UNIWERSYTET RZESZOWSKI
WYDZIAŁ BIOLOGICZNO-ROLNICZY

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ANTYOKSYDACYJNE WŁAŚCIWOŚCI POLIFENOLI I ZWIĄZKÓW SYNTETYCZNYCH

Praca doktorska wykonana w Zakładzie Biochemii Analitycznej
pod promotorską opieką: dr hab. Izabeli Sadowskiej-Bartosz, prof. UR

Obszar wiedzy: nauki przyrodnicze
Dziedzina nauki: nauki biologiczne
Dyscyplina naukowa: biologia

Rzeszów 2018

Pragnę serdecznie podziękować:

Promotorowi – dr hab. Izabeli Sadowskiej-Bartosz, prof. UR
za nieocenioną pomoc, cenne wskazówki, zaangażowanie i opiekę naukową,

prof. dr hab. Grzegorzowi Bartoszowi
za wnikliwe uwagi, owocne dyskusje i wszelką pomoc,

Kasi i Edycie
za życzliwą atmosferę, słowa wsparcia i mobilizację,

Rodzicom
za wsparcie, wyrozumiałość i cierpliwość,

Narzeczonemu – Łukaszowi
za nigdy niegasnącą wiarę we mnie.

SPIS TREŚCI

WYKAZ STOSOWANYCH SKRÓTÓW

ABTS	kwas 2,2'-azynobis-(3-etylobenzotiazolino-6-sulfonowy) [ang. *2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid*]
AAPH	dichlorowodorek 2,2'-azobis (2-amidynopropanu) [ang. *2,2'-Azobis(2-amidinopropane) dihydrochloride*]
DETAPA	kwas dietylenotriaminopentaoctowy (ang. *Diethylenetriaminepentaacetic Acid*)
DMEM	podłoże Dulbecco do hodowli komórek ssaczych (ang. *Dulbecco's Modified Eagle Medium*)
DU-145	linia komórkowa raka prostaty (ang. *human prostate cancer cell line*)
EPR	elektronowy rezonans paramagnetyczny (ang. *Electron Paramaqnetic Resonance*)
FBS	bydlęca surowica płodowa (ang. *Fetal Bovine Serum*)
FT-IR	spektroskopia w podczerwieni z transformacją Fouriera (ang. *Fourier-Transform Infrared Spectroscopy*)
FRAP	metoda oznaczania potencjału redukcji jonów żelaza (ang. *Ferric Reducing Antioxidant Potential*)
H_2DCF	2',7'-dichlorodihydrofluoresceina (ang. *2',7'-dichlorodihydrofluorescein*)
IC_{50}	stężenie powodujące 50% inhibicję procesu (ang. *Half Maximal Inhibitory Concentration*)
NADH	dinukleotyd nikotynoamidoadeninowy (ang. *Nicotinamide adenine dinucleotide*)
NBT	błękit nitrotetrazolowy (ang. *Nitro Blue Tetrazolium*)
NS	stres nitracyjny (ang. *nitration stress*)
OS	stres oksydacyjny (ang. *oxidative stress*)
PBS	zbuforowany roztwór soli fizjologicznej (ang. *Phosphate-Buffered Saline*)
PMS	metosiarczan fenazyny (ang. *Phenazine methosulfate*)
RFA/RNS	reaktywne formy azotu (ang. *Reactive Nitrogen Species*)
RFT/ROS	reaktywne formy tlenu (ang. *Reactive Oxygen Species*)
RXA	reaktywne formy chlorowców (ang. *Reactive Halogen Species*)
SIN-1	3-morfolinosydnonimina (ang. *3-morpholinosydnonimine*)
YNB	podstawowa pożywka do hodowli drożdży (ang. *Yeast Nitrogen Base*)
YPD	pożywka do hodowli drożdży zawierająca ekstrakt drożdży, pepton i dekstrozę (glukozę) (ang. *Yeast Extract-Peptone-Dextrose)*

INFORMACJE WPROWADZAJĄCE

ŹRÓDŁA FINANSOWANIA

Rozprawa doktorska była finansowana ze środków wewnątrzwydziałowych jak i ze środków zewnętrznych:

1. Dotacja celowa na finansowanie działalności polegającej na prowadzeniu badań naukowych lub prac rozwojowych oraz zadań z nimi związanych, służących rozwojowi młodych naukowców oraz uczestników studiów doktoranckich przyznana mi przez Wydział Biologiczno-Rolniczy UR w roku 2017, 2018.
2. Projekt Narodowego Centrum Nauki MAESTRO 6. Tytuł: „*Szereg Mocy Przeciwutleniającej jako narzędzie pozwalające na racjonalne projektowanie i ocenę właściwości prozdrowotnych żywności funkcjonalnej zawierającej przeciwutleniające związki fitochemiczne*”, nr rej.: 2014/14/A/ST4/00640 (kierownik: Prof. dr hab. Jacek Namieśnik).

DOROBEK NAUKOWY

PUBLIKACJE

Publikacje wchodzące w skład rozprawy doktorskiej:

1. **Grzesik M**., Naparło K., Bartosz G., Sadowska-Bartosz I. 2018. Antioxidant properties of catechins: comparison with other antioxidants. *Food Chemsitry* 241: 480-492 **(IF_{2017} = 4.946; Punkty MNiSW = 40)**.
2. **Grzesik M**., Bartosz G., Dziedzic A., Naróg D., Namieśnik J., Sadowska-Bartosz I. 2018. Antioxidant properties of ferrous flavanol mixtures. *Food Chemistry* 268: 567–576 **(IF_{2018} = 4.946; Punkty MNiSW = 40)**.
3. **Grzesik M**., Bartosz G., Stefaniuk I., Pichla M., Namieśnik J., Sadowska-Bartosz. I. 2019. Dietary antioxidants as a source of hydrogen peroxide. *Food Chemistry* 278: 692-699 **(IF_{2018} = 4.946; Punkty MNiSW = 40)**.

Sumaryczna wartość współczynnika **Impact Factor** publikacji wchodzących w skład rozprawy doktorskiej (zgodnie z rokiem opublikowania) wynosi **14,838** (**120** punktów **MNiSW**).

Pozostałe publikacje

4. Ciura J., Szeliga M., **Grzesik M**., Tyrka M. 2017. Next-generation sequencing of representational difference analysis products for identification of genes involved in diosgenin biosynthesis in fenugreek (*Trigonella foenum-graecum*). *Planta* 245(5): 977-991 **(IF: 3.249; punkty MNiSW: 40)**.

5. Adamczyk-Sowa M., Galiniak S., Żyracka E., **Grzesik M**., Naparło K., Sowa P., Bartosz G., Sadowska-Bartosz I. 2017. Oxidative Modification of blood serum proteins in multiple sclerosis after interferon beta and melatonin treatment. *Oxidative Medicine and Cellular Longevity* 2017: 7905148 **(IF: 4.936; punkty MNiSW: 30)**.
6. Ciura J., Szeliga M., **Grzesik M**., Tyrka M. 2018. Changes in fenugreek transcriptome induced by methyl jasmonate and steroid precursors revealed by RNA-Seq. *Genomics* 110: 267-276 **(IF: 2.910; punkty MNiSW: 30)**.
7. Gonos E., Kapetanou M., Sereikaite J., Naparło K., Grzesik M., Bartosz G., Sadowska-Bartosz I. 2018 Origin and pathophysiology of protein carbonylation, nitration and chlorination in age-related brain diseases and aging. *Aging (Albany NY):*10(5): 868-901 **(IF: 5.179; punkty MNiSW: 30)**.

Sumaryczna wartość współczynnika **IF** dorobku publikacyjnego łącznie z publikacjami wchodzącymi w skład rozprawy doktorskiej (zgodnie z rokiem opublikowania) wynosi **31,11 (250 punktów MNiSW)**.

Komunikaty zjazdowe

1. **Grzesik M**., Stefaniuk I., Namieśnik J., Bartosz G., Sadowska-Bartosz. I. *Generation of hydrogen peroxide by dietary antioxidants*. III Kongres BIO 2018. Gdańsk, 18-21 wrzesień 2018 r. (prezentacja posterowa).
2. **Grzesik M**., Namieśnik J., Bartosz G., Sadowska-Bartosz I. *Antioxidant properties of ferrous catechin complexes.* The 19th biennial meeting for the Society for Free Radical Research International (SFRRI). Lizbona, 4-7 czerwca 2018 r. (prezentacja posterowa).
3. **Grzesik M**., Naparło K., Bartosz G., Sadowska-Bartosz I. *Catechins and other flavonoids as protectors against undesired modifications of biomolecules.* Reactive Oxygen species and lipid Peroxidation in Human Health and Disease. Graz, 14-15.09.2017 r. (prezentacja posterowa).
4. **Grzesik M**., Naparło K., Żuberek M., Grzelak A., Bartosz G., Sadowska-Bartosz I. *Katechiny jako związki chroniące przed nitracją, chlorowaniem i utlenianiem w układach bezkomórkowych i w komórkach in vitro*. III Ogólnopolska Konferencja Doktorantów Nauk o Życiu BIOOPEN. Łódź, 11-12.05.2017 r. (prezentacja posterowa).
5. **Grzesik M**., Naparło K., Żuberek M., Grzelak A., Bartosz G., Sadowska-Bartosz I. *Comparison of antioxidant properties of catechins*. XLIV Szkoła Zimowa WBBiB „No stress – no life". Zakopane, 14-18.02.2017 r. (prezentacja ustna).

Udział w projektach badawczych

1. Charakter udziału w projekcie: **Asystent naukowy**

   Źródło finansowania: Regionalny Program Operacyjny Województwa Podkarpackiego 2007-2013

   Numer projektu: UDA-RPPK.01.03.00-18-018/13

   Tytuł projektu: „Wykorzystanie narzędzi molekularnych i proteomicznych do poszukiwania genów i enzymów o charakterze biotechnologicznym”

   Kierownik projektu: dr hab. inż. Mirosław Tyrka, prof. PRz

   Okres trwania projektu (lata): 2014-2015

2. Charakter udziału w projekcie: **Wykonawca**

   Źródło finansowania: Narodowe Centrum Badań i Rozwoju

   Numer projektu: PBS1/A8/1/2012

   Tytuł projektu: „Innowacyjny system selekcji jęczmienia i pszenżyta oparty o najnowsze osiągnięcia fenomiki i genomiki”

   Kierownik projektu: prof. dr hab. Marcin Rapacz

   Okres trwania projektu (lata): 2012-2015

3. Charakter udziału w projekcie: **Starszy technik**

   Źródło finansowania: Narodowe Centrum Nauki

   Numer projektu: 2014/14/M/NZ2/00519

   Tytuł projektu: „CDKG/Ph1: czy istnieje uniwersalny mechanizm regulujący stabilność genomu u traw?”

   Kierownik projektu: prof. dr hab. Robert Hasterok

   Okres trwania projektu (lata): 2015-2018

4. Charakter udziału w projekcie: **Wykonawca**

   Źródło finansowania: Narodowe Centrum Nauki

   Numer projektu: UMO-2014/14/A/ST4/00640

   Tytuł projektu: "Szereg Mocy Przeciwutleniającej jako narzędzie pozwalające na racjonalne projektowanie i ocenę właściwości prozdrowotnych żywności funkcjonalnej zawierającej związki fitochemiczne”

   Kierownik projektu: prof. dr hab. inż. Jacek Namieśnik

   Okres trwania projektu (lata): 2015-2020

STRESZCZENIE

Na rozprawę doktorską składa się cykl trzech prac opublikowanych w czasopiśmie *Food Chemistry* poświęconych (i) porównaniu właściwości antyoksydacyjnych flawanoli oraz innych antyoksydantów naturalnych i syntetycznych, (ii) określeniu wpływu wiązania jonów Fe^{2+} na właściwości antyoksydacyjne flawanoli oraz (iii) porównaniu wytwarzania nadtlenku wodoru przez różne antyoksydanty i uzyskaniu wglądu w mechanizm tego zjawiska.

Wykazałam, że badane flawanole [(+)-katechina, (-)-epikatechina, (-)-epigallokatechina, galusan (-)-epikatechiny i galusan (-)-epigallokatechiny] są bardzo dobrymi antyoksydantami w różnych układach bezkomórkowych, chroniąc przed działaniem takich utleniaczy jak nadtlenoazotyn, podchloryn i rodniki nadtlenkowe w testach hamowania utleniania dihydrorodaminy 123 i chronią fluoresceinę przed utlenianiem powodującym zanik fluorescencji. W większości tych testów, jak również w teście redukcji rodnika $ABTS^{\bullet}$, zdolność antyoksydacyjna flawanoli była znacznie większa w porównaniu z aktywnością standardowych antyoksydantów (glutationu i askorbinianu). Flawanole miały także działanie ochronne wobec erytrocytów poddanych działaniu utleniaczy.

Stwierdziłam, że jony Fe^{2+} tworzą kompleksy z flawanolami; stechiometria tych kompleksów jest różna dla różnych flawanoli. Kompleksy flawanoli tworzone przez dodanie substechiometrycznych ilości Fe^{2+} (1 mol Fe^{2+} na 4 mole flawanoli) cechowały się właściwościami antyoksydacyjnymi porównywalnymi z właściwościami wyjściowych flawanoli. Nie zaobserwowałam, by kompleksy flawanoli z Fe^{2+} wykazywały pseudoenzymatyczne aktywności dysmutazy ponadtlenkowej bądź katalazy.

Porównałam 54 naturalne i syntetyczne antyoksydanty pod kątem wytwarzania nadtlenku wodoru w pożywkach stosowanych do hodowli komórek. Stwierdziłam, że połowa z nich (27) wytwarza w tych pożywkach nadtlenek wodoru; generacja nadtlenku wodoru była niższa w pożywkach stosowanych do hodowli drożdży niż w pożywkach do hodowli komórek ssaków i w zbuforowanym roztworze soli fizjologicznej (PBS). Związkami generującymi najwięcej nadtlenku wodoru były: galusan propylu, pirogalol, galusan (-)-epigallokatechiny i kwercetyna. Askorbinian generował nadtlenek wodoru, lecz w oddziaływaniu z polifenolami zmniejszał ilość nadtlenku wodoru wytwarzanego przez te związki. Wydaje się, że nadtlenek wodoru powstaje głównie w wyniku dysmutacji anionorodnika ponadtlenkowego wytwarzanego w dwu jednoelektronowych reakcjach: utleniania polifenoli do rodników semichinonowych i następczego utleniania rodników semichinonowych. Świadczy o tym wykazanie powstawania rodników semichinonowych podczas utleniania galusanu propylu i galusanu (-)-epigallokatechiny techniką EPR i powstawania anionorodnika ponadtlenkowego, wykazane w reakcjach redukcji NBT i utleniania dihydrorodaminy 123, hamowanych przez dysmutazę ponadtlenkową. Czynnikami decydującymi o ilości nadtlenku wodoru wytwarzanego w różnych roztworach są: stężenie śladowych ilości jonów metali ziem

przejściowych, głównie żelaza i pH roztworu. Potwierdziłam wytwarzanie nadtlenku wodoru w herbacie i wykazałam, że dodatek cytryny do herbaty znacznie zmniejsza ilość wytworzonego nadtlenku wodoru.

Nadtlenek wodoru wytwarzany w pożywce DMEM przyczyniał się do cytotoksycznego działania galusanu propylu, galusanu (-)-epigallokatechiny i kwercetyny, gdyż obecność katalazy w pożywce obniżała cytotoksyczne działanie tych związków na komórki DU-145.

ABSTRACT

My doctoral dissertation consists of 3 papers published in the journal *Food Chemistry* devoted to (i) comparison of antioxidant properties of flavanols and other natural and synthetic antioxidants, (ii) studies of the effect of binding Fe^{2+} ions on the antioxidant properties of flavanols, and (iii) comparison of generation of hydrogen peroxide by various antioxidant and an insight into the mechanism of this phenomenon.

The flavanols studied [(+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate] are very good antioxidants in various cell-free systems, protecting against such oxidants as peroxynitrite, hypochlorite and peroxyl radicals in the tests of inhibition of dihydrorhodamine 123 oxidation and inhibition of oxidation-induced fluorescein bleaching. In the majority of these tests as well as in the test of reduction of $ABTS^{\bullet}$ radical, the antioxidant activities of the flavanols were much higher than those of standard antioxidants (glutathione and ascorbate). Flavanols protected also erythrocytes against oxidant-induced hemolysis.

Fe^{2+} ions were found to form complexes with flavanols; stoichiometry of these complexes was different for various compounds. Flavanol complexes formed by addition of substoichiometric amounts of Fe^{2+} (1 mol Fe^{2+} per 4 moles of a flavanol) had antioxidant properties similar to those of flavanols alone. Complexes of flavanols with Fe^{2+} did not show pseudoenzymatic activities of superoxide dismutase or catalase.

Comparison of hydrogen peroxide generation in cell culture media by 54 natural and synthetic antioxidants showed that half of them (27 compounds) produced hydrogen peroxide. Generation of hydrogen peroxide was lower in media used for yeast culture than in media used for culture of mammalian cells and phosphate-buffered saline (PBS). The highest amounts of hydrogen peroxide was generated by propyl gallate, pyrogallol, (-)-epigallocatechin gallate and quercetin. Ascorbate generated hydrogen peroxide but when combined with polyphenols it decreased hydrogen peroxide generation by the polyphenols.

Apparently, hydrogen peroxide is formed mainly by dismutation of the superoxide radical anions generated in two successive one-electron reaction of polyphenol oxidation to semiquinone radicals and subsequent oxidation of semiquinone radicals. In agreement with this mechanism, the formation of semiquinone radicals during oxidation of propyl gallate and (-) epigallocatechin gallate was demonstrated by EPR spectroscopy and superoxide formation was demonstrated by superoxide-dismutase inhibitable NBT reduction and dihydrorhodamine 123 oxidation.

The factors determining the amount of hydrogen peroxide are: the concentration of trace amounts of transition metal ions and pH of the solution. Generation of hydrogen peroxide in the tea was confirmed. It was found that addition of lemon to the tea considerably decreases the amount of hydrogen peroxide generated.

Hydrogen peroxide generated in the DMEM medium contributed to the cytotoxic action of propyl gallate, (-)-epigallocatechin gallate and quercetin since the presence of catalase in the medium decreased the cytotoxic action of these compounds on DU-145 cells.

WSTĘP

Równowaga pomiędzy niekontrolowanymi reakcjami utleniania a aktywnością antyoksydacyjną jest ważnym aspektem homeostazy na poziomie komórki i organizmu. Oprócz endogennych niskocząsteczkowych antyoksydantów i enzymów antyoksydacyjnych, w równowadze tej uczestniczą także związki o właściwościach antyoksydacyjnych pochodzenia egzogennego. Organizm człowieka bezwzględnie potrzebuje takich antyoksydantów jak kwas askorbinowy i tokoferole, które są witaminami (odpowiednio C i E) oraz ß-karoten, który pełni rolę prowitaminy A. W pożywieniu i w napojach spożywamy wiele innych antyoksydantów, zwłaszcza flawonoidów, a także antyoksydantów syntetycznych, stosowanych jako dodatki do produktów spożywczych łatwo ulegających utlenieniu. Dokładne zbadanie mechanizmów działania tych antyoksydantów i ich oddziaływania z komórkami organizmu jest więc istotne i może dostarczyć ważnych wskazówek dietetycznych.

Antyoksydanty pochodzenia roślinnego zdominowały w ostatnich dwóch dekadach badania przekładające się na praktyczne zastosowania (ang. „*translational research*") w dziedzinie badań nad żywnością i żywieniem, profilaktyką chorób cywilizacyjnych, a także kosmetologią. W szerokim obiegu antyoksydanty roślinne funkcjonują jako synonim nutraceutyków i nutrikosmetyków, przynajmniej wśród konsumentów i producentów. Popularność ta i związany z nią sukces komercyjny jest pochodną badań prowadzonych w różnych modelach badawczych. Wyniki tych badań wskazują na udział stresu oksydacyjnego (OS) i nitracyjnego (NS) w etiologii chorób cywilizacyjnych, ale także w regulowaniu przez reaktywne formy tlenu i azotu (RFT/RFA) wielu istotnych procesów

odpowiedzialnych za prawidłowe funkcjonowanie organizmów eukariotycznych na wszystkich etapach ich rozwoju. Związki chemiczne o charakterze redukującym od dawna były stosowane w przemyśle spożywczym w celu ochrony składników żywności przed utlenieniem. Potencjalnie prozdrowotne właściwości przeciwutleniających substancji fitochemicznych, z którymi wiązano i wiąże się ogromne nadzieje postrzegane są jako obiecujący element strategii przeciwdziałania zagrożeniom chorobami cywilizacyjnymi [1].

Stres oksydacyjny definiowany jest jako brak równowagi pomiędzy wytwarzaniem i akumulacją reaktywnych form tlenu (ang. *Reactive Oxygen Species*, ROS) w komórkach i tkankach, a zdolnością układu biologicznego do neutralizacji i eliminacji tych produktów. Do reaktywnych form tlenu zaliczamy zarówno wolne rodniki posiadające niesparowany elektron [np. anionorodnik ponadtlenkowy ($O_2^{\bullet -}$), rodnik hydroksylowy ($HO^{\bullet}$)] jak również indywidua chemiczne nie będące wolnymi rodnikami: tlen singletowy [$^1O_2$], ozon [O_3], nadtlenek wodoru [H_2O_2]. Dodatkowo nieodłącznym elementem tlenowego metabolizmu komórkowego jest wytwarzanie reaktywnych form azotu (ang. *Reactive Nitrogen Species*, RNS) i chlorowców (ang. *Reactive halogen species*, RXS), m. in. nadtlenoazotynu ($ONOO^-$), ditlenku azotu (NO_2), chlorku nitrylu (NO_2Cl) czy kwasu podchlorawego (HOCl). Ich źródłem są produkty pośrednie utleniania przenośników elektronów, związki wytwarzane w czasie reakcji zapalnych, peroksydacja lipidów oraz reakcje katalizowane przez oksydazy i jony metali (Fe^{3+}, Cu^{2+}).

Reaktywne formy tlenu i azotu są produkowane w komórkach w warunkach fizjologicznych, jako cząsteczki gwarantujące homeostazę wewnątrzkomórkową. Odgrywają one kluczową rolę m.in. w procesie transdukcji sygnałów wewnątrzkomórkowych [2]. Jednak na skutek działania czynników zewnętrznych, takich jak promieniowanie jonizujące, zanieczyszczenia, metale ciężkie czy różnego typu ksenobiotyki może dojść do znacznego zwiększenia produkcji ROS/RNS co może skutkować zachwianiem równowagi, doprowadzając do uszkodzenia komórek i tkanek [3].

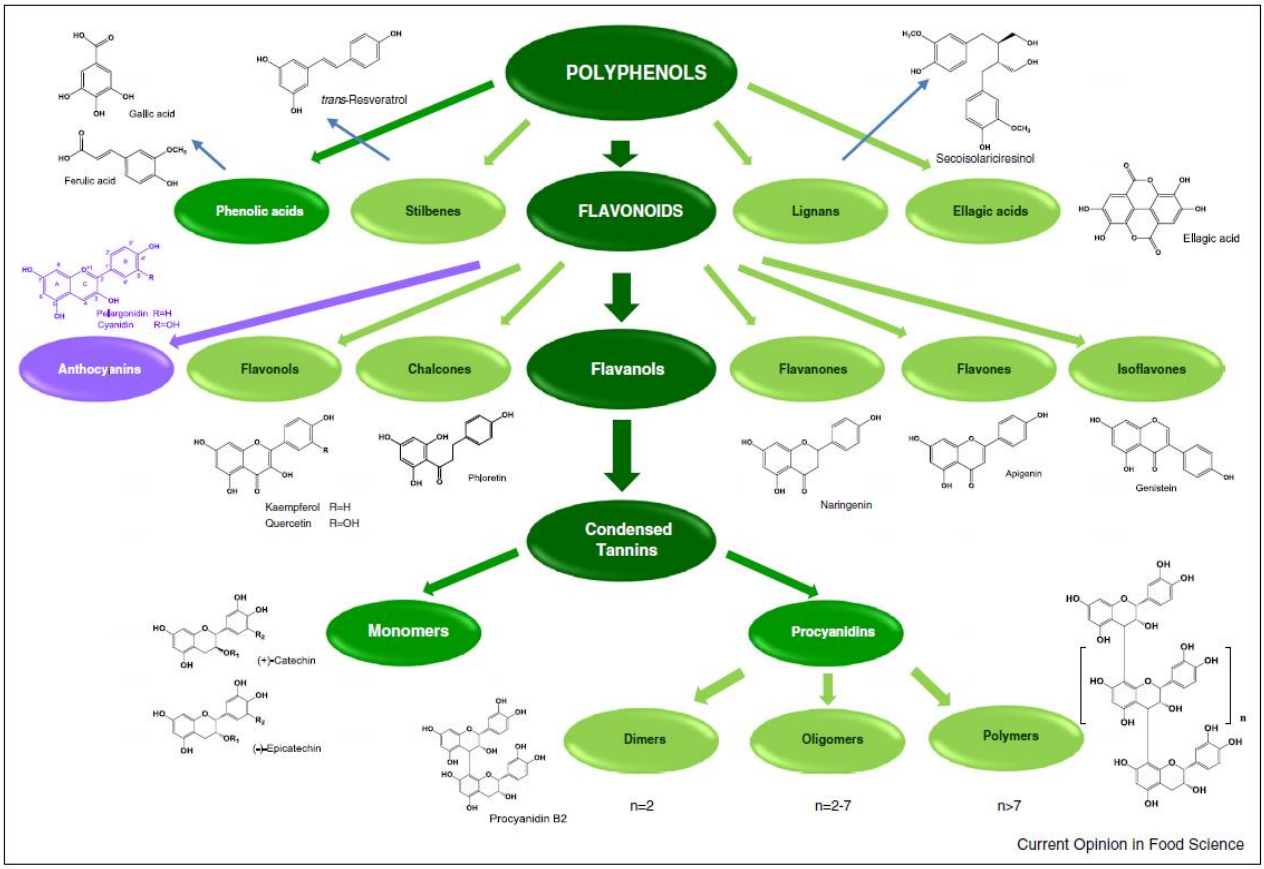
Pod wpływem reaktywnych form tlenu, azotu i chloru dochodzi do uszkodzeń wielu związków biologicznie czynnych – lipidów, kwasów nukleinowych oraz białek, które są głównym celem ataku wolnych rodników. Przy dużej aktywności reaktywnych form tlenu i azotu, a zmniejszonej skuteczności układów antyoksydacyjnych i proteolitycznych, dochodzi do nagromadzenia utlenionych produktów białkowych. Może to prowadzić do wielu zmian patologicznych oraz jest jednym z czynników przyczyniających się do procesu starzenia się organizmów [4].

W ostatnich latach nastąpił wzrost zainteresowania naturalnymi substancjami o właściwościach przeciwutleniających, które w znacznym stopniu chronią przed negatywnym działaniem stresu oksydacyjnego. Do związków tych należą m.in. polifenole.

Polifenole są naturalnie występującymi związkami znajdującymi się głównie w owocach, warzywach i zbożach. Owoce takie jak winogrona, jabłka czy wiśnie zawierają do 200-300 mg

polifenoli na 100 gramów świeżej masy. Co istotne, produkty pochodzące z przetwórstwa owoców i warzyw również zawierają znaczące ilości tychże związków np. kieliszek czerwonego wina czy filiżanka herbaty lub kawy zawierają około 100 mg polifenoli.

Związki polifenolowe są wtórnymi metabolitami roślinnymi o zróżnicowanej budowie chemicznej. Produkowane są w odpowiedzi na stres, uszkodzenie, infekcję grzybową lub promieniowanie ultrafioletowe (UV). Zidentyfikowano ponad 8 000 polifenoli występujących u różnych gatunków roślin [5]. W zależności od liczby pierścieni aromatycznych oraz sposobu ich połączenia, dzieli się je na kilka głównych klas: flawonoidy, kwasy fenolowe, stilbeny, lignany i kwasy elagowe (Ryc. 1).

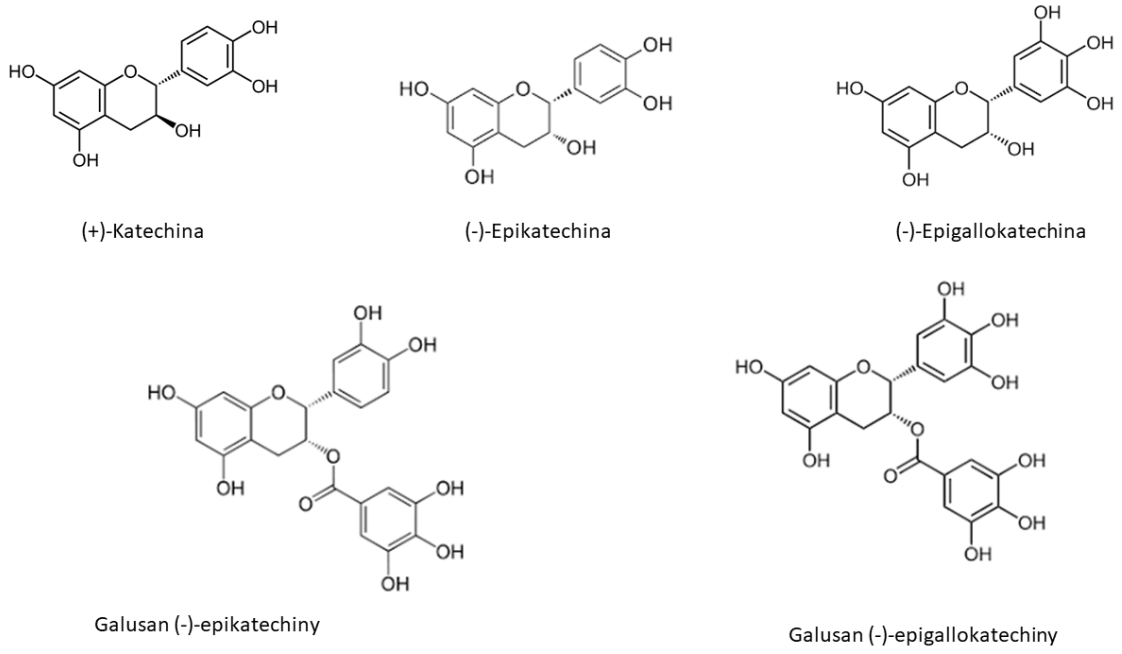


Ryc.1. Klasyfikacja polifenoli [wg Zhang, 2016].

Spośród szerokiej grupy związków polifenolowych na szczególną uwagę zasługują flawan-3-ole (katechiny). Związki te poza doskonałymi właściwościami przeciwutleniającymi posiadają także właściwości przeciwnowotworowe, przeciwmutagenne, przeciwbakteryjne i przeciwzapalne. Dzięki szerokiej gamie właściwości biologicznych mogą one zapobiegać rozwojowi lub wspomagać walkę z chorobami takimi jak nowotwory, choroby neurodegeneracyjne i sercowo- naczyniowe.

Flawanole są obecne w szczególnie dużych ilościach w herbacie (zwłaszcza zielonej), kakao, czekoladzie i winie, występują także w wielu owocach i warzywach. Moje badania skupiały się na analizie właściwości antyoksydacyjnych pięciu flawanoli: (+)-katechiny, (-)-epikatechiny, (-)

epigallokatechiny, galusanu (-)-epikatechiny i galusanu (-) epigallokatechiny. Ten ostatni flawanol jest głównym flawonoidem występującym w herbacie, mogącym stanowić do $^1/_3$ suchej masy herbat zielonych (Ryc. 2).



Ryc. 2. Struktury badanych flawanoli.

Antyoksydanty różnią się stechiometrią i szybkością reakcji z reaktywnymi formami tlenu i azotu, co skutkuje zróżnicowaną efektywnością ich przeciwutleniającego działania w różnych układach doświadczalnych. Porównanie tych właściwości jest istotnym elementem charakterystyki antyoksydantów.

Istotną właściwością przeciwutleniającego działania antyoksydantów jest ich oddziaływanie z metalami. Wiele antyoksydantów, w szczególności flawonoidy, wiąże jony metali, co może hamować prooksydacyjne działanie tych jonów, ale niekiedy wzmagać ich prooksydacyjne efekty [7, 8]. Zbadanie zachowania się antyoksydantów w obecności jonów metali jest więc także ważnym elementem charakterystyki tych związków.

Polifenole, ale także inne klasy antyoksydantów, zapobiegając reakcjom utleniania, same ulegają utlenieniu. Ich utlenianie w obecności tlenu prowadzi do redukcji tlenu i wytwarzania anionorodnika ponadtlenkowego oraz nadtlenku wodoru. Ten efekt może komplikować doświadczenia z użyciem antyoksydantów, zwłaszcza doświadczenia prowadzone na komórkach *in vitro*, bowiem nadtlenek wodoru może mieć wkład w obserwowaną toksyczność badanych związków i powodować efekty mutagenne i klastogenne [9, 10]. Zbadanie stosowanych antyoksydantów pod kątem generacji przez nie reaktywnych form tlenu jest kolejnym istotnym elementem ich charakterystyki.

CELE PRACY

Celem mojej pracy wykonanej w ramach przygotowania rozprawy doktorskiej było:

1. Porównanie właściwości antyoksydacyjnych wybranych związków obecnych w żywności, szczególnie flawan-3-oli i antyoksydantów syntetycznych z wzorcowymi antyoksydantami (glutation, kwas askorbinowy).
2. Ustalenie, czy wyselekcjonowane związki o właściwościach antyoksydacyjnych wykazują działanie prooksydacyjne.
3. Ocena właściwości antyoksydacyjnych mieszanin flawan-3-oli z żelazem i porównanie ich z właściwościami flawanoli w formie niezwiązanej.
4. Porównanie generacji nadtlenku wodoru przez ponad 50 naturalnych i syntetycznych antyoksydantów oraz uzyskanie wglądu w mechanizm tego zjawiska.

METODY

Badania rozpoczęto od porównania właściwości antyoksydacyjnych wybranych flawanoli z innymi naturalnymi i syntetycznymi antyoksydantami, w tym z głównymi antyoksydantami wewnątrzkomórkowymi (glutation, kwas askorbinowy). Oznaczenia przeprowadzono zarówno w układach bezkomórkowych jak również na modelowym układzie komórkowym (erytrocyty) [Publikacja 1: Grzesik M**.,** Naparło K., Bartosz G., Sadowska-Bartosz I. Antioxidant properties of catechins: comparison with other antioxidants. *Food Chemistry* 2018; 241: 480-492].

W celu zbadania aktywności przeciwutleniającej testowanych związków użyto testów spektrofotometrycznych, mierząc kinetykę i stechiometrię redukcji rodników $ABTS^{\bullet+}$ [11] oraz zdolność do redukcji jonów Fe^{3+} przy użyciu metody FRAP [12].

Właściwości antyoksydacyjne badanych związków określono za pomocą kilku testów: testu hamowania reakcji utleniania dihydrorodaminy 123 przez SIN-1 (3-morfolinosydnoniminę) generującą nadtlenoazotyn ($ONOO^-$) (jeden z ważniejszych czynników utleniających i nitrujących w organiźmie), testu ochrony fluoresceiny przed utratą fluorescencji wywołaną przez podchloryn sodu i testu ochrony fluoresceiny przed utratą fluorescencji wywołaną przez AAPH [dichlorowodorek 2,2'-azobis(2- metylopropionoamidyny)]. Rozpad AAPH daje rodniki alkilowe $R^{\bullet}$, które w reakcji z tlenem tworzą rodniki nadtlenkowe $ROO^{\bullet}$ [13,14].

W układzie komórkowym, na komórkach izolowanych z krwi ludzkiej, oznaczono wpływ wybranych substancji na hemolizę erytrocytów wywołaną przez AAPH oraz podchloryn sodu. Dodatkowo sprawdzono, czy wybrane substancje chronią zlokalizowaną wewnątrzkomórkowo sondę fluorescencyjną H_2DCF (2',7'-dichlorodihydrofluoresceinę) przed utlenianiem indukowanym przez AAPH [15]. Uzyskane wyniki odnoszono do antyoksydacyjnego/chroniącego przed chlorowaniem działania wzorcowych antyoksydantów (glutation, kwas askorbinowy).

Ze względu na fakt, iż flawanole charakteryzowały się doskonałym działaniem antyoksydacyjnym wobec różnych, istotnych z fizjologicznego punktu widzenia utleniaczy, postanowiono sprawdzić czy obok właściwości antyoksydacyjnych związki te charakteryzują się właściwościami prooksydacyjnymi. W tym celu wykorzystano jedną z właściwości flawanoli - zdolność do wiązania jonów metali przejściowych i porównano właściwości antyoksydacyjne wybranych flawanoli z ich mieszaninami z żelazem. Dodatkowo sprawdzono również czy flawanole i ich mieszaniny z żelazem wykazują aktywność podobną do aktywności dysmutazy ponadtlenkowej oraz do aktywności katalazy. Tak jak poprzednio, oznaczenia przeprowadzono zarówno w układach bezkomórkowych jak i w modelowym układzie komórkowym (erytrocyty) [Publikacja 2: Grzesik M., Bartosz G., Dziedzic A., Naróg D., Namieśnik J., Sadowska-Bartosz I. Antioxidant properties of ferrous flavanol mixtures. *Food Chemistry* 2018; 268: 567–576]. Badania rozpoczęto od wyznaczenia

stechiometrii wiązania żelaza przez wybrane flawanole. W tym celu roztwory flawanoli miareczkowano spektrofotometrycznie roztworem żelaza (II) (sól Mohra). Do oceny powstałych wiązań pomiędzy flawanolami, a żelazem użyto techniki spektroskopii w podczerwieni z transformacją Fouriera. Przy użyciu skaningowego mikroskopu elektronowego scharakteryzowano topografie badanego materiału oraz zbadano jego skład chemiczny. Średnicę hydrodynamiczną powstałych cząstek zmierzono stosując spektroskopię korelacji fotonów, wykorzystując do tego celu technikę dynamicznego rozpraszania światła.

W celu porównania właściwości antyoksydacyjnych flawanoli z ich kompleksami z Fe^{2+} wykonano szereg testów spektrofotometrycznych: zbadano i porównano stechiometrię redukcji kationorodników $ABTS^{\bullet}$ i jonów Fe^{3+} oraz porównano zdolność flawanoli i ich mieszanin z żelazem do ochrony fluoresceiny przed utratą fluorescencji wywołaną przez podchloryn sodu.

Sprawdzono również czy poszczególne mieszaniny flawanoli z żelazem wykazują aktywność podobną do aktywności dysmutazy ponadtlenkowej i katalazy. Oznaczenia rozpoczęto od testu w układzie PMS-NADH (metosiarczan fenazyny - dinukleotyd nikotynoamidoadeninowy). W układzie tym pod wpływem reakcji PMS z NADH wytwarzany jest anionorodnik ponadtlenkowy, który redukuje NBT (błękit nitrotetrazolowy) [16].

Sprawdzono czy badane katechiny i ich mieszaniny z żelazem wykazują aktywność wychwytywania anionorodników i tym samym czy chronią NBT przed redukcją. W kolejnym teście zbadano ochronny wpływ flawanoli i ich mieszanin z żelazem na fotochemiczną redukcję NBT przy udziale ryboflawiny [17]. Sprawdzono również czy badane substancje i ich mieszaniny z żelazem hamują utlenianie dwóch związków chemicznych: pirogalolu [18] i adrenaliny [19]. Następnie sprawdzono czy flawanole, a zwłaszcza ich mieszaniny z żelazem, mają aktywność podobną do działania katalazy, inkubując je zarówno z niskimi (mikromolarnymi) jak i wysokimi (milimolarnymi) stężeniami nadtlenku wodoru [20, 21].

W układzie komórkowym, na erytrocytach izolowanych z krwi ludzkiej, porównano wpływ wybranych katechin oraz ich mieszanin z żelazem na hemolizę wywołaną przez podchloryn sodu. Dodatkowo sprawdzono również czy wybrane flawanole i ich kompleksy z żelazem chronią lipidy (zawarte w żółtku jaj kurzych) przed peroksydacją wywołaną przez AAPH i jony żelaza (Fe^{2+}).

