**UNIWERSYTET RZESZOWSKI**
**KOLEGIUM NAUK PRZYRODNICZYCH**

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# OCHRONA MODELOWYCH KOMÓREK PRZED STRESEM OKSYDACYJNYM PRZEZ POLIFENOLE ORAZ ZWIĄZKI SYNTETYCZNE

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

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## DOROBEK NAUKOWY

**Publikacje wchodzące w skład rozprawy doktorskiej:**

1. **Naparlo K.,** Zyracka E., Bartosz G., Sadowska-Bartosz I. 2019. Flavanols protect the yeast *Saccharomyces cerevisiae* against heating and freezing/thawing injury. *Journal of Applied Microbiology,* 126(3):872-880. **($IF_{2019}$ = 2,683; Punkty $MNiSW_{2019}$ = 70)**
2. **Naparlo K.,** Bartosz G., Stefaniuk I., Cieniek B., Soszynski M., Sadowska-Bartosz I. 2020. Interaction of catechins with human erythrocytes. *Molecules,* 25(6):1456. **($IF_{2020}$ = 3,267; Punkty $MNiSW_{2020}$ = 100)**
3. **Naparlo K.,** Soszynski M., Bartosz G., Sadowska-Bartosz I. 2020. Comparison of antioxidants: the limited correlation between various assays of antioxidant activity. *Molecules,* 25(14):E3244. **($IF_{2020}$ = 3,267; Punkty $MNiSW_{2020}$ = 100)**

Sumaryczna wartość współczynnika **Impact Factor** publikacji wchodzących w skład rozprawy doktorskiej (zgodnie z rokiem opublikowania) wynosi **9,217** (**270** punktów **MNiSW**).

**Pozostałe publikacje**

1. Grzesik M., **Naparlo K**., Bartosz G., Sadowska-Bartosz I. 2018. Antioxidant properties of catechins: comparison with other antioxidants. *Food Chemistry,* 241: 480-492. **($IF_{2017}$ = 4,946; Punkty $MNiSW_{2017}$ = 40)**
2. Adamczyk-Sowa M., Galiniak S., Zyracka E., Grzesik M., **Naparlo 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. **($IF_{2017}$ = 4,936; punkty $MNiSW_{2017}$ = 30)**
3. Gonos E., Kapetanou M., Sereikaite J., Naparlo 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.* **($IF_{2018}$ = 5,179; punkty $MNiSW_{2018}$ = 30)**.

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**Komunikaty zjazdowe**

1. **Naparlo K.,** Bartosz G., Zyracka E., Sadowska-Bartosz I. *Antioxidants protect yeast S. cerevisiae against DMSO-induced oxidative stress.* The 44th FEBS Congress „From molecules to living systems". Kraków, 6-11.07.2019 r. (prezentacja posterowa)
2. **Naparlo K.,** Zyracka E., Grzesik M., Bartosz G., Sadowska-Bartosz I. *Influence of catechins on saccharomyces cerevisiae subjected to thermal stress*. The 19th biennial meeting for the Society for Free Radical Research International (SFRRI). Lizbona 4-7.06.2018 r. (prezentacja posterowa)
3. Grzesik M., **Naparlo K**., Bartosz G., Sadowska-Bartosz I. *Catechins and other flavonoids as protectors against undesired modifications of biomolecules.* Meeting of the International HNE-Club and the University of Graz "Reactive Oxygen species and lipid Peroxidation in Human Health and Disease" Graz, 14- 15.09.2017 r. (prezentacja posterowa)
4. Grzesik M., **Naparlo K**., Zuberek 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., **Naparlo K**., Zuberek 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**

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**STRESZCZENIE**

Na rozprawę doktorską składa się cykl trzech prac opublikowanych w czasopismach *Journal of Applied Microbiology* oraz *Molecules* poświęconych (I) ocenie ochronnych właściwości wybranych katechin względem drożdży przed stresem oksydacyjnym, (II) określeniu właściwości antyoksydacyjnych wybranych katechin względem erytrocytów poddanych działaniu stresu oksydacyjnego, (III) porównaniu właściwości ochronnych wybranych polifenoli przed peroksydacją lipidów zawartych w żółtku jaja i błonach erytrocytów oraz utlenieniem składników erytrocytów (hemoglobiny i glutationu).

Wykazaliśmy, że badane flawanole [(+)-katechina i galusan (-)-epigallokatechiny] zwiększały przeżywalność drożdży poddanych działaniu wysokiej temperatury i zamrażanych. Markery stresu oksydacyjnego takie jak produkcja reaktywnych form tlenu, całkowita zdolność antyoksydacyjna czy karbonylacja białek miały niższe wartości w przypadku drożdży poddanych działaniu tych flawanoli. Dzięki temu można wnioskować, że związki te pełnią funkcję ochronną dla drożdży przed skutkami działania stresu oksydacyjnego.

Nasze badania pozwoliły wykazać, że wybrane katechiny ((+) - katechina, epigallokatechina (EGC) i galusan epigallokatechiny (EGCG)) chronią ludzkie erytrocyty przed stresem oksydacyjnym. Wszystkie badane katechiny chroniły erytrocyty przed hemolizą wywołaną nadmanganianem, utlenianiem grup tiolowych białek erytrocytów oraz peroksydacją lipidów błonowych, co pozwala na poznanie korzystnego wpływu katechin obecnych w żywności i napojach pochodzenia roślinnego na organizm ludzki.

Praca miała także na celu ocenę ochronnego wpływu szeregu syntetycznych i naturalnych antyoksydantów przed peroksydacją lipidów żółtka jaja i błon erytrocytów. Większość z badanych związków wykazywała takie działanie w obydwu układach jednak niektóre w interakcji z erytrocytami ujawniały swoje działanie prooksydacyjne, co pokazuje zróżnicowanie działania antyoksydantów w różnych układach.

**ABSTRACT**

My doctoral dissertation consists of three papers published in the *Journal of Applied Microbiology* and *Molecules* devoted to (I) assessment of the protective properties of selected catechins against oxidative stress, (II) determination of the antioxidant properties of selected catechins against erythrocytes subjected to oxidative stress, (III) comparison of the protective properties of selected polyphenols against peroxidation of lipids in egg yolk and erythrocyte membranes and oxidation of chosen erythrocyte components (hemoglobin and glutathione).

We showed that studied flavanols [(+) - catechin and (-) - epigallocatechin gallate] increased the survival of yeasts subjected to thermal stress. Oxidative stress markers such as production of reactive oxygen species, total antioxidant capacity and protein carbonylation had lower values in yeast treated with these flavanols. It can be concluded that these compounds protect yeast against the effects of oxidative stress.

Next, we checked whether selected catechins [(+) - catechin, epigallocatechin (EGC) and epigallocatechin gallate (EGCG)] affect human erythrocytes and if they protect against damage caused by oxidative stress. All tested catechins protected erythrocytes against hemolysis caused by permanganate, oxidation of thiol groups of erythrocyte proteins and peroxidation of membrane lipids, which show us beneficial effect of catechins present in plant food and drink on the human body.

We also compared the protective effect of a number of synthetic and natural antioxidants on the peroxidation of lipids in egg yolk and erythrocyte membranes. Most of the tested compounds showed this activity in both systems, but some revealed their pro-oxidative activity in interaction with erythrocytes, which tells us that antioxidant activity may differ in different systems.

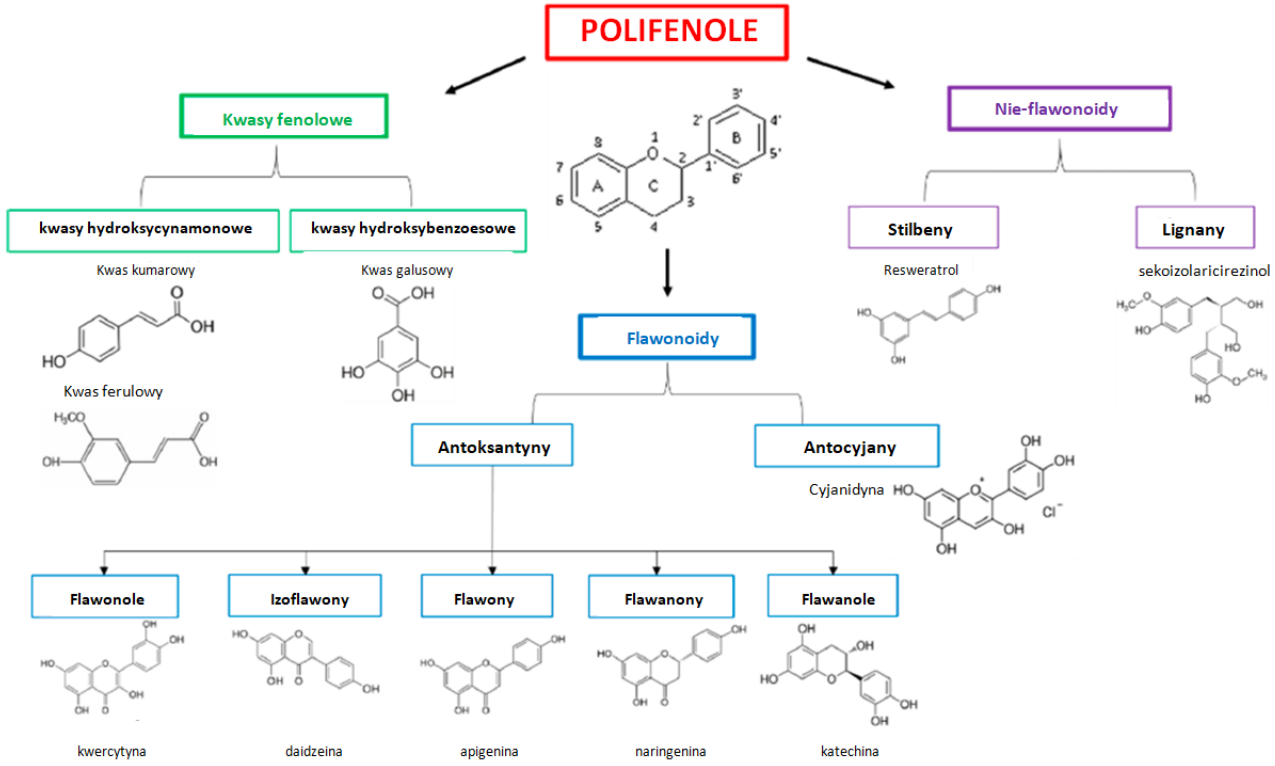
**WSTĘP**

Rodniki ponadtlenkowe ($O_2^{\bullet -}$), nadtlenek wodoru ($H_2O_2$), rodniki hydroksylowe ($^{\bullet}OH$) i tlen singletowy ($^1O_2$) są najczęściej występującymi reaktywnymi formami tlenu (ROS) i są one wytwarzane jako uboczne produkty licznych procesów metabolicznych w organizmach. Obecność ROS w komórkach jest niezbędna do zachodzenia wielu reakcji, takich jak regulacja fosforylacji białek, aktywacja licznych czynników transkrypcyjnych, apoptoza, reakcje odpornościowe, różnicowanie czy zwalczanie drobnoustrojów chorobotwórczych [1,2]. Gdy produkcja ROS wzrasta w komórkach, zaczynają one wykazywać szkodliwy wpływ na ważne struktury komórkowe, takie jak białka, lipidy i kwasy nukleinowe [1].

Stres oksydacyjny to zaburzenie homeostazy prowadzące do wzrostu stężeń reaktywnych form tlenu. Prowadzi to do zaburzenia równowagi prooksydacyjno-antyoksydacyjnej w kierunku reakcji utlenienia. Tak długo, jak system obrony przeciwutleniającej jest zdolny do zmiatania wytworzonych reaktywnych form tlenu, zwiększona produkcja ROS lub reaktywnych form azotu (RNS) nie powoduje patologicznych zmian w organizmie. W starzejącym się organizmie dochodzi do zwiększonego wytwarzania wolnych rodników przy zmniejszonej wydajności endogennego systemu obrony antyoksydacyjnej. Stres oksydacyjny jest charakterystyczny nie tylko dla starzenia się organizmu, ale występuje także w przypadku wielu chorób takich jak nowotwory, choroba Parkinsona, miażdżyca, zawał mięśnia sercowego, zespół Downa, depresja czy cukrzyca [3]. Organizmy w obronie przed szkodliwym działaniem utleniaczy wytworzyły szereg związków o charakterze antyoksydantów. Do najważniejszych należą: niektóre kofaktory enzymów (NADPH, NADH), kwas moczowy, różnorodne składniki pokarmowe, w tym witaminy A, C, E, flawonoidy oraz jony metali ($Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$). Ważną rolę w tym procesie pełnią również liczne enzymy, m.in. dysmutaza ponadtlenkowa czy peroksydaza glutationowa [4].

Syntetyczne antyoksydanty mogłyby być stosowane jako potencjalne środki zapobiegające i wspomagające leczenie chorób związanych ze stresem oksydacyjnym. Jednak ze względu na potencjalną szkodliwość oraz możliwą toksyczność i rakotwórczość nie są wskazane jako dobre dodatki do żywności. Dlatego antyoksydanty naturalne budzą duże zainteresowanie jako czynniki mogące chronić żywność przed utlenianiem i wspomagać antyoksydacyjny system obronny organizmu [5]. Zatem dokładne zbadanie mechanizmów działania antyoksydantów i ich oddziaływania z komórkami organizmu jest niezmiernie istotne [6].

Flawonoidy to duża klasa polifenolowych wtórnych metabolitów roślin, w tym flawonoli, flawonów, izoflawonów, antocyjanów i chalkonów [7]. Polifenole można podzielić na grupy ze względu na liczbę pierścieni fenolowych, które zawierają i na podstawie elementów konstrukcyjnych, które łączą te pierścienie ze sobą. Trzy główne klasy obejmują: kwasy fenolowe, flawonoidy i nie-flawonoidy (stilbeny i lignany ) (ryc. 1) [8, 9].

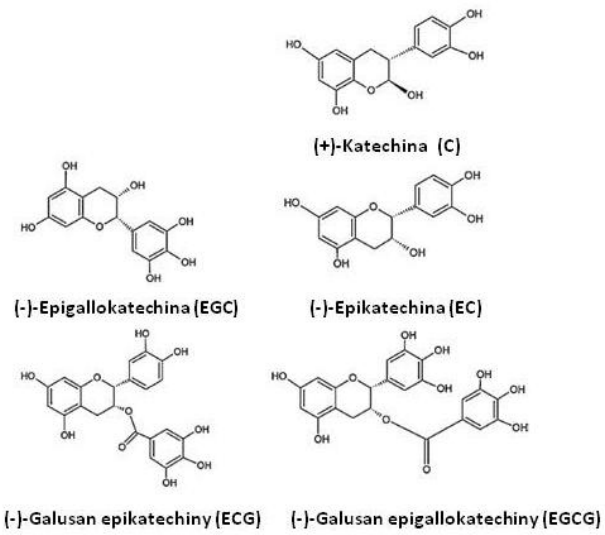



**Ryc. 1.** Schematyczna klasyfikacja polifenoli i przykłady struktur chemicznych [9;zmodyfikowano].

Nie są one niezbędne u większości roślin do prawidłowego wzrostu i rozwoju. Są one bardzo zróżnicowane strukturalnie, a wiele z nich występuje tylko u ograniczonej liczby gatunków. Większość flawonoidów to skuteczne naturalne przeciwutleniacze. Ich rola, między innymi właśnie ze względu na działanie przeciwutleniające, stała się ważna dla badań nad żywieniem człowieka. Wiele danych wskazuje na ich działanie prozdrowotne, które może być związane z ich właściwościami antyoksydacyjnymi lub niezależne od nich – m. in. zmniejszenie zachorowalności na nowotwory i choroby przewlekłe takie jak choroby sercowo-naczyniowe i cukrzyca typu II oraz ochrona przed upośledzeniem funkcji poznawczych [10, 11].

Związki fenolowe wykazują silne działanie przeciwutleniające *in vitro* i *in vivo* związane z ich zdolnością do usuwania wolnych rodników, przerywania rodnikowych reakcji łańcuchowych i

chelatowania jonów metali [7]. Katechiny z kolei to grupa związków polifenolowych należąca do klasy flawonoidów, występująca w dużych stężeniach w różnych owocach, warzywach i napojach pochodzenia roślinnego (ryc.2). Spośród związków w tej grupie, katechiny zyskały w ostatnim czasie dużą popularność dzięki odkryciu ich licznych cech prozdrowotnych, przy braku toksycznego działania i innych skutków ubocznych dla organizmu człowieka [12].




**Ryc. 2.** Struktury chemiczne wybranych katechin [12;zmodyfikowano].

**CELE PRACY**

Celem mojej pracy wykonanej w ramach przygotowania rozprawy doktorskiej było:

- ocena właściwości ochronnych katechin względem komórek drożdży przed stresem oksydacyjnym wywołanym czynnikami fizycznymi i chemicznymi;
- określenie czy katechiny chronią przed peroksydacją lipidów i utlenianiem białek w erytrocytach człowieka;
- porównanie skuteczności wybranych polifenoli w hamowaniu peroksydacji lipidów w dwóch układach modelowych - żółtku jaja kurzego i błonach erytrocytów oraz ochronie wybranych składników erytrocytów przed utlenianiem.

**METODY**

Zbadano aktywność ochronną wybranych związków przed stresem oksydacyjnym. W tym celu wykorzystano szczep drożdży BY4741 (wt) oraz mutanta BY4741 *Δsod1*. BY4741 jest częścią zestawu szczepów pochodzących ze szczepu S288C, w których usunięto powszechnie używane selekcyjne geny znacznikowe w celu zminimalizowania lub wyeliminowania homologii do odpowiednich genów markerowych w powszechnie używanych wektorach bez istotnego wpływu na sąsiednie ekspresje genu. BY4741 *Δsod1* jest izogenicznym względem niego szczepem pozbawionym miedziowo-cynkowej dysmutazy ponadtlenkowej.

W celu określenia cytotoksyczności badanych związków względem wybranych szczepów drożdży wykorzystano testy spektrofotometryczne mierząc zmiany turbidancji co było miarą szybkości wzrostu hodowli i tym samym liczby podziałów komórek drożdży w ciągu 24 h. Oba szczepy drożdży potraktowano różnymi dawkami flawonoidów w zakresie od 5 do 1000 μM.

Kolejno sprawdzono wrażliwość drożdży na działanie wybranych oksydantów: menadionu, chlorku sodu, podchlorynu sodu, nitroprusydku sodu, dichlorowodorku 2,2'-azobis(2-amidynopropanu) (AAPH), chlorku 5-amino-3-(4-morfolinylo)-1,2,3-oksadiazolium (Sin-1), wodoronadtlenku tert-butylu i nadtlenku wodoru. Obydwa szczepy drożdży traktowano różnymi dawkami oksydantów w zakresie od 0,5 do 500 mM.

Następnie określono wpływ wybranych nitroksydów, czyli stabilnych rodników organicznych o małej masie cząsteczkowej posiadających grupę nitroksylową (>N-O), na obydwa szczepy drożdży poddając je działaniu różnych dawek tych związków (w zakresie od 5 do 2000 μM): 4-hydroksy-TEMPO, 4-acetamido–TEMPO, 4-okso-TEMPO, 4-karboksy-TEMPO, 4-metoksy-TEMPO, 3-karbomoilo-PROXYLu.

Stres indukowano czynnikami fizycznymi w celu wyeliminowania interakcji pomiędzy badaną substancją a czynnikami oksydacyjnymi. W tym celu zbadano wpływ flawonoidów na uszkodzenie drożdży pod wpływem wysokiej temperatury (50°C). Obydwa szczepy hodowano do wczesnej fazy logarytmicznej, a następnie inkubowano przez 2 godziny z różnymi dawkami galusanu epigallokatechiny (EGCG) oraz katechiny (C) (10,20,50 μM), po czym umieszczono poszczególne serie próbek w termobloku o temperaturze 50°C na 10, 20, 30 i 40 min. Wybrano temperaturę 50°C na podstawie pracy [13].

Zbadano również wpływ flawonoidów na uszkodzenie drożdży pod wpływem niskiej temperatury. Podobnie jak w teście z wysoką temperaturą obydwa szczepy zostały poddane

2-godzinnej inkubacji z różnymi stężeniami flawonoidów (galusanem epigallokatechiny oraz katechiny, 10,20,50 μM), a następnie zamrożono poszczególne serie w temperaturze -20°C. Po 1h, 2h, 3h i 5h próbki rozmrażano i wykonywano oznaczenia. Zarówno wysoka temperatura, jak i zamrażanie/rozmrażanie indukują stres oksydacyjny w komórkach drożdży [13, 14].

[Publikacja 1. Naparlo K, Zyracka E, Bartosz G, Sadowska-Bartosz I. 2019. Flavanols protect the yeast Saccharomyces cerevisiae against heating and freezing/thawing injury. *J Appl Microbiol.,* 126(3):872-880.]

W kolejnej pracy, na podstawie wyników uzyskanych na modelu drożdżowym, sprawdziliśmy wpływ tych samych katechin (C, EGCG) oraz epigallokatechinę (EGC) na erytrocyty izolowane z krwi ludzkiej. Oznaczyliśmy wpływ wybranych substancji na hemolizę wywołaną przez AAPH, podchloryn sodu oraz nadmanganian potasu. Sprawdzono również ochronny wpływ tych substancji przed peroksydacją lipidów i utlenianiem białek zawartych w błonach izolowanych z erytrocytów.

Przygotowano serię rozcieńczeń zawiesiny erytrocytów do której dodano wybrane związki - C, epigallokatechinę (EGC) lub EGCG tak, aby otrzymać ich końcowe stężenie w próbce 50 μM. Próbki inkubowano przez 1h w temperaturze pokojowej z ciągłym mieszaniem, a następnie je zwirowano i zmierzono fluorescencję supernatantu (wzbudzenie: 230 nm, emisja: 290 nm). Liczba cząsteczek flawanoli związanych przez erytrocyty przedstawiono jako $10^{16}$ cząsteczek związanych na $10^{9}$ liczba erytrocytów.

Następnie sprawdzono wpływ wybranych substancji (C, EGC lub EGCG) na oporność osmotyczną erytrocytów. Do 10% zawiesiny erytrocytów dodawano katechiny (stężenie końcowe 50 μM). Następnie natychmiast po dodaniu lub po 90 min inkubacji w temperaturze 37$^{o}$C pobierano 50 μl zawiesiny i dodawano do roztworów o różnym stężeniu NaCl (0.34 - 0.45%). Próbkę kontrolną stanowiło 50 μl zawiesiny erytrocytów dodane do 950 μl wody destylowanej. Wszystkie próbki następnie odwirowano i zmierzono absorbancję supernatantu przy długości fali 540 nm. Z krzywej wzorcowej oporności osmotycznej określono stężenie NaCl powodujące 50% hemolizy (**c50**).

Czynnikiem powodującym hemolizę erytrocytów, choć rzadziej stosowanym w badaniach erytrocytów jest również nadmanganian potasu ($KMnO_4$), który wykorzystano w badaniu dotyczącym określenia ochronnego wpływu wybranych związków (C, EGC lub EGCG)

na hemolizę erytrocytów. W tym celu do zawiesiny erytrocytów dodano odpowiednią ilość badanego związku tak, aby końcowe stężenie wynosiło od 1-25 μM i próbki inkubowano 15 min w temperaturze 37$^{o}$C z mieszaniem, po czym do każdej z nich dodano nadmanganian potasu, tak aby jego końcowe stężenie wynosiło 100 μM. Pomiar absorbancji próbek przy długości fali 700 nm prowadzono co 2 min przez 120 min. Kontrolę stanowiła próbka zawierająca tylko erytrocyty i $KMnO_4$. Z wykresów zależności zmian turbidancji zawiesiny erytrocytów w funkcji czasu wyznaczano wartość czasu połowicznej hemolizy (t50) czyli czas odpowiadający zmniejszeniu turbidancji do 50% wartości początkowej. Dla wszystkich oznaczeń czas połowicznej hemolizy (w sekundach) i względny czas połowicznej hemolizy w stosunku do kontrolnych erytrocytów (przyjęty jako 100%) obliczono jako 100% x (t50 w sekundach w obecności badanego związku) / (t50 w sekundach dla kontroli).

Sprawdzono również wpływ badanych związków na aktywność acetylocholinoesterazy znajdującej się w błonach erytrocytów. Aktywność enzymu mierzono metodą kolorymetryczną opisaną przez Ellmana z niewielkimi modyfikacjami, stosując jako substrat jodek acetylotiocholiny (AcTCh). W tym celu do 5 μl zawiesiny błon erytrocytów dodano badane związki do końcowego stężenia 0, 1, 2, 3, 5, 10, 20, 30 lub 50 μM oraz 0,1 M bufor fosforanowy o pH 7.4, 0,5 mM kwas 5,5'-ditiobis(2-nitrobenzoesowy) (DTNB; odczynnik Ellmana) i 0,5 mM jodek acetylotiocholiny. Prowadzono pomiar kinetyczny absorbancji przy długości fali 412 nm co 10 sekund przez 3 minuty.

