinedisulfonic acid disodium salt (BCS) were dissolved in distilled water. Hematoxylin was dissolved in DMSO and its working solution (0.25 mM) was usable for no longer than 90 min. All flavonoids were dissolved in DMSO, while trientine in distilled water.

Experiments were performed in 15 mM buffers, acetate (pH 4.5 and 5.5) and HEPES (pH 6.8 and 7.5).

Luteolin-4′-*O*-glucoside was purchased from Extrasynthese (France). Mosloflavone and negletein were synthesized by a convergent synthesis starting from chrysin according to the previous report.[19] All other chemicals were purchased from Sigma-Aldrich (Germany).

Copper chelation assessment

Two spectrophotometric methods were used for the screening of copper-chelating properties: an assay based on BCS and a complementary assay using hematoxylin. These methods were previously reported in the details.[18]

The hematoxylin assay: different concentrations of a tested compound were mixed for 2 min with cupric ions in the presence of a buffer. Non-reacted cupric ions were mixed for next 3 min with the indicator hematoxylin and the absorbance was measured thereafter and after other 4 min. Different wavelengths were used according to pH: 595 nm (pH 5.5), 590 nm (pH 6.8), and 610 nm (pH 7.5), as reported earlier.[18]

A similar approach was used in the BCS assay: different concentrations of a tested compound were mixed with cupric or cuprous ions in an excess of buffer and mixed for 2 min. In the case of cuprous ions, hydroxylamine was added before the copper solution in order to retain copper in its reduced state. In the case of cupric ions, hydroxylamine was added after mixing by virtue of reduction of non-reacted cupric ions. The non-chelated copper was then evidenced in both cases by the indicator BCS and absorbance was read immediately and after 5 min at 484 nm.

All experiments were performed in 96-well microplates, at least in duplicates, at room temperature. A Synergy HT Multi-Detection Microplate Reader (BioTec Instruments, Inc., USA) was used for all spectrophotometric measurements.

Statistical analysis

The amount of remaining copper was calculated from the difference of absorbance between the tested sample (with the indicator) and its corresponding blank (without indicator) divided by the difference of the control sample (the known amount of copper without the tested substance) and its control blank.

The dose-dependent curves with 95% confidence (prediction) intervals were constructed by GraphPad Prism 6 for Windows (GraphPad Software, USA). Each curve of an efficient substance was composed from at least four points: first minimal point was approximately 0–5% chelation, last point was maximal chelation. Maximal chelation was considered 95–100% for high-affinity chelation or lower in cases when plateau bellow this value was found. The efficiency of the copper

chelation was calculated at the concentration ratio 1 : 1 and 10 : 1, substance : copper, respectively, according to the curve equations:

$$y = \max/(1 + 10\exp((\log ER_{50} - x) \times k))$$

where y is the amount of chelated copper ions in per cent; max the maximal chelation of copper ions in per cent; ER_{50} the effective substance : copper concentration ratio needed to chelate 50% of the copper; x the common logarithm of the substance : copper concentration ratio; and k the slope of the curve.

In the hematoxylin assay, ER_{50} were used for the comparison of the chelation potencies. The corresponding SD values were computed from the relation between confidence intervals and SD.[20]

Because the used indicators act virtually as copper chelators, the competition between the tested compound and the indicator occurs. When the compound forms unstable complexes, it is possible to assess the relative stability of the substance : copper complex. The value of relative stability s was calculated as follows:

$$s = y_2/y_0$$

where y_2 is the percentage of chelated copper in the second measurement (after 4 or 5 min in the hematoxylin or BCS methodology, respectively), and y_0 is the amount of chelated copper ions at first measurement.

Data are expressed as mean ± SD. Differences were considered significant at $p < 0.05$, unless stated otherwise. The differences in chelation and relative stability of copper complexes were performed by one-way ANOVA test followed by Bonferroni's multiple comparisons test.

Results

Firstly, we assessed copper-chelating properties of all 26 flavonoids (for chemical structures see Fig. S1†) by the use of the hematoxylin assay because the methodology is less competitive than that of BCS and therefore less efficient copper chelators may be revealed as well.[18]

Hematoxylin assay

All tested flavonoids excluding non-substituted flavone and 7-hydroxyflavone were able to chelate copper in this complementary assay (Fig. 1). Although this methodology is not principally aimed at the determination of the stoichiometry, the stoichiometry could be assessed in the case of potent copper chelators. *E.g.*, if ER_{50} equals to 1, this mean that at the ratio 1 : 1 about 50% of copper was chelated, thus at the ratio 2 : 1, flavonoid to copper, respectively, about 100% of copper will be chelated and therefore the stoichiometry of the complex would be 2 : 1. Analogously, ER_{50} of 0.25 suggests the stoichiometry of 1 : 2 and 0.5 that of 1 : 1. In the case of low-affinity chelators, the ER_{50} is apparently higher to fit in a possible chelation stoichiometry and thus the stoichiometry cannot be assessed in such cases.

Flavones luteolin and baicalein and flavonol myricetin were the most potent compounds. They were more active than trientine and their ER_{50} values were approximately 0.25 suggesting the complex stoichiometry 1 : 2 (flavonoid : copper). Trientine is well known to form a complex 1 : 1 stoichiometry and herein the expected ER_{50} of 0.5 was confirmed. In contrast to trientine which formed the same complex at all pH conditions, luteolin formed likely the complex 1 : 1 at pH 5.5 while the stoichiometry of complexes with baicalein and myricetin was on average between 1 : 1 and 1 : 2 at this pH and thus it could not be established with certainty from the current experiment. Quercetin formed likely complexes with stoichiometry 1 : 2 at pH 6.8 as well but such complexes were not stable at pH 7.5 and 5.5. At the latter pH, apparently 1 : 1 stoichiometry was observed.

The ER_{50} around 0.5 was observed in the case of negletein and baicalin at pH 6.8 and 7.5 and in rutin at pH 6.8 suggesting that these flavonoids are able to form complexes with stoichiometry 1 : 1. Again, their affinity for cupric ions at pH 5.5 was lower in comparison to slightly acidic or physiological pH conditions.

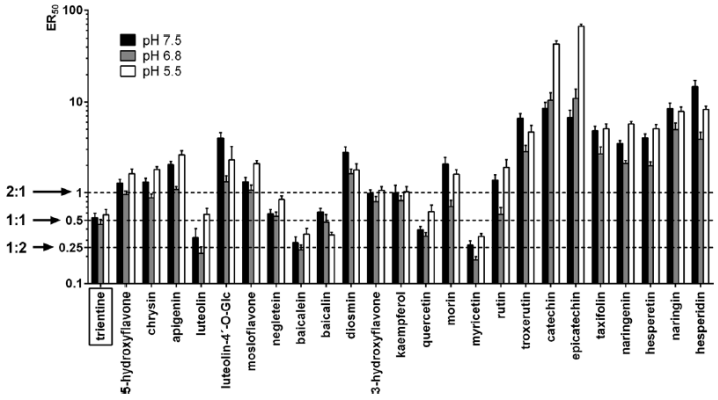

Fig. 1 The chelation of cupric ions by flavonoids and trientine according to the hematoxylin assay. Data are expressed as ER_{50} values.

The ER_{50} of 1, implicating stoichiometry 2 : 1, was observed in the case of flavonols without the catechol ring B (3-hydroxyflavone and kaempferol) at all tested pH conditions. Flavones without the catechol ring B including 5-hydroxyflavone, chrysin, apigenin, mosloflavone and luteolin-4′-*O*-glucoside formed the complexes with cupric ions at pH 6.8 with stoichiometry 2 : 1.

In the cases of flavanones and flavanols, the stoichiometry cannot be established because these flavonoids have high ER_{50} indicating their lower affinity to copper.

The active flavonoids formed stable complexes at pH 5.5 and 6.8, however at pH 7.5, the relative stability depended on the ratio of the flavonoid to copper ions and it dropped with decreasing ratio (Fig. S2†). The exceptions were baicalein, 3-hydroxyflavone and luteolin which formed the stable complexes even at pH 7.5.

Structure–activity relationship

The 5,6,7-trihydroxyl or 6,7-dihydroxyl; 3′,4′-dihydroxyl; 3-hydroxyl-4-keto or 5-hydroxy-4-keto functional groups were associated with cupric chelation. In order to highlight the relationships between structural modifications and the activity, following schemes (Fig. 2–4) were prepared.

In the hematoxylin methodology, the chelating groups were evidently responsible for a majority of the differences in copper chelation, *e.g.*:

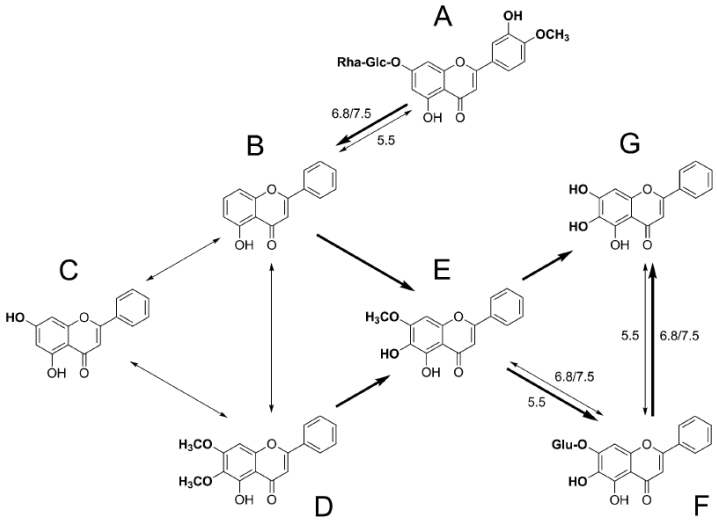

Fig. 2 The effect of different substitution in ring A in flavones. (A) Diosmin, (B) 5-hydroxyflavone, (C) chrysin, (D) mosloflavone, (E) negletein, (F) baicalin, and (G) baicalein. The direction of an arrow shows the relationship between compounds: a bold one-way arrow means a significant difference ($p < 0.05$), a two-way arrow the same potency. Glc: glucose, Glu: glucuronic acid, Rha: rhamnose.

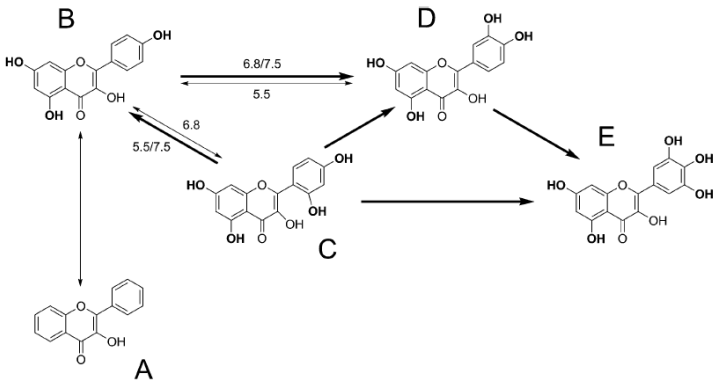

Fig. 3 The effect of a hydroxyl substitution in flavonols. (A) 3-Hydroxyflavone, (B) kaempferol, (C) morin, (D) quercetin, and (E) myricetin. The direction of an arrow shows the relationship between compounds: a bold one-way arrow means a significant difference ($p < 0.05$), a two-way arrow the same potency.

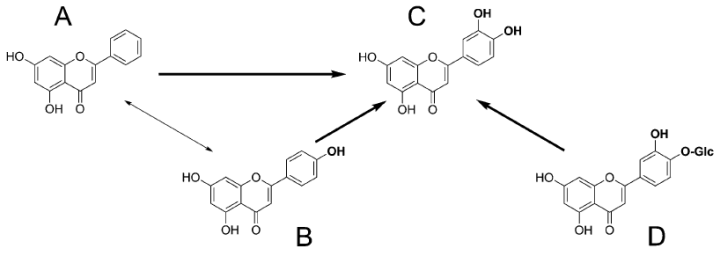

Fig. 4 The effect of the substitution in ring B in flavones. (A) Chrysin, (B) apigenin, (C) luteolin, and (D) luteolin-4′-*O*-glucoside. The direction of an arrow shows the relationship between compounds: a bold one-way arrow means a significant difference ($p < 0.05$), a two-way arrow the same potency.

• three adjacent hydroxyl groups in ring A or in ring B were associated with better copper chelation than two adjacent hydroxyl groups (Fig. 2 and 3);

• the catechol moiety in ring B increased the chelation in comparison with an isolated hydroxyl group, in particular at pH 6.8 and 7.5 (Fig. 3 and 4);

• the 3-hydroxy-4-keto chelation site apparently improved copper chelation as the blockade of the 3-hydroxyl group decreased the copper chelation potential (quercetin was more potent than rutin and troxerutin). The effect of that chelation site was confirmed even in the absence of the 2,3-double bond: flavanols with the catechol ring B were less potent than taxifolin possessing both catechol ring B and the 4-keto group under acidic conditions (Fig. 1);

• similarly, the 5-hydroxy-4-keto chelation site was associated with the copper chelation activity (*e.g.* non-chelating flavone *vs* chelating 5-hydroxyflavone).

The non-chelating groups influenced only slightly the resulting chelation effect with few exceptions:

• the adjacent hydroxyl group in the position 2′ decreased the chelation potential of the 3-hydroxy-4-keto chelation site (Fig. 3C *vs.* 3D);

• in flavanones, a sugar in the position 7 significantly reduced the copper chelation potential (Fig. 1), similarly, a sugar in the position 7 in flavones decreased the chelation potential of the 5-hydroxy-4-keto chelation site (Fig. 2F *vs.* 2G);

• contrarily, a sugar in the position 7 was more advantageous than a methoxyl group in this position at least at pH 5.5 (Fig. 2F *vs.* 2E).

BCS assay

In this assay, all 24 flavonoids which chelated copper ions in the hematoxylin method were tested. In contrast to the former assay, only the members of flavones and flavonols were able to substantially chelate copper in this competitive approach including BCS. This suggested that the 3′,4′-dihydroxyl group alone was insufficient for copper chelation in the competitive conditions in contrast to the previously mentioned slightly competitive hematoxylin approach. A blocking of the catechol group in the case of luteolin-4′-*O*-glucoside or diosmin abolished absolutely the remaining activity. For an overview, the cupric chelation ability of active flavonoids was compared at the ratios of 1 : 1 and 10 : 1 (flavonoid : copper) (Fig. 5). All tested compounds showed generally higher activity towards cupric ions, but the results are in general analogous for cuprous ions (Fig. S3†).

In most cases, the chelation ability significantly dropped with increasing acidity of the environment and the majority of flavonoids chelated hardly any or no copper ions at pH 4.5. Interestingly, in contrast to the hematoxylin method or the chelation curves of trientine, the majority of tested flavonoids were not able to chelate 100% of the copper even at very high concentration ratios (the representative examples are shown in Fig. S4†). Rather a plateau was observed in majority of flavonoids. This is apparent from the Fig. 5 where the copper chelation of 3-hydroxyflavone or kaempferol increased from the ratio of 1 : 1 to 10 : 1 while baicalein mostly reached its maximal chelation approximately at the ratio of 1 : 1.

None of the tested flavonoids was more potent than trientine at the physiological or slightly acidic conditions even if 3-hydroxyflavone and partly as well kaempferol were similarly active at the ratio of 10 : 1. However, under the acidic conditions, the both mentioned flavonols were more potent than trientine at the ratio of 10 : 1 for chelation of the both cupric and cuprous ions. Baicalein, due to its inability to chelate 100% of copper, surpassed trientine only at pH 4.5 (Cu^{2+} at the ratio of 1 : 1, Cu^{+} at the ratio of 10 : 1) while at pH 5.5 it was more active chelator of cuprous ions and non-inferior chelator of cupric ions. All other tested flavonoids were less efficient than trientine at all conditions.

An additional analysis was performed with active copper chelators, *i.e.* those with chelation higher than 30% at the ratio of 10 : 1 (Fig. S5†). The relative stability of flavonoids was independent on pH, the only exception was baicalein which formed less stable complexes at more acidic conditions. 3-Hydroxyflavone, kaempferol, quercetin and myricetin formed stable complexes at all tested pH conditions with the relative stability comparable with complexes of trientine at pH 6.8/7.5 (Fig. S5†). On the other hand, the complexes of other assessed flavones were significantly less stable than in the case of trientine. The described phenomenon was independent on the oxidation state of copper ions.

Structure–activity relationship

In contrast to the hematoxylin methodology, the impact of different substitutions was more complex and markedly

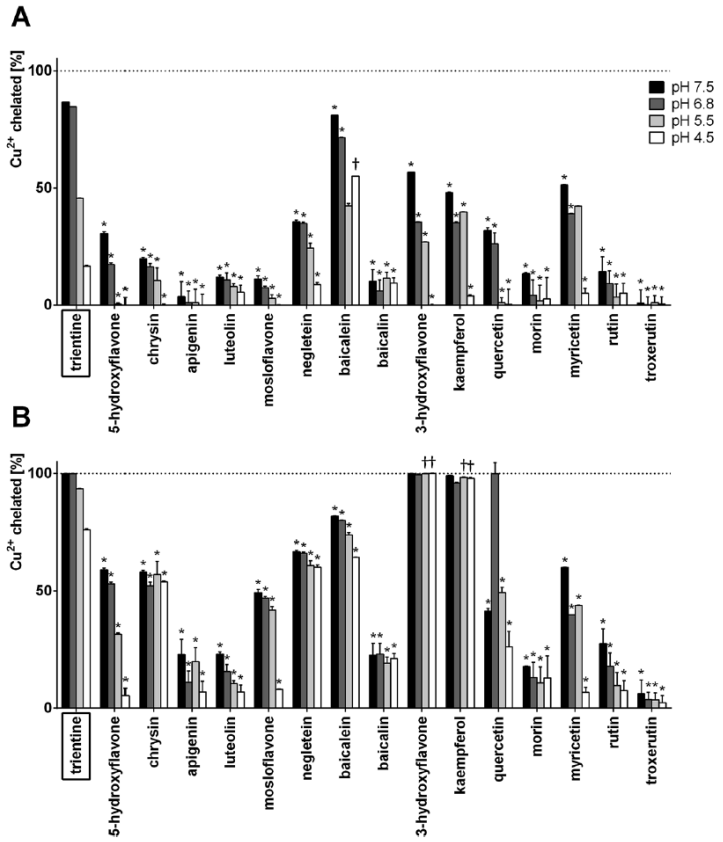

Fig. 5 The chelation of cupric ions by flavonoids in comparison with trientine, at the ratios of 1 : 1 (A) and 10 : 1 (B) (flavonoid : copper). * Less potent than trientine, † more potent than trientine ($p < 0.05$).

dependent on copper oxidation state and pH (Fig. 6–9). The most important findings are:

- a substitution of the free 3-hydroxyl group was associated with a marked decrease in the chelation activity (Fig. 6);
- a presence of adjacent hydroxyl groups in ring A increased the chelation potency while a methoxyl group or a sugar had negative influence on it (Fig. 7). On the contrary, a hydroxyl substitution in ring B had no influence (Fig. 8A *vs.* 8B) or even deteriorated the activity (Fig. 8 and 9);

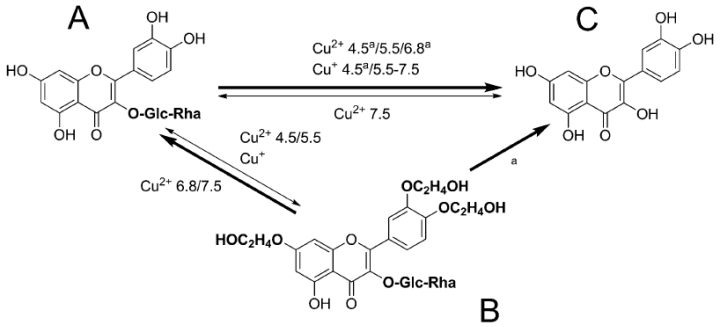

Fig. 6 The effect of the 3-hydroxyl group and additional hydroxyl groups in flavonols. (A) Rutin, (B) troxerutin, (C) quercetin, [a] significance was found for pH 4.5 and cuprous ions at the ratios higher than 10 : 1 (flavonoid : copper, respectively). The direction of an arrow shows the relationship between compounds: a bold one-way arrow means a significant difference ($p < 0.05$), a two-way arrow the same potency. Glc: glucose, Rha: rhamnose.

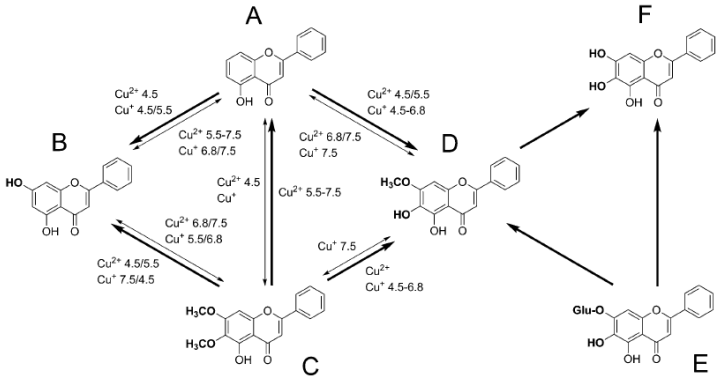

Fig. 7 The effect of different substitution in ring A in flavones. (A) 5-Hydroxyflavone, (B) chrysin, (C) mosloflavone, (D) negletein, (E) baicalin, and (F) baicalein. The direction of an arrow shows the relationship between compounds: a bold one-way arrow means a significant difference ($p < 0.05$), a two-way arrow the same potency. Glu: glucuronic acid.

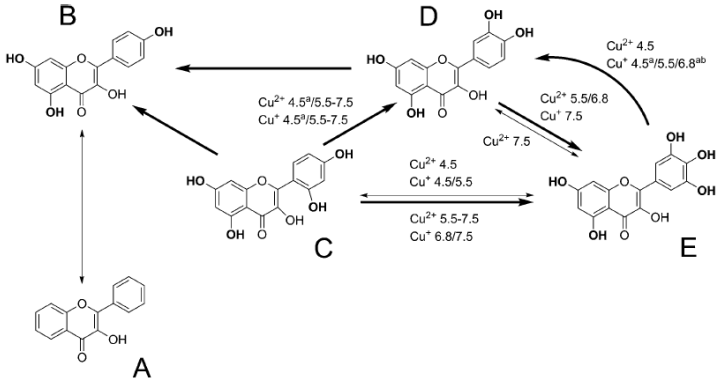

Fig. 8 The effect of hydroxyl substitution in flavonols. (A) 3-Hydroxyflavone, (B) kaempferol, (C) morin, (D) quercetin, (E) myricetin, [a] significance was found at the ratios higher than 10 : 1 (flavonoid : copper), [b] at the ratios lower than 4 : 1 (flavonoid : copper) myricetin was more potent than quercetin. The direction of an arrow shows the relationship between compounds: a bold one-way arrow means a significant difference ($p < 0.05$), a two-way arrow the same potency.

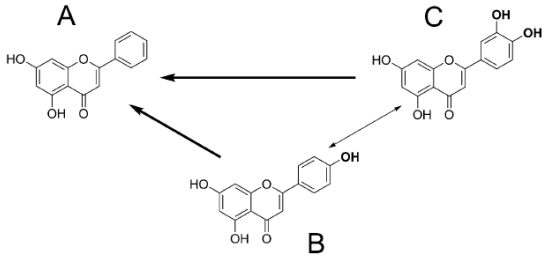

Fig. 9 The effect of substitution in ring B in flavones. (A) Chrysin, (B) apigenin, and (C) luteolin. The direction of an arrow shows the relationship between compounds: a bold one-way arrow means a significant difference ($p < 0.05$), a two-way arrow the same potency.

• a hydroxyl group in the position 2′ weakened the effect of the essential 3-hydroxy-4-keto chelation site (Fig. 9C *vs.* 9B).

Discussion

While flavonoids-iron interactions have been studied in a detailed way,[21–30] such attention has not been focused on copper. This study demonstrated that flavonoids across different subclasses were able to chelate copper ions, however, in a very variable manner. The chelating sites for copper does not substantially differ from that of iron or other metals.[22,31] In harmony with published data on the both copper and iron chelation, the most important chelation sites were 3-hydroxy-4-keto, 5-hydroxy-4-keto, 3′,4′-dihydroxyl (*i.e.* the catechol ring B), and (5),6,7-(tri)dihydroxyl substitution in ring A. Isolated keto, hydroxyl and methoxyl groups were not associated with metal chelation.

Contrary to the widespread application of a direct spectrophotometry in the analysis of metal–chelator interactions,[32,33] we used an indirect spectrophotometric approach.[18] Contrary to the direct spectrophotometry, a competition between the indicator, which is in fact a strong cuprous chelator BCS, and a tested compound can be useful for the assessment of the formed complex relative stability. While the complementary hematoxylin method is suitable for basic screening of copper chelation ability and suggestion of complex stoichiometry, the assay employing BCS reveals the potency of a chelator.

Although there are important drawbacks of the hematoxylin method including decreasing affinity of hematoxylin for copper with decreasing pH and resulting impossibility to use the method at pH 4.5, its outcomes concerning suggestion of the stoichiometry appears to be real and in agreement with available literary sources.[18] Because the affinity of hematoxylin for copper is high at pH 7.5, only very active copper chelators are able to form stable complexes at this pH. This was true for powerful copper chelators of flavonoid class as 3-hydroxyflavone and baicalein. Contrarily, the assessment of copper–flavonoid stoichiometry can be easily measured as well in moderately active chelators at pH 6.8 or pH 5.5 because of lower affinity of hematoxylin for copper at these pH conditions. This is supported by the fact that all flavonoid–copper complexes were stable at pH 5.5 and 6.8 in contrast to pH 7.5 (Fig. S2†).

The stoichiometry of the complexes is apparently based on the number and type of functional groups. At slightly acidic pH of 6.8, 3-hydroxyflavone and kaempferol with only one possible 3-hydroxy-4-keto chelation site formed the complexes of 2 : 1, flavonoid to copper, similarly to flavones with analogous 5-hydroxy-4-keto chelation group (5-hydroxyflavone, chrysin, apigenin, mosloflavone and luteolin-4′-glycoside). Luteolin and quercetin possessing both (3-hydroxy)-5-hydroxy-4-keto and the catechol ring B were able to chelate 2 copper atoms per molecule similarly to myricetin equipped with three adjacent hydroxyl groups in ring B and 3-hydroxy-5-hydroxy-4-keto chelation site. Rutin, although possessing the identical chelation site as luteolin, formed only complexes with 1 : 1 stoichiometry. The likely reason is the steric hindrance of the sugar in the position 3. Specific cases are flavones with 5,6- and 7-hydroxyl groups forming complexes of 1 : 1 stoichiometry in case of the presence of only two hydroxyl groups (baicalin and negletein) or chelating 2 molecules of copper per unit in case of the presence of all three hydroxyl groups (baicalein).

By decreasing pH from 6.8 to 5.5, a marked drop in the chelation activity of flavonoids with the catechol ring B, the 5-hydroxy-4-keto and even the 5,6-dihydroxyl/5,6,7-trihydroxyl chelation sites was observed among all classes of flavonoids. The catechol ring B is promoted by the majority of researchers as the most important due to its best direct scavenging potential.[34,35] However, its metal chelating activity is substantial only at physiological pH and particularly at alkaline conditions[9,22,31] in contrast to other possible chelation sites, which arises from different pK_a values of hydroxyl groups in the flavonoid structure.[36]

A similar instability is apparently valid for chelation sites in ring A or between the rings A and C. The only pH-independent chelation site was the 3-hydroxy-4-keto site as was clearly demonstrated by 3-hydroxyflavone and kaempferol, whose chelation activity was stable at all pH including pH 7.5. Interestingly, myricetin, the only flavonoid tested in this study with three adjacent hydroxyl groups in ring B behaved as well as a pH-independent copper chelator. Sparse data from the literature confirm our findings. Quercetin formed complexes of 1 : 1 or 1 : 2 stoichiometry (flavonoid : copper). A complex of 2 : 1 was not documented by potentiometric and spectrophotometric measurements but may exist likely in a smaller amount at more acidic conditions, as was documented by highly sensitive electrospray ionisation mass spectrophotometry (ESI-MS).[7,8,10] Rutin is firstly forming complexes with 1 : 1 stoichiometry followed by a disproportionation process leading to 2 : 1 (rutin : copper) complex.[7] Other groups found complexes of quercetin with copper of 1 : 1 stoichiometry in non-buffered conditions which may be associated with the fact that the catechol ring B may not be available at these conditions in contrast to physiological, slightly basic pH where deprotonation of catechol hydroxyls is taking place.[10] It must be mentioned too that the study of de Souza reported complexes of flavonoids with copper in the stoichiometry of 1 : 2 for quercetin, 2 : 3 for rutin and 1 : 1 for 3-hydroxyflavone. The reason for this discrepancy is not known but authors documented identical stoichiometries for flavonoid–iron complexes and those are not in agreement with other studies.[22,37]

Although flavanols and flavanones tested in this study possess the catechol ring B and/or the 5-hydroxy-4-keto or even the 3-hydroxy-4-keto-5-hydroxy chelation site, their copper chelation activity was clearly low and unequivocally lower than that of their close congeners from flavone or flavonol groups. Similarly, spectrophotometric approach did not detect spectral changes in the case of adding cupric ions to flavanones naringenin and naringin at pH 5.5 and 7.4 while the spectrum of flavanol catechin was changed only in the case of pH 7.4. Interestingly, no change was surprisingly observed in the case of taxifolin,[9] which possesses identical substitution pattern to catechin and 4-keto group in addition, and was shown to be more active than catechin.[38] In contrast, highly sensitive ESI-MS even at pH 3 found some complexes of catechin and naringenin.[8] Although authors did not test other mentioned flavonoids, it is apparent, that these flavonoids are able to form only unstable complexes with copper. The reason consists in the lack of the 2,3-double bond, which influences the planarity of the molecule and enables delocalization of π–electronic system.[39] It is suggested that copper ions prefer planar or tetrahedral conformation in contrast to octahedral geometries in the case of iron.[6] This may be relevant, as the stoichiometric complex of 3 : 1, flavonoid to copper, was not observed in this study. Such a complex was found by ESI-MS in low quantities and its formation is clearly less probable.[8,40]

The instability of the catechol ring B and of the 5-hydroxy-4-keto chelation site was further confirmed by the highly competitive BCS methodology. Compounds possessing only the catechol ring B were almost inefficient while those with the latter were apparently less active than in the hematoxylin method. Such finding was observed earlier in smaller sets of

flavonoids, where copper was removed from luteolin and rutin complexes after addition of EDTA while only partly from quercetin.[5,10] Although, a decrease in the copper chelation potency comparing the hematoxylin and the BCS methodology was observed in chelators with the 3-hydroxy-4-keto group as well, these flavonoids were clearly the most powerful chelators followed by baicalein with three hydroxyl groups in ring A. The importance of the 3-hydroxy-4-keto group is supported by:

(1) mathematical calculations which supposed that the 3-hydroxy-4-keto group is the preferred copper chelation site even in the simultaneous presence of the 5-hydroxyl group or the 3′,4′-dihydroxyl group in ring B;[40]

(2) other studies analysing the effect of flavonoids on other metals which confirmed the importance of the 3-hydroxy-4-keto group, although in alkaline media, the catechol might be preferred.[22,31]

Interestingly, the hydroxyl groups in ring B may represent a disadvantage in contrast to the direct scavenging potential. This was demonstrated in the highly competitive approach with BCS. Neither a catechol nor a pyrogallol moiety in ring B improved the chelation. Moreover, these substituents even deteriorated the ability (Fig. 8 and 9). The reason may be paradoxical but important. Probable explanation of this negative effect lies in a reduction of Cu^{2+} ions, which is substantial by a catechol or a pyrogallol structure in ring B.[9] Because the affinity for cuprous ions is lower in flavonoids and very high in the case of BCS, flavonoids with highly reducing properties are not able to retain the cuprous ions in the complex structure in competition with the strong indicator BCS. This is in a clear contrast to powerful copper chelators like trientine at pH 6.8/7.5 or chloroxine at all tested pH conditions.[18] We are currently performing experiments on copper reduction and in line with previous data,[9] copper reduction is highly dependent on number of hydroxyl groups in ring B and may therefore influence the copper chelation too.

Similarly, the reduction may explain the failure of baicalein to be the most active copper chelator like in the case of iron, where its affinity for iron was comparable to the standard iron chelator deferoxamine.[22] Although the chelation ability was growing with the number of free hydroxyl groups in ring A (Fig. 7) and baicalein with the 5,6,7-trihydroxyl groups represented the most potent flavone, it was clearly less efficient than 3-hydroxyflavone. The major difference is that 3-hydroxyflavone or kaempferol were able to chelate 100% of copper in highly competitive environment with BCS while baicalein did not. The influence of methoxylation of hydroxyl groups in ring A was more pronounced than in the case of iron chelation. On one hand, a methoxyl group instead of the corresponding hydroxyl group abolished the effect of the hydroxyl group which could be observed in the series baicalein > negletein > mosloflavone, but on the other hand mosloflavone possessing the 6,7-dimethoxyl groups was even weaker chelator than the basic flavone, 5-hydroxyflavone, under certain conditions. Steric hindrance was probably responsible for this finding. This may be supported by the fact that baicalin with the attached glucose moiety in the position 7 was less potent in comparison with baicalein and even negletein. The 7-hydroxyl group in chrysin favoured the chelation especially under the acidic conditions when compared with 5-hydroxyflavone and mosloflavone.