Badania do kolejnej pracy dotyczącej generacji nadtlenku wodoru na skutek samoutleniania wybranych antyoksydantów, rozpoczęto od porównania zdolności tych antyoksydantów do generowania nadtlenku wodoru i wyselekcjonowania związków, naturalnie występujących w napojach czy żywności, które wytwarzają najwięcej nadtlenku wodoru [Publikacja 3: Grzesik M., Bartosz G., Stefaniuk I., Pichla M., Namieśnik J., Sadowska-Bartosz I. Dietary antioxidants as a source of hydrogen peroxide. *Food Chemistry* 2019; 278: 692–699].

W tym celu zastosowano metodę spektrofotometryczną polegającą na utlenianiu jonów żelazawych Fe^{2+} do żelazowych Fe^{3+} przez nadtlenki w środowisku kwaśnym, a następnie na ich kompleksowaniu przez oranż ksylenolowy [21]. W celu upewnienia się czy produktem reakcji jest nadtlenek wodoru, przed końcem trzygodzinnej inkubacji, do próbek została dodana katalaza w stężeniu 10 µg/ml. Dodatkowo określono również kinetykę generowania nadtlenku wodoru przez wyselekcjonowane substancje, mierząc ilość powstałego nadtlenku po różnych czasach inkubacji. Oznaczenie produkcji nadtlenku wodoru przez wybrane substancje zostały powtórzone w roztworze soli fizjologicznej buforowanej fosforanem (PBS) oraz innych podłożach hodowlanych (medium do hodowli komórek ssaczych: DMEM/F12, DMEM/Glutamax; do hodowli komórek drożdży: YPD; podłoże minimalne: YNB). Dodatkowo przeprowadzono oznaczenia mające na celu pomiar wytwarzanych wolnych rodników podczas utleniania związków polifenolowych (rodników semichinonowych), przy użyciu spektrometru EPR [22]. Do oceny tworzenia anionorodnika ponadtlenkowego podczas samoutleniania polifenoli posłużyła również metoda polegająca na pomiarze redukcji NBT oraz utlenienia dihydroetydyny w obecności dysmutazy ponadtlenkowej - enzymu rozkładającego anionorodnik ponadtlenkowy [23]. Przy użyciu metody badającej redukcję rodników $ABTS^{\bullet}$ wyznaczono całkowitą pojemność antyoksydacyjną używanych mediów (DMEM, YPD, YNB itp.). Oznaczano również zdolność wybranych mediów do zmiatania rodnika ponadtlenkowego, stosując test hamowania utleniania pirogalolu [24]. Aktywność zmiatania anionorodnika ponadtlenkowego zdefiniowano jako ilość badanego medium zmniejszająca szybkość redukcji NBT o 50%. Sprawdzono również czy badane media rozkładają nadtlenek wodoru. W tym celu do badanych mediów dodano roztwór nadtlenku wodoru (końcowe stężenie 30 µM) i zmierzono jego ilość przed i po 3h inkubacji w 37°C. Wykonano również test sprawdzający, czy badane podłoża hodowlane posiadają zdolność wiązania jonów żelaza Fe^{2+}. Do tego celu wykorzystano ferrozynę, która charakteryzuje się zdolnością wiązania jonów żelaza, stąd dodana do reakcji konkuruje z badanym medium o jony żelaza znajdujące się w mieszaninie reakcyjnej.

W układzie komórkowym na komórkach linii raka prostaty (DU-145) wykonano test sprawdzający, czy generowany przez wybrane antyoksydanty nadtlenek wodoru wpływa na cytotoksyczność tych związków. Linia raka prostaty DU-145 (stadium II) pochodzi z komórek wyizolowanych z przerzutów do mózgu 69-letniego mężczyzny rasy kaukaskiej, o grupie krwi 0Rh+. Komórki te charakteryzują się morfologią nabłonkową, są adherentne. Linia nie jest wrażliwa na hormony, nie wytwarza antygenu prostaty. Ultrastrukturalne analizy, zarówno linii komórkowej jak i oryginalnego guza ujawniły obecność mikrokosmków, tonofilamentów, desmosomów, mitochondriów, dobrze rozwinięte aparaty Golgiego i heterogenne lizosomy.

Komórki wysiano, a następnie hodowano w kompletnym medium wzrostowym (DMEM-F12 HAM) wzbogaconym inaktywowaną termicznie bydlęcą surowicą płodową (FBS) – 10% v/v, z

dodatkiem antybiotyków: streptomycyny [10 000 U/ml] i penicyliny [10 mg/ml] (1% v/v), w warunkach standardowych w inkubatorze CO_2 (temperatura 37°C, 95% powietrza, 5% CO_2, 100% wilgotności względnej). Po 24h inkubacji wymieniono medium na świeże z dodatkiem badanych antyoksydantów o różnym zakresie stężeń oraz z dodatkiem lub bez katalazy w stężeniu 10 µg/ml. Komórki hodowano przez 24h, a następnie inkubowano przez 1h z 2% roztworem czerwieni obojętnej, a po przemyciu określono ich przeżywalność, mierząc absorbancję poddanych lizie komórek przy długościach fal 540/620 nm. Czerwień obojętna przechodzi na drodze transportu biernego do cytoplazmy jedynie komórek żywych i gromadzi się w lizosomach.

Wszystkie eksperymenty powtórzono trzykrotnie na różnych preparatach.

Istotność statystyczną różnic oceniano testem *t* Studenta, testem U Manna-Whitneya lub testem Kruskala-Wallisa, czy testem Dunnetta.

WYNIKI

Antyoksydacyjne właściwości flawanoli i innych antyoksydantów

Oznaczenie zdolności badanych antyoksydantów do zmiatania rodnika $ABTS^{\bullet}$ wykazało, że badane flawanole cechują się bardzo dużą zdolnością zmiatania tego rodnika. Jedna cząsteczka galusanu epikatechiny jest zdolna do reakcji z prawie 8 rodnikami $ABTS^{\bullet}$, jedna cząsteczka galusanu epigallokatechiny z prawie 6 rodnikami $ABTS^{\bullet}$, a jedna cząsteczka katechiny z czterema rodnikami $ABTS^{\bullet}$. Zdolność flawanoli (i niektórych innych flawonoidów) do zmiatania rodnika $ABTS^{\bullet}$ jest więc znacznie większa w porównaniu z wzorcowymi antyoksydantami, takimi jak glutation czy askorbinian, których cząsteczki są zdolne do reakcji z jednym rodnikiem $ABTS^{\bullet}$. Zdolność flawanoli do redukcji jonów Fe^{3+} oznaczana metodą FRAP jest niższa w porównaniu z ich zdolnością do redukcji rodnika $ABTS^{\bullet}$ i w przypadku galusanu epikatechiny i galusanu epigallokatechiny jest porównywalna ze zdolnością askorbinianu do redukcji jonów Fe^{3+} (około 2 jonów/cząsteczkę), zaś w przypadku katechiny, epikatechiny i epigallokatechiny (około 1 jon/cząsteczkę) niższa w porównaniu z askorbinianem. Warto jednak podkreślić, że zdolność drugiego standardowego antyoksydanta, glutationu, do redukcji jonów Fe^{3+} jest bardzo niska (**Publikacja 1**, Tabela 2).

Moryna, rezweratrol i rutyna okazały się najbardziej skuteczne w ochronie dihydrorodaminy 123 przed utlenianiem przez SIN-1. Wartości stężeń tych związków hamujące utlenianie dihydrorodaminy 123 w 50% (IC_{50}) były o około 2 rzędy wielkości niższe w porównaniu ze standardowymi antyoksydantami – glutationem i askorbinianem. Galusan epikatechiny, moryna i epigallokatechina najlepiej chroniły fluoresceinę przed odbarwieniem indukowanym przez podchloryn. W tym przypadku wartości IC_{50} glutationu i askorbinianu były o co najmniej rząd wielkości wyższe. Rezweratrol, naringenina i moryna okazały się najbardziej efektywne w ochronie

fluoresceiny przed odbarwieniem wywołanym przez AAPH. Również w tym przypadku wszystkie katechiny były dużo bardziej skuteczne niż askorbinian i glutation (**Publikacja 1**, Tabela 3).

Większość badanych antyoksydantów przejawiała działanie ochronne wobec erytrocytów eksponowanych na działanie AAPH i podchlorynu, co przejawiało się wydłużeniem czasu potrzebnego do 50% hemolizy erytrocytów poddanych działaniu utleniaczy w obecności tych związków. Kwas pirogronowy wykazał brak skuteczności w przypadku hemolizy indukowanej zarówno przez AAPH, jak i przez podchloryn (**Publikacja 1**, Ryc. 1 i 2).

Badane antyoksydanty zmniejszały utlenianie H_2DCF przez wolne rodniki generowane przez AAPH wewnątrz erytrocytów. W tym przypadku najbardziej skutecznymi związkami były: galusan epigallokatechiny, galusan epikatechiny i kwas chlorogenowy (**Publikacja 1**, Tabel 4).

Właściwości antyoksydacyjne kompleksów flawanole-Fe^{2+}

Badania wiązania Fe^{2+} przez flawanole wykazały zróżnicowaną stechiometrię wiązania jonów żelazawych przez te związki. Katechina i epikatechina wiązały jony Fe^{2+} w stosunku 3 mole flawanolu/mol Fe^{2+}. Stechiometria wiązania Fe^{2+} przez epigallokatechinę i galusan epikatechiny odpowiadała stosunkom odpowiednio 1:0,62 i 1:0,82. Galusan epigallokatechiny wiązał żelazo w stosunku molowym 1:1 (**Publikacja 2**, Ryc. 1). Wiązanie żelaza prowadziło do powstawania kompleksów, o czym świadczy pojawienie się nowego maksimum pochłaniania w widmie FT-IR, odpowiadającego liczbie falowej 1382 cm^{-1} (**Publikacja 2**, Ryc. 2)[1]. Obrazy katechin i ich kompleksów uzyskane za pomocą skaningowego mikroskopu elektronowego wykazały obecność struktur o średnicy 5-10 µM (**Publikacja 2**, Ryc. S1)[2]. Podejrzewając, że tworzenie takich struktur może być artefaktem powstającym podczas suszenia próbek, określono wielkość agregatów tworzonych w roztworze przez flawanole i ich kompleksy z jonami Fe^{2+} techniką dynamicznego rozpraszania światła[3]. Uzyskane wyniki wskazują na tworzenie przez badane związki w roztworze wodnym struktur o średnicy hydrodynamicznej w granicach (zależnie od badanego związku) 241-430 nm; średnica tych agregatów zwiększała się w wyniku interakcji z jonami Fe^{2+}. Tworzenie przez flawanole i ich kompleksy z jonami żelazawymi wielocząsteczkowych agregatów może być przyczyną nieoczekiwanej stechiometrii wiązania jonów Fe^{2+} przez te związki.

Dla uniknięcia artefaktów związanych z obecnością nieskompleksowanych jonów żelaza w sytuacji zróżnicowanej stechiometrii wiązania żelaza przez poszczególne flawanole, badano kompleksy tych związków z jonami Fe^{2+} tworzone w stosunku molowym 4:1 (w niektórych doświadczeniach, dla porównania, także w stosunku molowym 2:1 i 1:1).

W porównaniu z wyjściowymi flawanolami, kompleksy flawanoli z jonami Fe^{2+} wykazywały niezmienioną zdolność zmiatania rodnika $ABTS^{\bullet}$ (epigallokatechina, galusan epikatechiny),

zmniejszoną zdolność zmiatania $ABTS^{\bullet}$ (katechina, galusan epigallokatechiny) lub lekko zwiększoną zdolność zmiatania $ABTS^{\bullet}$ (epikatechina). Porównanie odpowiednich wartości IC_{50} wskazuje, że wiązanie jonów Fe^{2+} nie wpływało znacząco na zdolność flawanoli do ochrony fluoresceiny przed utlenianiem prze AAPH i NaOCl (katechina, epikatechina, epigallokatechina), zmniejszało tę zdolność (galusan epikatechiny, galusan epigallokatechiny – kompleks 1:1, utlenianie fluoresceiny przez NaOCl) bądź też ją zwiększało (galusan epigallokatechiny). Badano także ochronę lipidów jaja kurzego przez flawanole i ich kompleksy z jonami Fe^{2+} przed peroksydacją indukowaną przez AAPH i nadmiar jonów Fe^{2+}. W większości przypadków związanie nie wpływało w znaczący sposób na zdolność flawanoli do przeciwdziałania peroksydacji lipidów z wyjątkiem epigallokatechiny (zwiększenie zdolności przeciwdziałania peroksydacji indukowanej przez AAPH), galusanu epikatechiny (zmniejszenie zdolności przeciwdziałania peroksydacji indukowanej przez AAPH) oraz galusanu epigallokatechiny (zmniejszenie zdolności przeciwdziałania peroksydacji indukowanej przez NaOCl) (**Publikacja 2**, Tabela 2).

Badanie hamowania przez flawanole hemolizy erytrocytów indukowanej przez NaOCl nie wykazało zdecydowanego wpływu wiązania Fe^{2+} na tę aktywność. Kompleks katechina-Fe^{2+} w stężeniu 5 µM chronił skuteczniej przed hemolizą niż 5 µM katechina, kompleks epikatechina- Fe^{2+} w stężeniu 2,5 µM chronił gorzej niż sama epikatechina w tym stężeniu, 1 µM kompleks galusanu epigallokatechiny (utworzony w stosunku 4:1) był bardziej skuteczny niż 1 µM galusan epigallokatechiny, ale 5-15 µM kompleks galusanu epigallokatechiny z Fe^{2+} utworzony w stosunku molowym 1:1 chronił przed hemolizą gorzej niż sam galusan epigallokatechiny w tych stężeniach. (**Publikacja 2**, Ryc. 3).

Ogólnie można więc stwierdzić, że wiązanie jonów Fe^{2+} przez flawanole w stosunku molowym 4:1 nie prowadzi w znakomitej większości przypadków do obniżenia ich właściwości antyoksydacyjnych.

Ze względu na doniesienia literaturowe o wykazywaniu przez kompleksy flawonoidów pseudoenzymatycznej aktywności dysmutazy ponadtlenkowej [25,26] sprawdzono, czy aktywność taką wykazują badane przeze mnie kompleksy. Badanie hamowania przez flawanole i ich kompleksy z Fe^{2+} w różnych układach reakcyjnych, w których anionorodnik ponadtlenkowy był mediatorem tworzenia produktu (redukcja NBT zależna od metosiarczanu fenazyny – Publikacja 2, Tabela 3, fotochemiczna redukcja NBT – Publikacja 2, Ryc. S2, utlenianie pirogalolu, utlenianie adrenaliny – **Publikacja 2**, Ryc. S3) nie wykazało systematycznego podwyższenia zdolności do hamowania tych reakcji przez kompleksy flawanole-Fe^{2+} oraz kompleksy flawanole-Fe^{3+} i flawanole-Cu^{2+} (te ostatnie kompleksy stosowane były, by odtworzyć układy doświadczalne stosowane przez innych Autorów) w porównaniu z wyjściowymi flawanolami. W przypadku kompleksów galusanu epigallokatechiny z Fe^{3+}

obserwowano nawet działanie prooksydacyjne w reakcji utleniania adrenaliny (zwiększenie szybkości utleniania) (**Publikacja 2**, Ryc. S3).

Nie stwierdziłam także, by kompleksy flawanoli z Fe^{2+} posiadały zdolność rozkładu nadtlenku wodoru.

[1]Wyniki uzyskane we współpracy z dr Dorotą Naróg na Wydziale Chemii Politechniki Rzeszowskiej im. I. Łukasiewicza.
[2]Wyniki uzyskane we współpracy z dr Andrzejem Dziedzicem w Katedrze Fizyki Doświadczalnej Uniwersytetu Rzeszowskiego.
[3]Wyniki uzyskane we współpracy z dr hab. Ivanem Ionov'em w Katedrze Biochemii Ogólnej Uniwersytetu Łódzkiego.

Wytwarzanie nadtlenku wodoru przez antyoksydanty

Stwierdziłam, że 27 spośród 54 przebadanych naturalnych i syntetycznych antyoksydantów wytwarza podczas inkubacji z pożywką stosowaną do hodowli komórek ssaków *in vitro* (DMEM) produkt dający pozytywną reakcję z odczynnikiem zawierającym oranż ksylenolowy, stosowanym do oznaczania nadtlenków [20, 21]. Reakcja ta nie zachodziła po inkubacji próbek z katalazą, co dowodzi, że wytwarzaną substancją był nadtlenek wodoru. Kinetyka wytwarzania nadtlenku wodoru różniła się dla różnych substancji (**Publikacja 3**, Ryc. 1), zdecydowałam się więc na oznaczanie stężenia wytworzonego nadtlenku wodoru po 3-godzinnej inkubacji w temperaturze 37^{o}C. Najwięcej nadtlenku wodoru wytwarzały: galusan propylu, pirogalol (który nie jest stosowany jako antyoksydant, ale został użyty w celach porównawczych), galusan epigallokatechiny i kwercetyna. Nadtlenek wodoru wytwarzany był także wskutek utleniania m. in. askorbinianu, rezweratrolu i etoksychiny (**Publikacja 3**, Tabela 1).

Nadtlenek wodoru wytwarzany był także w innych pożywkach stosowanych do hodowli komórek ssaków i pożywkach używanych do hodowli drożdży (choć w tych ostatnich pożywkach w dużo mniejszych ilościach). Inkubacja w PBS trzech antyoksydantów wytwarzających najwięcej nadtlenku wodoru w DMEM skutkowała wytwarzaniem porównywalnych z inkubacją w DMEM ilości nadtlenku wodoru (**Publikacja 3**, Tabela 2).

Choć askorbinian wytwarzał nadtlenek wodoru w pH zbliżonym do obojętnego, inkubacja polifenoli w obecności askorbinianu prowadziła do zależnego od stężenia aksorbinianu zmniejszenia ilości wytwarzanego nadtlenku wodoru (**Publikacja 3**, Ryc. 2). Nadtlenek wodoru wytwarzany był także w herbacie, która cechuje się dużą zawartością flawanoli i innych polifenoli. Dodanie plasterka cytryny do herbaty znacznie zmniejszało wytwarzanie nadtlenku wodoru (**Publikacja 3**, Ryc. 3).

Wytwarzanie nadtlenku wodoru przez polifenole zachodzi najprawdopodobniej w wyniku dysmutacji anionorodnika ponadtlenkowego wytwarzanego w dwu reakcjach: (i) jednoelektronowego utleniania polifenolu do rodnika semichinonowego i (ii) jednoelektronowego utleniania rodnika semichinonowego. Za słusznością tego mechanizmu przemawia detekcja

tworzenia rodnika semichinonowego podczas utleniania galusanu propylu i galusanu epigallokatechiny metodą elektronowego rezonansu paramagnetycznego (EPR)[4] (**Publikacja 3**, Ryc. S1) oraz wykrycie powstawania rodnika ponadtlenkowego towarzyszącego utlenianiu galusanu propylu i galusanu epigallokatechiny. Powstawanie rodnika wykrywane było poprzez hamowaną przez dysmutazę ponadtlenkową redukcję NBT i hamowane przez dysmutazę ponadtlenkową utlenianie dihydroetydyny (**Publikacja 3**, Ryc. S2).

Utlenianie antyoksydantów wydaje się być katalizowane przez jony metali przejściowych, zwłaszcza żelaza, o czym świadczy obniżenie ilości wytwarzanego nadtlenku wodoru po poddaniu PBS stosowanego do reakcji działaniu złoża Chelex X-100 (wyłapującego jony żelaza) oraz po dodaniu do środowiska reakcji chelatorów żelaza – desferrioksaminy i DETAPA (**Publikacja 3**, Tabela S2), a także wytwarzanie większych ilości nadtlenku wodoru przez herbatę przygotowaną na wodzie kranowej, zawierającą jony żelaza, w porównaniu z wodą destylowaną (**Publikacja 3**, Ryc. 3).

Dla uzyskania informacji o czynnikach wpływających na szybkość utleniania antyoksydantów w różnych środowiskach, porównywano całkowitą zdolność antyoksydacyjną różnych pożywek, ich zdolność do wiązania jonów Fe^{2+}, zdolność do zmiatania anionorodnika ponadtlenkowego oraz zdolność do zmiatania nadtlenku wodoru. Żaden z wymienionych czynników nie wydawał się mieć decydującego wpływu na ilość wytwarzanego nadtlenku wodoru (**Publikacja 3**, Tabela S3). Czynnikami determinującymi ilość wytwarzanego przez dany związek nadtlenku wodoru w różnych środowiskach są, jak się wydaje, stężenie śladowych ilości jonów żelaza (lub innych metali przejściowych) i pH roztworu (kwaśne pH hamuje utlenianie antyoksydantów).

Wykazano, że nadtlenek wodoru wytwarzany w pożywce może przyczyniać się do cytotoksycznych efektów galusanu propylu, galusanu epigallokatechiny i kwercetyny, gdyż dodanie katalazy do pożywki zmniejszało cytotoksyczne efekty tych związków[5] (**Publikacja 3**, Ryc. 4).

[4]Wyniki uzyskane we współpracy z dr Ireneuszem Stefaniukiem w Centrum Dydaktyczno-Naukowym Mikroelektroniki i Nanotechnologii Uniwersytetu Rzeszowskiego.

[5]Wyniki uzyskane we współpracy z mgr Moniką Pichlą w Zakładzie Biochemii Analitycznej Uniwersytetu Rzeszowskiego.

DYSKUSJA

Wyniki moich badań potwierdzają, że flawonoidy, a w szczególności flawanole są świetnymi antyoksydantami. Należy jednak zwrócić uwagę, że porównanie „mocy" antyoksydantów zależy od rozważanego parametru. W Wikipedii można znaleźć stwierdzenie, że galusan epigallokatechiny „jest antyoksydantem 100 razy silniejszym niż witamina C i 25 razy silniejszym niż witamina E" [27]. Takie stwierdzenie jest nieuprawnione, jeśli nie jest odniesione do konkretnego parametru, na podstawie którego jest ono dokonywane. Uzyskane przeze mnie wyniki wskazują np. (**Publikacja 1**), że galusan

epigallocatechiny jest 5,48 razy lepszym antyoksydantem niż glutation, jeśli porównamy zmiatanie rodnika $ABTS^{\bullet}$ przez oba związki, 1,11 razy lepszym antyoksydantem od askorbinianu, jeśli porównamy zdolność tych związków do redukcji jonów Fe^{3+}, 12,11 razy lepszym antyoksydantem niż askorbinian, jeśli porównamy wartości IC_{50} dla hamowania reakcji utleniania dihydrorodaminy 123 przez SIN-1, a 5,50 razy lepszym antyoksydantem niż askorbinian, jeśli porównamy wartości IC_{50} dla hamowania utleniania fluoresceiny przez AAPH.

Porównania te dotyczą jednak zachowania antyoksydantów w prostych układach bezkomórkowych. Rozpatrując układy komórkowe trzeba zwrócić uwagę na szereg innych aspektów, głównie zdolność związku do penetracji błony i jego lokalizację w komórce. Rozważając znaczenie antyoksydanta na poziomie organizmu, należy wziąć pod uwagę jego biodostępność i metabolizm. Te aspekty nie są atutami flawonoidów. Biodostępność flawonoidów jest niska. W badaniu klinicznym, po spożyciu sproszkowanej cebuli, maksymalne stężenie kwercetyny w osoczu krwi wynosiło 273 ng/ml czyli ok. 0,9 µM, a po spożyciu bardziej niż kwercetyna biodostępnego 4'-O-glukozydu kwercetyny osiągało 2,1 µg/ml czyli ok. 7 µM [28]. Biodostępność flawonoidów choć może być zwiększona przez podawanie tych związków zamkniętych w liposomach czy nanocząstkach, jednak wysokie stężenia flawonoidów mogą wykazywać niekorzystne efekty biologiczne niezwiązane z aktywnością antyoksydacyjną. Po wchłonięciu flawonoidy szybko ulegają metylacji i O-metyloglukuronidacji [29] i te metabolity winny być przedmiotem dalszych badań.

Trudno oczekiwać, by flawonoidy osiągające w organizmie stężenia submikromolarne mogły mieć istotny wkład w całkowitą aktywność antyoksydacyjną komórek i płynów pozakomórkowych, gdyż endogenne antyoksydanty są tam obecne w stężeniach wyższych o co najmniej trzy rzędy wielkości. Nie można jednak wykluczyć silniejszego lokalnego działania antyoksydacyjnego tych związków w miejscu ich akumulacji. Istotne działanie antyoksydacyjne mogą wykazywać flawanole i inne flawonoidy w przewodzie pokarmowym, gdzie ich stężenia są stosunkowo duże, a inne antyoksydanty ulegają preferencyjnemu wchłanianiu.

W pożywieniu, a zatem i w świetle jelita, ale także w innych środowiskach możliwy jest kontakt flawanoli z jonami metali ziem przejściowych, zwłaszcza Fe^{2+}. Pojawia się więc problem, jak wiązanie jonów wpływa na antyoksydacyjne właściwości flawanoli. Wyniki uzyskane przeze mnie świadczą jednoznacznie, że wiązanie substechiometrycznych ilości Fe^{2+} nie upośledza w znaczącym stopniu antyoksydacyjnych właściwości flawanoli, można więc oczekiwać, że flawanole zachowują swoje właściwości antyoksydacyjne w świetle przewodu pokarmowego mimo możliwego uwalniania żelaza z trawionych produktów. Ta ich właściwość może przyczyniać się do przeciwdziałania powstawaniu raka jelita grubego przez pokarmy bogate we flawonoidy [30]. Wiązanie Fe^{2+} nie prowadzi do nabycia przez flawanole pseudoenzymatycznych właściwości dysmutazy ponadtlenkowej ani katalazy.

Ważnym elementem charakterystyki antyoksydantów jest określenie możliwości wytwarzania przez nie nadtlenku wodoru. Ten efekt jest przyczyną artefaktów w doświadczeniach na komórkach w hodowli, gdzie utleniacz wytwarzany wskutek utleniania antyoksydanta interferuje z efektem antyoksydacyjnym. Stwierdzono, że polifenole, tiole i askorbinian wytwarzają nadtlenek wodoru w pożywkach stosowanych do hodowli komórek ssaków *in vitro* [31,32], w piśmiennictwie brak jednak porównania generacji nadtlenku wodoru przez tak dużą liczbę substancji, jak w moich badaniach. W dalszej pracy zamierzam porównać wytwarzanie nadtlenku wodoru przez wybrane antyoksydanty w warunkach normoksji i w warunkach hipoksji (odpowiadającym warunkom *in vivo*), bowiem można przypuszczać, że obserwowana w dotychczasowych doświadczeniach generacja nadtlenku wodoru jest z fizjologicznego punktu widzenia artefaktem warunków znacznie odbiegających od tych, jakie panują w tkankach czy nowotworach *in vivo*.

Zwróciłam uwagę, że pożywki stosowane do hodowli komórek mają zdolność do rozkładu nadtlenku wodoru, więc mierzona szybkość wytwarzania nadtlenku wodoru przez dodane substancje jest zaniżona.

Nadtlenek wodoru wytwarzany jest wskutek utleniania polifenoli, głównie flawanoli, w tak popularnych napojach jak herbata, a także kawa [33]. Nie sądzę, by odkrycie tego efektu winno nas zniechęcić do spożywania tych napojów. Niskie stężenia nadtlenku wodoru (< 1 mM) mogą mieć łagodne działanie odkażające w przewodzie pokarmowym i nie wydaje się, by miały szkodliwy wpływ na zdrowie. Jeśli jednak nie chcemy spożywać nadtlenku wodoru w herbacie lub zminimalizować jego spożycie, wystarczy dodać do herbaty plasterek cytryny, jak najszybciej po zaparzeniu, by łączne działanie askorbinianu i obniżonego pH drastycznie obniżyło wytwarzanie w herbacie nadtlenku wodoru.

WNIOSKI

1. Flawanole są świetnymi antyoksydantami, wykazującymi w wielu reakcjach aktywność antyoksydacyjną znacznie wyższą niż standardowe antyoksydanty (glutation i askorbinian).
2. Wiązanie substechiometrycznych ilości Fe^{2+} przez flawanole nie obniża w istotnym stopniu ich właściwości antyoksydacyjnych.
3. Kompleksy flawanoli z jonami Fe^{2+} nie wykazują pseudoenzymatycznych aktywności dysmutazy ponadtlenkowej i katalazy.
4. Połowa spośród przebadanych 54 antyoksydantów wytwarza nadtlenek wodoru w pożywkach stosowanych do hodowli komórek i w zbuforowanym roztworze soli fizjologicznej.
5. Ilość nadtlenku wodoru wytworzona przez antyoksydant w roztworze zależy od stężenia śladowych ilości jonów metali przejściowych i od pH roztworu.
6. Wytwarzanie nadtlenku wodoru ma wkład w cytotoksyczność galusanu propylu, galusanu epigallokatechiny i kwercetyny wobec komórek w hodowli *in vitro*.

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OŚWIADCZENIA WSPÓŁAUTORÓW

Rzeszów, dnia 04.12.2018

mgr inż. Michalina Grzesik

Wydział Biologiczno-Rolniczy

Promotor: dr hab. Izabela Sadowska-Bartosz, Prof. nadzw. UR

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PUBLIKACJE Z SUPLEMENTAMI

Food Chemistry 241 (2018) 480–492

Contents lists available at ScienceDirect

Food Chemistry

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

Antioxidant properties of catechins: Comparison with other antioxidants

Michalina Grzesik[a], Katarzyna Naparło[a], Grzegorz Bartosz[b], Izabela Sadowska-Bartosz[a,*]

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ARTICLE INFO

Keywords:
Catechins
Flavonoids
FRAP
Hemolysis

ABSTRACT

Antioxidant properties of five catechins and five other flavonoids were compared with several other natural and synthetic compounds and related to glutathione and ascorbate as key endogenous antioxidants in several *in vitro* tests and assays involving erythrocytes. Catechins showed the highest ABTS$^{\cdot}$-scavenging capacity, the highest stoichiometry of Fe^{3+} reduction in the FRAP assay and belonged to the most efficient compounds in protection against SIN-1 induced oxidation of dihydrorhodamine 123, AAPH-induced fluorescein bleaching and hypochlorite-induced fluorescein bleaching. Glutathione and ascorbate were less effective. (+)-catechin and (−)-epicatechin were the most effective compounds in protection against AAPH-induced erythrocyte hemolysis while (−)-epicatechin gallate, (−)-epigallocatechin gallate and (−)-epigallocatechin protected at lowest concentrations against hypochlorite-induced hemolysis. Catechins [(−)-epigallocatechin gallate and (−)-epicatechin gallate)] were most efficient in the inhibition of AAPH-induced oxidation of 2′7′-dichlorodihydrofluorescein contained inside erythrocytes. Excellent antioxidant properties of catechins and other flavonoids make them ideal candidates for nanoformulations to be used in antioxidant therapy.

1. Introduction

Plant antioxidants are generally recognized as synonyms of nutriceuticals, at least among consumers and producers. Chemical compounds displaying reductive properties have been long used in food industry to protect foods against oxidation; however, the current interest stems from their ability to combat oxidative stress (OS) in the human organism.

Oxidative stress is an imbalance between antioxidant defence system and the production of reactive oxygen species (ROS). The collective term “reactive oxygen species” includes both free radicals [molecules having an odd electron, like superoxide radical anion ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$)] and species that are not free radicals, like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3). Reactive nitrogen species (RNS) include, i.a., nitric oxide ($^{\cdot}NO$) and peroxynitrite ($ONOO^-$) formed in a reaction of $^{\cdot}NO$ with $O_2^{\cdot -}$. Reactive halogen species (RXS) include such species as HOCl, HOBr, HOJ, chlorine, bromine, iodine etc. Hypohalogenous acids are formed in the body mainly by oxidation of halogen ions by myeloperoxidase. The imbalance between the production of ROS, RNS or RXS, and the antioxidant defense, in favor of prooxidants, is called oxidative, nitr(os) ative and halogenative stress, respectively. Although at physiological concentrations ROS, RNS and RXS can function as signaling molecules regulating cell proliferation, growth, differentiation and apoptosis (Barbieri & Sestili, 2012; Bartosz, 2009), they react with and damage all classes of endogenous macromolecules including proteins, nucleic acids, lipids and carbohydrates (Sadowska-Bartosz & Bartosz, 2015). OS has a devastating effect causing cell death and tissue damage and is commonly observed in several conditions such as cardiovascular diseases, diabetes, neuronal disorders, and in aging (Kandikattu et al., 2015; Treml & Šmejkal, 2016). Recently, there has been an increase in interest in natural substances with antioxidant properties, which reduce or prevent negative effects of OS on living tissues, and inhibit aging processes and the development of many diseases, especially polyphenols (Stolarzewicz, Ciekot, Fabiszewska, & Białecka-Florjańczyk, 2013).

Polyphenols or phenolic compounds are one of the most important groups of secondary metabolites of plants. They are widely distributed in the plant kingdom (Losada-Barreiro & Bravo-Díaz, 2017). Dietary polyphenols comprise a wide range of aromatic compounds that are responsible for organoleptic characteristics of plant-derived food and beverages. The polyphenols that are present in foods can be divided into two main groups: non-flavonoids and flavonoids. Non-flavonoids are mostly monocyclic acids and can be further divided into two main sub-classes: phenolic acids and stilbenes (e.g. resveratrol). Phenolic acids are subdivided into benzoic acids and hydroxycinnamic acids. Flavonoids share a common nucleus consisting of two phenolic rings and an oxygenated heterocycle. They form a diverse range of

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http://dx.doi.org/10.1016/j.foodchem.2017.08.117
Received 27 June 2017; Received in revised form 30 August 2017; Accepted 31 August 2017
Available online 01 September 2017
0308-8146/

compounds and can be categorized into many classes, such as anthocyanins, flavonols (e.g. morin), flavanols (e.g. catechins), flavones, and chalcones. Catechins are present in many dietary products, plants, fruits (such as apples, blueberries, gooseberries, grape seeds, kiwi, strawberries), green and black tea, red wine, beer, cacao liquor, chocolate and cocoa. The main catechins present in the green tea include (−)-epigallocatechin gallate, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate and (+)-catechin (Zeeb, Nelson, Albert, & Dalluge, 2000). One gram of dried green tea leaves contains more than 200 mg catechins (Yen & Chen, 1996), although total catechin content varies widely depending on species, variety, growing location, season, illumination, and altitude. The best studied catechin is (−)-epigallocatechin gallate, the major polyphenol in green tea (Fujiki, 2005). The antioxidant properties of polyphenols are mostly due to their redox properties, which let them act as reducing agents, hydrogen donors and singlet oxygen quenchers (Lima, Vianello, Corrêa, da Silva Campos, & Borguini, 2014). In contrast to glutathione (GSH), polyphenols cannot be synthesized by humans, but are obtained through the diet; so, free radicals that are originated during body metabolism can be better neutralized by regular intake of foods containing a high content of fruits and vegetables (Milella et al., 2011).

Glutathione is present in the cytoplasm in millimolar concentrations. Reduced glutathione is a linear tripeptide of L-glutamic acid, L-cysteine, and glycine, which plays a key role in the cellular antioxidant system, and is the main determinant of the intracellular redox state (Schafer & Buettner, 2001).

The purpose of this study was to compare the antioxidant properties of catechins with those of other natural and synthetic antioxidants (Table 1) including main intracellular antioxidants (GSH and ascorbic acid). The antioxidant activities were estimated by commonly-used methods of scavenging of a model radical, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\cdot}$), and ferric reducing antioxidant power (FRAP) assays. We also checked the ability of the compounds studied to protect against dihydrorhodamine 123 (DHR123) oxidation by 3-morpholinosydnonimine (SIN-1) and against fluorescein bleaching by hypochlorite and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), a model source of peroxyl radicals ($ROO^{\cdot}$), in cell-free systems. Moreover, we investigated the anti-hemolytic activity of the selected antioxidant against hypochlorite or AAPH-induced OS of human erythrocytes and inhibition of oxidation of intracellular fluorogenic probe 2′,7′-dichlorofluorescein (H_2DCF-DA) by AAPH. This study is a part of a larger project aimed at comprehensive comparison of beneficial and adverse effects of natural and synthetic antioxidants.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO; purity: ≥99.9% Sterile Filtered) produced by BioShop Canada Inc. (Burlington, Ontario, Canada) was purchased from Lab Empire (Rzeszów, Poland).

3-Morpholinosydnonimine (SIN-1) was obtained from Tocris Bioscience (Bristol, United Kingdom). 3-Morpholinosydnonimine stock solutions (1 mM) were prepared in phosphate-buffered saline (PBS: 145 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4), and aliquots were frozen immediately at −80 °C until use. Under these conditions, SIN-1 was stable for several months, as assessed by HPLC analysis. 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Polysciences (Warrington, PA, USA). A stock solution of AAPH was freshly prepared in PBS before each experiment.

Dihydrorhodamine 123, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and rutin trihydrate were purchased from Thermo Fisher Scientific (Warsaw, Poland). Fluorescein and sodium hypochlorite (NaOCl, 15% active chlorine basis) were obtained from CHEMPUR (Piekary Slaskie, Poland). A stock solution of NaOCl was diluted in 0.1 M NaOH and its concentration was determined spectrophotometrically at 290 nm using the molar absorption coefficient of $\varepsilon_{290\ nm} = 350\ M^{-1}\ cm^{-1}$ (Morris, 1966). Under such conditions NaOCl exists exclusively as OCl^-. A stock solution of NaOCl was diluted in PBS, before use. At pH 7.4 the both forms, HOCl and OCl^- are present in the solution at comparable concentrations.

Selected compounds such as (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate were obtained from Extrasynthese (Genay, France). Curcumin and hydrocinnamic acid were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Glutathione was obtained from Fluka Chemica-Biochemika (Buchs, Switzerland). For every assay, experiments concerning the effect of solvent alone were always performed.

All other reagents, if not mentioned otherwise, were purchased from Sigma (Poznan, Poland) and were of analytical grade. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Fluorometric and absorptiometric measurements were done in a Tecan Infinite 200 PRO multimode reader (Tecan Group Ltd., Männedorf, Switzerland). All measurements were performed in triplicate and repeated at least three times. Selected compounds were dissolved in PBS, DMSO or ethanol (in studies of the effects of NaOCl). Minimal amounts of the solvents present in the samples had a small effect on the protection (up to several %). The effect of DMSO or ethanol was subtracted from the effects of substances introduced in this solvent. In cell-free systems, GSH and ascorbate served as reference antioxidants.

2.2. Experiments in cell-free systems

2.2.1. Antiradical activity of selected exogenic and endogenic compounds

2.2.1.1. $ABTS^{\cdot}$ assay. The ability of selected compounds to scavenge the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{\cdot}$) was measured according to a procedure previously proposed by us (Sadowska-Bartosz, Gajewska, Skolimowski, Szewczyk, & Bartosz, 2015), modified for application in a microplate. Briefly, appropriate amounts of compounds were added to a solution of $ABTS^{\cdot}$, diluted so that 200 µl of the solution had absorbance of 1.0 in a microplate well, at 734 nm. The decrease in $ABTS^{\cdot}$ absorbance was measured after 1 min ("fast" scavenging) and between 10 and 30 min ("slow" scavenging) of incubation at ambient temperature (21 ± 1 °C). From the plots of the dependence of absorbance decrease (ΔA) on the compound concentration, the value of ΔA/mM was calculated for the compounds tested.

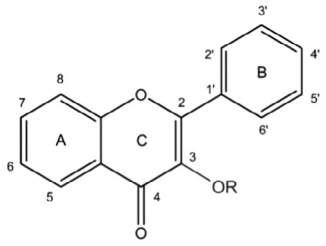
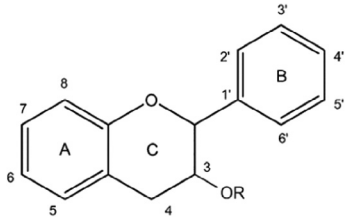
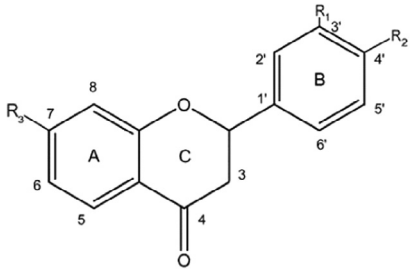
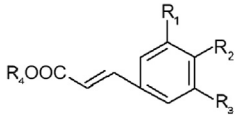
2.2.1.2. FRAP assay. The Ferric Reducing Antioxidant Potential (FRAP) was determined with 0.3 M acetate buffer (pH = 3.6), 0.01 M TPTZ (2,4,6-tripyridyl-*s*-triazine) in 0.04 M HCl and 0.02 M $FeCl_3 * 6H_2O$ mixed in a 10:1:1 vol ratio (Benzie & Strain, 1996). Absorbance was measured at the wavelength of 593 nm after 20-min incubation at room temperature.