Oszacowano kinetykę hamowania acetylocholinoesterazy poprzez sporządzenie dwóch serii próbek: jedna zawierała 0,1 M bufor fosforanowy o pH 7.4, odczynnik Ellmana, różne ilości substratu i wybrane flawanole o końcowym stężeniu 50 μM, druga zaś różniła się brakiem flawanoli. Pomiar absorbancji prowadzono przy długości fali 412 nm. Obliczono aktywność acetylocholinoesterazy i na podstawie uzyskanych średnich wartości sporządzono wykres Lineweavera-Burka (1 / (prędkość reakcji enzymatycznej) vs. 1 / (stężenie substratu)) pod nieobecność i w obecności badanych związków. Na tej podstawie określono rodzaj inhibicji.

Wykorzystując spektroskopię elektronowego rezonansu paramagnetycznego (EPR) określono płynność błon erytrocytów. W tym celu do 200 μl błon erytrocytów (2,7 mg białka/ml) dodano katechiny do końcowych stężeń 0, 50, 100 i 250 μM. Do każdej próbki dodano 2 μl wybranych 10 mM sond: kwasu 5-doksylostearynowego (5DS) i kwasu 16-

doksylostearynowego (16DS) rozpuszczonych w dimetylosulfotlenku (DMSO). Po 10 minutowej inkubacji wykonano pomiary EPR z wykorzystaniem kapilar mikrohematokrytowych (nieheparynizowane probówki mikrohemokrytowe ~75 μl; 1,55x75 mm) w wieloczęstotliwościowym rezonansowym aparacie BRUKER FT-EPR ELEXSYS E580. Kapilary z próbkami umieszczono w kwarcowej probówce EPR i wyśrodkowano w komorze mikrofalowej. Widma EPR rejestrowano i analizowano przy użyciu oprogramowania Xepr 2.6b.74.

Określono również ochronny wpływ badanych katechin na uszkodzenia białek i peroksydację lipidów zawartych w błonach erytrocytów. Uszkodzenia białek oszacowane zostały na podstawie ilości powstałych grup tiolowych. W tym celu błony erytrocytów (1 mg białka/ml) zawieszone w 0,1 M buforze fosforanowym o pH 7.4, traktowano podchlorynem sodu o stężeniu końcowym 500 μM przez 30 minut, przy braku lub w obecności różnych stężeń badanych związków. Następnie oznaczono ilość grup tiolowych w próbkach i zmierzono absorbancję przy długości fali 412 nm. Procent ochrony obliczono jako 100% x ((ilość grup tiolowych w próbce potraktowanej NaOCl i dane stężenie katechiny) - (ilość grup tiolowych w próbce potraktowanej NaOCl przy braku jakiegokolwiek środka ochronnego)) / ( (ilość grup tiolowych w próbce kontrolnej) - (ilość grup tiolowych w próbce potraktowanej NaOCl przy braku jakiegokolwiek środka ochronnego)).

W zawiesinie błon erytrocytów traktowanych podchlorynem sodu oznaczono także peroksydację lipidów. W tym celu zawiesinę błon traktowano zimnym 10% kwasem trichlorooctowym i 0,67% kwasem tiobarbiturowym. Probówki ogrzewano w temperaturze 100°C przez 20 min, a następnie zmierzono absorbancję supernatantów przy długości fali 532 nm. Procent ochrony obliczono jako 100% x ((ilość produktów peroksydacji lipidów w próbce traktowanej NaOCl i danym stężeniem katechiny) - (ilość produktów peroksydacji lipidów w próbce traktowanej NaOCl przy braku jakiegokolwiek środka ochronnego)) / ((ilość produktów peroksydacji lipidów w próbce kontrolnej) - (ilość produktów peroksydacji lipidów w próbce potraktowanej NaOCl przy braku jakiegokolwiek środka ochronnego))
[Publikacja 2. Naparlo K., Bartosz G., Stefaniuk I., Cieniek B., Soszynski M., Sadowska-Bartosz I. 2020. Interaction of Catechins with Human Erythrocytes. *Molecules,* 24;25(6)]

W trzeciej pracy postanowiliśmy rozszerzyć zakres testowanych związków z grupy polifenoli, nie tylko katechiny, ale także inne naturalne i syntetyczne antyoksydanty. Badania dotyczące ochronnych właściwości wybranych polifenoli przed peroksydacją lipidów

rozpoczęto od oznaczenia peroksydacji lipidów w systemie bezkomórkowym. 10% zawiesinę żółtka jaja traktowano AAPH w obecności wybranych antyoksydantów w zakresie stężeń 0,01 – 5 mM. Do monitorowania peroksydacji lipidów wykorzystano intensywnie fluoryzującą sondę BODIPY®581/591. Prowadzono pomiar kinetyczny wzrostu fluorescencji (wzbudzenie: 485 nm, emisja: 526 nm) i na tej podstawie obliczono procent ochrony przed peroksydacją lipidów przez wybrane antyoksydanty.

Kolejno określono ochronny wpływ wybranych związków na peroksydację lipidów błon erytrocytów, do tego celu również wykorzystano sondę BODIPY® 581/591. Błony traktowano wybranymi związkami w zakresie stężeń 10–1000 mM. Fluorescencję mierzono co 2 minuty przez 150 minut. Procent ochrony przed peroksydacją lipidów przez wybrane antyoksydanty obliczono jak powyżej.

Na podstawie przeprowadzonych badań wybrano pięć związków do dalszych oznaczeń. Do badania hemolizy wybrano metodę turbidymetryczną, która, choć jest mniej precyzyjna niż metoda oparta na wirowaniu zawiesin erytrocytów i pomiarze uwolnionej hemoglobiny, jest znacznie prostsza, może być wykonana w czytniku mikropłytek i jest zadowalająca dla celów porównawczych.

Sprawdzono ich wpływ na inhibicję hemolizy erytrocytów wywołaną przez wolne rodniki, których wytwarzanie indukowano przez AAPH. W wyniku rozpadu tego związku powstają rodniki alkilowe $R^{\bullet}$, które w reakcji z tlenem tworzą rodniki nadtlenkowe $ROO^{\bullet}$ . Zawiesinę erytrocytów traktowano roztworem odpowiedniego antyoksydantu do końcowego stężenia w zakresie 25–1000 μM i inkubowano z wytrząsaniem w obecności/nieobecności 50 mM AAPH w temperaturze 37°C. Absorbancję (700 nm) mierzono co 1 godzinę przez 12 godzin przy użyciu czytnika Tecan Spark. Dla wszystkich oznaczeń czas hemolizy i procent czasu hemolizy obliczono jako: 100% [czas (min) dla badanego związku/średni czas (min) dla próbki kontrolnej zawierającej tylko erytrocyty i AAPH].

Dioctan 2',7'-dichlorodihydrofluoresceiny ($H_2$DCF-DA, znany również jako dioctan 2',7'-dichlorofluorescyny) jest chemicznie zredukowaną formą dioctanu 2',7'-dichlorofluoresceiny stosowaną jako wskaźnik ilości ROS w komórkach. Po rozszczepieniu grup octanowych przez wewnątrzkomórkowe esterazy, a następnie po utlenieniu, niefluorescencyjny $H_2$DCF-DA przekształca się w wysoce fluorescencyjną 2',7'-dichlorofluoresceinę (DCF). 10 mM $H_2$DCF-DA dodano do 10 % zawiesiny erytrocytów do

końcowego stężenia 10 μM i inkubowano w 37°C przez 30 min. Następnie traktowano je odpowiednim roztworem antyoksydantu w PBS do uzyskania końcowego stężenia w zakresie 25–1000 μM. Kolejno dodawano AAPH i prowadzono pomiar fluorescencji (wzbudzenie: 485 nm, emisja: 529 nm). Obliczano sumę wartości fluorescencji uzyskanych w kolejnych pomiarach i na tej podstawie wyznaczono $IC_{50}$ (stężenie przeciwutleniacza hamujące tworzenie RFT o 50%).

Kolejno sprawdzono wpływ antyoksydantów na utlenianie hemoglobiny. W tym celu do 10 % zawiesiny erytrocytów dodano antyoksydant (końcowy zakres stężeń 5–1000 μM) i AAPH (końcowe stężenie 50 mM) i inkubowano w temperaturze 37°C przez 5h. Kontrolę stanowiły próbki zawierające zawiesinę erytrocytów i antyoksydanty, ale bez AAPH. Zmierzono absorbancję przy długości fali 540 nm oraz 630 nm i na tej podstawie wyznaczono stosunek absorbancji A630 / A540 jako miarę utlenienia hemoglobiny.

Oznaczono także zawartość glutationu (GSH) w komórkach traktowanych antyoksydantami i AAPH. W tym celu 2% zawiesinę erytrocytów traktowano różnymi stężeniami wybranych przeciwutleniaczy i AAPH i inkubowano przez 1 godz. w temperaturze 37°C. Następnie oznaczono poziom glutationu w próbkach stosując metodę fluorymetryczną opartą na reakcji z aldehydem *o*-ftalowym [15] i odczytano stężenie GSH z krzywej kalibracyjnej.

[Publikacja 3. Naparlo K., Soszynski M., Bartosz G., Sadowska-Bartosz I. 2020. Comparison of antioxidants: the limited correlation between various assays of antioxidant activity. *Molecules,* 25; 3244]

**WYNIKI**

Na podstawie badań cytotoksyczności do kolejnych testów wybrano flawonoid, w obecności którego badane komórki drożdży wykazały największą intensywność wzrostu: galusan epigallokatechiny (EGCG) . Do badań wykorzystano także drugi flawonoid: katechinę (C) który również wspomagał wzrost komórek drożdży.

Poddanie drożdży działaniu temperatury 50°C spowodowało zależne od czasu obniżenie żywotności drożdży. Traktowanie ich flawanolami (50 μM) spowodowało statystycznie znaczącą ochronę przed utratą żywotności zarówno w szczepie typu dzikiego jak i mutanta. Zamrożenie drożdży w temperaturze -20°C i rozmrożenie po 1 lub 2 godzinach przechowywania w stanie zamrożonym również zmniejszyło żywotność drożdży. W tym przypadku zarówno EGCG jak i C zapewniały istotną statystycznie ochronę przed utratą żywotności w obu szczepach.

Zarówno ogrzewanie, jak i zamrażanie-rozmrażanie drożdży powodowało pojawienie się u nich objawów stresu oksydacyjnego: zwiększone tworzenie reaktywnych form tlenu (ROS) i zwiększoną karbonylację białek oraz spadek całkowitej zdolności antyoksydacyjnej (TAC).

Pomiar wewnątrzkomórkowego poziomu ROS za pomocą $H_2$DCFDA wykazał brak znaczących zmian w komórkach typu dzikiego ogrzewanych przez 7 minut w temperaturze 50°C oraz znaczący wzrost poziomu ROS w komórkach poddanych zamrażaniu. (+)-Katechina obniżyła poziom ROS w komórkach kontrolnych (nie poddanych działaniu wysokiej temperatury). Galusan epigallokatechiny zwiększał poziom ROS w komórkach traktowanych temperaturą 50°C (w odniesieniu do komórek nie poddanych działaniu flawanolu); oba flawanole obniżały poziom ROS w komórkach poddanych zamrażaniu-rozmrażaniu (w odniesieniu do komórek nie poddanych działaniu flawanolu).

W przypadku mutanta *Δsod1* zaobserwowano wzrost poziomu ROS jedynie w komórkach traktowanych temperaturą -20°C i tylko tu pojawił się istotny statystycznie wpływ EGCG na zmniejszenie ilości ROS.

Inkubacja erytrocytów z katechinami spowodowała wychwyt tych związków przez komórki. Badanie zależności ilości pobieranych katechin od ilości erytrocytów wskazało na wystąpienie efektu wysycenia erytrocytów związkiem. Przedstawienie danych w postaci podwójnego wykresu zależności odwrotności liczby pobranych cząsteczek flawanoli od

odwrotności liczby erytrocytów wykazało podobny wychwyt wszystkich badanych związków przez erytrocyty. Liczba cząsteczek badanych związków pobranych przez $2,5x10^8$ erytrocytów z 500 μl 50 μM roztworów flawanoli (te wartości odpowiadają stężeniu erytrocytów we krwi) wynosiły $(1,19±0,03)x10^6$ dla katechiny, $(1,23±0,21)x10^6$ dla epigallokatechiny (EGC) i $(1,18±0,14)x10^6$ dla EGCG.

Z krzywych oporności osmotycznej określono wartości stężenia NaCl wywołujące 50% hemolizy (c50). Gdy erytrocyty o hematokrycie 10% inkubowano z 50 μM wybranymi flawanolami w 37°C przez 90 minut, katechiny zmniejszały oporność osmotyczną erytrocytów, o czym świadczy wzrost wartości c50. Niemniej jednak, po dodaniu katechin do erytrocytów i natychmiastowej ocenie oporności osmotycznej związki te chroniły erytrocyty przed hemolizą osmotyczną, o czym świadczą podwyższone wartości c50.

Na podstawie pomiarów widm EPR kwasu 5-doksylostearynowego (5DS) i 16-doksylostearynowego (16DS) można wywnioskować, że katechiny mają tendencję do zwiększania czasu korelacji rotacyjnej $\tau_c$ 16DS oraz parametru uporządkowania (S) obu sond osadzonych w lipidach błon erytrocytów.

Katechina w stężeniach do 50 μM nie miała zauważalnego wpływu na aktywność acetylocholinoesterazy błonowej erytrocytów. EGC i EGCG hamowały enzym w sposób zależny od stężenia, wywołując odpowiednio około 30% i 35% hamowania przy stężeniu 50 μM. Wykres Lineweavera-Burka dla hamowania acetylocholinoesterazy przez 50 μM EGC i EGCG wskazał na mieszany typ hamowania reakcji w obu przypadkach.

Krzywe zmian w funkcji czasu turbidancji zawiesin erytrocytów poddanych działaniu 100 μM nadmanganianu potasu w obecności różnych stężeń katechiny wykazały, że połowiczny czas hemolizy przy braku badanych związków wyniosło 19,9±1,9 min, a katechiny wydłużyły czas niezbędny do osiągnięcia 50% hemolizy. Innym sposobem oceny ilościowej hemolizy było zsumowanie kolejnych wartości zmętnienia podczas 2-godzinnych pomiarów i także ten parametr wykazał ochronne działanie katechin.

Badanie mikroskopowe erytrocytów poddanych działaniu nadmanganianu wykazało całkowitą hemolizę komórek pod nieobecność badanych flawanoli po ponad 20 min i ochronę przed hemolizą przez wybrane flawanole. Co ciekawe, podczas gdy początkowo erytrocyty traktowane flawanolami monomerycznymi wykazywały echinocytozę, później ta echinocytoza zniknęła, najwyraźniej z powodu utlenienia badanych związków i słabszej interakcji produktów utleniania flawanoli monomerycznych z błonami erytrocytów.

Wszystkie badane związki wykazywały zależną od dawki ochronę przed indukowanym podchlorynem utlenianiem błonowych białkowych grup tiolowych i peroksydacją lipidów.

Porównaliśmy szereg syntetycznych i naturalnych antyoksydantów pod względem skuteczności hamowania peroksydacji lipidów indukowanej przez AAPH w dwóch układach: zawiesinie żółtka jaja i błonach erytrocytów. Szybkość peroksydacji mierzono za pomocą BODIPY® 581/591. Sonda ta emituje czerwoną fluorescencję przy 595 nm. Utlenianiu sondy rodnikami nadtlenkowymi towarzyszy zanik czerwonej fluorescencji i pojawienie się zielonej fluorescencji przy około 520 nm. Zmniejszenie intensywności czerwonej fluorescencji, zmiany stosunku intensywności zielonej i czerwonej fluorescencji lub wzrost intensywności zielonej fluorescencji są wykorzystywane przez różnych autorów jako wskaźniki peroksydacji [16–18]. Tempo wzrostu intensywności zielonej fluorescencji było najbardziej powtarzalną i miarodajną miarą szybkości peroksydacji dlatego zostało wykorzystane w tych badaniach. W zawiesinie żółtka jaja *tert*-butylohydrochinon (t-BHQ) był najbardziej skutecznym antyoksydantem, wykazując najniższą wartość stężenia wymaganego do zahamowania peroksydacji w 50% (IC50). Niektóre naturalne substancje, w tym kwas galusowy, galusan epikatechiny (ECG) i melatonina wykazały porównywalną skuteczność. Mniejszą skuteczność wykazywały antyoksydanty hydrofilowe takie jak glutation (GSH), cysteina, naringina, hesperetyna i kwas askorbinowy. W innym układzie, tj. błonach erytrocytów ECG był najbardziej skuteczny, niemniej jednak kolejność skuteczności pozostałych związków była inna. Podczas gdy niektóre przeciwutleniacze, takie jak ECG lub resweratrol, miały podobne wartości IC50 w obu układach, wartości dla większości związków różniły się znacząco w obu systemach, w większości przypadków były wyższe dla błon erytrocytów. Współczynnik korelacji liniowej Pearsona między wartościami IC50 w obu systemach wynosił zaledwie 0,30. Różnice te mogą wynikać z faktu, że w bardziej złożonej błonie erytrocytów niektóre antyoksydanty mogą wiązać się z białkami błonowymi i mogą nie być dostępne lub mogą być dostępne tylko częściowo dla reakcji z rodnikami nadtlenkowymi. Stosunek wagowy białek do lipidów wynosi około 1 : 1 w błonach erytrocytów [19] i jest znacznie niższy (około 0,5 : 1) w żółtku jaja kurzego [20], a skład białek różni się znacznie w obu materiałach. Wyniki te pokazują, że odnoszenie wyników oceny zdolności do hamowania peroksydacji lipidów z jednego układu do drugiego może być mylące.

Do dalszych eksperymentów (ochrona hemoglobiny i glutationu w erytrocytach przed utlenieniem wywołanym przez AAPH) wybrano pięć skutecznych antyoksydantów

rozpuszczalnych w PBS (w celu uniknięcia wpływu rozpuszczalnika organicznego na błonę erytrocytów): EGCG, C, kwas galusowy, kwas gentyzynowy i kwas askorbinowy.

Większość wybranych przeciwutleniaczy częściowo wiąże się z błonami, ale są one również obecne w środowisku wodnym komórki, podczas gdy askorbinian jest praktycznie całkowicie obecny w roztworze, więc wszystkie badane przeciwutleniacze mogą wchodzić w interakcje z rodnikami generowanymi przez AAPH poza komórkami i wewnątrz komórek. W odniesieniu do zmiatania tych ostatnich rodników można oczekiwać, że polifenole będą bardziej reaktywne niż kwas askorbinowy, ze względu na większą liczbę reaktywnych grup hydroksylowych. Chociaż wszystkie badane antyoksydanty były skuteczne, najwyższe stężenia oddziałujących z błoną przeciwutleniaczy wydłużały czas niezbędny do uzyskania 50% spadku turbidancji zawiesin erytrocytów do ponad 1000%, podczas gdy 1 mM kwas askorbinowy wydłużał względny czas hemolizy tylko do około 300% . Do skuteczności hydrofilowego antyoksydantu, jakim jest kwas askorbinowy, może przyczynić się jego zdolność do regeneracji rodników innych przeciwutleniaczy powstających w procesie hamowania peroksydacji lipidów.

AAPH wytwarzał w erytrocytach duże ilości RFT reagujących z H2DCF-DA. Wszystkie zastosowane antyoksydanty w sposób zależny od dawki obniżyły poziom RFT w erytrocytach. Co ciekawe, EGCG okazał się najskuteczniejszym przeciwutleniaczem w tym teście, C i kwas galusowy wykazywały podobną skuteczność, podczas gdy kwas gentyzynowy okazał się najmniej skuteczny pomimo podobieństwa strukturalnego do kwasu galusowego.

RFT generowane przez AAPH powodowały utlenianie hemoglobiny, która jest obecna w bardzo wysokim stężeniu wewnątrz erytrocytów (ponad 30% wagowych); dlatego tylko wysokie stężenia antyoksydantów były skuteczne w zapobieganiu utlenianiu hemoglobiny. Jednak EGCG i kwas galusowy były skuteczne tylko w pewnym zakresie stężeń, podczas gdy efekt ochronny zanikał wraz z dalszym wzrostem stężenia antyoksydantów. Ponieważ efekt ten był powtarzalny, sprawdziliśmy, czy badane przeciwutleniacze same utleniają hemoglobinę. Wyniki uzyskane dla najwyższego stężenia przeciwutleniaczy wykazały, że rzeczywiście EGCG i kwas galusowy były w stanie utleniać hemoglobinę, co wyjaśnia zaobserwowaną dziwną zależność od dawki. Wcześniej odnotowano utlenianie hemoglobiny do methemoglobiny przez ekstrakty z czarnej i zielonej herbaty zawierające EGCG [8]. Zarówno kwas galusowy, jak i EGCG zawierają resztę kwasu trihydroksybenzoesowego, która może być odpowiedzialna za ich prooksydacyjny wpływ na hemoglobinę.

Inkubacja zawiesiny erytrocytów z roztworem AAPH przez 1 godzinę spowodowało zmniejszenie stężenia glutationu (GSH) w erytrocytach z wartości 1875 ± 164 µM do 865 ± 233 µM, tj. o 54%. Kwas askorbinowy i katechina w zależności od dawki chroniły przed utlenianiem GSH. Przeciwny efekt wystąpił w przypadku EGCG, kwasu galusowego i kwasu gentyzynowego, które znacznie zwiększyły utratę GSH w erytrocytach. Gdy erytrocyty inkubowano z antyoksydantami w tych samych warunkach, ale bez AAPH, kwas askorbinowy, C i kwas gentyzynowy nie obniżały poziomu GSH, podczas gdy wyższe stężenia EGCG i kwasu galusowego powodowały znaczną utratę GSH. Badania innych autorów wykazały, że kwas galusowy utlenia GSH w erytrocytach [21], a bogate w EGCG ekstrakty czarnej i zielonej herbaty także obniżają poziom GSH w tych komórkach [22]. Zatem dane dotyczące EGCG i kwasu galusowego są zrozumiałe (utrata GSH indukowana przez sam ten związek nakłada się na utratę wywołaną AAPH). Wyniki dla kwasu gentyzynowego są trudniejsze do wyjaśnienia. Można postulować, że reaktywne związki powstałe w wyniku interakcji AAPH z kwasem gentyzynowym mogą wykazywać właściwości prooksydacyjne, których nie wykazuje natywny kwas gentyzynowy. Wyniki badań innych autorów pokazują, że inne przeciwutleniacze, melatonina i resweratrol, miały działanie prooksydacyjne wobec dehydrogenazy gliceroaldehydo-3-fosforanowej, gdy były stosowane razem z tlenkiem azotu; efekt ten był spowodowany tworzeniem się ich reaktywnych pochodnych, a mianowicie rodnika fenoksylowego w przypadku resweratrolu [23,24]. Ponieważ rodnik fenoksylowy powstaje również podczas utleniania kwasu gentyzynowego [25], mechanizm prooksydacyjnego działania kwasu gentyzynowego może być podobny.

Oczywistym wynikiem tego badania jest ograniczona korelacja lub brak korelacji między wynikami różnych testów zdolności antyoksydacyjnej tych samych związków. EGCG i kwas galusowy, które są dobrymi antyoksydantami w teście hamowania peroksydacji lipidów i hemolizy, powodowały utlenianie hemoglobiny i glutationu w nieobecności innych czynników utleniających.

**DYSKUSJA**

Wyniki naszych badań potwierdzają, że flawonoidy są dobrymi antyoksydantami. Drożdże poddane działaniu flawanoli takich jak EGC-G i C wykazywały zwiększoną przeżywalność w odpowiedzi na czynniki fizyczne i chemiczne indukujące stres oksydacyjny. Sposób działania tych substancji wymaga dalszych badań, jednak najprostszym wyjaśnieniem jest to, że ochrona wynika z bezpośredniego działania antyoksydacyjnego flawanoli, które są doskonałymi przeciwutleniaczami. Flawonoidy można uznać za związki zwiększające stabilność drożdży w ekstremalnych warunkach temperaturowych i prawdopodobnie w innych warunkach indukujących stres oksydacyjny, takich jak przechowywanie preparatów zawierających drożdże.