Although these findings may be relevant to both pharmacology and physiology because flavonoids are common part of our food, the influence of flavonoids on copper pharmacokinetics in human will need additional studies. The fact that 3-hydroxyflavone and kaempferol were powerful copper chelators and their effect was under the acidic conditions even more pronounced than that of trientine, a copper chelator used in a clinical practice for the treatment of Wilson's disease,[41] may be of a clinical relevance. Indeed, trientine is active particularly at neutral and slightly acidic conditions[18] and because various physiological and pathophysiological aspects (*e.g.* GIT absorption, tumours, inflamed or ischaemic tissue) influence the acidity of different compartments in the human body, these flavonoids may be possibly advantageous in several pathophysiological situations. However, their pharmacokinetics, which may result in the inactivity, must be taken into account.[42] None of the tested flavonoids was selective to either oxidation state of copper ions, the majority of them showed higher affinity for the oxidized form. Similar finding was documented in the study with the iron chelation.[22] But in contrast to the fate of iron in the organism, which is quite well known, the copper pharmacokinetics and in particular the importance of its oxidation state is a subject of debate.[43] At the moment, even the data on copper absorption are divergent. It appears that the copper transporter CTR1 recognize cuprous ions but the absorption of copper in the form of cupric ions cannot be fully excluded.[43] Complexes of copper with very active flavonoids 3-hydroxyflavone and kaempferol may lead to either decreased or increased copper absorption depending on possible transport mechanisms and charge. While 3-hydroxyflavone is a synthetic compound and serves as a model molecule, kaempferol is commonly found in plant-derived food and in plants used in traditional medicine[44] and thus may have potential influence on copper (patho)physiology. On the other hand, other flavonoids with low copper chelation ability together with powerful copper reducing properties may reduce cupric ions present in food into cuprous ones and thus influence the absorption. Some studies analysing these factors are currently being performed in our laboratory. Limited available experimental data in rats give rather equivocal results: feeding with tea or its polyphenols did not affect the absorption of copper,[45] neither did the administration of rutin and catechin[46] while in another study, rutin decreased copper content in the liver.[47] Another interesting factor is the anti- or pro-oxidant activity of copper–flavonoid complexes. A recent study found that copper complexes of flavonoids were more potent antioxidants than non-complexed flavonoids.[10] However, there is no accordance on this finding in the literature,[6] and this fact together with possible influence of flavonoids on the copper driven Fenton reaction should be analysed in the future.

Conclusion

This study demonstrated that the potency of copper chelation is very different among various flavonoids. Although almost all

flavonoids are able to chelate copper, in the presence of the competitive indicator, their copper chelation potency was rather low in majority of cases. Some compounds, particularly those containing the 3-hydroxyl group in association with the 4-keto group and the 2,3-double bond (3-hydroxyflavone, kaempferol) or possessing the 5,6,7-trihydroxyl substitution (baicalein) were, however, very potent even in highly competitive environment. Their activity in lower pH conditions was even higher than clinically used copper chelator trientine. On the other hand, the catechol ring B did not represent significant advantage for the activity, moreover, it may deteriorate the copper chelation under certain conditions, probably through participating in reduction of copper ions. The copper reduction properties must be taken into account for the complex evaluation of flavonoid–metal interactions.

Abbreviations

BCS	Bathocuproinedisulfonic acid disodium salt
ESI-MS	Electrospray ionisation mass spectrophotometry
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
ROS	Reactive oxygen species

Acknowledgements

This study was supported by the grant of Czech Science Foundation (project no. P303/12/G163) and by Charles University in Prague (grants no. 605712C and 1220314B; and project no. SVV 260 064).

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IN VITRO EVALUATION OF COPPER-CHELATING PROPERTIES OF FLAVONOIDS

SUPPLEMENTARY DATA

6 pages

Michal Říha, Jana Karlíčková, Tomáš Filipský, Kateřina Macáková, Liliana Rocha, Paolo Bovicelli, Ilaria Proietti Silvestri, Luciano Saso, Luděk Jahodář, Radomír Hrdina and Přemysl Mladěnka*

*Corresponding author:

Assoc. Prof. Přemysl Mladěnka, Ph.D.

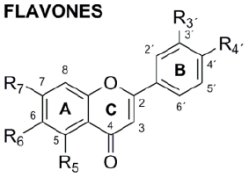
Department of Pharmacology and Toxicology

Faculty of Pharmacy in Hradec Králové

Charles University in Prague

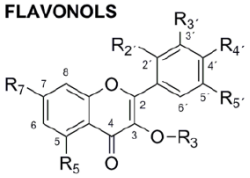
Heyrovského 1203

FLAVONES



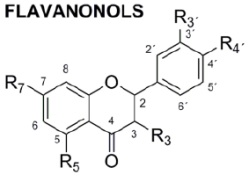
	R_5	R_6	R_7	$R_{3'}$	$R_{4'}$
flavone	H	H	H	H	H
5-hydroxyflavone	OH	H	H	H	H
7-hydroxyflavone	H	H	OH	H	H
chrysin	OH	H	OH	H	H
apigenin	OH	H	OH	H	OH
luteolin	OH	H	OH	OH	OH
luteolin-4′-O-Glc	OH	H	OH	OH	O-Glc
mosloflavone	OH	OCH_3	OCH_3	H	H
negletein	OH	OH	OCH_3	H	H
baicalein	OH	OH	OH	H	H
baicalin	OH	OH	O-Glu	H	H
diosmin	OH	H	O-Glc-Rha	OH	OCH_3

FLAVONOLS



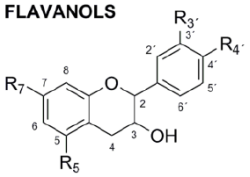
	R_3	R_5	R_7	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
3-hydroxyflavone	H	H	H	H	H	H	H
kaempferol	H	OH	OH	H	H	OH	H
quercetin	H	OH	OH	H	OH	OH	H
morin	H	OH	OH	OH	H	OH	H
myricetin	H	OH	OH	H	OH	OH	OH
rutin	Glc-Rha	OH	OH	H	OH	OH	H
troxerutin	Glc-Rha	OH	O-C_2H_4-OH	H	O-C_2H_4-OH	O-C_2H_4-OH	H

FLAVANONES/ FLAVANONOLS



	R_3	R_5	R_7	$R_{3'}$	$R_{4'}$	configuration
naringenin	H	OH	OH	H	OH	2RS
naringin	H	OH	O-Glc-Rha	H	OH	2RS
hesperetin	H	OH	OH	OH	OCH_3	2S
hesperidin	H	OH	O-Glc-Rha	OH	OCH_3	2S
taxifolin	OH	OH	OH	OH	OH	2R, 3R

FLAVANOLS



	R_5	R_7	$R_{3'}$	$R_{4'}$	configuration
(-)-epicatechin	OH	OH	OH	OH	2R, 3R
(+)-catechin	OH	OH	OH	OH	2R, 3S

Fig. S1 Chemical structures of the tested flavonoids. Glc: glucose, Glu: glucuronic acid, Rha: rhamnose.

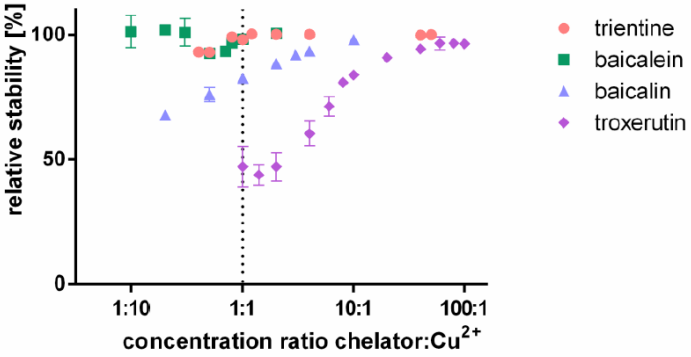


Fig. S2 The representative examples of stable (trientine, baicalein) and unstable (baicalin, troxerutin) copper-chelator complexes, evaluated by the hematoxylin method. The values express mean percent relative stability of flavonoids at all tested pH conditions and that of trientine at pH 6.8 and 7.5 due to instability of its complexes under acidic conditions.

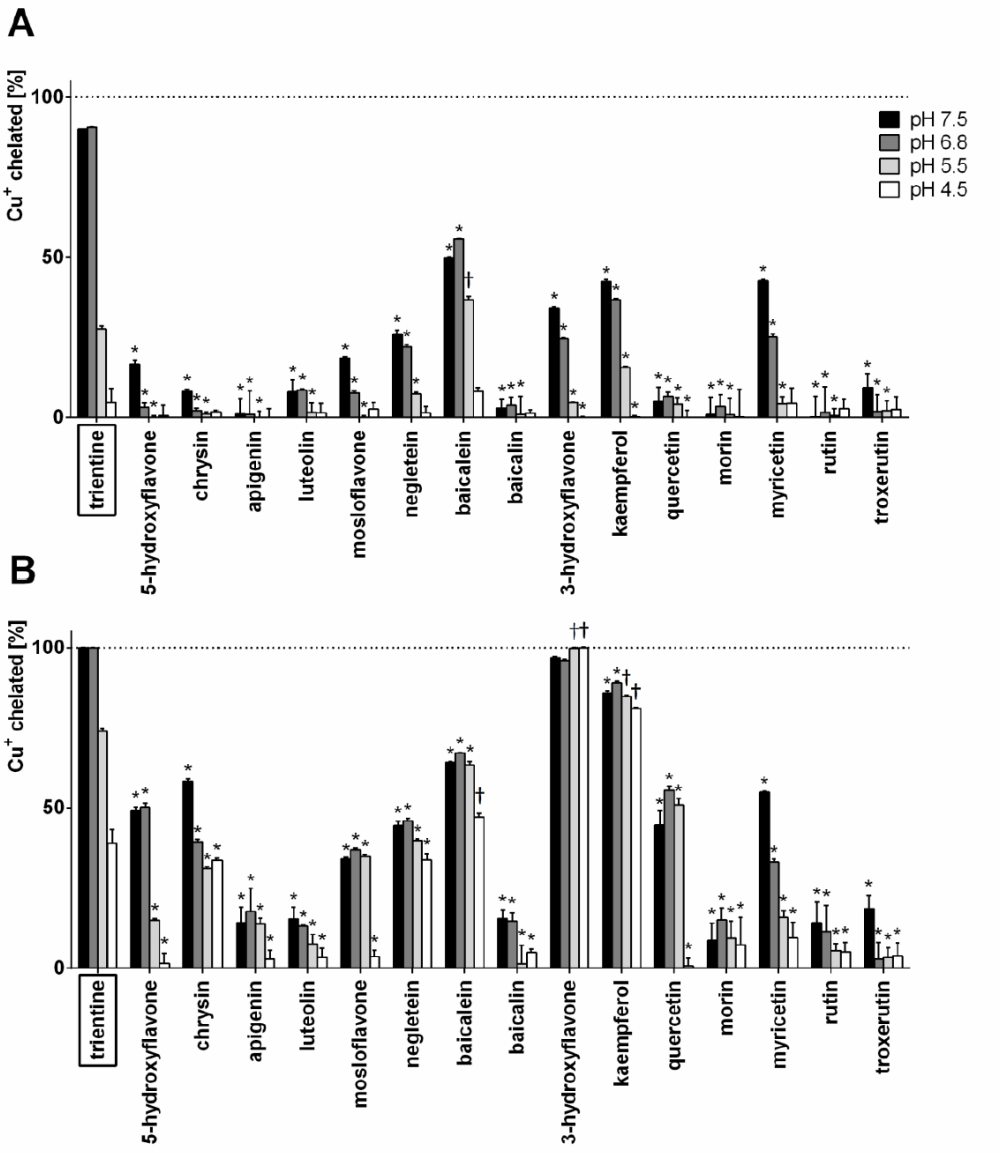


Fig. S3 The chelation of cuprous ions by flavonoids, compared with trientine, at the ratios of 1:1 (A) and 10:1 (B) (flavonoid:copper). * Less potent than trientine, † more potent than trientine ($p < 0.05$).

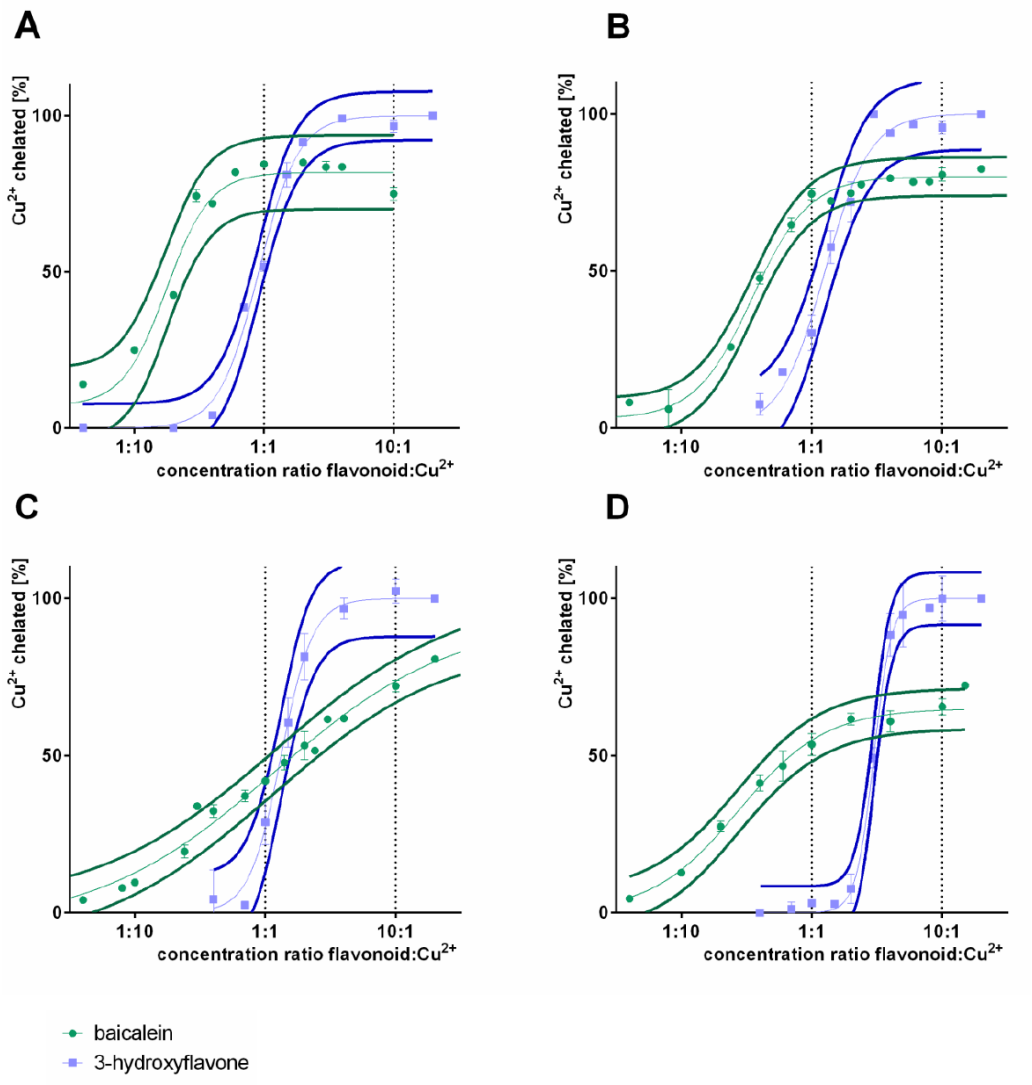


Fig. S4 The comparison of cupric chelation properties of baicalein and 3-hydroxyflavone according to the pH conditions: pH 7.5 (A), 6.8 (B), 5.5 (C), and 4.5 (D). Different character of chelation curves was observed: while the chelation by baicalein reached a plateau at the concentration ratio of 1:1, flavonoid to copper, respectively, in most cases, the chelation of 3-hydroxyflavone increased up to 100% between the concentration ratios of 1:1 and 10:1.

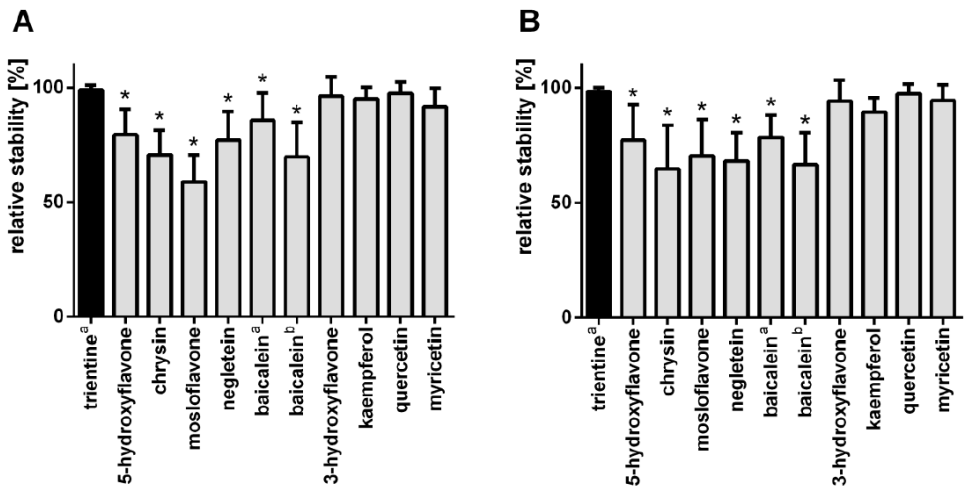


Fig. S5 The relative stability of complexes compared with those of trientine. (A) Cupric ions, (B) cuprous ions, [a] pH 6.8 and 7.5, [b] pH 4.5 and 5.5. * Less stable than the complexes of trientine ($p < 0.05$).

4.4. Iron reduction potentiates hydroxyl radical formation only in flavonols

MACÁKOVÁ, K.; MLADĚNKA, P.; FILIPSKÝ, T.; ŘÍHA, M.; JAHODÁŘ, L.; TREJTNAR, F.; BOVICELLI, P.; PROIETTI SILVESTRI, I.; HRDINA, R.; SASO, L. Iron reduction potentiates hydroxyl radical formation only in flavonols. *Food Chemistry*. 2012, **135**(4), 2584-2592.

(IF 2013: 3,259)

Food Chemistry 135 (2012) 2584–2592

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

Iron reduction potentiates hydroxyl radical formation only in flavonols

Kateřina Macáková [a], Přemysl Mladěnka [b,*], Tomáš Filipský [b], Michal Říha [b], Luděk Jahodář [a], František Trejtnar [b], Paolo Bovicelli [c], Ilaria Proietti Silvestri [c,d], Radomír Hrdina [b], Luciano Saso [e]

[a] Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[c] Institute of Biomolecular Chemistry of CNR, Piazzale Aldo Moro 5, 00185 Rome, Italy
[d] Department of Chemistry, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy
[e] Department of Physiology and Pharmacology "Vittorio Erspamer", Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy

ARTICLE INFO

Article history:
Received 17 April 2012
Received in revised form 13 June 2012
Accepted 27 June 2012
Available online 14 July 2012

Keywords:
Flavonoids
Iron
Pro-oxidant
Antioxidants
Fenton reaction
Flavonols
Hydroxyl radical

ABSTRACT

Flavonoids, substantial components of the human diet, are generally considered to be beneficial. However, they may possess possible pro-oxidative effects, which could be based on their reducing potential. The aims of this study were to evaluate the ability of 26 flavonoids to reduce ferric ions at relevant pH conditions and to find a possible relationship with potentiation of hydroxyl radical production. A substantial ferric ions reduction was achieved under acidic conditions, particularly by flavonols and flavanols with the catecholic ring B. Apparently corresponding bell-shaped curves displaying the pro-oxidant effect of flavonols quercetin and kaempferol on iron-based Fenton reaction were documented. Several flavonoids were efficient antioxidants at very low concentrations but rather inefficient or pro-oxidative at higher concentrations. Flavonols, morin and rutin were progressively pro-oxidant, while 7-hydroxyflavone and hesperetin were the only flavonoids with dose-dependent inhibition of hydroxyl radical production. Conclusively, administration of flavonoids may lead to unpredictable consequences with few exceptions.

1. Introduction

Flavonoids represent substantial components of human diet and although GIT metabolism and absorption largely limits their bioavailability, the daily food intake can cause significant increase in their human plasma levels (Scalbert & Williamson, 2000). Flavonoids have been largely promoted for their broad potential health benefits in the past and thus markedly consumed by patients in the form of herbal medicines or dietary supplements. However, no definite and unequivocal evidence concerning their clinical effects has been reported so far (Chahar, Sharma, Dobhal, & Joshi, 2011; Galati & O'Brien, 2004; Mladenka, Zatloukalova, Filipsky, & Hrdina, 2010b). Their uncritically assessed positive effects were mostly attributed to their reactive oxygen species (ROS) scavenging activity, which was confirmed by many different antioxidant assays. On the other hand, substantial data have been published about their pro-oxidative properties too (Galati & O'Brien, 2004; Prochazkova, Bousova, & Wilhelmova, 2011; Sakihama, Cohen, Grace, & Yamasaki, 2002). Probably due to different approaches, it is still not clear which flavonoids and conditions can be associated with pro-oxidative properties. Moreover, the mechanism(s) of pro-oxidation action are still a topic of discussion. The involvement of redox-active transient metals, namely copper and iron, or enzymes in the generation of ROS by flavonoids has been suggested (Cao, Sofic, & Prior, 1997; Galati & O'Brien, 2004; Prochazkova et al., 2011; Sakihama et al., 2002). Interestingly, antioxidant effects are sometimes measured by ferric ions reduction at acidic pH (originally designed as "ferric reduction ability of plasma", today usually named "ferric reducing antioxidant power" or simply FRAP). There are very good correlations between this assay and redox potential and sufficient correlations with other antioxidant assays (Firuzi, Lacanna, Petrucci, Marrosu, & Saso, 2005; Zhang et al., 2011). Thus, although this assay clearly reflects the reducing potential of the tested compounds and the majority of antioxidants expresses a certain degree of activity in this assay (Pulido, Bravo, & Saura-Calixto, 2000), one very important factor is neglected – possible risk of potentiation of Fenton chemistry. During the Fenton reaction, ferrous ion catalyses production of hydroxyl radical, while being simultaneously oxidised to ferric ion. Therefore, ferrous ions recovery by a reductant may intensify Fenton reaction (Aruoma, Murcia, Butler, & Halliwell, 1993), hence rendering FRAP assay pathophysiologically less relevant. Similar pathways of reactions were documented for copper as well (Sakihama et al., 2002). It need not be

* Corresponding author. Tel.: +420 495067295; fax: +420 495067170.
E-mail address: mladenkap@faf.cuni.cz (P. Mladěnka).

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http://dx.doi.org/10.1016/j.foodchem.2012.06.107

emphasised that the reducing properties of ascorbic acid are commonly used for the potentiation of the Fenton reaction (Aruoma et al., 1993; Laughton, Halliwell, Evans, & Hoult, 1989).

The first aim of this study was to evaluate ferric ions reducing properties of flavonoids at selected (patho)physiologically relevant pHs. The second aim was to find the relationship between ferric ions reducing properties of flavonoids and potentiation of the Fenton reaction. The choice of the tested pHs was based on the fate of iron and flavonoids in the human organism and relevant pathological states. For example, low pH facilitates iron absorption in the proximity of the gastro-duodenal junction, lysosomes are important organelles for iron kinetics and ischaemia markedly disturbs physiological pH. Similarly, lower pH was documented in tumours (Ambrosio, Zweier, Jacobus, Weisfeldt, & Flaherty, 1987; Donovan & Andrews, 2004; Kurz, Terman, Gustafsson, & Brunk, 2008; Parolini et al., 2009).

2. Materials and methods

2.1. Reagents

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium salt (ferrozine), ferrous sulphate heptahydrate, ferric chloride hexahydrate, hydroxylamine, dimethyl sulphoxide (DMSO), sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), HEPES sodium salt, salicylic acid, 1,4-dioxane, 2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids and all tested flavonoids (Supplementary data, Fig. S1), with exception of negletein and mosloflavone, were purchased from Sigma–Aldrich (Steinheim, Germany). Negletein and mosloflavone were synthesised through a convergent synthesis starting from chrysin, as was previously reported (Righi et al., 2010). Deferoxamine was purchased from Novartis (Basle, Switzerland) and methanol for HPLC was from JT Baker (Phillipsburg, NJ).

2.2. Assessment of ferric ions reduction potential

The degree of iron reduction was established by use of ferrozine methodology as previously reported by us (Mladenka et al., 2010a). Ferrozine forms a magenta-coloured complex with ferrous ions and this reaction is specific to these ions because ferric ions do not react with ferrozine (Stookey, 1970). Hence, the assessment of ferric ions reduction potential can be easily established in experiments, in which ferric ions were mixed with flavonoids together, and afterwards, the amount of reduced ferrous ions was evidenced by ferrozine.

Briefly, various 15 mM buffers (acetate buffers for pH 4.5 and 5.5 and HEPES buffers for pH 6.8 and 7.5) were firstly mixed with different concentrations of the tested compound dissolved in DMSO. Freshly prepared aqueous solution of ferric ions (50 μl, 250 μM) was added and mixture was stirred for 2 min. Afterwards, aqueous solution of ferrozine (50 μl, 5 mM) was added and absorbance was measured at 562 nm 5 min later by Anthos Reader 2010 (Anthos Labtec Instruments, Salzburg, Austria). The assay for non-buffered conditions was identical with the exception that no buffer was added into the mixture. In all sets of experiments, hydroxylamine (50 μl, 10 mM) was used as positive control (100% iron reduction) (Mladenka et al., 2010a).

2.3. Measurement of inhibition of iron mediated production of hydroxyl radicals

As generally known, ferrous ions react with hydrogen peroxide to produce hydroxyl radical (the Fenton reaction) (Halliwell & Gutteridge, 1999). The formed radical can be trapped by salicylic acid and its ensuing products (2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids) can be detected by HPLC (Nappi & Vass, 1998).

Briefly, ferrous ions were mixed with the tested compounds dissolved in methanol in different concentration ratios for 2 min. Salicylic acid and hydrogen peroxide (concentration of the both substances was 7 mM) were added subsequently, and afterwards, the mixture was analysed by HPLC (Philips PU 4100 pump, Philips, UK), Eclipse Plus C18 column (4.6 × 100 mm, 3.5 μm, Agilent, Santa Clara, CA), with UV–vis detector (Ecom LCD 2083; Ecom, Prague, Czech Republic), using 40% methanol and 0.085% aqueous solution of phosphoric acid as mobile phase. The only exception was taxifolin, because the peak of taxifolin interferes with dihydroxybenzoic acids at the abovementioned settings, the procedure of analysis was modified: mobile phase consisted of 0.1% aqueous solution of formic acid and methanol, gradient elution was used by increasing the methanol content from 5% to 15% within 5 min and afterwards to 40% within 10 min. All experiments were checked by addition of 'internal standard', i.e., known amounts of 2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids.

2.4. Statistical analysis

Results are expressed as mean ± SD. The differences among the tested substances were compared by use of one-way ANOVA test followed by Dunnett's multiple comparison test. Correlations were analysed by Pearson's test. All statistical analysis was performed by GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA).

3. Results

Firstly, ferric ions reductive properties of flavonoids were established at four (patho)physiologically relevant pHs and under non-buffered conditions as well. None of the tested flavonoids was able to reduce ferric ions at pH 7.5 or pH 6.8. At pH 5.5 only flavanols catechin, epicatechin and partly taxifolin were efficient reducing agents. At pH 4.5 and at non-buffered conditions the number of reducing flavonoids increased markedly. Only flavone, all tested monohydroxyflavones, flavones without the adjacent hydroxyl groups in ring A and the catecholic group in ring B (chrysin, apigenin, mosloflavone, diosmin), isoflavones (daidzein and genistein) and troxerutin were not able to significantly reduce ferric ions at any tested conditions. Comparing pH 4.5 and non-buffered conditions, the results were similar but not equal (full curves of the efficient flavonoids are depicted in Supplementary data, Figs. S2 and S3). Baicalein, which was a very potent reducing agent at non-buffered conditions but a relatively weak reducing agent at pH 4.5, and partly rutin, which demonstrated the opposite behaviour, were exceptions. The results summarising the maximal ferric ions reducing potential of flavonoids are shown in Fig. 1. The statistical analysis confirmed that the most potent reducing agents were epicatechin and catechin at all tested conditions.

The detailed statistical analysis among flavonols and flavanols (Fig. 2A) and flavones (Fig. 2B) emphasised that there was no direct relationship between the number of hydroxyl groups in ring A and/or ring B, and reducing activity. More likely, the localisation of the hydroxyl groups had the major role. The most efficient structural features included the 3-hydroxy group with catecholic ring B and 5,6-dihydroxy substitution. The role of the 2,3-double bond was variable depending on conditions. Its presence increased the reducing potential at pH 4.5 and decreased it at pH 5.5 and had no influence at non-buffered conditions. Contrarily, the presence of the 4-keto group diminished the ferric ions reducing potential of flavonoids under all conditions. The 3-hydroxyl group alone was not associated with reduction but its presence enhanced the reducing potential of reducing group(s). Interestingly, its

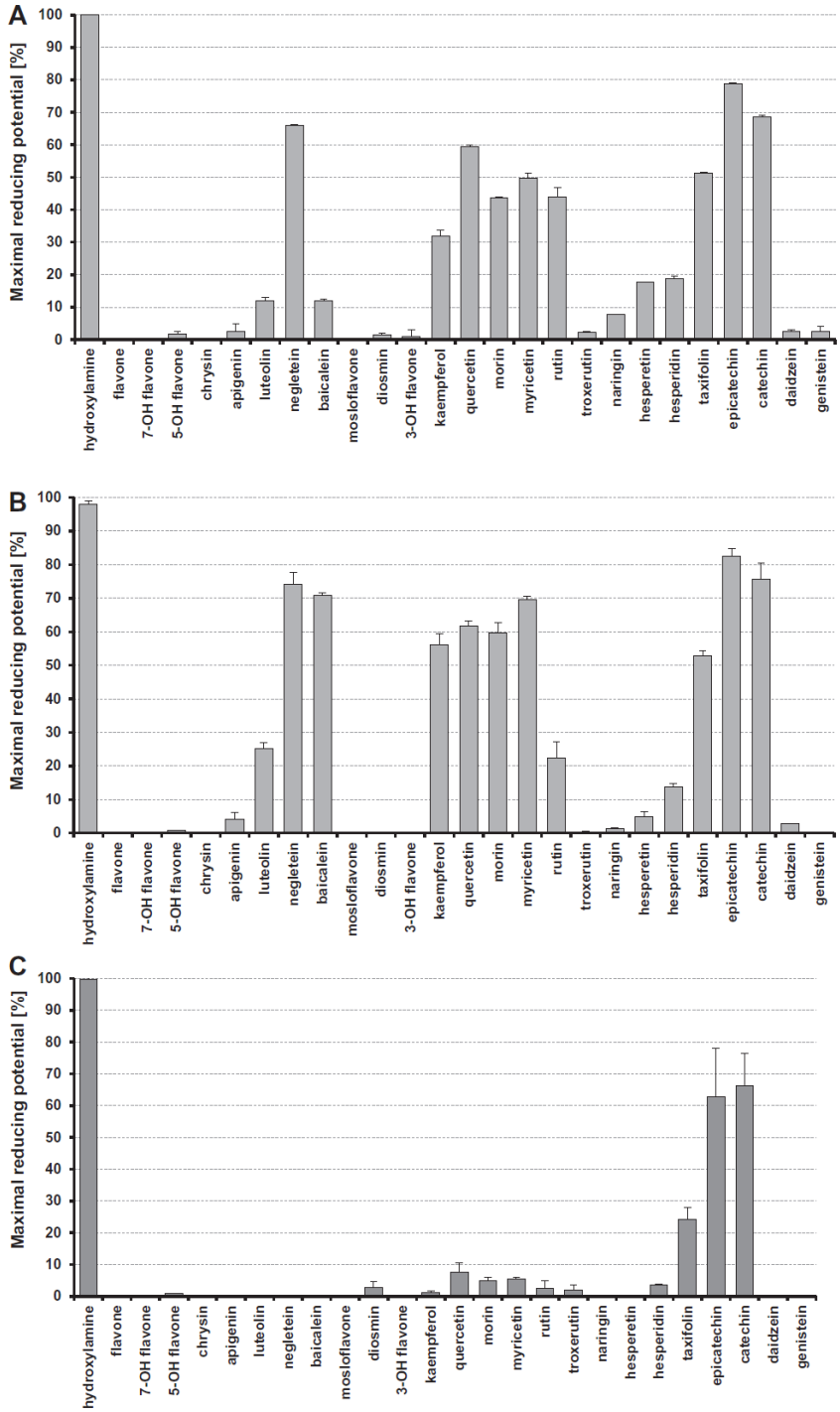

Fig. 1. Summary of the maximal ferric reducing potential of the tested flavonoids. (A) pH 4.5, (B) non-buffered conditions and (C) pH 5.5. The bars show the maximal percentage of the reduced ferric ions. The statistical comparison among the relevant flavonoids is shown in Fig. 2 for better clarity. Naringenin is not shown because the colour of its complex with iron interfered with ferrozine assay.

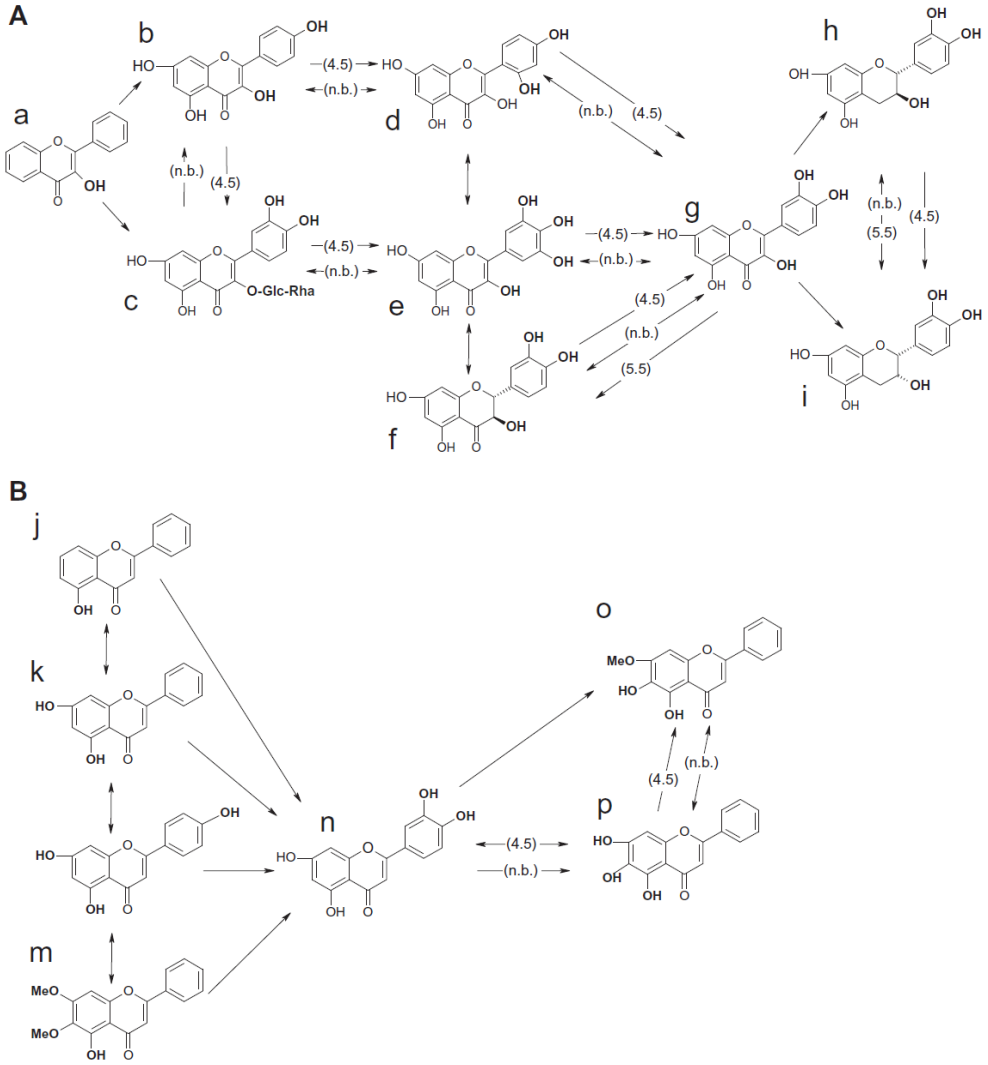

Fig. 2. Differences in the maximal ferric ions reducing potential of flavonols and flavanols (A) and flavones (B). a: 3-hydroxyflavone, b: kaempferol, c: rutin, d: morin, e: myricetin, f: taxifolin, g: quercetin, h: catechin, i: epicatechin, j: 5-hydroxyflavone, k: chrysin, l: apigenin, m: mosloflavone, n: luteolin, o: negletein and p: baicalein. Direction of arrows shows more powerful ferric ions reducing agent (at $p < 0.05$), double-head arrow means no significant difference. Flavonoids on the left side (a, j–m) did not produce any significant reduction potential. Because most of the tested flavonoids did not significantly reduce iron at pH 5.5, comparison at this pH is shown only among taxifolin, quercetin, catechin and epicatechin. n.b. – non-buffered conditions; 4.5 – at pH 4.5 and 5.5 – at pH 5.5.

configuration may possess some influence, since epicatechin was more efficient at pH 4.5 than catechin. The presence of three hydroxyl groups in ring B did not improve the reducing potential, rather a decrease was observed at pH 4.5. The 2′-hydroxyl group increased the reducing potential under acidic conditions but not under non-buffered conditions.