In each case, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; CID: 40634) was used as control to obtain the standard curve and value was calculated with respect to the activity of Trolox and expressed as Trolox equivalents.

2.2.2. Protection against oxidation of dihydrorhodamine 123

Dihydrorhodamine 123 (DHR123) is a model substance that is easily oxidized by various oxidants, among them SIN-1 [a compound generating $ONOO^-$-anion of peroxynitrous acid (HONOO)], to the fluorescent rhodamine 123. SIN-1 is an active metabolite of the vasodilator drug 3-morpholinosydnonimine, which decomposes spontaneously in solution. In the presence of O_2, $^{\cdot}NO$ and $O_2^{\cdot -}$ are released, generating $ONOO^-$. Prevention of DHR123 oxidation by a compound is a measure of its property to react with a given oxidant. The lower is the concentration of a compound inhibiting DHR123 oxidation by 50%, the higher is the antioxidant potency of this compound with respect to a

Table 1
Classification and structure of flavonoids and model polyphenols used in this study.

Class of compounds	General structure	Compounds studied
Flavonols		Morin (3,5,7,2′,4′-OH) Rutin (5,7,3′,4′-OH, R = rutinose)
Flavanols (catechins)		(+)-Catechin (5,7,3′,4′-OH, R = H) (−)-Epicatechin (5,7,3′,4′-OH, R = H) (−)-Epicatechin gallate (5,7,3′,4′-OH, R = gallate) (−)-Epigallocatechin (5,7,3′,4′,5′-OH, R = H) (−)-Epigallocatechin gallate (5,7,3′,4′,5′-OH, R = gallate)
Flavanones		Hesperidin (5-OH, R_1 = OH, R_2 = OCH_3, R_3 = *O*-rutinoside) Naringenin (5-OH, R_1 = H, R_2 = R_3 = OH) Naringin (5-OH, R_1 = H, R_2 = OH, R_3 = *O*-neohesperidoside)
Hydroxycinnamic acids		Caffeic acid (R_1 = R_4 = H, R_2 = R_3 = OH) Chlorogenic acid (R_1 = H, R_2 = R_3 = OH, R_4 = quinic acid) *p*-Coumaric acid (R_1 = R_3 = R_4 = H, R_2 = OH) Ferulic acid (R_1 = R_4 = , R_2 = OH, R_3 = OCH_3) Hydrocinnamic acid (R_1,R_2,R_3, R_4 = H) Sinapic acid (R_1 = R3 = $OCH3$, R_2 = OH, R_4 = H)
Others		Ascorbic acid Butylated hydroxyanisole *tert*-Butylhydroquinone 2,6-di-*tert*-Butyl-1-4-methylphenol Carnosine Curcumin Deferoxamine Glutathione Mangiferin Propyl gallate Pyruvic acid Resveratrol Spermine Uric acid

given oxidant.

Dihydrorhodamine 123 (190 μl of 1 μM solution in 0.1 M phosphate buffer, pH 7.4) was added to each well of a 96-well plate containing the compounds studied in a range of concentrations (0.005–500 μM). The final volume of a sample was 200 μl. SIN-1 chloride (1 μl of 1 mM solution) was added to each well and kinetic measurement of fluorescence increase was carried using the excitation/emission wavelengths of 460/528 nm at 37 °C for 2 h. From the area under curve values of fluorescence, IC_{50} values were determined.

2.2.3. Protection of fluorescein against bleaching induced by NaOCl or AAPH

The fluorescence of fluorescein can be bleached by various oxidants, including hypochlorite or AAPH due to oxidation. Inhibition of fluorescein bleaching is thus a measure of a given compound to prevent reactions of oxidation by selected oxidants. AAPH is a water-soluble azo compound which is used extensively as a free radical generator. Decomposition of AAPH produces molecular nitrogen and two carbon radicals. The carbon radicals may combine to produce stable products or react with molecular oxygen to give $ROO^{\cdot}$. The half-life of AAPH is about 175 h (37 °C at neutral pH), making the rate of free radical generation essentially constant during the first several hours in solution.

Various amounts of hypochlorite were added to a well containing 0.2 μM fluorescein dissolved in PBS and the solution was mixed immediately. The amount of hypochlorite required to decrease fluorescence down to ca 5–10% of the initial value was determined (17.5 μM hypochlorite). These conditions were used for subsequent measurements, in which compounds dissolved in PBS or DMSO in a range of concentrations (usually 0.125–5 μM), were present in the fluorescein solution before addition of hypochlorite, keeping the volume of the sample constant (100 μl). Fluorescence was measured after 15 min incubation at room temperature at the excitation/emission wavelengths of 485 and 538 nm, respectively.

Table 2
Reducing activity of the antioxidants studied. Comparison with glutathione scavenging activity: [a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$ (Dunnett test).

Compound	PubChem CID	$ABTS^{\cdot}$ scavenging activity [mol TE/mol]	FRAP [mol TE/mol]
Standard antioxidants			
Glutathione	124886	1.027 ± 0.004	0.010 ± 0.000
Synthetic antioxidants			
Ascorbic acid	54670067	1.114 ± 0.017	1.982 ± 0.016[a]
Butylated hydroxyanisole	8456	1.080 ± 0.006	0.756 ± 0.003[a]
tert-Butylhydroquinone	16043	0.951 ± 0.003	0.933 ± 0.005[a]
2,6-di-*tert*-Butyl-1-4-methylphenol	31404	1.016 ± 0.004	0.010 ± 0.001
Propyl gallate	4947	1.881 ± 0.028[b]	1.122 ± 0.004[a]
Catechins			
(+)-Catechin	9064	3.965 ± 0.067[a]	0.793 ± 0.004[a]
(−)-Epicatechin	72276	2.800 ± 0.051[a]	0.917 ± 0.004[a]
(−)-Epigallocatechin	72277	2.939 ± 0.037[a]	1.032 ± 0.007[a]
(−)-Epicatechin gallate	107905	7.800 ± 0.037[a]	2.335 ± 0.006[a]
(−)-Epigallocatechin gallate	65064	5.632 ± 0.027[a]	2.211 ± 0.006[a]
Other flavonoids and derivatives			
Morin	16219651	2.497 ± 0.027[a]	1.169 ± 0.003[a]
Naringenin	932	1.507 ± 0.007	0.011 ± 0.002
Naringin	442428	1.086 ± 0.018	0.030 ± 0.009[c]
Rutin	16218542	2.074 ± 0.006[a]	1.156 ± 0.011[a]
Hesperidin	10621	0.849 ± 0.034	0.101 ± 0.001[a]
Hydroxycinnamic acids and derivatives			
p-Coumaric acid	637542	2.123 ± 0.006[a]	0.008 ± 0.002
Caffeic acid	689043	0.965 ± 0.015	1.018 ± 0.004[a]
Ferulic acid	445858	1.560 ± 0.041	0.687 ± 0.002[a]
Sinapic acid	637775	1.618 ± 0.004	1.230 ± 0.008[a]
Chlorogenic acid	1794427	0.926 ± 0.056	1.061 ± 0.012[a]
Hydrocinnamic acid	107	0.264 ± 0.016[b]	0.011 ± 0.004
Other natural antioxidants			
Curcumin	969516	1.685 ± 0.019[c]	0.709 ± 0.002[a]
Resveratrol	445154	2.738 ± 0.023[a]	0.619 ± 0.002[a]
Mangiferin	5281647	2.376 ± 0.030[a]	1.719 ± 0.022[a]

TE, trolox equivalents.

Different concentrations of AAPH were added to a well containing 0.2 μM fluorescein dissolved in PBS and the solution was mixed immediately. The concentration of AAPH required to decrease fluorescence down to ca 5–10% of the initial value after 60 min was determined (10 mM AAPH). These conditions were used for subsequent measurements, in which compounds dissolved in PBS or DMSO in a range of concentrations (usually 0.25–100 μM), were present in the fluorescein solution before addition of AAPH, keeping the volume of the sample constant (100 μl). Fluorescence was measured after 1 h incubation at 37 °C temperature at the excitation/emission wavelengths of 485 and 538 nm, respectively.

Percent of protection was calculated according to the formula and the concentration of a compound providing 50% protection (IC_{50}) against the fluorescein bleaching was calculated.

$$\%Protection = (F_n - F_0)/(F_c - F_0) \times 100\%$$

F_n – fluorescence of sample containing fluorescein, hypochlorite/AAPH and a compound studied; F_0 – fluorescence of fluorescein treated with hypochlorite/AAPH; F_c – fluorescence of non-treated fluorescein.

2.2.4. Iron chelating assay

To examine the strong iron chelating properties of the compounds studied, the deoxyribose test was performed as described previously by Sadowska-Bartosz, Galiniak, and Bartosz (2017).

2.2.5. Comparison with standard antioxidants

In order to evaluate the antioxidant power of compounds studied, standard antioxidants, glutathione and ascorbic acid, were assayed in parallel.

2.3. Erythrocytes hemolysis assays

2.3.1. Ethical approval

The study was approved by the Research Bioethics Committee of the University of Łódź (Poland).

2.3.2. Preparation of erythrocytes

Eight ml of peripheral blood from a healthy donor (lab volunteer, a 39-year-old woman) was collected in EDTA tubes and used within the day of its collection. Erythrocytes were isolated by centrifugation for 10 min at 3000 rpm, at 4 °C. The plasma and buffy coat were removed by aspiration. The red blood cells (RBCs) were washed four times with ice-cold PBS. Washed RBCs were suspended to a final hematocrit of 10% in PBS.

2.3.3. The assay of AAPH-induced hemolysis

The inhibition of free radical-induced RBCs hemolysis was performed by a modification of a previously published method (Wang, Sun, Cao, & Tian, 2009), in which hemolysis was monitored turbidimetrically. The erythrocyte hemolysis was induced by thermal decomposition of AAPH as an alkyl radical initiator. The protective effect of exogenic/endogenic compound against AAPH-induced hemolysis was measured only for compounds dissolved in PBS. The RBCs suspension was mixed with a selected endogenic or exogenic compound solution at the final concentration range 0.05–1 mM or 0.025–0.5 mM, respectively and incubated with shaking in the presence/absence of 75 mM AAPH, as optimal concentration to induce hemolysis at 37 °C. The turbidance (600 nm) was measured every 15 min for 10 h using an automated Bioscreen C turbidity reader [Oy Growth Curves Ab Ltd. (Helsinki, Finland)]. For all determinations, hemolysis time and percentage of hemolysis time with respect control erythrocytes were calculated 100% * [time (seconds) for test compound/mean time (seconds) for control sample containing RBCs and AAPH only].

2.3.4. The assay of hypochlorite-induced hemolysis

Aliquots of erythrocyte suspensions in PBS were mixed with a selected exogenic/endogenic compound solution at the final concentration range 5–25 μM (a final volume of 200 μl) and incubated 15 min with shaking at 37 °C. Then 0.15 mM hypochlorite (final), as optimal concentration to induce hemolysis, was added and turbidance (700 nm) was measured every 2 min for 240 min using a Tecan Infinite 200 PRO multimode reader. We prefer monitoring hemolysis at 700 nm whenever possible, as hemoglobin has some absorbance at 600 nm which may interfere with turbidance measurements especially when hemoglobin is oxidized. For all determinations, hemolysis time (seconds) and percentage of hemolysis time with respect control RBCs were calculated [100% * time (seconds) for test compound/mean time (seconds) for control sample containing RBCs and NaOCl only].

2.3.5. Determination of the intracellular ROS generation

2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (also known as 2′,7′-dichlorofluorescin diacetate) is a chemically reduced form of 2′7′-dichlorofluorescein diacetate used as an indicator for ROS in cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent H_2DCF-DA is converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Wang et al., 2017). The indicator H_2DCF-DA [dissolved in ethanol (0.4 mM), 10 μM final concentration] was added to the erythrocyte suspension (10% final hematocrit) with subsequent incubation at 37 °C for 30 min. The samples were centrifuged, and supernatant was discarded to remove excess H_2DCF-DA. Selected compounds were added in a range of

Table 3
Protection against dihydrorhodamine 123 oxidation by SIN-1 and against fluorescein bleaching by hypochlorite and AAPH (IC_{50} values). IC_{50} values compared to glutathione: [a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$ (Dunnett test).

Compound	Protection against DHR123 oxidation (IC_{50}, μM)	Protection against fluorescein bleaching by NaOCl (IC_{50}, μM)	Protection against fluorescein bleaching by AAPH (IC_{50}, μM)
Standard antioxidants			
Ascorbic acid	13.900 ± 1.012	> 5[a]	11.850 ± 0.001[a]
Glutathione	14.070 ± 0.359	3.519 ± 0.048	15.440 ± 0.009
Synthetic antioxidants			
Butylated hydroxyanisole	0.933 ± 0.055[a]	0.594 ± 0.080[a]	1.503 ± 0.090[a]
tert-Butylhydroquinone	0.456 ± 0.028[a]	0.486 ± 0.037[a]	2.093 ± 0.081[a]
2.6-di-*tert*-Butyl-1-4-methylphenol	105.924 ± 8.844[a]	1.743 ± 0.109[a]	83.350 ± 0.854[a]
Propyl gallate	0.435 ± 0.012[a]	0.503 ± 0.022[a]	2.923 ± 0.233[a]
Catechins			
(+)-Catechin	0.805 ± 0.072[a]	0.341 ± 0.002[a]	0.671 ± 0.037[a]
(−)-Epicatechin	1.359 ± 0.044[a]	0.511 ± 0.026[a]	1.076 ± 0.060[a]
(−)-Epicatechin gallate	1.207 ± 0.049[a]	0.246 ± 0.034[a]	0.658 ± 0.025[a]
(−)-Epigallocatechin	1.076 ± 0.039[a]	0.307 ± 0.011[a]	1.872 ± 0.074[a]
(−)-Epigallocatechin gallate	1.147 ± 0.002[a]	0.355 ± 0.007[a]	2.156 ± 0.170[a]
Other flavonoids and derivatives			
Hesperidin	4.988 ± 0.748[a]	0.332 ± 0.027[a]	0.918 ± 0.033[a]
Morin	0.183 ± 0.014[a]	0.280 ± 0.007[a]	0.598 ± 0.010[a]
Naringenin	4.910 ± 0.374[a]	0.693 ± 0.048[a]	0.483 ± 0.011[a]
Naringin	40.507 ± 22.813[a]	0.458 ± 0.037[a]	1.478 ± 0.120[a]
Rutin	0.324 ± 0.009[a]	0.338 ± 0.009[a]	0.966 ± 0.014[a]
Hydroxycinnamic acids and derivatives			
Caffeic acid	0.390 ± 0.020[a]	0.625 ± 0.123[a]	1.662 ± 0.021[a]
Chlorogenic acid	0.718 ± 0.166[a]	0.506 ± 0.017[a]	1.138 ± 0.024[a]
p-Coumaric acid	4.252 ± 0.199[a]	0.627 ± 0.014[a]	0.976 ± 0.014[a]
Ferulic acid	4.694 ± 0.294[a]	0.595 ± 0.020[a]	1.132 ± 0.067[a]
Hydrocinnamic acid	> 500[a]	2.781 ± 0.160[a]	282.703 ± 0.009[a]
Sinapic acid	0.359 ± 0.018[a]	0.614 ± 0.041[a]	1.254 ± 0.033[a]
Other natural antioxidants			
Curcumin	0.903 ± 0.086[a]	0.328 ± 0.045[a]	0.793 ± 0.038[a]
Deferoxamine**	5.403 ± 3.301[b]	2.462 ± 0.055[a]	2.183 ± 0.032[a]
Mangiferin	0.412 ± 0.017[a]	0.339 ± 0.022[a]	0.879 ± 0.036[a]
Resveratrol	0.295 ± 0.025[a]	0.346 ± 0.007[a]	0.353 ± 0.009[a]
Uric acid*	0.880 ± 0.100[a]	5.243 ± 0.155[a]	6.469 ± 0.194[a]

* CID:1175;
** CID:62881.

concentrations (25–200 μM for uric acid, hydroxycinnamic acid, spermine, deferoxamine and pyruvic acid, 0.10–25 μM for other selected substances). The samples were maintained at 37 °C in a dark environment in a shaking incubator for 20 min. AAPH (25 mM final concentration) was then added with good mixing. The fluorescence (485 nm/535 nm) was measured every 1 min for 30 min. The reaction percent and IC_{50} were calculated.

2.4. Statistical analysis

Statistical significance of differences was evaluated using the Dunnett's test or paired Student's "t" test. Statistical analysis of the data was performed using STATISTICA software package (version 12, StatSoft Inc. 2014, Tulsa, OK, USA, www.statsoft.com).

3. Results

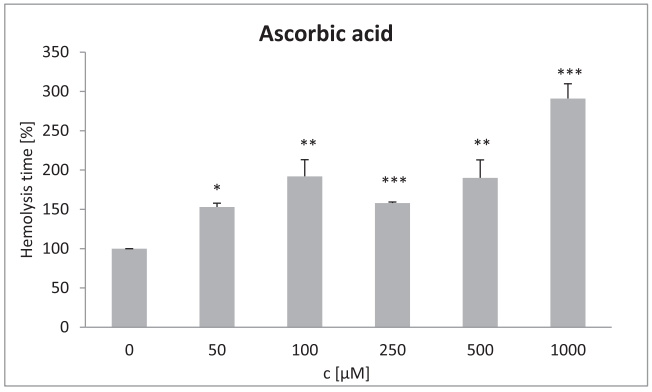
Free radical reducing capacity estimated by reduction of $ABTS^{\cdot}$ and Fe^{3+}-reducing capacity estimated by the FRAP assay of the compounds studies are compared in Table 2. From among the compounds studied, catechins showed the highest stoichiometry of $ABTS^{\cdot}$ reduction, (−)-epicatechin gallate being 7.8 times as efficient as Trolox, followed by (−)-epigallocatechin gallate (5.6 times), (+)-catechin (4 times), (−)-epigallocatechin (2.9 times) and epicatechin (2.8 times). Other antioxidants studied, including flavonoids and standard antioxidants, GSH and ascorbic acid, showed lower $ABTS^{\cdot}$-scavenging activity than catechins on the molar basis. The reactivity of both GSH and ascorbic acid was close to that of Trolox (about 1 mol Trolox equivalents/mol).

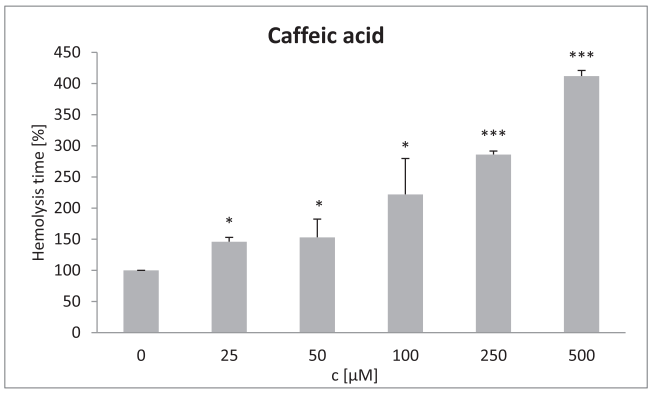
The reactivity of the antioxidants studied was generally lower in the FRAP assay than in the $ABTS^{\cdot}$ decolorization assay. However, some hydrocinnamic acids (caffeic acid and chlorogenic acid) showed comparable activity in both assays. The reactivity of catechins did not exceed 2.3 Trolox equivalents/mol and was much higher than that of GSH and comparable with that of ascorbic acid. In general, the correlation between the results of $ABTS^{\cdot}$ decolorization assay and FRAP assay within the groups of compounds studied was modest (0.615).

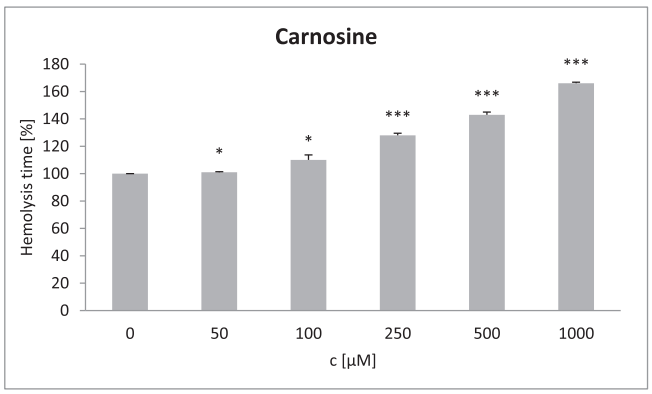
In view of many studies on the metal ion-binding properties of flavonoids, we checked catechins and other polyphenols studied for strong binding of ferrous ions using a recently developed assay (Sadowska-Bartosz et al., 2017). No compound studied showed the ability for strong ferrous iron binding except for deferoxamine (not shown).

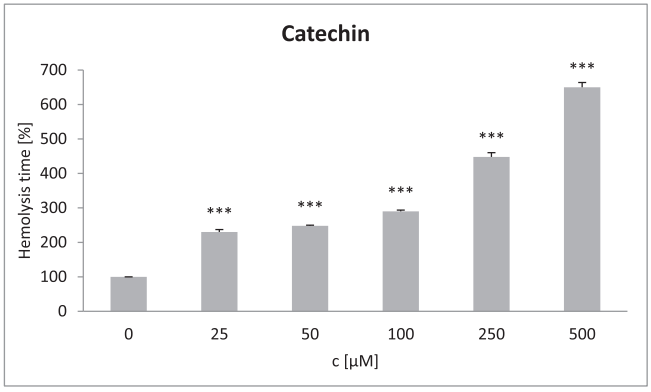
We compared the efficiency of selected antioxidants to react with other physiologically relevant oxidants such as $ONOO^-$, hypochlorite and $ROO^{\cdot}$. SIN-1 was used as a source of $ONOO^-$ and AAPH as a source of $ROO^{\cdot}$. Peroxyl radical formed via the reaction of carbon-centered radical with oxygen is a biologically relevant active species, because of its likelihood to damage cellular constituents. Furthermore, the pathological effects of $ROO^{\cdot}$ have received much attention in connection with the chain-propagation mechanism of lipid peroxidation.

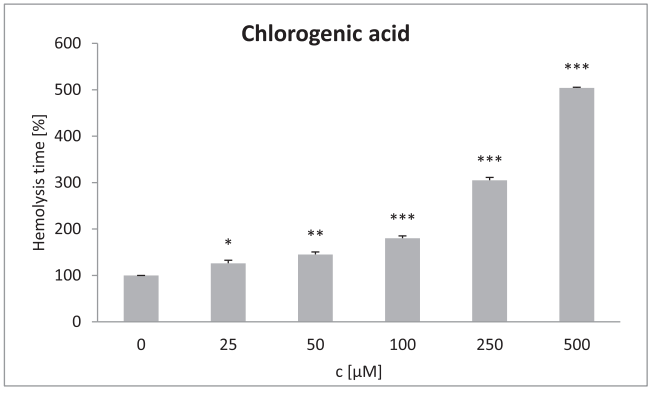
From plots of the dependence of DHR123 oxidation rate on the antioxidant concentration, IC_{50} values were derived. Morin was the most effective compound in protection against SIN-1 induced oxidation of DHR123, followed by rutin and hydroxycinnamic acids: sinapic acid











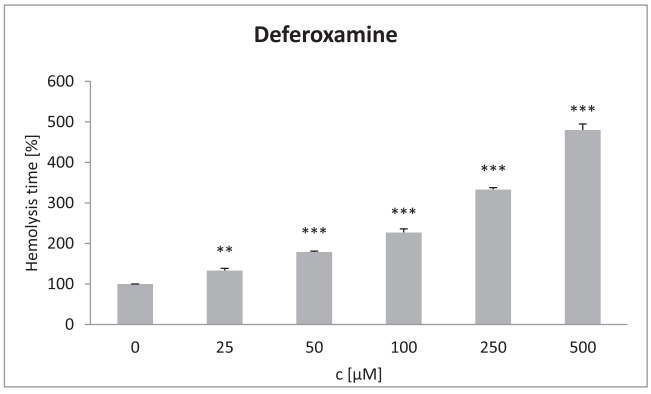


Fig. 1. Protection against AAPH-induced hemolysis by selected antioxidants.

Fig. 1. (*continued*)

and caffeic acid. Catechins showed reactivity much higher in comparison with GSH and ascorbic acid (IC_{50} values of about 1 μM, as compared with 14.1 and 13.9 μM, respectively). Interestingly, the reactivity of naringin was much lower in comparison with its aglycone naringenin (about 10-fold difference in IC_{50} values). This results point out to the important potential role of hydrolysis of flavonoid glycosides in the biological efficiency of these food components (Table 3).

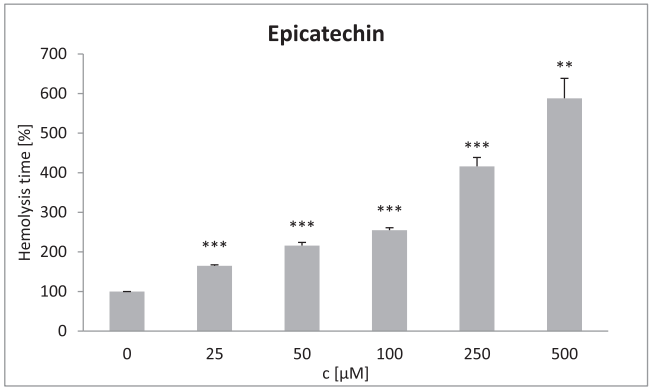
Resveratrol was the most effective in protection against AAPH-induced fluorescein bleaching, followed by naringenin and morin. Catechins, especially (+)-catechin and (−)-epicatechin gallate, showed also high reactivity, much higher than GSH and ascorbic acid. Also in this case, naringenin was more effective than naringin.

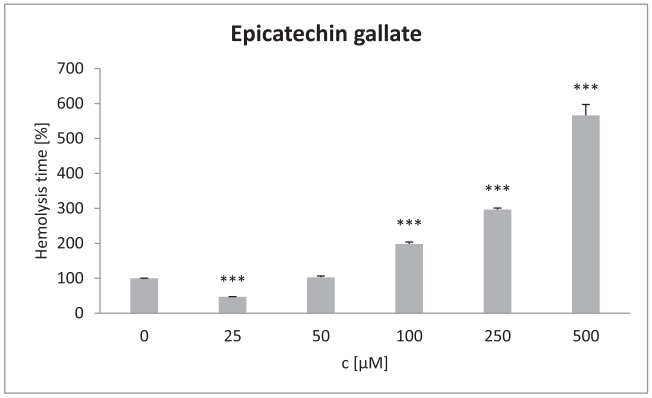
(−)-Epicatechin gallate was the most effective in protection against hypochlorite-induced fluorescein bleaching, followed by morin and (−)-epigallocatechin. Catechins, especially (+)-catechin and (−)-epicatechin gallate, showed also high reactivity, much higher than GSH and ascorbic acid; the list of other most effective compounds included flavonoids studied, especially morin, curcumin, resveratrol, as well as hydroxycinnamic acids, BHT and *tert*-butylhydroquinone. Glutathione and ascorbic acid were less effective (Table 3).

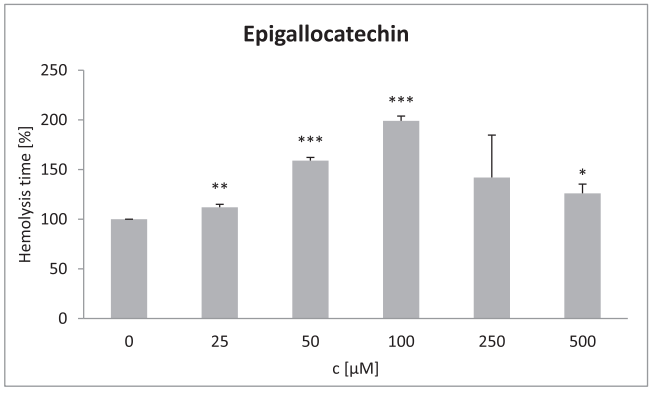
The cell-free assays are valuable for comparison of antioxidant reactivities with various oxidants, but they may not reflect the behavior of antioxidants in cellular systems where other factors such as membrane permeability for antioxidants and their location within the cell may considerably affect their effects. Using RBCs as model cells, we studied protection by antioxidants against hemolysis induced by hypochlorite and AAPH, and against intracellular ROS formation induced by AAPH. In these experiments, only water soluble antioxidants were used in order to avoid the cellular, mainly membrane effects of DMSO.

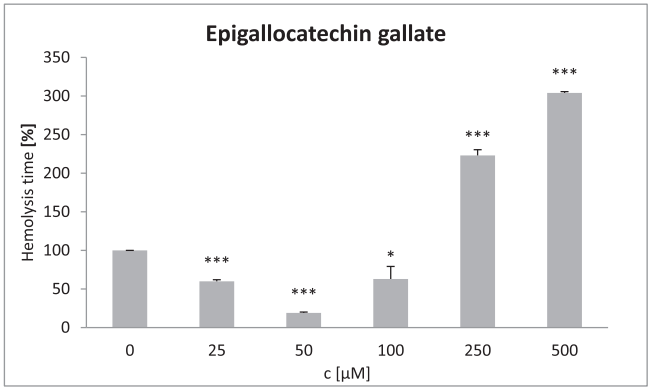
Protection by various antioxidants against hemolysis induced by AAPH is shown in Fig. 1. Catechins showed good protection. The lowest concentration of some catechins used [25 μM (+)-catechin, 50 μM (−)-epicatechin] prolonged the time of hemolysis by more than 100%. The same effect was achieved by 100 μM caffeic acid and deferoxamine, 250 μM (−)-epicatechin gallate, (−)-epigallocatechin gallate and chlorogenic acid, 1000 μM ascorbic acid and uric acid. Other antioxidants tested, including GSH, did not reach this level of protection.

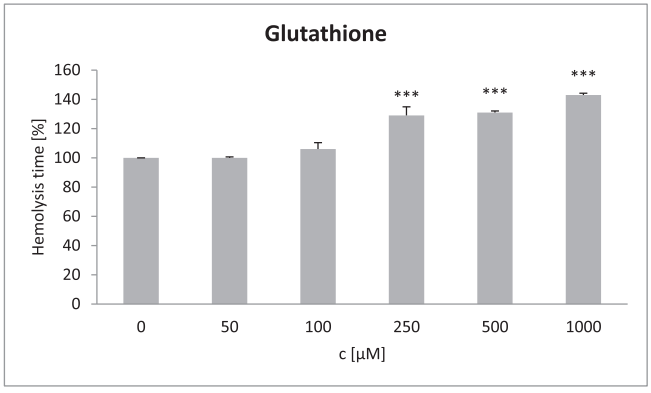
Protection against hemolysis induced by hypochlorite is presented in Fig. 2. Prolongation of the hemolysis time by more than 100% was achieved by 0.5 mM (−)-epicatechin, 1 μM (+)-catechin, 5 μM (−)-epigallocatechin and (−)-epicatechin gallate, 10 μM glutathione and epigallocatechin gallate, 15 μM spermine and 20 μM deferoxamine,











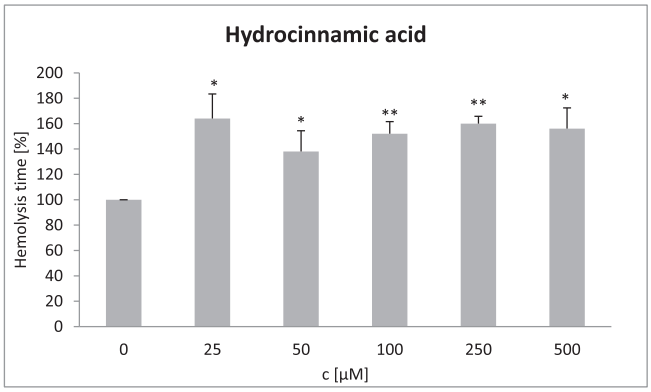


Fig. 1. (*continued*)

Fig. 1. (*continued*)

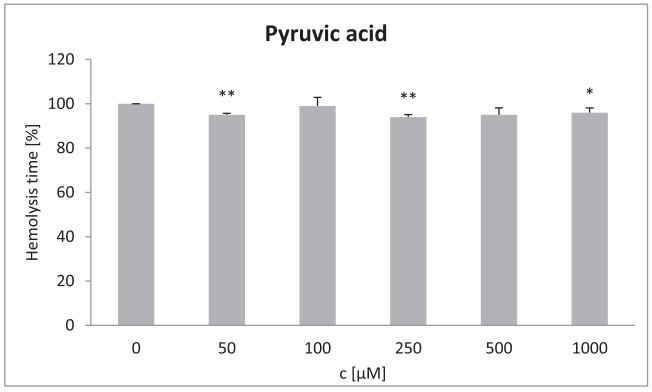
carnosine and uric acid.

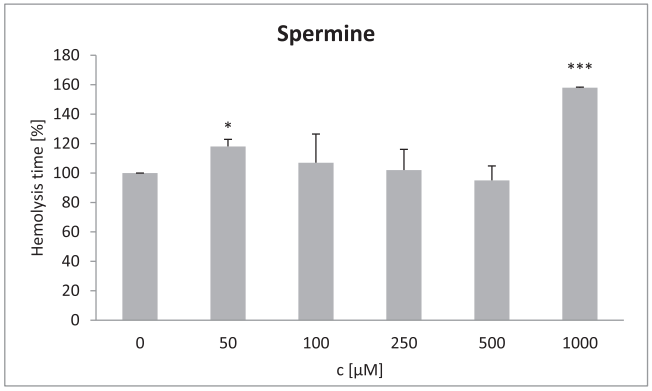
We tested the protective intracellular activity of antioxidants using a modification of the method proposed by Wang et al. (2017) in which RBCs are preloaded with H_2DCF-DA and challenged with AAPH in the absence and in the presence of the antioxidants tested. From the dependence of on the concentration of antioxidants, the concentrations providing 50% protection against intracellular H_2DCF-DA oxidation were estimated. In this assay, catechins, hydroxycinnamic acids and ascorbic acid showed the highest effectiveness [(−)-epigallocatechin gallate > (−)-epicatechin gallate > chlorogenic acid > ascorbic acid > (−)-epigallocatechin > caffeic acid] having IC_{50} values below or equal to 500 nM. The IC_{50} values of other antioxidants tested were in the range of about 5–100 μM (Table 4).

4. Discussion

There is increasing interest in the beneficial health effects of compounds present in food and beverages. Numerous studies have been devoted to the antioxidant properties of flavonoids, including catechins (Higdon & Frei, 2003; Lambert & Elias, 2010; Prior & Cao, 1999; Re et al., 1999). In particular, catechins have been found to be good chain-breaking antioxidants, inhibiting lipid peroxidation in low-density lipoprotein (LDL) induced by metmyoglobin at submicromolar concentrations, (−)-epicatechin gallate being the most effective (Rice-Evans, 1995), and bind metal ions catalyzing oxidation reactions (Lambert & Elias, 2010). The antioxidant action of catechins is well-established in various *in vitro* and *in vivo* systems. Many studies have reported that the scavenging effects of galloylated catechins were stronger than those of nongalloylated catechins and the scavenging effects of [(−)-epigallocatechin] was stronger than those of [(−)-epicatechin] and [(+)-catechin] (Jovanovic, Hara, Steenken, & Simic, 1995; Nanjo et al., 1996; Yoshida et al., 1989). Lee, Kim, Kim, and Kim (2014) have also found that (−)-epigallocatechin gallate has the highest antiradical capacity.

The present study demonstrates that catechins have remarkable antioxidant properties with respect to model free radical $ABTS^{\cdot}$, $ROO^{\cdot}$, ferric ions and other physiologically relevant oxidants such as $ONOO^-$ and hypochlorite, both in pure chemical systems and in protection of RBCs against hemolytic membrane damage and intracellular generation of free radicals. Catechins proved to be among the best antioxidants studied. This property of catechins may be of importance since after ingestion they are transported by blood, are in contact with RBCs and





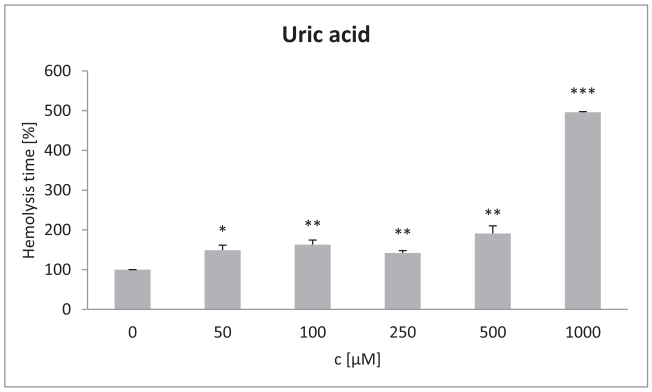
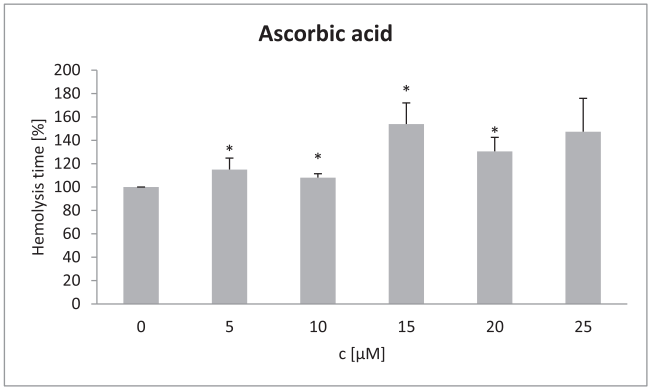
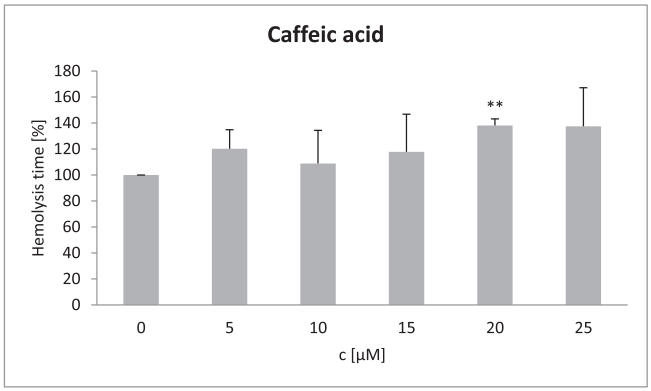


Fig. 1. (*continued*)





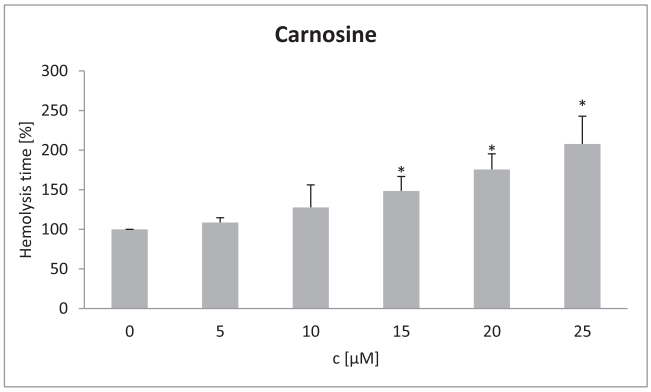


Fig. 2. Protection against hypochlorite-induced hemolysis by selected antioxidants.

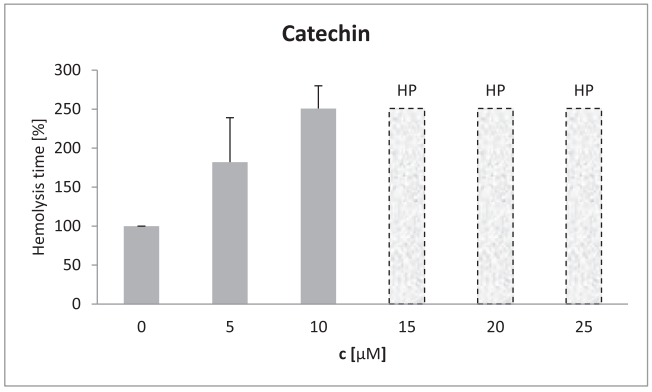
are bound by these cells.