Podobne wnioski można wysnuć na podstawie badań interakcji katechin zawartych w żywności, a erytrocytami. Zastosowano stężenia, które nie są możliwe do osiągnięcia *in vivo* jednak pozwala to na wnioskowanie, że podobne efekty, choć w znacznie mniejszym stopniu, zachodzą *in vivo*. Ochrona erytrocytów przed hemolizą przez ekstrakt z zielonej herbaty i EGCG zostało już wcześniej opisane [8]. Nasze badania potwierdziły te obserwacje nie tylko dla EGCG, ale i dla wszystkich badanych flawanoli.

W kolejnych testach porównałam większą ilość substancji z grupy flawonoidów pod względem ich aktywności antyoksydacyjnej. Uzyskane wyniki pokazały, że wiele związków z tej grupy było skutecznych. Korelacja pomiędzy efektywnością substancji w poszczególnych testach była niewielka, a niektóre z nich ujawniły prooksydacyjne działanie antyoksydantów, indukując utlenianie pod nieobecność oksydantu i wzmacniając ten proces w obecności oksydantu, co nie było widoczne w przypadku innych testów. Dlatego przy określaniu zdolności antyoksydacyjnej danej substancji należy pamiętać, że mierzone parametry są zależne od zastosowanych warunków i nie należy bezkrytycznie odnosić wyników jednego testu na inne testy.

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## OŚWIADCZENIA WSPÓŁAUTORÓW

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Rzeszów, dnia 21.07.2020

**OŚWIADCZENIE**

W związku z przygotowywaniem przeze mnie rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów, oświadczam niniejszym, że wkład mojej pracy naukowej, a tym samym pracy pozostałych współautorów w opublikowaniu poniższych artykułów, które zamierzam przedstawić jako własną dysertację doktorską jest następujący:

1. Naparło Katarzyna, Żyracka Ewa, Bartosz Grzegorz, Sadowska-Bartosz Izabela. **„Flavanols protect the yeast Saccharomyces cerevisiae against heating and freezing/thawing injury".** *Journal of Applied Microbiology,* 2019; 126(3):872-880. doi: 10.1111/jam.14170. $IF_{2019}$ = **2.683;** Punkty MNiSW = **30** (obecnie 70 punktów)

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2. Naparło Katarzyna, Bartosz Grzegorz, Stefaniuk Ireneusz, Cieniek Bogumił, Soszyński Mirosław, Sadowska-Bartosz Izabela. **„Interaction of Catechins with Human Erythrocytes".** *Molecules*, 2020;25(6):1456. doi: 10.3390/molecules25061456. $IF_{2020}$=**3,267;** Punkty MNiSW=**100.**

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Editor's Choice

ORIGINAL ARTICLE

# Flavanols protect the yeast *Saccharomyces cerevisiae* against heating and freezing/thawing injury

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**Keywords**
catechin, epigallocatechin gallate, freeze-thawing injury, heat lethality, oxidative stress, *Saccharomyces cerevisiae*.

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## Abstract

**Aims:** The aim of the study was to check whether two flavanols ((−)-epigallocatechin gallate and (+)-catechin) can ameliorate oxidative stress (OS) accompanying and contributing to the lethal effects of heating (50°C) and freezing-thawing on the yeast *Saccharomyces cerevisiae*.
**Methods and Results:** The flavanols studied increased yeast survival during heating and freezing-thawing, estimated by the colony forming assay. They improved also such indices of OS as increased production of reactive oxygen species, decrease of total antioxidant activity of yeast cell extracts and increase in the level of protein carbonyls.
**Conclusions:** Amelioration of OS by flavanols increases the survival of the yeast subjected to high temperature and freezing-thawing.
**Significance and Impact of the Study:** Flavanols may be considered as means of enhancing yeast survival under extreme temperature conditions and probably in other conditions involving OS.

## Introduction

Flavanols (flavan-3-ols), the main components of tea extracts, present also in cocoa and wine and other plant products, rise increasing interest due to their diverse biological effects. They have strong antioxidant properties, mainly due to reactions with reactive oxygen, nitrogen and halogen species and to binding transition metal ions (Rice-Evans 1995; Grzesik *et al.* 2018a,b). However, in the body they exert a plethora of other effects which can be only partly or not at all related to their antioxidant action. They have been reported to lower blood pressure, especially in prehypertensive and hypertensive individuals, modulate metabolism and respiration including maximal oxygen uptake, $O_2$ cost of exercise and energy expenditure, and reduce inflammation, resulting in increased skeletal muscle efficiency and endurance capacity (Al-Dashti *et al.* 2018), positively modulate blood platelet aggregation (Peluso *et al.* 2015), improve endothelial function (Aprotosoaie *et al.* 2016) as well as protect against obesity (Akhlaghi *et al.* 2018). Flavanols influence also positively insulin signalling by relieving insulin-signalling pathways from oxidative stress (OS) and inflammation and/or via a heightened incretin response (Strat *et al.* 2016). Protective effects of long-term flavanol consumption on cognition and behaviour, including age- and disease-related cognitive decline, were seen in animal models of normal ageing, dementia and stroke (Sokolov *et al.* 2013). Flavanols induce release of nitric oxide (˙NO) through activation of endothelial ˙NO synthase which, in turn, accounts for vasodilation and cardioprotective effects, and switch on some important signalling pathways such as toll-like receptor 4/nuclear factor κβ/signal transducer and activator of transcription (Magrone *et al.* 2017). They were reported to have beneficial effects in type 2 diabetic patients by enhancing insulin secretion, improving insulin sensitivity in peripheral tissues and exerting a lipid-lowering effect (Ramos *et al.* 2017). Flavanols may inhibit digestive enzymes and glucose transporters, causing a reduction in glucose absorption, which helps patients with metabolic disorders to maintain glucose homeostasis. Unabsorbed flavanols and those which undergo enterohepatic recycling exert prebiotic effects on the gut microbiota. Interactions with the

gut microbiota may improve gut barrier function, resulting in attenuated endotoxin absorption (Strat *et al.* 2016). Thus, flavanols can be regarded as dietary bioactives, that is, food constituents that although not essential to human life and procreation, may nevertheless play an important role in disease risk reduction, primary disease prevention and healthy ageing (Ottaviani *et al.* 2018). Interestingly, studies of activation of Nrf2-, FoxO- and PPAR$\gamma$-dependent signalling pathways by other flavonoids showed that the activity of the flavonoids to activate gene expression shows no correlation with their antioxidant activities found in *in vitro* tests (Pallauf *et al.* 2017). Therefore, it is not easy to ascertain antioxidant effects of flavanols in human and animal systems and to distinguish them from other effects of these compounds.

In order to study the antioxidant effects of flavanols on the cellular level, a simpler system, in which antioxidant effects are more directly evaluated and less dependent on whole-body mechanisms, is more convenient. The budding yeast *Saccharomyces cerevisiae* may constitute such a system. This eukaryotic system is relevant from ecophysiological and biotechnological point of view since both under natural conditions and in biotechnological processes the yeast is exposed to OS and may be in contact with flavanols.

The aim of this study was to examine the capacity of selected flavanols ((−)-epigallocatechin gallate (EGC-G) and (+)-catechin (C)) to protect *S. cerevisiae* against freeze-thaw injury and heat-induced lethality at 50°C. These modalities were chosen as both conditions were shown to induce OS as demonstrated by increased production of reactive oxygen species (ROS) in yeast cells during heating (Davidson *et al.* 1996) and formation of oxygen radicals during freezing-thawing of yeast cells as detected by spin trapping (Park *et al.* 1998); moreover, OS contributes to cell killing induced by both treatments since CuZn-superoxide dismutase (SOD) deficient mutants of the yeast were more sensitive to both freeze-thaw injury and heating (Davidson *et al.* 1996; Park *et al.* 1998).

Both wild-type and *Δsod1* mutant were used in the study to compare the effects of flavanols on a normal yeast strain and on a strain of increased sensitivity to OS.

## Materials and methods

### Yeast strains and culture

Two yeast strains were used: wild-type BY4741 (genotype MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and its mutant devoid of CuZn-SOD BY4741*Δsod1* (genotype MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sod1). They were grown usually in a liquid YPD medium (2% glucose, 1% yeast extract, 1% bacto peptone) on a rotary shaker or on solid medium of the above composition but with 2% agar, at 28°C. The liquid cultures were used for experiments in the early exponential phase.

### Materials

All substances were purchased from Sigma-Aldrich (Poznań, Poland) except for sodium chloride (Chempur, Piekary Śląskie, Poland), D-(+)-glucose as well as ethanol (Polskie Odczynniki Chemiczne, Gliwice, Poland), Bacto™ Yeast Extract, Bacto Peptone and Difco™ Agar Granulated (obtained from Becton Dickinson (BD) Biosciences, Warsaw, Poland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA). 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 distilled water.

### Effects of flavonoids on yeast growth

Yeast cultures of density of $2 \times 10^7$ cells per ml were aliquoted to wells of a multiwell plate, added with appropriate concentrations of the flavanols tested and their growth was monitored under constant shaking by measuring turbidity in a Bioscreen C Automated Growth Curve System (Oy Growth Curves AB Ltd, Raisio, Finland) at 600 nm. As EGC-G concentrations higher than 50 $\mu$mol l$^{-1}$ inhibited the growth of the *Δsod1* mutant (Fig. 1b,d), the flavanol concentration of 50 $\mu$mol l$^{-1}$ was chosen for further experiments.

### Estimation of colony forming units

Control yeast and yeast subjected to appropriate treatments were diluted to a density $10^3$ cells per ml and 200 $\mu$l of such suspensions was applied to a Petri dish containing solid YPD medium. The plates were incubated at 28°C for 48 h, and the number of colonies formed was counted.

### Heating and freezing of the yeast

Cells (except for control) were preincubated with the flavanols (50 $\mu$mol l$^{-1}$) in the YPG medium for 1 and 2 h and then washed with flavanol-free medium. Aliquots (200 $\mu$l) of yeast suspensions in YPD of density of $10^7$ cells per ml were heated in a water bath at 50·0 ± 0·1°C for various time intervals or frozen at −20°C for 1, 2 or 3 h and thawed in air at room temperature.

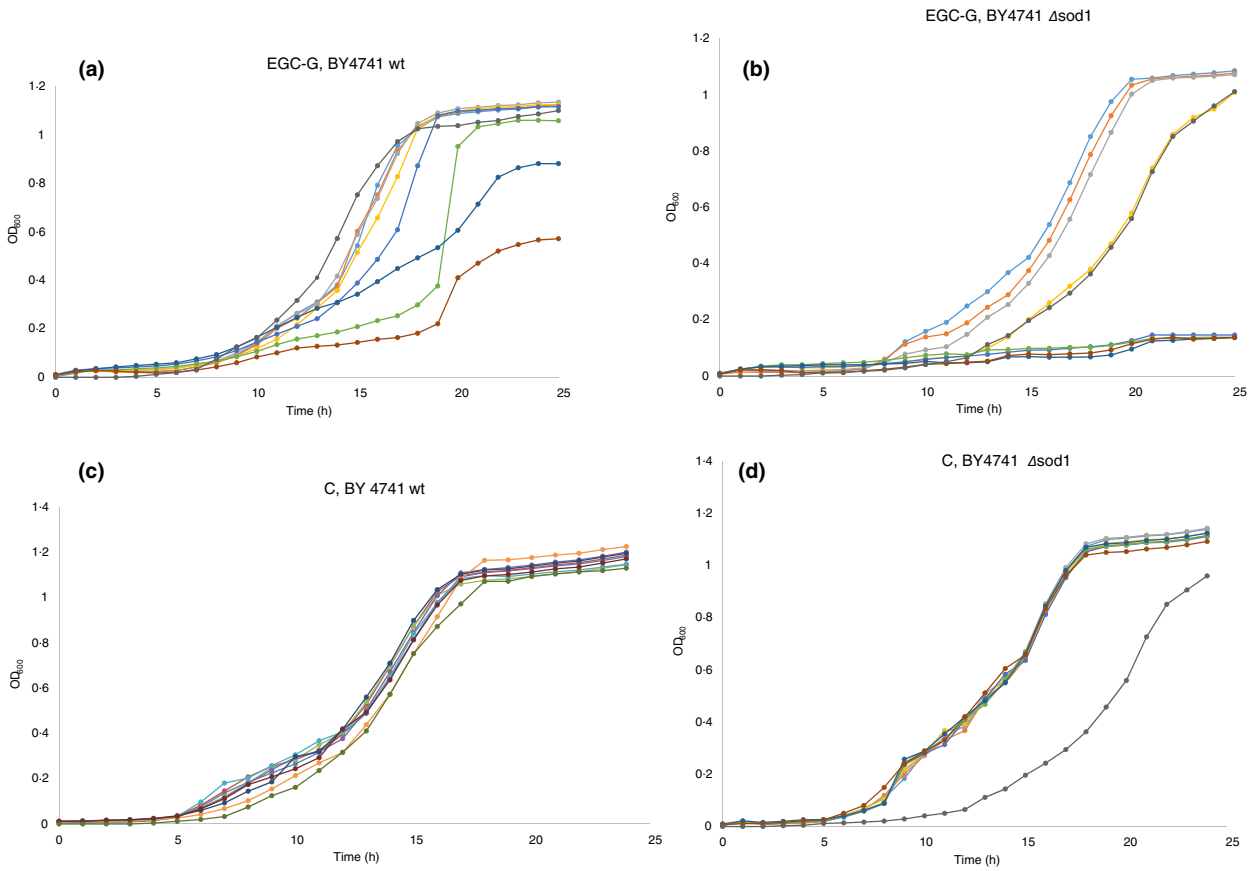


**Figure 1** Effect of EGC-G (a, b) and C (c, d) on the growth of yeast, wild-type BY4741(a, c) and BY4741*Δsod1* mutant (b, d) in the liquid YPD medium. Yeast growth was monitored under constant shaking by measuring turbidity in a Bioscreen C Automated Growth Curve System at 600 nm (— 5 μmol $l^{-1}$; — 10 μmol $l^{-1}$; — 20 μmol $l^{-1}$; — 50 μmol $l^{-1}$; — 100 μmol $l^{-1}$; — 200 μmol $l^{-1}$; — 500 μmol $l^{-1}$; — 1000 μmol $l^{-1}$; — control). [Colour figure can be viewed at wileyonlinelibrary.com]

### Estimation of ROS production

Yeast suspensions ($2 \times 10^7$ cells) were centrifuged and suspended in 200 $\mu$l of 100 mmol $l^{-1}$ phosphate buffer, pH = 7·0, containing 0·1% glucose and 1 mmol $l^{-1}$ EDTA, applied to wells of a 96-well plate and added with 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) to a final concentration of 20 $\mu$mol $l^{-1}$ or dihydroethidine (DHE) to a final concentration of 18 $\mu$mol $l^{-1}$. Fluorescence was measured at excitation/emission wavelengths of 495 nm/525 nm for $H_2$DCF-DA and at 405 nm/570 nm for DHE (to increase the specificity of the assay for superoxide (Nazarewicz *et al.* 2013)), after 10-min incubation at 28°C.

### Estimation of total antioxidant capacity of yeast extracts

Total antioxidant capacity (TAC) of yeast extracts was estimated by a modified method of Re *et al.* (1999). Briefly, yeast was sedimented by centrifugation, washed and suspended in 100 mmol $l^{-1}$ phosphate buffer, pH 7·0 containing 1 mmol $l^{-1}$ EDTA to a density of $10^9$ cells per ml. The acid-soluble fraction was obtained by treatment with 5% (final) trichloroacetic acid, incubation on ice (20 min) and centrifugation. Appropriate amount of the supernatant was added to a cuvette containing the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical (ABTS*) solution of absorbance of 1·0 at 414 nm. After 10 s, the decrease in absorbance was measured. Total antioxidant capacity was calculated on a basis of a standard curve obtained with Trolox.

### Estimation of carbonyl group content

Protein carbonyls were estimated by ELISA using OxiSelectTM Protein Carbonyl Fluorometric Assay kit (Cell Biolabs Inc., San Diego, CA) according to the instruction of the manufacturer.

### Protein assay

Protein concentration was assayed using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, Rockford, IL).

### Statistical analysis

Statistical significance of differences was evaluated using the paired Student's '*t*' test. Statistical analysis of the data was performed using STATISTICA software package (ver. 13.1; StatSoft Inc. 2016, Tulsa, OK: www.statsoft.com).

## Results

We examined the effects of two flavanols, EGC-G and C, on the growth of yeast in a liquid medium and found no apparent toxicity of both flavanols at concentrations lower than 500 $\mu$mol $l^{-1}$ for the wild-type strain (Fig. 1a, c). Interestingly, the growth of the *Δsod1* strain was enhanced by low concentrations (5–20 $\mu$mol $l^{-1}$) of EGC-G and all C concentrations used (5–1000 $\mu$mol $l^{-1}$), up to the level of that of the wild-type strain. (−)-Epigallocatechin gallate was more toxic for the *Δsod1* strain, concentrations higher than 50 $\mu$mol $l^{-1}$ inhibited the growth of the mutant (Fig. 1b,d).

Exposure of the yeast to the temperature of 50°C resulted in a time-dependent decrease in yeast viability. Pretreatment of yeast with flavanols (50 $\mu$mol $l^{-1}$) resulted in a statistically significant protection against the viability loss both in the wild-type strain and in the *Δsod1* mutant (Fig. 2).

Freezing of yeast at −20°C and thawing after 1 or 2 h storage in the frozen state also reduced yeast viability. In this case, both EGC-G as well as C (50 $\mu$mol $l^{-1}$) offered protection against viability loss in both strains (Fig. 3).

Both heating and freezing-thawing of the yeast induced symptoms of OS: increased formation of ROS and protein carbonyls, and decrease in the content of TAC.

Measurement of intracellular ROS level with $H_2$DCF-DA demonstrated a lack of significant changes in wild-type cells heated for 7 min at 50°C and a significant increase in ROS level in cells subjected to freezing-thawing. (+)-Catechin decreased the ROS level in control cells (not subjected to any treatment). (−)-Epigallocatechin gallate increased the ROS level in cells heated at 50°C (with respect to cells containing no flavanol); both flavanols decreased the ROS level in cells subjected to freezing-thawing (with respect to cells containing no flavanol). In the *Δsod1* mutant, due to higher scatter, only increase in the ROS level in freeze-thawed cells and its attenuation by EGC-G were significant (Fig. 4).

The ROS level estimated with DHE, reflecting mainly the level of superoxide ($O_2^{\cdot -}$) was higher in the *Δsod1* mutant than in the wild-type strain ($P < 0{\cdot}001$) and increased in yeast cells subjected to both heating at 50°C and to freezing/thawing in both strains. In both strains, the flavanols decreased the ROS level in cells not subjected to any treatment. In the wild-type strain, C decreased the level of ROS in cells subjected to heating while EGC-G increased this level (an effect seen also with DA-$H_2$DCF although without statistical significance for C). In the *Δsod1* strain, both flavanols decreased the ROS level in cells subjected to freezing/thawing, while C (but not EGC-G) decreased the ROS level in cells subjected to heating (Fig. 5).

Total antioxidant capacity of yeast extracts was decreased by both heating and freezing/thawing of yeast cells of both strains. Lower decrease was observed in the presence of EGC-G or C (Fig. 6).

Another index of OS, the level of protein carbonyls, increased in yeast cells subjected to heating and freezing/thawing in both strains studied. Flavanols decreased the level of protein carbonyls both in control cells and in cells subjected to temperature treatments (Fig. 7).

## Discussion

As SODs are critical for the survival in aerobic atmosphere (Fridovich 1974), mutants of *S. cerevisiae* deficient in SODs are more sensitive to OS. The *Δsod1* mutant, devoid of cytoplasmic SOD, cannot grow in the atmosphere of pure oxygen, has decreased replicative and chronological lifespan, and is hypersensitive not only to superoxide-generating agents but also to a plethora of other factors inducing OS (Biliński *et al.* 1985; Wawryn *et al.* 2002; Fabrizio and Longo 2003). Antioxidants can compensate for the effects of lack of CuZn-SOD; their protective effect is usually more discernible in the mutant, which is therefore a better sensor of OS and antioxidant effects than the wild-type strain (Koziol *et al.* 2005; Zyracka *et al.* 2005). For this reason, the *Δsod1* mutant was employed in this study, apart from the wild-type strain.

The flavanols studied: EGC-G at low concentrations and C in the whole concentration range used restored the defective growth of the *Δsod1* strain in the liquid medium (Fig. 1b,d), apparently compensating for the lack of an important antioxidant enzyme or some secondary consequences of this mutation, perhaps by stimulation of biosynthesis of some elements of the antioxidant barrier.

In order to examine the antioxidant action of flavanols on the yeast, treatments with high and low temperature were chosen, procedures involving OS, but avoiding the use of external chemicals. Such an approach was chosen

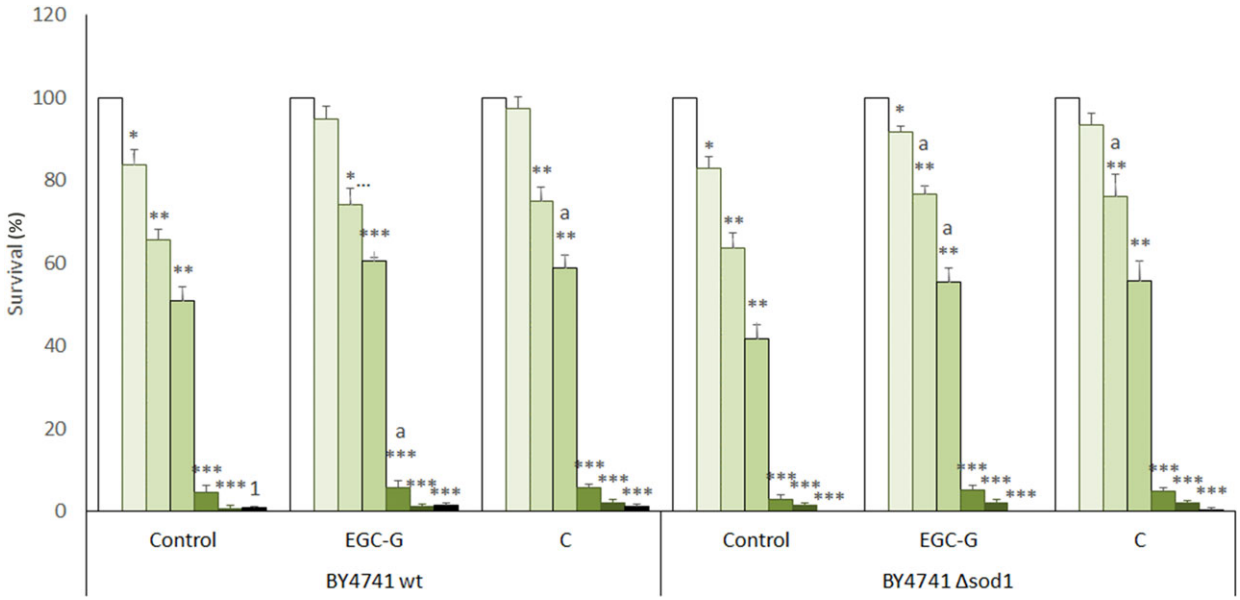


**Figure 2** Effect of EGC-G and C on the survival of yeast heated at a temperature of 50°C for various time intervals. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ (effect of temperature); $^{a}P<0{\cdot}05$ (effect of flavanols) (□ 0 min; □ 2 min; □ 5 min; □ 7 min; ■ 10 min; ■ 20 min; ■ 30 min). [Colour figure can be viewed at wileyonlinelibrary.com]

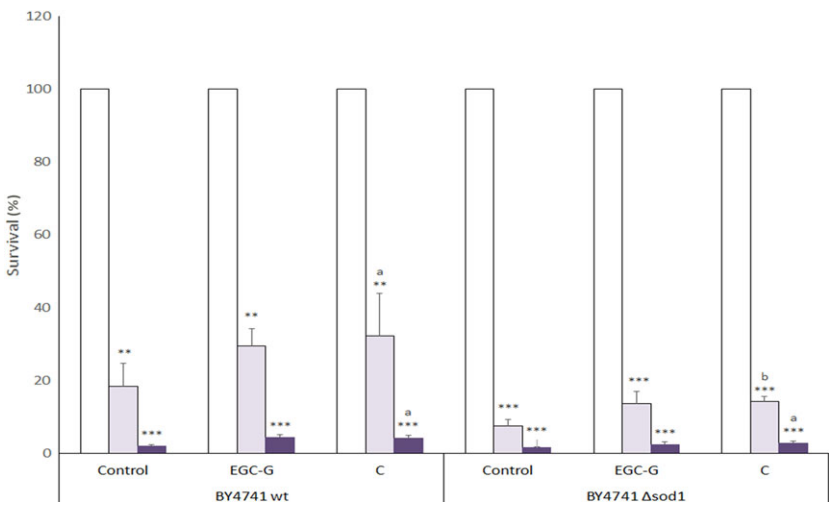


**Figure 3** Effect of EGC-G and C on the survival of yeast subjected to freezing and thawing after 1 or 2 h at −20°C. $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ (effect of temperature); $^{a}P < 0{\cdot}05$, $^{b}P < 0{\cdot}01$ (effect of flavanols) (□ 0 h; □ 1 h; ■ 2 h). [Colour figure can be viewed at wileyonlinelibrary.com]

in order to avoid situations, which may point to artifactual protection by antioxidants caused by neutralization of antioxidants outside the cells. If OS accompanies and contributes to the lethal effects of both heating and freezing-thawing, antioxidants should provide protection against lethality caused by both procedures. At the concentration of 50 $\mu$mol $l^{-1}$, both flavanols used proved effective in small, but significant protection against both heating at 50°C and freezing-thawing injury in both strains studied (Figs 2 and 3).