Similar to flavonols, the catecholic B ring was associated with enhanced ferric ions reduction in flavones. But interestingly, in contrast to flavonols, the monohydroxylated ring B did not evoke any significant reduction, supporting the role of the 3-hydroxyl group. The presence of the 5,6-dihydroxy group was linked with a significantly higher ferric ions reducing potential than the catecholic B ring in flavones. Again, the three adjacent hydroxyl groups in ring A did not improve the ferric ions reducing potential. Moreover, a marked decrease was observed under acidic conditions.

The curves illustrating the ferric ions reducing potential of different molar ratios of the tested flavonoid:Fe^{3+} (log scale) followed in most cases the bell-shaped character. This is because their ferric ions reducing potential rose with increasing concentration of the flavonoids up to a peak and thereafter dropped in a similar manner with further increase in the concentration (examples are shown in Fig. 3 and all curves of reducing flavonoids in Supplementary data, Figs. S2 and S3). At pH 4.5 the maximum reduction potential was achieved at a molar ratio of 1:1 (flavonoid:iron). Exceptions were flavones and flavonols with the catecholic or pyrogallol ring B (quercetin, myricetin and luteolin) where the maximum was

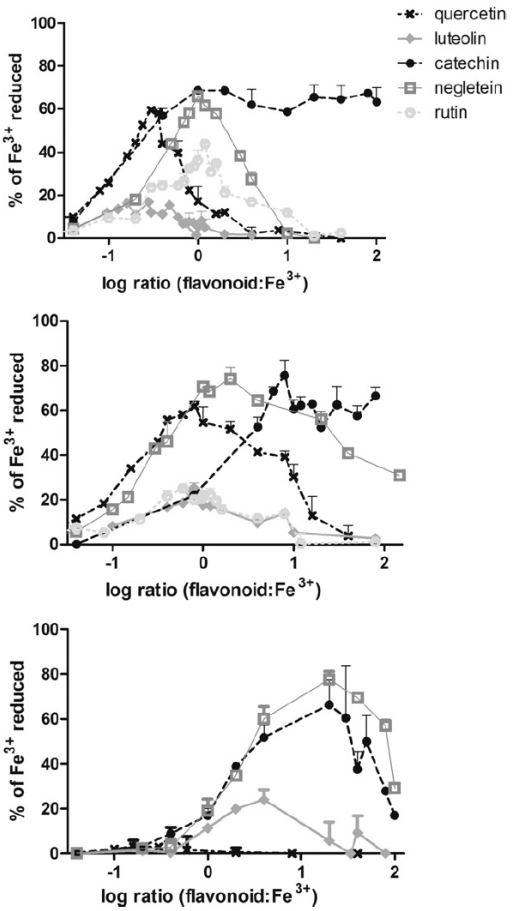

Fig. 3. Representative examples of the curves showing the relationship between log molar concentration ratio (flavonoid:Fe^{3+}) and reducing potential at pH 4.5 (A), at non-buffered conditions (B) and at pH 5.5 (C).

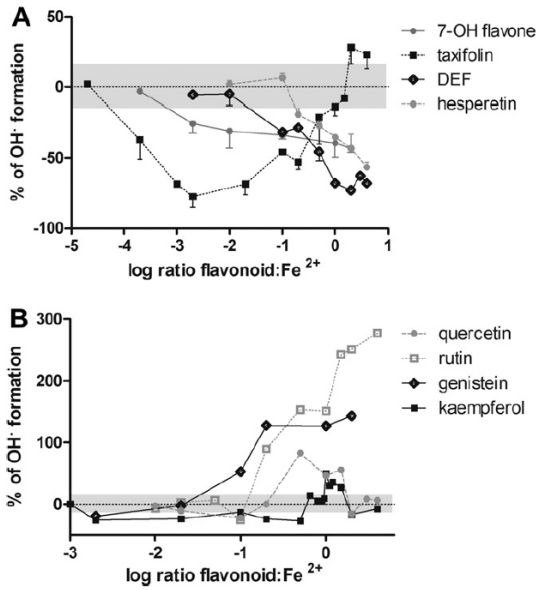

Fig. 4. Prototypical curves of flavonoids and deferoxamine (DEF) showing the influence on the iron-mediated Fenton reaction. (A) Antioxidant effects, (B) pro-oxidant behaviour. In Fig. 4B, SDs are not shown for increased clarity. Grey area indicates the error of the method.

shifted to the left, i.e., between the molar ratio 1:10 and 1:1 of flavonoid:iron (Fig. 3A). Under non-buffered conditions, the maximum reduction potential was mostly seen at the molar ratio 1:1 (Fig. 3B). At pH 5.5 the curves of efficient reducing agents, namely flavanols and taxifolin, had the maximal reducing peak at higher concentration ratios (Fig. 3C).

Because of the considerable reducing potential of the tested flavonoids, we performed additional experiments to assess whether these properties correspond to the intensification of the iron-catalysed Fenton reaction.

There were five types of responses:

- *Progressive antioxidant effect:* the antioxidant effect increased with the concentration of flavonoid. This was a typical behaviour for iron-chelator deferoxamine as a standard substance, but on the other hand, it was rather exceptional in flavonoids. The only cases were 7-hydroxyflavone and hesperetin (Fig. 4A).
- *Low antioxidant:* the antioxidant effects were seen only at very low ratios of flavonoid to iron, but the curve slowly reverted, usually in the proximity of ratio 1:1. Such examples are 3-hydroxyflavone, taxifolin or isoflavonoid daidzein (Fig. 4A, Supplementary data, Figs. S4 and S5).
- *Bell-shaped curves:* low concentrations had low or no antioxidant effects while comparable concentrations of iron and flavonoids (i.e. in the proximity of concentration ratio 1:1) were pro-oxidant and further increases in the concentration of flavonoid led to a decrease in pro-oxidation effect or to an antioxidant effect. This behaviour was seen mainly in flavonols; typical examples were kaempferol and quercetin (Fig. 4B). Interestingly, flavone chrysin seemed to follow the same pattern (Supplementary data, Fig. S4C).
- *Progressive pro-oxidant effect:* flavonoids morin, rutin, naringin and genistein fit into this class (Fig. 4B, Supplementary data, Figs. S4 and S5).
- No or negligible effect at all tested concentration ratios – troxerutin and flavanols catechin and epicatechin (Supplementary data, Fig. S5).

While the behaviour of various flavonoids was clearly different from the standard iron chelator deferoxamine, we compared their effects on the Fenton reaction at three different ratios (Fig. 5):

(a) at the concentration ratio 1:10 (compound:iron); under these conditions deferoxamine was only partly efficient since it cannot chelate all iron at this ratio (Mladenka et al., 2010a),
(b) at the concentration ratio 1:1 where deferoxamine should chelate all iron, since it forms complexes with 1:1 stoichiometry. Interestingly, several flavonoids can form complexes with iron at the same stoichiometry (Mladenka et al., 2011),
(c) at the concentration ratio 2:1 since many flavonoids chelate iron at this ratio under acidic conditions (Mladenka et al., 2011). From Fig. 4A it is clear, that in the case of deferoxamine, there was no significant difference between ratios 1:1 and 2:1. This is likely because the excess of deferoxamine was not associated with improved Fenton reaction inhibition.

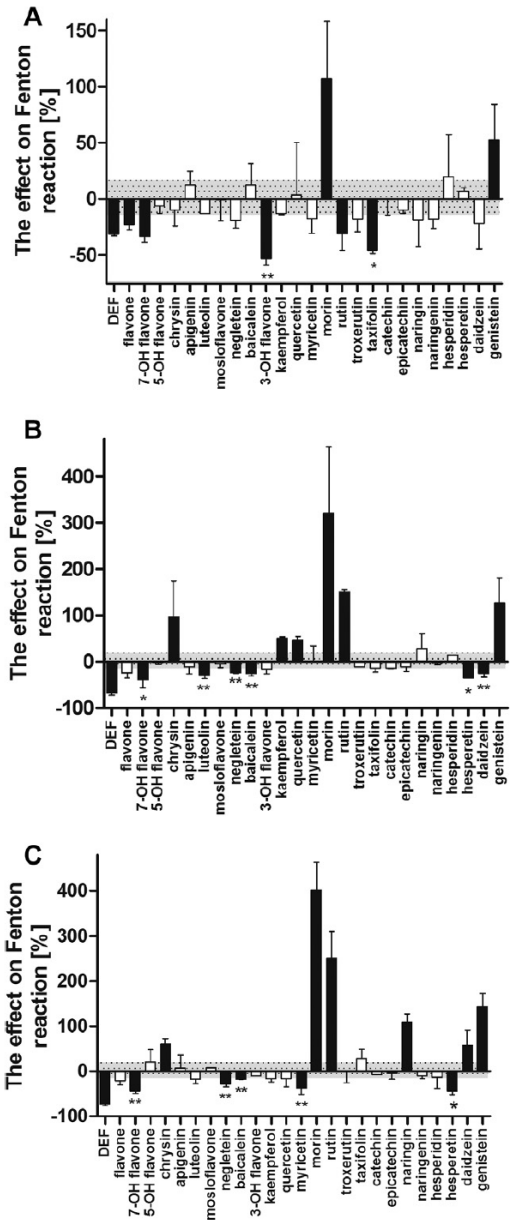

Fig. 5. Effects of flavonoids and deferoxamine (DEF) on the Fenton reaction at the concentration ratios (flavonoid or DEF:iron) 1:10 (A), 1:1 (B) and 2:1 (C), respectively. White bars mean that flavonoid was neither pro-oxidant nor antioxidant at a given ratio (error of the method is shown as grey dotted area). The statistical significance is shown for antioxidant activity vs. deferoxamine: $^{*}p < 0.01$, $^{**}p < 0.001$.

Summarising the described data concerning the Fenton reaction it appeared that monohydroxylated flavonoids, as well as simple flavone, were similarly efficient inhibitors of the Fenton reaction at low ratios; in particular, 3-hydroxyflavone was more efficient than deferoxamine in the ratio 1:10 (compound:iron). Taxifolin was an apparent exception. It was the most efficient inhibitor of the Fenton reaction even at very low concentration ratios (Fig. 4A, 1:1000 – 1:100; taxifolin:iron). But its inhibitory potential dropped with increasing concentrations, although at concentration ratio 1:10 remained more potent than deferoxamine. At the concentration ratio 1:1 and 2:1 (compound:iron), none of the tested flavonoids were more potent than deferoxamine. However, 7-hydroxyflavone, negletein, baicalein and hesperetin were efficient at both mentioned ratios. Contrarily, some flavonoids, in particular morin and genistein, were apparently pro-oxidant at all three selected ratios.

Comparing the data from ferric ions reduction and the Fenton reaction assays, it was apparent that the direct relationship between reduction of ferric ions and potentiation of the Fenton reaction was not confirmed for the majority of flavonoids. Real exceptions were flavonols (Fig. 6 and Supplementary data, Table 1). Flavonols with bell-shaped curves (kaempferol and quercetin) correlated or tended to have correlations between ferric ions reducing properties and intensifying effects on the Fenton reaction. Progressively pro-oxidant flavonols (morin and rutin) showed such correlation at concentrations up the concentration ratio flavonol:iron 1:1 (axis *x*, log = 0 in Fig. 6), i.e. up to their maximal reduction peaks.

4. Discussion

In the past, the antioxidant effects of flavonoids were almost exclusively linked to their hydrogen-donating effects and considered to be identical with their reducing properties. To date, many studies have confirmed and compared the reducing potential of different flavonoids. Because the majority of these studies used the FRAP assay, which is principally based on measuring the amount of reduced ferric ions in their marked excess, the reducing potential of flavonoids appears to be linear (Firuzi et al., 2005). The relevance of this assay in relation to iron is rather low since plasma or cellular concentrations of free iron are negligible at physiological conditions. Even a marked release of free iron after ischaemia (during reperfusion) can hardly be considered as a multiple excess of free iron (Berenshtein et al., 2002). Moreover, in the substantial excess of iron, the FRAP assay is not able to relevantly consider iron-chelating properties of flavonoids, in contrast to the ferrozine methodology analysing both the low and high concentration ratios of the tested compound to iron. Thus we suppose that the bell-shaped curves measured in this study are more relevant for flavonoid-iron interactions. Recently, we have reported that chelation of ferrous ions at pH 4.5 is low with exception of baicalein. On the other hand, some flavonoids, especially those with the free 3-hydroxyl group, are able to chelate ferric ions at the concentration ratio 2:1 (Mladenka et al., 2011). On the contrary, complexes at the concentration ratio 1:1 seem to be unstable at this pH, as can be deduced from the maximal ferric ions reduction (Fig. 5A, Supplementary data, Fig. S2). The importance for the different ratios can be supported by the study of Mira et al. (2002) who measured the reducing potential by the ferrozine methodology at pH 5.5 only at the concentration ratio 1:4 (flavonoid:iron). Hence, the outcome of that study is not identical with our data.

In spite of differences in the reducing curves, the FRAP values appear to correspond with the maximum percentage of reduced ferric ions at both non-buffered condition and pH 4.5. In this assay, flavone or other flavonoids with only one isolated hydroxyl group, either in position 3 or in other positions, did not exhibit any significant reduction potential. Similarly, isolated hydroxyl groups in ring A in flavones (5,7-dihydroxy group in chrysin and methoxy–hydroxy substitution in mosloflavone) or aliphatic hydroxyl groups in troxerutin did not reduce ferric ions, as well. FRAP assays con-

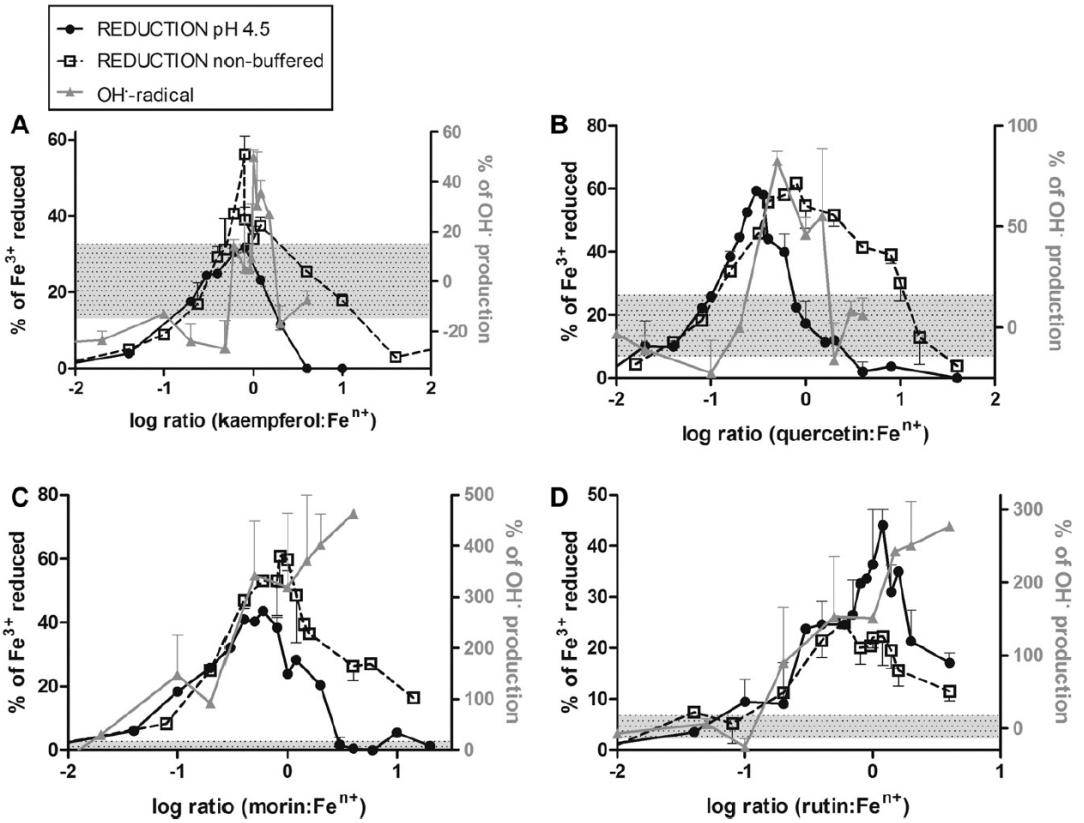

Fig. 6. Relationship between ferric ions reduction and effect on the Fenton reaction in flavonols. (A) kaempferol, (B) quercetin, (C) morin and (D) rutin. Left axis *y* and black curves represent the percentage of ferric ions reduced, right axis *y* and grey line show the influence of the compound on the Fenton reaction. Dotted grey area represents the error of the Fenton reaction assay.

firm that 5-hydroxyflavone and 7-hydroxyflavone together with chrysin do not reduce iron at all but some moderate iron reduction is observed with 3-hydroxyflavone (Firuzi et al., 2005). The difference may consist in the lower pH used in the FRAP methodology. An isolated hydroxyl group in ring B did not reduce ferric ions (apigenin and the tested isoflavonoids), but the presence of 3-hydroxyl group converted non-reducing apigenin into reducing kaempferol. These results are also in accordance with the FRAP results, in which a negligible or no activity is found for apigenin, a very low activity is exerted by isoflavonoids, daidzein and genistein, and a marked activity is observed in the case of kaempferol (Firuzi et al., 2005; Zhang et al., 2011). Flavonoids with the catecholic ring B, in particular flavonols or flavanols with the free 3-hydroxyl group, were very potent reducing agents. In comparison to the catecholic ring, the pyrogallol ring B or A did not increase the reduction potential. Again, those results agree with FRAP assay (Firuzi et al., 2005; Zhang et al., 2011). Similar to pH 4.5 in our assay, myricetin with pyrogallol ring B is less potent than quercetin with catecholic ring B (Firuzi et al., 2005; Zhang et al., 2011). In addition, the methoxy–hydroxy substitution of ring B is on one hand less potent than catecholic ring B, but on the other hand has comparable activity with the pyrogallol ring B (Zhang et al., 2011). The FRAP assay shows that the influence of the 5-hydroxyl group is rather neutral, since fisetin, a quercetin congener without the mentioned hydroxyl group, is only slightly less active than quercetin (Firuzi et al., 2005). Both catecholic rings A and B showed similar reduction potentials. Interestingly, in flavanones, the presence of methoxy–hydroxy substitution of ring B carried some limited reduction potential, which was higher in comparison to one isolated hydroxyl group in ring B. Similar finding shows FRAP (Firuzi et al., 2005; Zhang et al., 2011). The absence of both the 2,3-double bond and 4-keto group improved the reduction potential (quercetin vs. catechin and epicatechin) in our reduction assays but not in the FRAP assay, in which quercetin is by far the most potent reducing agent (Firuzi et al., 2005; Pulido et al., 2000; Zhang et al., 2011). The difference could be probably explained by different pH conditions used in the assays, since pH has been shown to influence the redox potential of polyphenols (Hagerman et al., 1998). It should be also mentioned that endogenous antioxidants are less potent than flavonoids in FRAP assay: (1) uric acid shows only limited reducing potential comparable to that of flavanones with methoxy–hydroxy ring B and (2) ascorbic acid is roughly three times less active than quercetin (Firuzi et al., 2005; Pulido et al., 2000).

One marked exception is baicalein, which was a similarly active reducing agent at non-buffered conditions to its analogue negletein with blocked 7-hydroxyl group by a methyl group. Contrarily, at acidic pH, baicalein was a slightly active reducing agent while negletein remained very active. This difference could be explained by the fact that baicalein is a very active iron chelator, even in comparison to the standard iron chelator deferoxamine under acidic conditions (Mladenka et al., 2011). But its chelating potential under non-buffered conditions is much lower (unpublished data).

Whereas the reduction activity of the tested flavonoids is in sufficient agreement with published studies, on the other hand, effects on the Fenton reaction or, in general, on metal-based oxidation are very different to the published papers, particularly in relation to the used methodology and transient metals (Cao et al., 1997; Laughton et al., 1989; Sugihara, Arakawa, Ohnishi, & Furuno, 1999). Chiefly, studies reported potentiation of the Fenton reaction at neutral pH by use of EDTA–ferric ions. However, this may be pathophysiologically less relevant because pure ferric salt or ferric-ADP or ferric citrate do not intensify the Fenton reaction in the presence of flavonoid, in contrast to EDTA–ferric ions (Laughton et al., 1989). The reason may lie in the solubility. Ferric ions have very low solubility at neutral pH, but EDTA improves it. Indeed, in this study, no reduction of ferric ions by flavonoids was observed at neutral or slightly acidic pH. We aimed to compare the ferric ions reduction potential of the tested flavonoids at relevant pHs with potentiation of the Fenton reaction. This hypothesis was confirmed only in the cases of flavonols, namely quercetin and kaempferol. Both flavonols had similar ferric ions reducing potential curves and the Fenton reaction curves depicting the changes in production of hydroxyl radical (Fig. 6). This behaviour could be explained by the fact that except for their reducing potentials, they are active iron chelators even under acidic conditions (Mladenka et al., 2011). Therefore, at lower concentration they are reducing iron, while at higher one, they are able to firmly chelate iron. In contrast, morin and rutin showed similar ferric ions reduction curves and, at the same time, they had pro-oxidant properties. Rutin, in contrast to all previously mentioned flavonols, does not contain the free 3-hydroxyl group and is less potent iron chelator at acidic conditions. Morin contains the 2′-hydroxyl group which lies in the proximity of the 3-hydroxyl group. Hence, it seems that the presence of the free 3-hydroxyl group is very important in this issue. Although not all outcomes from the study of Sugihara et al. (1999) are in agreement with our study, morin was similarly to this study clearly pro-oxidant in ferrous ions-based (without hydrogen peroxide) lipid peroxidation. Myricetin was an exception since it has not been pro-oxidant at any concentration ratio implying that the presence of the pyrogallol ring B can scavenge hydroxyl radical or inhibit its formation, notwithstanding its powerful ferric ions reducing properties, which were the base of pro-oxidant behaviour in other experiments (Laughton et al., 1989). The manner of action of flavonoids described by the bell-shaped curve was published in few studies in the past; e.g., ferric ions/EDTA/hydrogen peroxide-based assay showed the bell-shaped behaviour of quercetin, with the maximum pro-oxidation peak in the proximity of the concentration ratio 1:1 (quercetin:iron). The pro-oxidation effect with rather a plateau in the case of morin and a dose dependent pro-oxidation effect of naringenin is observed (Yen, Duh, Tsai, & Huang, 2003). The bell-shaped DNA degradation in bleomycin–ferric ions assay is well documented in the case of quercetin and myricetin (Laughton et al., 1989). Interestingly, concerning the copper, flavonoids have been shown to rather inhibit copper-based oxidation, in contrast to ferrous-based oxidation, although reverse bell-shaped curves have been also observed (Cao et al., 1997; Sugihara et al., 1999).

Generally, behaviour of flavonoids towards the Fenton reaction was very different in comparison to the standard iron chelator deferoxamine. Deferoxamine progressively blocked the Fenton reaction in apparent relation to its iron-chelating properties. A dose-dependent inhibition of the Fenton reaction was observed also in the case of 7-hydroxyflavone. There are two important differences: (1) 7-hydroxyflavone is not able to chelate iron (Mladenka et al., 2011) and (2) 7-hydroxyflavone decreased hydroxyl radical production in much lower concentrations, e.g., 1:1000 flavonoid:iron, respectively. Therefore in accordance to the literature, the probable explanation is that 7-hydroxyflavone and other flavonoids are direct scavengers of hydroxyl radical (Bochoráková, Paulová, Slanina, Musil, & Táborská, 2003). In fact, many flavonoids were active inhibitors of the Fenton reaction in very low ratios while pro-oxidant or ineffective in the proximity of ratio 1:1. This likely reflects the fact that the amount of the formed complex in very low ratios did not markedly influence the chemistry of the Fenton reaction and the decisive factor is their scavenging potential. Contrarily, at higher ratios, the complexes of iron with flavonoids are capable of redox cycling and this may balance or even prevail over their scavenging potential. Of interest is the influence of the 7-hydroxyl group since 7-hydroxyflavone and hesperetin were the only mentioned dose-dependent antioxidants. Any modification of these structures, e.g. by a saccharide moiety in the case of hesperidin or addition of the 5-hydroxyl group in chrysin absolutely abolished the inhibitory effect on hydroxyl radical production. The effect of 5-hydroxyl group is of particular interest since hesperetin contains also the 5-hydroxyl group like chrysin but the behaviour is completely opposite. The likely explanation is the different stereochemistry of the molecule. Nearly planar flavones with 5-hydroxyl-4-keto group and 2,3-double bond are able to form unstable complexes with iron even at low pH, in contrast to non-planar flavanones with 5-hydroxyl-4-keto group but without 2,3-double bond, which do not chelate almost any iron in that condition (Mladenka et al., 2011). In addition, the concomitant substitution of ring B markedly modified the activity as naringenin was inactive at most concentrations and its glycoside with blocked 7-hydroxyl group was even pro-oxidant. Although pro-oxidant effects of chrysin were documented by others too, it should be mentioned that hesperetin was shown to be pro-oxidative in a ferric ions/EDTA/hydrogen peroxide based assay (Sugihara et al., 1999; Yen et al., 2003). We suppose that non-physiological addition of EDTA influences the assay since, as was mentioned, it improves solubility of ferric ions at used neutral pH. In general, slight modification of flavonoid structure was associated with very different behaviour, e.g., both flavone apigenin and its corresponding isoflavone genistein did not reduce ferric ions but genistein was apparently pro-oxidant in comparison to neutral behaviour of apigenin. A low effect of apigenin on iron-based pro-oxidation has been documented by others, as well (Sugihara et al., 1999).

Previously, we have shown that baicalein exerted similar potent iron-chelating properties to deferoxamine (Mladenka et al., 2011), and therefore we have suggested it as a promising iron chelator. However, in this study, its inhibition of hydroxyl radical formation was rather moderate, probably reflecting its ferric ions reducing properties. Of interest is that very active reducing agents negletein, catechin and epicatechin did not promote the Fenton reaction. Similarly, another study documented that the influence of catechin on hydroxyl radical formation was rather low (Hagerman et al., 1998). However, depending on the methodological approach, the effect of catechin may be very different, e.g., any effect in the presence of ferric ions and hydrogen peroxide, dose-dependent antioxidant effect in the presence of Fe^{3+}/H_2O_2/ascorbic acid, and pro-oxidant in the presence of Fe^{3+}/EDTA (Chobot, Huber, Trettenhahn, & Hadacek, 2009). In contrast to flavanols, taxifolin with the 4-keto group was a very efficient reductant and its $OH^{\cdot}$-scavenging potential was very high and significant even at ratios 1:1000 (taxifolin:iron). The marked potency of taxifolin and its close congeners in comparison to other flavonoids was also documented towards the copper-based Fenton reaction (Cao et al., 1997; Sugihara et al., 1999). However it seemed to be lost or even reversed at higher taxifolin:iron concentration ratios (Fig. 4A).

Although many flavonoids can behave as pro-oxidant elements, they can be still useful in specific pathological or pharmacological conditions:

(1) Iron absorption is achieved in the proximity of the gastroduodenal junction, where the pH is lower than in the intestine (Donovan & Andrews, 2004). Thus, it is worth considering the results from pH 4.5 or pH 5.5, in which ferric ions were reduced into ferrous, and hence, the improvement in iron absorption may take place. In particular, flavanols catechin and epicatechin seem to be suitable since their reducing potential was preserved at pH 5.5 and their influence on the Fenton reaction was neutral.
(2) As mentioned above, ischaemia with subsequent reperfusion leads to the release of free iron which is redox active and its participation in the Fenton reaction has been observed (Berenshtein et al., 2002). The previous idea that flavonoids may decrease the Fenton reaction consequences does not seem to be absolutely correct since many flavonoids were able to potentiate the chemistry of the Fenton reaction in this study. The 7-hydroxyflavone and hesperetin are the only promising candidates for this pathological state.
(3) The situation in cancer is contrary. The idea that the anticancer activity of flavonoids may be, at least partly, associated with increased ROS production has been suggested (Galati & O'Brien, 2004). The fact that morin and rutin efficiently induced cell apoptosis (Romero, Paez, Ferruelo, Lujan, & Berenguer, 2002) supports outcomes from this study and suggests that pro-oxidant flavonoids may act by this mechanism.
(4) Iron overload conditions – flavonoids are mostly potent iron chelators (Mladenka et al., 2011) and they did not appear to reduce ferric ions at physiological pH.

Interestingly quercetin was shown to extend the life span of nematode *Caenorhabditis elegans* in low doses but to shorten it in higher concentration (Pietsch et al., 2011). This hormetic effect was explained by influence on ROS production. It would be thus of interest to test other flavonoids with different influence on Fenton chemistry using the same model.

Conclusively, a systemic administration of flavonoids with the exceptions of 7-hydroxyflavone and hesperitin appears to have unpredictable impact, because low levels may have antioxidant and higher ones pro-oxidant effects. This may, at least, partly explain the controversy among various studies analysing the effects of flavonoids.

Acknowledgements

The authors wish to thank Mrs. Irena Rejlová for her excellent technical assistance. This work was supported by Czech Science Foundation project No. P303/12/G163. T.F. and M.R. acknowledge support from the Charles University in Prague (SVV 265 003/2012).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.06.107.

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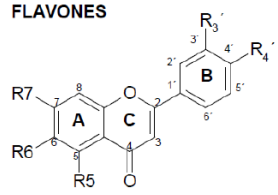
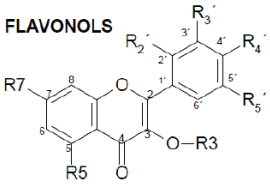
SUPPLEMENTARY DATA

FLAVONES



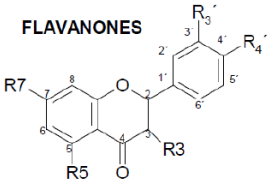
flavones	R_5	R_6	R_7	R_3'	R_4'
flavone	H	H	H	H	H
5-hydroxyflavone	OH	H	H	H	H
7-hydroxyflavone	H	H	OH	H	H
chrysin	OH	H	OH	H	H
apigenin	OH	H	OH	H	OH
luteolin	OH	H	OH	OH	OH
negletein	OH	OH	OCH_3	H	H
baicalein	OH	OH	OH	H	H
mosloflavone	OH	OCH_3	OCH_3	H	H
diosmin	OH	H	O-Glc-Rha	OH	OCH_3

FLAVONOLS



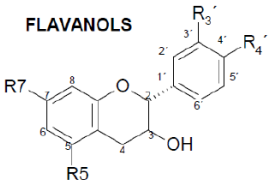
flavonols	R_3	R_5	R_7	R_2'	R_3'	R_4'	R_5'
3-hydroxyflavone	H	H	H	H	H	H	H
kaempferol	H	OH	OH	H	H	OH	H
quercetin	H	OH	OH	H	OH	OH	H
morin	H	OH	OH	OH	H	OH	H
myricetin	H	OH	OH	H	OH	OH	OH
rutin	-Glc-Rha	OH	OH	H	OH	OH	H
troxerutin	-Glc-Rha	OH	O-C_2H_4-OH	H	O-C_2H_4-OH	O-C_2H_4-OH	H

FLAVANONES



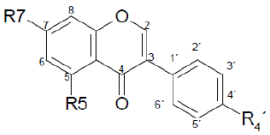
flavanones	R_3	R_5	R_7	R_3'	R_4'	configuration
naringenin	H	OH	OH	H	OH	2RS
naringin	H	OH	O-Glc-Rha	H	OH	2RS
hesperetin	H	OH	OH	OH	OCH_3	2S
hesperidin	H	OH	O-Glc-Rha	OH	OCH_3	2S
taxifolin	OH	OH	OH	OH	OH	2R, 3R

FLAVANOLS



flavanols	R_5	R_7	R_3'	R_4'	configuration
(-)-epicatechin	OH	OH	OH	OH	2R, 3R
(+)-catechin	OH	OH	OH	OH	2R, 3S

ISOFLAVONOIDS



isoflavonoids	R_5	R_7	R_4'
daidzein	H	OH	OH
genistein	OH	OH	OH

Fig. S1. Chemical structures of the tested flavonoids.