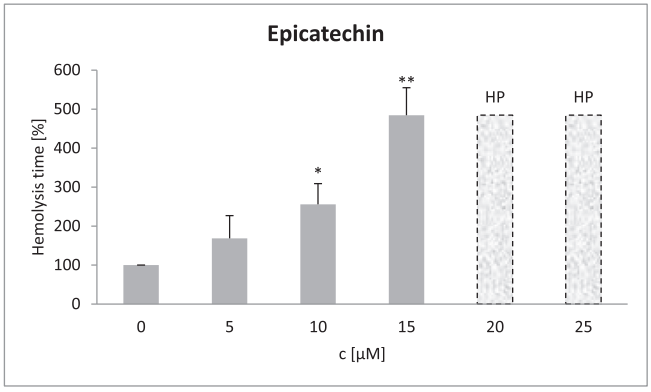
Selected compounds were assayed for antioxidant and free radical scavenging activities using the FRAP and ABTS˙ assays respectively. Although both the FRAP and ABTS˙ assays are based on electron transfer mechanisms, FRAP method measures the direct capacity of selected compounds to reduce ferric iron to ferrous iron as a measure of the antioxidant power (Benzie & Strain, 1996) and the ABTS˙ assay is based on the capacity of compounds to reduce the stable ABTS˙ radical in aqueous solutions (Re et al., 1999). Both activities were compared with that of Trolox, a water soluble derivative of vitamin E. FRAP and also ABTS˙ assays results indicate the highest antioxidant activity for the flavan-3-ols (catechins) as (−)-epicatechin gallate and (−)-epigallocatechin gallate, which have the galloyl moiety at the C-3 position.

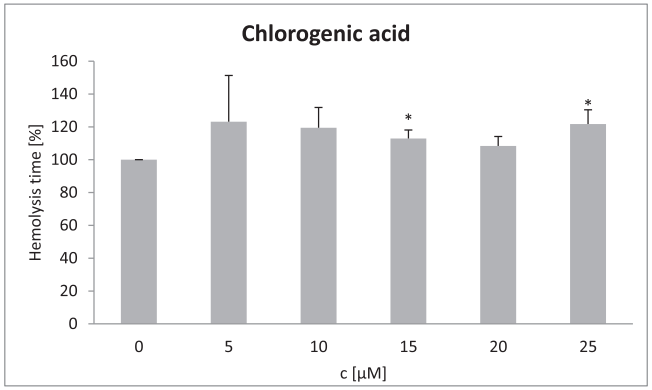
Generally, the stoichiometry of reaction of the antioxidants studied was lower in the FRAP assay than in the ABTS˙ decolorization assay. This is obvious for thiol-containing compounds such as GSH, but true also for many other antioxidants, including flavonoids. Similar differences in the reactivity of catechins with ABTS˙ and FRAP assays were found by Lee et al. (2014).

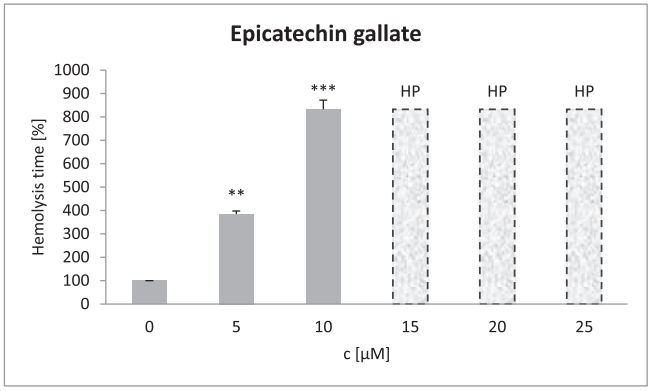
Comparison of structures and reactivity of hydroxycinnamic acids suggest that the presence of the second hydroxyl group in the phenolic ring is the main determinant of reactivity in the FRAP assay. *p*-Coumaric acid which has only one hydroxyl group shows very low reactivity, similar to that of hydroxycinnamic acid, lacking hydroxyl group. Caffeic acid (3,4-dihydroxy-cinnamic acid) having two hydroxyl groups and its ester, chlorogenic acid, show a FRAP reactivity of about 1 mol Trolox equivalents/mol. Methylation of the second hydroxyl group lowers the reactivity: ferulic acid (3-methoxy-4-hydroxycinnamic acid) has a reactivity of 0.6–0.7 mol Trolox equivalents/mol while sinapic acid, having one hydroxyl and two methoxy groups has a reactivity of about 1.2 mol Trolox equivalents/mol. Reactivity of hydroxycinnamic acids with ABTS˙ is more difficult to explain since even *p*-coumaric acid, having one hydroxyl group in the phenyl ring, shows a reactivity of about 2 mol Trolox equivalents/mol, suggesting a more complex reaction mechanism.

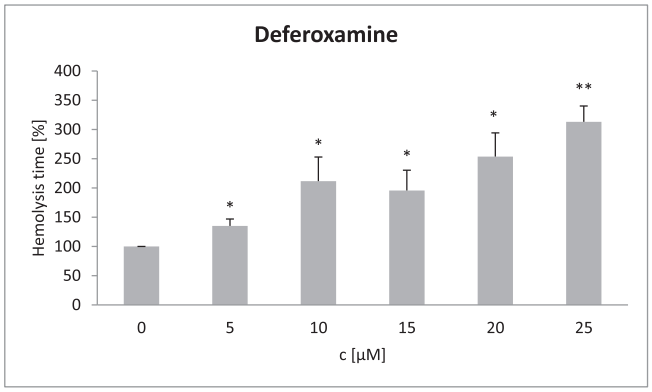
In flavonoids, the number of hydroxyl group in the A ring seems to determine the reactivity in the FRAP assay, the presence of the second hydroxyl group being again a prerequisite for reactivity. Naringin and naringenin, having one hydroxyl group in the A ring, show negligible reactivity, like hesperidin, which has one hydroxyl group and one glycosylated hydroxyl group. (+)-Catechin, (−)-epicatechin, (−)-epigallocatechin, morin and rutin, having two hydroxyl groups in the A











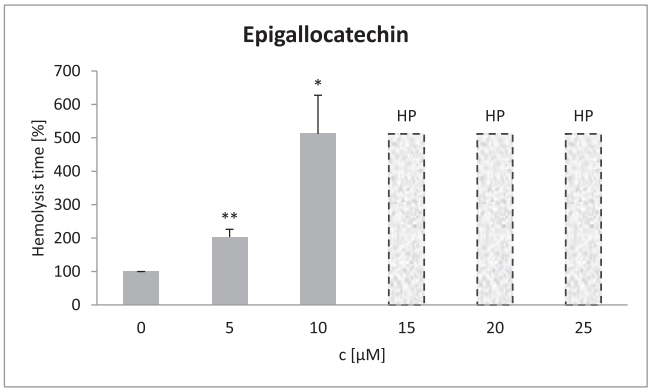


Fig. 2. (*continued*)

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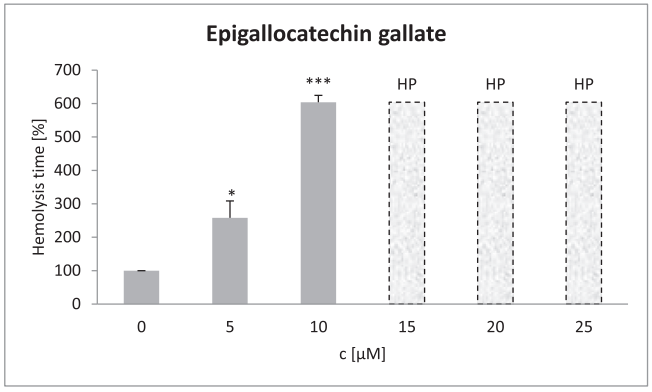
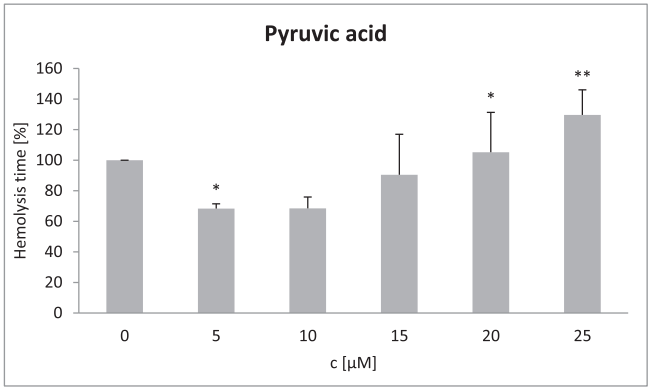
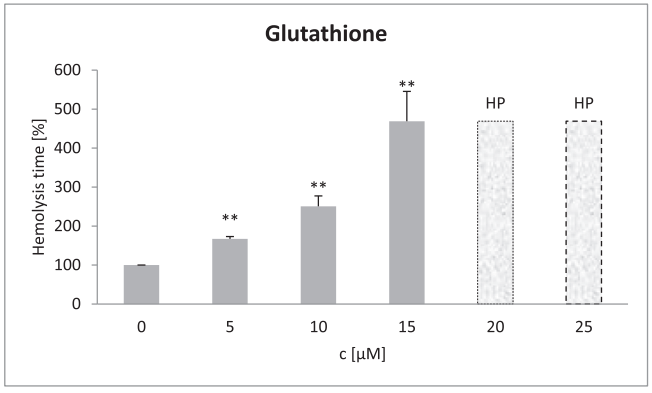
ring, have a reactivity of about 1 mol Trolox equivalents/mol. These data suggest that Fe^{3+} binding by *ortho*-hydroxyl groups in the A ring is necessary for iron reduction. The number of hydroxyl groups in the B ring does not seem to affect the flavonoid reactivity as it can be inferred, i.a., from the lack of significant difference between the reactivity of (−)-epigallocatechin (three hydroxyl groups in the B ring) and other flavonoids mentioned (two hydroxyl groups in the B ring). In flavonoid esters, hydroxyl groups of the gallic acid seem to account for the increased FRAP reactivity. Again, the $ABTS^{\cdot}$ reactivity of flavonoids is more difficult to explain. $ABTS^{\cdot}$ is a promiscuous radical in its reactivity and it could be expected that each hydroxyl group of flavonoids is able to scavenge $ABTS^{\cdot}$. However, (−)-epicatechin gallate, which has a total of 7 hydroxyl groups shows $ABTS^{\cdot}$ reactivity of about 8 mol Trolox equivalents/mol, higher than (−)- epigallocatechin gallate, which has a total of 8 hydroxyl groups. (+)-Catechin, having two hydroxyl groups in the A ring and two hydroxyl groups in the B ring shows higher reactivity, (ca 4 mol Trolox equivalents/mol) than (−)-epicatechin having the same number of hydroxyl groups and (−)-epigallocatechin having two hydroxyl groups in the A ring and three hydroxyl groups in the B ring (ca 3 mol Trolox equivalents/mol). An explanation may lie in the complex reaction of catechins with $ABTS^{\cdot}$. It has been demonstrated that during this reaction covalent adducts are formed, which further scavenge $ABTS^{\cdot}$ (Osman, Wong, Hill, & Fernyhough, 2006). These reactions are not completed within one minute (the time period of our measurement), what further complicates the comparisons. In line with these findings, Sang et al. (2003) characterized the reaction products of (−)-epicatechin with $ROO^{\cdot}$ generated by thermolysis of the azo initiator azo-*bis*-isobutyronitrile. Eight reaction products were isolated and identified using high-field 1D and 2D NMR spectral analysis. This study demonstrated that the B-ring is the initial site for formation of reaction products in the peroxyl radical oxidant system.

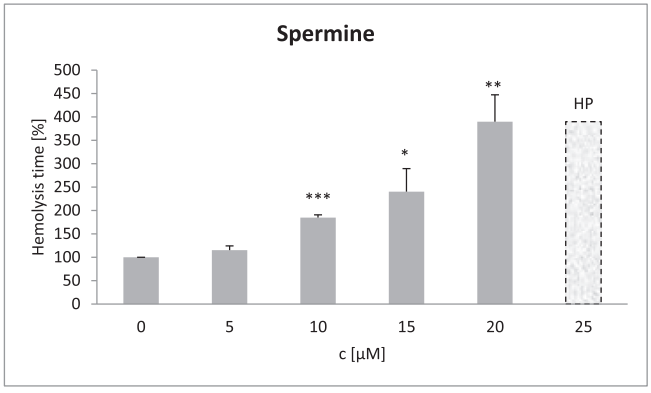
Reactivity of catechins in the FRAP assay confirms their ability to reduce metal ions reported previously (Lambert & Elias, 2010; Nagle, Ferreira, & Zhou, 2006). However, our study demonstrates that these compounds (like other natural compounds studied) are unable to bind ferrous ions strongly enough to prevent their participation in the Fenton reaction. It explains the prooxidant behavior of catechins mediated by metal ions (Lambert & Elias, 2010).

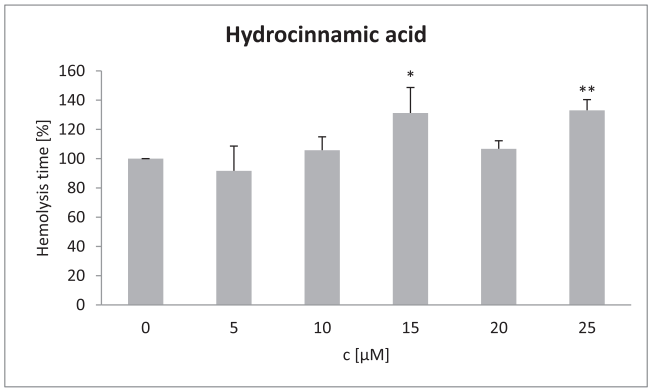
In most cases, catechins were the most effective among the compounds studies, as well as more effective than the standard antioxidant GSH, ascorbate and synthetic antioxidants tested. They inhibited the SIN-1 induced oxidation of DHR123, and fluorescein bleaching induced











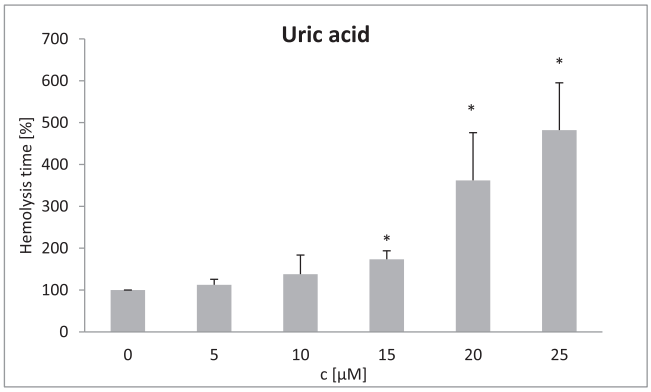


Fig. 2. (*continued*)

Fig. 2. (*continued*)

by AAPH and hypochlorite at concentrations of about 1 μM, or lower, i.e. at concentrations attainable *in vivo*. The same concentrations of some catechins were able to ameliorate hemolysis induced by AAPH and hypochlorite and diminish intracellular free radical reactions. Thus, catechins are versatile antioxidants able to protect against various oxidants formed *in vivo*, which may underlie their health-promoting effects. Martínez, Ugartondo, Vinardell, Torres, and Mitjans (2012) reported that (−)-epicatechin and its conjugates spread out in the core of the bilayer, but might also interact with its external part. They stabilize the membrane through a decrease in lipid fluidity, blocking the access of the $ROO^{\cdot}$ to the interior of RBCs membranes, which may contribute to their ability to inhibit oxidative hemolysis.

The studies presented herein demonstrated also that (−)-epicatechin gallate and (−)-epigallocatechin gallate exerted the most significant cellular protective activity against intracellular H_2DCF-DA oxidation as well as hemolysis induced by hypochlorite.

(−)-Epicatechin gallate and (−)-epigallocatechin gallate have high octanol/water partition coefficients, i.e., 23.9 and 16.0, respectively, as compared to 0.5 for (−)-epigallocatechin, suggesting mainly membrane localization of the esters in erythrocytes. The pK_a value of (−)-epigallocatechin gallate is estimated to be at 7.59 and that of (−)-epicatechin gallate at 7.74 (Kumamoto, Sonda, Nagayama, & Tabata, 2001), which suggests that their cellular absorption is, most probably, in the undissociated, nonionized form by diffusion through the phospholipid bilayer of the plasma membrane. Yet, surface receptors have been identified for (−)-epigallocatechin gallate and (−)-epicatechin gallate (Babich, Krupka, Nissim, & Zuckerbraun, 2005).

Red blood cells are considered a prime target for free radical attack due to their oxygen transport, which is a potent promoter of ROS and the presence of high contents of polyunsaturated fatty acid in their membrane. Exposure of RBCs to oxidative conditions results in successive free radical-mediated reactions that ultimately lead to cell lysis. Among the studied antioxidants dissolved in PBS, (+)-catechin was the most potent protector against AAPH ($ROO^{\cdot}$) damage, followed by (−)-epicatechin, caffeic acid, deferoxamine, (−)-epicatechin gallate, (−)-epigallocatechin gallate, chlorogenic acid, ascorbic acid and uric acid.

Several facets of interaction of antioxidants with erythrocytes, and most probably their resultant action, seem to be critical for their antioxidant effects in these cells: penetration into erythrocytes, partition into the erythrocyte membrane and interaction with the membrane

Table 4
The inhibitory effect of selected antioxidants on AAPH-induced ROS production in erythrocytes. IC_{50} values compared to glutathione: [a] $P < 0.001$; [b] $P < 0.01$ (Dunnett test).

Compound	IC_{50} [μM]
Standard antioxidants	
Ascorbic acid	0.40 ± 0.00[a]
Glutathione	19.15 ± 1.28
Catechins	
(+)-Catechin	4.95 ± 0.25[b]
(−)- Epicatechin	12.02 ± 1.29
(−)-Epicatechin gallate	0.36 ± 0.06[a]
(−)-Epigallocatechin	0.41 ± 0.11[a]
(−)-Epigallocatechin gallate	0.19 ± 0.07[a]
Hydroxycinnamic acids and derivatives	
Caffeic acid	0.50 ± 0.08[a]
Chlorogenic acid	0.37 ± 0.10[a]
Hydrocinnamic acid	45.00 ± 1.48[a]
Other natural antioxidants	
Carnosine*	19.47 ± 1.80
Deferoxamine	80.53 ± 8.13[a]
Pyruvic acid***	74.16 ± 3.16[a]
Spermine**	106.39 ± 4.85[a]
Uric acid	52.92 ± 2.11[a]

* CID: 62881;
** CID: 1103;
*** CID: 1060.

surface.

Most antioxidants, including flavonoids (Fiorani & Accorsi, 2005), are able to diffuse into erythrocytes or be transported by appropriate transport systems.

In a comparative study concerning catechins, (−)-epicatechin gallate showed the highest interaction with the lipid bilayer, followed by (−)-epigallocatechin gallate, (−)-epicatechin and (−)-epigallocatechin as reflected by their hydrophobicity (partition coefficient in the system of *n*-octanol/PBS), incorporation into lipid bilayer of liposomes and quenching of 2-anthroyloxystearic acid (Nakayama, Hashimoto, Kajiya, & Kumazawa, 2000). However, the sequence of protective efficiency against hemolysis induced by AAPH and hypochlorite (Figs. 1 and 2) does not correlate with the above sequence.

Another factor may consists in the localization of antioxidants in the membrane. It has been proposed that the high antioxidant capacity of some galloylated catechins such as (−)-epicatechin gallate could be partially due to the formation of membrane structures showing resistance to detergent solubilization and in which the phospholipids have tightly packed acyl chains and highly hydrated phosphate groups (Caturla, Vera-Samper, Villalaín, Mateo, & Micol, 2003). Interaction of antioxidants with the membrane surface protects the membrane from attack by oxidants present in the aqueous phase while interaction with both the membrane surface and hydrophobic interior protects the membrane from hydrophilic and hydrophobic oxidants. (−)-Epigallocatechin gallate, e. g., was demonstrated to interact both with the hydrophobic and hydrophilic regions of lipid bilayers (Oteiza, Erlejman, Verstraeten, Keen, & Fraga, 2005).

(−)-Epicatechin gallate was found to be the most effective compound in protection against fluorescein bleaching induced by NaOCl and in inhibiting AAPH-induced ROS formation in RBCs. In other cell-free systems (protection against DHR123 oxidation and protection against fluorescein bleaching by AAPH), another compound, the stilbene resveratrol (*trans*-3,5,4′-trihydroxystilbene), present e.g. in red wine showed the highest protection. Moderate consumption of red wine reduces the risk of heart disease and extends lifespan, but the relative contribution of wine polyphenols to these effects is still unclear.

More recent studies suggested to use of peanut sprouts as a functional food (Wang et al., 2017). However, our results showed that catechins are more potent against oxidative stress-induced RBCs hemolysis to compare with caffeic acid, which is greatly enriched in peanut sprout. In our opinion only comparative analysis of antioxidants and also, employment of more than one test method specific to a radical species, gives a better estimate of antioxidant potential of a tested compound.

Apart from the excellent antioxidant properties of catechins and other flavonoids, they may show synergistic interaction with endogenous antioxidants and act as indirect antioxidants. Pereira, Sousa, Costa, Andrade, and Valentão (2013) reported that flavonoid showed synergistic interaction in the $DPPH^{\cdot}$ scavenging assay; the presence of a catechol group in the B ring was demonstrated to be essential for synergisms with GSH, except when an OH group at C6 is also present. Moreover, adducts formed at C2′ and C5′ of the B ring seemed to be more important for the antioxidant capacity than adducts formed at C6 and C8 of the A ring.

In addition to their direct antioxidant activity, polyphenols exhibit indirect antioxidant action, consisting in induction of synthesis of endogenous antioxidants and antioxidant enzymes, and inhibition of biosynthesis of prooxidant proteins. Basu et al. (2013) reported that green tea beverage and green tea extract significantly increased plasma antioxidant capacity (from 1.5 mmol/l to 2.3 mmol/l and from 1.2 mmol/l to 2.5 mmol/l respectively) and whole blood glutathione (from 1783 to 2395 μg/g hemoglobin and from1905 to 2751 μg/g hemoglobin, respectively) versus controls at 8 weeks. Catechin intake has been also reported to increase the activity of glutathione *S*-transferase and decrease the activities of prooxidant enzymes such as xanthine oxidase or nitric oxide synthase (Butt et al., 2014). More recently, Yokotani and Umegaki (2017) reported that the administration of (−)-epigallocatechin gallate (500 mg/kg) to rats increased plasma (−)-epigallocatechin gallate (4 μmol/l as free form) and ascorbic acid levels (1.7-fold), as well as oxygen radical absorbance capacity (1.2-fold) and FRAP (3-fold) values.

It should be noted that (+)-catechin, (−)-epicatechin and (−)-epigallocatechin were classified as the least toxic for normal cells (HGF-2 fibroblasts cells from the human oral cavity; IC_{50} values > 500 μM)) to compare with (−)-epigallocatechin gallate (moderately toxic, IC ≈ 250 μM) or (−)-epicatechin gallate and catechin gallate (highly toxic, IC ≥ 100 μM) (Babich et al., 2005). However, such high concentrations of catechins are not attainable *in vivo* and significant antioxidant effects were observed at much lower concentrations in this study. (−)-Epigallocatechin gallate has been reported to exert selective toxicity to tumor cells but not to normal epithelial cells (Hsu & Liou, 2011). It was found that normal but not malignant cells expresses large amount to (−)-epigallocatechin gallate-binding protein called “Fas-like decoy proteins” which decreases the concentration of free (−)-epigallocatechin gallate, resulting in resistance to apoptosis (Suzuki, Miyoshi, & Isemura, 2012). By this mechanism, green tea catechins block carcinoma and help in modulating signal transduction pathways pertaining to cell proliferation, transformation, inflammation, and metastasis (Butt, Ahmad, Sultan, Qayyum, & Naz, 2015).

Rosenkranz et al. (2002) suggested catechin-mediated inhibition of the platelet-derived growth factor beta receptor signaling, which plays a critical role in the pathogenesis of atherosclerosis; it offers another molecular explanation for the “French paradox”.

Intestinal absorption of catechins in humans depends on their properties and is higher for catechins of lower molecular weight (Kanwar et al., 2012). The ability of catechins to cross the blood-brain barrier rose interest in using their antioxidant properties of polyphenols to prevent and treat neurodegenerative diseases (Mandel, Amit, Reznichenko, Weinreb, & Youdim, 2006). Recent evidence reveals that catechins may be a key mediator in cardiovascular health via mechanisms of blood pressure reduction, flow-mediated vasodilation and atherosclerosis attenuation (Mangels & Mohler, 2017). Japanese and Chinese people who often have the habit of drinking tea have a very low incidence of coronary heart disease.

The low bioavailability of flavonoids is the main problem in their prophylactic and pharmaceutical use. However, contemporary nanotechnology may overcome this restriction, by production of flavonoid-containing nanoparticles (Maity, Mukhopadhyay, Kundu, & Chakraborti, 2017). The versatile antioxidant properties of catechins make them ideal candidates for formulation of nanoparticles devoted to efficient antioxidant supplementation, especially in cases requiring antioxidant participation in therapy.

Acknowledgements

This study was performed within the project 2014/14/A/ST4/00640 ,Antioxidant Power Series as a tool for rational design and assessment of health promoting properties of functional food based on antioxidant phytochemicals" of the National Science Center of Poland. We are grateful to Edyta Bieszczad-Bedrejczuk, M.Sc., for her excellent technical help.

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.2017.08.117.

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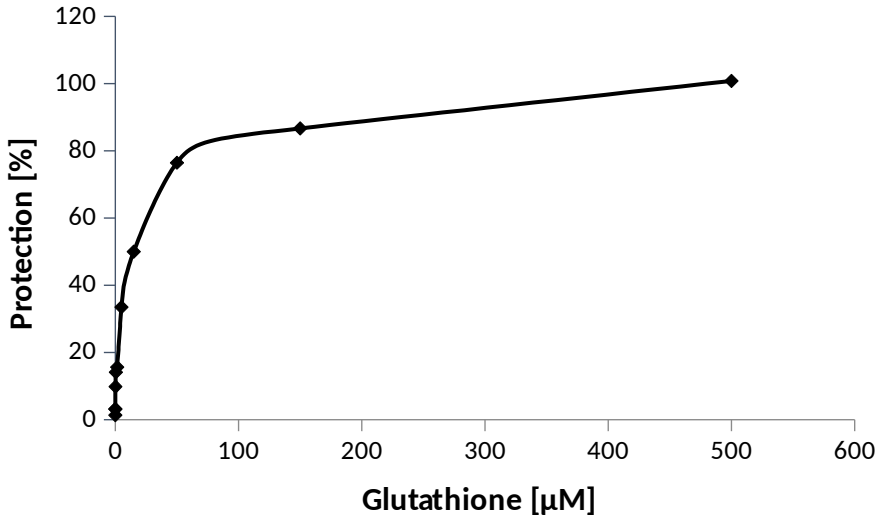

Fig. S1. Inhibition of SIN-1 induced DHR113 oxidation on the concentration of glutathione.

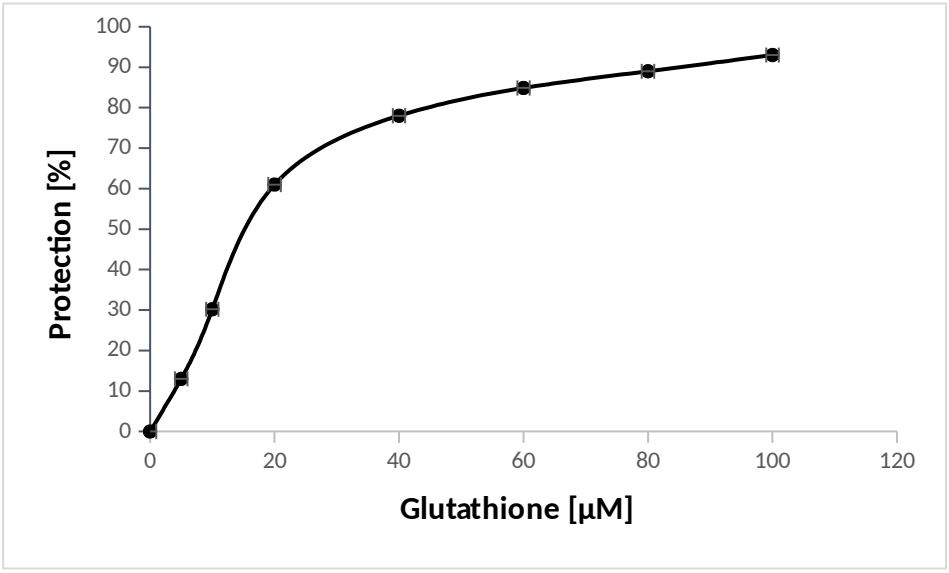

Fig. S2. Concentration dependence of the inhibition of AAPH-induced fluorescein bleaching by glutathione.

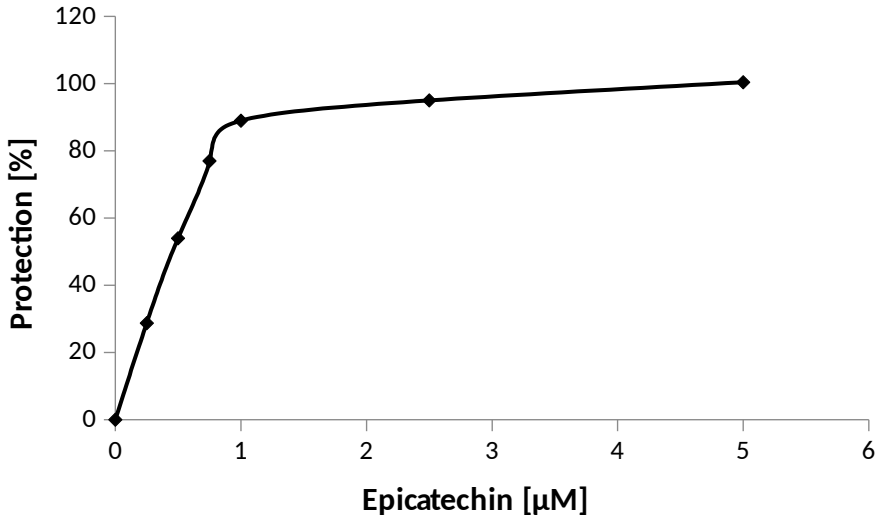

Fig. S3. Concentration dependence of the inhibition of NaOCl-induced fluorescein bleaching by epicatechin.

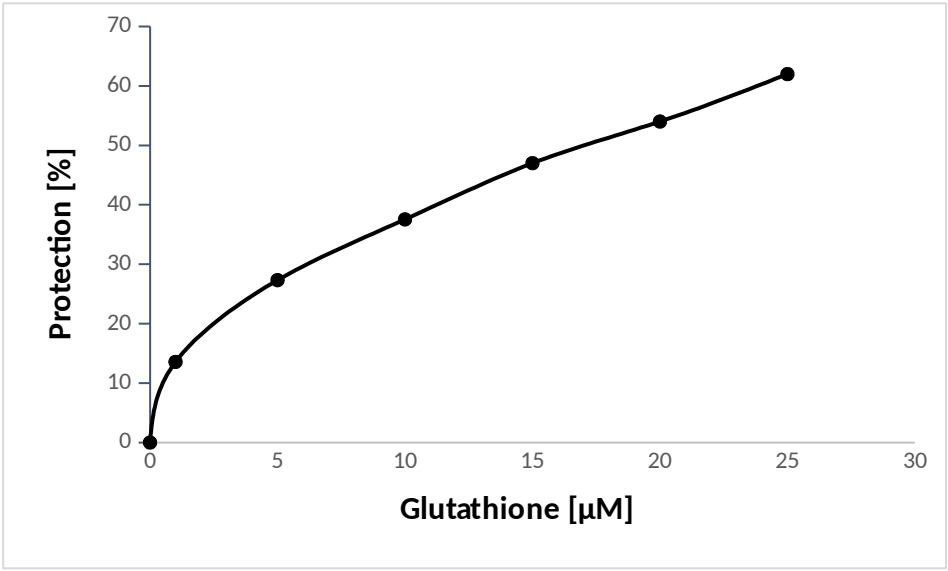

Fig. S4. Concentration dependence of protection by glutathione against AAPH-induced oxidation of intracellular H_2-DCF.

Food Chemistry 268 (2018) 567–576

Contents lists available at ScienceDirect

Food Chemistry

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Antioxidant properties of ferrous flavanol mixtures

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ARTICLE INFO

Chemical compounds studied in this article:
(+)-Catechin, CID: 9064
(−)-Epicatechin, CID:72276
(−)-Epigallocatechin, CID: 72277
(−)-Epicatechin gallate, CID:107905
(−)-Epigallocatechin gallate, CID: 65064

Keywords:
AAPH
ABTS
Catechin
Epicatechin
Epigallocatechin
Epicatechin gallate
Epigallocatechin gallate
Hemolysis
Hypochlorite

ABSTRACT

Interaction of metal, especially iron ions with flavanols is considered as an important feature of these compounds and is believed to contribute to their both antioxidant and prooxidant properties. The aim of this study was to examine how Fe^{2+} binding to form a 4:1 (flavanol:Fe^{2+}) mixtures affects the antioxidant properties of flavanols. ABTS* scavenging, protection against fluorescence bleaching induced by AAPH and hypochlorite, protection against lipid peroxidation and protection against hypochlorite-induced hemolysis demonstrated that flavonol-Fe^{2+} mixtures retain antioxidant properties, although, in most cases, they are lower with respect to the flavanols alone. No superoxide dismutase-like or catalase-like activity of the mixtures was revealed.

1. Introduction

Flavanols (flavan-3-ols), the main components of tea extracts, rise increasing interest due to their biological effects. Tea (*Camellia sinensis*) is the second most common beverage in the world next to water; a typical brewed green tea beverage (250 ml) contains 50–100 mg of flavanols (Wei et al., 1999). Tea extracts and pure flavanols has been reported to have antioxidant, antiviral, antibacterial, and anticancer activities, to decrease blood pressure as well as blood glucose level. Lipid metabolism studies have revealed that tea extracts and individual flavanols lower triacylglycerol and total cholesterol concentrations, inhibit hepatic and body fat accumulation, and stimulate thermogenesis (Nagao et al., 2005). Flavanols have been reported to penetrate the blood brain barrier and to protect against neuronal death in a wide array of cellular and animal models of neurological diseases (Mandel, Amit, Reznichenko, Weinreb, & Youdim, 2006). For example, (−)-epigallocatechin gallate (EGC-G) was reported to protect mice from dopamine neuron loss in *substantia nigra* caused by the neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in that model of Parkinsońs disease (Levites, Weinreb, Maor, Youdim, & Mandel, 2001).

Flavanol effects are complex and partly mediated by their properties not directly related to the antioxidant action (Kim, Quon, & Kim, 2014). However, these compounds are, first of all, natural antioxidants and most of their pharmacological actions is considered to be mainly due to their antioxidant activity (Ames, Gold, & Willett, 1995), understood as the ability to scavenge free radicals generated endogenously and formed by various xenobiotics, UV and ionizing radiation. We have recently characterized the antioxidant properties of flavanols demonstrating their excellent antioxidant action against various physiologically relevant oxidants (Grzesik, Naparło, Bartosz, & Sadowska-Bartosz, 2018). Nevertheless, flavanols, like most plant polyphenols possess also prooxidant properties (Hadi, Asad, Singh, & Ahmad, 2000).

Another important property of flavanols is the capacity to bind metal ions, especially iron ions under physiologic conditions. The interaction of flavanols with metal ions may contribute to both prooxidant and antioxidant properties of these compounds. On one hand,

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https://doi.org/10.1016/j.foodchem.2018.06.076
Received 6 January 2018; Received in revised form 12 June 2018; Accepted 18 June 2018
Available online 19 June 2018
0308-8146/

these compounds may reduce transition metal ions and induce the Fenton reaction (Nakagawa et al., 2002). Hydrogen peroxide, necessary for this reaction, is formed i.a. by autoxidation of catechins (Mochizuki, Yamazaki, Kano, & Ikeda, 2002). The prooxidant activity of flavanols has been associated with their interactions with metal ions. Interaction of EGC-G and (−)-epicatechin (EC) with copper leads to superoxide and hydroxyl radical formation and oxidative DNA degradation. This prooxidant property of flavanols was implied in the apoptogenic and anticancer activity of these compounds (Azam, Hadi, Khan, & Hadi, 2004). On the other hand, sequestering especially iron and copper by flavanols has been recognized as one mode of their antioxidant action (Bao et al., 2013). Under physiological conditions, formation of iron mixtures of flavanols may be of significance, both in the digestive tract where ingested flavanols may interact with iron released from metalloproteins and other sources, and also within the cells. Application of flavanols has been proposed in cases of iron overload (Thephinlap et al., 2007).

Adverse effects of iron binding by flavanols include impairment of intestinal iron absorption and lead to iron deficiency (Kim, Ham, Shigenaga, & Han, 2008; Tamilmani & Pandey, 2016). Nevertheless, high levels of free iron in the gastro-intestinal tract is associated with disease and carcinogenesis (Werner et al., 2011; Radulescu et al., 2012), so iron binding by flavanols in the intestine may be also beneficial.

Iron complex of another flavonoid, quercetin, was reported to be internalized by RKO cells (poorly differentiated colon carcinoma cell line) under conditions of low extracellular iron. The iron complexed to quercetin did not associate with the labile iron pool and cells behaved as though they were iron deficient (Horniblow, Henesy, Iqbal, & Tselepis, 2017). Iron complexes of flavonoids were postulated to be involved in the formation of molecular assemblies due to the facilitation of membrane adhesion and fusion, protein–protein and protein–membrane binding, and other processes responsible for the regulation of cell metabolism and protection against environmental hazards (Tarahovsky, Kim, Yagolnik, & Muzafarov, 2014).

As a significant fraction of flavanols may occur in the form of iron complexes *in vivo*, properties of flavanol-Fe^{2+}complexes are of considerable interest. The aim of this study was to evaluate the antioxidant properties of ferrous mixtures of flavanols in comparison with native compounds. We expected that flavanol ferrous mixtures retain most of the antioxidant activity of parent flavanols. Moreover, we intended to check whether these mixtures show superoxide dismutase or catalase activity. The following flavanols were used in the study: (+)-catechin (C), EC, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (EC-G) and EGC-G. They represent the main flavanols present in the green tea (Zeeb, Nelson, Albert, & Dalluge, 2000). We checked the stoichiometry of flavanol complex formation and, in order to avoid excess iron, 4:1 (flavanol:Fe^{2+}) ferrous mixtures of flavanols were used in this study unless stated otherwise.

2. Materials and methods

2.1. Materials

Nitro Blue Tetrazolium (NBT) produced by BioShop Canada Inc. (Burlington, Ontario, Canada) and dimethyl sulfoxide (DMSO) were purchased from Lab Empire (Rzeszów, Poland). 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) was from Polysciences (Warrington, PA, USA). Stock solutions of NBT and AAPH were freshly prepared in phosphate buffered saline (PBS) before each experiment. Fluorescein and sodium hypochlorite (NaOCl, 15% active chlorine basis) were obtained from CHEMPUR (Piekary Slaskie, Poland). A stock solution of NaOCl was diluted in 0.1 M NaOH and its concentration was determined spectrophotometrically at 290 nm using the molar absorption coefficient of $\varepsilon_{290nm} = 350\ M^{-1}\,cm^{-1}$ (Morris, 1966). Under such conditions NaOCl exists exclusively as OCl^-. The stock solution of NaOCl was diluted in PBS before use. At pH 7.4 the both forms, HOCl and OCl^- are present in the solution at comparable concentrations. 2-Thiobarbituric acid (TBA) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). A stock solution of TBA was prepared in 0.1 M NaOH at a concentration of 0.67%. Selected flavanols and all other reagents, if not mentioned otherwise, were purchased from Sigma (Poznan, Poland) and were of analytical grade. Mohr's salt (purity of 99.997%) was obtained from Sigma-Aldrich. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Fluorometric and absorptiometric measurements were done in a Tecan Infinite 200 PRO multimode reader or a Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). All measurements were performed in triplicate and repeated at least three times on different preparations. Selected flavanols were dissolved in PBS (EGC-G and EGC) or in DMSO/PBS (EC-G, EC, C) (1 ml of 5 mM flavanol solution was obtained by dissolving flavanol in 5 µl DMSO and then 995 µl PBS was added.

2.2. Spectrophotometric titration of flavanols with iron (II)

100 µl samples of 1 mM flavanol solutions were titrated with 5 µl aliquots of 2 mM $Fe_2(NH_4)_2SO_4$ (Mohr's salt) in 1 mM HCl and absorbance was measured at a wavelength corresponding to absorption maximum of the complex, established on the basis of difference in absorbance spectra of 0.5 mM selected flavanol before and after addition of 0.5 mM Mohr's salt solution. From the dependence of absorbance at this wavelength on the amount of Fe^{2+} added, the stoichiometry of complex formation was determined (Fig. 1a–e).