The protective action of flavanols was most probably due to their antioxidant action and amelioration of OS since these compounds attenuated changes in several markers of OS. While generation of ROS estimated with $H_2$DCF-DA provided partly inconclusive results (Fig. 4), ROS level estimated with DHE was increased by both heating and freezing/thawing of the cells, confirming earlier findings (Davidson *et al.* 1996; Park *et al.* 1998). Interestingly, the basic ROS level estimated with DHE, under conditions optimal for detection of 2-

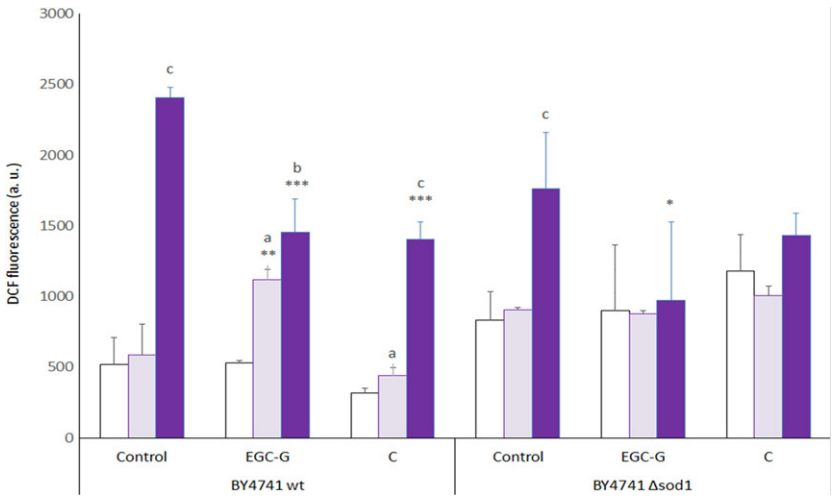


**Figure 4** Effect of heating (50°C) for 7 min or freezing/thawing (1 h) on the ROS level in wild-type and *Δsod1* yeast cells detected by 2′,7′-dichlorofluorescein fluorescence. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ with respect to cells containing no flavanol subjected to the same treatment; $^{a}P < 0{\cdot}05$, $^{b}P < 0{\cdot}01$, $^{c}P < 0{\cdot}001$ with respect to control cells or cells treated with a flavonoid, not subjected to any treatment (□ 0 min; ▨ 7 min/50°C; ■ 1 h/−20°C). [Colour figure can be viewed at wileyonlinelibrary.com]

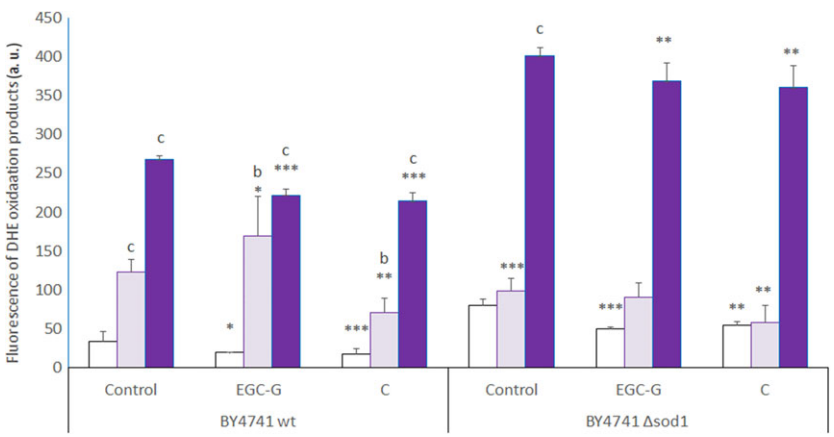


**Figure 5** Effect of heating (50°C) for 7 min or freezing (1 h)/thawing on the ROS level in wild-type and *Δsod1* yeast cells detected by fluorescence of DHE oxidation products. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ with respect to cells containing no flavanol subjected to the same treatment; $^{a}P < 0{\cdot}05$, $^{b}P < 0{\cdot}01$, $^{c}P < 0{\cdot}001$ with respect to control cells or cells treated with a flavonoid, not subjected to any treatment (□ 0 min; ▨ 7 min/50°C; ■ 1 h/−20°C). [Colour figure can be viewed at wileyonlinelibrary.com]

hydroxyethidium, the specific product of reaction with superoxide (Nazarewicz *et al.* 2013), was higher in the *Δsod1* strain in comparison with the wild-type strain, according to expectations (Fridovich 1974). In the wild-type strain, the effects of both flavanols on the increase in ROS production induced by heating were opposite. While C diminished/tended to diminish the increased in ROS production detected by both fluorogenic probes used, EGC-G further increased the ROS level. These results suggest an additional mode of action of C with respect to EGC-G. In the case of freeze/thaw injury, both probes indicated a protective role of C and EGC-G against the increase in the ROS level (Figs 4 and 5).

Total antioxidant capacity of acid-soluble yeast extracts, a measure of activity of low-molecular weight antioxidants present in yeast cells, decreased after both heating and freezing/thawing, the decrease being reduced by the flavanols (Fig. 6). The level of protein carbonyls, a measure of oxidative damage to cellular proteins, was elevated after both treatments, and this elevation was inhibited by the flavanols (Fig. 7).

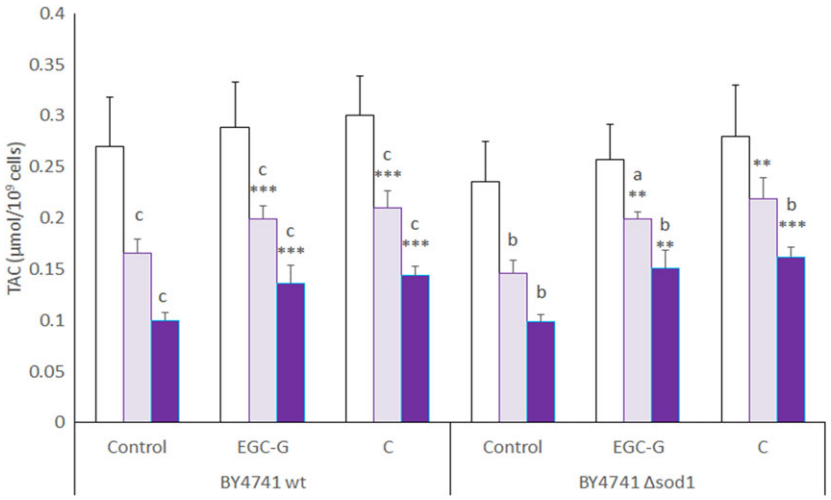


**Figure 6** Effect of heating (50°C) for 7 min or freezing/thawing (1 h) on the total antioxidant capacity in wild-type and *Δsod1* yeast cells. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ with respect to cells containing no flavanol subjected to the same treatment; $^{a}P < 0{\cdot}05$, $^{b}P < 0{\cdot}01$, $^{c}P < 0{\cdot}001$ with respect to control cells or cells treated with a flavonoid, not subjected to any treatment (□ 0 min; ▨ 7 min/50°C; ■ 1 h/−20°C). [Colour figure can be viewed at wileyonlinelibrary.com]

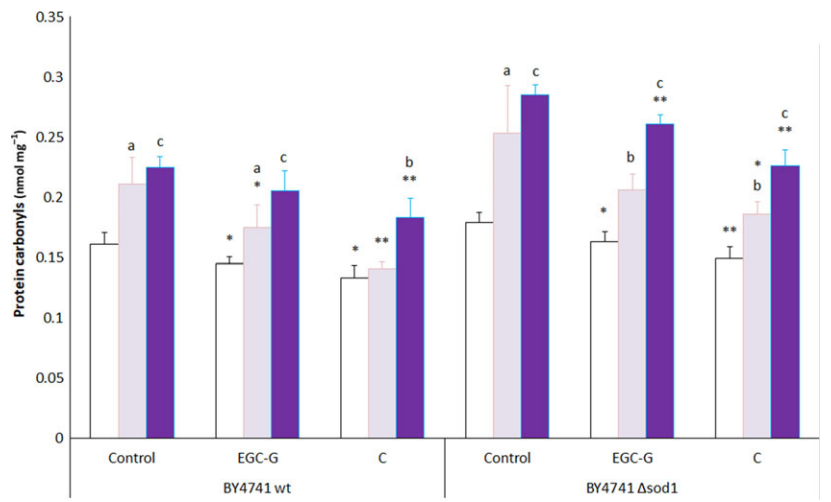


**Figure 7** Effect of heating (50°C) for 7 min or freezing/thawing (1 h) on the protein carbonyl level in wild-type and *Δsod1* yeast cells. $^{*}P < 0{\cdot}01$, $^{**}P < 0{\cdot}01$ with respect to cells containing no flavanol subjected to the same treatment; $^{a}P < 0{\cdot}05$, $^{b}P < 0{\cdot}01$, $^{c}P < 0{\cdot}001$ with respect to control cells or cells treated with a flavonoid, not subjected to any treatment (□ 0 min; ▨ 7 min/50°C; ■ 1 h/−20°C). [Colour figure can be viewed at wileyonlinelibrary.com]

Flavanols have been reported to protect the yeast against OS generated by other agents. (+)-Catechin protected the yeast against OS induced by hydrogen peroxide, carbon tetrachloride and cadmium (Dani *et al.* 2008). (−)-Epigallocatechin gallate and flavanol-rich green tea extract could protect yeast against light- and singlet oxygen-dependent toxicity (Mitrica *et al.* 2012).

The mode of action of flavanols on the viability of yeast subjected to heating and freezing/thawing requires further studies. The simplest explanation is that the protection is due to the direct antioxidant action of the flavanols which are excellent antioxidants (Grzesik *et al.* 2018a,b). However, it has been also reported that EGC-G and other tea polyphenols activate the Yap1 transcription factor mounting elevated expression of endogenous antioxidant proteins (Maeta *et al.* 2007).

Interestingly, the flavanols decreased the level of ROS detectable with DHE and the level of protein carbonyls also in control cells upon 2-h incubation with the cells, not subjected to temperature treatment, indicating that they attenuate OS also under typical cell culture conditions.

The vulnerability of yeast to both high temperature and freezing/thawing may be of crucial importance for their survival in natural environment as well as for output of various biotechnological processes. Flavanols are compounds that yeast can encounter both in nature and in biotechnology; in the latter cases materials rich in flavonoids can be considered as means of enhancing yeast stability under extreme temperature conditions and probably in other conditions involving OS such as storage of yeast-containing preparations.

## Conflict of Interest

The authors declare no conflicts of interest.

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# Interaction of Catechins with Human Erythrocytes

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**Abstract:** The aim of this study was to characterize the interaction of chosen catechins ((+)-catechin, (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG)) with human erythrocytes and their protective effects against oxidative damage of erythrocytes. Uptake of the catechins by erythrocytes was studied by fluorimetry, their interaction with erythrocyte membrane was probed by changes in erythrocyte osmotic fragility and in membrane fluidity evaluated with spin labels, while protection against oxidative damage was assessed by protection against hemolysis induced by permanganate and protection of erythrocyte membranes against lipid peroxidation and protein thiol group oxidation. Catechin uptake was similar for all the compounds studied. Accumulation of catechins in the erythrocyte membrane was demonstrated by the catechin-induced increase in osmotic resistance and rigidification of the erythrocyte membrane detected by spin labels 5-doxyl stearic acid and 16-doxyl stearic acid. (−)-Epigallocatechin and EGCG inhibited erythrocyte acetylcholinesterase (mixed-type inhibition). Catechins protected erythrocytes against permanganate-induced hemolysis, oxidation of erythrocyte protein thiol groups, as well as membrane lipid peroxidation. These results contribute to the knowledge of the beneficial effects of catechins present in plant-derived food and beverages.

**Keywords:** erythrocyte; monometric flavanols; catechin; (−)-epigallocatechin; (−)-epigallocatechin gallate; hemolysis; membrane rigidity; antioxidant

## 1. Introduction

Catechins (monomeric flavanols) and their gallate derivatives are a class of flavonoids mainly present in fruits and vegetables and derived products like fruit juices or jams [1]. Monomeric flavanols are abundant in teas derived from the tea plant *Camellia sinensis*, as well as in some cocoas and chocolates (made from the seeds of *Theobroma cacao*) [2]. Catechins are also present in wine and are found in many other plant species. One gram of dried green tea leaves contain more than 200 mg catechins [3], although total catechin content varies widely depending on species, variety, and growth conditions. The main catechins present in green tea, as well as in black tea, are (−)-epigallocatechin (EGC) and the ester of epigallocatechin and gallic acid, (−)-epigallocatechin gallate (EGCG) [4,5]. (+)-Catechin is present in tea in minor amounts but is the main catechin in broad bean, black as well as white grape, plum, rhubarb, and raisins [6]. The food sources of catechins (tea, chocolate, apples, pears, grapes, and red wine) are very popular and highly consumed. Tea catechins consumed by volunteers at a total dose of 240 mg have been found to reach a concentration of up to 0.5 μM in blood plasma [7].

Consumption of eight cups of black tea every 2 h daily by volunteers has brought their blood plasma catechin level up to 1 μM [8].

As the consumption of catechins is significant, these compounds raise considerable interest with respect to their health effects. Numerous beneficial health effects of catechins have been demonstrated. These compounds have been claimed to reduce cardiovascular disease mortality by inhibiting endothelial dysfunction [9], to disrupt inflammation mediated by lipid and cholesterol oxidation [10], prevent onset and complications of diabetes [11], as well as prevent and slow down the development of metabolic syndrome [12]. They are thought to exert renoprotective actions that may be of importance in diseases, such as glomerulonephritis, diabetic nephropathy, and chemically-induced kidney insufficiency [13], have antihypertensive and neuroprotective actions [14] and improve exercise performance and recovery [15]. The action of catechins is mainly, though by no means exclusively, ascribed to their direct or indirect antioxidant activities. Previously, we demonstrated excellent antioxidant properties of selected catechins to compare with several other natural and synthetic compounds and related to glutathione and ascorbate as key endogenous antioxidants [16].

The effects of catechins are critically dependent on their cellular action. We studied the protective effect of (+)-catechin and (−)-epigallocatechin gallate on the yeast *Saccharomyces cerevisiae* with respect to cellular mortality dependent on oxidative stress [17]. There are reports on the effects of catechins on erythrocytes. (+)-Catechin has been found to protect human erythrocytes against pentachlorophenol-induced oxidative damage [18]. Tea catechins have been demonstrated to show significant protection to erythrocyte against oxidative stress induced by *tert*-butyl hydroperoxide. The effect is more pronounced in the older age group compared to the lower age group [19]. We demonstrated that selected monomeric catechins, including (+)-catechin and EGCG, protected erythrocytes against oxidative hemolysis induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and hypochlorite [16]. Protection against hypochlorite-induced hemolysis has also been confirmed by others [20]. Black tea extract is found to protect erythrocytes against damage induced by γ-radiation [21]. EGCG has protected erythrocyte membrane $Ca^{2+}$-ATPase and ($Na^+$, $K^+$)-ATPase against inactivation induced by *tert*-butyl hydroperoxide [22], protected erythrocytes against oxidative effects of cigarette smoke [23], and partly prevented hemolysis induced by cyclosporine [24]. (+)-Catechin has protected erythrocyte membranes against lipid peroxidation induced by hydrogen peroxide [25] and erythrocytes against hemolysis induced by cumene hydroperoxide [26]. Catechins, including EGCG and (+)-catechin, among other flavonoids, have been reported to increase the activity of a plasma membrane redox system (PMRS) that transfers electrons from intracellular substrates to extracellular electron acceptors by entering erythrocytes and donating electrons to PMRS [27].

However, EGCG and other catechins have been found to inhibit catalase in a cell-free system and in K562 cells, thus contributing to oxidative stress and an increase in the intracellular level of reactive oxygen species [28]. Prooxidative effects of tea catechins, including EGCG (decrease in the level of reduced glutathione, increase in the ratio of oxidized/reduced glutathione, and elevation in methemoglobin level), have been observed in glucose-6-phosphate dehydrogenase-deficient erythrocytes, leading to a recommendation against excessive consumption of concentrated tea polyphenolic products by glucose-6-phosphate dehydrogenase-deficient subjects [29].

The aim of this study was to further characterize the interaction of chosen catechins (Figure 1) with model human cells, viz. erythrocytes, which are considered a prime target for oxidant attack due, i.e., to their function as oxygen carriers and the presence of high contents of polyunsaturated fatty acid in their membranes. These simple cells allow for easy studies of the effects of xenobiotics on the cellular membrane, studies of their transport, and examination of antioxidant effects not complicated by the intermediacy of cellular effects dependent on protein synthesis de novo. Erythrocytes interact with xenobiotics circulating in blood after ingestion in the intestine. We wanted to quantify and compare the uptake of catechins by human erythrocytes, which, according to our best knowledge, has not been studied quantitatively. Due to their hydrophobicity, catechins can be expected to accumulate partly in the erythrocyte membrane; if so, a decrease in osmotic fragility of erythrocytes should be expected

due to membrane expansion, and we intended to verify this expectation. Moreover, the accumulation of catechins in the membrane should affect membrane fluidity, and this effect was checked by EPR spectroscopy using fatty acid spin probes. Although antioxidant effects of flavonoids on erythrocytes have already been studied, we wanted to provide additional data in this respect by studying the protection of erythrocytes against oxidative hemolysis induced by potassium permanganate and protection of erythrocyte membranes against lipid peroxidation and protein thiol oxidation.

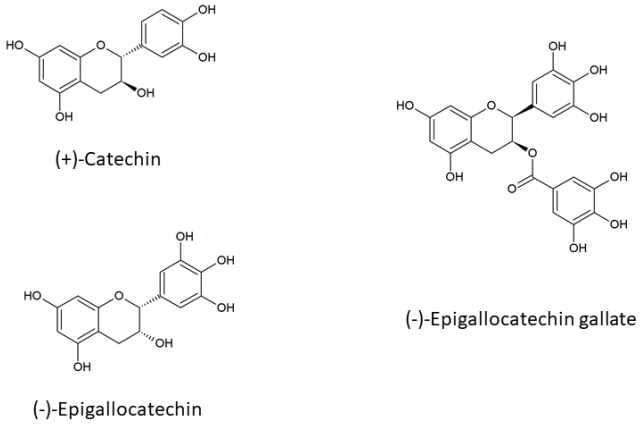


**Figure 1.** Structures of monomeric flavanols used in this study.

## 2. Results

### *2.1. Catechin Uptake by Erythrocytes*

Incubation of erythrocytes with catechins resulted in the uptake of these compounds by the cells. The study of the dependence of the amount of catechins taken up on the amount of erythrocytes showed a saturation behavior. Presentation of the data in the form of a double reciprocal plot of dependence of the number of molecules of monomeric flavanols taken up on the number of erythrocytes (Figure 2) demonstrated similar uptake of all compounds tested. The number of molecules of the studied compounds taken up by $2.5 \times 10^8$ erythrocytes from 500 µL of 50 µM solutions of monomeric flavanols (these values correspond to erythrocyte concentration in the blood) was $(1.19 \pm 0.03) \times 10^6$ for catechin, $(1.23 \pm 0.21) \times 10^6$ for epigallocatechin (EGC), and $(1.18 \pm 0.14) \times 10^6$ for EGCG.

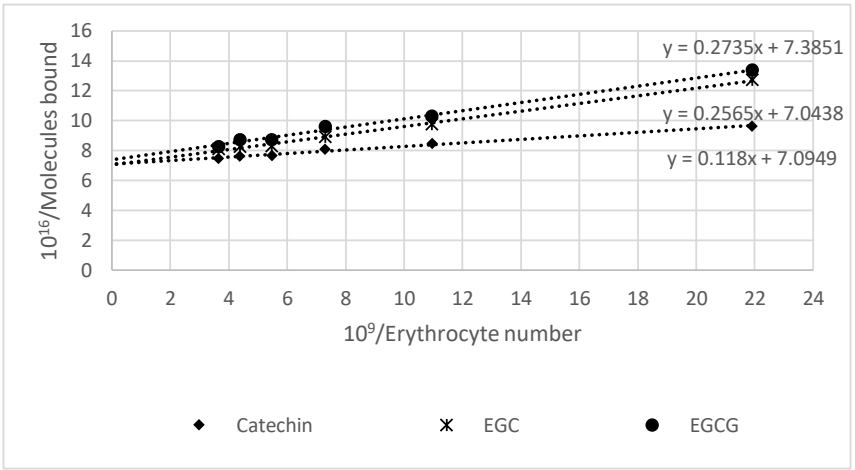


**Figure 2.** The double reciprocal plot of the uptake of selected flavanols by human erythrocytes. EGC, epigallocatechin; EGCG, epigallocatechin gallate.

### 2.2. Effect of Selected Flavanols on Osmotic Fragility of Erythrocytes

From osmotic fragility curves, the values of NaCl concentration evoking 50% hemolysis ($c_{50}$) were determined (Figure S1). When erythrocytes (hematocrit of 10%) were incubated with 50 μM selected flavanols at 37 °C for 90 min, catechins increased erythrocyte's osmotic fragility, as evidenced by an increase in $c_{50}$ values. Nevertheless, when catechins were added to erythrocytes, and osmotic fragility was assessed immediately (the contact of catechins with erythrocytes was about 5 min at room temperature), these compounds protected erythrocytes against osmotic hemolysis, as evidenced by decreased values of $c_{50}$ (Table 1).

**Table 1.** Effect of selected catechins on NaCl concentration causing 50% hemolysis ($c_{50}$) of erythrocytes (mM). Mean ± SD, $n = 3$.

| Compound | $c_{50}$ (mM) (Measurement after Incubation (37 °C, 90 min)) | $c_{50}$ (mM) (Measurement Asap) |
|---|---|---|
| Control | 65.4 ± 0.4 | 65.2 ± 0.8 |
| C | 66.5 ± 0.5 * | 62.8 ± 0.8 * |
| Epigallocatechin (EGC | 67.0 ± 0.7 * | 63.5 ± 0.7 * |
| Epigallocatechin gallate (EGCG) | 66.1 ± 0.4 * | 62.7 ± 0.8 ** |

* $p < 0.05$, ** $p < 0.01$.

### 2.3. Effect of Selected Catechins on Membrane Fluidity

Examples of EPR spectra of 5-doxyl stearic acid (5DS) and 16-doxyl stearic acid (16NS) embedded in erythrocyte membranes in the absence and in the presence of EGCG are shown in Figure S2. The catechins had generally a tendency to increase the rotational correlation time $\tau_c$ of 16DS (Table 2) and order parameter (S) (Table 3) of both probes embedded in erythrocyte membrane lipids.

**Table 2.** Effect of catechins on the rotational correlation time (in nanoseconds) of 16-doxyl-stearic acid in erythrocyte membranes. Mean values ± SD, $n \geq 3$.

| Compound | Rotational Correlation Time $\tau_c$ (ns) | | |
|---|---|---|---|
| **Concentration (μM)** | **Catechin** | **EGC** | **EGCG** |
| 0 | | 1.73 ± 0.03 | |
| 50 | 1.62 ± 0.08 | 1.78 ± 0.005 | 1.77 ± 0.10 |
| 100 | 1.73 ± 0.28 | 1.86 ± 0.03 ** | 1.83 ± 0.20 |
| 250 | 1.81 ± 0.15 | 1.99 ± 0.23 | 1.79 ± 0.01 * |

* $p < 0.05$, ** $p < 0.01$.