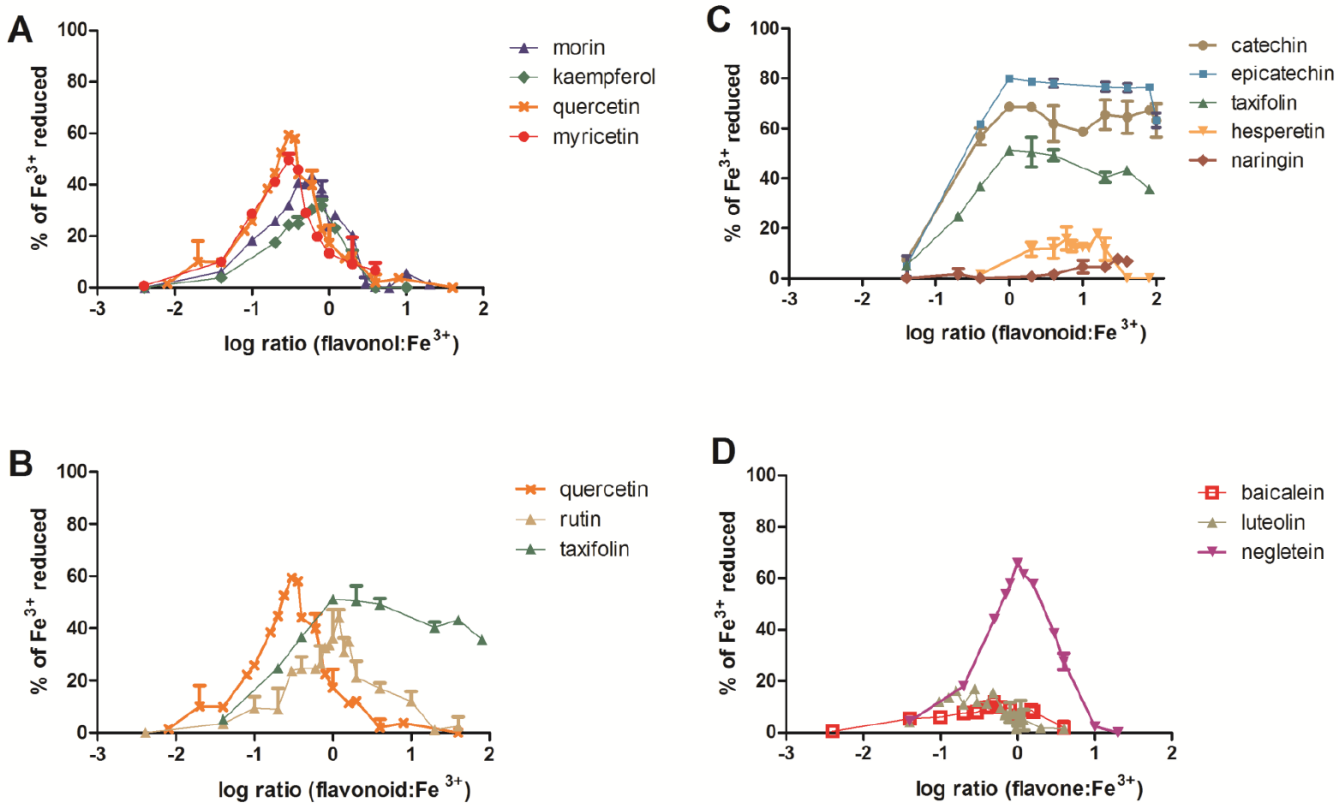


Fig. S2. Iron reduction at pH 4.5. Figure shows active reducing flavonoids (maximum reduction >5%). **A**: flavonols, **B**: flavanols and taxifolin, **C**: effect of 3-OH group and 2,3-double bond, **D**: flavones. Data represents mean ± SD.

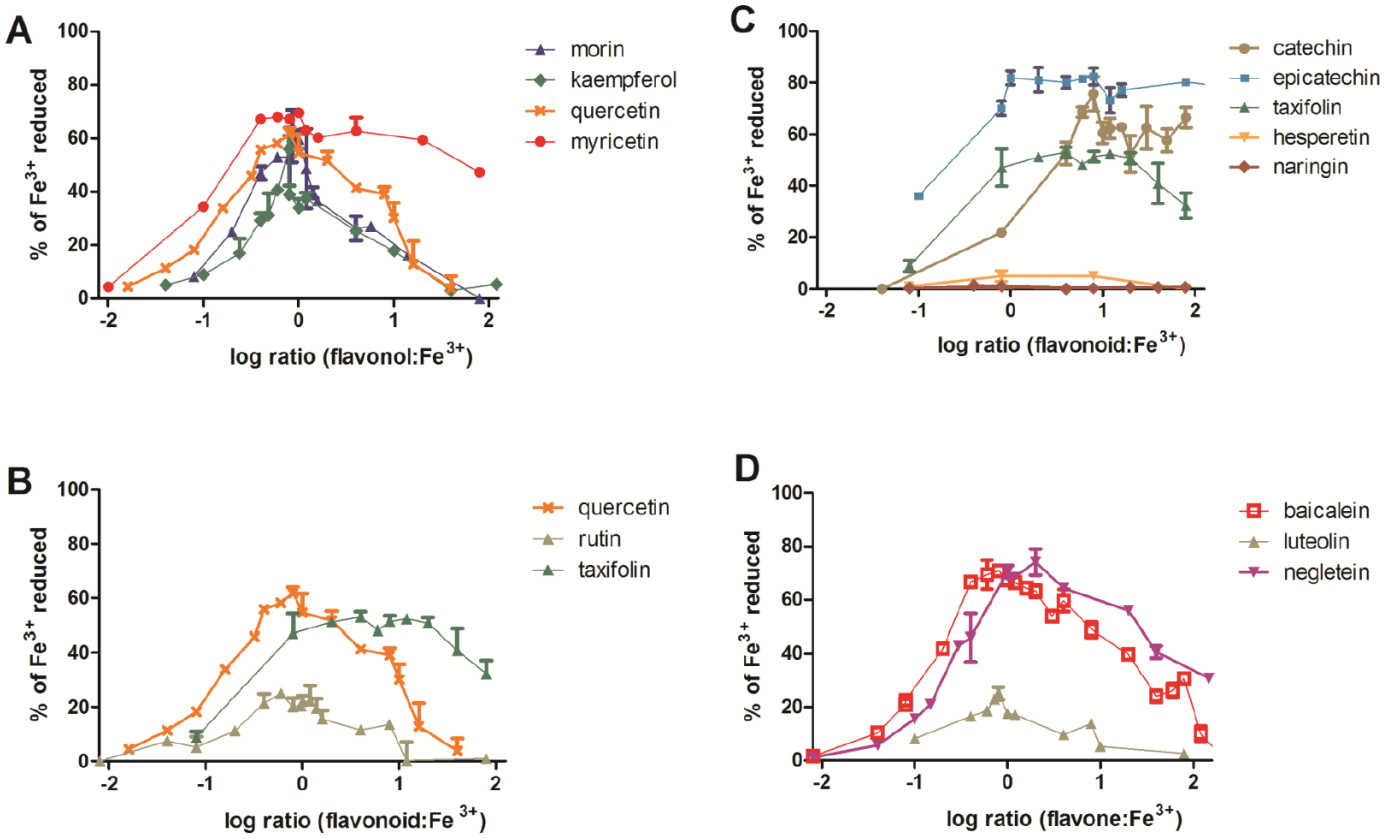


Fig. S3. Iron reduction in non-buffered conditions. Figure shows active reducing flavonoids (maximum reduction >5%). **A**: flavonols, **B**: flavanols and taxifolin, **C**: effect of 3-OH group and 2,3-double bond, **D**: flavones. Data represents mean ± SD.

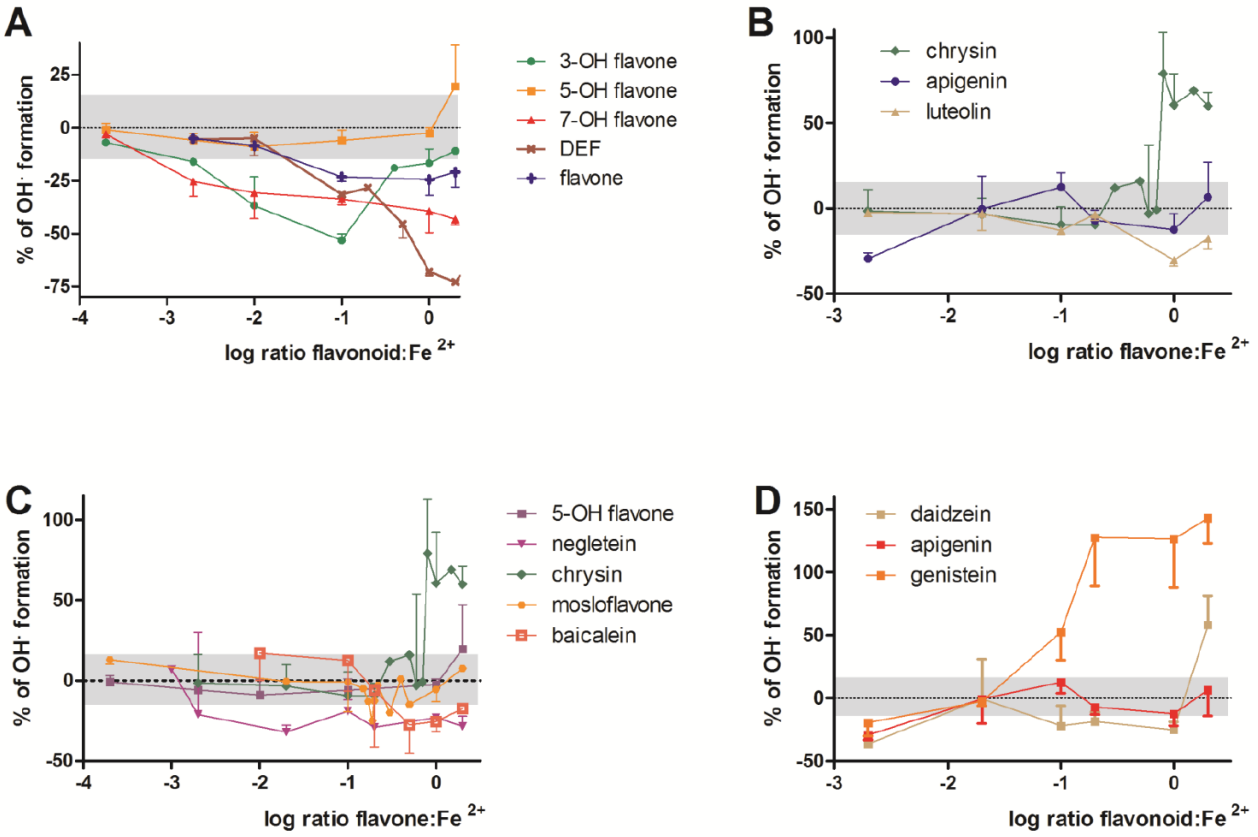


Fig. S4. Effect of flavonoids on Fenton chemistry I. **A**: monohydroxyflavones, flavones and deferoxamine, **B**: influence of hydroxyl groups in ring B in flavones, **C**: influence of A-ring substitution in flavones. **D**: isoflavonoids and apigenin. Data represents mean ± SD.

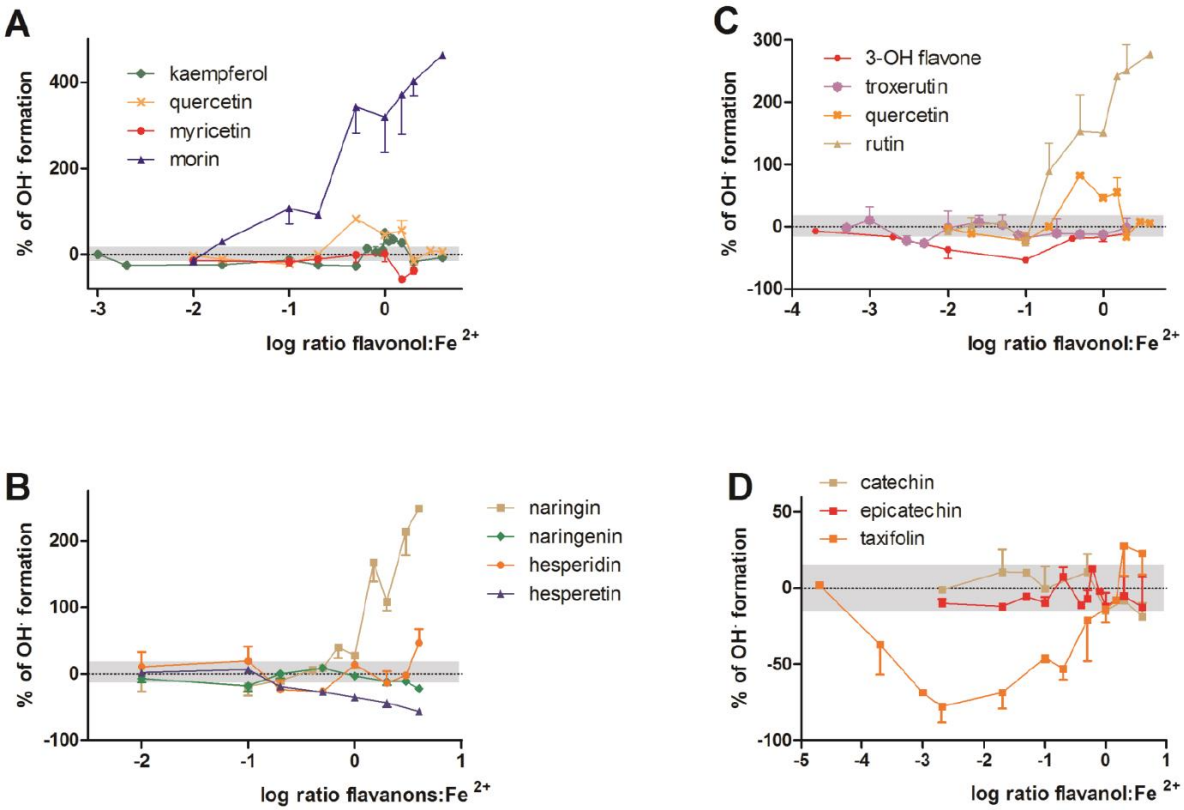


Fig. S5. Effect of flavonoids on Fenton chemistry II. **A**: influence of hydroxyl groups in ring B in flavonols, **B**: influence of the 3-hydroxyl group and 2,3-double bond, **C**: flavanones. **D**: flavanols and taxifolin. Data represents mean ± SD.

Table 1. Correlations between ferric ions reducing potential of flavonols at pH 4.5 or in non-buffered conditions and effect on the iron-catalysed Fenton reaction.

flavonol	**kaempferol**	**quercetin**	**morin**	**rutin**
concentration ratio range	whole tested range	whole tested range	up to 1:1 morin:Fe	up to 1:1 rutin:Fe
pH 4.5	0.66 (p=0.05)	0.31 (p=0.41)	0.82 (p=0.09)	0.87 (p=0.01)
non-buffered conditions	0.46 (p=0.26)	0.64 (p=0.36)	0.91 (p=0.03)	0.84 (p=0.04)

Table summarizes Pearson correlation coefficients as well as their significance. The corresponding curves are shown in the main document *(Fig. 6)*.

4.5. Mathematical calculations of iron complex stoichiometry by direct UV-Vis spectrophotometry

FILIPSKÝ, T.; ŘÍHA, M.; HRDINA, R.; VÁVROVÁ, K.; MLADĚNKA, P. Mathematical calculations of iron complex stoichiometry by direct UV-Vis spectrophotometry. *Bioorganic Chemistry*. 2013, **49**, 1-8.

(IF 2013: 2,141)

Bioorganic Chemistry 49 (2013) 1–8

Contents lists available at SciVerse ScienceDirect

Bioorganic Chemistry

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

Mathematical calculations of iron complex stoichiometry by direct UV–Vis spectrophotometry

Tomáš Filipský [a], Michal Říha [a], Radomír Hrdina [a], Kateřina Vávrová [b], Přemysl Mladěnka [a,*]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] Department of Inorganic and Organic Chemistry, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 15 November 2012
Available online 11 June 2013

Keywords:
Iron
Chelator
Complex
Job's method
Stoichiometry
Spectrophotometry

ABSTRACT

The effects of iron-chelating agents on miscellaneous pathologies are currently largely tested. Due to various indications, different properties for chelators are required. A stoichiometry of the complex in relation to pH is one of the crucial factors. Moreover, the published data on the stoichiometry, especially concerning flavonoids, are equivocal.

In this study, a new complementary approach was employed for the determination of stoichiometry in 10 iron-chelating agents, including clinically used drugs, by UV–Vis spectrophotometry at relevant pH conditions and compared with the standard Job's method.

This study showed that the simple approach based on absorbance at the wavelength of complex absorption maximum was sufficient when the difference between absorption maximum of substance and complex was high. However, in majority of substances this difference was much lower (9–73 nm). The novel complementary approach was able to determine the stoichiometry in all tested cases. The major benefit of this method compared to the standard Job's approach seems to be its capability to reveal a reaction stoichiometry in chelators with moderate affinity to iron.

In conclusion, using this complementary method may explain several previous contradictory data and lead to a better understanding of the underlying mechanisms of chelator's action.

1. Introduction

In the past decades, there has been a rapid development of novel iron-chelating agents. Many of them have shown a promising potential in the therapy of iron overload and non-iron overload pathologies [1,2]. Despite of the enormous interest of investigators, to date, a limited number of iron chelators is clinically used. Deferoxamine, deferasirox or deferiprone are indicated in patients, who require a long-term treatment with blood transfusions in haematological disorders [3,4]. Moreover, in epidemiologically rare cases, deferoxamine can be clinically used for the management of acute iron intoxication [5]. Dexrazoxane is an approved cardioprotective agent that effectively protects against anthracycline-induced cardiotoxicity although the involvement of its iron-chelating product has recently been questioned [6,7].

Apart from the above mentioned clinically used iron chelators, there is a wide spectrum of structurally different substances of the both natural and synthetic origin sharing the ability for chelation of Fe(II) and/or Fe(III) [8]. Beyond iron overload conditions, iron-chelating agents are experimentally tested in the prevention/treatment of acute myocardial infarction, neurodegenerative diseases and cancer [1,4,7,9–11].

Thus, in relation to the mentioned approved or examined indications, different properties for iron chelators are required. Activity of iron chelator may be affected by many factors, e.g. pH. Differences in pH due to both physiological (e.g. in upper part of the intestine, where the absorption of iron occurs) and pathological (e.g. ischaemic myocardial tissue and cancer) aspects may significantly influence chelation of iron [12–14].

Moreover, low stability of the complex may allow or even potentiate the reaction of iron with hydrogen peroxide (Fenton chemistry) with the known generation of the most harmful biological oxidant, hydroxyl radical [15]. In order to prevent this reaction, the complex chelator-iron has to be very stable. Complexes with a lower stoichiometry, where all coordination sites are not fully occupied, are more prone to the production of hydroxyl radical, but on the other hand, such complexes may be useful in the therapy of cancer [1,15]. Hence the knowledge of the stoichiometry of the complex may be also of potential clinical significance.

To date, the characterizations of Fe(II)/Fe(III) complexes at different pH conditions are rather scarce. This may be likely associated with difficulties in a methodological approach, e.g. apparently contradictory findings have been published concerning the stoichiometry of the complexes in flavonoids [16,17]. Therefore, the aim of

* Corresponding author. Fax: +420 495 067 170.
E-mail address: mladenkap@faf.cuni.cz (P. Mladěnka).

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http://dx.doi.org/10.1016/j.bioorg.2013.06.002

this study was to develop a simple, precise and rapid UV–Vis spectrophotometric approach usable at different (patho)physiologically relevant pH. Novel mathematical calculations of the stoichiometry, and a standard method of continuous variation, also known as the Job's method, were employed and their advantages and disadvantages disclosed.

2. Materials and methods

2.1. Reagents

Deferoxamine was purchased from Novartis (Switzerland). Deferasirox was isolated from Exjade tablets (Novartis, Switzerland) by extraction with hot ethanol and then precipitation by water. NMR (Varian Mercury-Vx BB 300 instrument, operating at 300 MHz for 1H, 75 MHz for 13C, Palo Alto, CA, USA) and MS spectra (Agilent 500 Ion Trap LC/MS, Santa Clara, CA, USA) of the product were in accordance with literature and elemental analysis (Fisons EA 1110, Milano, Italy) revealed its sufficient purity (calcd: C 67.56, H 4.05, N 11.25; found: C 67.46, H 4.14, N 11.29) [18]. Deferiprone was a kind gift from ApoPharma Inc. (Apotex Inc., Canada), ethylenediaminetetraacetic acid disodium salt (EDTA), 8-hydroxyquinoline, chloroxine, quercetin and rutin were purchased from Sigma–Aldrich Inc. (USA). Pyridoxal isonicotinoyl hydrazone (PIH) and salicylaldehyde isonicotinoyl hydrazone (SIH) were synthesized as was described previously [19]. The tested iron-chelating agents are depicted in Fig. 1.

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazinedisulphonic acid sodium salt (ferrozine), ferrous sulphate heptahydrate, ferric chloride hexahydrate, ferric tartrate, hydroxylamine hydrochloride, sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), HEPES sodium salt were purchased from Sigma–Aldrich Inc. (USA) and methanol from J.T. Baker (Avantor Performance Materials, Inc., USA). Ultrapure water was used throughout this study.

2.2. Assessment of iron concentration in stock solutions

Before each experiment, a concentration of iron in stock solutions was routinely checked by a spectrophotometric reagent – ferrozine. Ferrozine specifically reacts with Fe(II) and gives a stable magenta coloured complex with a single absorption maximum at 562 nm [20]. Therefore, an aqueous solution of ferrozine (final concentration 1.67 mM) was used for a direct determination of Fe(II) concentration, which linearly corresponds to the absorbance of the formed complex with ferrozine.

Moreover, the above-mentioned approach was slightly modified for an assessment of Fe(III) by an addition of a reducing agent – hydroxylamine (final concentration 3.33 mM). Afterwards, Fe(III)

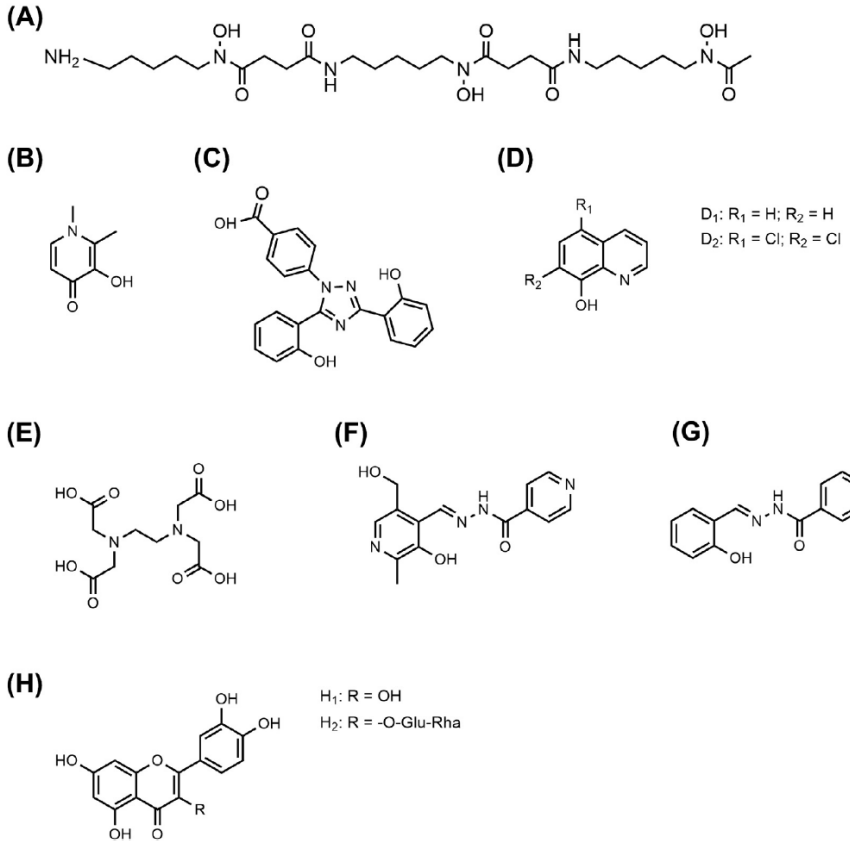

Fig. 1. Iron-chelating agents tested in this study. Deferoxamine (A), deferiprone (B), deferasirox (C), 8-hydroxyquinoline (D_1), chloroxine (D_2), EDTA (E), PIH (F), SIH (G), quercetin (H_1) and rutin (H_2).

was reduced into Fe(II) and its concentration was subsequently determined by ferrozine.

2.3. Iron and pH conditions

The assessment of stoichiometry was performed at four (patho)physiologically relevant pH values (4.5, 5.5, 6.8 and 7.5). For the both lower pHs, 15 mM acetate buffers were used, while 15 mM HEPES buffer was used for pH 6.8. Because oxidation of Fe(II) significantly raises in the course of time at pH 7.5, hydroxylamine was added in the final concentration of 5 mM to the HEPES buffer in order to prevent Fe(II) oxidation [21]. For the determination of Fe(III) chelation at pH 7.5, HEPES buffer without hydroxylamine was used.

Two kinds of ferric solutions, i.e. ferric chloride hexahydrate and ferric tartrate, were tested because of low solubility of Fe(III) at higher pHs. In this study, ferric chloride hexahydrate was used at pHs 4.5 and 5.5 and ferric tartrate at pHs 6.8 and 7.5.

2.4. Ultraviolet–visible spectrophotometry

All experiments were performed in semi-micro polystyrene or ultraviolet-transparent cuvettes (BrandTech Scientific Inc., The United Kingdom) and absorbance was measured by the use of spectrophotometer Helios Gamma equipped with VisionLite Software 2.2 (ThermoFisher Scientific Inc., USA).

2.4.1. Assessment of absorption maxima of iron-chelating agent and its complex

Firstly, absorption spectra ranging from 220 to 800 nm with wavelength(s) of absorption maximum(a) of a tested substance (λ_{Smax}) were determined at all pH conditions. Molar absorption coefficients of the substance (ε_S) were calculated according to the Lambert–Beer law. Similarly, a determination of the wavelength(s) of absorption maximum(a) of the complex (λ_{Cmax}) and the corresponding molar absorption coefficients (ε_C) were accomplished by the use of iron excess at different concentration ratios ranging from 1:6 to 1:50 (substance:iron). The blank was composed from a buffer and a solvent of the substance (methanol or water) at the ratio 2:1 in the case of Fe(II). Because the absorbance of Fe(III) disturbed the measurement, the assessment of ferric complexes was slightly modified by an inclusion of ferric aqueous solution into the blank.

2.4.2. Job's method

The Job's method, also known as the method of continuous variation, is a simple analytical approach which is used to the determination of stoichiometry of two interacting components. In this method, the total molar concentration of two reactants is kept constant while their molar concentration ratios are continuously varied throughout the series of samples (Fig. 2A) [22].

Briefly, an aqueous solution of Fe(II) or Fe(III) was mixed for 3 min with a methanolic/aqueous solution of a tested substance at different molar concentration ratios ranging from 1:3 to 6:1 (substance:iron) at all tested pHs and afterwards absorption spectra were immediately measured. The blank was composed from a buffer and a solvent at the ratio 2:1, respectively.

2.4.3. Complementary methods based on mathematical calculations of the stoichiometry

In addition to the standard Job's approach, complementary mathematical calculations were employed. Compared to the Job's method, the total molar concentration of the tested substance was continuously varied, while the molar concentration of Fe(II) or Fe(III) was kept constant throughout the series of samples (Fig. 2B).

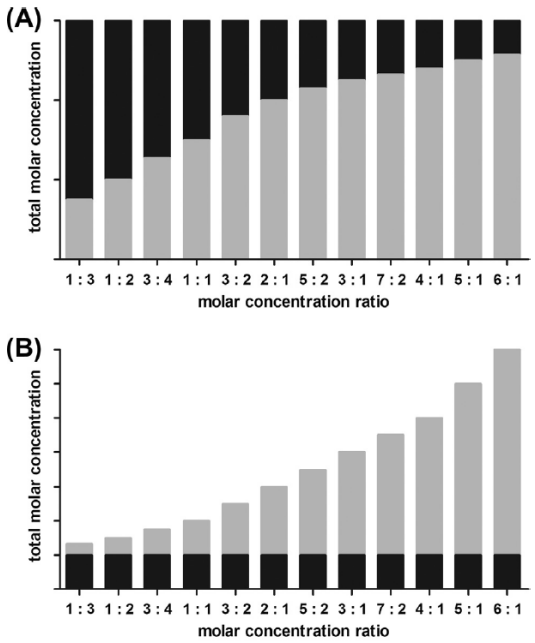

Fig. 2. A schematic depiction of the Job's method (A) and the complementary method (B). The grey columns correspond to the molar concentration of a substance and the black columns correspond to the molar concentration of iron. In the Job's method, the molar concentration ratios of the substance to iron are continuously changing while the total molar concentration is kept constant. In the complementary method, there was a constant molar concentration of iron while a molar concentration of the substance continuously varied. The ratios signify substance to iron.

The preparation of different molar concentration ratios ranging from 1:3 to 6:1 (substance:iron) was identical to the above described protocol.

2.4.3.1. Absorbance at absorption maximum of the complex (Method I). A determination of the stoichiometry according to the Method I was based on a simple evaluation of absorbance of a series of samples at the wavelength of absorption maximum of the complex λ_{Cmax}.

2.4.3.2. Symmetry of the absorption maximum of the complex (Method II). This method was based on a simple assumption that in the theory the absorption maximum of a complex is symmetric, if there are no interfering proximal absorption maxima (Fig. 3). Thus, at similar distance (d) from λ_{Cmax} to the left (λ_{sym1}) or to the right (λ_{sym2}), the absorbance of the complex $A_{C_{\lambda sym1}}$ was proposed to be the same as the absorbance of the complex $A_{C_{\lambda sym2}}$ (Eqs. (1)–(3)).

$$\lambda_{sym1} = \lambda_{Cmax} - d \quad (1)$$

$$\lambda_{sym2} = \lambda_{Cmax} + d \quad (2)$$

$$A_{C_{\lambda sym1}} = A_{C_{\lambda sym2}} \quad (3)$$

According to the known additive character of absorbance, a measured absorbance was additively composed from the absorbance of the formed complex and the non-reacted substance at any wavelength. Therefore in the theory, if the absorbance of the

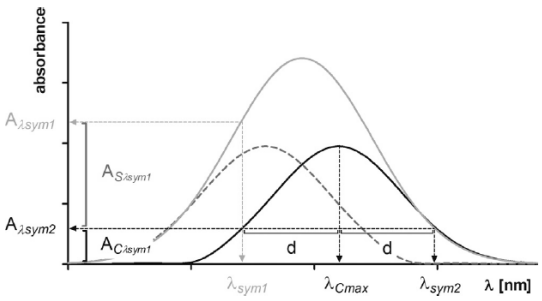

Fig. 3. The method of the symmetry of absorption maximum of the complex. The measured absorption spectrum (grey curve) is the sum of the absorption spectrum of the non-reacted substance (dark grey dashed curve) and the absorption spectrum of the formed complex (black curve).

complex $A_{C_{\lambda sym2}}$ was sufficient (>0.1) and the absorbance of the substance $A_{S_{\lambda sym2}}$ was zero, the measured absorbance $A_{\lambda sym2}$ should be equal to the absorbance of the complex $A_{C_{\lambda sym2}}$ (Eqs. (4) and (5)).

$$A_{\lambda_{sym2}} = A_{S_{\lambda sym2}} + A_{C_{\lambda sym2}} \tag{4}$$

$$A_{\lambda_{sym2}} = A_{C_{\lambda sym2}} \tag{5}$$

Thus, the measured absorbance $A_{\lambda sym1}$ was directly used for the assessment of the molar concentration of the non-reacted substance (Eqs. (6) and (7)):

$$A_{\lambda_{sym1}} = A_{S_{\lambda sym1}} + A_{C_{\lambda sym1}} \tag{6}$$

and because of Eqs. (3) and (5):

$$A_{\lambda_{sym1}} = A_{S_{\lambda sym1}} + A_{\lambda_{sym2}} \tag{7}$$

Considering the Lambert–Beer law (Eq. (8)), the molar concentration of the non-reacted substance (c_S) was calculated as follows (Eq. (9)):

$$A_{S_{\lambda sym1}} = c_S \times \varepsilon_{S_{\lambda sym1}} \times \ell \tag{8}$$

in which ℓ was the known width of cuvette and $\varepsilon_{S_{\lambda sym1}}$ was the molar absorption coefficient of the substance at λ_{sym1},

$$c_S = \frac{A_{\lambda_{sym1}} - A_{\lambda_{sym2}}}{\varepsilon_{S_{\lambda sym1}} \times \ell} \tag{9}$$

Afterwards, the chelation ratio (X) was calculated according to the Eq. (10), in which c_{S_0} was the initial molar concentration of a substance and c_{Fe} was the final molar concentration of iron in the sample.

$$X = \frac{c_{S_0} - c_S}{c_{Fe}} \tag{10}$$

2.4.3.3. Calculation using the absorption maximum of the substance (Method III). A calculation of the stoichiometry using the absorption maximum of the substance was based on the determination of the molar concentration of the non-reacted substance (c_S) likewise in the previous methodology. However, the absorbance at the wavelength of absorption maximum of substance ($A_{\lambda_{Smax}}$) was used (see Supplementary data Fig. S1A). Similarly, $A_{\lambda_{Smax}}$ was the sum of the absorbance of the non-reacted substance and the formed complex (analogously to the Eq. (6)). Hence, considering the Lambert–Beer law, the molar concentration of the non-reacted substance (c_S) was calculated as follows (Eqs. (11)–(13)):

$$A_{\lambda_{Smax}} = c_S \times \varepsilon_{S_{\lambda Smax}} \times \ell + c_C \times \varepsilon_{C_{\lambda Smax}} \times \ell \tag{11}$$

in which $\varepsilon_{S_{\lambda Smax}}$ and $\varepsilon_{C_{\lambda Smax}}$ were the molar absorption coefficients of the substance and the formed complex, respectively, at the wavelength of absorption maximum of the substance λ_{Smax}. The unknown molar concentration of the complex (c_C) was substituted by conversion to the molar concentration equivalents of the substance:

$$c_C + c_S = c_{S_0} \tag{12}$$

And hence the concentration of the non-reacted substance (c_S) was:

$$c_S = \frac{\frac{A_{\lambda_{Smax}}}{\ell} - \varepsilon_{C_{Smax}} \times c_{S_0}}{\varepsilon_{S_{\lambda Smax}} - \varepsilon_{C_{\lambda Smax}}} \tag{13}$$

Afterwards, the calculation of the stoichiometry was accomplished according the Eq. (10).