2.3. Preparing flavanol mixtures with iron (II)

Flavanol-Fe^{2+} mixtures were prepared by mixing aqueous Mohr's salt solution with an excess of a flavanol. In order to obtain mixtures for all selected flavanols in the ratio 4:1, 50 µl of 1 mM Mohr's salt in 1 mM HCl was added with 200 µl of 1 mM flavanol solution and incubated for 10 min. For some experiments, flavanol mixtures with Fe^{3+} and Cu^{2+} were prepared in the same manner, using $FeCl_3$ and $CuSO_4$, respectively. An 1:1 mixture of EGC-G-Fe^{2+} was also prepared, using a slight (5%) flavanol excess with respect to Fe^{2+} to avoid appearance of free Fe^{2+}. Then the solutions were diluted with PBS to the final flavanol concentration of 0.5 mM. In experiments concerning superoxide scavenging activity generated by the PMS-NADH, in addition to 4:1 mixtures, 2:1 (flavanol:Fe^{2+}) mixtures were also prepared, as described by Kostyuk, Potapovich, Strigunova, Kostyuk, and Afanas'ev (2004).

2.4. Fourier transform infra-red spectroscopy (FTIR)

Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used to evaluate the bonds formed between iron and flavanols. FTIR Spectrometer was controlled by OMNIC software. Samples for FTIR measurement were prepared by mixing a sample (approx. 2 mg) with 200 mg potassium bromide (KBr) powder in an agate mortar then a transparent pellet was prepared with a hand press. FT-IR spectra (KBr pellet) were recorded for the substrates (flavanols and Mohr's salt) and the flavanol-Fe^{2+} mixtures. An average of 32 scans was collected for each measurement at a resolution of 2 cm^{-1}.

2.5. Scanning electron microscopy (SEM)

The morphology of the flavanols and flavanol mixtures with Mohr's salt was visualized using scanning electron microscope (SEM) with energy-dispersive X-ray spectroscopy (EDS) analyzer – Quanta™ 3D 200i (FEI Co. Field Emission Instruments, Hillsboro, OR, USA).

2.6. Measurement of particle hydrodynamic size

The particle size and size distribution in flavanol-Fe^{2+} mixtures in solution were measured by the dynamic light scattering (DLS) technique using a photon correlation spectrometer Malvern Zeta-Sizer Nano-ZS (Malvern Instruments, Worcestershire, United Kingdom) in DTS0012 plastic cells (Malvern Instruments, Worcestershire, United Kingdom). The refraction factor was 1.33, at a detection angle of 90, and a wavelength was set at 633 nm. Selected flavanols and flavanol-Fe^{2+} mixtures were measured at the flavanol concentration of 1 mM. Particle size was measured from the average of 11 × 3 cycles at 25 °C. To analyze the data Malvern software was used.

2.7. Antioxidant activity of flavanols and flavanol-Fe^{2} mixtures in cell-free systems

2.7.1. Antiradical activity: $ABTS^{*}$ scavenging.

The ability of selected flavanols and their Fe^{2+} mixtures to scavenge the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{*}$) was measured according to a procedure previously proposed by us (Grzesik et al., 2018).

Appropriate amounts of the studied compounds were added to a solution of $ABTS^{*}$, diluted so that 200 µl of the solution had absorbance of 1.0 in a microplate well, at 734 nm. The decrease in $ABTS^{*}$ absorbance was measured after 1 min ("fast" scavenging) and between 10 and 30 min ("slow" scavenging) of incubation at room temperature

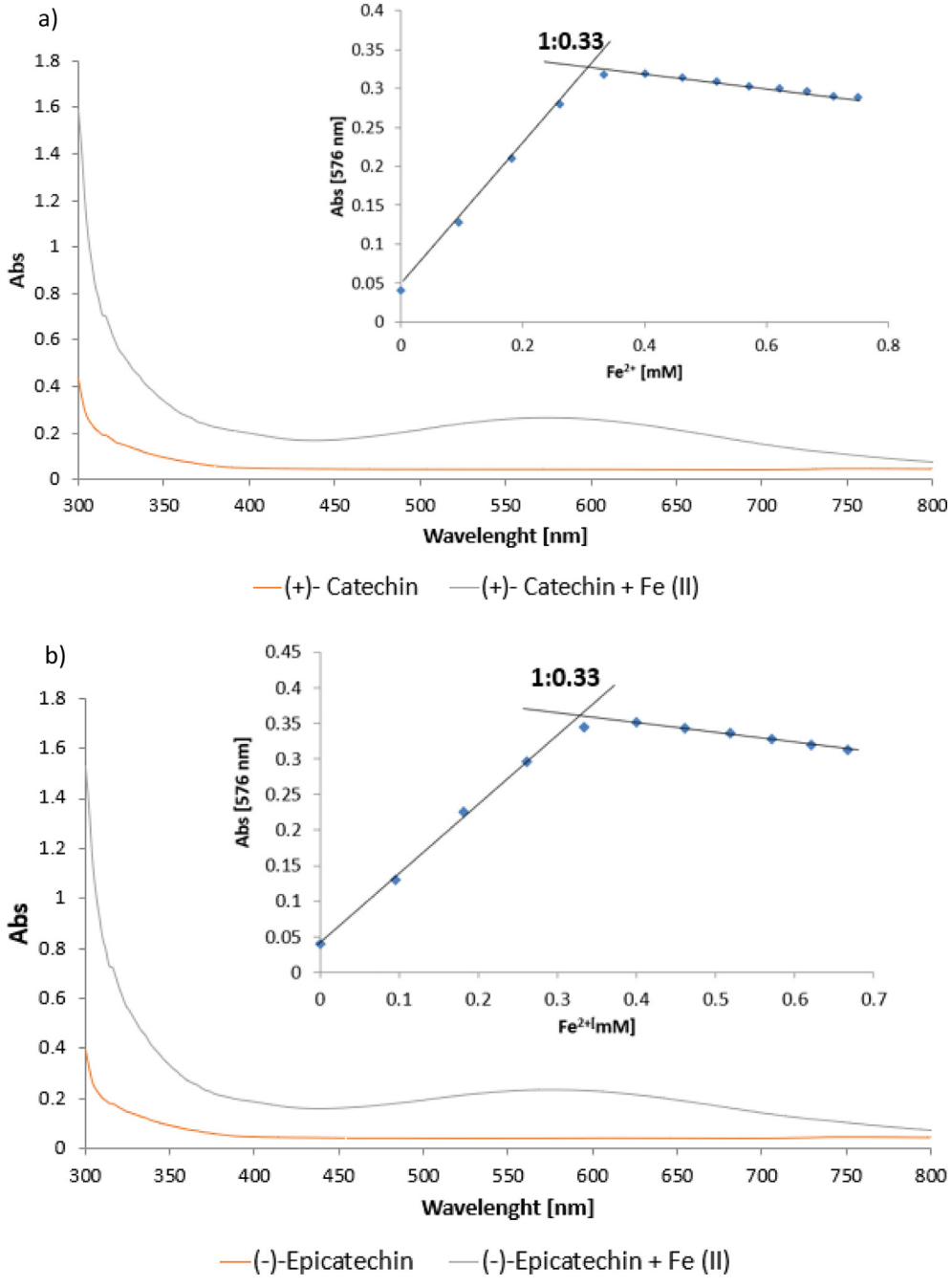

Fig. 1. Absorption spectra of flavanols and their Fe^{2+} mixtures: a) (+)-Catechin, b) (−)-Epicatechin, c) (−)-Epigallocatechin, d) (−)-Epicatechin gallate, e) (−)-Epigallocatechin gallate. Inset: Titration curves obtained from changes in the absorbance of flavanol: Fe^{2+} mixtures at a wavelength corresponding to maximum absorbance of the complex.

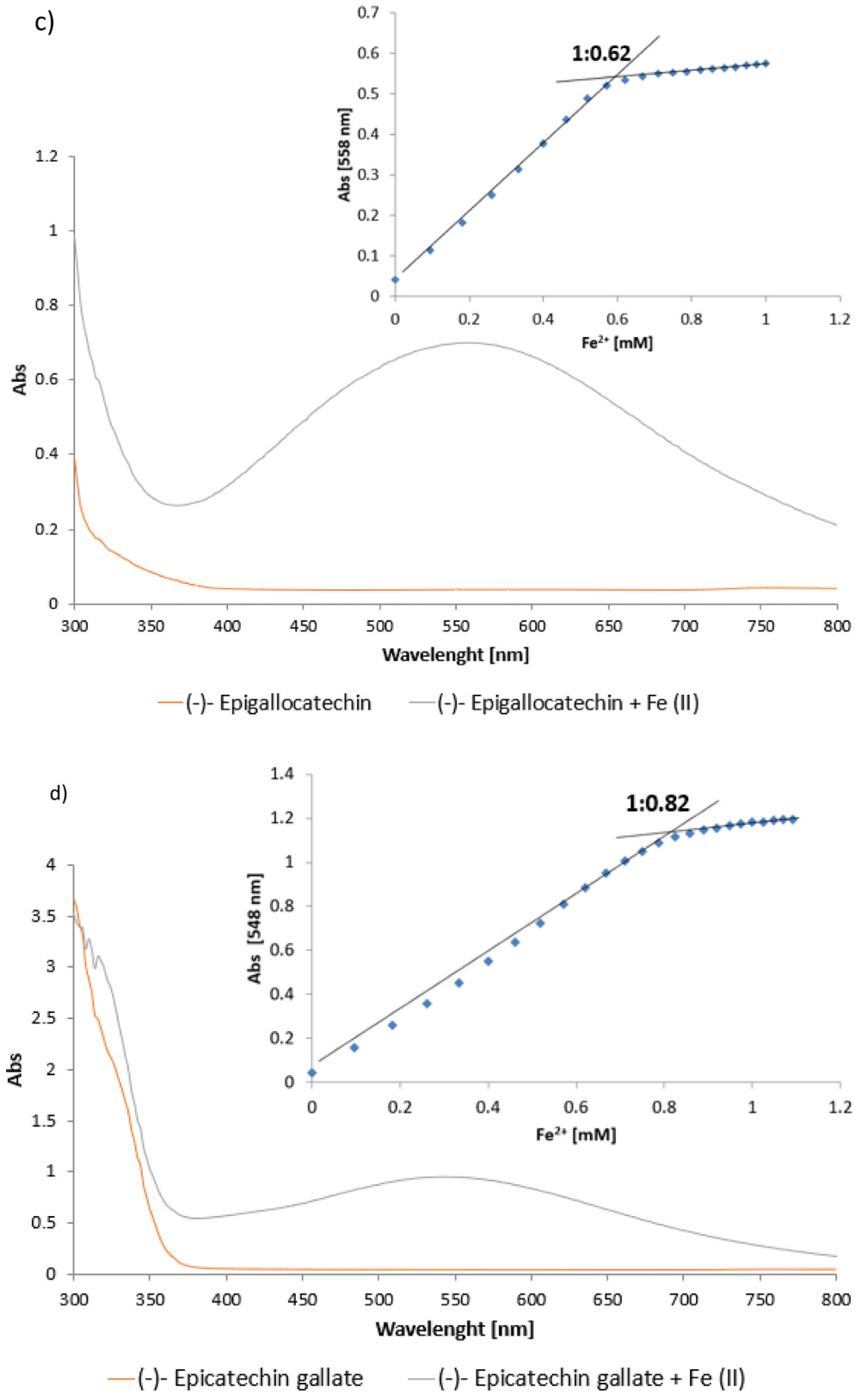

Fig. 1. (*continued*)

(21 ± 1 °C). From the plots of the dependence of absorbance decrease (ΔA) on the compound concentration, the value of ΔA/mM was calculated for the compounds tested.

2.7.2. Protection of fluorescein against bleaching induced by NaOCl or AAPH

Inhibition of fluorescein bleaching was determined with a method proposed by us (Grzesik et al., 2018).

Briefly, aliquot of hypochlorite was added to a microplate well

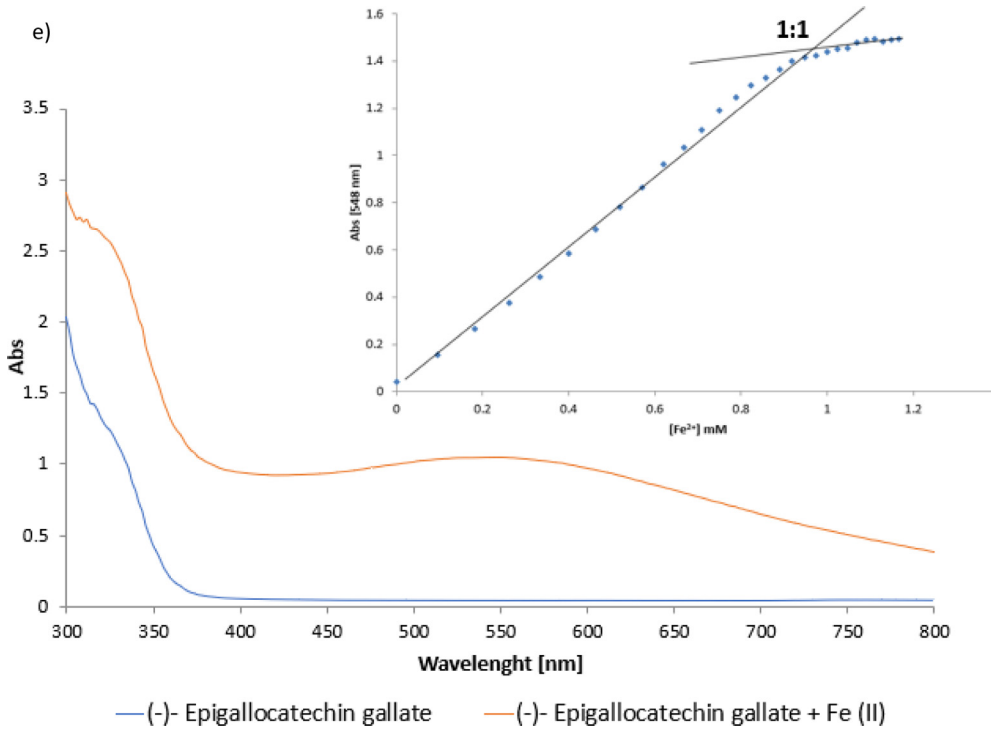

Fig. 1. (*continued*)

containing 100 µl of 0.2 µM fluorescein dissolved in PBS and the solution was mixed immediately. The amount of hypochlorite required to decrease fluorescence down to ca 5–10% of the initial value was determined (1.75 nmol of hypochlorite). Flavanols/flavanol-Fe^{2+} mixtures in a range of concentrations (0.125–5 µM in flavanol), were added to the fluorescein solution before addition of hypochlorite, keeping the volume of the sample constant (100 µl). Fluorescence was measured after 15 min incubation at room temperature at the excitation/emission wavelengths of 485 and 538 nm, respectively.

10 mM AAPH (final concentration) was added to a well containing 0.2 µM fluorescein dissolved in PBS and the solution was mixed immediately. Flavanols/flavanol-Fe^{2+} mixtures in a range of concentrations (0.25–10 µM in flavanol) were added to the fluorescein solution before addition of AAPH, keeping the volume of the sample constant (100 µl). Fluorescence was measured (excitation 485 nm, emission 538 nm) after 1 h incubation at 37 °C. Percent of protection was calculated according to the formula:

$$\%\ Protection = (Fn - Fo)/(Fc - Fo) \times 100\%$$

where F_n – fluorescence of a sample containing fluorescein, hypochlorite/AAPH and a compound studied; F_o – fluorescence of fluorescein treated with hypochlorite/AAPH; F_c – fluorescence of non-treated fluorescein.

From the concentration dependence of protection, the concentrations of compounds providing 50% protection (IC_{50}) against the fluorescein bleaching was calculated.

2.7.3. Superoxide scavenging activity

Superoxide scavenging activity was estimated using several tests:

2.7.3.1. Pyrogallol autoxidation assay. The procedure was modified from Marklund and Marklund (1974). 10 mM pyrogallol solution in 10 mM HCl was added to 50 mM Tris-HCl buffer, pH 8.5, containing flavanols or their Fe^{2+} mixtures in the flavanol concentration range of 50–500 µM. Increase in absorbance at 420 nm was monitored for 10 min.

2.7.3.2. Photochemical reduction of NBT. Flavanols or their Fe^{2+} mixtures were added, at a flavanol concentration range of 1–20 µM, to a solution of 10 mM methionine, 20 µM NBT and 1.5 µM riboflavin. The plates were illuminated for different time periods (up to 2 h) and increase of absorbance at 560 nm was measured (procedure modified from Beauchamp and Fridovich (1971)).

2.7.3.3. Adrenalin autoxidation assay. The procedure was modified from Misra and Fridovich (1972). 10 mM adrenalin solution in 10 mM HCl was added to 50 mM sodium carbonate buffer, pH 10.2, containing flavanols or their 4:1 Fe^{2+}, Fe^{3+} and Cu^{2+} mixtures in the flavanol concentration range of 5–50 µM. Maximal rate of increase in absorbance due to adrenochrome formation was estimated at 480 nm during 5 min.

2.7.3.4. PMS-NADH assay. Superoxide anion was generated nonenzymatically with a phenazine methosulfate (PMS)-NADH system. The reaction mixture consisted of 50 mM sodium carbonate buffer (pH 10.0), 2.5 mM NBT, various amount of sample solution (flavanols/flavanol-Fe^{2+} mixtures) and 8 mM NADH. The reaction was started by the addition of 150 µl of PMS, and the absorbance at 560 nm was recorded for 20 min. As the control, A. dest. was used. The reaction rate was calculated from the proportional increase of absorbance, and scavenging activity of sample was expressed as percent inhibition (modified from Ewing & Janero, 1995).

2.7.4. Hydrogen peroxide scavenging activity

100 µM solution of hydrogen peroxide in 100 mM phosphate buffer, pH 7.4, were added with flavanols/flavanol-Fe^{2+} mixtures to concentrations of 10–50 µM. After various times (15, 30 or 60 min) concentration of hydrogen peroxide was estimated in a reaction with Xylenol Orange (Gay, Collins, & Gebicki, 1999).

Alternatively, flavanols/flavanol-Fe^{2+} mixtures at flavanol concentrations of up to 15 µM were added to 25 mM hydrogen peroxide solution in 100 mM phosphate buffer, pH 7.4. Absorbance of hydrogen peroxide was monitored at 240 nm for up to 30 min.

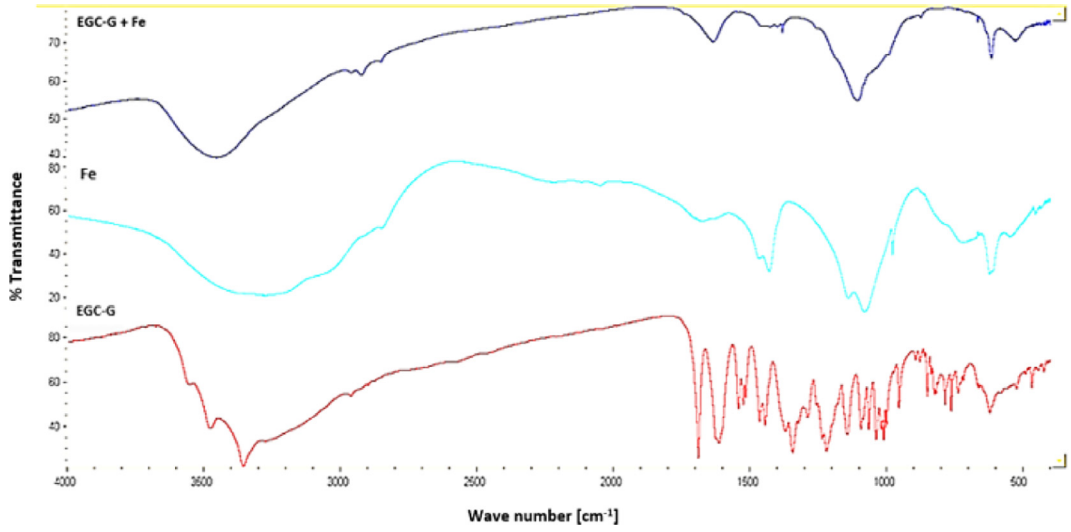

Fig. 2. Comparison of FT-IR spectra of EGC-G-Fe^{2+} mixture, iron, and EGC-G (KBr pellet).

2.7.5. Lipid peroxidation

Protection of egg yolk lipids against peroxidation induced by AAPH and Fe^{2+} was studied. 200 µl of 10% egg yolk suspension in PBS (1 ml of egg yolk suspended vigorous vortexing in PBS) was treated with 10 mM AAPH (final concentration) or 1 mM Fe^{2+} (final concentration) in the presence of flavanols or flavanol/Fe^{2+} mixtures. The concentration range of flavanols/flavanol-Fe^{2+} mixtures was 0.2–1.18 mM or 1–10 µM (with respect to flavanol), for experiments with Mohr's salt and AAPH respectively. The samples were incubated at 37 °C for 3 h under conditions of oxygen access. Then, the samples were added with an equal volume (250 µl) of 10% trichloroacetic acid (TCA) and 250 µl of 0.67% thiobarbituric acid (TBA). The samples were heated at 100 °C for 20 min, cooled, centrifuged, and absorbance of supernatants was measured at 532 nm.

Per cent protection against lipid peroxidation by flavanols/flavanol-Fe^{2+} mixtures was calculated as

$$\%\,\text{Protection} = 100\%[1-(A_n-A_c)/(A_o-A_c)]$$

where A_o – absorbance of sample incubated with AAPH of Fe^{2+} without any flavanol/flavanol-Fe^{2+} mixture, A_n – absorbance of a sample containing a protective agent, A_c – absorbance of a sample not treated with an oxidizing agent.

From the dose-dependent curve relationship of the protection, flavanol/flavanol-Fe^{2+} IC_{50} values were determined.

2.8. Protection against hypochlorite-induced hemolysis

2.8.1. Ethical approval of experiments with blood

The study was approved by the Research Bioethics Committee of the University of Łódź (Poland).

2.8.2. Preparation of erythrocytes

Eight ml of peripheral blood from a healthy donor (lab volunteer, a 40-year-old woman; ISB) was collected in Citrate Tubes containing 3.2% buffered sodium citrate solution and used within the day of its collection. Erythrocytes were isolated by centrifugation for 10 min at 3000 rpm, at 4 °C. The plasma and buffy coat were removed by aspiration. The red blood cells (RBCs) were washed four times with ice-cold PBS. Washed RBCs were suspended to a final hematocrit of 10% in PBS.

2.8.3. Hypochlorite-induced hemolysis

Aliquots of RBC suspensions in PBS were mixed with flavanols/flavanol-Fe^{2+} mixtures at the final flavanol concentration range of 5–25 µM (final volume of 200 µl) and incubated for 15 min with shaking at 37 °C. Then 0.15 mM hypochlorite (final concentration), as optimal concentration to induce hemolysis, was added and turbidity (700 nm) was measured every 2 min for 240 min. The time required to decrease the turbidity to 50% of the initial value (about 1.0) was defined as hemolysis time. Relative hemolysis times were calculated as [100% × (hemolysis time in the presence of a test compound/mean hemolysis time for control sample containing erythrocytes and NaOCl only)].

2.9. Statistical analysis

Statistical significance of differences was evaluated using the Kruskal-Wallis test (when all samples were compared) or paired Student's "t" test (when pairs of results were compared). Statistical analysis of the data was performed using STATISTICA software package (version 13.1, StatSoft Inc. 2016, Tulsa, OK, USA, www.statsoft.com).

3. Results

The flavanols studied differed in the stoichiometry of Fe^{2+} binding, as shown by Fig. 1a–e. (+)-Catechin and EC bound Fe^{2+} at a ratio of 3:1 (flavanol/Fe^{2+}) (Fig. 1a–b). The apparent ratios of Fe^{2+} binding by EGC and EC-G were: 1.61:1 and 1.22:1 respectively, while the apparent ratio of Fe^{2+} by EGC-G was 1:1 (Fig. 1c–e). Thus, mixing Fe^{2+} with fourfold excess of flavanols allowed to form mixtures, in which Fe^{2+} was totally bound by these compounds.

The formation of flavanol-Fe^{2+} complexes was confirmed by FI-IR (KBr pellet) analyses (Fig. 2). For EGC-G, the wave number of spectra of the pure flavanol at 700–900, 1150, 1250 , 1450–1350, 1600–1430, 1700 and 3300–3600 cm^{-1} could be assigned to Ar-H out-of-plane bond, C—O stretch, Ar—O—C, OH bond aromatic, C=C aromatic ring stretch, C=O and O—H stretch respectively. The absorption bands at 1450–1350 cm^{-1} were absent in Fe-EGC-G spectra. A new peak with maximum at wave number at 1382 cm^{-1} was observed in Fe-EGC-G spectra. This band does not appear in the spectrum of Mohr's salt and may be assigned to stretching vibration of Fe—O. The O—H stretching is shifted to higher wave number (Manna, Saha, & Ghoshal, 2014). Analogous results were obtained for other flavanols (not shown).

The vacuum-dried iron-flavanol mixtures were characterized by SEM combined with energy dispersive X-ray (EDX) analysis in order to characterize the morphology of the particles and to examine global their chemical composition. The average particle size of particles was about 5 µm (C with Mohr's salt; Fig. 1Sc–d) or about 10 µm (EC, EC-G,

Table 1
Hydrodynamic diameters d of flavanols and their Fe^{2+} (4:1) mixtures in PBS. Statistical significance of differences between flavanols and their Fe^{2+} mixtures: $^{**}P < 0.01$, $^{***}P < 0.001$.

Compound/Mixture	d [nm]
(+)-C	240.7 ± 24.5
(+)-C + Fe^{2+}	406.7 ± 17.6***
(−)-EC	276.0 ± 9.7
(−)-EC + Fe^{2+}	549.4 ± 25.4***
(−)-EGC	429.6 ± 55.8
(−)-EGC + Fe^{2+}	444.9 ± 28.5
(−)-EC-G	387.8 ± 98.9
(−)-EC-G + Fe^{2+}	760.7 ± 49.5**
(−)-EGC-G	326.6 ± 92.2
(−)-EGC-G + Fe^{2+}	1454.0 ± 51.5***

EG-C and EGC-G with Mohr's salt) (Fig. 1Sf, h, j–k). The presence of iron in the mixture was detected in EDX spectra (not shown).

Taking into account that SEM pictures may be affected by artifacts of drying, we estimated also the hydrodynamic size of particles formed by the flavanols and their mixtures. The results shown in Table 1 demonstrate that the flavanols form aggregates in solution, of average diameters in the nanometer range (< 1 μm) and that addition of Fe^{2+} increases the size of flavanol aggregates. This increase is the most significant in the case of EGC-G, the size of the resulting aggregates exceeding 1 μm (Table 1).

The ABTS* scavenging activity of Fe^{2+} mixture of C was slightly lower in comparison with C, that of EC-Fe^{2+} slightly higher with respect to EC and that of EGC-G-Fe^{2+} mixtures significantly lower with respect to ECG-C (lower for a 1:1 than for a 4:1 mixture). The ABTS* scavenging activities of Fe^{2+} mixtures of EGC and EC-G did not differ significantly from those of respective flavanols (Table 2). Free Fe^{2+} (1–20 μM) did not decrease absorbance of ABTS* (not shown).

Flavanol-Fe^{2+} mixtures of C, EC and EGC retained of the ability of flavanols to protect fluorescein against bleaching induced by AAPH and NaOCl, the IC_{50} values being not changed significantly for the mixtures with respect to those for flavanols alone. The EC-G-Fe^{2+} mixture protected less effectively than EC-G against fluorescein bleaching induced by both agents as evidenced by higher IC_{50} values. Interestingly, the 4:1 EGC-G-Fe^{2+} mixture was more effective than EGC-G in protection against AAPH-induced fluorescein bleaching (for NaOCl-induced fluorescence bleaching, the difference in IC_{50} values was not significant), but the 1:1 mixture was significantly less effective than EGC-G in protection against AAPH-induced and NaOCl-induced fluorescein bleaching. Free Fe^{2+} (0.125–5 μM) did not offer any protection (not shown).

Flavanols as well as their Fe^{2+} mixtures protected against lipid peroxidation induced by AAPH and Fe^{2+}. No significant differences were observed between the mixtures and their parent flavanols except for EC-G (smaller protection against AAPH-induced peroxidation by the Fe^{2+} mixture) and EGC-G (smaller protection against Fe^{2+}-induced peroxidation by the mixture; Table 2).

Flavanols did not inhibit pyrogallol autoxidation. Flavanol-Fe^{2+} mixtures slightly, while flavanol-Fe^{3+} mixtures significantly increased the rate of pyrogallol autoxidation (not shown). Flavanols inhibited dose-dependently photochemical riboflavin-mediated NBT reduction. The extent of reduction decreased in time (probably reflecting flavanol oxidation). Flavanol-Fe^{2+} mixtures showed lower extent of inhibition turning into acceleration of NBT reduction at longer times, as shown for EGC-G in Fig. 2S.

Adrenalin autoxidation at alkaline pH was slightly inhibited by 5–50 μM flavanols, while their Fe^{2+} and Cu^{2+} mixtures did not inhibit this reaction and Fe^{3+} mixtures increased the autoxidation rate, as shown for EGC-G mixtures in Fig. 3S.

Both flavanols and their Fe^{2+} mixtures inhibited PMS-mediated NBT reduction and in general the activities of flavanols and their mixtures were mostly similar. In some cases the inhibitory activity of Fe^{2+} mixtures was higher with respect to flavanols, but in most cases it was lower or not significantly different for 4:1 flavanol:Fe^{2+} mixtures (Table 3).

Table 2
Comparison of antioxidant properties of flavanols and their Fe^{2+} mixtures (4:1) in various assays. For EGC-G, results for a 1:1 mixture are also shown. Statistically significant differences between a flavanol and a corresponding flavanol-Fe^{2+} mixtures: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The statistical significance of differences was evaluated by using the paired Student's "t" test.

Compound/Mixture	ABTS* scavenging activity [mol Trolox equivalents/mol]	Protection against fluorescein bleaching by AAPH (IC_{50}, μM)	Protection against fluorescein bleaching by NaOCl (IC_{50}, μM)	Protection against lipid peroxidation by AAPH (IC_{50}, μM)	Protection against lipid peroxidation by Fe^{2+} (IC_{50}, μM)
(+)-C	2.86 ± 0.05	0.68 ± 0.06	1.43 ± 0.11	9.03 ± 0.10	1121.4 ± 1.9
(+)-C + Fe^{2+}	2.72 ± 0.03*	0.63 ± 0.04	1.30 ± 0.04	7.31 ± 0.64	1123.8 ± 19.4
(−)-EC	2.34 ± 0.08	0.98 ± 0.02	0.55 ± 0.04	3.00 ± 0.24	1086.9 ± 12.9
(−)-EC + Fe^{2+}	2.60 ± 0.03**	0.96 ± 0.02	0.56 ± 0.03	3.90 ± 0.40	1096.3 ± 9.4
(−)-EGC	4.65 ± 0.02	5.35 ± 0.15	1.29 ± 0.01	9.64 ± 0.15	786.3 ± 26.7
(−)-EGC + Fe^{2+}	4.73 ± 0.03	5.31 ± 0.23	1.14 ± 0.05	7.96 ± 0.45*	906.0 ± 29.2
(−)-EC-G	5.19 ± 0.01	1.67 ± 0.05	0.69 ± 0.06	1.93 ± 0.10	821.8 ± 54.8
(−)-EC-G + Fe^{2+}	5.17 ± 0.00	2.07 ± 0.03**	1.05 ± 0.06**	3.14 ± 0.11**	1159.4 ± 22.0
(−)-EGC-G	4.98 ± 0.01	0.98 ± 0.02	0.64 ± 0.02	4.43 ± 0.28	464.2 ± 11.4
(−)-EGC-G + Fe^{2+}	3.96 ± 0.03***	0.78 ± 0.04**	0.58 ± 0.04	4.29 ± 0.21	549.0 ± 37.0*
(−)-EGC-G + Fe^{2+} 1:1	3.48 ± 0.04***	7.56 ± 0.39***	1.49 ± 0.01***	3.40 ± 1.05	591.9 ± 69.5*

Table 3
Protection against PMS-mediated NBT reduction by flavanols and flavanol-Fe^{2+} mixtures. Statistically significant differences between a flavanol and the corresponding flavanol-Fe^{2+} mixture: $^{*}P < 0.05$, $^{**}P < 0.01$.

Compound	Flavanol (3 μM)	[Flavanol:Fe^{2+}] (3 μM:0.75 μM)	Flavanol (7.5 μM)	[Flavanol:Fe^{2+}] (7.5 μM:1.875 μM)	Flavanol (15 μM)	[Flavanol:Fe^{2+}] (15 μM:3.75 μM)
(+)-C	13.46 ± 5.10	23.07 ± 5.11	42.23 ± 4.76	56.17 ± 8.54	63.81 ± 5.59	82.57 ± 5.21*
(−)-EC	37.60 ± 9.21	48.70 ± 4.62	61.43 ± 7.51	73.53 ± 1.96	79.69 ± 0.91	81.31 ± 2.55
(−)-EGC	42.04 ± 6.31	34.45 ± 3.89	78.35 ± 2.75	68.10 ± 6.26*	89.61 ± 0.23	91.11 ± 2.24
(−)-EC-G	8.37 ± 7.21	19.64 ± 2.84*	33.02 ± 3.35	26.69 ± 8.27	71.45 ± 0.26	60.24 ± 8.79
(−)-EGC-G	33.10 ± 2.12	10.11 ± 7.91	61.60 ± 3.41	56.58 ± 3.92	85.90 ± 2.77	73.90 ± 1.03**

We attempted to see whether flavanols and, especially, their Fe^{2+} mixtures have catalase-like activity, incubating them with both low (μmolar) and high (millimolar) concentrations of hydrogen peroxide for up to 1 h. No detectable catalase-like activity of the flavanols or their Fe^{2+} mixtures was found (not shown).

Both flavanols and their Fe^{2+} mixtures protected erythrocytes against lysis induced by HOCl. There were no significant differences between the action of flavanol 4:1 mixtures with respect to flavanols alone, with the exception of C (higher protection by C-Fe^{2+} than C at concentrations of 2.5 μM and 5 μM; Fig. 3a), EC (lower protection by EC-Fe^{2+} than by EC at the concentration of 2.5 μM; Fig. 3b) and EGC-G (higher protection by EGC-G-Fe^{2+} than by EGC-G at the concentration of 1 μM; Fig. 3e). In contrast, 1:1 EGC-G-Fe^{2+} mixture was significantly less effective than EGC-G alone in protection against NaOCl-induced hemolysis, pointing again to different antioxidant behavior of flavanol mixtures of various flavanol:Fe^{2+} ratio (Fig. 3f). Interestingly, Fe^{2+} alone sensitized erythrocytes to the hemolytic action of NaOCl at a low concentration (1 μM) while higher Fe^{2+} concentrations show a weak protective effect (Fig. 3g).

4. Discussion

Flavanol complexes with iron cations are interesting because both components are present in the human body. Flavanols are naturally occurring chemicals found in foods such as fruits, some types of grain, wine and tea (Kim et al., 2008). Iron plays an important role in electron transfer, cellular respiration, cell proliferation and differentiation, and regulation of gene expression. However, iron exposure is directly associated with the pathogenesis of many disorders, such as atherosclerosis, cancer and inflammation, mainly via the production of free radicals (Matsui, Tanaka, & Iwahashi, 2017).

Flavanols are known to chelate metal ions. Chelation activity of flavanols is mainly related to a flavanol groups in the B ring while redox behavior of ligands in complexes depends on the presence of the 3-hydroxy group in their structure. In ring C, the 3-hydroxyl-4-carbonyl and 5-hydroxyl-4-carbonyl groups can also participate in metal chelation (Tarahovsky et al., 2014; Kostyuk et al., 2004). Some flavonoids were found to react with two Fe^{2+} ions at two separate binding sites (Ryan & Hynes, 2007). Additionally, flavonoids can also chelate potentially toxic transition metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}) preventing metal-catalysed free radical generation reactions. Among these reactions iron chelation is of particular interest since binding of iron to the antioxidant flavonoids can reduce the accessibility of the iron to oxygen molecules and consequently diminish its high toxicity. Iron chelation can also serve as an effective tool in modulating cellular iron homoeostasis, under physiologically relevant conditions (Kostyuk et al., 2004; Tarahovsky, Yagolnik, Muzafarov, Abdrasilov, & Kim, 2012; Tarahovsky et al., 2014; Mladěnka et al., 2011).

There exist diverse data in the literature concerning the stoichiometry of flavonoid:Fe^{2+} interactions (Horniblow et al., 2017; Kostyuk et al., 2004). We titrated the flavanols studied with Fe^{2+} to determine their binding stoichiometry. Although it is often assumed that flavanols form a 1:1 complex with Fe^{2+} (Kostyuk et al., 2004), we found an apparent 1:1 stoichiometry only for EGC-G. An apparent stoichiometry of 3 flavanol molecules/Fe^{2+} was found for C and EC, while for EGC and EC-G apparent fractional stoichiometry between 1:1 and 2:1 was found. This result can be explained as due to heterogeneity of complexes formed; nonetheless it should be taken into account that the flavanols tested were not present as a homogenous solution, but rather as aggregates of a hydrodynamic size of several hundred nm; SEM showed the presence of particles of even higher size (several μm). It should be mentioned that flavanol-iron complexes can change under conditions of flavanol excess and as a function of pH, as it has been demonstrated for other flavonoids (Filipsky, Riha, Hrdona, Vavrowa, & Mladenka, 2013). The presence of such structures must affect the binding properties of flavanols.

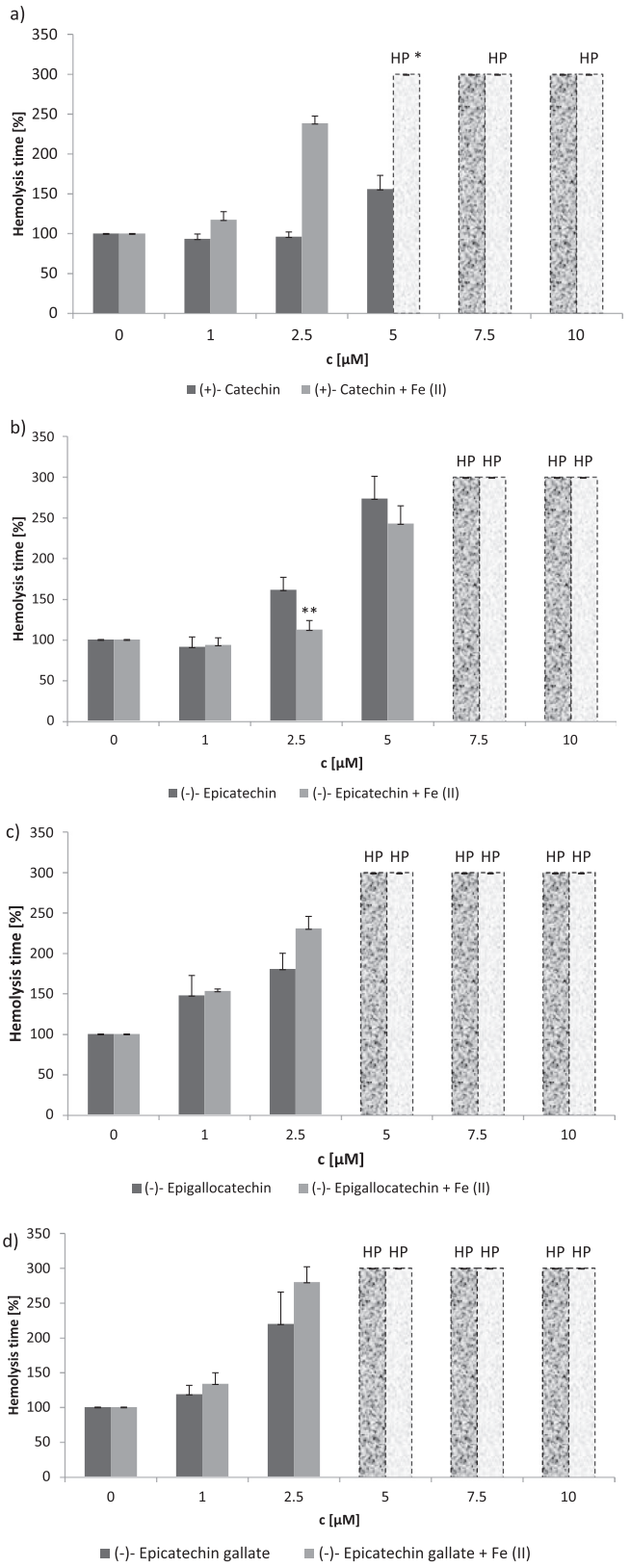

Fig. 3. Protection of erythrocytes against NaOCl-induced hemolysis by flavanols, flavanol-Fe^{2+} mixtures (4:1) and Fe^{2+}.