**Table 3.** Effect of catechins on the order parameter of 5-doxyl stearic acid (5DS) and 16-doxyl-stearic acid (16DS) in erythrocyte membranes. Mean values ± SD, $n \geq 3$.

| 5DS | | | |
|---|---|---|---|
| **Compound** | Order Parameter S | | |
| **Concentration (μM)** | **Catechin** | **EGC** | **EGCG** |
| 0 | | 0.610 ± 0.006 | |
| 50 | 0.616 ± 0.007 | 0.616 ± 0.007 | 0.616 ± 0.007 |
| 100 | 0.617 ± 0.012 | 0.617 ± 0.012 | 0.617 ± 0.012 |
| 250 | 0.618 ± 0.008 | 0.618 ± 0.008 | 0.618 ± 0.008 |

**Table 3.** *Cont.*

| 16DS | | | |
|---|---|---|---|
| **Compound** | | **S** | |
| **Concentration (μM)** | **Catechin** | **EGC** | **EGCG** |
| 0 | | 0.145 ± 0.001 | |
| 50 | 0.150 ± 0.002 ** | 0.148 ± 0.003 | 0.147 ± 0.001 * |
| 100 | 0.152 ± 0.003 ** | 0.150 ± 0.004 * | 0.147 ± 0.002 |
| 250 | 0.153 ± 0.002 *** | 0.156 ± 0.010 * | 0.150 ± 0.002 ** |

Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

## 2.4. Effect of Catechins on Membrane Acetylcholinesterase

Catechin at reasonable concentrations (up to 50 μM) did not have any discernible effect on the activity of erythrocyte membrane acetylcholinesterase (not shown). EGC and EGCG inhibited the enzyme in a concentration-dependent manner, evoking a ca 30% and 35% inhibition, respectively, at a concentration of 50 μM. Lineweaver–Burk plot of inhibition of acetylcholinesterase by 50 μM EGC and EGCG pointed to a mixed type of inhibition in both cases (Figure 3, Table 4).

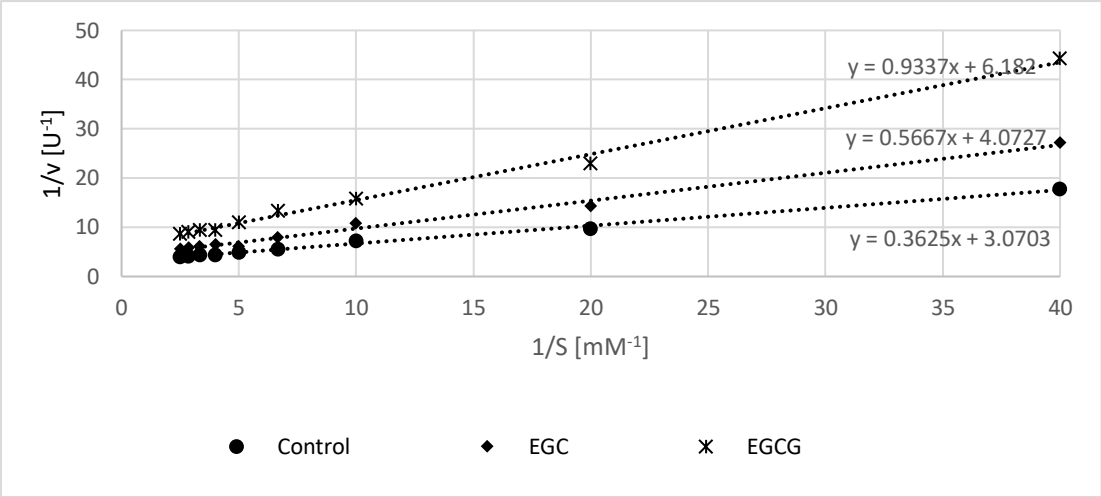


**Figure 3.** Lineweaver–Burk plot of erythrocyte membrane acetylcholinesterase activity in the absence and in the presence of 50 μM (−)-epigallocatechin (EGC) and 50 μM (−)-epigallocatechin gallate (EGCG).

**Table 4.** Effect of EGCG on the kinetic parameters of erythrocyte membrane acetylcholinesterase. Mean values ± SD, $n \geq 3$.

| | **Michaelis Constant $K_m$ (μM)** | **Maximal Velocity $V_m$ (U/g Protein)** |
|---|---|---|
| Control | 118 ± 9 | 4.79 ± 0.34 |
| +50 μM EGC | 139 ± 16 | 3.66 ± 0.45 * |
| +50 μM EGCG | 151 ± 25 * | 2.41 ± 0. 28 ***♣♣ |

* $p < 0.05$, *** $p < 0.001$ with respect to catechin, ♣♣ $p < 0.01$ with respect to ECG.

## 2.5. Protection against Oxidative Hemolysis

We chose the turbidimetric method of monitoring hemolysis, which, although being less precise than the approach based on the centrifugation of erythrocyte suspensions and measurement of

released hemoglobin, is much simpler, can be executed in a microplate reader, and is satisfactory for comparative purposes.

An example of the time course of turbidity of erythrocyte suspensions subjected to the action of 100 µM potassium permanganate in the presence of various concentrations of catechin is shown in Figure 4. Hemolysis of half-time (time corresponding to a decrease of turbidance to 50% of the initial values) in the absence of studied compounds was 19.9 ± 1.9 min. Catechins increased the time necessary to reach 50% hemolysis (Figure 5). Another means of quantifying the extent of hemolysis was the summation of subsequent turbidance values during 2-h measurements. Also, this parameter demonstrated the protective effect of catechins (Figure 6).

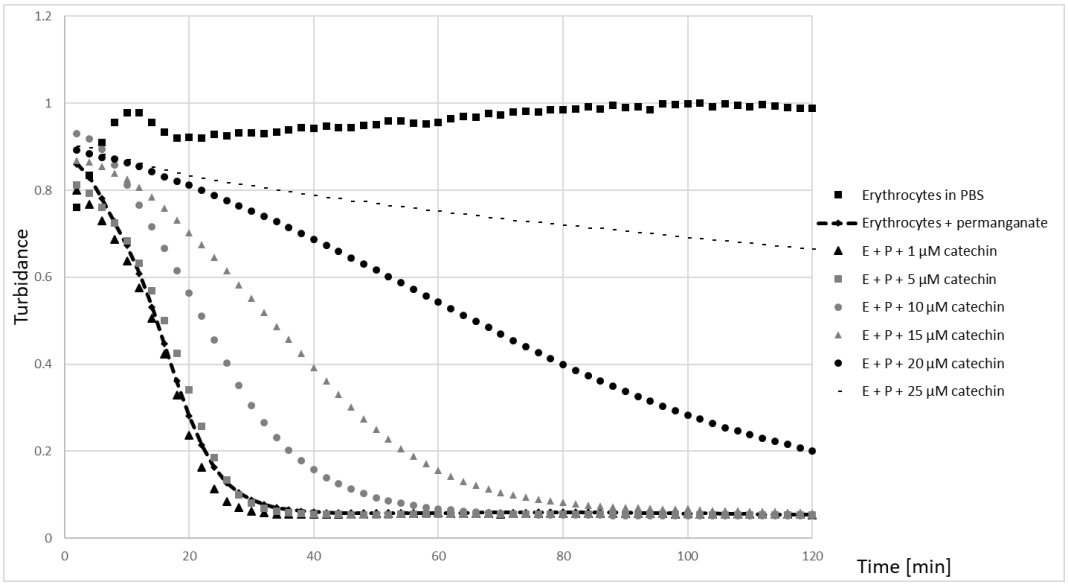


**Figure 4.** The exemplary curve of permanganate-induced hemolysis in the presence of various concentrations of catechin. E—erythrocytes; P—permanganate.

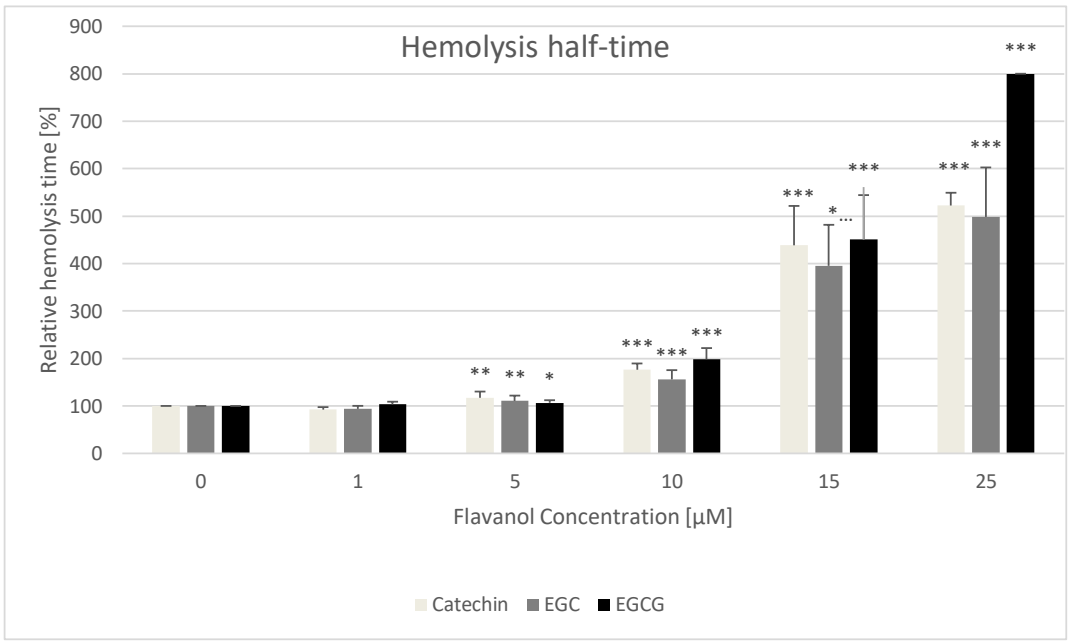


**Figure 5.** Effect of monomeric flavanols on the relative hemolysis half-time of erythrocytes. Half-time of hemolysis of control samples assumed as 100%. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (with respect to control).

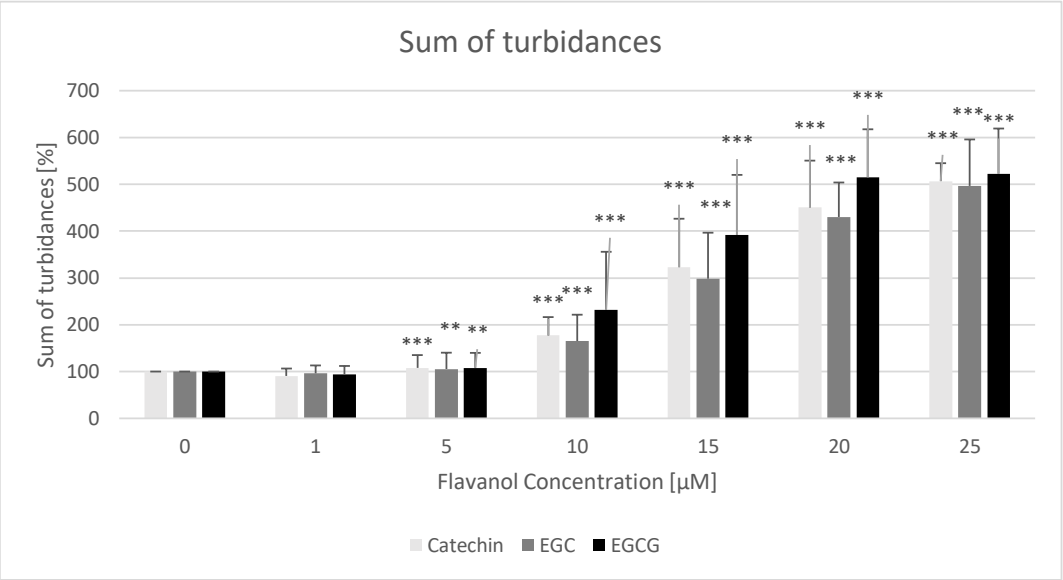


**Figure 6.** Effect of monomeric flavanols on the hemolysis of erythrocytes estimated from the sum of turbidance values during 120-min measurements (every 2 min). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (with respect to control). Values for control samples were assumed as 100%.

Microscopic examination of erythrocytes subjected to the action of permanganate showed complete hemolysis of cells in the absence of studied flavanols after >20 min (Figure S3b) and protection from hemolysis by selected flavanols. Interestingly, while initially erythrocytes treated with monomeric flavanols showed echinocytosis (Figure S3e), later on, this echinocytosis disappeared (Figure S3f), apparently due to oxidation of the compounds studied by peroxymanganate and weaker interaction of oxidation products of monomeric flavanols with erythrocyte membranes.

### *2.6. Protection against Oxidation of Erythrocyte Membrane Components*

All the compounds studied tested dose-dependently protected against hypochlorite-induced oxidation of membrane protein thiol groups (Table 5) and lipid peroxidation (Table 6).

**Table 5.** Protection by monomeric flavanols against hypochlorite-induced oxidation of erythrocyte membrane protein thiol groups. * $p < 0.05$, ** $p < 0.01$ (with respect to control).

| Compound | Catechin | EGC | EGCG |
|---|---|---|---|
| 0.1 | 10.3 ± 1.1 * | 11.1 ± 1.9 * | 13.6 ± 1.2 * |
| 0.2 | 16.7 ± 3.4 * | 17.7 ± 2.1 * | 18.2 ± 2.0 * |
| 0.5 | 32.8 ± 3.8 * | 28.3 ± 3.4 * | 31.5 ± 8.6 * |
| 1 | 58.4 ± 13.5 ** | 44.6 ± 9.9 ** | 50.2 ± 5.7 * |

**Table 6.** Protection by monomeric flavanols against hypochlorite-induced erythrocyte membrane lipid peroxidation. * $p < 0.05$, ** $p < 0.01$ (with respect to control).

| Compound | Catechin | EGC | EGCG |
|---|---|---|---|
| 0.1 | 19.0 ± 2.1 * | 16.8 ± 1.3 * | 24.8 ± 2.0 * |
| 0.2 | 36.1 ± 5.7 * | 27.7 ± 2.2 * | 41.1 ± 1.9 * |
| 0.5 | 44.7 ± 1.5 * | 37.4 ± 2.6 * | 46.8 ± 3.2 * |
| 1 | 60.6 ± 2.0 * | 59.7 ± 1.5 ** | 62.9 ± 3.3 ** |

## 3. Discussion

The present study was aimed at the characterization of the interaction of three chosen catechins with the human erythrocyte and the protective effect of the catechins against oxidant-induced damage. Erythrocytes are a main component of blood, and ingested catechins can interact with these cells. Moreover, erythrocytes are a simple and convenient model to study the cellular effects of catechins, especially effects on the plasma membrane.

The catechins studied were bound by erythrocytes at similar amounts (Figure 1), corresponding to about $1.12 \times 10^6$ molecules, when $2.5 \times 10^8$ erythrocytes interacted with 50 μM solutions of monomeric flavanols in a total volume of 500 μL (this erythrocyte concentration corresponds roughly to that in the blood). Such a high concentration of catechins is not attainable in vivo; nevertheless, these model conditions allow for an easy examination of the effect of monomeric flavanols on erythrocytes, while fractions of these effects can take place in vivo.

Due to their considerable hydrophobicity, catechins are expected to accumulate predominantly in the plasma membrane of the erythrocyte. Scanning electron microscopy observations showed that EGCG induced morphological alterations in human erythrocytes from their normal discoid form to crenated echinocytes. We confirmed this observation also for other catechins (Figure S3). According to the bilayer couple hypothesis [30], the shape changes induced in human erythrocytes by foreign molecules are due to differential expansion of the two monolayers of the red blood cell membrane. When the exogenous molecules locate into the outer membrane leaflet, echinocytosis occurs. NMR studies have shown that EGCG molecules are preferably located in regions near to the surface of the lipid bilayer with their B-ring and the galloyl moiety at the level of the trimethylammonium groups of phosphatidylcholines [31].

Accumulation of catechins in the erythrocyte membrane should lead to membrane expansion and an increase in the osmotic resistance of erythrocytes. According to the Ponder's classical description of hemolysis [32], erythrocytes (which in isotonic solutions have a surface excess with respect to the spherical shape) swell in hypotonic solutions until attaining a spherical shape. Since the erythrocyte membrane is essentially inextensible, hemolysis then occurs. If the membrane surface is augmented (e.g., by incorporation of exogenous substances), a spherical shape is attained at a lower osmolarity of the medium, i.e., erythrocyte osmotic fragility is decreased. The protection of erythrocytes against osmotic hemolysis by green tea extract and EGCG has been reported [33]. We confirmed this observation for all catechins studied (Table 1).

Interestingly, the protective effect of catechins on erythrocyte osmotic fragility was observed only under conditions of short (up to 5 min) incubation of catechins with erythrocytes. Prolonged incubation (90 min at 37 °C) evoked a reverse effect, viz. increase in osmotic fragility of erythrocytes by the catechins studied. It is a surprising effect, pointing to the cautious design of experiments and the interpretation of experimental results. We could suggest two reasons for an explanation of this effect (i) during prolonged incubation, more and more catechins are taken up by erythrocytes, which may lead to increase in the osmolarity of cell interior and thus easier hemolysis in hypotonic solutions due to a greater difference in osmolarity between cell interior and exterior; however, this effect does not seem significant at low concentration of catechins employed; (ii) as demonstrated previously, catechins generate hydrogen peroxide during prolonged incubation [34,35]. Hydrogen peroxide may damage the erythrocyte membrane and increase its fragility, including osmotic fragility.

Accumulation of exogenous compounds in the erythrocyte membrane can lead to changes in the ordering of membrane lipids. We observed generally an increase in the rotational correlation time of 16DS (Table 2) and order parameter of membrane lipids (Table 3), estimated with two spin labels—5DS and 16DS. The use of these spin probes is complicated by the fact that $pK_a$ values of fatty acid spin probes are in the range of near-neutral pH, which leads to the appearance of two components in their EPR spectra, corresponding to non-ionized and ionized molecules [36,37]. However, the resolution of both components is not easy in standard EPR spectra, so we restricted our analysis to the dominant component, corresponding to non-ionized probes (Figure S2). Both probes evidenced an increase in the

rigidity of the hydrophobic core of the membrane. Our results pointed to the rigidification of membrane lipids at high catechin concentrations, not attainable in vivo. Apparently, such concentrations were saturated with respect to the effects of catechins on membrane fluidity, which could explain the lack of concentration dependence of the effect. Nevertheless, other reports document membrane rigidification by monomeric flavanols also at their lower concentrations. EGCG-induced increase in erythrocyte membrane anisotropy measured with DPH, known to be located in the hydrophobic zone of the erythrocyte, has been found [33]. In isolated human erythrocyte membranes, EGCG has induced a moderate increase in laurdan general polarization, a result that implies a decrease in the molecular dynamics at the hydrophobic–hydrophilic interphase, which indicates an increase in the rigidity of this region of the membrane [20]. The rigidifying effect of catechins can contribute to their antioxidant effect. As initiation and propagation of free radical reactions depend on the mobility of membrane components, increased membrane rigidity hampers lipid peroxidation [38,39].

Inhibition of acetylcholinesterase by natural compounds, including polyphenols, is of interest as acetylcholine deficit accompanies Alzheimer's disease (AD). During the progression of AD, many different types of neurons deteriorate, although there is a profound loss of forebrain cholinergic neurons, accompanied by a progressive decline in acetylcholine, and acetylcholinesterase inhibitors provide effective temporary relief of symptoms at least in some patients [40]. Although the function of acetylcholinesterase on the erythrocyte surface remains a mystery, this membrane-bound enzyme is very useful for model studies of acetylcholinesterase inhibitors. Inhibition of erythrocyte membrane acetylcholinesterase by EGCG has been reported [41,42], although the kinetic aspects of this inhibition have not been reported to our best knowledge. Our results pointed to a mixed-type inhibition of erythrocyte membrane acetylcholinesterase activity by EGC and EGCG.

An association between the consumption of tea, rich in catechins, and the reduced risk of AD have been reported. Tea has been suggested to have anti-amyloid effects [43] and ameliorate cognitive dysfunction [44]. These effects have been mainly ascribed to the antioxidant and anti-inflammatory effects of tea catechins, but the inhibition of acetylcholinesterase by EGCG may contribute to this effect. The EGCG concentration attainable in the blood is low, but the octanol/PBS partition coefficient of this compound is about 26 [45], so it can reach much higher concentrations in the membranes where acetylcholinesterase is located.

We found a protective effect of all catechins studied on the hemolysis induced by potassium permanganate (Figures 3–5). Potassium permanganate is a strong oxidizing agent that can induce hemolysis and methemoglobinemia [46,47]. Protection by catechins against other oxidants inducing hemolysis has been reported previously [16,20,24]. Catechins protected also against oxidation of erythrocyte membrane protein thiol groups and membrane lipid peroxidation. In all cases, the effects of three catechins studied were similar, speaking against a significant contribution of the gallate group of EGCG to the antioxidant properties of this compound.

The parameters of membrane oxidative damage studied (lipid peroxidation and membrane thiols) are typically used in studies of oxidative damage and antioxidant effects. In these experiments, we used hypochlorite, which is a physiologically relevant oxidant. HOCl is generated by the reaction of $H_2O_2$ with chloride ions ($Cl^-$) catalyzed by myeloperoxidase. Up to 80% of the $H_2O_2$ generated by activated neutrophils may be used to produce local concentrations as high as 20–400 μM HOCl within an hour [48]. Relatively high concentrations of the catechins were used in order to observe the significant effects of membrane protection. However, other parameters may be more sensitive to demonstrate the protective effects of lower antioxidant concentrations. One such parameter is the rate of Band 3-mediated sulfate transport, which has been demonstrated to be affected by hydrogen peroxide under conditions inducing neither lipid peroxidation nor thiol oxidation [49].

## 4. Materials and Methods

### *4.1. Chemicals and Equipment*

Dimethyl sulfoxide (DMSO; DMS555.500, Purity: ≥ 99.9%), sodium phosphate monobasic (SPM306.500; purity ≥ 98–103%), sodium phosphate dibasic (SPD579.500; purity ≥ 98–102%), as well as phosphate-buffered saline (PBS tablets; PBS404.200), produced by BioShop Canada Inc. (Burlington, Ontario, Canada), were purchased from Lab Empire (Rzeszow, Poland). Sodium chloride (NaCl; 31434, purity ≥ 99.5%), produced by Honeywell Speciality Chemicals (Seelze, Germany), was purchased from Alchem (Rzeszow, Poland). Sodium hypochlorite (1.05614) was purchased from Merck (Darmstadt, Germany). Trichloroacetic acid (TCA; 115779700, purity ≥ 98%) was purchased from Chempur (Piekary Slaskie, Poland). 2-Thiobarbituric acid (TBA) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). TBA was dissolved in 0.1 M NaOH at a concentration of 0.67%. Spin probes [5-doxyl stearic acid (5DS) and 16-doxyl stearic acid (16DS)] and all other reagents, if not stated otherwise, were purchased from Sigma-Alrich (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 Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). All measurements were performed in triplicate and repeated at least three times on different preparations. (+)-Catechin (C; 43412, purity ≥ 99%), (−)-Epigallocatechin gallate (EGCG; E4143, purity ≥ 95%) and (−)-Epigallocatechin (EGC; E3768, purity ≥ 95%) were purchased from Sigma-Aldrich (Poznan, Poland). They were dissolved in PBS.

### *4.2. Ethical Approval*

The study was approved by the Bioethics Committee of the University of Lodz (Permit No. KBBN-UŁ/I/3/2013).

### *4.3. Preparation of Erythrocytes*

Eight milliliters of peripheral blood from healthy donors (lab volunteers) were 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 at various hematocrit from 4% to 60%.

### *4.4. Preparation of Erythrocyte Ghosts*

Erythrocyte ghosts were prepared from washed erythrocytes according to the method of Dodge et al. [50] with some modifications. Briefly, after incubation, erythrocytes were hemolyzed on ice with 20 volumes of 20 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 4 °C at 20,000× *g* for 20 min. The ghosts were resuspended in ice-cold 10 mM and then 5 mM phosphate buffer, pH 7.4 containing 1 mM EDTA, centrifuged, and this process was continued until the ghosts were free from residual hemoglobin. Finally, the erythrocyte ghosts were resuspended in 20 mM phosphate buffer, pH 7.4. The protein concentration was estimated by the method of Lowry et al. [51].

### *4.5. Transport of Catechins into Erythrocytes*

The solution of a selected flavanol was incubated with different volumes of erythrocytes, and, after centrifugation, the catechin concentration in the supernatant was determined by measurements of fluorescence of the monomeric flavanol.

#### 4.5.1. Determination of Optimal Excitation and Emission Wavelengths of Catechins

The optimal excitation and emission wavelengths for 50 μM C, EGCG, as well as EGC, were determined. The standard curve was prepared to determine the concentration dependence of the fluorescence of a studied compound.