2.4.3.4. Calculation using the absorption maximum of the complex (Method IV). The calculation of the stoichiometry using the absorption maximum of the complex was analogous to the Method III with one exception that the absorbance was measured at the wavelength of absorption maximum of the complex (λ_{Cmax}) (see Supplementary data Fig. S1B).

2.4.3.5. Theoretical determination of absorbance of the complex at the wavelength of its absorption maximum (Method V). This method was based on a construction of theoretical lines mimicking absorbance of the most probable stoichiometries. Basically, because the molar concentration of iron was stable throughout the complementary approach (Fig. 2B), absorbance was firstly raising dependently on the formation of complex as long as all added substance reacted with iron and formed the complex (Eqs. (14) and (15)):

$$A_{\lambda Cmax} = A_{C_{\lambda Cmax}} \tag{14}$$

$$A_{C_{\lambda Cmax}} = c_{S_0} \times \varepsilon_{C_{\lambda Cmax}} \times \ell \tag{15}$$

In a certain point, at which all iron was exhausted, the absorbance $A_{\lambda Cmax}$ raised only dependently on the absorbance of the further added (non-reacted) substance $A_{S_{\lambda Cmax}}$ (Eqs. (16) and (17)):

$$A_{\lambda Cmax} = A_{C_{\lambda Cmax}} + A_{S_{\lambda Cmax}} \tag{16}$$

$$A_{\lambda Cmax} = c_{eq} \times \varepsilon_{C_{\lambda Cmax}} \times \ell + (c_{S_0} - c_{eq}) \times \varepsilon_{S_{\lambda Cmax}} \times \ell \tag{17}$$

The point of the molar concentration equilibrium (c_{eq}) was at $c_{S_0} = c_{Fe}$ for stoichiometry 1:1, $c_{S_0} = 2 \times c_{Fe}$ for stoichiometry 2:1, etc. The comparison of the measured absorbance with these theoretical lines was able to reveal the searched stoichiometry or

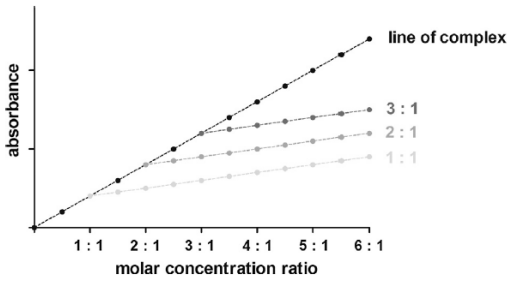

Fig. 4. Theoretical determination of absorbance (Method V). The black line corresponds to the absorbance of the formed complex at the excess of iron. The light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron.

Table 1
Summarized wavelengths of absorption maxima of the tested substances and their complexes with iron.

Substance	λ_{Smax} (nm)	λ_{Cmax} (nm)	λ shift (nm)
8-Hydroxyquinoline	240 ± 0 307 ± 1	250 ± 0 355 ± 0 456 ± 0 575 ± 0	10–268
Chloroxine	247 ± 0 278 ± 1 316 ± 2	261 ± 1	14
Deferasirox	246 ± 0 293 ± 1	309 ± 2	14
Deferiprone	280 ± 0	227 ± 1 289 ± 3	9
Deferoxamine	225 ± 1	430 ± 0	205
EDTA	×	256 ± 0	×
PIH	218 ± 2 296 ± 1 342 ± 1	233 ± 3 310 ± 2 367 ± 2 463 ± 2	14–123
Quercetin	370 ± 0	434 ± 2[a] 443 ± 3[b]	64–73[ab]
Rutin	356 ± 2	401 ± 5	45
SIH	218 ± 5 288 ± 0 330 ± 0	231 ± 7 303 ± 0 350 ± 0	13–20

λ shift means the difference between absorption maximum(a) of the tested substance and the complex with iron.
× – no absorption maximum of EDTA was found.
[a] Quercetin: at pH 4.5–6.8.
[b] Quercetin: at pH 7.5.

Table 2
Summarized results of the described methodological approaches. ✓ successful method, (✓) partially efficient method, × unsuccessful method, – the analysis was not performed.

Substance/method	Job's	I	II	III	IV	V	VI
8-Hydroxyquinoline	✓	✓	–	–	–	–	–
Chloroxine	✓	✓	✓	✓	✓	✓	✓
Deferasirox	✓	×	×	×	×	✓	✓
Deferiprone	✓	×	✓	×	×	×	✓
Deferoxamine	✓	✓	–	–	–	–	–
EDTA	✓	✓	–	–	–	–	–
PIH	✓	✓	(✓)	×	×	✓	✓
Quercetin	✓	✓	(✓)	(✓)	(✓)	✓	✓
Rutin	✓	×	(✓)	(✓)	(✓)	✓	✓
SIH	✓	×	(✓)	×	×	✓	✓

Partially efficient method means that the method was able to suggest the correct stoichiometric ratio but the measured points were not in the full agreement with the theoretical lines.

even the reaction stoichiometry at different molar concentration ratios (Fig. 4). At the wavelength of absorption maximum of the substance, the identical approach was used.

2.4.3.6. Theoretical determination of the sum of absorbance of the non-reacted substance and complex at absorption maximum of substance (Method VI). This calculation was similar to the previous methodology based on the construction of theoretical lines mimicking the absorbance of the most probable stoichiometries.

The principle was the same, i.e. firstly, the absorbance depended on the formation of complex up to the point, in which the whole iron was consumed for the complex formation, and thereafter the absorbance was dependent only on the added (non-reacted) substance (Eqs. (14)–(16)). But in the contrast to the Method V, it presumed that diverse complexes with different molar absorption coefficients were formed in the excess of iron. Therefore, the absorbance was not rising linearly up to the concentration equilibrium. Thus, the lines depending only on the non-reacted substance were constructed directly from the measured absorbance at the most probable chelation ratios (1:1, 2:1, 3:1, etc.).

The identical approach was used at the wavelength of the absorption maximum of the complex.

2.5. Data analysis

The majority of experiments, in particular those that determined unclear stoichiometry, were performed at least in duplicates with two different stock solutions. On the other hand, some experiments, which gave unequivocal outcomes, were performed as a single measurement after the concentration of both reagents was calibrated.

Data are expressed as means ± SD. In appropriate cases, a single measurement is depicted for better lucidity in figures.

3. Results and discussion

First, the absorption spectra of all 10 analyzed substances and their iron complexes were measured and compared. The tested substances varied markedly in the positions of their absorption maxima and in the arithmetic differences between the absorption maxima of the pure substance and its corresponding complex. In almost all tested cases at pH ⩾ 5.5, there were no apparent differences in absorption maxima between Fe(II) or Fe(III) complexes. This suggests that only one type of iron-substance complex was formed and thus the data were summarized. Since ferrous ions may be oxidized in the complex with strong iron chelators under physiological pH, ferric complexes were likely formed [23].

Quercetin was an exception because there was a marked difference between the absorption maximum of the complex at different

pH conditions (Table 1), which is in agreement with the previously published data [24].

At pH 4.5 the iron-chelating activity differed among the tested substances. 8-Hydroxyquinoline, SIH, PIH, quercetin and rutin had low affinity for Fe(II) but not for Fe(III) at this pH.

In three iron-chelating agents, there were marked shifts of the absorption maximum(a) of the pure substance and the formed complex (deferoxamine and 8-hydroxyquinoline) or the pure substance (EDTA) did not absorb in the measured range of wavelengths. In these cases, the analytical approach was not complicated. The Job's method or Method I was sufficient for the assessment and no additional approaches were necessary (see summarized data in Table 2). Examples are shown for deferoxamine (Fig. 5 and see Supplementary data Fig. S2), EDTA (see Supplementary data Fig. S3) and 8-hydroxyquinoline (see Supplementary data Fig. S4). The EDTA-iron complex's absorption maximum was localized at the low wavelength (256 nm), at which Fe(III) is known to exert some absorbance. But this fact did not interfere with the assessment in low concentrations of iron and EDTA. The resulting stoichiometries are in full accordance with available literary data, since both deferoxamine and EDTA are hexadentate iron chelators, and hence 1:1 ratios, as expected, were confirmed in this study as well [1,15]. 8-Hydroxyquinoline formed 3:1 complexes which is in agreement with the bidentate nature of this iron-chelating agent [25].

In all other tested substances, all described methodological approaches were applied (see summarized data in Table 2).

The Method I, based on the use of the wavelength of the absorption maximum of complex (λ_{Cmax}), was able to successfully determine the chelation stoichiometry in several other cases due to the character of the absorption spectra (chloroxine, quercetin and PIH), but not in other tested chelators (deferasirox, rutin and SIH; Supplementary data Figs. S5, S7 and S9).

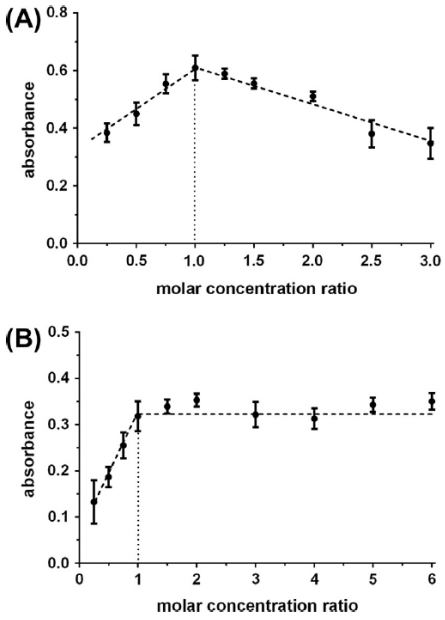

Fig. 5. Assessment of deferoxamine-Fe(II) complex. Figure shows the standard Job's method (A) and simple method I (B) at pH 7.5. Absorbance was read at λ_{Cmax} (430 nm). The ratios signify substance to iron. The total molar concentration of deferoxamine and iron was 0.5 mM for the Job's method. In the Method I, the final molar concentration of deferoxamine was from 0.025 to 0.6 mM while that of iron was constantly 0.1 mM. The assessment was performed in duplicates.

The Method II was able to clearly identify the stoichiometry in chloroxine (Supplementary data Fig. S8E) and deferiprone. In several cases (Table 2), the method suggested apparently the correct stoichiometric ratio, but the resulting graph did not fit in the expected scheme. This was the case of quercetin or SIH, where the ratio 1:1 and 2:1, respectively, was suggested but the chelation lines were not identical with the theoretical lines (Fig. 6A and Supplementary data Fig. S7E). In the case of deferasirox, the Method II completely failed. The Methods III and IV were even less efficient and gave the clear result only in the case of chloroxine (Supplementary data Fig. S8).

The reasons for the success/failure of the Methods II, III and IV likely consisted in three factors: (a) a small difference between wavelengths of absorption maxima of the substance and its complex with iron, (b) a presence of another absorption maximum in the proximity of analyzed absorption maximum and (c) a low

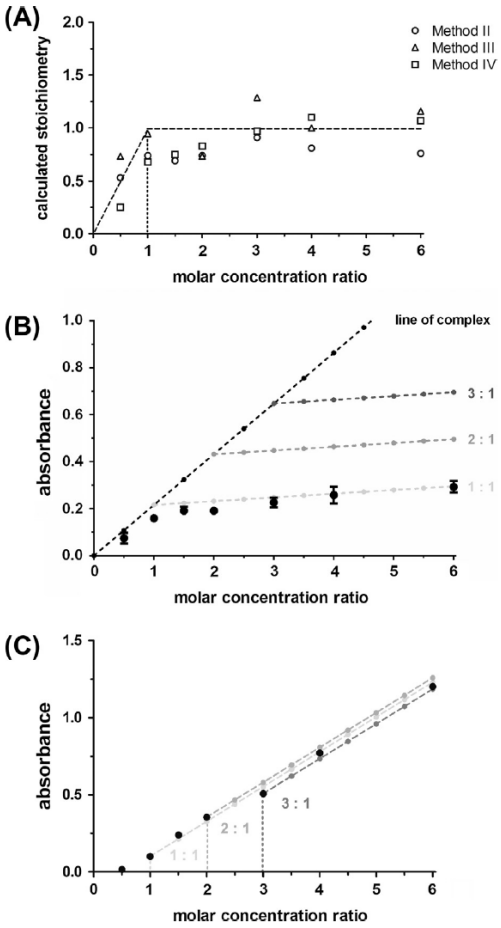

Fig. 6. Quercetin and Fe(III) at pH 7.5. Complementary approach – the plots of Method II, III and IV (A), the plot of Method V (B) and the plot of Method VI (C), in which all lines are almost identical suggesting the formation of the complex at the stoichiometry 1:1. The final molar concentration of iron was 0.01 mM and the final molar concentrations of quercetin were 0.005–0.06 mM. In Fig. 6B, the light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron. In this figure, tsingle measurements are depicted for better lucidity. In this figure, single measurements are depicted for better lucidity.

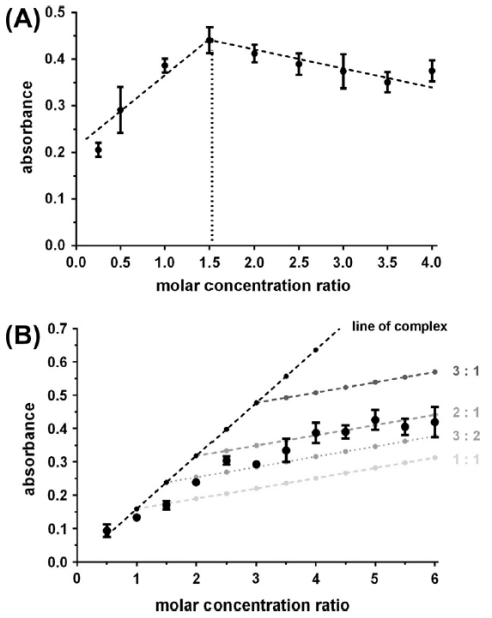

Fig. 7. Rutin and Fe(II) at pH 6.8. Job's method – the Job's plot at λ_{Cmax} (404 nm) (A). The total molar concentration of rutin and iron was 0.05 mM. Complementary approach – the plot of Method V (B). The final molar concentration of iron was 0.01 mM and the final molar concentrations of rutin were 0.005–0.06 mM. In Fig. 7B, the black line corresponds to the absorbance of the formed complex at the excess of iron. The light grey line mimics the absorbance at the stoichiometry 1:1, the dotted grey line at 3:2, the dashed grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron. The assessment was performed with 4 new stock solutions.

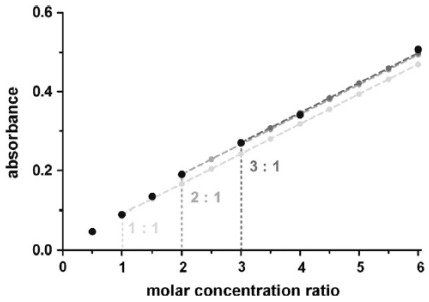

Fig. 8. PIH and Fe(III) at pH 7.5. Complementary approach – the plot of Method V. The final molar concentration of iron was 0.01 mM and the final molar concentrations of PIH were 0.005–0.06 mM. The light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The line 3:1 is identical with the line 2:1 suggesting the latter complex. The ratios signify substance to iron. In this figure, a single measurement is depicted for better lucidity.

difference between molar absorption coefficients of the substance and its complex at analyzed wavelength(s).

The stoichiometries of the both tested flavonoids, rutin and quercetin, were identified by all Methods II–VI suggesting that the wavelength difference of 45 nm between the tested substance and its complex was sufficient for these methodologies. In all other compounds (chloroxine, deferasirox, deferiprone, SIH and PIH), the difference was much lower ranging approximately from 9 to 25 nm. One exception was PIH, in which a distant absorption maximum at 463 nm (difference 121 nm) was observed as well. But at this wavelength, absorbance of the complex was very low (see Supplementary data Tab. S1) which did not enable the precise calculation. As mentioned previously, the Methods III and IV failed with an exception of chloroxine in all mentioned substances suggesting that the difference between wavelengths (factor a) is the principal factor for the applicability of those methods.

Although the difference between wavelengths in the case of chloroxine was quite small, comparable to deferiprone, and smaller than PIH or SIH, these methods were applicable. The reason likely lay in the steepness of the absorption maxima, i.e. the differences between the molar absorption coefficients (factor c; Supplementary data Tab. S1). The second factor (b), the presence of a close absorption maximum, may substantially contribute to the failure as well. This was apparently true for deferasirox, PIH and SIH (Supplementary data Figs. S5A, S7A and S10A, respectively).

The Methods V and VI were the most efficient and were able to reveal the stoichiometry in all cases. The only exception was deferiprone in the case of Method V. The reason for failure of Methods III, IV and V in the case of deferiprone, in contrast to successful Methods II and VI, can be likely explained by the dependence of the former methods on the assessment of molar absorption coefficients of complex. The probable explanation was the very small difference between the wavelengths of pure deferiprone and its complex (9 nm), which blunted the correct calculation of the molar absorption coefficient of the complex. Indeed, there was almost no difference between the molar absorption coefficient of pure deferiprone and its complex at the λ_{Cmax} (Supplementary data Tab. S1).

Using the Job's method and the complementary mathematical calculations for the determination of chelation stoichiometry, it was disclosed that all tested substances chelated iron in various manners and the obtained ratios were consistent with the previously published data – deferasirox 2:1 (Fig. S5) [15,26]; quercetin 1:1 (Fig. 6 and Supplementary data Fig. S6) [27]; SIH 2:1 (Supplementary data Fig. S7) [28–30]; chloroxine 3:1 (Supplementary data Fig. S8) [25]; rutin 1:1, 3:2 and 2:1 (Fig. 7 and Supplementary data Fig. S9) [16,17]; PIH 2:1 (Fig. 8, Supplementary data Fig. S10) [28–30]; and deferiprone 3:1 (Supplementary data Fig. S11) [28].

Another feature of the theoretical methods, especially of the Method V, was demonstrated within the assessment of rutin's stoichiometry at pH 6.8. Comparing the proposed values for absorbance with the measured ones, it was found that rutin may chelate iron at various chelation ratios (1:1, 3:2 and 2:1, rutin to iron, respectively) depending on its concentration (Fig. 7B). On the other hand, the Job's method showed only one ratio 3:2 (Fig. 7A). Comparing these two different methodological approaches led to a slight superiority of the complementary approach. Therefore, this finding may explain the diverse results in flavonoids from the different studies [16,17].

This method is not able to determine the iron oxidation status in the complex. Notwithstanding this limitation may be of importance for chemical screening of novel specific ferrous chelators, this is of lower importance for a pharmacological study. The main question in the later is the efficacy of a tested substance to chelate ferrous or ferric iron while the oxidation status of the complex is of secondary importance.

4. Conclusion

This study reported the novel calculations for the assessment of stoichiometry of chelators with iron. This approach can be useful in the confirming of the chelation stoichiometry and moreover, it may reveal the reaction stoichiometry in chelators with a moderate affinity to iron. Since the data on stoichiometry of the com-

plexes of several substances were apparently different among miscellaneous studies, particularly in flavonoids, we suggest that for the correct stoichiometry calculation, the both Job's and the complementary approaches should be used.

Acknowledgments

This study was supported by the Charles University (GAUK 605712C and SVV 265 003/2012). We thank to Dr. John Connelly (ApoPharma Inc.) for kind providing deferiprone.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.06.002.

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Bioorganic Chemistry

Mathematical calculations of iron complex stoichiometry by direct UV-Vis spectrophotometry

SUPPLEMENTARY DATA

(13 pages)

Tomáš Filipský[a], Michal Říha[a], Radomír Hrdina[a], Kateřina Vávrová[b] and Přemysl Mladěnka[a]✉

Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

[a]Department of Pharmacology and Toxicology, [b]Department of Inorganic and Organic Chemistry

✉**Corresponding author:**

Přemysl Mladěnka, Ph.D., Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

Tel.: +420-495-067-295, Fax: +420-495-067-170, Email: mladenkap@faf.cuni.cz

Calculations using absorption maximum of substance
and absorption maximum of complex
(Method III and Method IV)

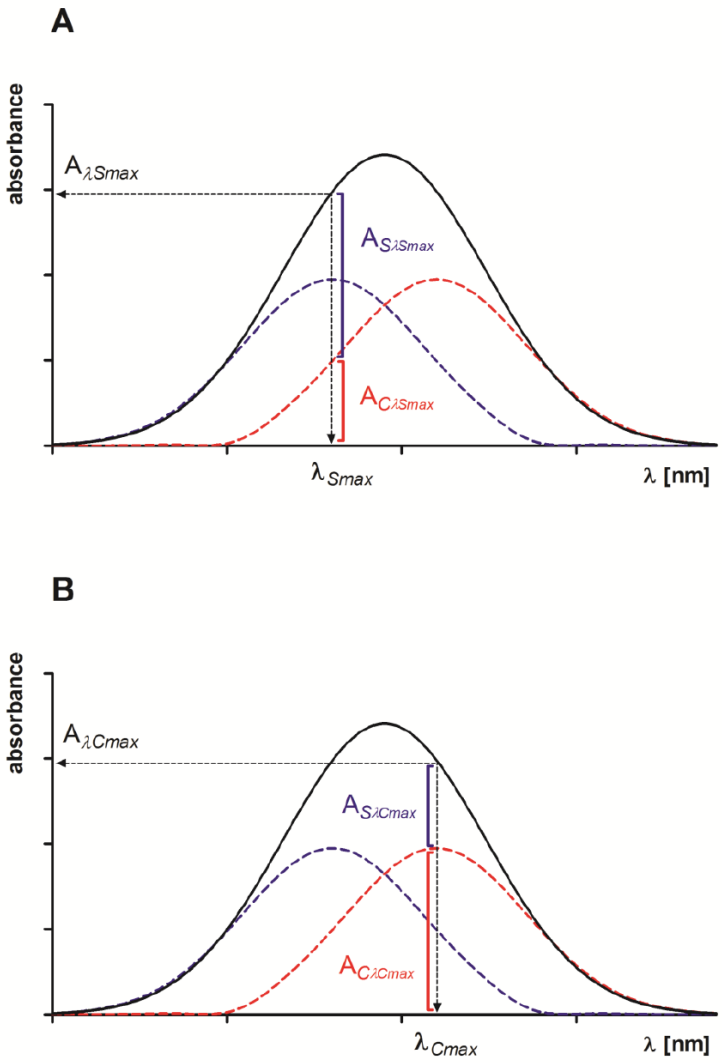

Fig. S1: Calculations using the absorption maximum of a substance (**A**) and the absorption maximum of the complex (**B**). The measured absorption spectrum (black curve) is the sum of the absorption spectrum of the non-reacted substance (blue dashed curve) and the absorption spectrum of the formed complex (red dashed curve).

Deferoxamine and Fe(II) at pH 7.5

Job's method and Complementary approach – Method I

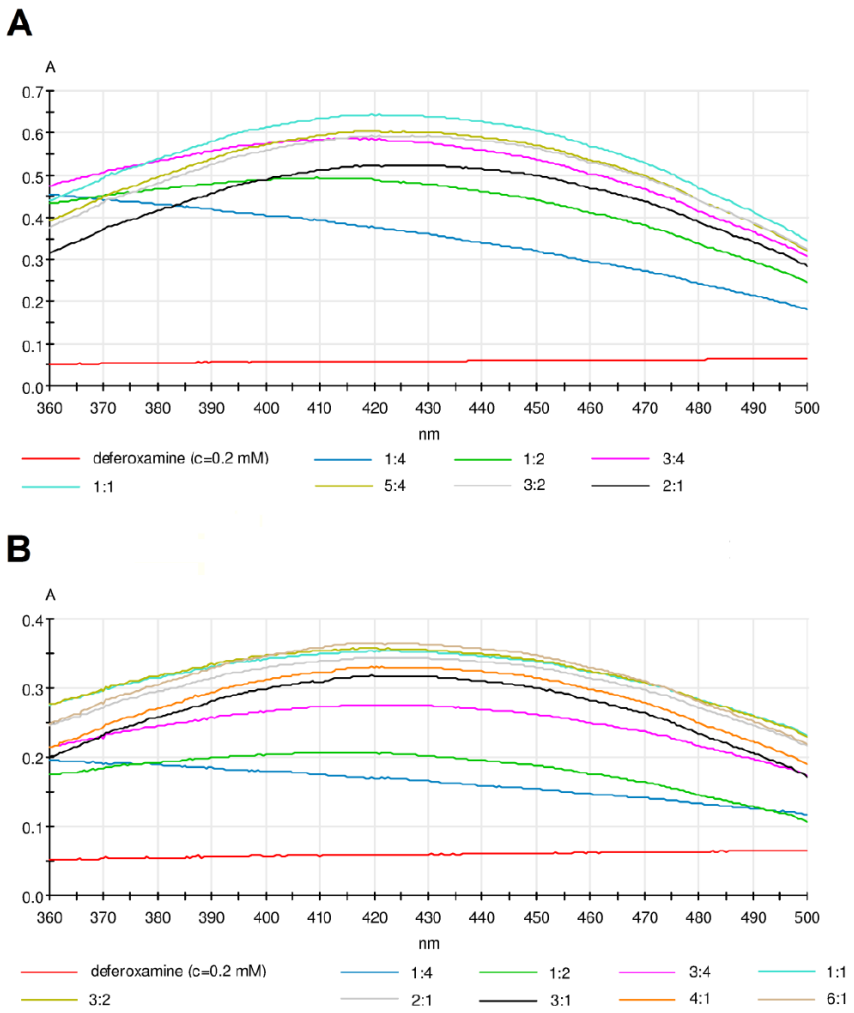

Fig. S2: Deferoxamine and Fe(II) at pH 7.5.

Job's method – the absorption spectra (**A**). The total molar concentration of deferoxamine and iron was 0.5 mM.

Complementary approach – the absorption spectra (**B**). The final concentration of iron was 0.1 mM and the final concentrations of deferoxamine were 0.025-0.6 mM.

The ratios signify substance to iron. In this figure, the absorption spectra of single measurements are depicted.

EDTA and Fe(II) at pH 7.5

Job's method and Complementary approach – Method I

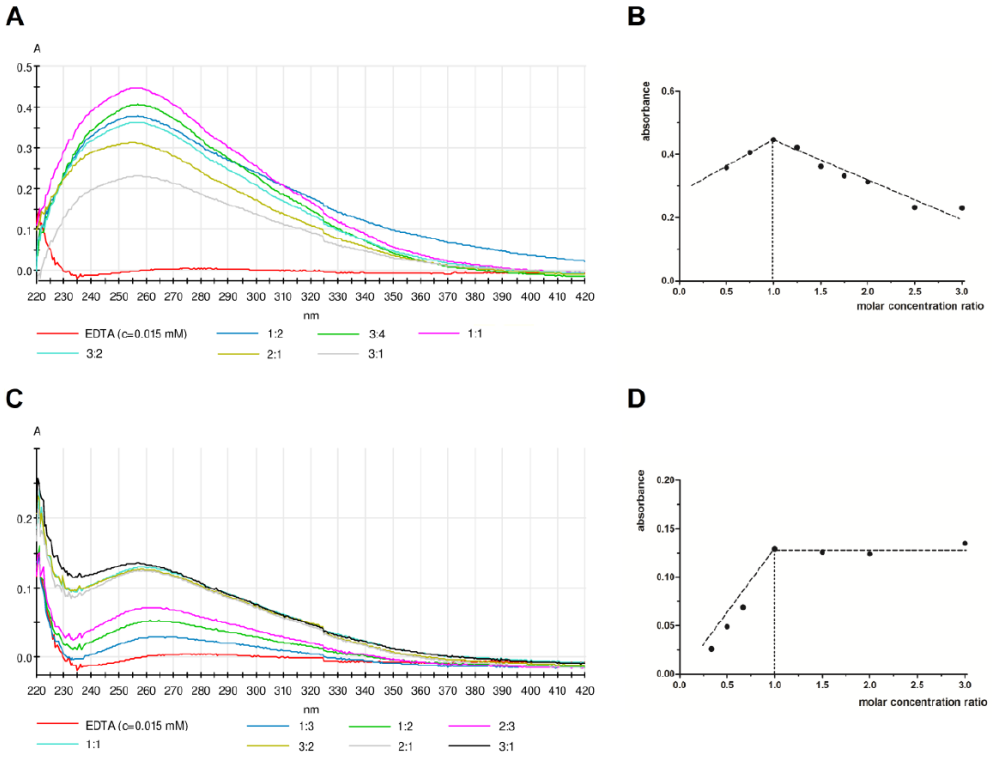

Fig. S3: EDTA and Fe(II) at pH 7.5.

Job's method – the absorption spectra (**A**) and the Job's plot at λ_{Cmax} (256 nm) (**B**). The total molar concentration of EDTA and iron was 0.1 mM.

Complementary approach – the absorption spectra (**C**) and the plot of Method I (**D**). The final molar concentration of iron was 0.015 mM and the final molar concentrations of EDTA were 0.005-0.045 mM.

The ratios signify substance to iron. In this figure, single measurements are depicted for better lucidity.

8-Hydroxyquinoline and Fe(III) at pH 7.5

Job's method and Complementary approach – Method I

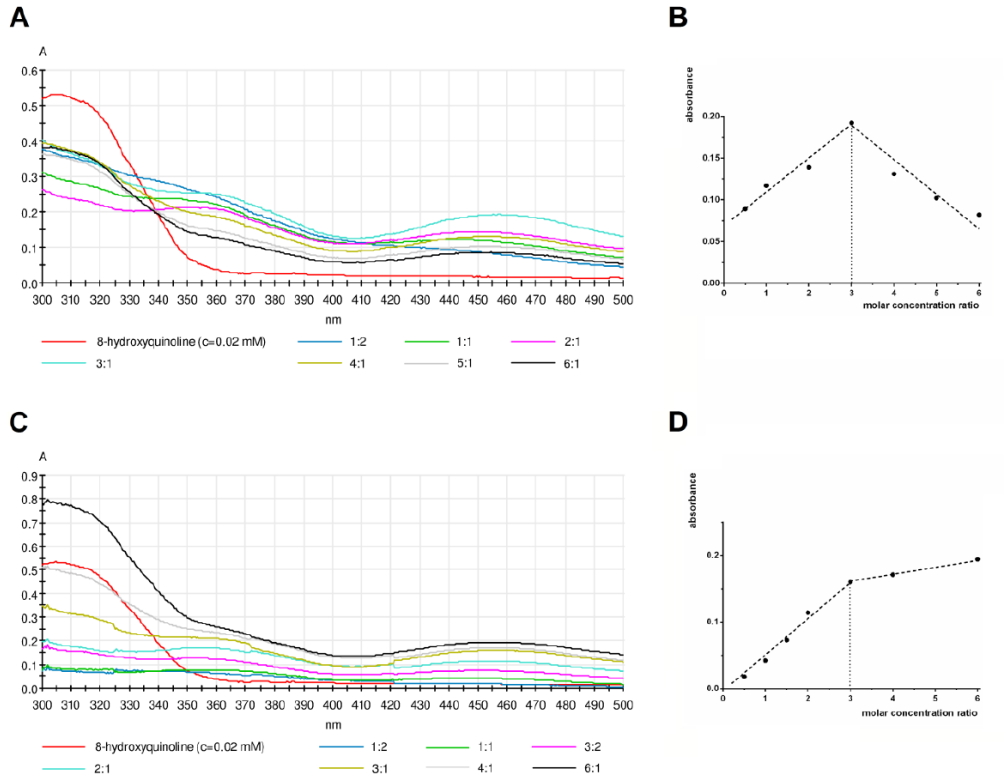

Fig. S4: 8-Hydroxyquinoline and Fe(III) at pH 7.5.

Job's method – the absorption spectra (**A**) and the Job's plot at λ_{Cmax} (460 nm) (**B**). The total molar concentration of 8-hydroxyquinoline and iron was 0.2 mM.

Complementary approach – the absorption spectra (**C**) and the plot of Method I (**D**). The final molar concentration of iron was 0.05 mM and the final molar concentrations of 8-hydroxyquinoline were 0.025-0.3 mM.

The ratios signify substance to iron. In this figure, single measurements are depicted for better lucidity.

Deferasirox and Fe(II) at pH 7.5

Absorption spectra, Job's method and Complementary approach – Method I

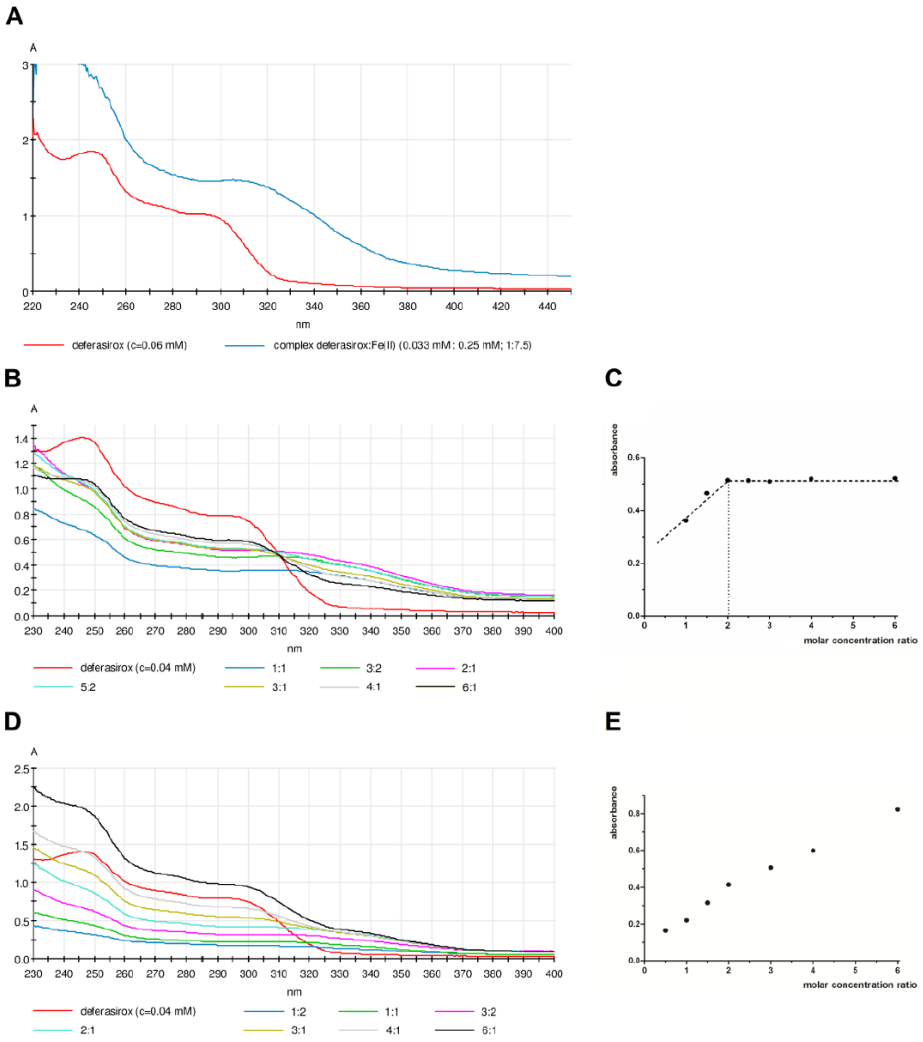

Fig. S5: Deferasirox and Fe(II) at pH 7.5.