We prepared 4:1 mixtures of flavanols with Fe^{2+} in order to avoid the occurrence of free Fe^{2+} and mimic the possible *in vivo* situations where flavanols are expected to be in excess over trace amount of Fe^{2+}. Therefore, we studied the effects of flavanol-Fe^{2+} mixtures on a

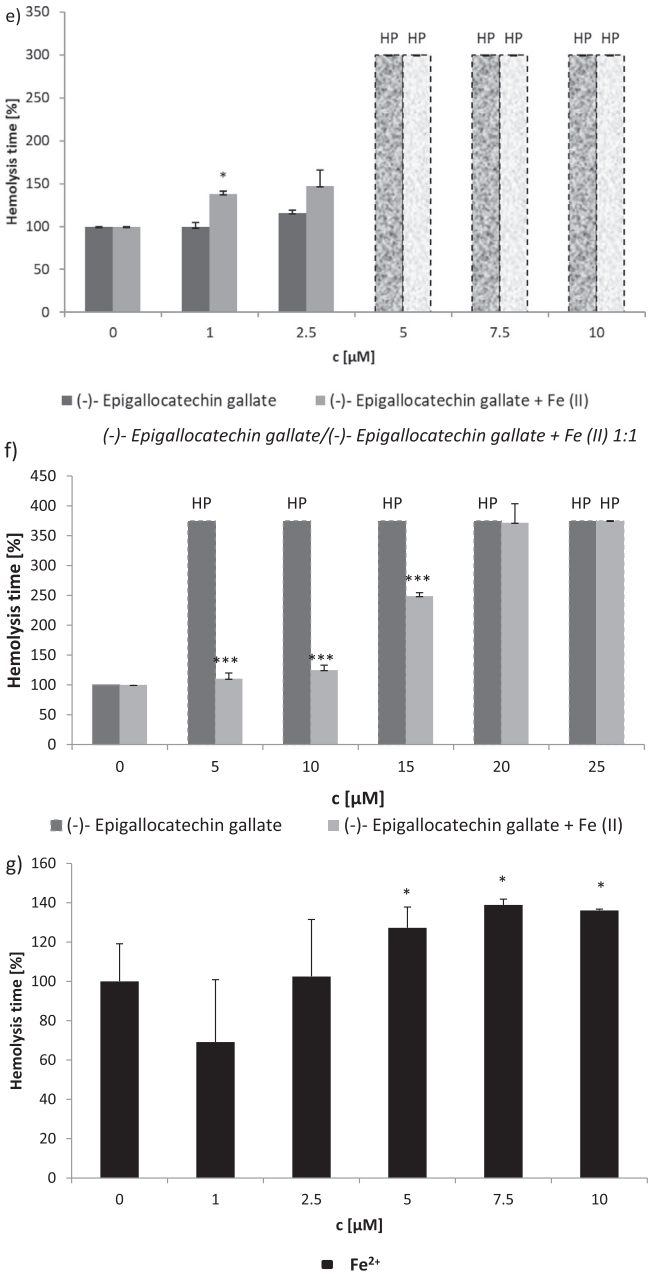

Fig. 3. (*continued*)

background of flavanols alone, so the effects of such mixtures were always compared with those of Fe^{2+}-free flavanols.

There are contradictory data in the literature on antioxidant properties of flavonoids-metal complexes in comparison with uncomplexed flavonoids. Quercetin-Co^{2+} complex (2:1) was found to be more effective than uncomplexed quercetin in scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH*) (Bukhari, Memona, Tahir, & Bhanger, 2008). However, quercetin-Sn^{2+} complex showed lower ferric reducing antioxidant power (FRAP) values and lower ABTS*-scavenging and DPPH*-scavenging activity than uncomplexed quercetin (Dehghan & Khoshkam, 2012). Similarly, the cadmium complex of quercetin was less effective in scavenging ABTS* and DPPH* than quercetin (Ravichandran, Rajendran, & Devapiriam, 2014). In a non-biological system, the reductive activity of green tea polyphenols for graphene oxide was increased by the presence of iron (Akhavan, Kalaee, Alavi, Ghiasi, & Esfandiar, 2012).

Our results show generally that flavanol-Fe^{2+} show antioxidant activity such as ABTS* scavenging, protection against fluorescein bleaching induced by AAPH and NaOCl and NaOCl-induced hemolysis, and protection against lipid peroxidation comparable to those of flavanols alone. It can be concluded that binding low amounts of Fe^{2+} does not significantly decrease, and in some cases even enhances the antioxidant activity of flavanols. Equimolar EGC-G-Fe^{2+} mixture shows significantly lower antioxidant activity than pure EGC-G and 4:1 EGC-G-Fe^{2+} mixture, as it is seen in decreased ABTS* scavenging, decreased protection against fluorescein bleaching induced by AAPH and NaOCl, and decreased protection of erythrocytes against NaOCl-induced hemolysis. Therefore, flavanols can be expected to act as efficient antioxidants in the gastrointestinal tract in spite of contact with ferrous irons released from the food.

We checked the Fe^{2+} mixtures of flavanols for superoxide dismutase-like activity. Such an activity of flavonoid-metal complexes has been reported. Moridani, Pourahmad, Bui, Siraki and O'Brien (2003) demonstrated superoxide scavenging in a system of hypoxanthine-xanthine oxidase-NBT by 2:1 complexes of flavonoids (including catechin) with Fe^{2+}, Fe^{3+} or Cu^{2+}. This activity was the highest for Cu^{2+}complexes and the lowest, but still discernible for Fe^{2+} complexes. Kostyuk et al. (2004) found that activities of metal complexes of flavonoids, including EC, inhibited NBT reduction by photoactivated riboflavin and IC_{50} values for inhibition of this photoreduction were lower for Cu^{2+}, Fe^{2+} and Fe^{3+} 1:1 complexes than for uncomplexed flavonoids. For EC, the IC_{50} values for inhibition of this reaction were similar for all metal complexes. A postulated mechanism for the pseudo-superoxide dismutase activity of flavanol-iron complexes is bases on a reversible reduction and oxidation of iron bound to hydroxyl groups in the positions 4′ and 5′ of the B ring. This activity was suggested as a reason for the protective effect of the complexes on hepatocytes against hypoxia:reoxygenation injury (Zhao, Khan, & O'Brien, 1998).

We were unable to demonstrate the superoxide dismutase-like activity of iron (and in some systems copper) mixtures of flavanols. Flavanol mixtures did not inhibit (Fe^{2+} mixtures) or accelerated (Fe^{3+} mixtures) pyrogallol and adrenalin autoxidation and were less rather than more effective than flavanols alone in the inhibition of photochemical NBT reduction. In the case of PMS-mediated NBT reduction, Fe^{2+} mixtures of C and EC were in some cases significantly more effective in the inhibition of the reaction than C and EC alone, but the situation was changeable, mainly opposite for Fe^{2+} mixtures of other flavanols. It should be mentioned that all the assays of superoxide dismutase activity employed are indirect and based on complex reactions generally catalyzed by metal ions (Marklund & Marklund, 1974; Misra & Fridovich, 1972; Beauchamp & Fridovich, 1971; Ewing & Janero, 1995) and metal ions chelators are usually included in the assay media. We did not use chelators to avoid metal ion removal from the flavanol mixtures. In such mixture systems, antioxidants may not only scavenge superoxide, but also interfere with reactions leading to superoxide formation, which makes the systems less predictable and complicates interpretation of results. We checked also if Fe^{2+} mixtures acquire catalase activity, but the results obtained do not confirm such a possibility.

It should be remembered that reactions of flavonoids with iron ions are complex and may involve both reduction of Fe^{3+}, as well as oxidation of Fe^{2+} (Yoshino & Murakami, 1998). Therefore, Fe^{3+} ions can appear in flavanol-Fe^{2+} mixtures, especially during prolonged incubation, and may express prooxidant properties, as revealed in this study in the case of pyrogallol autoxidation.

The relevance of this *in vitro* study with respect to the *in vivo* situation may seem limited since, during the absorption in the jejunum, most of flavanols is subject to methylation and *O*-methylglucuronidation (Kuhnle et al., 2000; Del Rio et al., 2013). Nevertheless, interaction of iron with flavanols in the gastrointestinal tract may affect their bioavailability and susceptibility to the action of glucuronyl-transferases, methyl transferases and other metabolizing enzymes. This subject deserves further studies.

In summary, our results demonstrate that flavanol-Fe^{2+} mixtures retain most of antioxidant activity of the parent flavanols so, even after complexing subsaturating amounts of iron, flavanols are still potent antioxidants. However, no gain of antioxidant function of the mixtures was detected.

Acknowledgements

This study was performed within the project 'Antioxidant Power Series as a tool rational design and assessment of health promoting properties of functional food based on antioxidant phytochemicals" (2014/14/A/ST4/00640) financed by National Science Centre, Poland within a programme 'MAESTRO 6" and research fund of the University of Rzeszów, Poland (grant WBR/ZBA/PB/1/2017). We are grateful to Ivan Ionov (University of Łódź), Dr. hab., for measurements of hydrodynamic aggregate size and Edyta Bieszczad-Bedrejczuk (University of Rzeszów), M.Sc., for her excellent technical help.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.06.076.

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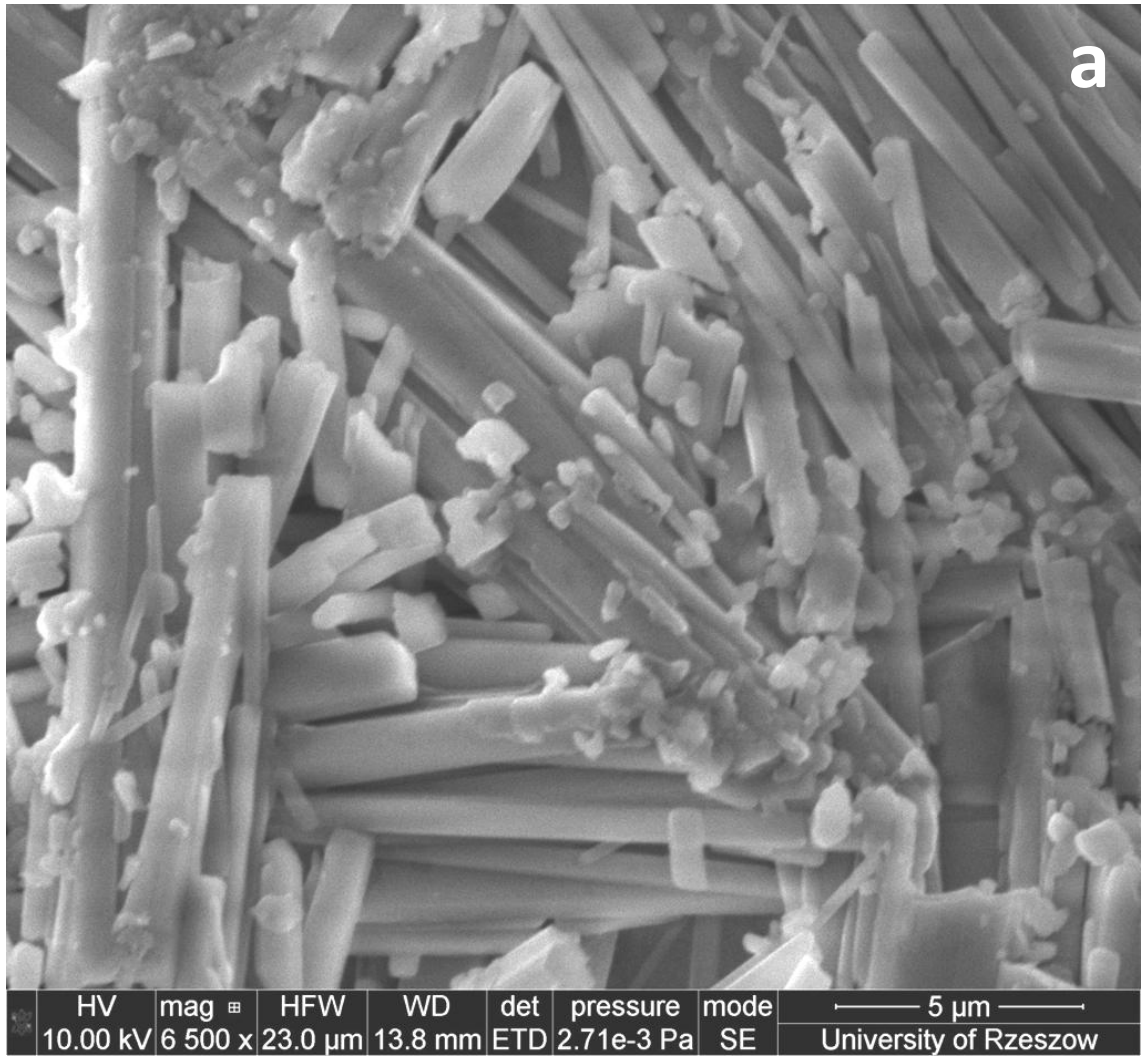
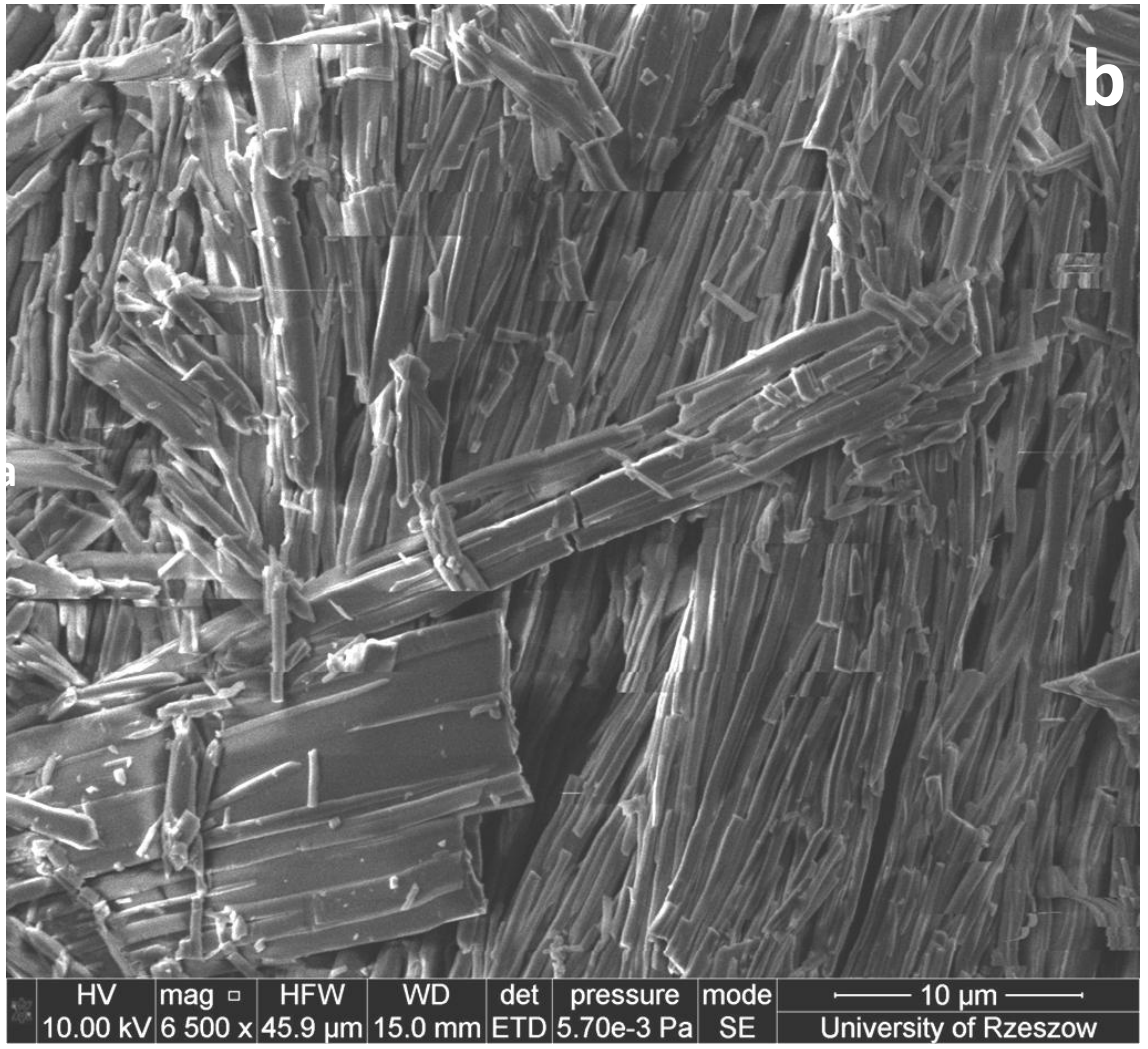
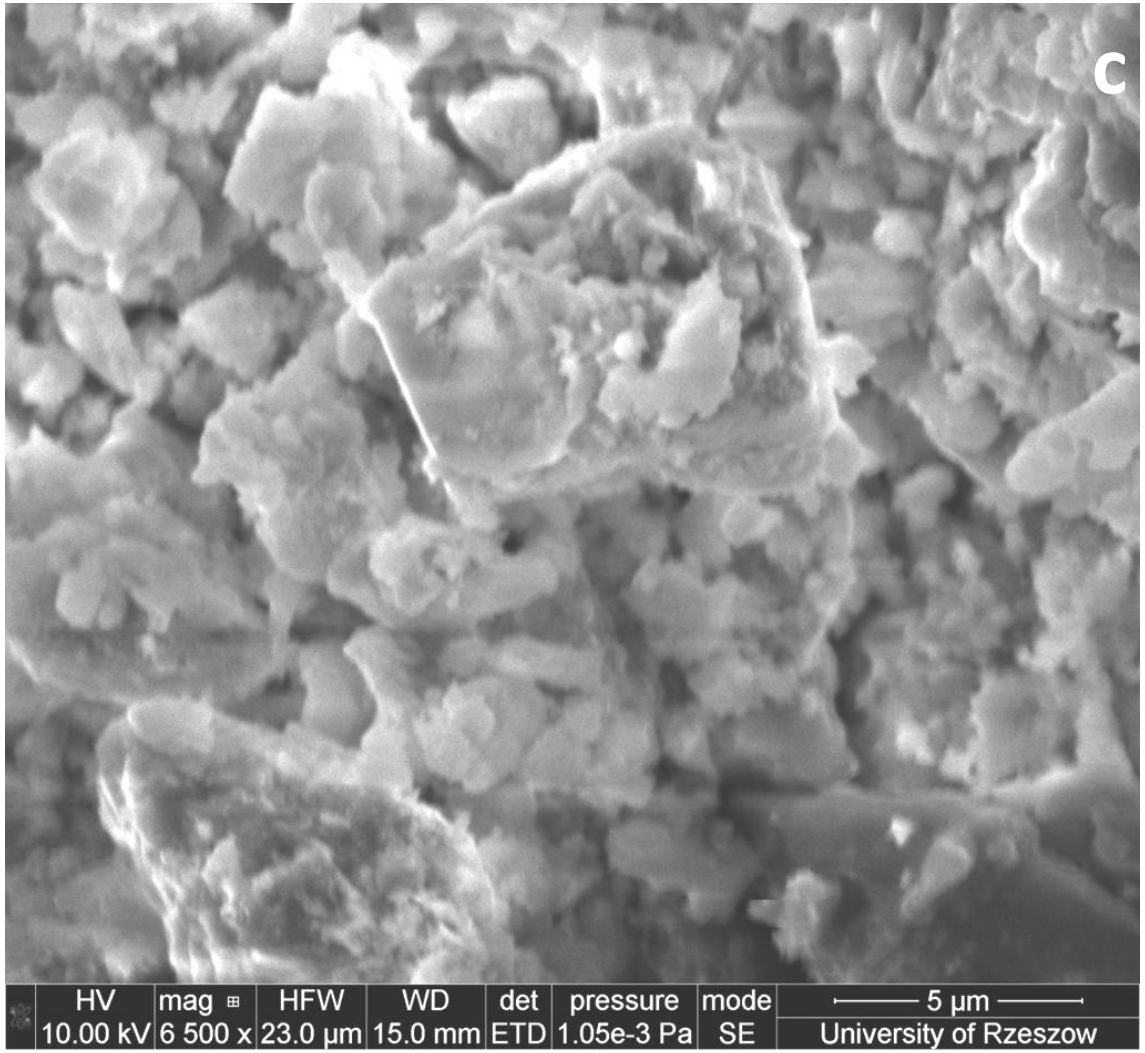
Fig. 1S. SEM images of flavanols and flavanol:Fe^{2+} mixtures (4:1).

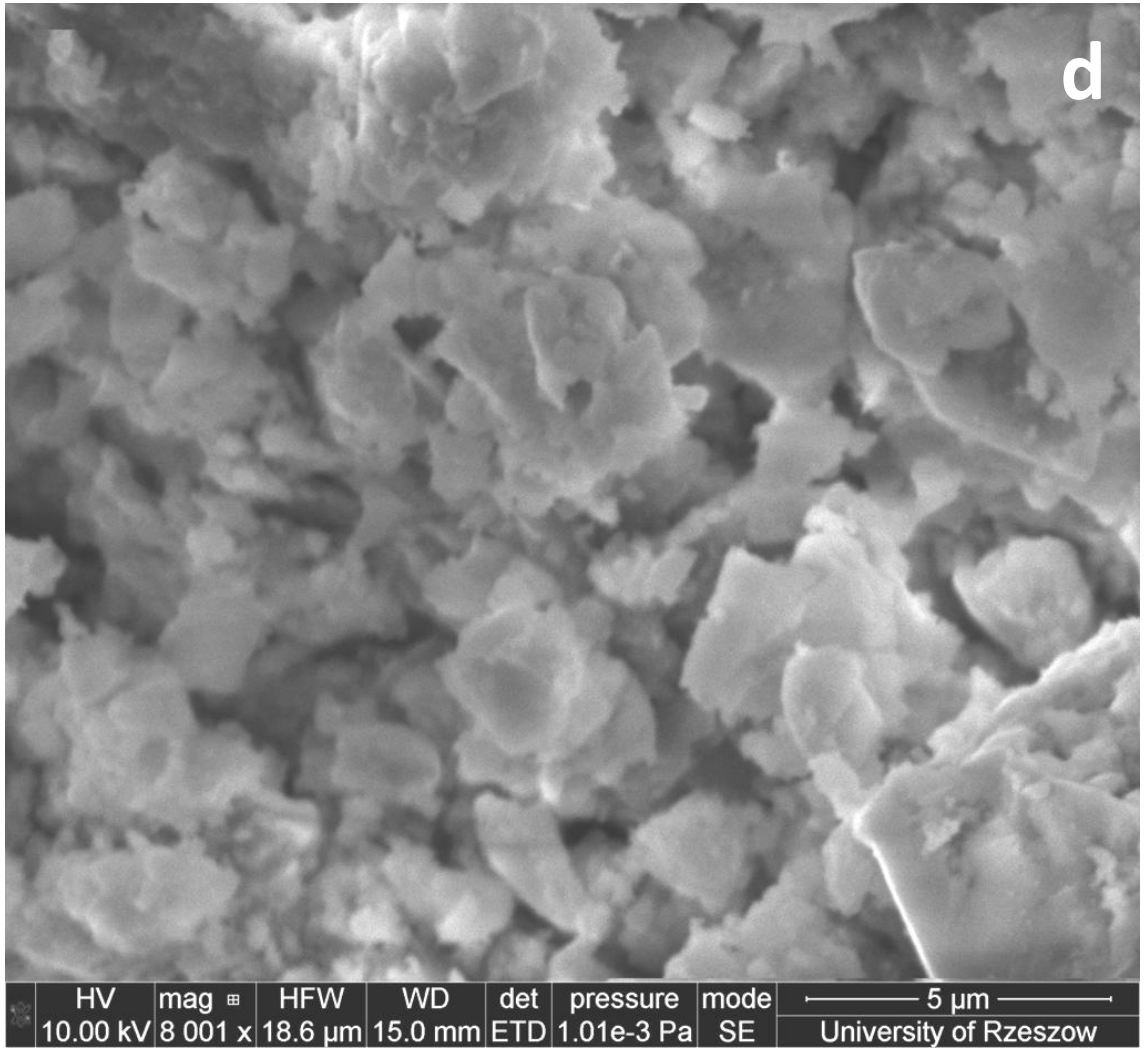
SEM images: a) Mohr's salt b) (+)-Catechin