#### 4.5.2. Uptake of Catechins by Human Erythrocytes

A series of erythrocytes suspensions in PBS were prepared (total volume 450 μL), and 50 μL of the solution of a selected compound (catechin, EGC, or EGCG) in PBS was added to obtain a final catechin concentration of 50 μM. Then, the samples were mixed and incubated for 1 h at room temperature (22–25 °C) with constant mixing. Finally, the samples were centrifuged (10 min, 5000 rpm), and the fluorescence was measured for 100 μL of selected supernatant mixed with 100 μL of DMSO (excitation 230 nm, emission 290 nm). The amount of catechins taken up was dependent on the amount of erythrocytes, and the reciprocal plots (plots of the (1/(number of molecules bound) as a function of 1/(erythrocyte number)) were linear. They were presented as $10^{16}$/(number of molecules bound by erythrocytes in the sample) vs. $10^{9}$/(number of erythrocytes).

### *4.6. Effect of (+)-Catechin, (−)-Epigallocatechin, and (−)-Epigallocatechin Gallate on the Osmotic Fragility of Human Erythrocytes*

Washed RBCs were suspended at 10% hematocrit and added with catechins to a final concentration of 50 μM. Immediately or after 90-min incubation at 37 °C, 50 μL of erythrocyte suspensions were added to 950 μL solutions of various NaCl concentrations (0.34, 0.35, 0.36, 0.37, 0.38, 0.39, 0.40, 0.41, 0.43, 0.44, and 0.45%). The control sample consisted of 50 μL erythrocyte suspension and 950 μL of water. Immediately, the samples were centrifuged (5 min, 5000 rpm), and the absorbance of the supernatant was measured (540 nm). From the osmotic fragility curves, NaCl concentration causing 50% hemolysis ($c_{50}$) was determined.

### *4.7. Potassium Permanganate ($KMnO_4$)-Induced Hemolysis*

Aliquots of erythrocyte suspensions in PBS were mixed with selected catechin at a final concentration range of 1–25 μM (final volume of 200 μL) and incubated for 15 min with shaking at 37 °C. Then, 0.1 mM potassium permanganate (final concentration), as optimal concentration to induce hemolysis, was added, and turbidance (700 nm) was measured every 2 min for 120 min, with intermittent shaking.

For all determinations, hemolysis time (seconds) and relative hemolysis time with respect to that of control erythrocytes, assumed as 100%, were calculated as 100% × (time (seconds) for test compound)/(mean time (seconds) for control sample containing erythrocytes and $KMnO_4$ only) (Figure 5). As an alternative measure of the extent of hemolysis, the sums of turbidances of erythrocyte suspensions measured at 2-min intervals were calculated. Again, the value for control erythrocytes was assumed as 100%, and appropriate values for samples supplemented with catechins were expressed as a percent of this value (Figure 6).

### *4.8. Estimation of Acetylcholinesterase (AChE) Activity*

The activity of AChE was measured using the colorimetric method described by Ellman et al. [52] with slight modifications, employing acetylthiocholine iodide (AcTCh) as a substrate. Briefly, 5 μL of erythrocyte membranes were added to 0, 1, 2, 3, 5, 10, 20, 30 μM, or 50 μM (+)-catechin, (−)-epigallocatechin, or (−)-epigallocatechin gallate solutions with 0.1 M phosphate buffer pH 7.4, 0.5 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman reagent) (10 μL of 10 mM DTNB stock), as well as 0.5 mM acetylthiocholine iodide (20 μL of 5 mM acetylthiocholine iodide stock) (the final volume: 200 μL). The absorbance was measured in a plate reader, every 10 s for 3 min, at 412 nm. The membrane protein concentration in the assay mixture was 67 μg/mL.

Kinetics of inhibition of acetylcholinesterase was also estimated. Two measuring series (one with phosphate buffer, pH 7.4, Ellman reagent, different amounts of the substrate, and 50 µM selected flavanol (final volume 200 µL), another without the flavanol) were added to wells of a 96-well plate, and time course of the increase in absorbance at 412 nm was measured. Acetylcholinesterase activity was calculated, and based on the obtained average values, a Lineweaver–Burk graph ((1/(velocity of enzymatic reaction) vs. 1/(substrate concentration)) in the absence and in the presence of the studied compounds was made (Figure 2), and the type of inhibition was determined.

### *4.9. Assessment of Membrane Fluidity*

Briefly, 200 µL aliquots of erythrocyte membranes (2.7 mg protein/mL) were added with 10 mM catechins to desired concentrations (0, 50, 100, and 250 µM). Two microliters of selected 10 mM probes (5DS or 16DS) in DMSO were added to each sample, mixed, and after 10 minutes of incubation, electron paramagnetic resonance (EPR) measurements were performed using microhematocrit capillaries (non-heparinized microhematocrit tubes ~75 µL; 1.55 × 75 mm; Medlab Products, Raszyn, Poland) in a BRUKER multifrequency and multi resonance FT-EPR ELEXSYS E580 apparatus (BRUKER BIOSPIN, Billerica, MA, USA) [X-band(~9.5GHz) CW-EPR spectrometer consisting of an ER4119-HS cavity]. Sample capillaries were placed into a quartz EPR sample tube and centered in a microwave cavity. The following settings were used: central field, 3353 G; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 23.77 mW; power attenuation 2 dB; scan range, 100 G; conversion time, 25 ms; sweep time, 25.6 s. The spectra were recorded in 1024 channels, and the number of accumulated scans was 3. The EPR spectra were recorded and analyzed using the Xepr 2.6b.74 software. Xepr is a comprehensive software package of the ELEXSYS series, accommodating the needs of every user with highly developed acquisition and processing tools.

Rotational correlation time $\tau_c$ was calculated according to Schreier et al. [53]:

$$\tau_c = \frac{1}{2}\kappa W_0 \left( \sqrt{\frac{h_0}{h_{+1}}} + \sqrt{\frac{h_0}{h_{-1}}} - 2 \right) \quad (1)$$

where $\tau_c$—time when the spin probe undergoes full rotation, κ- constant equal to $1.19 \times 10^{-9}$ s, $W_0$—width of the mid-line spectrum, $h_0$—the height of the mid-line spectrum, $h_{+1}$—the height of the low-field line of the spectrum, $h_{-1}$—the height of the high-field line of the spectrum.

Order parameter (S) was calculated according to Hubbell and McConnell [54]:

$$S = \frac{2\,A - 2A}{2\left[A_{zz} - \left(A_{xx} + A_{yy}\right)/2\right]}, \quad (2)$$

where 2 $A_{\|}$and 2 $A_{\perp}$ are the separations between the outer and inner extrema, respectively, in the experimental spectrum, and $A_{xx}$, $A_{yy}$, and $A_{zz}$ are the values of the principal components of the hyperfine tensor ($A_{xx}$ = $A_{yy}$ = 6 G, $A_{zz}$ = 32 G) [53].

### *4.10. Estimation of the Protective Effects of Catechins on Erythrocyte Membrane Protein Thiol Groups*

Erythrocyte membranes (1 mg protein/mL) in 0.1 M sodium phosphate buffer, pH 7.4, were treated with 500 µM (final concentration) sodium hypochlorite for 30 min, in the absence or in the presence of various concentrations of the compounds studied. Then, 100 µL aliquots of membrane suspension were added to wells of a 96-well microplate, followed by 50 µL of 10% solution of sodium dodecyl sulfate (SDS), 50 µL of 0.2 M phosphate buffer, pH 8, and 10 µL of 10 mM DTNB solution. After 15-min incubation in the dark, absorbance was measured at 412 nm. Corrections were made for the absorbance of DTNB solution and absorbance of solutions of the compounds studied. Percent protection was calculated as 100% × ((amount of thiol groups in a sample treated with NaOCl and a given concentration of catechin) − (amount of thiol groups in a sample treated with NaOCl in the absence of any protective

agent))/((amount of thiol groups in a control sample) – (amount of thiol groups in a sample treated with NaOCl in the absence of any protective agent)) (Table 5).

### *4.11. Estimation of the Protective Effects of Catechins on Erythrocyte Membrane Lipid Peroxidation*

Erythrocyte membranes were treated as above. Then, 200 μL aliquots of membrane suspensions were pipetted to Eppendorf tubes, followed by 250 μL of cold 10% trichloroacetic acid and 250 μL of 0.67% thiobarbituric acid in 0.1 M NaOH. The tubes were heated at 100 °C for 20 min, cooled, centrifuged, and the absorbance of the supernatants was measured at 532 nm. Percent protection was calculated as 100% × ((amount of lipid peroxidation products in a sample treated with NaOCl and a given concentration of catechin) − (amount of lipid peroxidation products in a sample treated with NaOCl in the absence of any protective agent))/((amount of lipid peroxidation products in a control sample) − (amount of lipid peroxidation products in a sample treated with NaOCl in the absence of any protective agent)) (Table 6).

### *4.12. Statistical Analysis*

The error bars are standard deviation. The paired Student's t-test was performed to estimate the differences between samples and control. Kruskal–Wallis test was also performed to determine differences between antioxidant-treated and non-treated cells. $p \leq 0.05$ was considered as statistically significant. Statistical analysis of the data was performed using the STATISTICA software package (version 13.3, StatSoft Inc. 2016, Tulsa, OK, USA).

## 5. Conclusions

The presented results characterized quantitatively the interaction of catechins with human erythrocytes, showing the similarity of the interaction of catechin, EGC, and EGCG, confirmed their rigidifying effect on erythrocyte membrane, demonstrated and, characterized inhibition of erythrocyte acetylcholinesterase activity by EGC and EGCG, and extended the knowledge on the antioxidant effects of catechins on the erythrocyte as well as erythrocyte membranes. Antioxidant effects of catechins on the erythrocyte shown contribute to the understanding of the beneficial effects of catechins present in plant-derived food and beverages on human health.

**Supplementary Materials:** The following are available online, Figure S1. Osmotic fragility curves of control erythrocytes and erythrocytes treated with 50 μM catechins. Incubation with catechins ≤5 min. Figure S2. EPR spectra of 5DS and 16DS embedded in control erythrocyte membranes and erythrocyte membranes treated with 250 μM EGCG. Figure S3. Microscopic images of control erythrocytes and erythrocytes treated with 100 μM permanganate. Human erythrocytes were analyzed using Olympus CKX53 microscope with a U-TV0.5XC-3 digital microscope camera; (a) untreated erythrocytes; erythrocytes incubated with (b) 100 μM potassium permanganate, (c) 20 μM catechin and 100 μM potassium permanganate, (d) 20 μM EGC and 100 μM potassium permanganate, (e) 20 μM EGCG and 100 μM potassium permanganate (incubation time: 5 min), (f) 20 μM EGCG and 100 μM potassium permanganate (incubation time: 20 min).

**Author 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. She was also responsible for co-providing the funding for the study; K.N. performed main part of experiments and their statistical evaluation, as well as contributed reagents/materials/analysis tools; I.S. and B.C. carried out EPR measurements and interpreted the data; M.S. took part in the execution of experiments; G.B. participated in the revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Sample Availability:** Samples of monomeric flavanols are available from the authors.

**SUPLEMENT**

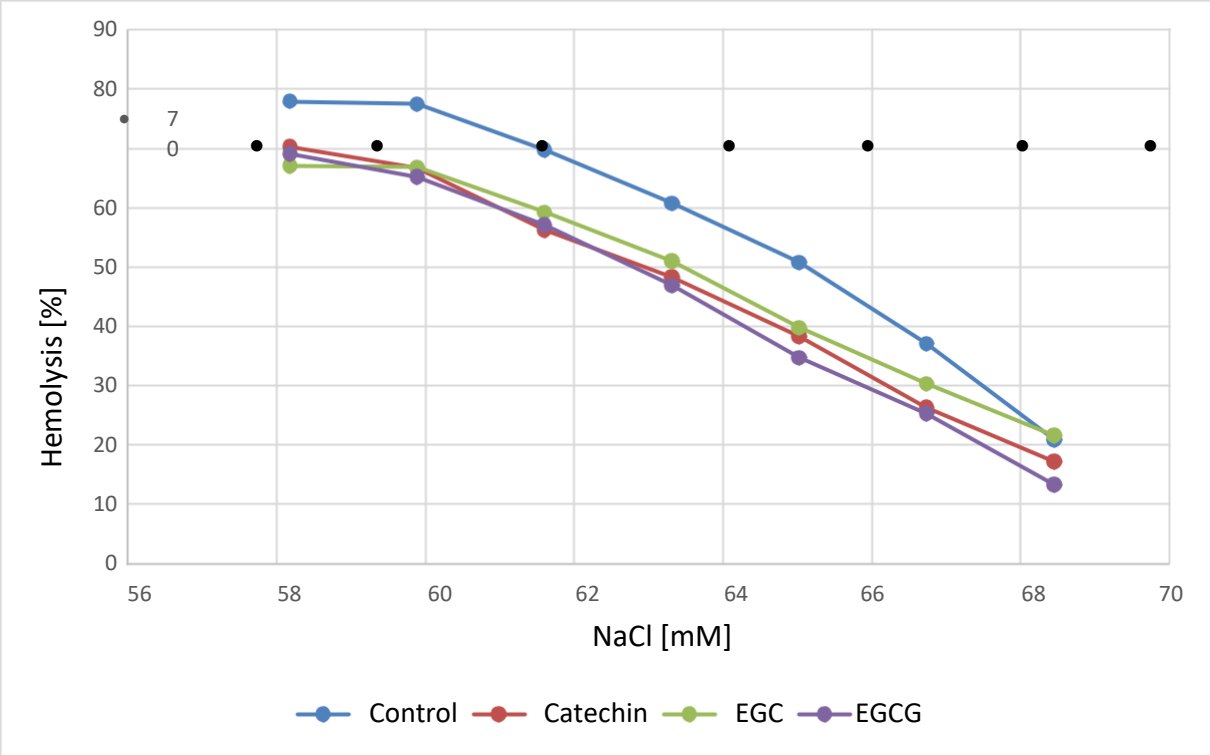
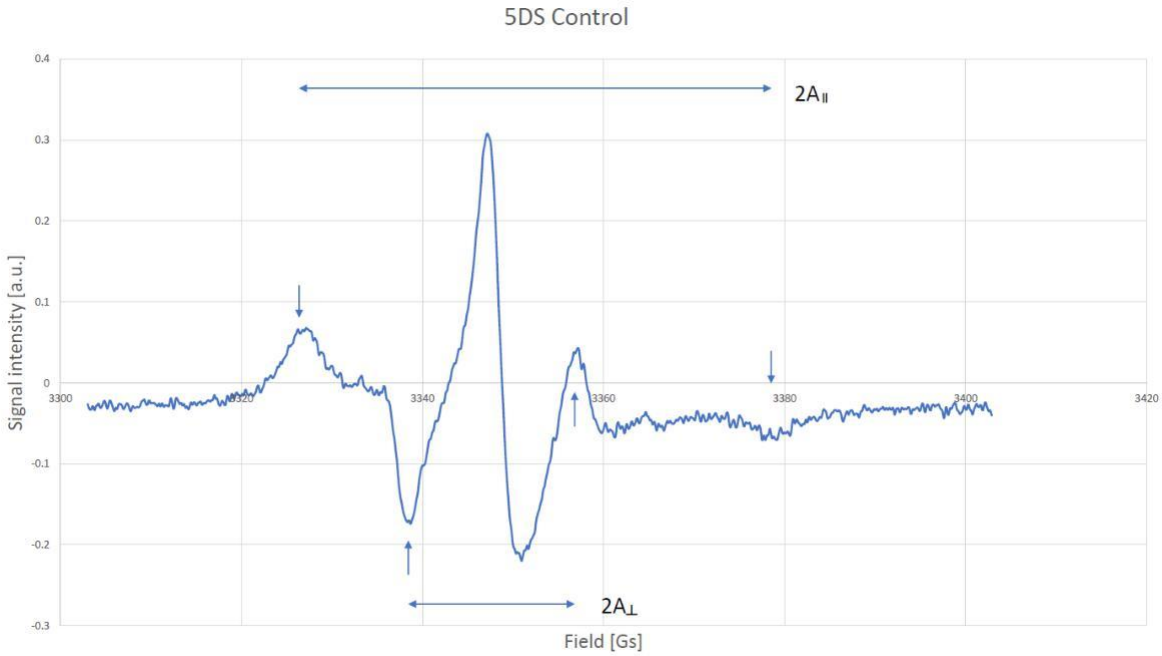
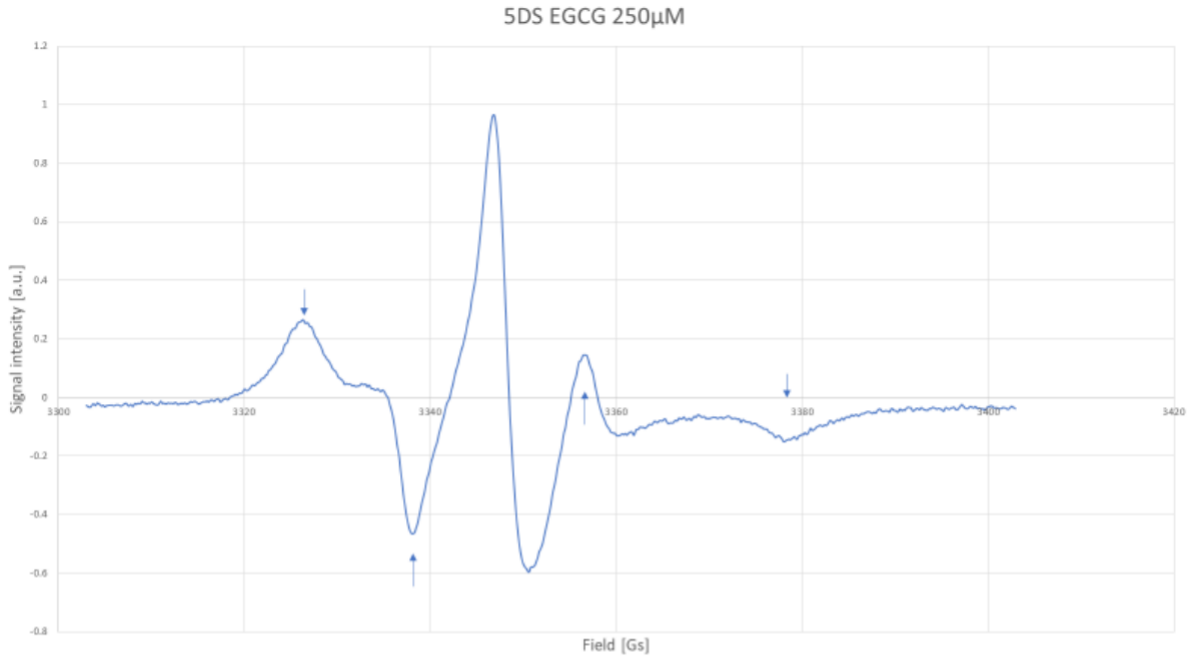



**Figure S1**. Osmotic fragility curves of control erythrocytes and erythrocytes treated with 50 μM catechins. Incubation with catechins ≤ 5min.

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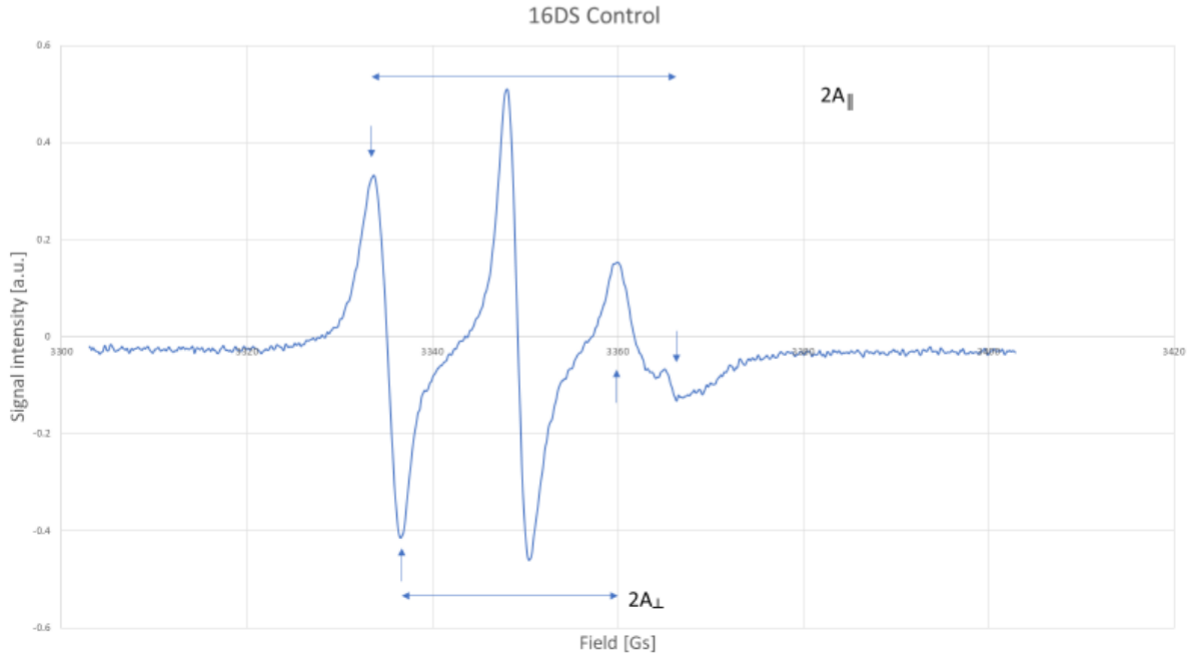
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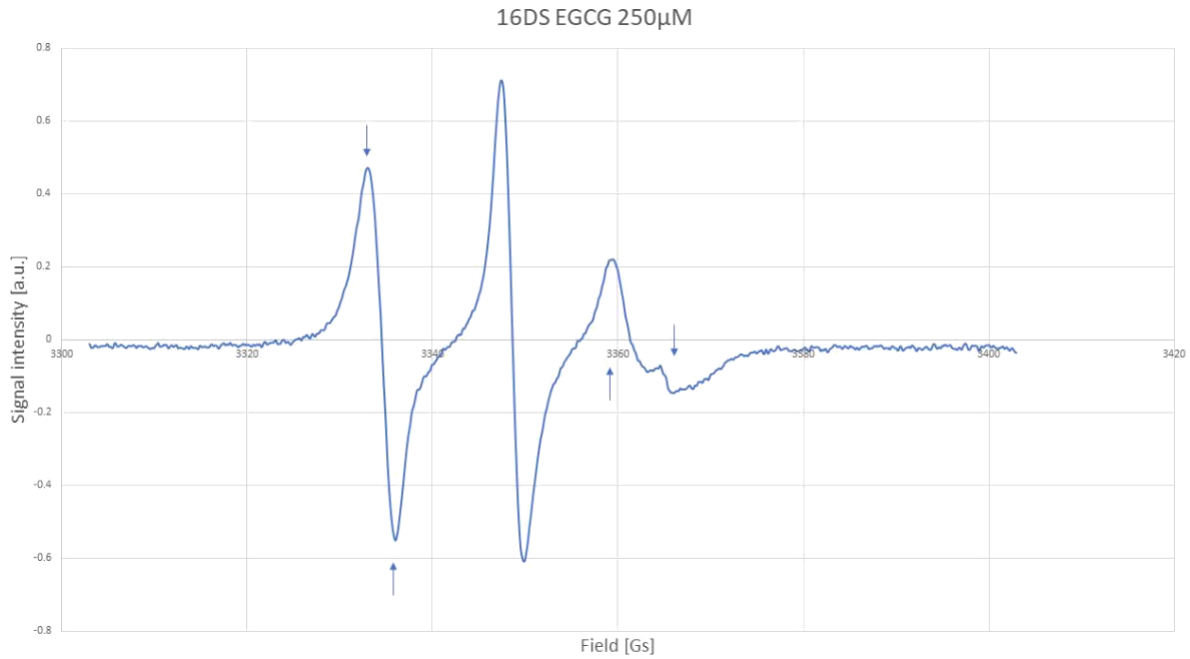
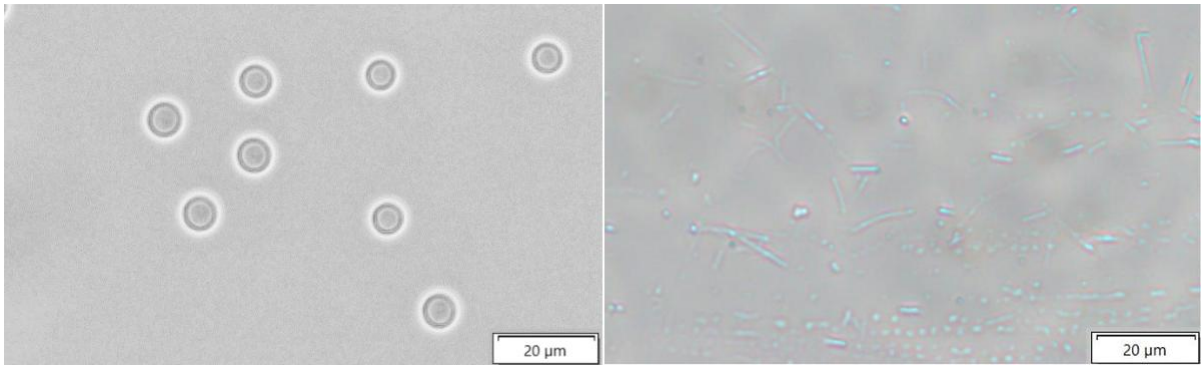



**Figure S2**. EPR spectra of 5DS and 16DS embedded in control erythrocyte membranes and erythrocyte membranes treated with 250 µM EGCG.