Absorption spectra – deferasirox and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (309 nm) (**C**). The total molar concentration of deferasirox and iron was 0.04 mM.

Complementary approach at λ_{Cmax} – the absorption spectra (**D**) and the plot of Method I (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of deferasirox were 0.005-0.06 mM. This method was unsuccessful.

The ratios signify substance to iron. In this figure, single measurements are depicted for better lucidity.

Quercetin and Fe(III) at pH 7.5

Absorption spectra, Job's method and Complementary approach – Method I

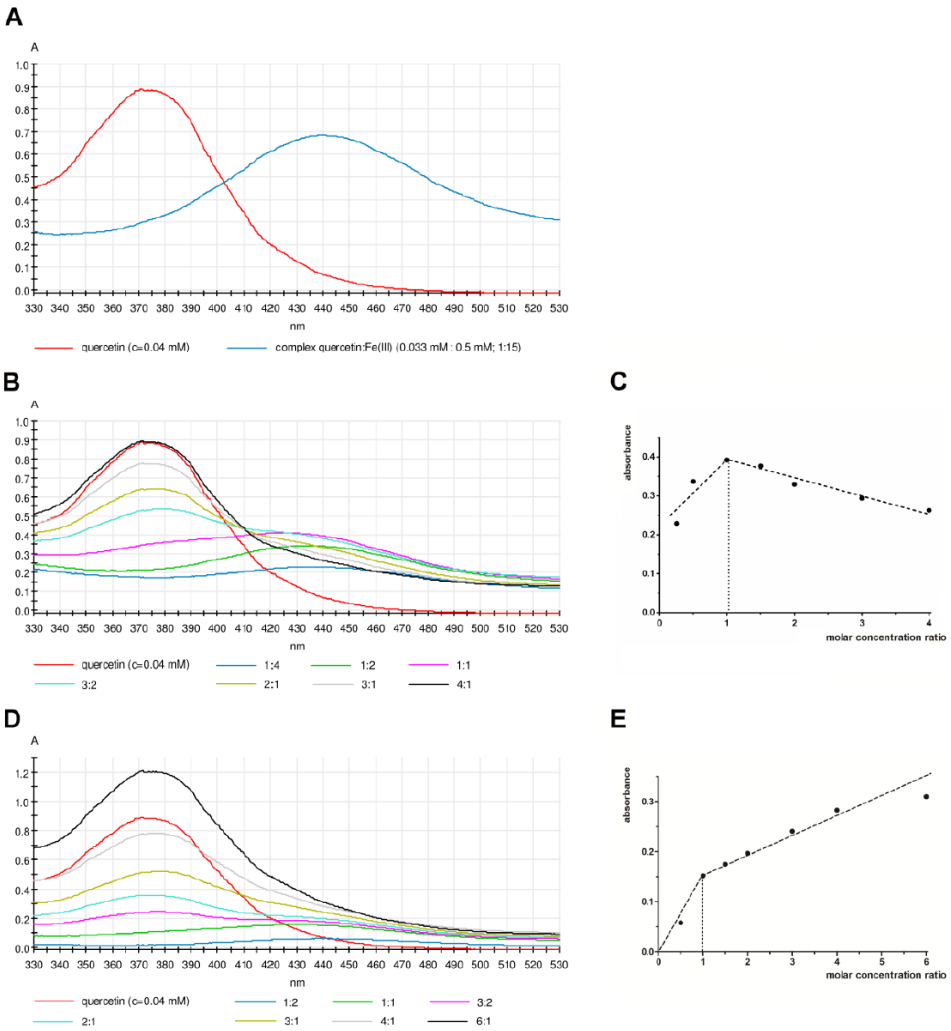

Fig. S6: Quercetin and Fe(III) at pH 7.5.

Absorption spectra – quercetin and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (443 nm) (**C**). The total molar concentration of quercetin and iron was 0.05 mM.

Complementary approach – the absorption spectra (**D**) and the plot of Method I (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of quercetin were 0.005-0.06 mM.

The ratios signify substance to iron. In this figure, single measurements are depicted for better lucidity.

SIH and Fe(II) at pH 7.5

Absorption spectra, Job's method and Complementary approach – Method II

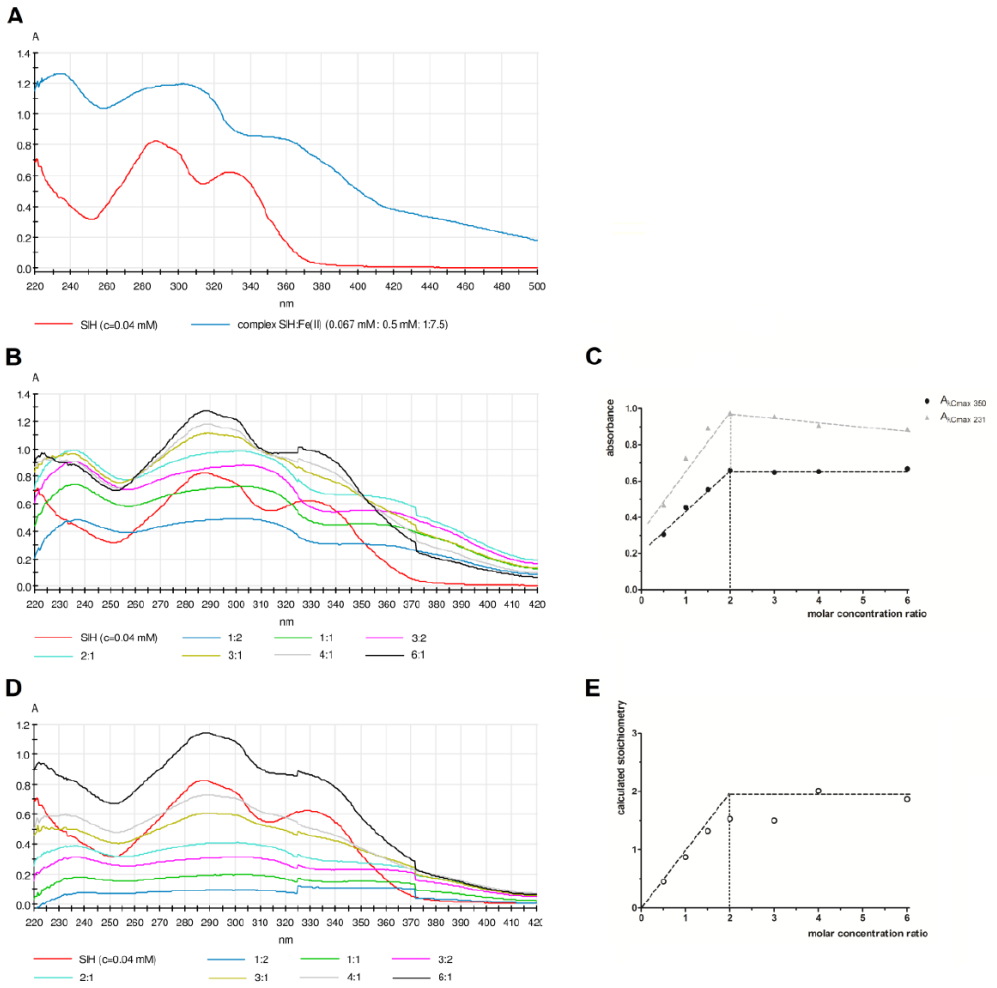

Fig. S7: SIH and Fe(II) at pH 7.5.

Absorption spectra – SIH and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plots at λ_{Cmax} (231 and 350 nm) (**C**). The total molar concentration of SIH and iron was 0.075 mM.

Complementary approach – the absorption spectra (**D**) and the plot of Method II (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of SIH were 0.005-0.06 mM.

The ratios signify substance to iron. In this figure, single measurements are depicted for better lucidity.

Chloroxine and Fe(II) at pH 5.5

Absorption spectra, Job's method and Complementary approach – Method II-IV

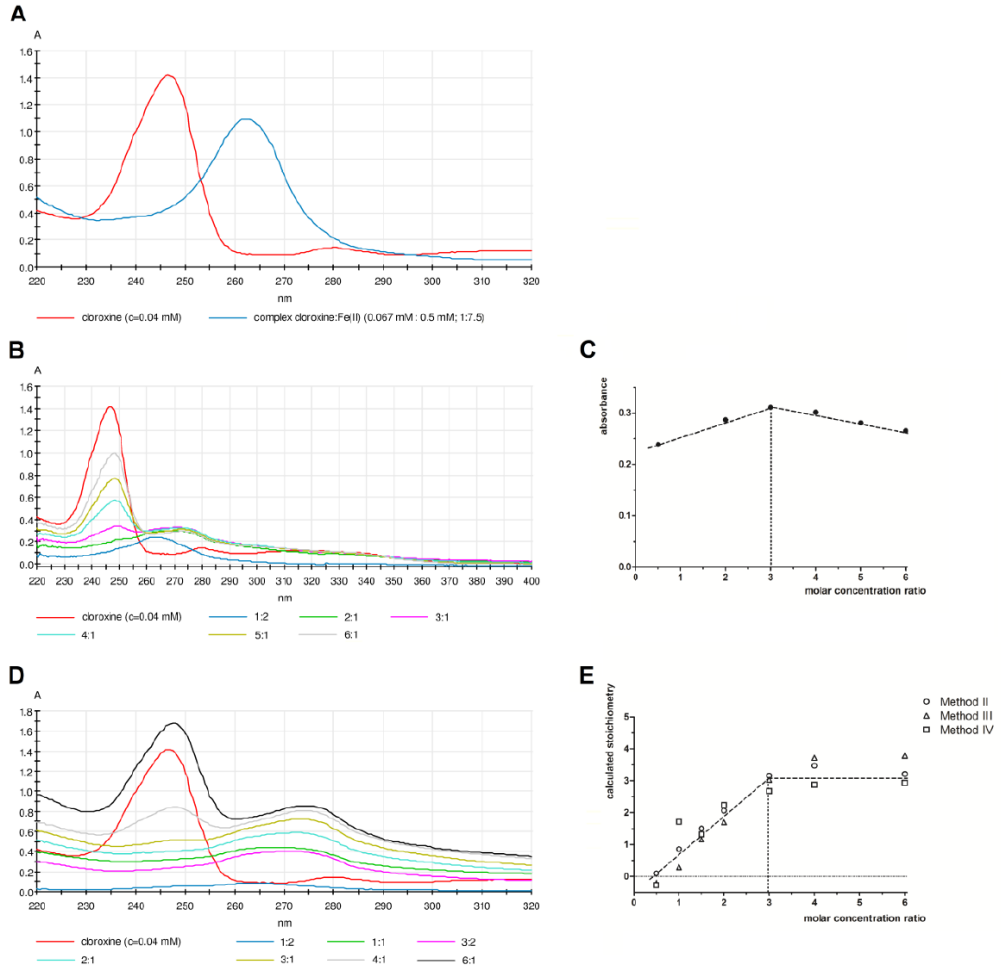

Fig. S8: Chloroxine and Fe(II) at pH 5.5.

Absorption spectra – chloroxine and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (262 nm) (**C**). The total molar concentration of chloroxine and iron was 0.05 mM.

Complementary approach – the absorption spectra (**D**) and the plots of Method II, III and IV (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of chloroxine were 0.005-0.06 mM.

The ratios signify substance to iron. In this figure, single measurements are depicted for better lucidity.

Rutin and Fe(II) at pH 6.8

Absorption spectra, Job's method and Complementary approach

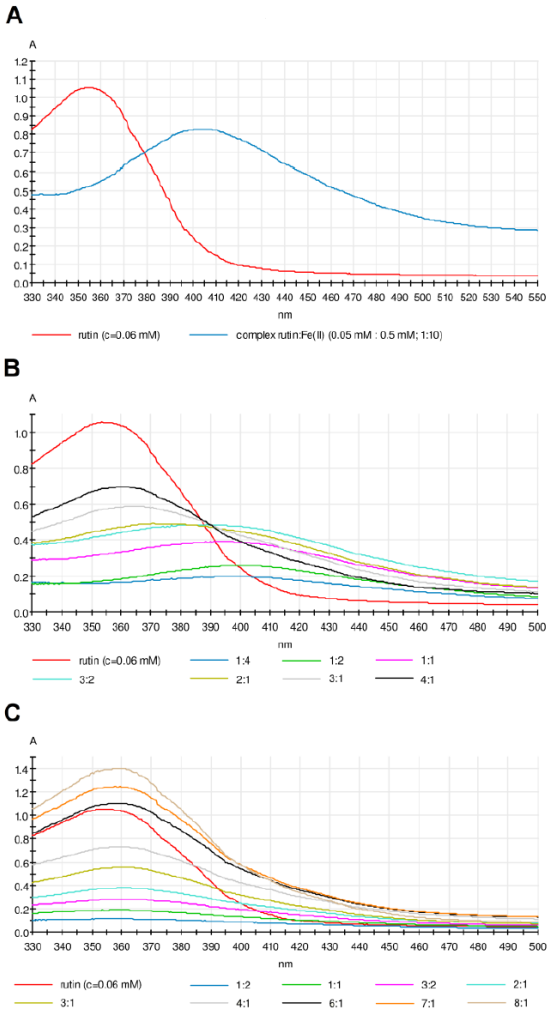

Fig. S9: Rutin and Fe(II) at pH 6.8.

Absorption spectra – rutin and its complex (**A**).

Job's method – the absorption spectra (**B**). The total molar concentration of rutin and iron was 0.05 mM.

Complementary approach – the absorption spectra (**C**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of rutin were 0.005-0.08 mM. Corresponding plots are shown in Fig. 7 in the main article.

The ratios signify substance to iron. In this figure, the absorption spectra of single measurements are depicted.

PIH and Fe(III) at pH 7.5

Absorption spectra, Job's method and Complementary approach

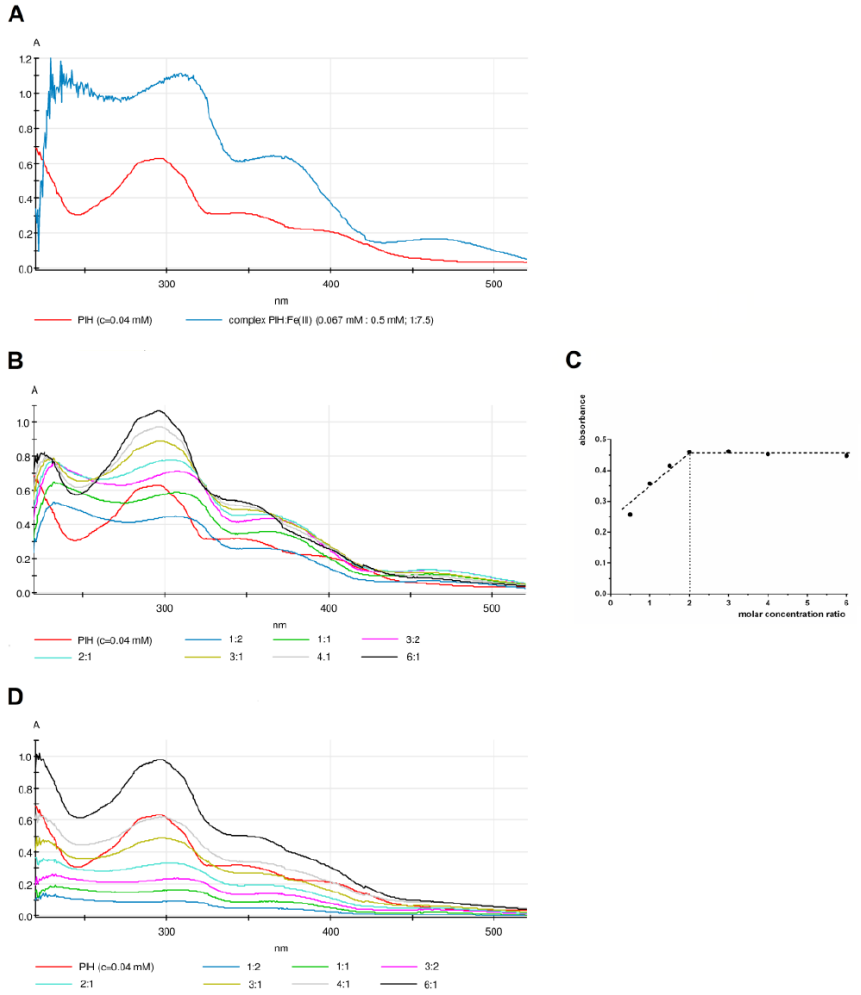

Fig. S10: PIH and Fe(III) at pH 7,5.

Absorption spectra – PIH and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (366 nm) (**C**). The total molar concentration of PIH and iron was 0.075 mM.

Complementary approach – the absorption spectra (**D**).The corresponding plot (Method VI) is shown in Fig. 8 in the main article. The final molar concentration of iron was 0.01 mM and the final molar concentrations of PIH were 0.005-0.06 mM.

The ratios signify substance to iron. In this figure, single measurements are depicted for better lucidity.

Deferiprone and Fe(III) at pH 7.5

Absorption spectra, Job's method and Complementary approach

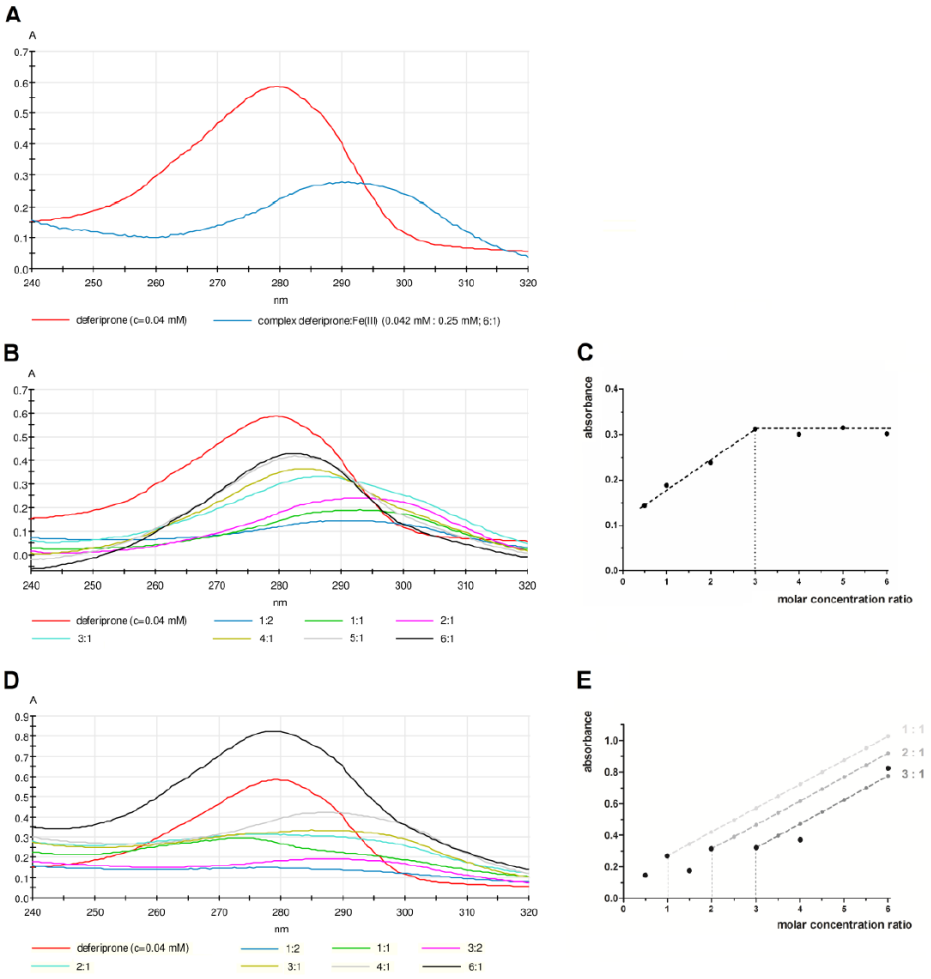

Fig. S11: Deferiprone and Fe(III) at pH 7.5.

Absorption spectra – deferiprone and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (305 nm) (**C**). The total molar concentration of deferiprone and iron was 0.05 mM.

Complementary approach – the absorption spectra (**D**) and the plot of Method VI (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of deferiprone were 0.005-0.06 mM.

The light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron. In this figure, single measurements are depicted for better lucidity.

Tab. S1: Summarized molar absorption coefficients and corresponding differences at the analyzed wavelengths.

substance	ε_S [$mol^{-1}.cm^{-1}.l$]	ε_C [$mol^{-1}.cm^{-1}.l$]	$\lvert \varepsilon_S - \varepsilon_C \rvert$ [$mol^{-1}.cm^{-1}.l$]
chloroxine	$\varepsilon_{\lambda 247}$ = 34 803 ± 4 033 $\varepsilon_{\lambda 261}$ = 9 059 ± 1 557	$\varepsilon_{\lambda 247}$ = 14 337 ± 6 700 $\varepsilon_{\lambda 261}$ = 21 973 ± 6 502	λ_{247} = 20 465 λ_{261} = 12 914
deferasirox	$\varepsilon_{\lambda 246}$ = 30 815 ± 518 $\varepsilon_{\lambda 309}$ = 10 771 ± 875	$\varepsilon_{\lambda 246}$ = 35 258 ± 9 196 $\varepsilon_{\lambda 309}$ = 18 666 ± 4 412	λ_{246} = 4 443 λ_{309} = 7 895
deferiprone	$\varepsilon_{\lambda 280}$ = 14 730 ± 1 093 $\varepsilon_{\lambda 289}$ = 9 800 ± 2 012	$\varepsilon_{\lambda 280}$ = 8 490 ± 1 853 $\varepsilon_{\lambda 289}$ = 9 948 ± 2 204	λ_{280} = 6 240 λ_{289} = 148
PIH	$\varepsilon_{\lambda 218}$ = 16 007 ± 1353 $\varepsilon_{\lambda 233}$ = 11 133 ± 1 644 $\varepsilon_{\lambda 296}$ = 16 425 ± 1 486 $\varepsilon_{\lambda 310}$ = 13 423 ± 1 391 $\varepsilon_{\lambda 342}$ = 7 909 ± 487 $\varepsilon_{\lambda 367}$ = 5 569 ± 381 $\varepsilon_{\lambda 463}$ = 260 ± 179	$\varepsilon_{\lambda 218}$ = 13 630 ± 3 298 $\varepsilon_{\lambda 233}$ = 14 900 ± 4 630 $\varepsilon_{\lambda 296}$ = 14 162 ± 2 090 $\varepsilon_{\lambda 310}$ = 14 627 ± 2 751 $\varepsilon_{\lambda 342}$ = 9 686 ± 1 191 $\varepsilon_{\lambda 367}$ = 10 732 ± 1 501 $\varepsilon_{\lambda 463}$ = 3 084 ± 879	λ_{218} = 2 377 λ_{233} = 3 766 λ_{296} = 2 263 λ_{310} = 1 205 λ_{342} = 1 777 λ_{367} = 5 164 λ_{463} = 2 824
quercetin	$\varepsilon_{\lambda 370}$ = 23 838 ± 887 $\varepsilon_{\lambda 434/443}$ = 983 ± 192	$\varepsilon_{\lambda 370}$ = 8 247 ± 1 713 $\varepsilon_{\lambda 434/443}$ = 15 887 ± 3 346	λ_{370} = 15 591 $\lambda_{434/443}$ = 14 905
rutin	$\varepsilon_{\lambda 356}$ = 17 210 ± 1 346 $\varepsilon_{\lambda 401}$ = 2 319 ± 706	$\varepsilon_{\lambda 356}$ = 10 219 ± 1 342 $\varepsilon_{\lambda 401}$ = 16 674 ± 1 924	λ_{356} = 6 991 λ_{401} = 14 356
SIH	$\varepsilon_{\lambda 218}$ = 16 875 ± 3 577 $\varepsilon_{\lambda 231}$ = 9 200 ± 1 700 $\varepsilon_{\lambda 288}$ = 18 020 ± 1 147 $\varepsilon_{\lambda 303}$ = 14 473 ± 1 325 $\varepsilon_{\lambda 330}$ = 13 725 ± 804 $\varepsilon_{\lambda 350}$ = 6 844 ± 688	$\varepsilon_{\lambda 218}$ = 17 195 ± 4 412 $\varepsilon_{\lambda 231}$ = 20 325 ± 3 631 $\varepsilon_{\lambda 288}$ = 16 243 ± 1 927 $\varepsilon_{\lambda 303}$ = 16 850 ± 1 533 $\varepsilon_{\lambda 330}$ = 11 726 ± 1 153 $\varepsilon_{\lambda 350}$ = 11 279 ± 1 110	λ_{218} = 320 λ_{231} = 11 125 λ_{288} = 1 777 λ_{303} = 2 378 λ_{330} = 1 999 λ_{350} = 4 435

ε_S – molar absorption coefficient of substance, ε_S – molar absorption coefficient of complex, $\lvert \varepsilon_S - \varepsilon_C \rvert$ – absolute difference between the coefficients

4.6. Oral administration of quercetin is unable to protect against isoproterenol cardiotoxicity

ŘÍHA, M.; VOPRŠALOVÁ, M.; PILAŘOVÁ, V.; SEMECKÝ, V.; HOLEČKOVÁ, M.; VÁVROVÁ, J.; PALICKA, V.; FILIPSKÝ, T.; HRDINA, R.; NOVÁKOVÁ, L.; MLADĚNKA, P. Oral administration of quercetin is unable to protect against isoproterenol cardiotoxicity. *Naunyn-Schmiedeberg´s Archives of Pharmacology*. 2014, **387**(9), 823-835.

(IF 2013: 2,360)

Naunyn-Schmiedeberg's Arch Pharmacol (2014) 387:823–835
DOI 10.1007/s00210-014-0995-z

ORIGINAL ARTICLE

Oral administration of quercetin is unable to protect against isoproterenol cardiotoxicity

Michal Říha · Marie Vopršalová · Veronika Pilařová · Vladimír Semecký · Magdalena Holečková · Jaroslava Vávrová · Vladimir Palicka · Tomáš Filipský · Radomír Hrdina · Lucie Nováková · Přemysl Mladěnka

Received: 16 May 2013 / Accepted: 20 May 2014 / Published online: 5 June 2014

Abstract Catecholamines are endogenous amines that participate in the maintenance of cardiovascular system homeostasis. However, excessive release or exogenous administration of catecholamines is cardiotoxic. The synthetic catecholamine, isoprenaline (isoproterenol, ISO), with non-selective β-agonistic activity has been used as a viable model of acute myocardial toxicity for many years. Since the pathophysiology of ISO–cardiotoxicity is complex, the aim of this study was to elucidate the effect of oral quercetin pretreatment on myocardial ISO toxicity. Wistar–Han rats were randomly divided into four groups: solvent or quercetin administered orally by gavage in a dose of 10 mg kg^{-1} daily for 7 days were followed by s.c. water for injection or ISO in a dose of 100 mg kg^{-1}. Haemodynamic, ECG and biochemical parameters were measured; effects on blood vessels and myocardial histology were assessed, and accompanying pharmacokinetic analysis was performed. Quercetin was unable to protect the cardiovascular system against acute ISO cardiotoxicity (stroke volume decrease, cardiac troponin T release, QRS-T junction elevation and histological impairment). The sole positive effect of quercetin on catecholamine-induced cardiotoxicity was the normalization of increased left ventricular end-diastolic pressure caused by ISO. Quercetin did not reverse the increased responsiveness of rat aorta to vasoconstriction in ISO-treated animals, but it decreased the same parameter in the control animals. Accompanying pharmacokinetic analysis showed absorption of quercetin and its metabolite 3-hydroxyphenylacetic acid formed by bacterial microflora. In conclusion, a daily oral dose of 10 mg kg^{-1} of quercetin for 7 days did not ameliorate acute ISO–cardiovascular toxicity in rats despite minor positive cardiovascular effects.

Keywords Cardiotoxicity · Catecholamine · Isoproterenol · Quercetin

M. Říha · M. Vopršalová · T. Filipský · R. Hrdina · P. Mladěnka (✉)
Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1 203, Hradec Králové 500 05, Czech Republic
e-mail: mladenkap@faf.cuni.cz

V. Pilařová · L. Nováková
Department of Analytical Chemistry, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1 203, Hradec Králové 500 05, Czech Republic

V. Semecký
Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1 203, Hradec Králové 500 05, Czech Republic

M. Holečková · J. Vávrová · V. Palicka
Faculty of Medicine in Hradec Králové, Charles University in Prague, Šimkova 870, Hradec Králové 500 38, Czech Republic

M. Holečková · J. Vávrová · V. Palicka
University Hospital Hradec Králové, Sokolská 581, Hradec Králové 500 05, Czech Republic

Introduction

Endogenous catecholamines are essential signal molecules in very low concentrations. However, they are well-known to be cardiotoxic in higher concentrations (Rona 1985; Costa et al. 2011). In harmony with this finding, the synthetic non-selective β-agonist isoprenaline (isoproterenol, ISO) has been used for more than 50 years for inducing a pathological state that mimics acute myocardial infarction in many respects (Rona et al. 1959). Administration of ISO in necrogenic doses is an adequate model for this purpose. A dose of 100 mg kg^{-1} s.c. evokes considerable histopathological changes in the myocardium which are associated with haemodynamic disturbances, marked release of cardiac troponin T, calcium overload, ST segment (J-point) elevations, R

amplitude decrease and with a mortality of around 30 % (Ramesh et al. 1998; Mladenka et al. 2009a; Zatloukalova et al. 2012). This level of mortality corresponds to the mortality rate of acute myocardial infarction before the introduction of current non-pharmacological treatment approaches (Widimský and Špaček 2004). Despite the plethora of research in this area, no single drug appears to be able to protect against all the consequences of catecholamine administration. Additionally, nor was a combination of α- and β-blockers able to completely protect against catecholamine injury notwithstanding the observed and expected effect on heart rate (Neri et al. 2007). This is very likely due to the fact that catecholamine cardiotoxicity is a complex mechanism involving both hyperstimulation of adrenoreceptors and catecholamine auto-oxidation leading to production of reactive compounds (Costa et al. 2011; Rona 1985; Dhalla et al. 2010). Moreover, the mentioned hyperstimulation of β-receptors leads to myocardial hypoxia which can exacerbate the myocardial impairment by additional generation of reactive oxygen species (ROS) (Blasig et al. 1985).

However, one group reported that a series of flavonoids appeared to be unexpectedly protective against each tested aspect of isoprenaline toxicity (Prince 2011; Prince and Sathya 2010; Karthick and Prince 2006). A few years ago, our group tested the direct intravenous administration of the flavonoid rutin in two doses. The findings were contradictory to the oral administration. A dose of 11.5 mg kg^{-1} i.v. was not protective while a dose of 46 mg kg^{-1} apparently aggravated the ISO injury (Mladenka et al. 2009b). Our previous idea was that metabolism had been responsible for this paradoxical effect since oral administration of rutin would not lead to absorption of the parent compound (Manach et al. 1995).

Due to serious discrepancies in this research, we decided to return to this issue. The main aim of this study was to confirm the suggested protection of oral quercetin in the form of chronic premedication and to elucidate its effect on major cardiovascular aspects of isoprenaline toxicity.

Materials and methods

Animals

Thirty-one Wistar–Han male rats were obtained from Meditox (Czech Republic), 23 rats were used in the basic study with isoprenaline and 8 rats in the pharmacokinetic study. The rats were housed in cages located in a special air-conditioned room with a periodic light–dark (12–12 h) cycle for 2 weeks. During this period, they were provided with free access to tap water and standard pellet diet for rodents. After the acclimatization period, the healthy rats weighing approximately 375 g (haemodynamic study)/456 g (pharmacokinetic study) were used for the experiments.

The study had the approval of the Ethics Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové and conformed to The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experimental design

Haemodynamic study

The rats were randomly divided into two groups. The first group received solvent (2 ml kg^{-1}) and the second one received quercetin (Sigma-Aldrich, Germany) in a dose of 10 mg kg^{-1} by gastric gavage in seven succeeding days. On the last day, animals from both groups were again randomly divided for s.c. administration of water for injection (2 ml kg^{-1}) or aqueous solution of ISO (100 mg kg^{-1}) 1 h after gavage. The experiment started 24 h following ISO/water administration. The experimental groups designated control and Q (oral pretreatment by solvent or quercetin, respectively) were followed by s.c. administration of water for injection, while the groups of ISO and Q+ISO (pretreated by solvent or quercetin, respectively) were exposed to s.c. injection of ISO.

Pharmacokinetic study

Similar to the foregoing, the rats were randomly divided into two groups and received either solvent or quercetin by gastric gavage in seven succeeding days in the same doses/concentrations. Pentobarbital (Sigma-Aldrich) in a dose of 45 mg kg^{-1} i.p. was used as anaesthetic in this study. On the last day, three animals from the quercetin group were anaesthetized, their right common carotid artery was cannulated and the cannula ran out through the skin on the back of the neck. After recovery, approximately 400 μl of blood was collected (time 0), and the last dose of quercetin was administered via gastric gavage. Additional blood samples were collected each hour up to 8 h. Three other quercetin and two control animals were treated in a slightly different way. They were anaesthetized 105 min after the last dose of quercetin/solvent. The right common carotid artery was cannulated, and blood samples were collected from 2 up to 8 h again at a 1-h interval. In these animals, the anaesthesia was maintained throughout.

Quercetin was firstly dissolved in a mixture of ethanol:DMSO (19:1), and the suspension finally containing 0.5 % DMSO was then prepared with water for injection. Control animals received solvent which was composed of DMSO (0.5 %), ethanol (9.5 %) and water (90 %). Neither control nor quercetin animals received more than 5 μL of DMSO in each gavage.