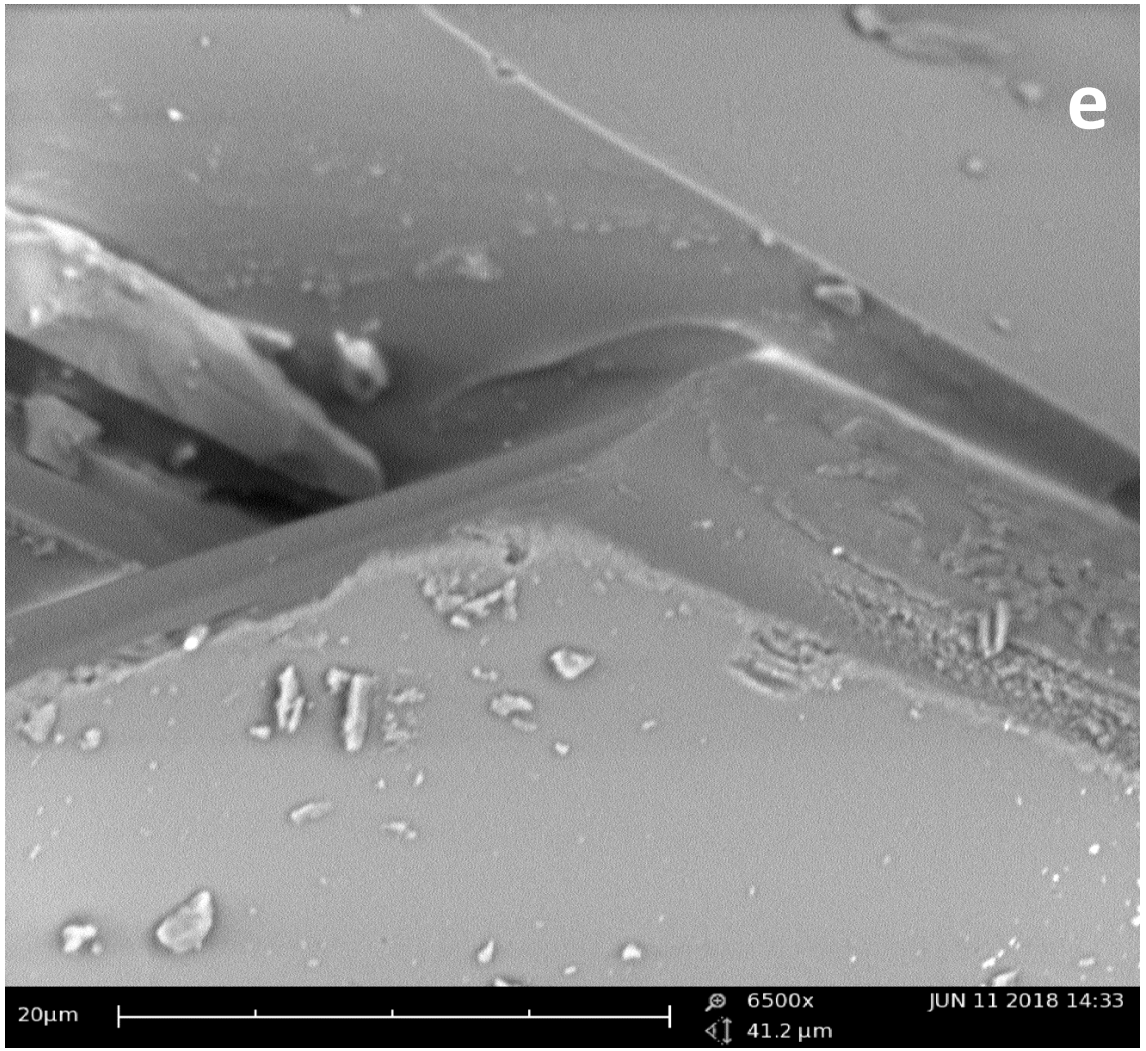


SEM images: c-d) (+)-Catechin with Mohr's salt

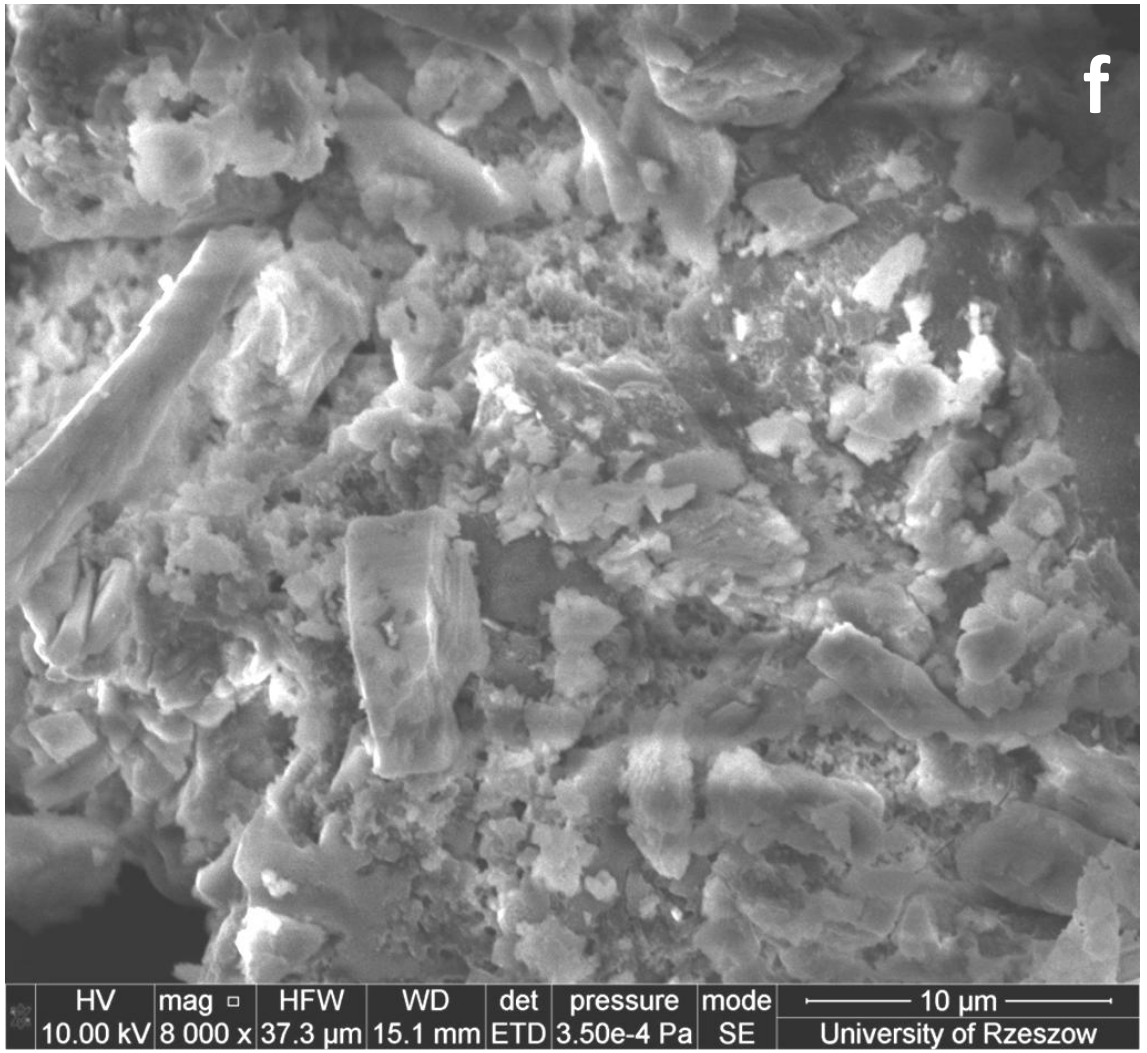




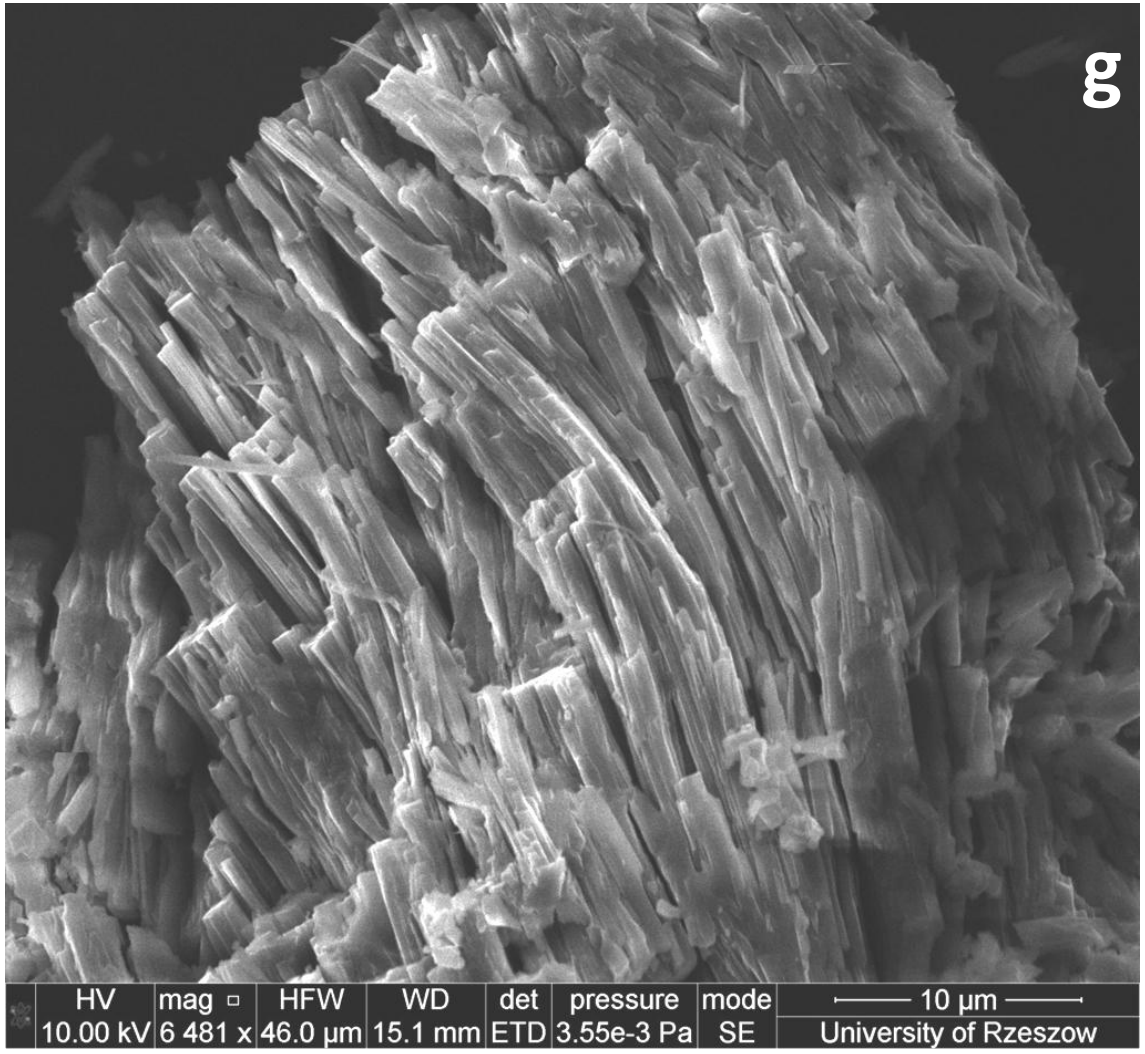
e) (-)-Epicatechin



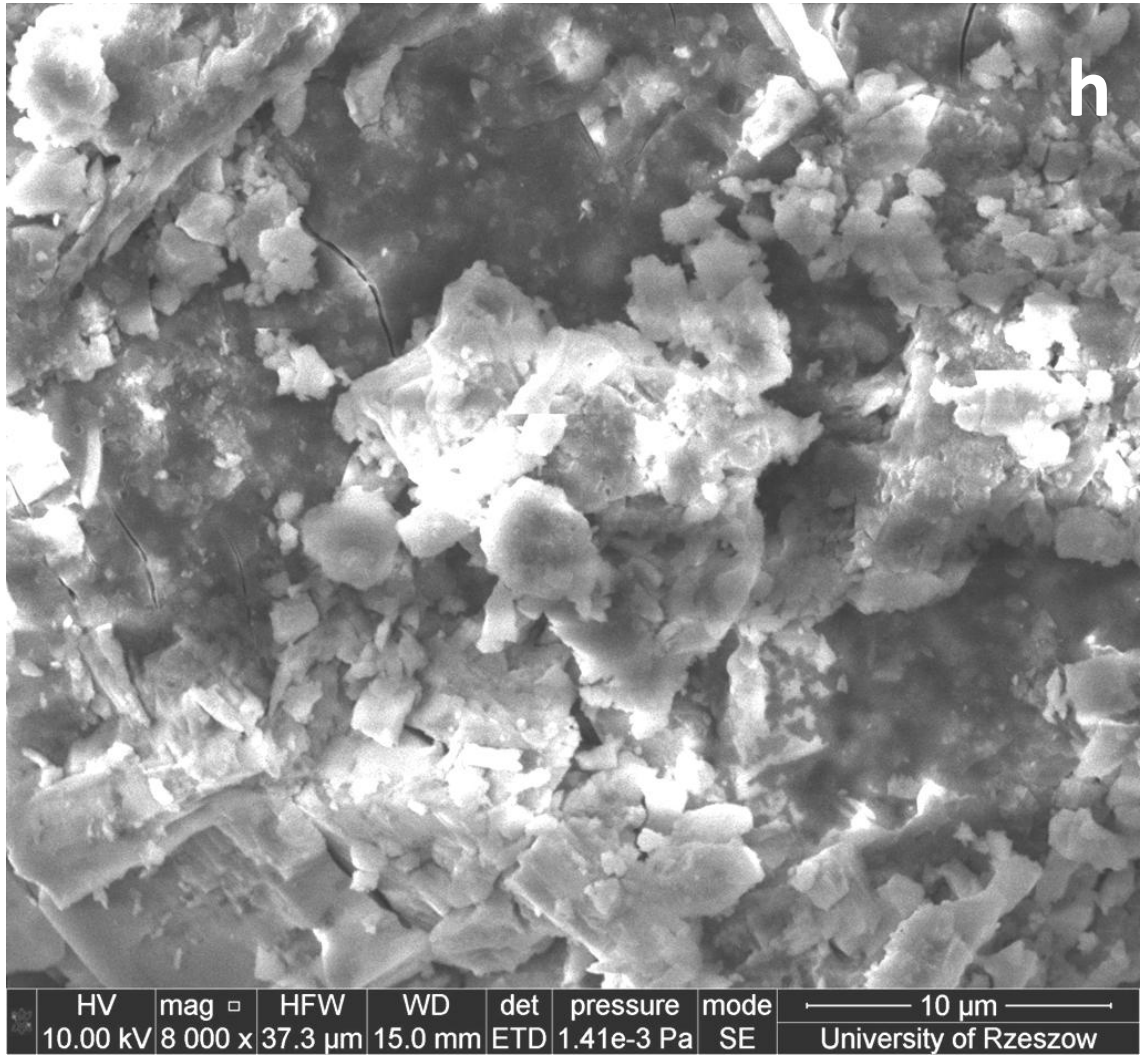
f) (-)-Epicatechin with Mohr's salt



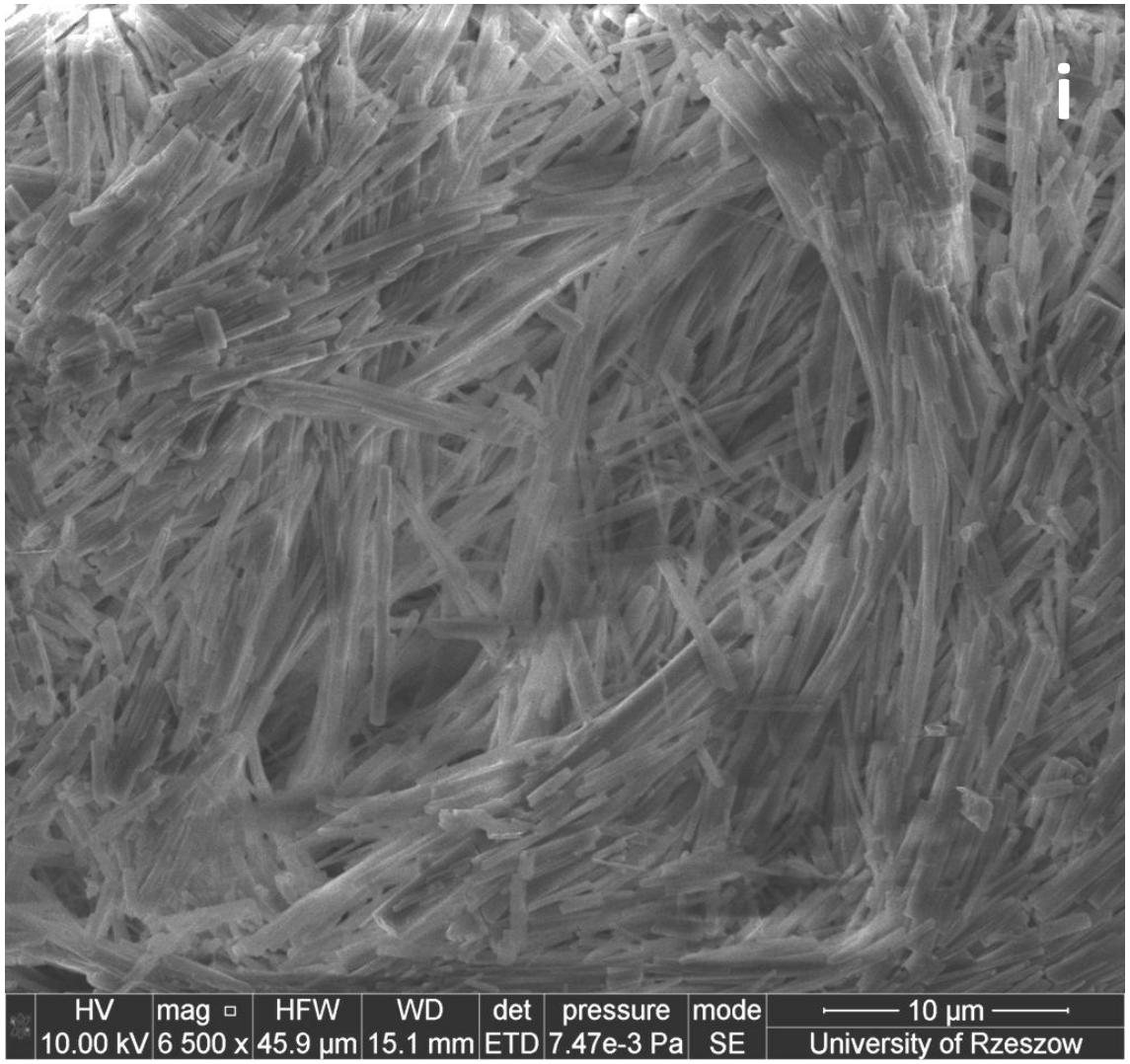
g) (-)-Epigallocatechin



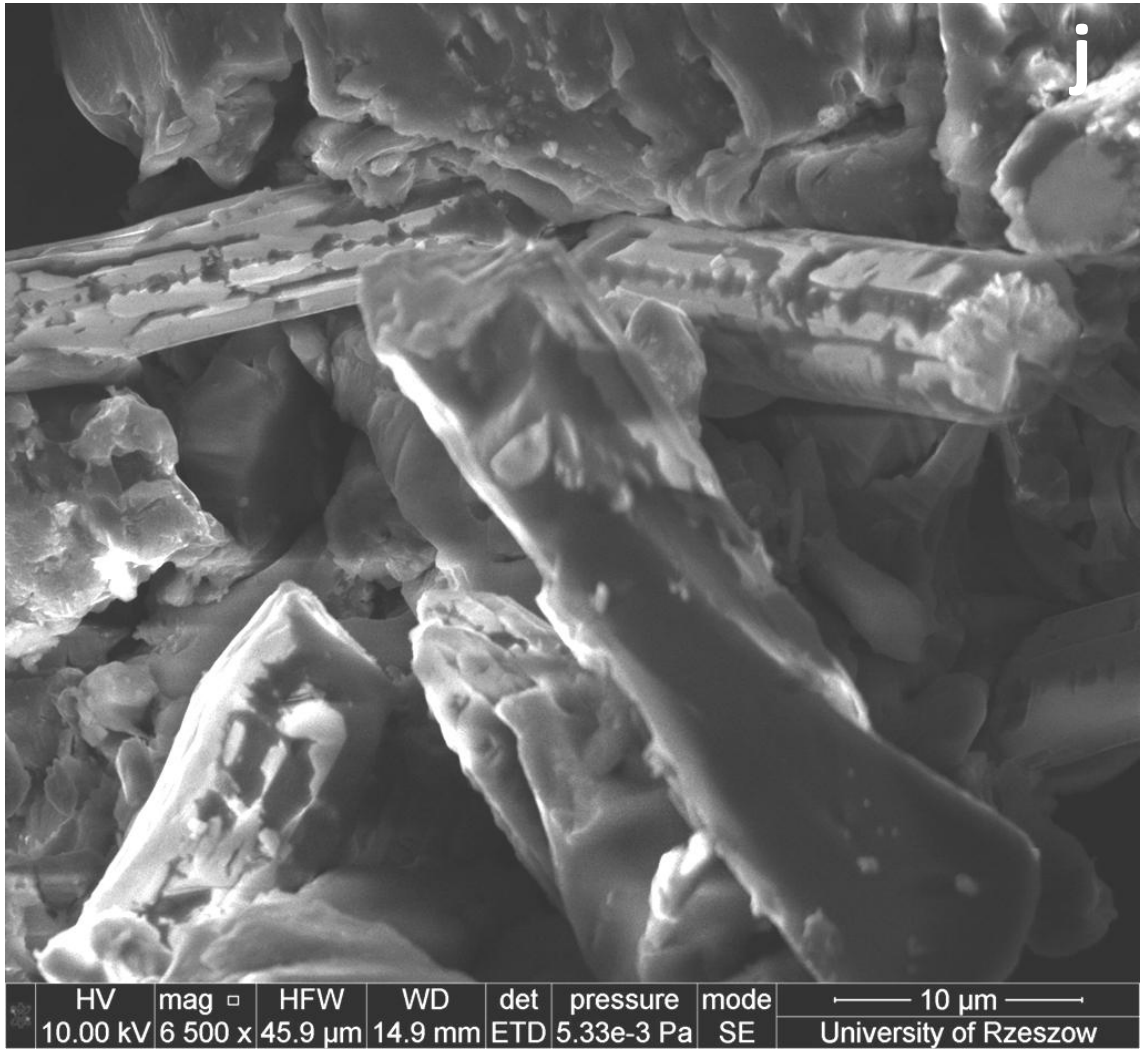
h) (-)-Epigallocatechin with Mohr's salt

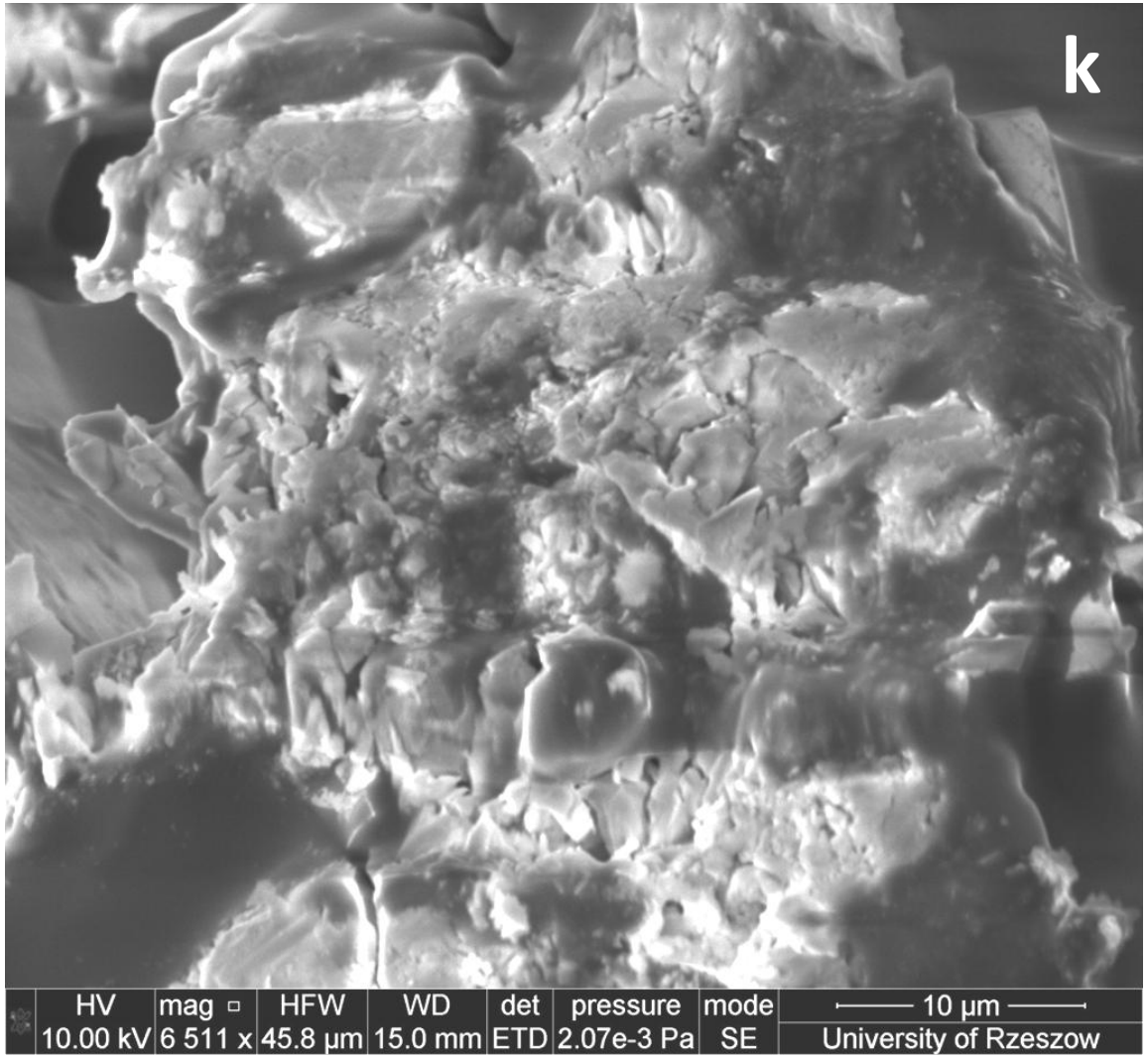


i) (−)-Epicatechin gallate

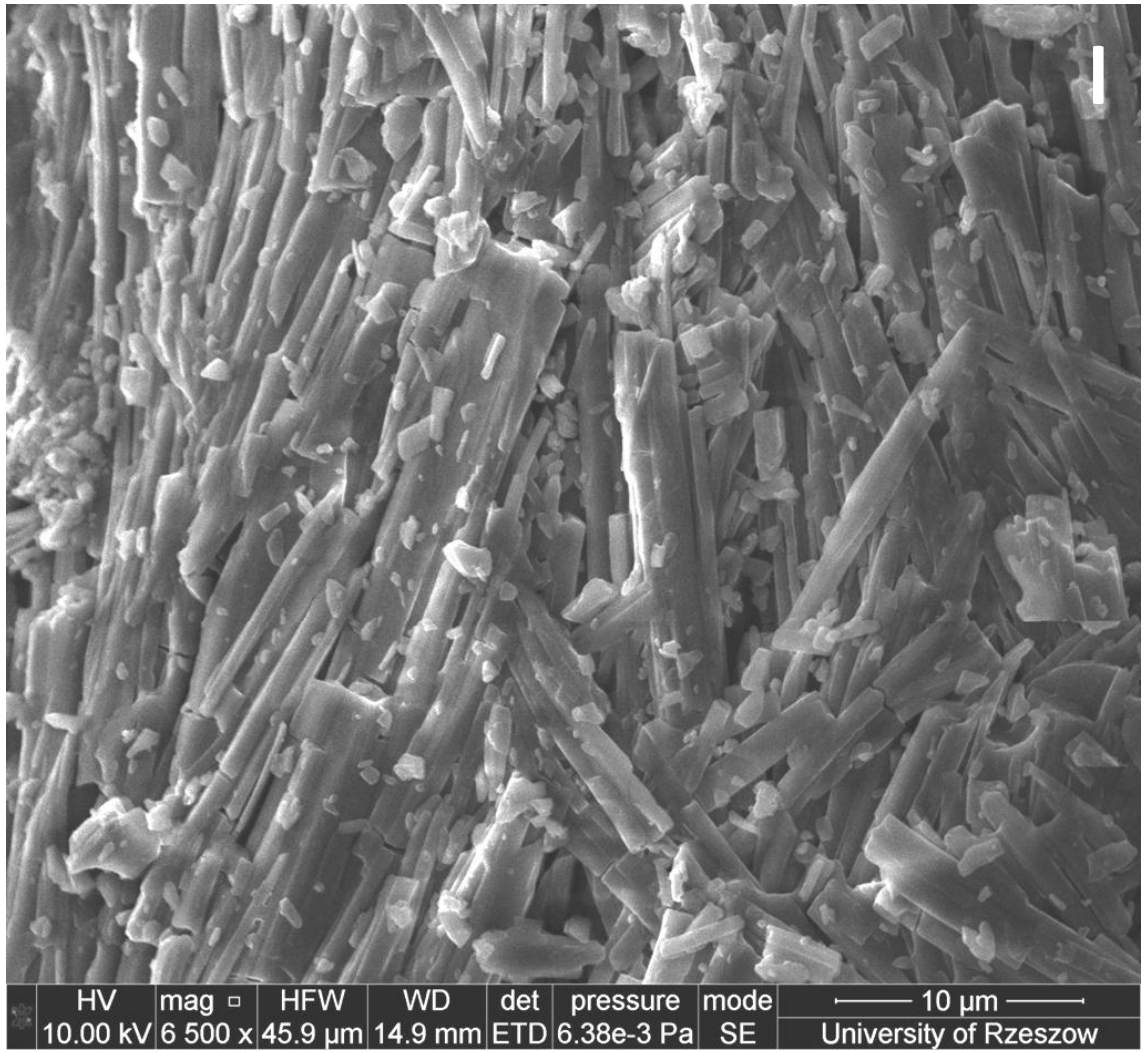


j-k) (−)-Epicatechin gallate with Mohr's salt





l) (−)-Epigallocatechin gallate



m) (-)-Epigallocatechin gallate with Mohr's salt

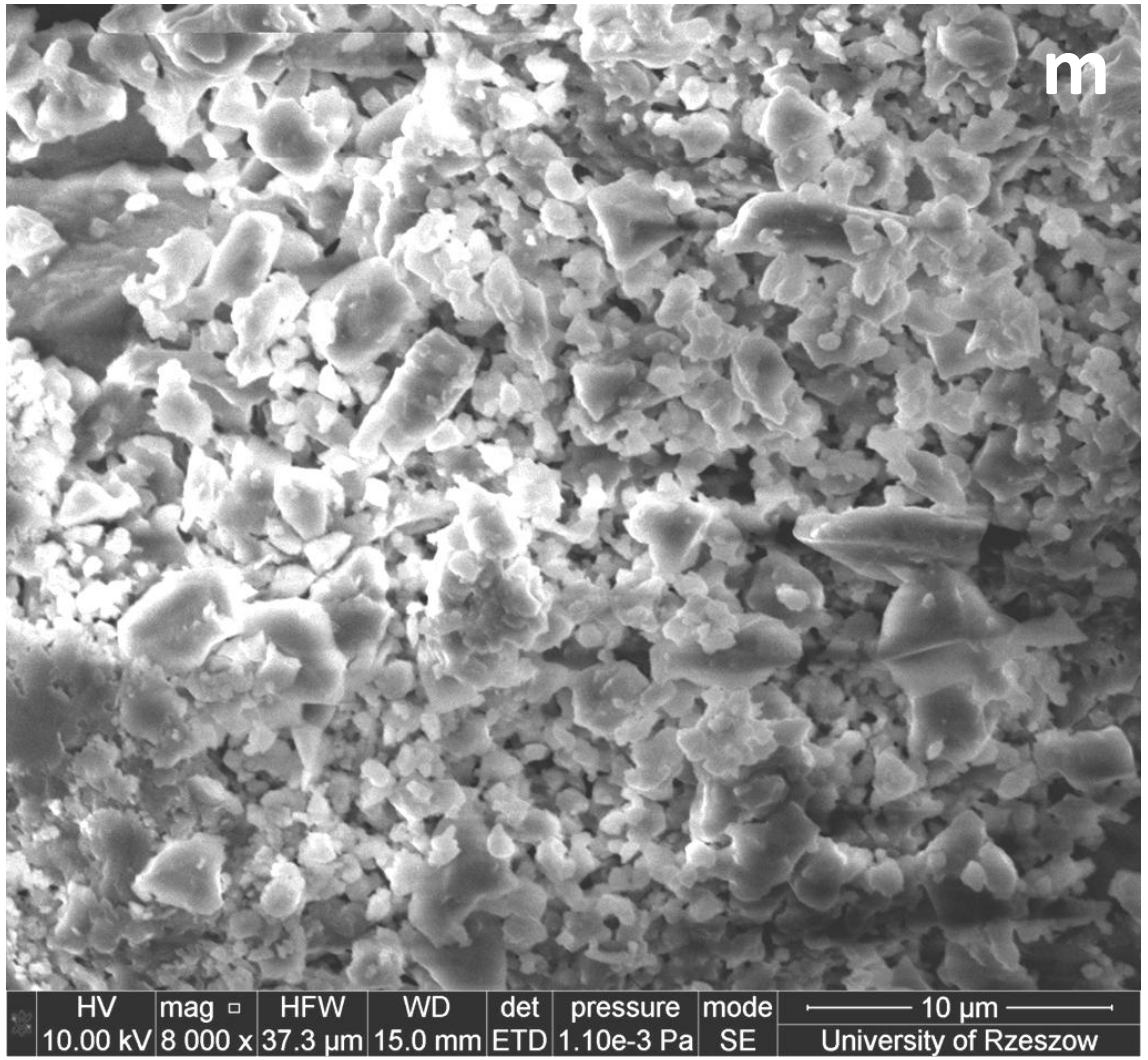


Fig. 2S. Protection against photochemical NBT reduction by EGC-G and its Fe^{2+} mixture. SD bars (not exceeding 5%) omitted for clarity.

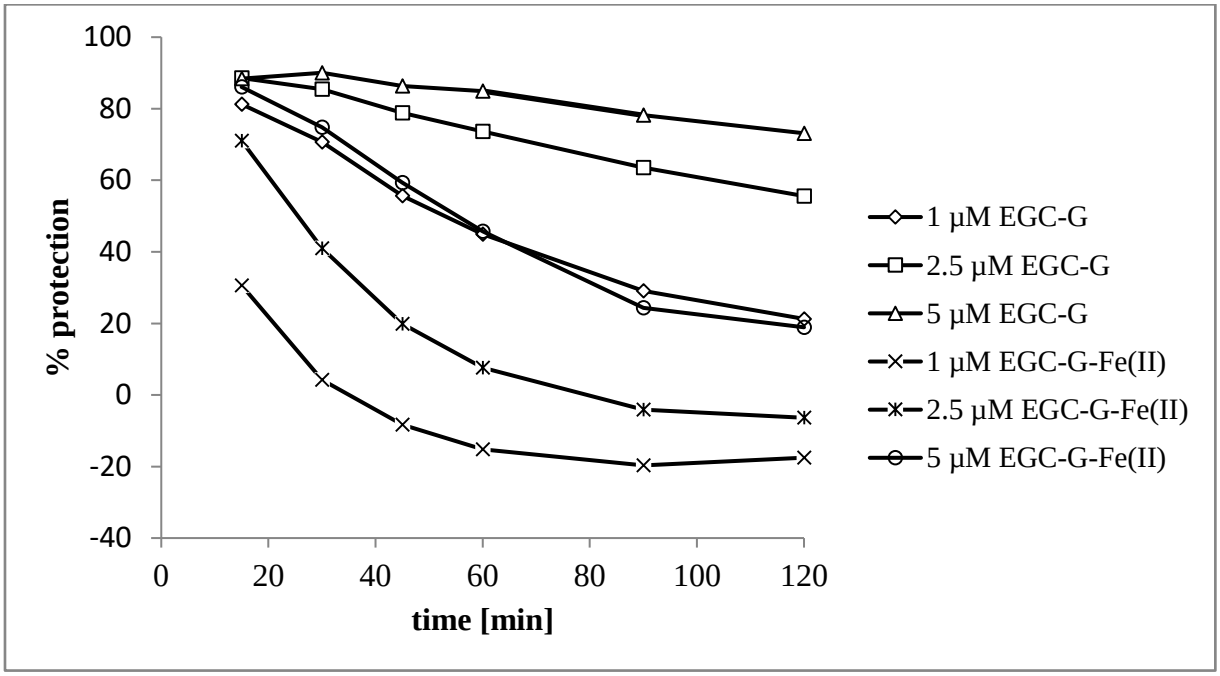
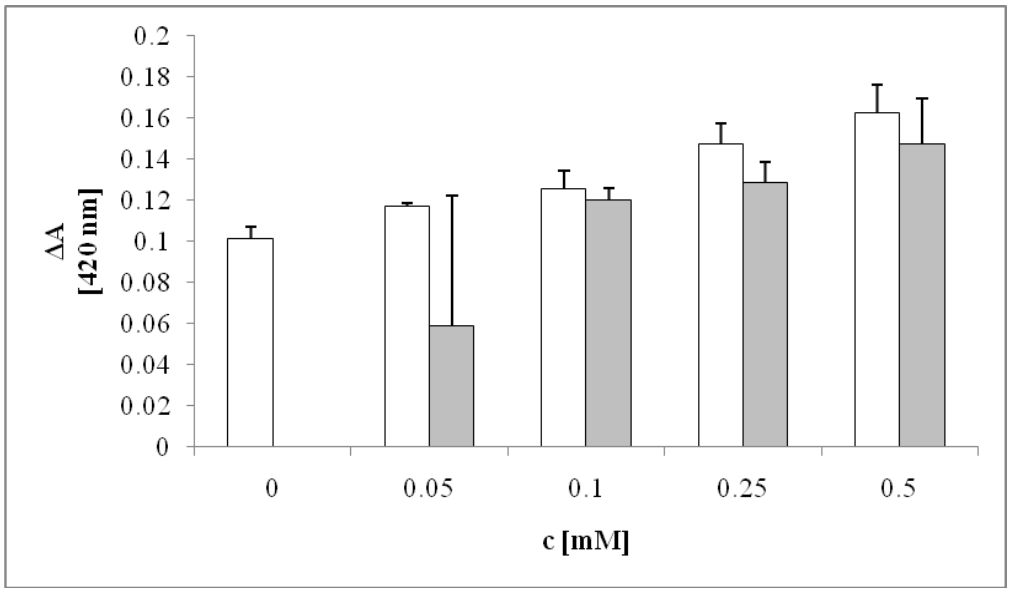
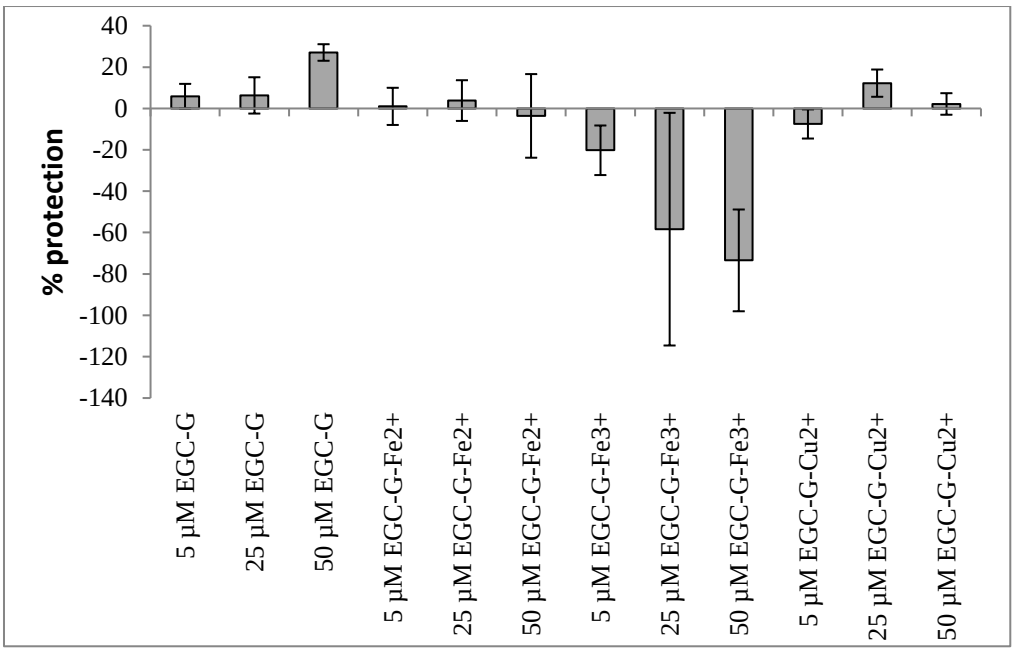


Fig. 3S. Effect of EGC-G and its Fe^{2+}, Fe^{3+} and Cu^{2+} mixtures on the autoxidation of pyrogallol (top) and adrenalin (bottom). Panel A: empty boxes, EGC-G, full boxes, EGC-G-Fe^{2+}.





Food Chemistry 278 (2019) 692–699

Contents lists available at ScienceDirect

Food Chemistry

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

Dietary antioxidants as a source of hydrogen peroxide

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ARTICLE INFO

Keywords:
Antioxidant
Autoxidation
Ascorbic acid
Hydrogen peroxide
Polyphenols
Tea

Chemical compounds studied:
N-Acetylcysteine, CID: 12035
Aminoguanidine hydrochloride, CID: 2734687
Apigenin, CID: 5280443
L-Ascorbic acid, CID: 54670067
Betanin, CID: 54600918
2,6-di-*tert*-Butyl-4-methylphenol, CID: 66609
tert-Buthylhydroquinone, CID: 16043
Buthylhydroxyanizole, CID: 24667
Caffeic acid, CID: 689043
β-Carotene, CID: 5280489
(+)-Catechin, CID: 9064
Chlorogenic acid, CID: 1794427
Citric acid, CID: 311
p-Coumaric acid, CID: 637542
Curcumin, CID: 969516
L-cysteine, CID: 5862
Daidzein, CID: 5281708
(−)-Epicatechin, CID: 72276
(−)-Epicatechin gallate, CID: 107905
(−)-Epigallocatechin, CID: 72277
(−)-Epigallocatechin gallate, CID: 65064
Ethoxyquin, CID: 3293
trans-Ferulic acid, CID: 445858
Gallic acid, CID: 370
Genistein, CID: 5280961
Gentisic acid, CID: 3469
L-Glutathione, CID: 124886

ABSTRACT

Studies of 54 antioxidants revealed that 27 of them, mainly polyphenols, generated hydrogen peroxide (H_2O_2) when added to Dulbecco's modified Eagle's medium (DMEM), other media used for culture of mammalian and yeast cells and phosphate-buffered saline. The most active antioxidants were: propyl gallate (PG), (−)-epigallocatechin gallate (EGCG) and quercetin (Q). Chelex treatment and iron chelators decreased H_2O_2 generation suggesting that transition metal ions catalyze antioxidant autoxidation and H_2O_2 production. Green tea also generated H_2O_2; tea prepared on tap water generated significantly more H_2O_2 than tea prepared on deionized water. Ascorbic acid decreased H_2O_2 production although it generated H_2O_2 itself, in the absence of other additives. Lemon added to the tea significantly reduced generation of H_2O_2. Hydrogen peroxide generated in the medium contributed to the cytotoxicity of PG, EGCG and Q to human prostate carcinoma DU-145 cells, since catalase increased the survival of the cells subjected to these compounds *in vitro*.

Abbreviations: ABTS˙, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical; ARE, Antioxidant Response Element; BHA, butylated hydroxyanisole; C, (+)-catechin; DETAPA, diethylenetriaminepentaacetic acid; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; H_2O_2, hydrogen peroxide; MEM, Eagle's Minimal Essential Medium; ESR, Electron Spin Resonance; NBT, Nitro Blue Tetrazolium; PBS, Phosphate-buffered saline; PG, Propyl Gallate; Q, quercetin; ROS, reactive oxygen species; SM, Yeast synthetic Minimal Medium; SOD, superoxide dismutase; YNB, Yeast Nitrogen Base; YPD, Yeast Peptone Dextrose; TAC, Total Antioxidant Capacity; TBHQ, *tert*-Butylhydroquinone; $O_2^{\cdot -}$, superoxide radical anion

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https://doi.org/10.1016/j.foodchem.2018.11.109
Received 6 September 2018; Received in revised form 19 November 2018; Accepted 22 November 2018
Available online 30 November 2018
0308-8146/

Glycitein, CID: 5317750
Hesperetin, CID: 72281
Hesperidin, CID: 10621
Hydrocinnamic acid (3-Phenylpropionic acid), CID: 107
D-Isoascorbic acid, CID: 54675810
Mangiferin, CID: 5281647
Melatonin, CID: 896
Metformin hydrochloride, CID: 14219
L-methionine, CID: 6137
Morin, CID: 5281670
Naringenin, CID: 932
Naringin, CID: 442428
Oxaloacetic acid, CID: 970
D-pantothenic acid hemicalcium, CID: 11306073
Propyl gallate, CID: 4947
Pyrogallol, CID: 1057
Pyruvic acid, CID: 1060
Quercetin, CID: 5280343
trans-Resveratrol, CID: 445154
Rutin, CID: 5280805
Sinapic acid, CID: 637775
Sodium ascorbate, CID: 23667548
Sodium succinate, CID: 9020
Trolox, CID: 40634
Vanillic acid, CID: 8468

1. Introduction

From a chemical point of view, an antioxidant can be defined as a substance which, when present at a low concentration with respect to that of an oxidizable substrate, prevents or significantly delays oxidation of this substrate (Halliwell, 1990). Although in biological systems several other mechanisms of action of antioxidants can be distinguished, including inhibition of oxidant-producing enzymes and chelation of metal ions catalyzing oxidation reactions, the main way of an antioxidant action is its sacrificial oxidation instead of the substrate. Such a reaction generates an oxidized form of the antioxidant compound and other reaction product(s). Under aerobic conditions, antioxidants are subject to oxidation by oxygen or reactive oxygen species (ROS), and this reaction may protect other substrates from oxidation. Other products of the reaction of antioxidant oxidation are reduced forms of oxygen: superoxide radical anion ($O_2^{\cdot -}$) in the case of one-electron oxidation or hydrogen peroxide (H_2O_2) in the case of two-electron oxidation. Eventually, dismutation of superoxide produces H_2O_2 so this product of oxygen reduction can be expected to accumulate as a result of oxidation of antioxidant compounds.

Generation of H_2O_2 due to oxidation of phenolic compounds, including gallic acid (Wee, Long, Whiteman, & Halliwell, 2003), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (+)-catechin (C), and quercetin (Q) (Long, Clement, & Halliwell, 2000; Halliwell, Clement, Ramalingam, & Long, 2000), hydroxytyrosol, delphinidin and rosmarinic acid (Long, Hoi, & Halliwell, 2010), ascorbic acid (Wee et al., 2003) as well as thiol compounds (cysteine, glutathione, N-acetylcysteine, gamma-glutamylcysteine, cysteinylglycine, cysteamine, homocysteine) in commonly used cell culture media, especially Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 Medium and Eagle's Minimal Essential Medium (MEM) (Hua Long & Halliwell, 2001) has been documented. Green and black tea, coffee and red wine, beverages rich in catechins, also produce H_2O_2 when added to cell culture media (Chai, Long, & Halliwell, 2003; Akagawa, Shigemitsu, & Suyama, 2003; Long, Lan, Hsuan, & Halliwell, 1999). Tea and coffee but not cocoa were shown to generate H_2O_2 to achieve levels of over 100 μM. Milk decreased net H_2O_2 production by beverages and showed some ability to remove H_2O_2 itself (Long et al., 1999). The production of H_2O_2 in these fluids was in good agreement with the content of phenolic compounds, suggesting that polyphenols are responsible for the generation of H_2O_2 in beverages (Akagawa et al., 2003).

Generation of H_2O_2 *in vivo* due to oxidation of antioxidant compounds has also been demonstrated. Holding green tea solution in the mouth or chewing green tea produces micromolar concentrations of H_2O_2 in the mouth (Lambert, Kwon, Hong, & Yang, 2007). Higher levels of H_2O_2 were found in urine of coffee drinkers and attributed to excretion of hydroxyhydroquinone from coffee and its oxidation in urine, resulting in H_2O_2 production (Hiramoto, Kida, & Kikugawa, 2002; Halliwell, Long, Yee, Lim, & Kelly, 2004). The H_2O_2 generated by autoxidation of antioxidants may not only introduce artefacts in cell culture experiments (Halliwell et al., 2000), but also contribute to bactericidal action and to paradoxical genotoxic and mutagenic activities of these substances, generally assumed to have beneficial effects on human health (Lluís et al., 2011; Gomes et al., 2018).

In spite of literature reports, the generation of H_2O_2 in culture media is still an underappreciated problem in model studies of biological effects of food components and antioxidants. In many cases it is unclear if the effects observed *in vitro*, contributed by H_2O_2 generated by antioxidant autoxidation, are relevant to *in vivo* conditions where this autoxidation is absent or strongly attenuated. Moreover, data on autoxidation and H_2O_2 production are available only for a limited number of antioxidants and data on a broader spectrum of antioxidants are lacking. Similarly, our knowledge of the H_2O_2 in food, beverages and our body due to autoxidation of food components is still limited. The aim of this study was to compare the propensity of over 50 of commonly used antioxidants, especially of natural origin (present in food and beverages) for autoxidation and generation of H_2O_2, and to get an insight into the mechanism of this effect.

2. Materials and methods

2.1. Materials

BD™ Difco™ Yeast Nitrogen Base and Bacto™ Peptone were purchased from Becton Dickinson Poland (Warsaw), D-(+)-glucose and Xylenol Orange were obtained from Polish Chemical Reagents (POCh, Gliwice, Poland), perchloric acid ($HClO_4$) was purchased from Chempur (Piekary Śląskie, Poland), phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO) were obtained from Lab Empire (Rzeszów,

Poland) and Dulbecco's Modified Eagle's Medium (DMEM), DMEM + GlutaMax Medium and DMEM/F12 Medium were purchased from Thermo Fisher Scientific (Warsaw, Poland). Nitro Blue Tetrazolium (NBT) was obtained from BioShop Canada Inc. (Burlington, Ontario, Canada). Stock solutions of NBT was freshly prepared in PBS before each experiment. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Selected antioxidants and all other reagents, if not mentioned otherwise, were purchased from Sigma (Poznan, Poland) and were of analytical grade.

Yeast Peptone Dextrose (YPD) medium had the following composition: Yeast Extract 1%, Bactopeptone 1%, glucose 2% in deionized water. Yeast synthetic minimal medium (SM) contained 0.67% Yeast Nitrogen Base and 2% glucose in deionized water. The media components were sterilized by autoclaving before use (glucose solution separately) and complete media were made by mixing of sterilized components.

Fluorometric and absorptiometric measurements were done in a Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). All measurements were performed in triplicate and repeated at least three times on different preparations. The compounds studies were dissolved in PBS or in DMSO/PBS (ECG, EC, C) (0.5 ml of 10 mM antioxidant solution was obtained by dissolving an antioxidant in 10 µl DMSO and then 490 µl PBS was added). Stock solutions of less water-soluble compounds were prepared in DMSO. The effect of DMSO was subtracted from the effects of antioxidants introduced in this solvent. Dimethyl sulfoxide at the concentration used did not generate detectable amounts of H_2O_2.

2.2. Assay of hydrogen peroxide generation

Standard protocol for evaluation of H_2O_2 generation by antioxidants consisted in addition of 18 µl of 10 mM antioxidant to 162 µl of DMEM or other media. The samples were incubated for various times (in the kinetic studies), and routinely for 3 h at 37 ± 1 °C with shaking and the peroxide content was estimated before and after incubation by the ferric-Xylenol Orange method (Gay & Gebicki, 2003). Catalase (10 µg/ml) was added to additive set of samples 15 min before end of incubation in order to check if the reaction product is H_2O_2. Then, to 180 µl samples, 20 µl of Xylenol Orange reagent was added [2.5 mM Xylenol Orange/2.5 mM Mohr's salt ($Fe_2(NH_4)_2SO_4$; purity of 99.997%) in 1.1 M perchloric acid]. After 30-min incubation at room temperature, absorbance of the samples was measured at 560 nm.

When estimating H_2O_2 production in the tea, one bag of green tea (Le speciale) purchased in a local food store was added with 200 ml of boiling water (deionized or tap water) and incubated for 3 h, without or in the presence of a slice of lemon (11.2 g).

2.3. Semiquinone detection

The semiquinone radical can be detected and quantified by electron paramagnetic resonance (EPR) spectrometry. For detection of semiquinone radical formation, 100 mM solutions of propyl gallate (PG) and EGCG were prepared in PBS containing 0.2 M zinc sulfate (to stabilize the semiquinone radicals) (Metodiewa, Jaiswal, Cenas, Dickancaité, & Segura-Aguilar, 1999). The Bruker multifrequency and multiresonance FT-EPR ELEXSYS E580 spectrometer (Bruker Analytische Messtechnik, Rheinstetten, Germany) operating at the X-band (9.837530 GHz). The following settings were used: central field, 3505.6 G; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 94.64 mW; power attenuation, 2.0 dB; scan range, 80 G; conversion time, 25 ms; and sweep time, 25.6 s. The spectra were recorded in 1024 channels; number of scans: 15.

2.4. Detection of superoxide formation

Superoxide formation during polyphenol autoxidation was assesses by superoxide dismutase (SOD)-inhibitable reduction of NBT and SOD-inhibitable oxidation of dihydroethidine. 1 mM PG, EGCG and Q were incubated in PBS in the presence of 100 µM NBT or 5 µM dihydroethidine, in the absence or in the presence of 10 µg/ml Cu,Zn-superoxide dismutase. Absorbance of formazan formed by NBT reduction was measured at 540 nm. Fluorescence of the reaction products of dihydroethidine oxidation was measured at the excitation/emission wavelengths of 405/570 nm, to maximize the fluorescence share of 2-hydroxyethidium (Nazarewicz, Bikineyeva, & Dikalov, 2013).

2.5. $ABTS^{\cdot}$ scavenging assay

Total antioxidant capacity (TAC) of the media was determined by reduction of the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{\cdot}$) (Grzesik, Naparło, Bartosz, & Sadowska-Bartosz, 2018), slightly modified with respect to the time of measurement. Briefly, 5-µl aliquots of the media were added to wells of a 96-well plate containing 245 µl of $ABTS^{\cdot}$ solution having absorbance of 1.00 at 734 nm. Absorbance decrease was measured after 1 and 30 min. The decrease in absorbance after 1 min was assumed to reflect the "*fast scavenging*" of $ABTS^{\cdot}$ while the decrease in absorbance between 1 min and 30 min to reflect "*slow scavenging*". Standard curve obtained for the scavenging of Trolox enabled to express the $ABTS^{\cdot}$ scavenging activity in Trolox equivalents.

2.6. Scavenging of superoxide and hydrogen peroxide by the media

Scavenging of superoxide by the media was estimated by a modified method of Minami and Yoshikawa (1979). The assay system consisted of 167 µM pyrogallol, 50 mM Tris-HCl buffer containing 100 µM diethylenetriaminepentaacetic acid (DETAPA) and various volumes of the media in a final volume of 300 µl. Absorbance was measured at 540 nm every minute in a plate reader for 10 min. One unit of superoxide scavenging activity was defined as the amount of the medium decreasing the rate of NBT reduction (0.005/min in the control) by 50%.