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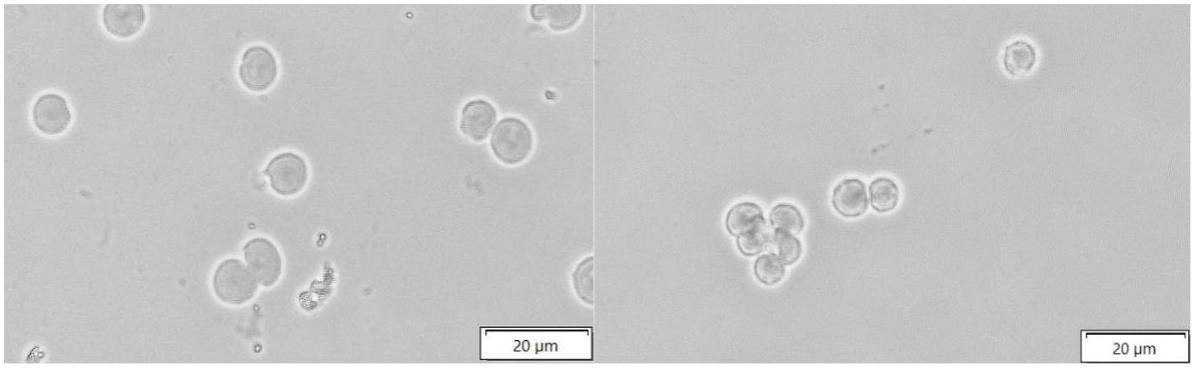
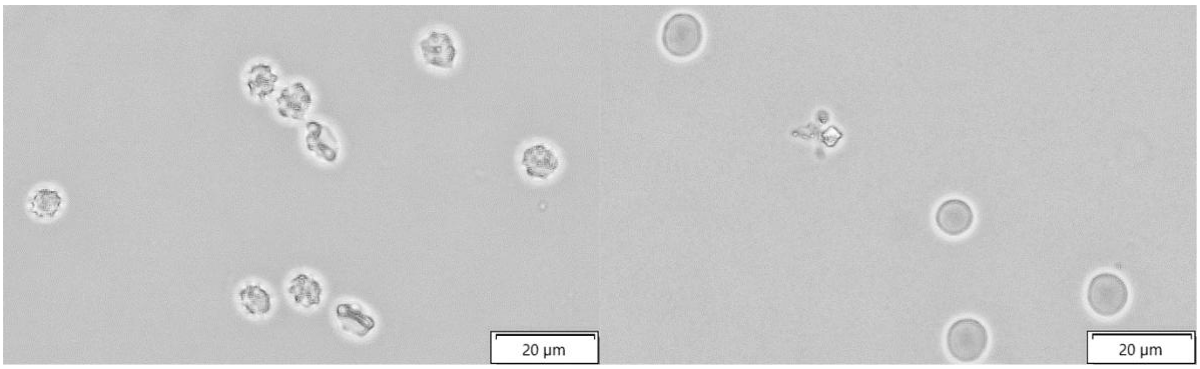



(c) (d)




(e) (f)

**Figure S3**. Microscopic images of control erythrocytes and erythrocytes treated with 100 µM potassium permanganate. Human erythrocytes were analyzed using Olympus CKX53 microscope with a U-TV0.5XC-3 digital microscope camera. Images: (a) untreated erythrocytes; erythrocytes incubated with (b)100 µM potassium permanganate, (c) 20 µM (+)-catechin and 100 µM potassium permanganate, (d) 20 µM EGC and 100 µM potassium permanganate, (e) 20 µM EGCG and 100 µM potassium permanganate (incubation time: 5 min), (f) 20 µM EGCG and 100 µM potassium permanganate (incubation time: 20 min).

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# Comparison of Antioxidants: The Limited Correlation between Various Assays of Antioxidant Activity

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**Abstract:** The inhibitory effects a range of synthetic and natural antioxidants on lipid peroxidation of egg yolk and erythrocyte membranes induced by a free radical generator 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was compared, with significant differences being found between both systems. When the protection by selected antioxidants against the effects of AAPH on erythrocytes (hemolysis, oxidation of hemoglobin and glutathione (GSH) and generation of reactive oxygen species (ROS)) was studied, most antioxidants were protective, but in some tests (oxidation of hemoglobin and GSH) some acted as prooxidants, inducing oxidation in the absence of AAPH and enhancing the AAPH-induced oxidation. These results demonstrate a diversified action of antioxidants in different systems and point to a need for careful extrapolation of any conclusions drawn from one parameter or experimental system to another.

**Keywords:** antioxidant; lipid peroxidation; AAPH; hemolysis; glutathione; reactive oxygen species; hemoglobin

## 1. Introduction

Antioxidants are indispensable for the proper functioning of aerobic organisms and are important in the food industry for the preservation of oxidizable products. They are also used in cosmetics, are believed to be prophylactic agents against various diseases as well as proposed to be potential therapeutics [1]. For food preservation, efficient synthetic antioxidants are usually employed, nevertheless there are concerns about their possible adverse effects [2–4]. Administration of antioxidants for the prevention and possible treatment of diseases imposes additional requirements such as bioavailability, lack of toxicity and the ability to penetrate the blood-brain barrier in the case of antioxidants aimed to ameliorate neurodegenerative diseases [5,6]. Often, rankings of antioxidants are created for different purposes based on their efficiency in particular systems. However, procedures proposed to determine antioxidant activity often yield widely divergent results [7]. Moreover, under certain conditions antioxidants may behave as prooxidants [5,8], which should be taken into account in more comprehensive antioxidant rankings.

The purpose of this work was to compare the protective effect of a range of antioxidants such as synthetic antioxidants, phenolic acids, flavonoids as well as other natural antioxidants against lipid peroxidation in two systems (egg yolk lipids and erythrocyte membrane lipids). The choice of antioxidants was a result of our previous participation in a project aimed at ranking of natural

antioxidants as potential health-promoting agents [9]. We employed two systems for studying protection against lipid peroxidation: a homogenous, but biologically relevant system of egg yolk and a model membrane system (erythrocyte membranes, which are easy to prepare in a pure form). Then, five effective PBS-soluble antioxidants [2,5-dihydroxybenzoic acid (gentisic acid), (−)-epigallocatechin gallate (EGCG), (+)-catechin, gallic acid as well as ascorbic acid] were selected to study the protective effect of these compounds against oxidative stress (OS) on erythrocytes employing four different parameters. 2,2-Azobis (2-amidinopropane) dihydrochloride (AAPH) was chosen as a model oxidant inducing lipid peroxidation. This compound decomposes in a temperature-dependent manner generating free radicals; the half-life of AAPH is about 175 h at neutral pH, at the temperature of 37 °C, and the rate of free radical generation is $1.3 \times 10^{-6}$ [AAPH]/s at these conditions [10]. Under aerobic conditions primary radicals are converted into peroxyl radicals, which induce oxidation of polyunsaturated lipids causing a chain reaction known as lipid peroxidation, and oxidation of other substrates [11–13].

This study demonstrates that for the same compounds, the relative antioxidant effects may differ even in simple model cell-free systems and prooxidant effects may be revealed at the level of a simple model cell such as the human erythrocyte even if they are not evident in other assays.

## 2. Results and Discussion

### *2.1. Inhibition of Lipid Peroxidation*

We compared a range of synthetic and natural antioxidants with respect to inhibition of AAPH-induced lipid peroxidation in two systems: egg yolk suspension and erythrocyte membranes. Peroxidation rate was measured with BODIPY® 581/591. This probe emits red fluorescence centered at 595 nm. Oxidation of the probe by peroxyl radicals is accompanied by decay of the red fluorescence and appearance of green fluorescence centered at about 520 nm. Decrease of the red fluorescence intensity, changes in the ratio of green and red fluorescence intensities or increase in the green fluorescence intensity have been used by various authors as indices of peroxidation [14–16]. In our hands, the rate of increase of the green fluorescence intensity was the most reproducible and reliable measure of the rate of peroxidation and was used in this study [16]. In egg yolk suspension, *tert*-butylhydroquinone (*t*-BHQ;) was the most efficient antioxidant, showing the lowest value of the concentration required for 50% inhibition of peroxidation ($IC_{50}$).

Some natural antioxidants, including gallic acid, (−)-epicatechin gallate and melatonin showed comparable efficiency. Generally, hydrophilic antioxidants such as glutathione (GSH), cysteine, naringin, hesperetin and ascorbic acid exhibited lower efficiency. In another system, i.e., erythrocyte membranes (−)-epicatechin gallate was the most efficient, nevertheless the sequence of efficiency was different. While some antioxidants, such as (−)-epicatechin gallate or resveratrol had similar $IC_{50}$ values in both system, the values for most compounds differed significantly in both systems, in most cases being higher for erythrocyte membranes (Table 1).

The Pearson linear correlation coefficient between $IC_{50}$ values in both systems was only 0.30 (Figure 1). These differences may be due to the fact that in the more complex erythrocyte membrane systems some antioxidants may associate with membrane proteins and may be not, or only partly available for reactions with peroxide radicals in the lipid phase. The weight ratio of proteins to lipids is about 1:1 in erythrocyte membranes [17] and is significantly lower (about 0.5:1) in hen egg yolk [18], and protein composition differs considerably in both materials.

These results show that it may be misleading to extrapolate even results of evaluation of antioxidant potency to inhibit lipid peroxidation from one system to another.

**Table 1.** Inhibition of BODIPY® 581/591 oxidation by synthetic and natural antioxidants.

| Compound | $IC_{50}$ Egg Yolk [μM] | $IC_{50}$ Erythrocyte Membranes [μM] |
|---|---|---|
| | Synthetic antioxidants | |
| *t*-BHQ | 12.04 ± 3.55 | 37.57 ± 1.91 *** |
| Pyrogallol | 60.79 ± 14.17 | 70.80 ± 6.46 |
| Trolox | 62.78 ± 2.90 | 87.46 ± 7.38 ** |
| BHA | 71.42 ± 15.54 | 50.04 ± 5.26 * |
| *N*-Acetyl-L-cysteine | 231.50 ± 2.21 | 554.99 ± 60.34 *** |
| | Phenolic acids | |
| Gallic acid | 15.33 ± 6.81 | 72.35 ± 4.09 *** |
| Caffeic acid | 15.45 ± 0.33 | 34.92 ± 0.40 *** |
| Propyl gallate | 19.60 ± 3.04 | 65.32 ± 3.27 *** |
| Chlorogenic acid | 22.92 ± 1.91 | 33.33 ± 1.22 *** |
| 2,5-Dihydroxybenzoic acid (Gentisic acid) | 29.38 ± 6.22 | 56.52 ± 0.60 *** |
| Vanillic acid | 34.67 ± 6.34 | 114.72 ± 17.69 *** |
| Ferulic acid | 80.80 ± 2.33 | 74.87 ± 1.12 ** |
| *p*-Coumaric acid | 89.28 ± 0.12 | 256.39 ± 31.65 *** |
| Sinapic acid | 124.71 ± 4.67 | 40.83 ± 1.6 *** |
| | Flavonoids | |
| (−)-Epicatechin gallate | 13.68 ± 0.51 | 14.46 ± 0.39 |
| (−)-Epicatechin | 16.45 ± 1.22 | 27.06 ± 1.04 *** |
| Quercetin | 17.89 ± 0.83 | 27.67 ± 1.29 *** |
| (−)-Epigallocatechin gallate | 18.16 ± 0.55 | 22.64 ± 0.03 *** |
| (+)-Catechin | 21.26 ± 0.67 | 30.51 ± 1.1 *** |
| Mangiferin | 21.98 ± 6.32 | 35.95 ± 3.59 * |
| (−)-Epigallocatechin | 28.35 ± 0.15 | 61.34 ± 2.48 *** |
| Rutin trihydrate | 34.40 ± 0.63 | 27.94 ± 1.12 *** |
| Daidzein | 67.37 ± 6.36 | 3331.1 ± 429.76 *** |
| Hesperidin | 84.41 ± 7.23 | 242.86 ± 3.72 *** |
| Naringenin | 91.67 ± 5.40 | 441.84 ± 30.72 *** |
| Naringin | 359.11 ± 59.28 | 1782.29 ± 76.34 *** |
| Hesperetin | 426.96 ± 18.13 | 165.58 ± 6.15 *** |
| | Other natural antioxidants | |
| Melatonin | 14.37 ± 4.29 | 1098.96 ± 65.19 *** |
| Resveratrol | 31.38 ± 2.79 | 35.57 ± 3.59 |
| β-Carotene | 66.19 ± 10.40 | 748.75 ± 20.35 *** |
| L-Ascorbic acid | 118.71 ± 5.38 | 138.19 ± 7.24 * |
| L-Cysteine | 241.95 ± 52.86 | 705.92 ± 32.68 *** |
| Glutathione | 341.85 ± 7.09 | 655.90 ± 41.89 *** |

Antioxidant concentrations providing 50% inhibition of the probe oxidation ($IC_{50}$) are presented (mean ± SD, $n > 3$). *t*-BHQ, *tert*-butylhydroquinone; BHA, butylated hydroxyanisole; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to the value obtained for egg yolk suspension.

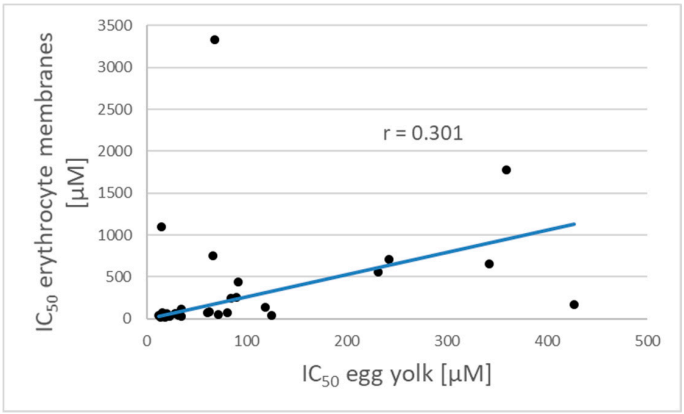


**Figure 1.** Correlation between $IC_{50}$ values of antioxidants presented in Table 1 in two systems of lipid peroxidation: egg yolk suspension and erythrocyte membranes.

### 2.2. Protection Against AAPH-Induced Hemolysis

For further experiments, five effective antioxidants soluble in PBS (to avoid the effects of organic solvent on the erythrocyte membrane), viz. (−)-epigallocatechin gallate, (+)-catechin, gallic acid, gentisic acid, and ascorbic acid were tested for their efficiency in preventing AAPH-induced oxidative damage on erythrocytes.

The hemolysis of erythrocytes has been extensively used as an ex vivo model in the study of ROS-induced disruption of cell membranes. AAPH is one of the most frequently studied compounds inducing oxidative hemolysis [19]. The mechanism of erythrocyte hemolysis induced by thermolysis of AAPH is not completely understood, but it has been correlated with lipid peroxidation and oxidation of membrane proteins. Accumulation of oxidized lipids around aggregated Band 3 protein most probably leads to the formation of hemolytic holes in the membrane [20].

Catechins (flavanols) are flavonoids that are found in black tea, green tea and other plant foods. They have been demonstrated to have numerous physiological effects, mostly dependent on their antioxidant action [21]. 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 [22]. In contrast to GSH, polyphenols cannot be synthesized by humans, but are ingested in the diet; so, free radicals that are originated during body metabolism can be better neutralized by regular intake of a diet containing a high content of fruits and vegetables [23].

The best studied catechin is EGCG, the major polyphenol in green and black tea [24]. (+)-Catechin is mostly found in cacao and tea constituents, as well as in *Vitis vinifera* grapes, and is among the main polyphenols present in fruit wine [25]. Gallic acid (3,4,5-trihydroxybenzoic acid) is a natural phenolic antioxidant belonging to the most abundant phenolic antioxidants in wines and green tea [26]. Gallic acid is a phenolic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark as well as other plants. This phytochemical is known for its antibacterial, anti-inflammatory, antimutagenic and chemopreventive properties. It is commonly used in the pharmaceutical industry as a standard for determination of the phenol content of various analytes by the Folin-Ciocalteau assay; results are usually reported in gallic acid equivalents [27,28]. Gentisic acid is a metabolite of acetylsalicylic acid, which shows a potent free radical scavenging activity with a minimal chelating effect. The antioxidant property of gentisic acid may partly account for the anti-atherogenic effects of aspirin [29]. It has been proposed that the antioxidant properties of gentisic acid are exerted by its phenoxyl group, leading to the formation of a phenoxyl radical. Ascorbate has been found to quench the phenoxyl radical of gentisic acid [30].

If the AAPH-induced oxidative hemolysis is mainly due to lipid peroxidation and membrane protein oxidation, membrane-interacting antioxidants should prevent hemolysis more effectively than those which do not interact with the membranes. All catechins are known to interact with the lipid bilayer, EGCG having higher affinity for membranes than non-esterified catechins [31]. However, AAPH is a water-soluble compounds and water-soluble antioxidants must be important in scavenging radicals generated by AAPH decomposition, while membrane-interacting antioxidants are crucial for inhibiting the chain process of lipid peroxidation [32].

Most of the chosen antioxidants are partly associated with membranes, but also present in the aqueous medium while ascorbate is practically totally present in solution, so all of the antioxidants tested can interact with AAPH-generated radicals outside the cells. With respect to scavenging of the latter radicals, polyphenols can be expected to be more reactive than ascorbic acid, due to the higher number of reactive hydroxyl groups [33]. Indeed, although all antioxidants studied were effective, the highest concentrations of membrane-interacting antioxidants prolonged the time necessary to obtain a 50% decrease in the turbidity of erythrocyte suspensions to more than 1000%, while 1 mM ascorbic acid increased the relative hemolysis time to only about 300% (Figure 2). The efficiency of a hydrophilic antioxidant, ascorbic acid, may be contributed by its property of regeneration of radicals of other antioxidants formed in the process of inhibition of lipid peroxidation [34,35].

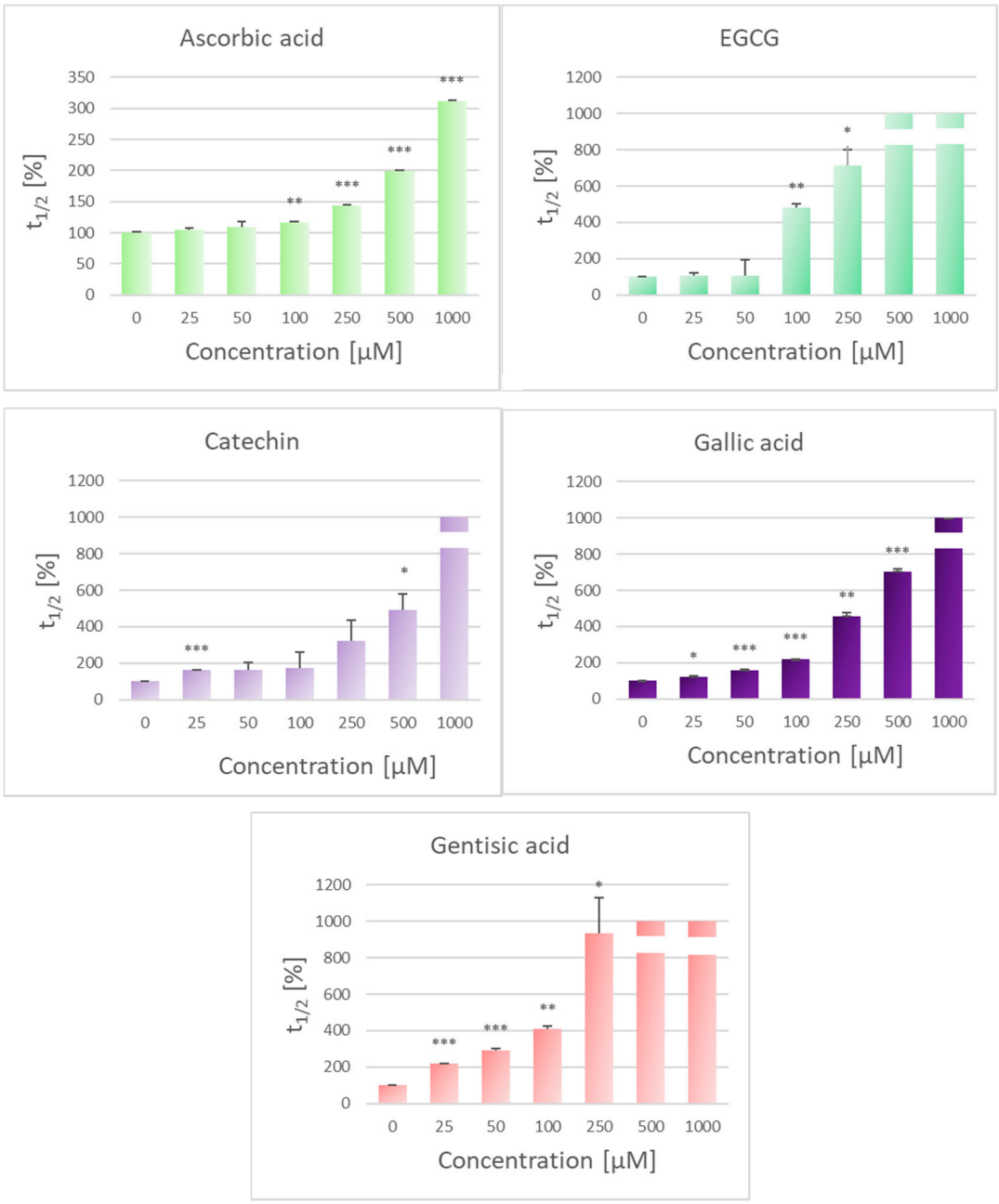


**Figure 2.** Effect of selected antioxidants on the AAPH-induced hemolysis of human erythrocytes. Hemolysis half-time expressed as percent of hemolysis half-time of "positive control" samples, containing no antioxidant ($t_{1/2}$ = 96.3 ± 1.4 min), assumed as 100%. The turbidity of "negative control" samples, not added with AAPH, did not change during the incubation time. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to control (100%).

### 2.3. Attenuation of ROS Level

Exposure to AAPH induced an abundant generation of ROS reacting with $H_2$DCF-DA. All the antioxidants used dose-dependently decreased the ROS level inside erythrocytes (Figure 3). The $IC_{50}$ values of the antioxidants tested for the inhibition of ROS formation in erythrocytes are given in Table 2. Interestingly, EGCG proved to be the most effective antioxidant in this test, catechin and gallic acid showed similar effectivity while gentisic acid was the least effective, in spite of its structural similarity to gallic acid.