Anaesthesia and surgery in the haemodynamic study

After 12 h fasting, the rats were anaesthetized with i.p. injection containing aqueous solution of urethane (Sigma-Aldrich) in a dose of 1.2 g kg^{-1}. Surgical and measuring procedures were identical to our previous studies (Zatloukalova et al. 2012; Filipsky et al. 2012) with minor modifications, briefly:

The left common iliac artery was connected to a pressure transducer MLT0380/D (AdInstruments, Australia) via a polyethylene catheter (0.5/1.0 mm filled with heparinized saline 50 IU/ml). A high-fidelity pressure-volume micromanometer catheter (Millar pressure-volume catheter SPR-838 2 F, 4E, 9 mm, Millar Instruments Inc., USA) was inserted into the left heart ventricle through the right common carotid artery. Both pressure transducer and Millar pressure-volume catheter together with subcutaneous electrodes for the ECG standard limb lead II MLA1215 (AdInstruments) were connected to PowerLab with LabChart 7 software (AdInstruments). Data were analyzed for 30 min, and necessary calibrations with hypertonic saline (2×20 μl of 25 % *w*/*w* sodium chloride solution) were performed at the end. A blood sample was collected from the abdominal aorta into a heparinized test tube (170 IU/10 ml). Following the experiment, all surviving animals were killed painlessly in anaesthesia by intravenous administration of 1 ml of 1 M aqueous solution of potassium chloride (Sigma-Aldrich).

Biochemical analysis

Cardiac troponin T (cTnT), vitamin C and vitamin E were measured in serum and total glutathione in the whole blood. cTnT was determined by high sensitive electrochemoluminescence immunoassay (Elecsys 2010, Roche), which employs two monoclonal antibodies specifically directed against cTnT. Capillary electrophoresis was used for separation of glutathione, which was measured by UV detection (PrinCE 750, Prince Technologies B.V., The Netherlands) at 200 nm. After deproteinization, analysis of vitamin E with fluorimetric detection was performed in an HPLC system LC-10A (Shimadzu, Japan). The analysis of vitamin C was performed after deproteinization by electrophoresis using UV detection (System P/ACE 5100, Beckman).

Malondialdehyde (MDA) was assessed in the hearts of the tested animals. The tissue was homogenized in 0.1 M sodium phosphate buffer (pH 7.4) using a Potter-Elvehjem homogenizer (B.Braun, Germany). The homogenate was centrifuged for 10 min at 2,600 g (centrifuge VWR Compact Star CS4, VWR International, LLC, USA). MDA was assessed in the samples adjusted to a final concentration of 0.05 or 0.01 mg/μl of the tissue in the buffer by the known spectrophotometric thiobarbituric acid method (Hendriks and Assmann 1990).

Quercetin metabolite analysis

The concentrations of flavonoids and phenolic acids in rat plasma were assessed using the UHPLC–MS/MS method in system consisting of Acquity UPLC (Waters, Czech Republic) and a Quattro Micro triple quadrupole mass spectrometer (Waters). The separation was performed on BEH Shield RP C18 (2.1×100 mm, 1.7 μm) using gradient elution with methanol and 0.1 % formic acid. All injected solutions were stored in the auto-sampler at 4 °C. The partial loop with needle overfill mode was set up to inject 5 μl. The analytical column was kept at 40 °C by column oven.

The MS conditions were finely tuned in positive/negative ESI polarity mode as follows: capillary voltage, +3,200 V/−2,000 V; ion source temperature, 130 °C; extractor, 3.0 V; RF lens, 0.5 V. The desolvation gas was nitrogen at a flow of 800 l h^{-1} and at a temperature of 450 °C. Nitrogen was also used as a cone gas (100 l h^{-1}). Argon was used as a collision gas. Analyses were performed in selected reaction monitoring (SRM) mode using the precursor ions $[M+H]^+$ or $[M-H]^-$ and the corresponding product ions. The cone voltage, collision energy and dwell time were carefully optimized for each compound and its transion individually. The most intensive product ion was selected for the SRM transition. MassLynx 4.1 software was used for MS control and data gathering. QuanLynx software was employed for data processing and peak integration. The sample pretreatment of plasma consisted in fast and simple protein precipitation. Fifty microliters of rat plasma was precipitated with 100 μl of acetonitrile. After 10 min, the sample was centrifuged for 10 min. The supernatant was then filtered through a PTFE membrane with 0.22-μm pores and injected into UHPLC system.

Standards, quercetin-3-glucuronide and 3-hydroxyphenylacetic acid, were purchased from Sigma-Aldrich and Toronto Research Chemicals (Canada), respectively.

Histological examination

The heart was excised immediately after the animal´s death and rapidly fixed in cold 10 % neutral buffered formaldehyde solution for at least 24 h. The cardiac muscle was then sliced transversally into four parts from the basis to the apex, and the fixed specimens were processed using the conventional paraffin-embedding technique. From the prepared paraffin blocks, 5-μm-thick sections were obtained and stained with haematoxylin and eosin for light microscopic examination. Photo documentation and image digitizing were performed with the Olympus AX 70 light microscope, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Canada) and image analysis software NIS (Laboratory Imaging, Czech Republic).

Isolated aorta

The thoracic aorta was gently excised and placed in Krebs solution. After removal of fat and connective tissue, the aorta was cut into rings (approximately 3-mm width). Aorta rings with endothelium were mounted between two stainless steel wire hooks. Then, they were transferred into the tissue bath chamber. The aortic rings were allowed to equilibrate at 37 °C in oxygenated (95 % O_2, 5 % CO_2) Krebs solution of the following composition: 135 mmol l^{-1} NaCl, 5.0 mmol l^{-1} KCl, 2.5 mmol l^{-1} $CaCl_2$, 1.3 mmol l^{-1} $MgSO_4$, 1.2 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} $NaHCO_3$ and 10 mmol l^{-1} glucose. The rings were equilibrated for 45 min at an initial resting tension of 2.0 g; the bathing solution was changed at 10 min intervals. After the equilibration period, the contractility of each arterial segment was assessed with 10 μmol l^{-1} norepinephrine (NE, Sigma-Aldrich). Changes in isometric tension were recorded using computer equipped with SPEL Advanced Kymograph Software (Experimetria Ltd., Hungary). The presence of functional endothelium was confirmed by the response to acetylcholine (10 μmol l^{-1}) as an induction of more than 50 % relaxation of aortic rings precontracted with 10 μmol l^{-1} NE. Preparations were then washed three times with Krebs solution and cumulative concentration–response curves to NE (1 pmol l^{-1}–500 μmol l^{-1}) were obtained. At the end of the experiment, KCl (75 mmol l^{-1}) was added to the bath to induce maximal contraction. Responses to NE were expressed as a percentage of the maximal contraction evoked by KCl.

Data analysis

Calculations were performed as previously described (Filipsky et al. 2012). Total peripheral resistance index (TPR) was calculated as mean arterial blood pressure divided by the cardiac output and adjusted to the weight of the animal. The double product was calculated as systolic blood pressure multiplied by heart rate. Tau (the time constant of left ventricular isovolumic pressure decay) was calculated by Weiss and Glantz methods (Weiss et al. 1976; Raff and Glantz 1981). Other parameters have common meaning.

Data are expressed as means±SD. Outliers were excluded by Grubb's test. Differences were compared by one-way ANOVA test followed by Fisher's LSD test, based on a set of individual *t* tests, or by 95 % confidence intervals (isolated aorta). Statistical software GraphPad Prism 5 for Windows (GraphPad Software, USA) was used for statistical analysis. Differences between groups were considered significant at $p \leq 0.05$ unless indicated otherwise.

Results

Mortality

No death occurred in any tested group, including ISO groups.

Haemodynamic parameters

ISO did not significantly modify mean blood pressure but significantly accelerated heart rate, increased the double product—the marker of myocardial oxygen consumption—and decreased the stroke volume 24 h after its administration (Fig. 1). Quercetin premedication had apparently no influence on the stroke volume, and it did not significantly affect double product or heart rate. Similar to the stroke volume, ISO significantly decreased the ejection fraction (39±15 %) when compared with both of the controls (solvent 67±18 %, quercetin 67±13 %). Quercetin premedication had no positive effect on this parameter (40±8 %). Such a drop in ejection fraction indicates heart failure. Therefore, additional parameters of heart function were analyzed.

ISO increased the left ventricular end-diastolic pressure and peripheral resistance and caused impairment of the diastolic isovolumic relaxation (Fig. 2), but did not modify the contractility (data not shown). Although there are some differences according to the calculation of the time constant of left ventricular isovolumic pressure decay (tau, Fig. 2c, d), it is apparent that quercetin premedication again failed to positively influence myocardial relaxation impairment. Quercetin did not influence the peripheral resistance either, but had some protective impact on the left ventricular end-diastolic pressure; there was no significant rise in this pressure in contrast to the ISO group (Fig. 2a).

To elucidate in detail the impact of quercetin on cardiac function, we analyzed maximal volume rise dV/dt_{max} (describes the peak filling rate in early diastolic filling) and the negative peak of dV/dt (characterizes the ejection phase). Likely, due to the variability of the data, although there were tendencies in quercetin to increase the negative dV/dt, the differences between quercetin vs control and quercetin+ISO vs ISO for this parameter were insignificant (Fig. 3a). No influence of quercetin on dV/dt_{max} was found either (Fig. 3b).

Morphological parameters and ECG

ISO administration induced a significant rise in cardiac wet ventricles weight index. Quercetin pretreatment did not affect the increase in this parameter (Fig. 4a). There was a marked QRS-T junction elevation/corresponding to

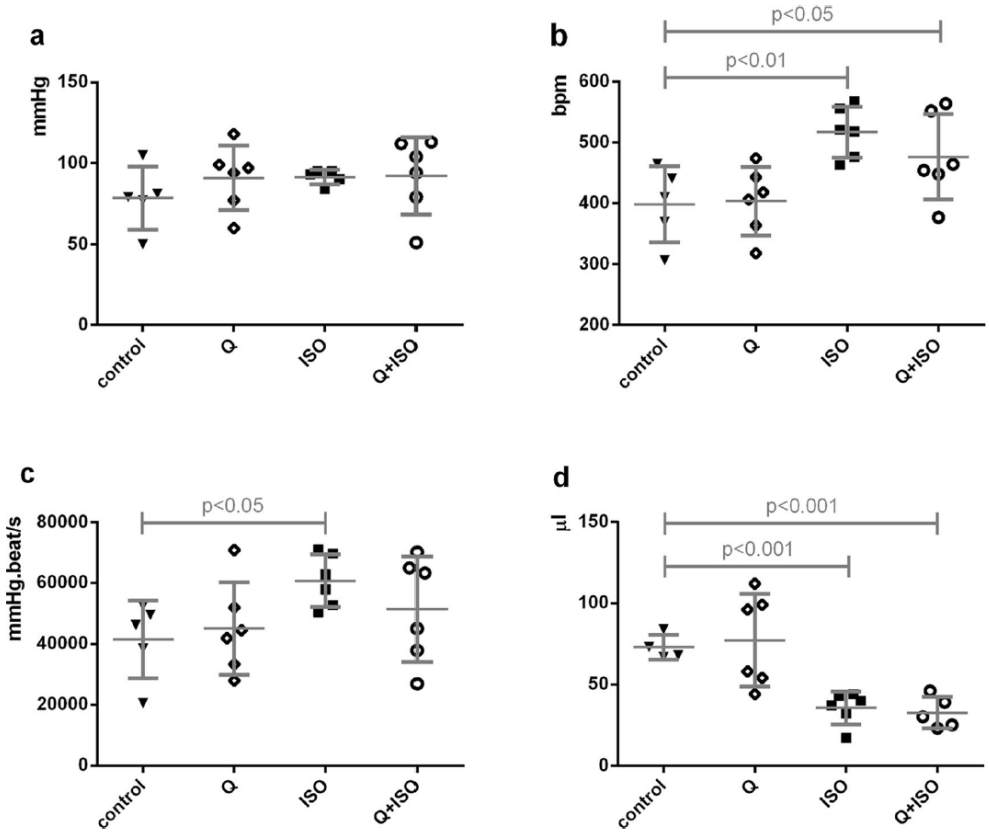

Fig. 1 Basic haemodynamic parameters: mean blood pressure (**a**), heart rate (**b**), double product (**c**) and stroke volume (**d**)

elevation of ST segment in human ECG (Beinfield and Lehr 1968)/in both of the ISO groups (Fig. 4b), which indicates again no protection of quercetin against myocardial ischaemia caused by ISO. All ECG from animals treated with ISO are shown (Fig. 5). It is clear that there is high variability, but the finding is unambiguous: quercetin cannot reverse ECG changes caused by ISO.

Biochemical markers

In agreement with previous data suggesting marked ISO cardiotoxicity, ISO induced a significant increase in serum levels of cardiac troponin T (Fig. 4c); quercetin pretreatment had no influence. Moreover, quercetin did not affect the significant decrease in serum concentration of vitamin C in ISO groups (Fig. 6a). Another marker of oxidative stress, vitamin E, did not change after ISO administration in comparison with the control group (Fig. 6b). Similarly, levels of malondialdehyde were not significantly elevated after ISO treatment in the heart samples (data not shown).

Histological findings

ISO administration caused frequent inflammatory infiltrates with the presence of oedema in widely expanded interstitial spaces and necrotic changes in cardiomyocytes including increased cytoplasmic eosinophilia, loss of myofibrilar striation and pycnotic damage of nucleus (Fig. 7). The lymphocytic infiltration including activated macrophages was mild to moderate from epicardial to subendocardial sections of the heart. The findings in quercetin+ISO-treated animals were apparently similar to that of ISO group (Fig. 7a vs b, c vs d). Animals treated with quercetin had, like the control animals, apparently healthy heart histology, with the exception of one animal from the quercetin group where mild focal interstitial infiltration of lymphocytes was found in the epicardium.

Effect on vascular smooth muscles

As a part of this study, the aortal reactivity on a vasoconstrictor was analyzed (Fig. 8). ISO-treated animals had markedly

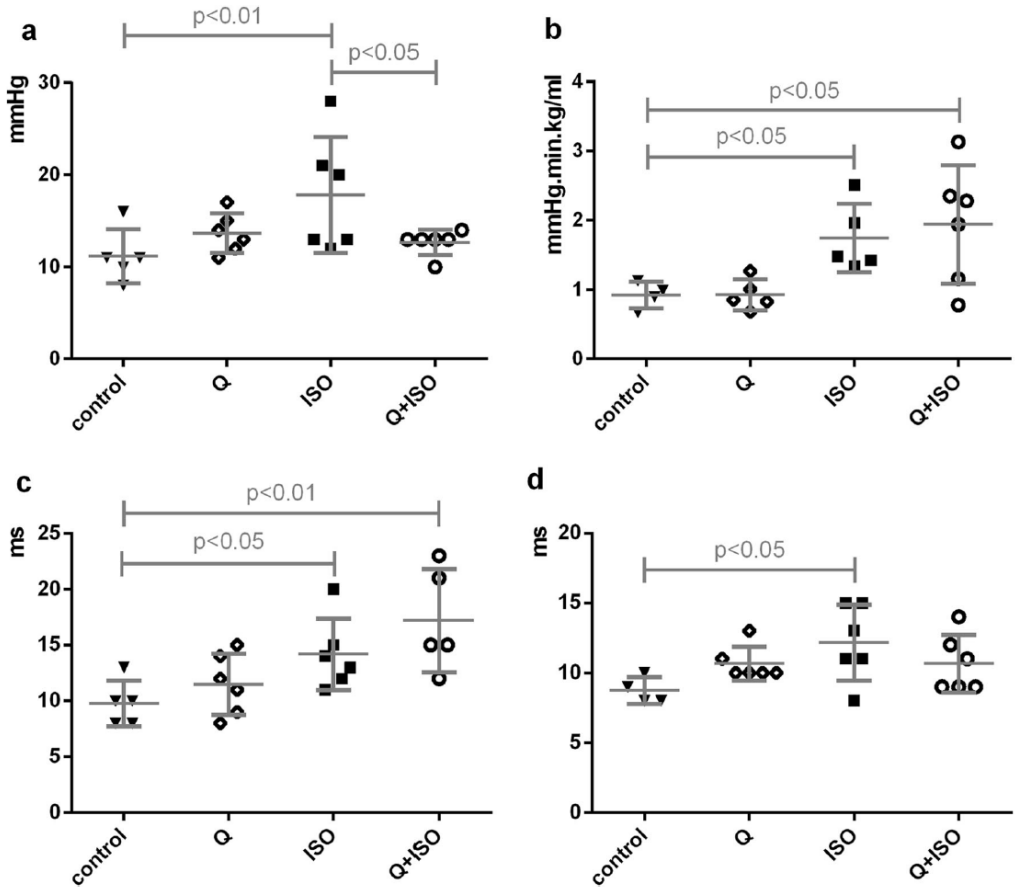

Fig. 2 Additional haemodynamic parameters: left ventricular end-diastolic pressure (**a**), peripheral resistance index (**b**) and the time constant of left ventricular isovolumic pressure decay calculated by the method of Weiss (**c**) and method of Glantz (**d**)

enhanced responsiveness on NE. There was no effect of quercetin pretreatment. However, control aorta from solvent and quercetin-treated animals reacted differently: quercetin premedication significantly decreased the NE vasoactive effects.

Quercetin pharmacokinetic analysis

Since the results showed, on the one hand, some positive effects on vascular smooth muscle cells, but on the other no positive influence on the isoprenaline model, we tested known metabolites produced by oral quercetin gavage. Two metabolites were clearly apparent in the MS analysis, quercetin-3-glucuronide and 3-hydroxyphenylacetic acid. In contrast, those metabolites were not present in the control animals at any time interval measured (data not shown).

The plasmatic profile of quercetin-3-glucuronide is shown in Fig. 9. Much higher variability was found in the case of 3-hydroxyphenylacetic acid. Since it has not been possible to

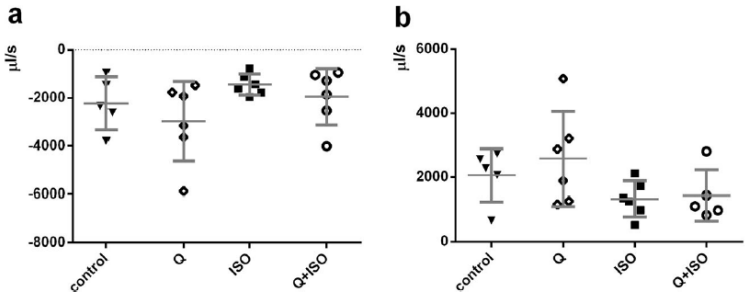

Fig. 3 Changes in the negative peak of dV/dt (**a**) and positive peak of dV/dt_{max} (peak filling rate, **b**)

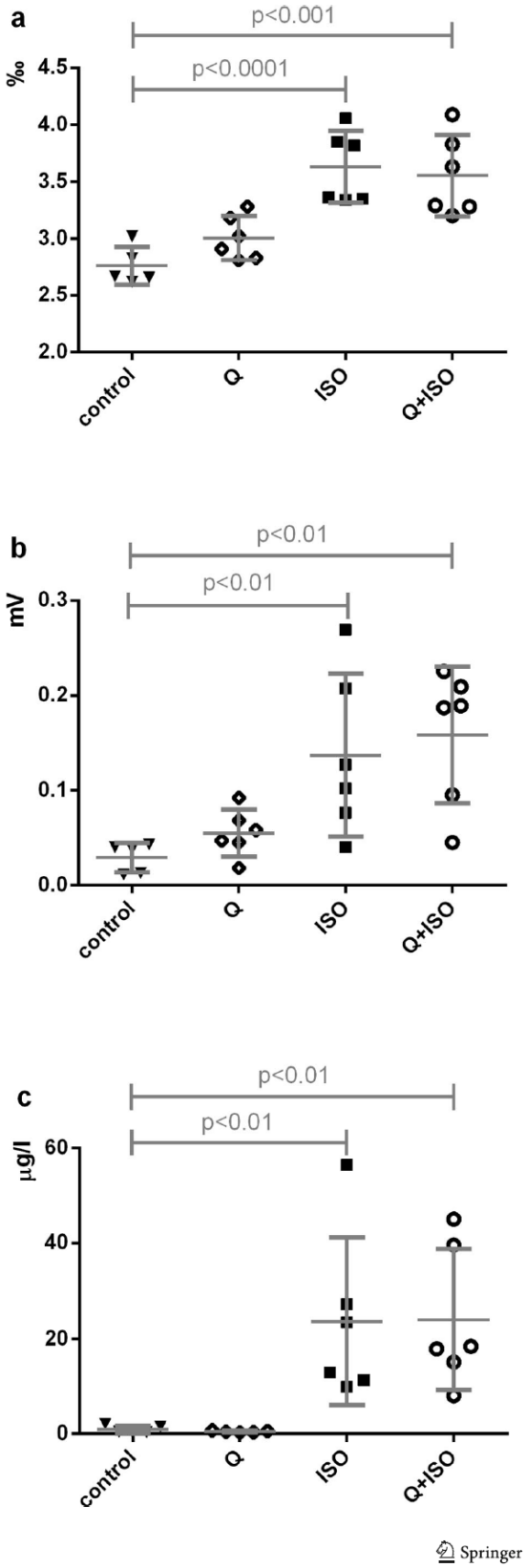

Fig. 4 Changes in cardiac wet ventricles weight index (**a**), elevation of the QRS-T junction (**b**) and serum levels of cardiac troponin T (**c**)

quantify precisely several samples due to low signal to noise ratio, the concentration–time curves were not prepared, but the metabolite was presented at least in low quantity in all samples from animals treated with quercetin. The absorption was clearly slower than in the case of quercetin-3-glucuronide and the rise of plasma concentration started apparently after 4 h from quercetin administration which is in the harmony with the necessity of bacterial cleavage of quercetin in the large intestine. In three rats, the concentration at 8 h from quercetin administration was found in a narrow range (28.8, 29.7 and 34.1 ng/ml).

Discussion

This study has produced two main novel findings. Firstly, chronic gastric gavage alone inhibited the ISO-induced mortality, and secondly, oral quercetin could not protect myocardium against the deleterious effect of ISO. The first finding is unexpected since in acute settings, ISO in a dose of 100 mg kg^{-1} s.c. causes about 30 % mortality in accordance to other studies with lower or higher ISO doses, e.g. 5 mg kg^{-1} s.c. caused about 20 % mortality while 1 g kg^{-1} s.c. approximately 50 % (Mladenka et al. 2009a, b; Ellison et al. 2007; Singal et al. 1982; Feng and Li 2010; Wexler and McMurtry 1981). Although, there are important differences among older (heavier) and younger animals (Joseph et al. 1981), this was not apparent in this case since the weight of animals was approximately the same as in our previous experiments (Mladenka et al. 2009a). It is possible that repeated daily gastric gavage represents a significant stress factor with consequent release of catecholamines. Adrenergic receptor desensitization by catecholamines is quite rapid and efficient (Doss et al. 1981; Hertel and Perkins 1984) and cannot be excluded as a reason for the survival of all animals. However, we have no available experimental data, and this needs to be investigated in the future in detail.

The second finding is in accordance with the current knowledge of the complex pathophysiology of catecholamine cardiotoxicity. For this reason, the outcome that one compound with antioxidant activity cannot reverse the complex pathophysiology of catecholamine cardiotoxicity is not very surprising. However, these data are not in agreement with the article of Prince and Sathya (2010). The reason for this is unclear as we used the same experimental setting in major aspects. In particular, the ECG findings published in that article and in another article from the same group are of note (Prince 2011; Prince and Sathya 2010). Although the authors

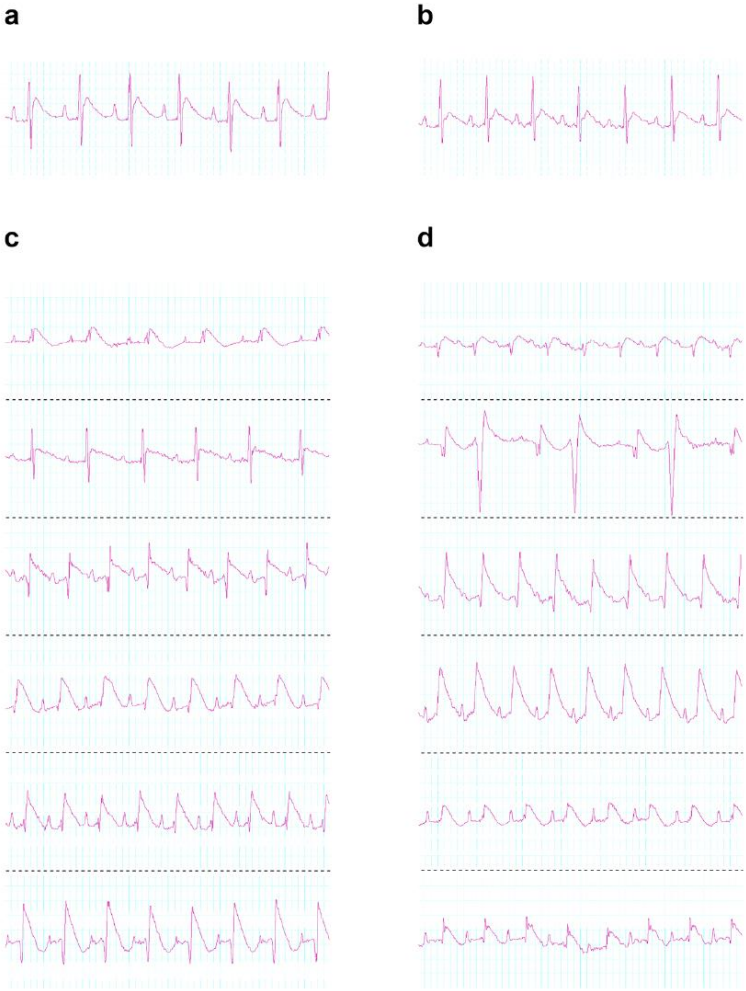

Fig. 5 ECG tracings of lead II. Normal ECG tracings are shown in quercetin- (**a**) and solvent-treated animals (**b**). All ECG from ISO groups, quercetin+ISO (**c**) and solvent+ISO (**d**), are shown. Prominent QRS-T junction changes are present in majority of ISO-treated animals (both ISO and Q+ISO). Bigeminy can be seen in one ISO-treated rat

did not state which lead was used, some aspects of the ECG findings deserve more detailed comment:

(1) ISO is associated with a decrease in R wave amplitude (Ramesh et al. 1998; Zatloukalova et al. 2012). It is therefore not clear why in the two mentioned publications, where the same dose of ISO was used, no change and an improbable increase in R amplitude were observed (Prince and Sathya 2010; Prince 2011). It has to be emphasized that R wave amplitude may be, in fact, higher in ISO-treated rats compared with the controls, but only in cases where the R wave, as a part of the QRS

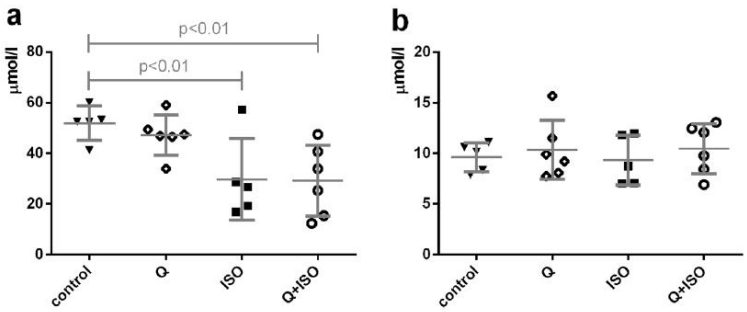

Fig. 6 Serum concentrations of vitamins C (**a**) and E (**b**)

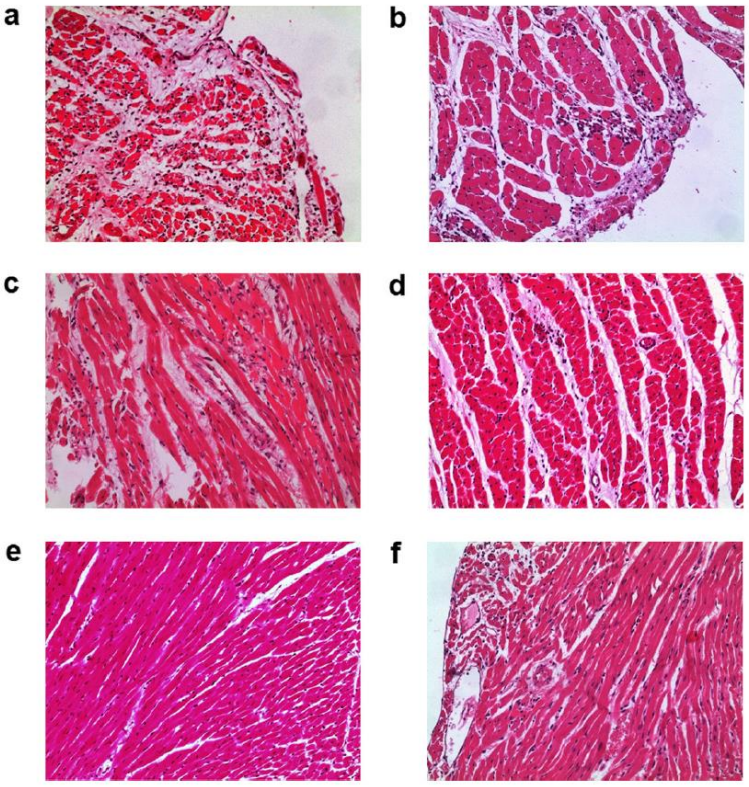

Fig. 7 Histological findings. Apparent ischaemic damage with necrotic myocytes, oedema in widely extended interstitium and presence of inflammatory cell infiltrates were observed in subendocardium (**a**) and myocardium (**c**) of ISO-treated animals. Similar findings were observed in combination (quercetin+ISO) group in the subendocardial tissue (**b**) and in the myocardium (**d**) or in the epicardium (**f**), where severe interstitial infiltration of lymphocytes and dilatation of subepicardial blood vessels were observed. The intact myocardium in the control group shows normal cardiac fibres without any changes (**e**). Staining: haematoxylin–eosin. Direct magnification, ×100

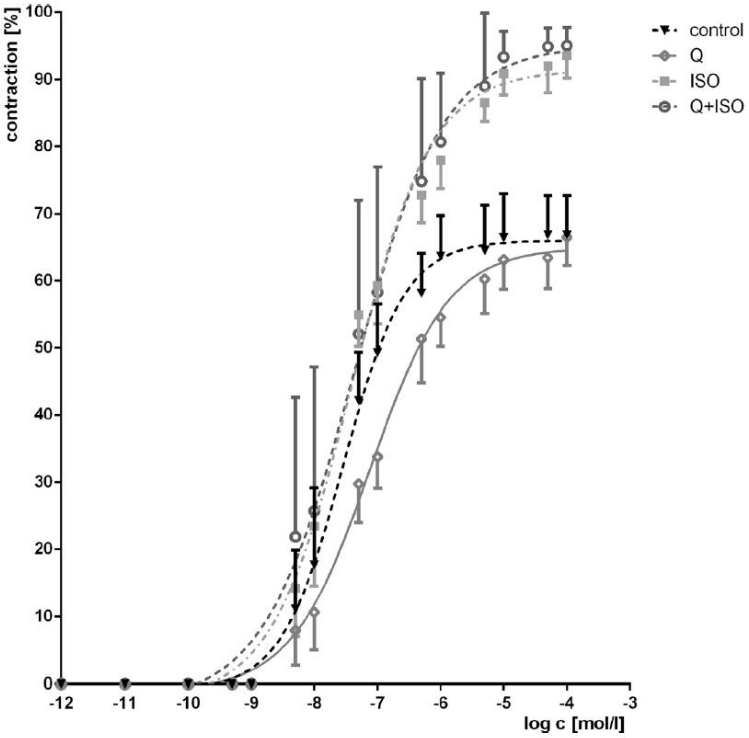

Fig. 8 Concentration–response curves to NE in aortic rings with endothelium. Responses to NE were expressed as a percentage of the maximal contraction evoked by KCl (75 mmol l^{-1})

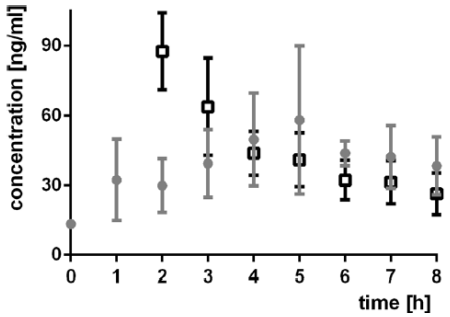

Fig. 9 Pharmacokinetic profile of quercetin-3-glucuronide in plasma of rats supplemented with quercetin or solvent for 7 days. Results shown as *circles* are from animals anaesthetized before the quercetin gastric gavage, *squares* depict animals anaesthetized 105 min after quercetin gavage. Controls are not shown since the concentration of this metabolite was zero at all time intervals

complex, is apparently joined with markedly elevated T wave. This can be seen in Fig. 5, but is not true for the publications mentioned, where both R and T waves were separated.

(2) The above authors referred to ST segment elevation, but this cannot be clearly deduced from their tracings. Moreover, S wave amplitude remained unchanged after ISO administration which is in clear contrast to the QRS-T junction elevation found in our study, where S wave was not usually present (Fig. 5).

(3) It is worth mentioning that the T wave amplitude apparently increased after ISO in both papers, but QRS-T junction, as mentioned above, was unchanged.

(4) Elevated heart rate indicates cardiac derangement and, thus, may represent an additional parameter. Indeed, an increase in heart rate was found even 24 h after ISO administration (Mladenka et al. 2009a). Although the heart rate was not reported in the article of Prince and Sathya (2010) and according to Fig. 1 in their article, it appears that quercetin in combination with ISO decreased the heart rate compared with the controls or ISO alone, and similarly, ISO alone had lower heart rate compared with the controls. It can be speculated that the second dose could modify the ISO effect on heart rate possibly due to discussed β-adrenoreceptor desensitization. This was probably not the case since a previous study showed that two doses of ISO caused even more pronounced effect on heart rate within 12 h after the second dose in comparison with the single dose (Ramesh et al. 1998).