To estimate scavenging of H_2O_2 by the media, H_2O_2 was added to the media to the final concentration of 30 µM. The peroxide concentration was estimated immediately and after 3 h incubation at 37 °C in the dark (unless stated otherwise), according to Gay and Gebicki (2003).

2.7. Fe^{2+}-binding capacity of the media

Fe^{2+}-binding capacity of the media was estimated by their competition for Fe^{2+} with ferrozine. Briefly, various amounts of the media were added to a solution containing 60 µM (final concentration) $Fe_2(NH_4)_2SO_4$ (Mohr's salt) in 20 mM sodium phosphate buffer, pH 7.0. Then ferrozine was added to a concentration of 200 µM, the solution was mixed and after 3 min absorbance of the solution was measured at 562 nm. From the dependence of absorbance of the volume of the medium, the medium volume decreasing absorbance of ferrozine to 50% of a value obtained without medium added. For some media, this method did not work due to interference with absorbance of the medium.

2.8. Assay of cytotoxicity of H_2O_2-generating antioxidants

Human prostate carcinoma DU-145 cells (ATCC® HTB-81™) were seeded into wells of a 96-well plate and cultivated in DMEM-F12 HAM (Sigma, St. Louis, MO) medium supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin and 10 µg/ml streptomycin in humidified atmosphere with 5% CO_2 at 37 °C for 24 h. Then, the medium was removed and replaced with a medium containing PG, EGCG or Q at various concentration, without or with 10 µg/ml catalase. The cells were cultivated for 24 h, then the medium was removed and replaced by 100 µl/well of a 2% solution of Neutral Red in the medium.

The cells were kept in an incubator for 1 h, washed twice with PBS, added with 100 µl/well of 50% ethanol/49% H_2O/1% glacial acetic acid and shaken for 15 min. For Q, the control contained 0.2% DMSO (concentration identical to that introduced with Q at the maximal concentration). Such a concentration of DMSO did not have any discernible effect on cell survival. Then, absorbance in the wells was measured at 540 nm vs 620 nm. All experiments were done in triplicate on different preparations.

2.9. Statistical analysis

The data are presented as mean ± standard deviation. The significance of differences of the examined parameters among samples was checked using a two-tailed Student's t test. Kruskal-Wallis test was performed to estimate differences between antioxidant treated and non-treated cells. Differences between antioxidant and antioxidant with catalase treated cells were tested using the U Mann-Whitney test. $P \leq 0.05$ was considered as statistically significant in both cases. Every test was performed in triplicates.

Statistical analysis of the data was performed using STATISTICA software package (version 13.1, StatSoft Inc. 2016, Tulsa, OK, USA, www.statsoft.com).

3. Results

Incubation of 54 various antioxidants in DMEM medium (37 °C, 3 h) revealed generation of a product reactive in the peroxide assay by 27 antioxidants, mainly natural food components. No generation of H_2O_2 was detected for other 27 antioxidants (Table 1). The kinetics of peroxide generation was not linear; the highest rate was observed in the initial phase of incubation; then the rate of generation decreased gradually and the H_2O_2 level reached practically a plateau (Fig. 1), probably as a result of equilibrium between the rate of generation and decomposition (see below). Since the kinetics differed for different compounds we decided to present data for 3 h when reaching a plateau level was probable for all compounds studied. Incubation of the reaction mixture with catalase (10 µg/ml) for 15 min resulted in the disappearance of this product demonstrating that the product is H_2O_2 (not shown). PG, EGCG and Q were the most active antioxidants generating H_2O_2 in the DMEM media; therefore, these substances were used in further comparative studies. Ascorbic acid and sodium ascorbate generated substantial amounts of H_2O_2; no H_2O_2 was generated, however, when not neutralized solution of ascorbic acid was added to the medium, decreasing the final pH.

Autoxidation of antioxidants produced H_2O_2 also in other media used for culture of mammalian cells (DMEM + GlutaMAX, DMEM/F12) and yeast cells (YPD and SM media) as well as in PBS, as demonstrated for three antioxidants most active in the DMEM medium. Much less peroxide was generated in media used for yeast culture (Table 2). The polyphenols studied produced in PBS amounts of H_2O_2 comparable to those DMEM media, so PBS was mainly used in further experiments. Inclusion of 10% fetal calf serum (FCS) to the media to imitate fully cell culture conditions increased the generation of H_2O_2 (Table S1).

In order to check the effects of possible interactions of antioxidants in the autoxidation process, we checked the effect of ascorbate on the H_2O_2 production by the three most active polyphenol antioxidants. Increasing concentrations of ascorbate decreased the production of H_2O_2, to zero for PG and EGCG, and to about 1/3 at 1 mM ascorbate for Q (Fig. 2). Ascorbic acid (neutralized to pH 7.0) or sodium ascorbate generated significant amounts of H_2O_2, but aqueous solution of ascorbic acid without neutralization did not generate detectable amounts of hydrogen peroxide.

Light did not accelerate the autoxidation of polyphenols; production of H_2O_2 by PG, EGCG and Q incubated in the DMEM medium amounted to 139 ± 11, 96 ± 12 and 123 ± 9%, respectively, of that observed upon incubation in a daylight.

Removal of the transition metal ions from PBS by Chelex treatment before incubation, as well as the presence of iron chelators, desferoxamine (DFO) and DETAPA decreased H_2O_2 production (Table S2).

Upon autoxidation of PG and EGCG, semiquinone radicals of these substances were detected by electron spin resonance (ESR) (Fig. S1). Formation of superoxide radicals was demonstrated by reduction of NBT and oxidation of dihydroethidine upon autoxidation of EGCG and PG, partly inhibited by SOD (Fig. S2). Measurement for NBT reduction accompanying Q autoxidation was not reliable, because of change of absorbance of the solution absorbing at 540 nm.

In order to get an insight into the factors responsible for differences in the amount of H_2O_2 generated in various media, TAC of the media, their capacity of iron binding, as well as scavenging of superoxide and scavenging of H_2O_2 was studied. Total "fast" antioxidant capacity of the YPD medium was higher in comparison to the DMEM media but that of the SM medium was significantly lower. The "slow" scavenging capacity of the medium was lower than those of the DMEM media and higher than that of the SM medium. Fe^{2+} binding was comparable for the DMEM and YPD media, nevertheless it was also significantly lower for the SM medium (Table S3).

DMEM showed the highest superoxide scavenging activity, while the superoxide scavenging activity of the YPD medium was the lowest. Interestingly, the media, except for the SM medium, decomposed added H_2O_2, the activity being comparable for all the media studied. None of the parameters studied could be correlated with the differences in the H_2O_2 generation in different media, and especially with the low H_2O_2 production in the media used for yeast culture. Another factor which could influence antioxidant autoxidation and H_2O_2 generation in

Table 1
Generation of hydrogen peroxide in DMEM medium [(mean ± SD; n ≥ 3 (independent samples)]. The following substances did not produce detectable amounts of H_2O_2: *N*-acetylcysteine, apigenin, betanin, *tert*-buthylhydroquinone, 2,6-di-*tert*-butyl-4-methylphenol, buthylhydroxyanizole, β-carotene, citric acid, p-coumaric acid, curcumin, L-cysteine, daidzein, ferulic acid, glutathione, glycitein, hesperetin, melatonin, metformin, morin, naringenin, naringin, oxaloacetic acid, D-panthotenic acid, pyruvic acid, sodium succinate, Trolox, vanillic acid.

Compound	H_2O_2 [µM]
Propyl gallate (PG)	95.2 ± 1.9***
Pyrogallol	94.5 ± 1.7***
(−)-Epigallocatechin gallate (EGCG)	90.2 ± 2.6***
Quercetin (Q)	76.4 ± 5.9**
(−)-Epicatechin	44.9 ± 2.2***
Isoascorbic acid	38.6 ± 1.3***
Ascorbic acid#	33.6 ± 0.9***
Sodium ascorbate	28.4 ± 5.1**
Kaempferol	23.9 ± 1.4***
Mangiferin	21.7 ± 0.7***
(−)-Epigallocatechin (EGC)	21.2 ± 3.0**
Caffeic acid	19.9 ± 0.5***
Resveratrol	13.7 ± 0.5***
Gentisic acid	14.7 ± 0.4***
Chlorogenic acid	11.3 ± 2.2**
(−)-Epicatechin gallate	11.0 ± 2.7**
Hydrocinnamic acid	7.1 ± 3.6*
Sinapic acid	7.0 ± 3.0*
Methionine	6.1 ± 0.9**
Gallic acid	5.2 ± 0.7**
Rutin	4.3 ± 2.0*
Genistein	3.3 ± 1.1*
Hesperidin	2.7 ± 0.3**
(+)-Catechin	2.6 ± 0.8*
Ethoxyquin	2.2 ± 0.3**
Aminoguanidine	1.6 ± 0.5*
p-Coumaric acid	0.6 ± 0.2*

#No H_2O_2 was generated when not neutralized ascorbic acid was used. Generation significantly different from 0: *P < 0.05, **P < 0.01, ***P < 0.001 (paired Student's "*t*" test).

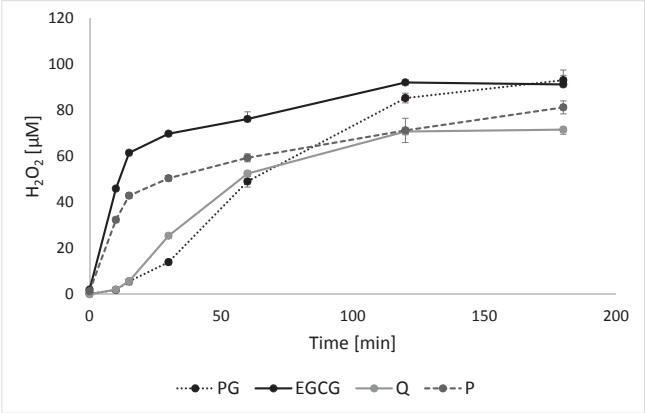

Fig. 1. Kinetics of hydrogen peroxide generation in DMEM medium by 1 mM compounds (propyl gallate, PG, epigallocatechin gallate, EGCG, quercetin, Q and pyrogallol P).

various media is their pH which, was lower for yeast culture media (YPD: 5.85, SM: 5.92) than for media used for mammalian cell culture (DMEM: 7.42, DMEM/F12: 7.25, DMEM + Glutamax: 7.33).

Since ECGC and other flavanols are present in high amounts in the tea, we studied H_2O_2 production in freshly prepared tea. Tea prepared on tap water generated much more H_2O_2 than that prepared on Milli-Q-filtered water. Ascorbic acid induced a concentration-dependent decrease in H_2O_2 production in tea prepared on deionized water. With tea prepared on tap water, low concentrations of ascorbic acid slightly increased H_2O_2 production, but 1 mM ascorbic acid decreased it. Addition of lemon, known to be rich in ascorbic acid, significantly decreased H_2O_2 production in the tea (Fig. 3).

In order to examine the effect of H_2O_2 generated by polyphenols in cell culture medium on cells, we compared the cytotoxicity of PG, EGCG and Q to cultured DU-145 cells. In all cases the survival of cells subjected to increasing concentrations of these compounds was significantly higher in the presence of catalase, a H_2O_2-decomposing enzyme (Fig. 4).

4. Discussion

Production of H_2O_2 due to autoxidation of some antioxidants, especially polyphenols, in cell culture media have been reported previously (Halliwell et al., 2000; Long et al., 2000, 2010) and regarded as one of the mechanisms of prooxidant effects of polyphenols (Kim, Quon, & Kim, 2014). The aim of this study was to extend the check of H_2O_2 production on a range of antioxidants studied by us within a framework of a project on, "*Antioxidant Power Series as a tool for rational design and assessment of health promoting properties of functional food based on antioxidant phytochemicals*". Results of this study indicate that, although antioxidants of various groups, including ascorbic acid, can produce H_2O_2, this feature is typical, first of all, for polyphenols. Interestingly, ethoxyquin, approved in some countries as a food and feed additive, generated relatively small but significant amounts of H_2O_2.

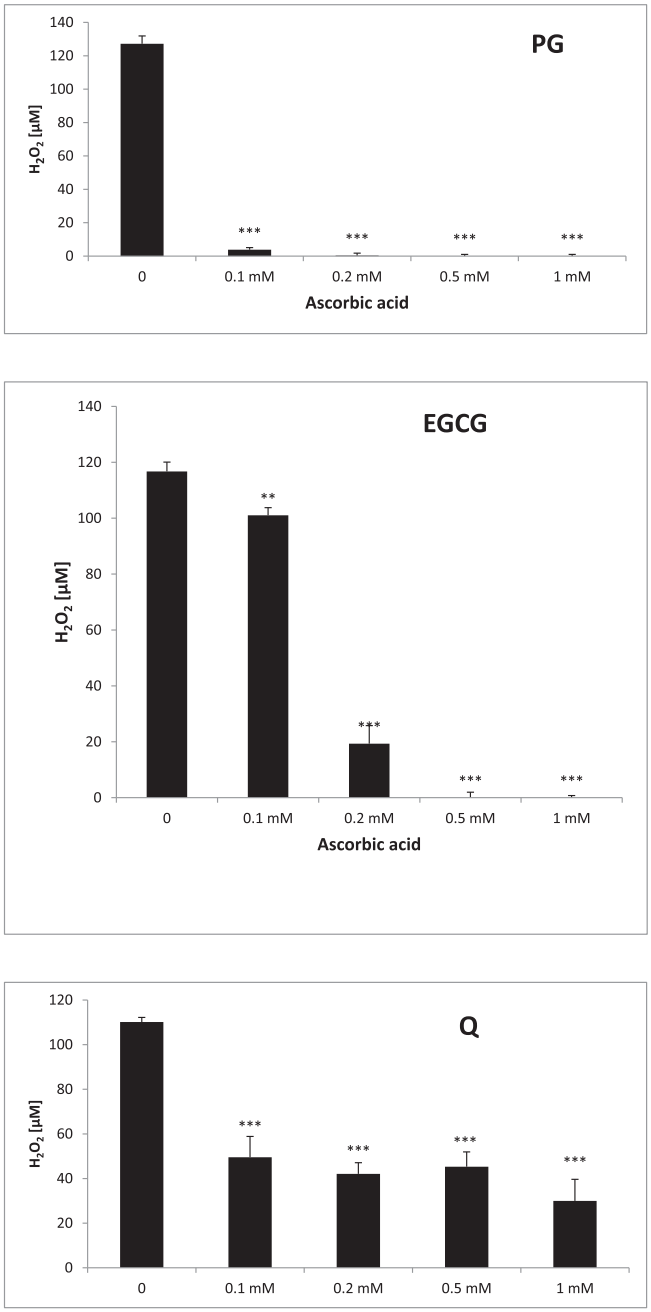

Fig. 2. Effect of ascorbate on the hydrogen peroxide production by autoxidation of PG, EGCG and Q in PBS. $^{**}P < 0.01$, $^{***}P < 0.001$ (with respect to PBS without ascorbic acid).

Table 2
Production of hydrogen peroxide in various media.

Compound	H_2O_2 [μM]				
	DMEM/F12	DMEM + Glutamax	YPG	SM	PBS
PG	90.4 ± 7.1	113.9 ± 7.5*	11.8 ± 0.9***	3.1 ± 0.2***	127.2 ± 4.7**
EGCG	140.9 ± 7.8**	157.9 ± 8.8**	0$^{**\#}$	0$^{**\#}$	116.7 ± 3.3**
Q	46.2 ± 9.7	41.8 ± 3.4	0$^{**\#}$	1.4 ± 1.2**	110.2 ± 2.0***

YPG medium: 0.5% Yeast nitrogen base (YNB), 1% yeast extract, 2% glucose: SM: 0.5% YNB, 2% glucose; PBS: phosphate-buffered saline; $^{\#}$ difference in absorbance between final and initial assay < 0. Peroxide generation significantly different from that in DMEM: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (paired Student's "*t*" test).

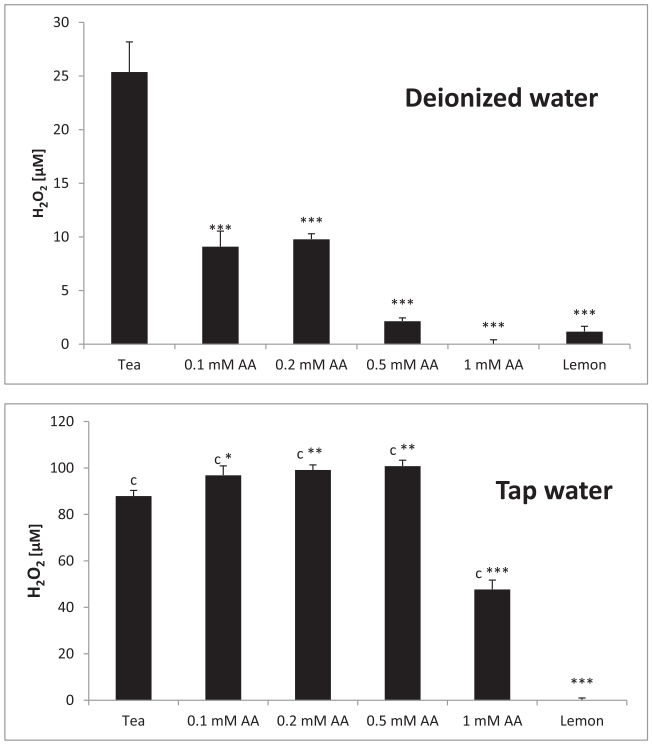

Fig. 3. Effect of ascorbic acid (AA) and lemon on generation of hydrogen peroxide in tea prepared on distilled and tap water. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to tea without additives; $^{c}P < 0.001$ with respect to analogous samples in deionized water.

The cell culture media containing FBS generated more H_2O_2 than media without FBS (Table S1). We hypothesize that this effect may be due to release of iron from iron-binding proteins (mainly transferrin), perhaps during freezing/thawing of the serum. This question deserves further study.

No correlation between the structure of the compounds studied and generation of H_2O_2 was apparent. As transition metal ions seem to catalyze the autoxidation of antioxidants (see below) the differences between different compounds observed may be affected by such a simple factor as the level of transition ion contamination of the commercial preparations. pH of the media may be another important factor, as the rate of autoxidation of many compounds increases with increasing pH. The case of ascorbic acid is especially instructive: addition of ascorbic acid from a not neutralized 10 mM stock solution did not induce generation of detectable amounts of H_2O_2, but addition of a solution neutralized to pH 7.0 or of sodium ascorbate resulted in production of significant amounts of H_2O_2. This factor could account for the lower rate of H_2O_2 generation in media used for yeast cell culture.

The most active antioxidants in our study were: PG, pyrogallol, EGCG and Q. The antioxidant compounds generated H_2O_2 in DMEM cell culture media and also in media used for yeast cell culture albeit at much lower yields, if any (pyrogallol was not studied in this respect since it is not generally employed as an antioxidant).

We have reported light-dependent production of ROS, which could contribute to the process of antioxidant autoxidation, in cell culture media (Grzelak, Rychlik, & Bartosz, 2001). However, this mechanism does not contribute to autoxidation of exogenous antioxidants since incubation in the darkness did not attenuate H_2O_2 formation by these compounds. H_2O_2 formation was comparable in PBS and in DMEM cell culture media, further suggesting that production of H_2O_2 by antioxidants is not due to interaction of these compounds with organic components of cell culture media, but rather to their autoxidation. Transition metal (presumably mainly iron) ions seem to catalyze this reaction as demonstrated by decrease in H_2O_2 production induced by pretreatment of PBS with Chelex X-100 and addition of iron chelators (DFO and DETAPA). Dulbecco's Modified Eagle's Medium contain ferric nitrate (0.1 mg/l) while DMEM/F12 and DMEM + GlutaMAX media contain 0.0012 mg/l cupric sulfate, 0.05 mg/l ferric nitrate and 0.417 mg/l ferric sulfate. Yeast Peptone Dextrose medium apparently contains iron ions but they may be tightly bound to other components of this medium. Yeast nitrogen base medium contains 0.04 mg copper sulfate, 0.2 mg ferric chloride and 0.4 mg manganese sulfate/l. Furthermore, all media may contain transition metal ions as trace contaminants of other components used to prepare the media. These ions may interact with polyphenols, catalyze their autoxidation and participate in the formation of H_2O_2. Probably the differences in the concentrations of transition metals, able to catalyze polyphenol autoxidation, are the main factors determining the rate of H_2O_2 generation due to autoxidation of polyphenols. Other parameters of the media studied (TAC, ability of Fe^{2+} binding, superoxide scavenging activity and hydrogen peroxide scavenging activity) did not correlate with the H_2O_2 generation. It is worthwhile to note that the cell culture media are able to scavenge H_2O_2 produced. This phenomenon may lead to underestimation of the amount of H_2O_2 formed by exogenous substances added to the media. Pyruvate was identified as one compound scavenging H_2O_2 (Long & Halliwell, 2009) and protecting cultured cells against the cytotoxic action of hydrogen peroxide (Rodemeister & Hill, 2017).

Polyphenol autoxidation is apparently a two-step reaction. First, polyphenol is oxidized to a semiquinone free radical $HQ^{\cdot}$ in a reaction coupled to reduction of molecular oxygen to the superoxide anion radical $O_2^{\cdot -}$ and then semiquinone is oxidized to quinone Q producing second superoxide radical:

$$H_2Q + O_2 \rightarrow HQ^{\cdot} + O_2^{\cdot -} + H^+$$

$$HQ^{\cdot} + O_2 \rightarrow Q + O_2^{\cdot -} + H^+$$

Semiquinone radicals can have extremely long half-lives (up to days at 37 °C) and tend to be neither reactive nor toxic. Problems can arise because of their propensity to donate the excess electron to molecular oxygen, thereby generating superoxide. The semiquinone radical is more susceptible to oxidation with O_2 than fully reduced catechins (Mochizuki, Yamazaki, Kano, & Ikeda, 2002). Dismutation of superoxide radical produces H_2O_2:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Autoxidation of polyphenols is pH-dependent and is accelerated by alkaline pH (Mochizuki et al., 2002). In agreement with this postulated mechanism, formation of $O_2^{\cdot -}$ during autoxidation of Q at alkaline pH has been demonstrated and used for determination of activity of superoxide dismutase (Kostyuk & Potapovich, 1989). As pH is an important factor in the polyphenol autoxidation, it should be expected that their autoxidation can be quite high in the intestine.

Autoxidation of polyphenols produces H_2O_2 in the tea, as reported previously (Long et al., 1999). Much higher H_2O_2 production was observed in tea prepared on tap water, containing transition metal ions, than in tea prepared on deionized water. It further suggests the role of contaminating transition metal ions as catalysts of polyphenol autoxidation. Interestingly, while ascorbate decreased H_2O_2 formation in cell culture medium, in PBS (prepared on deionized water) and in tea prepared on deionized water, low ascorbate concentrations augmented H_2O_2 production in tea prepared on tap water, containing transition metal ions. It has been reported that mixtures of ascorbate and phenolic compounds led to less H_2O_2 generation than it would be expected from the rates observed with ascorbate or phenolic compounds alone; this effect was ascribed to reduction of semiquinone radicals by ascorbate (Wee et al., 2003). Taking into account higher propensity for transition metal binding of polyphenols, compared to ascorbate (Perron & Brumaghim, 2009; Tamilmani & Pandey, 2016), such a transition requires higher concentrations of these ions as those required to catalyze

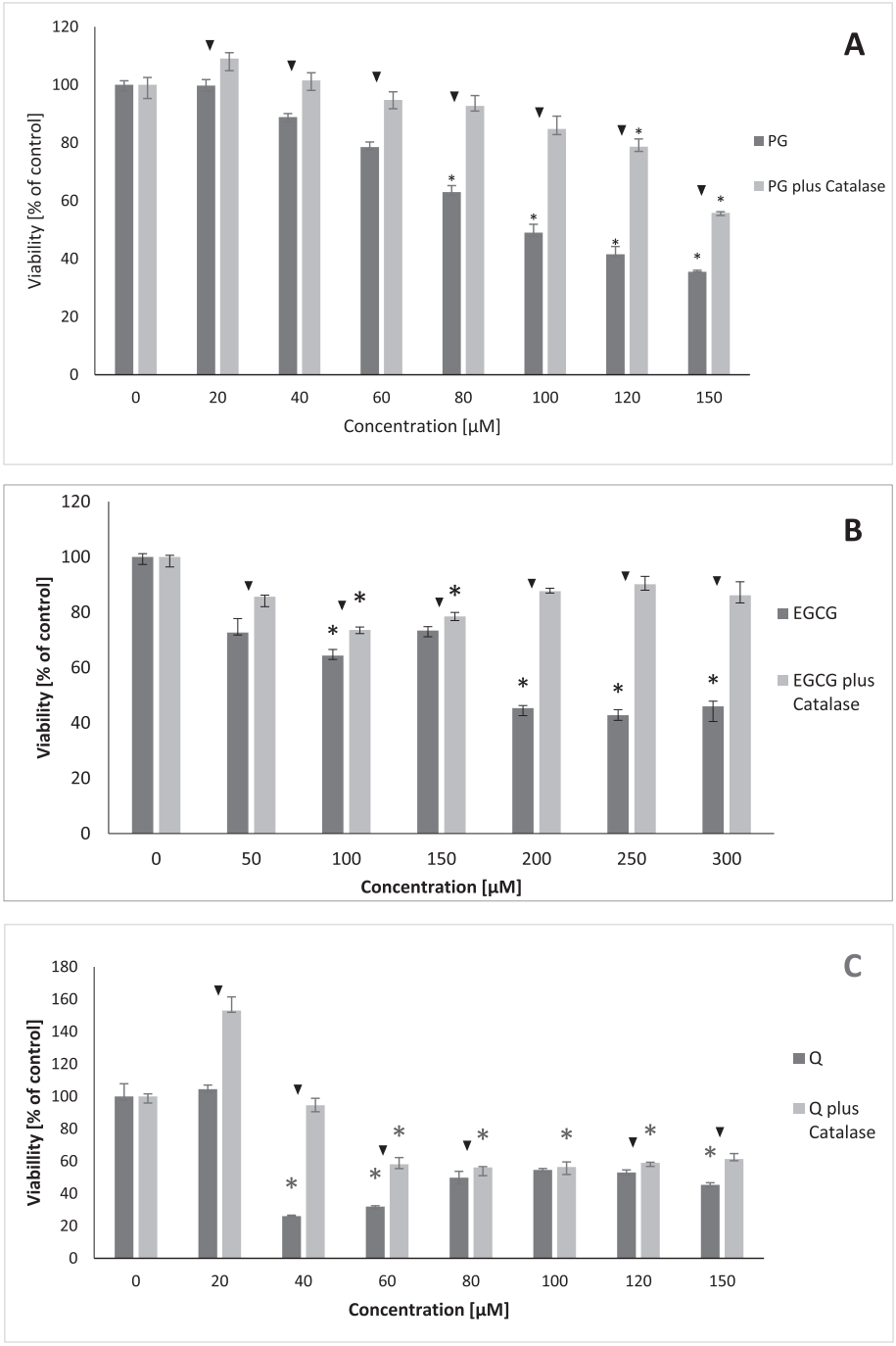

Fig. 4. Cytotoxicity of PG (A), EGCG (B) and Q (C) toward DU-145 cells after 24 h treatment in the absence and in the presence of catalase. *p ≤ 0.05 Kruskal–Wallis test against non-treated control. ▼ p ≤ 0.05 U-Mann-Whitney test (samples without vs in the presence of 10 µg/ml catalase). The whiskers are lower (25%) and upper (75%) quartile ranges.

polyphenol oxidation.

A practical conclusion from the study is that although tea generates and contains hydrogen peroxide, addition of lemon prevents H_2O_2 formation in the tea, an effect apparently due to reduction of semi-quinone radicals by and lowering of pH.

Hydrogen peroxide generated by PG, EGCG and Q contributes to the cytotoxicity of these compounds as the presence of catalase in the medium increased the survival of cells. The contribution of H_2O_2-mediated cytotoxicity is an artefact of cell culture conditions ex-aggerating the cytotoxicity of cells with respect to the *in vivo* conditions, where significant autoxidation of the compound studies does not seem to take place, due both to the lower oxygen level and to the presence of many reducing compounds. Interestingly, the cytotoxic and clastogenic action of EGCG on Chinese Hamster Ovary (CHO) cells correlated with the rate of H_2O_2 generation by EGCG in these media (Long, Kirkland, Whitwell, & Halliwell, 2007) while cytotoxicity of ascorbate is the higher, the higher is its autoxidation with concomitant H_2O_2 generation in various media (Clément, Ramalingam, Long, & Halliwell, 2001; Halliwell, 2018). Moreover, as H_2O_2 is involved in many intracellular signaling pathways (Rhee, 2006), activating, i. a., the redox-sensitive antioxidant response element (ARE) (Ho, Siu-wai, Siu, & Benzie, 2013), its penetration from the cell culture medium may alter cell behavior under *in vitro* conditions, though not necessarily *in vivo*.

5. Conclusions

A number of natural antioxidant compounds, mainly polyphenols, produce H_2O_2 upon autoxidation. This phenomenon may induce artefacts in cell culture experiments employing polyphenols as H_2O_2 produced in vitro, but most probably not *in vivo*, contributes to *in vitro* cytotoxicity of antioxidants and may affect cellular signaling. Autoxidation of polyphenols present in the tea also produces H_2O_2 in the tea which is diminished by lemon.

Authors' contributions

I. S.-B. was responsible for the concept of the study, design of experiments and supervision of experimental work, performed part of experiments as well as had a leading role in the analysis of the results and preparation of the manuscript. M. G. performed part of experiments in cell-free systems and their statistical evaluation as well as contributed reagents/materials/analysis tools. She further contributed to data acquisition and interpretation as well as wrote parts of the manuscript. I. S. carried out EPR measurements and interpreted the data. M. P. performed experiments in the cellular system and their statistical evaluation. G. B. critically discussed the research goals and participated in the revision and improvement of the manuscript. J. N. was responsible for providing the funding for the study. All authors have approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

This study was performed within the project, "Antioxidant Power Series as a tool for rational design and assessment of health promoting properties of functional food based on antioxidant phytochemicals" (2014/14/A/ST4/00640) financed by National Science Centre, Poland within a programme, MAESTRO 6 and research fund of the University of Rzeszów (Grant WBR/ZBA/PB/1/2017). We are grateful to Ms. Natalia Pienkowska (University of Rzeszów), M.Sc., for her excellent technical help.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.11.109.

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Table S1. Effect of fetal bovine serum (FBS) on the generation of hydrogen peroxide in DMEM (mean ± SD, n=6).

Compound	DMEM	DMEM with 10% FCS
PG	95.2 ± 1.9	109.4 ± 4.9***
EGCG	90.2 ± 2.6	159.5 ± 1.7***
Q	76.4 ± 5.9	110.2 ± 7.6***

***P < 0.001

Table S2. Effect of Chelex X-100 treatment and iron chelators.

Compound	H_2O_2 generated [µM]			
	PBS	Chelex-treated PBS	DFO 100 µM	DETAPA 100 µM
PG	95.2 ± 1.9	85.5 ± 4.3*	66.7 ± 1.4***	52.7 ± 2.4***
EGCG	53.1 ± 1.1	48.1 ± 1.3*	41.0 ± 3.2***	36.0 ± 0.6**
Q	51.9 ± 0.8	34.4 ± 1.9**	38.0 ± 5.3*	35.8 ± 7.2*

Differences significant with respect to PBS: *P<0.05, **P<0.01, ***P<0.001 (Student's "t" test).

Table S3. Total Antioxidant Capacity (TAC, Fe^{2+}-binding capacity, superoxide and hydrogen peroxide scavenging of media used for cell culture.

Medium	Fast ABTS* scavenging [mM]	Slow ABTS* scavenging [mM]	Fe^{2+} binding IV_{50} [µl]	Superoxide scavenging [units/l]	Hydrogen peroxide scavenging [µM/h]
DMEM	2.28 ± 0.26[b]	7.08 ± 0.05[c]	9.48 ± 0.37	18.4 ± 1.1[b]	3.4 ± 1.8[a]
DMEM/F12	1.54 ± 0.37*[a]	6.99 ± 0.21[c]	ND	10.2 ± 1.1*[b]	13.3 ± 1.6*[b]
DMEM GlutaMAX	1.43 ± 0.44[a]	6.15 ± 1.05	10.02 ± 0.38*	11.0 ± 0.9*[b]	7.7 ± 2.3[a]
YPD	4.84 ± 0.04**[c]	5.92 ± 0.04**[c]	35.21 ± 0.71***	6.5 ± 0.9**[a]	10.4 ± 1.1[b]
SM	0.006 ± 0.006**	0.22 ± 0.02***[b]	ND	9.2 ± 0.5**[c]	-2.3 ± 2.2

TAC is expressed in mM of Trolox equivalents; Fe^{2+}-binding capacity expressed as volume of the medium preventing Fe^{2+} binding by ferrozine in 50% (IV_{50}); ND – determination not possible. Generation significantly different from 0: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. Differences significant with respect to DMEM: *P<0.05, **P<0.01, ***P<0.001 (paired Student's "t" test).

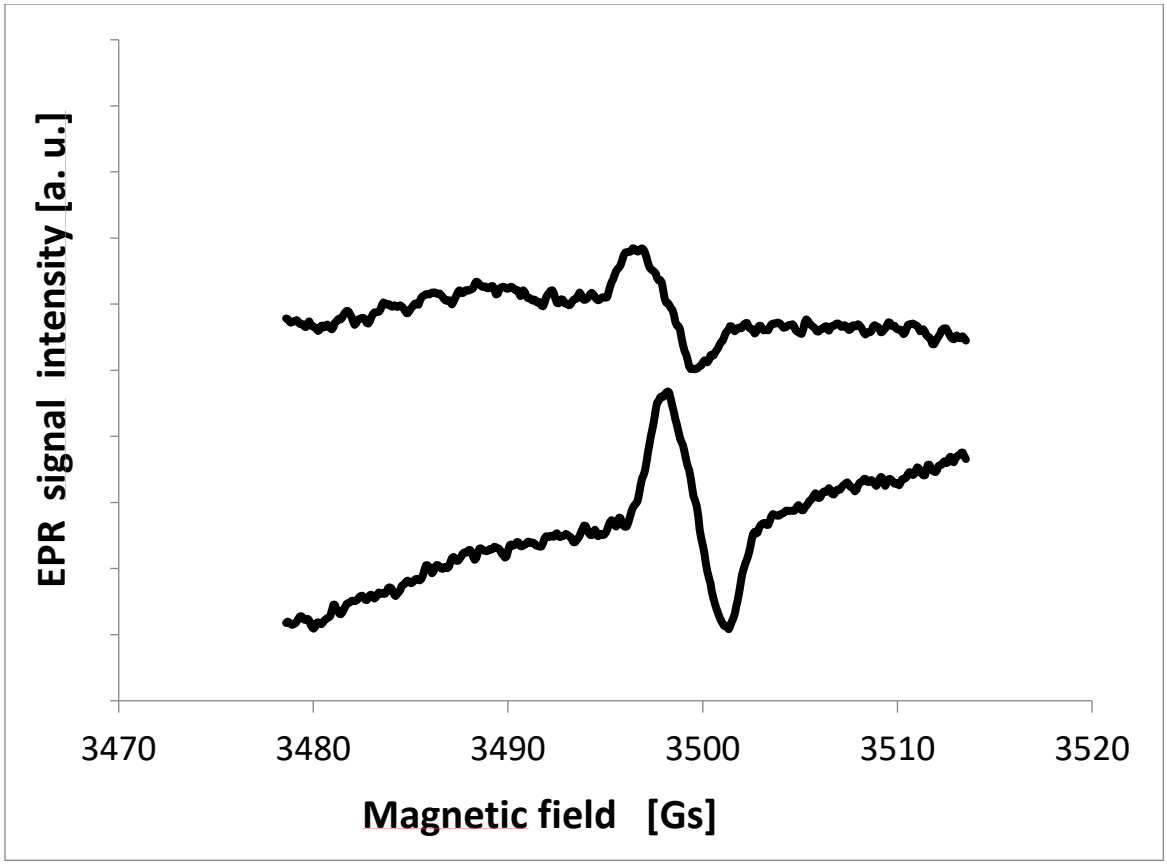

Fig. S1. EPR spectra of semiquinone radicals formed by autoxidation of EGCG (top) and PG (bottom) in PBS.

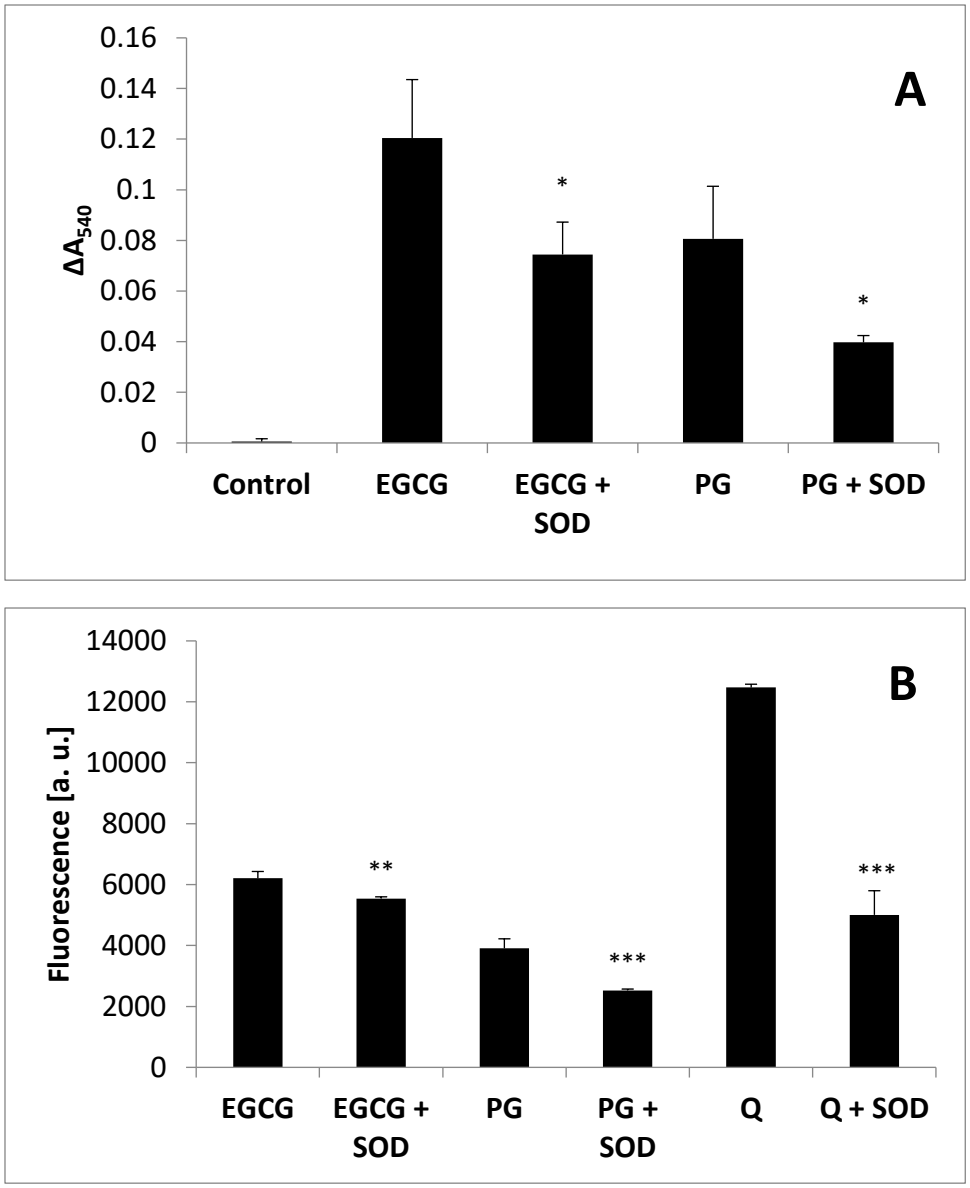

Fig. S2. Reduction of NBT (A) and oxidation of dihydroethidine (B) accompanying polyphenol autoxidation in the absence and in the presence of 10 µg/ml superoxide dismutase (SOD); *P < 0.05, **P<0.01, ***P<0.001.