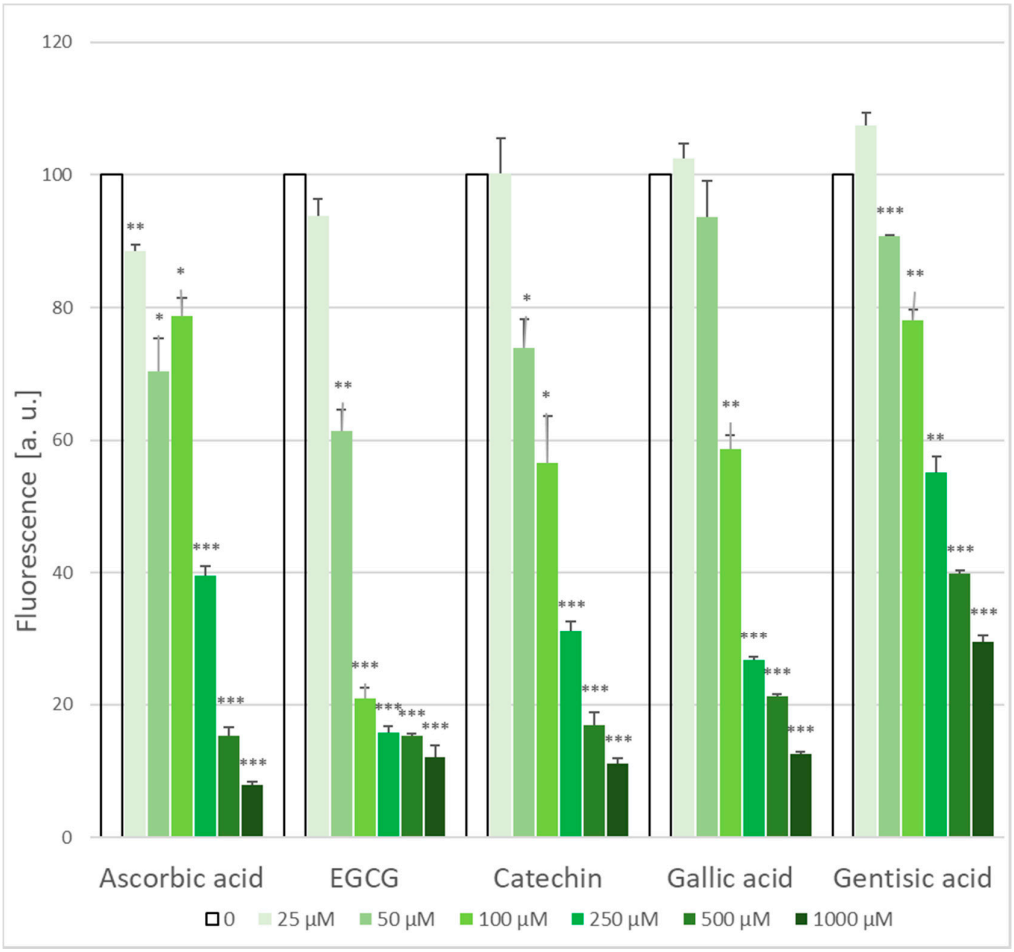


**Figure 3.** Attenuation of AAPH-generated intracellular ROS level by chosen antioxidants. The ROS level in the absence of any additive assumed as 100%. Endogenous ROS level in the absence of AAPH: 8.5 ± 0.3% of that found with AAPH. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to control (100%).

**Table 2.** $IC_{50}$ values of chosen antioxidants for inhibition of ROS formation in erythrocytes. Mean ± SD, $n \geq 3$.

| Compound | $IC_{50}$ [µM] |
|---|---|
| Ascorbic acid | 209.7 ± 35.2 |
| EGCG | 64.0 ± 18.2 |
| Catechin | 138.5 ± 29.4 |
| Gallic acid | 140.3 ± 20.0 |
| Gentisic acid | 333.3 ± 46.2 |

### *2.4. Protection against Hemoglobin Oxidation*

ROS generated by AAPH-induced oxidation of intracellular hemoglobin. As hemoglobin is present at very high concentration inside erythrocytes (over 30% by weight), it is obvious that only high concentrations of antioxidants were effective in preventing hemoglobin oxidation (Figure 4). However, EGCG and gallic acid were effective only in some concentration range while the protective effect disappeared with a further increase in the antioxidant concentration. As this effect was reproducible, we checked if the antioxidants studied oxidized hemoglobin themselves. The results obtained for the highest antioxidant concentration showed that, indeed, EGCG and gallic acid were able to oxidize hemoglobin (Figure 5), which explains the strange dose dependence observed.

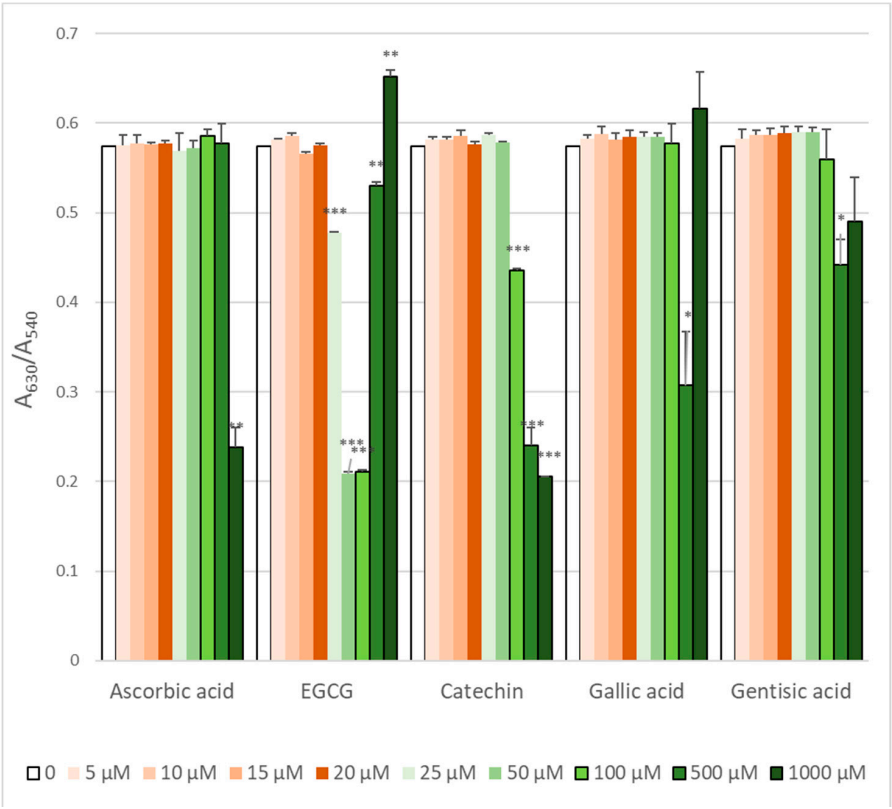


**Figure 4.** Prevention of AAPH-induced hemoglobin oxidation by selected antioxidants. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to control.

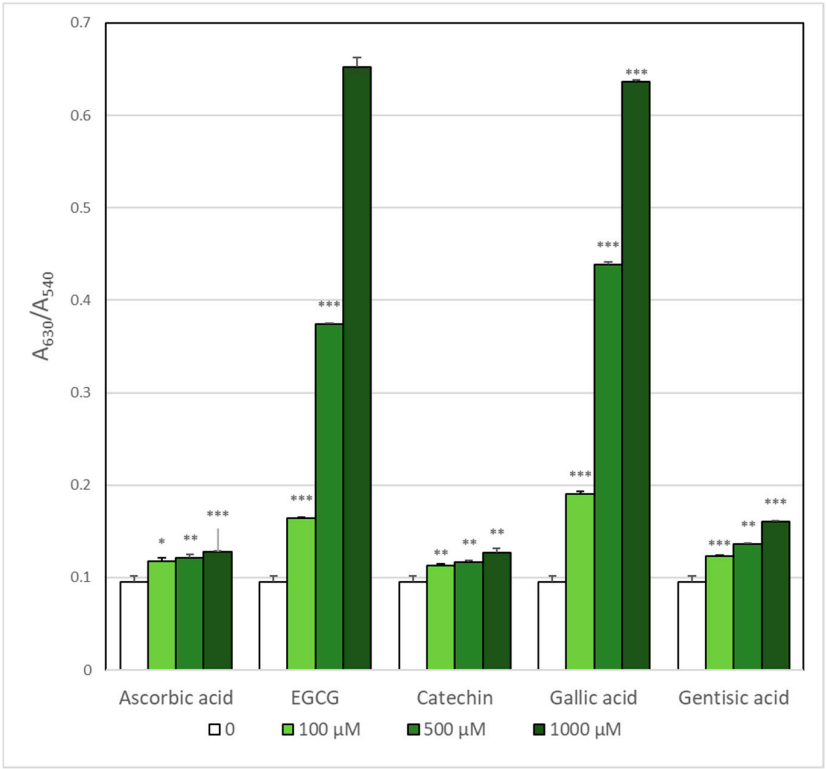


**Figure 5.** Oxidation of hemoglobin by selected antioxidants in the absence of AAPH. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to control.

Previously, oxidation of hemoglobin to methemoglobin by black and green tea extracts, containing EGCG, was noted [35]. Both gallic acid and EGCG contain the trihydroxybenzoic acid residue, which might be responsible for their prooxidative effect on hemoglobin.

### 2.5. Glutathione Loss

Treatment of erythrocyte suspension with AAPH solution for 1 h resulted in a decrease of erythrocyte glutathione concentration from a value of 1875 ± 164 µM to 865 ± 233 µM i.e., by 54%. Ascorbic acid and catechin dose-dependently protected against GSH loss. On the contrary, EGCG, gallic acid and gentisic acid significantly augmented GSH loss (Figure 6). When erythrocytes were incubated with antioxidants under the same conditions but without AAPH, ascorbic acid, catechin and gentisic acid did not decrease the GSH level, while higher concentrations of EGCG and gallic acid produced a considerable GSH loss (Figure 7). Gallic acid has been reported to oxidize glutathione in erythrocytes [19] and EGCG-rich extracts of black and green tea were found to decrease the level of erythrocyte GSH [35]. Thus, the data for EGCG and gallic acid are understandable (GSH loss induced by these compound alone is superposed on the AAPH-induced loss).

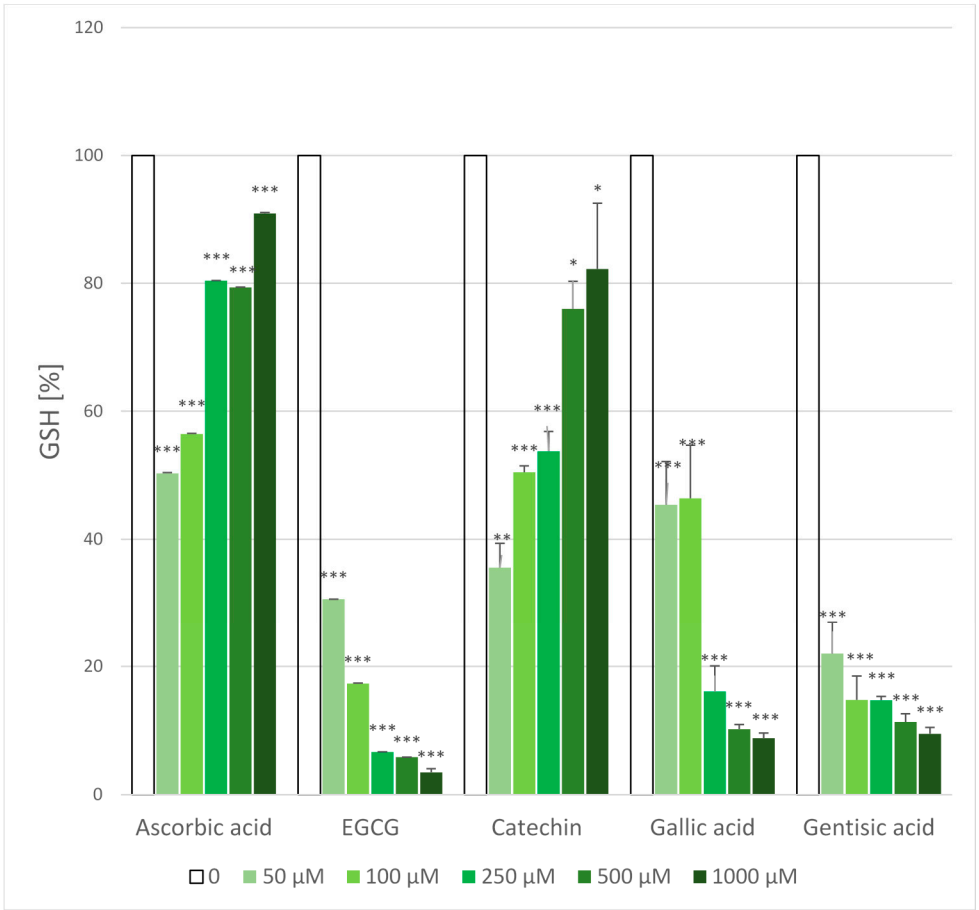


**Figure 6.** Effect of antioxidants on the AAPH-induced loss of erythrocyte glutathione (GSH). GSH level in AAPH-treated erythrocytes was assumed as 100%. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to control.

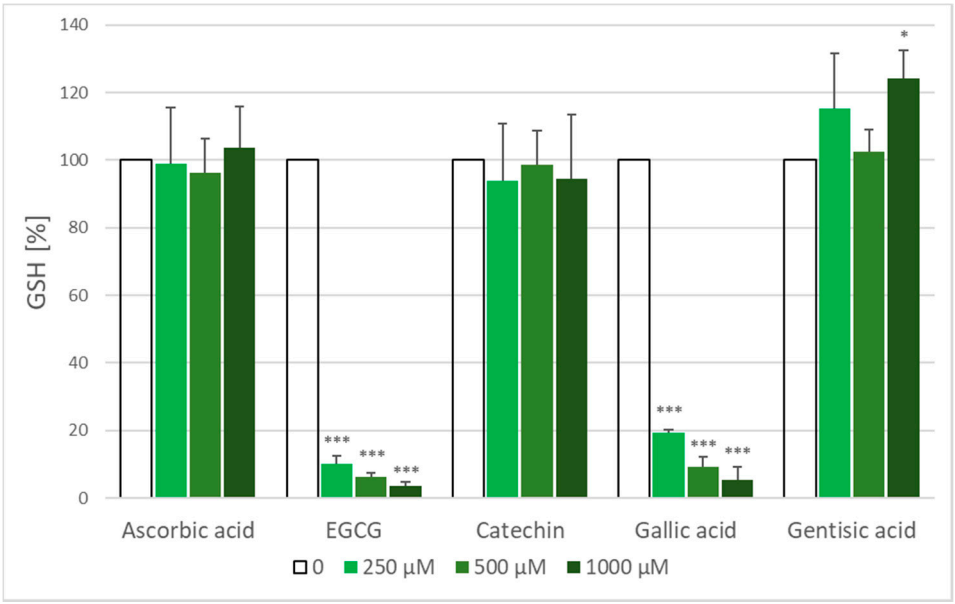


**Figure 7.** Effect of antioxidants on the GSH level in erythrocytes in the absence of AAPH. The level of GSH in control erythrocytes was assumed as 100%. * $p < 0.05$, *** $p < 0.001$ with respect to control.

The results for gentisic acid are more difficult to explain. It can be postulated that reactive species formed by interaction of AAPH with gentisic acid may exhibit prooxidative properties not shown by native gentisic acid. We and others have shown such that other antioxidants, melatonin and resveratrol showed prooxidant effects on glyceraldehyde 3-phosphate dehydrogenase when applied together with nitric oxide; this effect was due to formation of their reactive derivatives, viz. phenoxyl radical in the case of resveratrol [36,37]. As a phenoxyl radical is formed also during oxidation of gentisic acid [30], the mechanism of the prooxidant action of gentisic acid may be similar.

An obvious outcome of this study is the limited correlation or lack of correlation between results of various assays of antioxidant efficiency of the same compounds. EGCG and gallic acid, which are good antioxidants in the test of inhibition of lipid peroxidation and hemolysis, promoted hemoglobin oxidation and glutathione loss. However, another aspect of the prooxidant effects of some antioxidants is their physiological relevance. It should be noted that the latter effects were observed under model experimental conditions, with antioxidant concentrations not attainable in vivo and under oxygen concentrations much higher than those prevailing inside the body. Under conditions which can be physiologically achieved, the prooxidants effects of these compounds are most probably of negligible significance. From that point of view, limited bioavailability of many food antioxidants, may be not necessarily their disadvantage, preventing their undesired reactions in the body, and could be even an evolutionary adaptation.

## 3. Materials

*Chemicals and Equipment*

Dimethyl sulfoxide (DMSO; purity: ≥99.9% sterile filtered) produced by BioShop Canada Inc. (Burlington, ON, Canada) was purchased from Lab Empire (Rzeszów, Poland). 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. 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA), Rutin trihydrate and Lipid Peroxidation Sensor (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-un-decanoic acid; C11-BODIPY® 581/591) were purchased from Thermo Fisher Scientific (Warsaw, Poland). Daidzein was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

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 an Infinite 200 PRO multimode reader (C11-BODIPY fluorescent microplate assay) or a Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). All measurements were performed in triplicate and repeated at least three times. Other reagents were dissolved in PBS or DMSO. Minimal amounts of the solvents present in the samples had a small effect on the protection (up to a few %). The effect of DMSO was subtracted from the effects of substances introduced in this solvent.

## 4. Methods

### *4.1. Experiments in a Cell-Free System*

#### Lipid Peroxidation

Forty µL of 10% egg yolk suspension in PBS (1 mL of egg yolk suspended with vigorous vortexing in PBS) was treated with 50 mM AAPH (final concentration) in the presence of selected antioxidants. The concentration range of these compounds was 0.01–5 mM. The intensely fluorescent BODIPY®581/1591 (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-un-decanoic acid; C11-BODIPY) fluorophore was used for monitoring lipid peroxidation. BODIPY® 581/591 undecanoic acid (5 µL of 0.1 mM solution in DMSO) was added to each well of a black 96-well plate. The final volume of a sample was 200 µL. The kinetic measurement of fluorescence increase at 526 nm was carried using the excitation wavelength of 485 nm at 37 °C for 150 min and the rate of fluorescence increase was measured.

Percent protection against lipid peroxidation by selected antioxidants was calculated as:

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

where $A_o$—fluorescence of sample incubated with AAPH; $A_n$—fluorescence of a sample containing a protective agent; $A_c$—fluorescence of a sample not treated with an oxidizing agent.

### *4.2. Experiment with Erythrocytes*

#### 4.2.1. Ethical Approval

The study was approved by the Bioethics Committee of the University of Lodz (Permit No. KBBN-UŁ/I/3/2013).

#### 4.2.2. Preparation of Erythrocytes

Eight milliliters of peripheral blood from healthy donors (lab volunteers) were collected in EDTA tubes and used within the day of its collection. Erythrocytes were isolated by centrifugation for 10 min at 3000× *g* 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 at various hematocrit from 4% to 60%.

#### 4.2.3. Preparation of Erythrocyte Ghosts

Erythrocyte ghosts were prepared from washed erythrocytes according to the method of Dodge et al. [17] with some modifications. Briefly, after incubation, erythrocytes were hemolyzed on ice with 20 volumes of 20 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 4 °C at 20,000× *g* for 20 min. The ghosts were resuspended in ice-cold 10 mM and then 5 mM phosphate buffer, pH 7.4 containing 1 mM EDTA, centrifuged, and this process was continued until the ghosts were free from residual hemoglobin. Finally, the erythrocyte ghosts

were resuspended in 20 mM phosphate buffer, pH 7.4. The protein concentration was estimated by the method of Lowry et al. [38].

#### 4.2.4. Estimation of the Protective Effects of Selected Compounds on Erythrocyte Membrane Lipid Peroxidation

BODIPY® 581/591 undecanoic acid was dissolved in DMSO by adding 475 µL DMSO to 25 µL 2 mM BODIPY® 581/591 stock solution (dissolved also DMSO). First, PBS was pipetted so that the volume of each well of a black 96-well plate was 200 µL. Next, 40 µL aliquots of membrane suspensions were pipetted into the 96-well black plate, followed by selected compounds in a range of concentrations 10–1000 µM (10 mM GSH, *N*-acetyl-L-cysteine, L-ascorbic acid stock solutions in PBS or 10 mM BHA, mangiferin, naringenin, hesperetin, rutin trihydrate, naringin, *p*-coumaric acid, hesperidin, ferulic acid, chlorogenic acid, Trolox stock solutions in DMSO and then diluted with PBS to obtain concentrations of 5 or 1 mM), 5 µL of BODIPY® 581/591 (0.1 mM stock solution) as well as at the end 100 µL AAPH (100 mM stock solution). The fluorescence (480 nm/524 nm) was measured every 2 min for 150 min. Percent protection against lipid peroxidation by selected antioxidants was calculated as above.

#### 4.2.5. The Assay of AAPH-Induced Hemolysis

The inhibition of free radical-induced RBCs hemolysis was performed by a modification of a previously published method [39], in which hemolysis was monitored turbidimetrically. Hemolysis was induced by thermal decomposition of AAPH. The protective effect of selected antioxidants against AAPH-induced hemolysis was measured only for compounds dissolved in PBS. The RBCs suspension was added with appropriate antioxidant solution to a final concentration in the range of 25–1000 µM and incubated with shaking in the presence/absence of 50 mM AAPH, as optimal concentration to induce hemolysis at 37 °C. The absorbance (700 nm) was measured every 1 h for 12 h using the Tecan Infinite 200 PRO multimode reader. All measurements were performed in triplicate and repeated at least three times. For all determinations, hemolysis time and percentage of hemolysis time with respect to control erythrocytes were calculated as 100% × [time (min) for test compound/mean time (min) for control sample containing RBCs and AAPH only].

#### 4.2.6. Determination of Intracellular ROS Generation

2′,7′-Dichlorodihydrofluorescein diacetate ($H_2$DCF-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 as well as oxidation, the nonfluorescent $H_2$DCF-DA is converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [39,40]. The indicator $H_2$DCF-DA (10 mM stock solution in DMSO) was added to the erythrocyte suspension (10% final hematocrit) to a final concentration of 10 µM of with subsequent incubation at 37 °C for 30 min. Then, samples were washed 2 times with ice cold PBS by centrifugation at 4000× *g* for 5 min and supernatant was discarded to remove excess $H_2$DCF-DA. Afterwards, 10 µL of suspension of $H_2$DCF-DA-loaded erythrocytes (hematocrit of 10%) were pipetted into wells of a 96-well black plate containing appropriate amount of PBS to provide final volume of 200 µL, followed by addition of an appropriate antioxidant solution in PBS to obtain concentrations in the range of 25–1000 µM. AAPH (50 mM final concentration) was then added with good mixing. The fluorescence (485 nm/529 nm) was measured every 2 min for 120 min and sum of fluorescence values obtained in successive measurements was calculated. From these sums of fluorescence values, per cent inhibition of ROS formation and $IC_{50}$ values (antioxidant concentration inhibiting ROS formation by 50%) were calculated.

#### 4.2.7. Hemoglobin Oxidation

Aliquots of RBC suspensions in PBS (hematocrit of 10%) were added with appropriate antioxidant (final concentration range of 5–1000 µM) and AAPH (final concentration of 50 mM), and incubated with shaking at 37 °C for 5 h. A set of control samples contained antioxidants but no AAPH. After

incubation, 1.5 mL distilled water was added to the 0.4 mL samples, the samples were mixed and centrifuged (3 min, 8000× *g*). Next, 200 µL of supernatants were taken and added to a transparent 96-well plate. The absorbance was measured at the wavelengths of 540 and 630 nm. The absorbance ratio $A_{630}/A_{540}$ was calculated as a measure of hemoglobin oxidation.

#### 4.2.8. Glutathione Content

Suspensions of erythrocytes (RBCs) in PBS (final hematocrit of 2%) were treated with different concentration of selected antioxidants (5 mM stock solution) and/or AAPH (100 mM stock solution) and incubated for 1 h at 37 °C with continuous shaking. Then, all samples were centrifuged at 10,000× *g* for 3 min and the supernatants were discarded. Samples were washed with 300 µL of cold 1 × PBS (pH = 7.2). The erythrocyte pellet was precipitated with cold 100 µL RQB-TCA buffer (20 mM HCl, 5 mM diethylenetriaminepentaacetic acid, 10 mM ascorbic acid, 5% trichloroacetic acid), kept on ice for 10 min, centrifuged (13,000× *g*, 2 °C, 5 min) and the supernatant was taken for the GSH assay [41]. For GSH determination, 5 µL of deproteinized supernatant diluted by adding 25 µL RQB-TCA were put on two wells (denoted '−' and '+') of a 96-well black plate. The sample '−' was added with 4 µL of 7.5 mM N-ethylmaleimide in RQB-TCA, both samples added with 40 µL of 1 M potassium phosphate (pH = 7.0), mixed and incubated at room temperature for 5 min. Then 160 µL of 0.1 M potassium phosphate buffer (pH = 7.0), was added, followed by 25 µL of 0.5% *o*-phtalaldehyde in methanol and the plate was shaken (1 min). After 30-min incubation (room temperature) the fluorescence was read at 355 nm/460 nm. The value obtained for the '−' sample was subtracted from that obtained for the '+' value and GSH concentration was read from a calibration curve obtained with glutathione as a standard.

#### 4.2.9. Statistical Analysis

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

## 5. Conclusions

Results of this study demonstrate a limited correlation between various assays of antioxidant activity. Some assays reveal prooxidant effect of antioxidants which are not evident in other systems. Thus, rankings of antioxidants should not be based on a single parameter, but take into account their behavior in different systems.

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**Sample Availability:** Samples of the antioxidants are available from the authors on request.