There may be some objections concerning the slightly different study design. Firstly, animals used in this study were older, but this cannot explain marked differences in the results, and moreover, older animals are more relevant for research on cardiovascular diseases. Secondly, we used only single dose of ISO since this evokes severe injury (Mladenka et al. 2009a). If quercetin is not able to block this impairment, it could not likely block the pathophysiological changes caused by the double dose. Since oral quercetin had some obvious effect on blood vessels compared to the control animals (Fig. 7), it is apparent that quercetin or its metabolites reached the systemic circulation, and this was confirmed in this study (Fig. 9). We did not concentrate on detailed pharmacokinetic analysis owing to the complex metabolism of quercetin by human/rat intestinal and liver enzymes and by bacteria in the colon (Cermak et al. 2003; Graefe et al. 1999), but on the evidence for quercetin absorption. We selected two metabolites, quercetin-3-glucuronide and 3-hydroxyphenylacetic acid, as representatives of quercetin metabolites by rat/human and bacterial enzymes, respectively. It is well-known that the concentration of free quercetin is very low in contrast to its major human-conjugated metabolite quercetin-3-glucuronide (Bieger et al. 2008; Cialdella-Kam et al. 2013). Similarly, the majority of phenolic acid was found to be conjugated (Olthof et al. 2003), so the concentration of total 3-hydroxyphenylacetic acid may be higher. However, we have not yet measured it since the glucuronide of this acid is not available.

Considering the character of the pathophysiological changes associated with cardiotoxic doses of ISO, we focused on haemodynamic and biochemical markers. ISO effects on haemodynamics are rapid as can be demonstrated by very fast diastolic dysfunction and release of cardiac troponin T. Contractility derangement follows the diastolic impairment. The histopathological findings of heart damage appeared with some delay. Thus, the 24-h interval, when marked histological derangement and persistent biochemical markers were found, was selected in this study to assess the possible effect of quercetin (Chagoya de Sanchez et al. 1997; Pick et al. 1989; Filipsky et al. 2012; Mladenka et al. 2009a; Clements et al. 2010).

Although quercetin protective effects on ISO toxicity were clearly minor, its positive effect on left ventricular end-diastolic pressure is of note. The explanation of this is equivocal since ejection fraction was similar in both ISO groups. Furthermore, quercetin had no positive effect on the myocardial contractility and did not reverse depressed myocardial relaxation response. Quercetin has positive inotropic effect on isolated rat atria, but this effect is bell-shaped and the clinical situation can be different since quercetin administration will result mainly in systemic appearance of its conjugated metabolites (Erlund et al. 2000; Kubota et al. 2002). Even if the mechanism is not known, this finding may be of a clinical interest: Although quercetin cannot reverse ISO cardiotoxicity, it may have some positive effect on

progression of heart failure. In fact, quercetin supplementation decreased cardiac hypertrophy in rats with aortic constriction (Jalili et al. 2006).

Another interesting finding was the influence of quercetin on blood vessel responsiveness to vasoconstrictors. The data presented here seem to be in agreement with studies showing that flavonoids, including quercetin, have vasodilatory potential on isolated vessels and that oral administration of quercetin could affect the NO and endothelin-1 plasma concentrations in humans (Loke et al. 2008a; Ajay et al. 2003). Therefore, even if quercetin cannot revert acute cardiovascular injury caused by ISO, it may, in addition to the previously mentioned effect on heart failure, have some minor positive effects in arterial hypertension. Indeed, a decrease in arterial blood pressure has been documented in human after oral quercetin premedication (Edwards et al. 2007; Egert et al. 2009). It appears that the effect is mediated by phenolic acids produced by intestinal microflora rather than by quercetin itself or its close methylated or conjugated metabolites. In a recent study, oral administration of quercetin was more effective in reducing blood pressure than i.p. administration (Galindo et al. 2012). For this reason, we believe that some of the phenolic acids produced by quercetin cleavage by intestinal microflora may be responsible for the effect. One candidate may be the measured 3-hydroxyphenylacetic acid which has longer elimination half-life than other phenolic acids produced by quercetin cleavage (Sawai et al. 1987). Our next study will test the effect of quercetin bacterial cleavage products in greater detail.

The relationship between antioxidants, oxidative stress parameters and cardiovascular diseases is still equivocal (Strobel et al. 2011). The kinetics of changes of endogenous antioxidants after ISO administration is complicated and is highly dependent on time and likely on other factors too (Diaz-Munoz et al. 2006). Similarly, data on biomarkers of oxidative stress from our laboratory are not identical with our previous study (Mladenka et al. 2009a). In this study, serum vitamin E and myocardial malondialdehyde levels were not significantly changed after ISO administration, and vitamin C level drop caused by ISO was not positively influenced by quercetin, a known antioxidant. The lack of positive influence on oxidative stress biomarkers after quercetin oral administration is not a rare finding. Several previous studies reported that oral quercetin did not influence oxidized LDL, plasma/urinary F_2-isoprostanes and total plasma antioxidant capacity in humans (Edwards et al. 2007; Shanely et al. 2010; Egert et al. 2008; Loke et al. 2008a). This interesting finding may be due to several possible factors: (1) the dose of quercetin was low, (2) quercetin possesses both pro-oxidant and antioxidant activity depending on the concentration (Prochazkova et al. 2011) and (3) conjugates of quercetin formed in vivo have lower antioxidant activity (Loke et al. 2008b), and thus, oral quercetin may not be very active. We presume that the dose was not low. It is well-known that quercetin has antioxidant activity in very low doses, and in humans, the maximal concentration of total quercetin of 40 μg/l was found after a similar dose of 8 mg (Afanas'ev et al. 1989; Erlund et al. 2000). Our data are in a good agreement with this finding (Fig. 9). Such concentration could have some effect on vitamin C levels. The pro-oxidant effects of quercetin are not probable at this concentration because no negative effects on vitamin C or E levels were seen in this study. The third possibility appears to be the most probable since the majority of absorbed not cleaved quercetin is circulating in the plasma conjugated and/or bound on plasma proteins (Manach et al. 1995; Bieger et al. 2008). On the other hand, despite the very low concentration of free quercetin in plasma, the majority of quercetin is presented in its free, unconjugated form at least in some tissues (Bieger et al. 2008). The matter of oral quercetin metabolites both in plasma and tissue and their pharmacological activity deserves further study and is currently analyzed in our laboratory.

In conclusion, this study demonstrated that 7-day oral quercetin administration was not able to prevent acute manifestation of catecholamine cardiotoxicity; however, it could have some minor cardiovascular effects including decreased responsiveness of blood vessels to vasoconstrictors and normalization of left ventricular end-diastolic pressure.

Acknowledgments This study was supported by a grant from the Czech Science Foundation project no. P303/12/G163. V.P., M.H. and J.V. thank MH CZ-DRO and the programme PRVOUK P37/11. M.Ř. would like to thank Charles University (GAUK 605712C and SVV 267 003). The authors wish to thank Mrs. Pavlína Lukešová, Mrs. Anežka Kunová and Miss Renata Exnarová for their excellent technical assistance and to Dr. Alexander Oulton for the language correction.

Conflict of interest The authors declare that they have no conflict of interest.

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4.7. Is a highly linear relationship between the dose of quercetin and the pharmacological effect possible? – A comment on Liu, *et al*. Evaluation of antioxidant and immunity activities of quercetin in isoproterenol-treated rats. *Molecules* 2012, *17*, 4281-4291

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(IF 2013: 2,095)

Molecules **2014**, *19*, 9606-9609; doi:10.3390/molecules19079606

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molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Comment

Is a Highly Linear Relationship Between the Dose of Quercetin and the Pharmacological Effect Possible? — A Comment on Liu, *et al*. Evaluation of Antioxidant and Immunity Activities of Quercetin in Isoproterenol-Treated Rats. *Molecules* 2012, *17*, 4281–4291

Přemysl Mladěnka [1,*], Radomír Hrdina [1], Tomáš Filipský [1], Michal Říha [1] and Vladimir Palicka [2,3]

[1] Department of Pharmacology and Toxicology, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

[2] Charles University in Prague, Faculty of Medicine in Hradec Králové, Šimkova 870, 500 38 Hradec Králové, Czech Republic

[3] University Hospital Hradec Králové, Sokolská 581, 500 05 Hradec Králové, Czech Republic

* Author to whom correspondence should be addressed; E-Mail: mladenkap@faf.cuni.cz; Tel.: +420-495-067-295; Fax: +420-495-067-170.

Received: 19 March 2014 / Accepted: 1 July 2014 / Published: 7 July 2014

We wish to offer some comments on the article by H. Liu *et al*. entitled "Evaluation of antioxidant and immunity activities of quercetin in isoproterenol-treated rats", published in *Molecules* in 2012 [1]. First of all, there are several important points which are not adequately explained in the article and require clarification by the authors:

- No information is provided on how the quercetin was dissolved and administered.
- There are no illustrative ECG measurements nor any comment on how the ECG signals were measured, this necessitates comments due to the measured T wave depression data.
- Heart index is not specified and since this is not a very common parameter, how was it calculated?
- The statistical test used by the authors in the comparison of data is not mentioned.
- Did all isoprenaline-treated animals survive the experiment?

Secondly, the design of the study and thus the possible outcome may be misleading since it appears no control groups which only received quercetin were included in the study. This may markedly influence the study results, e.g., it is possible that oral administration of quercetin might result in gradual increases in antioxidant levels and decreases in pro-inflammatory factors, and this may

counterbalance the effect of isoprenaline leading to the opposite processes. Therefore it is not clear if quercetin had acute effects by blocking the activity of isoprenaline or if it chronically decreases oxidative stress.

The third and the most important issue is an unnaturally precise linear relationship reported between the dose of quercetin and its pharmacological effects. We have plotted all measured data from the tables of the discussed article in graphs (Figure 1) with the exception of IL-10, where no protection was found.

Figure 1. Linear relationship between the daily dose of quercetin and the percentage of inhibition of isoprenaline effect. **A**: T wave-amplitude, **B**: heart rate, **C**: AST, **D**: CK-MB, **E**: LDH, **F**: TNF α, **G**: NO, **H**: nitric oxide synthase, **I**: IL-1, **J**: IL-8, **K**: Na^+/K^+-ATPase, **L**: Ca^{2+}/Mg^{2+}-ATPase, **M**: myeloperoxidase, **N**: TBARS, **O**: glutathione, **P**: superoxide dismutase, **Q**: catalase and **R**: glutathione peroxidase. Coefficients of linear regression (R^2) were calculated using GraphPad software version 6.0 (San Diego, CA, U.S.A.).

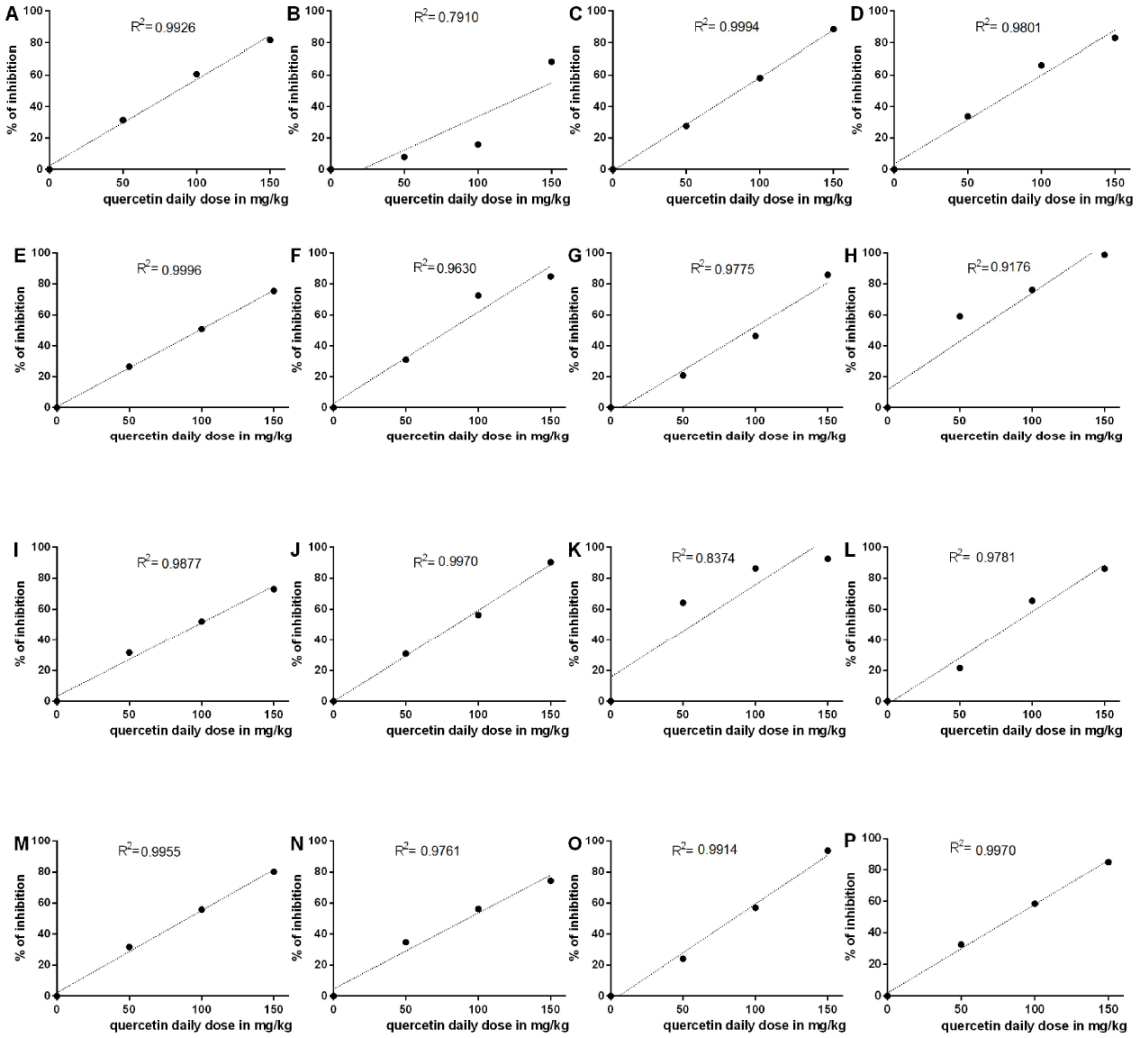
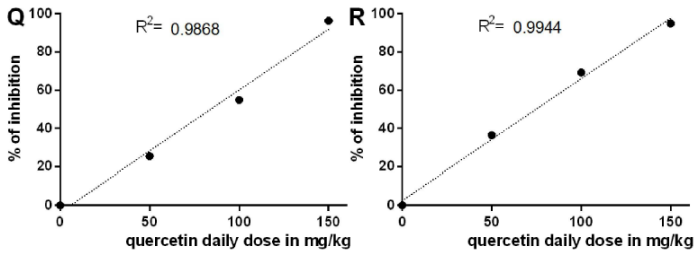


Figure 1. *Cont.*



The data were thus elaborated in a way that enabled comparison among the different measured parameters. In short, control values were considered 100% (healthy) and isoprenaline animals were considered 0% (pathological state), and the effect of quercetin was expressed as per cent inhibition of the isoprenaline effect. The blockade of isoprenaline activity was always dose dependent and moreover in eight out of 18 cases (44%) the linear regression coefficient was higher than 0.99 and in a total of 14 cases out of 18 (78%) the mentioned coefficient was higher than 0.975. It is not very probable to achieve such value in so many measured parameters. These results do not resemble *in vivo* experiments, but rather calibration curves. Moreover, according to literature precedents a dose dependent and clearly linear normalization of T wave amplitude is highly improbable. Notwithstanding it is not clear which ECG lead was recorded and the T wave amplitude units were not defined, their values do not correspond to the normal rat ECG. In a respected paper of Beinfeld and Lehr the following mean values of normal T wave amplitude in millivolts were described: lead I = 0.070, lead II = 0.145, lead III = 0.130, aVF = 0.150, aVL= −0.045 and aVR = −0.105 [2]. The mean value given in the article of Liu *et al.* of 2.68 (no units given) clearly does not fit in the above-mentioned range of normal amplitude T wave values. Additionally, it is well known that pharmacokinetics of oral quercetin are nonlinear [3–6] and thus a strictly linear relationship between the dose and the effect can be excluded with a high probability. We thus appeal to authors to promptly clarify the mentioned discrepancies.

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5. Souhrnný komentář k publikacím zahrnutým v disertační práci

Disertační práce je předkládána jako soubor sedmi prací publikovaných v recenzovaných odborných časopisech s impaktním faktorem, z nichž jedna představuje přehledový článek, jedna komentář k publikované studii a ostatní jsou původními experimentálními pracemi.

Uvedené publikace tvoří komplexní soubor prací, na různé úrovni se zabývající problematikou chelátorů přechodných kovů železa a mědi. Předložená práce navazuje na předchozí studie uskutečněné v rámci dlouhodobého výzkumu realizovaného skupinou Kardiovaskulární a respirační farmakologie a toxikologie. Vzhledem k úzké spolupráci s Katedrou farmaceutické botaniky a ekologie a klinickému významu jsou předmětem zájmu zejména přírodní látky ze skupiny kumarinů a flavonoidů.

Mimo rešeršní práce, týkající se antioxidačního působení kumarinů (publikace 4.1.), a komentáře k článku (publikace 4.7.) se jedná o *in vitro* a *in vivo* studie, které zahrnují:

- vývoj metodiky pro screening chelátorů mědi, včetně zhodnocení jejich redukční schopnosti (publikace 4.2.),
- vlastní screening chelatační, popřípadě i redukční aktivity vůči mědi u látek syntetického či přírodního původu, s důrazem na vztahy mezi strukturou a účinkem (publikace 4.2. a 4.3.),
- testování schopnosti flavonoidů redukovat ionty železa a ovlivnit tvorbu volných radikálů v železem katalyzované Fentonově reakci (publikace 4.4.),

- studium stechiometrie komplexů vybraných látek se železem, včetně vývoje nové metodiky pro její stanovení (publikace 4.5.),
- komplexní zhodnocení vlivu perorální premedikace kvercetinem na akutní poškození kardiovaskulárního systému vyvolané isoprenalinem (publikace 4.6.).

Chelátory železa a mědi mají kromě úzkého, avšak významného uplatnění v současné klinické praxi celou řadu potenciálních aplikací, jež jsou předmětem rozsáhlého výzkumu. Z tohoto důvodu je žádoucí jednak vyhledávat nové látky chelatující přechodné kovy, jednak blíže objasňovat další vlastnosti mající vztah k jejich výslednému účinku.

Podle výsledků předchozí *in vitro* studie jsou kumariny s určitými strukturními předpoklady účinné chelátory železa (Mladěnka *et al.* 2010a). Podle dostupné literatury se kromě přímého zhášení reaktivních forem kyslíku a dusíku (RONS) a inhibice enzymů produkujících RONS (xantinoxidasa, lipoxygenasa a další) na antioxidačním působení kumarinů podílí právě chelatace přechodných kovů. Většina prací studovala interakci kumarinů se železem, a to nepřímo, ovlivněním oxidačního stresu navozeného přechodným kovem. Strukturní požadavky pro zhášení RONS i chelataci kovů jsou obdobné – ideální uspořádání představuje 6,7- nebo 7,8-dihydroxysubstituce. Především u takto substituovaných kumarinů je dokumentována i významná schopnost redukovat přechodné kovy, což je mnohdy považováno za podklad antioxidačního působení. V reálných podmínkách však může být efekt přesně opačný, prooxidační, v důsledku podpory redoxního cyklu přechodného kovu. Obdobný efekt vykazuje katecholová skupina i v případě námi testovaných flavonoidů (viz dále).

Pro svoji rychlost, jednoduchost a přesnost je vyvinutá spektrofotometrická metodika využívající bathokuproindisulfonát vhodná pro screening chelátorů mědi v obou oxidačních stavech. Navíc po jednoduché modifikaci slouží ke stanovení

redukce mědi, takže dovoluje komplexněji zhodnotit interakci látky s kovem. Oproti hojně využívané přímé spektrofotometrii vyjadřuje toto nepřímé stanovení kompetici mezi testovanou látkou a indikátorem, která vypovídá o schopnosti látky udržet kov v redoxně neaktivní formě. V některých případech je možno na základě získaných dat odhadnout stechiometrii komplexu. Metoda byla validována pro spektrum pH odrážející fyziologické i patofyziologické poměry v organismu (pH 4,5-7,5). Přístup s použitím odlišného indikátoru, hematoxylinu, je vhodný zejména pro základní screening chelatační aktivity u měďnatých iontů, neboť se jedná o slabě kompetitivní prostředí, a v případě účinného chelátoru také může sloužit k odhadu stechiometrie komplexu. Uvedenými metodami byly zkoušeny látky používané jak v klinické praxi (D-penicilamin, trientin), tak experimentálně (skupina 8-hydroxychinolinů). Testováno bylo také celé spektrum flavonoidů napříč jednotlivými skupinami. Za povšimnutí jistě stojí za studovaných podmínek poměrně slabá chelatační aktivita D-penicilaminu v porovnání s ostatními látkami, zejména kliochinolem, kloroxinem a EDTA. Vyjma kyselého prostředí vykázal vysokou účinnost trientin. Pozorovaná vysoká redukce mědi D-penicilaminem souvisí s jeho mechanismem účinku při terapii Wilsonovy choroby (Peisach a Blumberg 1969).

Flavonoidy tvoří velmi rozsáhlou skupinu přírodních látek s komplexním působením na lidské zdraví. Diskutované jsou zejména kardiovaskulární účinky, na nichž se jistou měrou podílí i interakce s přechodnými kovy (Mladěnka *et al.* 2010b). Ve slabě kompetitivním prostředí je schopna chelatovat ionty mědi většina námi testovaných flavonoidů, pouze některé flavony a flavonoly však chelatovaly měď v silně kompetitivním prostředí. Lze konstatovat, že strukturní prvky flavonoidů důležité pro chelataci mědi se neliší významně od těch, které byly zjištěny v případě železa (Mladěnka *et al.* 2011). Esenciální je dvojná vazba mezi polohami 2 a 3

základního kruhu, nejúčinnější uspořádání reprezentují 3-hydroxy-4-ketoskupina u flavonolů a 5,6-dihydroxy- či 5,6,7-trihydroxyskupina u flavonů. Na druhou stranu katecholový kruh B (3′,4′-dihydroxyskupina) vykázal pouze slabou aktivitu. Jeho význam navíc klesal se vzrůstající kyselostí prostředí a v některých případech tato skupina ovlivnila účinek negativně. Na rozdíl od kyselého prostředí, v němž 3-hydroxyflavon, kempferol a částečně baikalein byly účinnější než trientin, žádný ze zkoušených flavonoidů nepřekonal trientin v přibližně neutrálním nebo mírně kyselém pH. Flavonoidy s vhodnými strukturními znaky tak mohou mít vliv nejen na patofyziologické procesy v organismu, ale vzhledem k jejich nezanedbatelné přítomnosti v lidské stravě také na metabolismus přechodných kovů, zejména absorpci. Ačkoliv přesný mechanismus průniku iontů mědi do enterocytů není zcela objasněn, předpokládá se nutnost redukce měďnatých iontů za účasti metaloreduktas nebo složek potravy (van den Berghe a Klomp 2009). Byl též dokumentován pozitivní vliv kyseliny askorbové na absorpci mědi (Lee *et al.* 2002). Výsledky ojedinělých studií týkajících se flavonoidů jsou poněkud rozporuplné (Říha *et al.* 2014). Nezbytný faktor, který je třeba brát v potaz, je metabolismus flavonoidů při jejich perorálním podání či konzumaci (Manach a Donovan 2004).

Flavonoidy mohou působit prooxidačně (Sakihama *et al.* 2002, Procházková *et al.* 2011). V další studii jsme se proto zabývali hodnocením redukčního účinku flavonoidů vzhledem k železu a jejich vlivu na železem katalyzovanou Fentonovu reakci. Výhodou použité ferrozinové metody ve stanovení redukční aktivity je použití různých koncentračních poměrů testované látky a kovu, do výsledné křivky se totiž promítne i chelatační působení látky. To je rozdíl od hojně používané metody FRAP (z angl. ferric reducing antioxidant power). Významná redukce kovu byla zjištěna pouze v kyselém prostředí, a to zejména u zástupců flavonolů a flavanolů s katecholovým

kruhem B. Z této skutečnosti vyplývá potenciální vliv takových látek na absorpci železa v tenkém střevě, neboť železo musí být redukováno pro transport do enterocytu, uskutečněný DMT1 (Ganz 2013). Zajímavě s ohledem na klinickou praxi se jeví především flavanoly s výraznou redukcí při pH 5,5 a neutrálním vlivem na Fentonovu reakci. Zatímco standardní chelátor železa deferoxamin inhiboval Fentonovu reakci v závislosti na koncentraci v souladu s jeho chelatačními vlastnostmi, některé flavonoidy působily jako účinné antioxidanty pouze při velmi nízkých koncentracích, ale působily neutrálně nebo dokonce prooxidačně při koncentracích vyšších. Morin a rutin vykázaly progresivně prooxidační působení. Následky v reálném biologickém systému jsou tedy obtížně predikovatelné. Látky podporující oxidační stres by však mohly mít potenciální využití v léčbě nádorových onemocnění (viz kapitola 2.2.5.).

Stechiometrie komplexu patří mezi jeho základní charakteristiky a její znalost má význam pro klinické uplatnění chelátoru. Na základě kompetitivních chelatačních experimentů lze za určitých okolností odhadnout stechiometrii vytvořeného komplexu. Její stanovení je však, mimo jiných metod (Fernandez *et al.* 2002), možné na základě měření absorpčních spekter látek a jejich odpovídajících komplexů s kovy v ultrafialové a viditelné oblasti. Přestože je takový postup poměrně dlouho znám – jedná se o metodu kontinuální variace, tzv. Jobovu metodu (Job 1928) – zejména u látek přírodního původu stechiometrie není mnohdy známa nebo jsou údaje nejednoznačné. Je to zejména z důvodu, že tyto látky nejsou většinou silnými chelátory přechodných kovů. V naší práce jsme se zaměřili na vývoj nového přístupu, jehož základní rozdíl oproti Jobově metodě tkví ve skutečnosti, že všechny vzorky obsahují stejnou koncentraci kovu a mění se pouze koncentrace testované látky. Pro případy malého rozdílu absorpčního maxima mezi látkou a odpovídajícím komplexem byly vytvořeny doplňující matematické výpočty pro zjištění stechiometrie. Tyto postupy byly

validovány na deseti látkách se známou schopností chelatovat železo. Metoda je výhodná zejména pro stanovení stechiometrie právě u látek se středně silnou afinitou k železu, umožňuje navíc odhalit změny ve stechiometrii komplexu v závislosti na koncentraci chelátoru.

Látky schopné chelatovat přechodné kovy mohou mít příznivý vliv na ischemické poškození myokardu a některé modelové studie tento efekt potvrdily (viz kapitola 2.2.4.). Vzhledem k možnému prooxidačnímu působení je výsledný účinek flavonoidů na ischemickou tkáň nejasný. Experimentálně je již mnoho let používán model katecholaminové kardiotoxicity, který v mnoha aspektech připomíná akutní infarkt myokardu u lidí (Rona *et al.* 1959). Tímto *in vivo* modelem se naše výzkumná skupina v minulosti do detailu zabývala (Filipský *et al.* 2012). Z důvodu publikovaného velmi příznivého působení kvercetinu na akutní kardiotoxicitu vyvolanou isoprenalinem (Prince a Sathya 2010), která ovšem nemá jasné mechanistické opodstatnění, jsme provedli studii v základních rysech shodnou s touto prací. Potkani Wistar:Han byli premedikováni kvercetinem po dobu sedmi dnů gastrickou sondou (kumulativní dávka 70 $mg.kg^{-1}$). Kvercetin však v žádném z hlavních aspektů nebyl schopen ochránit kardiovaskulární systém před akutním poškozením (uvolnění srdečního troponinu T, pokles tepového objemu, elevace QRS-T junkce, histologické vyšetření). Pozorován byl pouze příznivý vliv flavonolu na isoprenalinem zvýšený end-diastolický tlak v levé komoře a efekt na hladké svalstvo cév – kvercetin v kontrolní skupině snížil odpověď aorty na vazokonstrikční působení noradrenalinu. Nelze tedy vyloučit, že kvercetin (či jeho metabolity) tak může (mohou) mít potenciální význam při srdečním selhání nebo zejména u arteriální hypertenze. Za povšimnutí také stojí, že samotná aplikace rozpouštědla v kontrolní skupině zcela zabránila mortalitě způsobené isoprenalinem (100 $mg.kg^{-1}$), která se udává kolem 30 % (Ramesh *et al.* 1998, Mladěnka *et al.* 2009a,

Zatloukalová *et al.* 2012). Je potřeba také zdůraznit, že v této oblasti bylo publikováno mnoho studií, ale bohužel nelze vždy potvrdit, zda se zakládají jen na reálných výsledcích. Naše skupina podrobila nedávno kritice jednu práci týkající se kardio-protektivního účinku třicetidenní premedikace kvercetinem a zjistila velmi pochybné aspekty, které uznala i redakční rada časopisu Molecules (Liu *et al.* 2012). Autoři článku ovšem na výzvu redakce nereagovali. Proto byly provedeny i další práce, které mají do dané problematiky vnést více světla. Naše skupina totiž v minulosti prokázala, že flavonoid rutin při i.v. podání může naopak zhoršovat katecholaminovou kardiotoxicitu (Mladěnka *et al.* 2009b). Tyto výsledky byly nedávno znovu prověřeny naší skupinou s cílem analyzovat mechanismus pozorovaného toxického působení. V současné době jsou uvedené výsledky v recenzním řízení. Navíc je velmi zajímavé zhodnotit účinky D-penicilaminu na daný model kardiotoxicity, zejména s ohledem na zjištění překvapivě nízké chelatační účinnosti této látky vůči mědi. V současné době probíhá příprava nové publikace na toto téma.

6. Podíl kandidáta na jednotlivých publikacích

4.1. Antioxidant effects of coumarins include direct radical scavenging, metal chelation and inhibition of ROS-producing enzymes

- rešerše literatury týkající se chelatace a redukce železa a mědi kumariny, sepsání této kapitoly rukopisu

4.2. Novel method for rapid copper chelation assessment confirmed low affinity of D-penicillamine for copper in comparison with trientine and 8-hydroxyquinolines

- provedení většiny experimentů týkajících se jak vývoje metodik, tak vlastních chelatačních a redukčních pokusů
- zpracování a analýza dat
- příprava manuskriptu

4.3. *In vitro* evaluation of copper-chelating properties of flavonoids

- provedení podstatné části experimentů
- zpracování a analýza dat
- příprava rukopisu

4.4. Iron reduction potentiates hydroxyl radical formation only in flavonols

- podíl na zpracování dat
- účast na finalizaci rukopisu

4.5. Mathematical calculations of iron complex stoichiometry by direct UV-Vis spectrophotometry

- provedení části experimentů
- podíl na přípravě manuskriptu

4.6. Oral administration of quercetin is unable to protect against isoproterenol cardiotoxicity

- hlavní podíl na provedení *in vivo* experimentů, včetně aplikace léčiv, měření hemodynamických parametrů a odběrů vzorků, kromě kinetické analýzy metabolitů kvercetinu, histologické analýzy a experimentů s izolovanou cévou
- zpracování a analýza většiny dat
- příprava podstatné části manuskriptu

4.7. Is a highly linear relationship between the dose of quercetin and the pharmacological effect possible? – A comment on Liu, *et al*. Evaluation of antioxidant and immunity activities of quercetin in isoproterenol-treated rats. *Molecules* 2012, *17*, 4281-4291

- účast na finalizaci rukopisu

7. Použité zkratky

3-AP	3-aminopyridin-2-karbaldehyd-thiosemikarbazon
CYBRD1	duodenální cytochrom-b-reduktasa 1
DMT1	transportér pro dvojmocné kovy, z angl. divalent metal transporter 1
EDTA	ethylendiamintetraoctová kyselina
Fe-Tf	transferin
hCTR	human copper transporter
HIF	hypoxia inducible factor
IRE	iron-responsive element
PIH	pyridoxal-isonikotinoylhydrazon
RONS	reaktivní formy kyslíku a dusíku, z angl. reactive oxygen and nitrogen species
ROS	reaktivní formy kyslíku, z angl. reactive oxygen species
Steap	six-transmembrane epithelial antigen of the prostate
TfR	transferinový receptor

8. Seznam obrázků

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10. Závěr

Chelátory železa a mědi, kromě zásadního postavení v terapii přetížení organismu těmito kovy, představují skupinu látek s relativně širokým terapeutickým potenciálem. Nalézat nové chelátory s vhodnými vlastnostmi je tedy žádoucí. Dílčí závěry práce jsou následující:

- Významný podíl na biologických účincích kumarinů má jejich schopnost interagovat s oběma přechodnými kovy, přičemž nejvyšší aktivita je spojena s 6,7- a 7,8-dihydroxysubstitucí.

- Vyvinutá spektrofotometrická *in vitro* metoda pro stanovení chelatace a redukce iontů mědi byla použita pro screening účinků u řady přírodních a syntetických látek. Zatímco D-penicilamin vykázal poměrně slabou afinitu k mědi a vysoký redukční potenciál, mezi flavonoidy byly nalezeny látky se silným chelatačním potenciálem, a to i v kyselém prostředí. Tyto přírodní látky mají komplexní interakce s přechodnými kovy, neboť v závislosti na struktuře je mohou i redukovat, zejména za nízkého pH prostředí, a/nebo podpořit tvorbu volných radikálů.

- Pomocí nového přístupu na bázi přímé spektrofotometrie a s použitím matematických výpočtů byla ověřena stechiometrie komplexu několika látek. S výhodou může být tento přístup využit u látek slaběji vázajících kov.

- Komplexnímu kardiotoxickému působení isoprenalinu na *in vivo* modelu nebyla schopna zabránit perorální premedikace kvercetinem, přes jeho dokumentované interakce s přechodnými kovy. Byly však pozorovány účinky flavonolu na hladké svalstvo cév a některé hemodynamické parametry.

Nové poznatky shrnuté v disertační práci, včetně vyvinutých *in vitro* metod, by měly přispět k dalšímu výzkumu v této oblasti, pochopení dopadu přírodních látek na lidské zdraví nebo širšímu uplatnění chelátorů železa a mědi v klinické praxi.

11. Přehled odborných publikací

11.1. Recenzované publikace v odborných časopisech s impaktním faktorem

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11.2. Přednášky na konferencích

3. Postgraduální a 1. Postdoktorandská vědecká konference Farmaceutické fakulty v Hradci Králové, Univerzity Karlovy v Praze
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Hradec Králové, 3.-4. 2. 2015

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