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mgr farm. Magdalena Rutkowska
(nazwisko rodowe Matczak)

Analiza profilu polifenolowego i aktywności biologicznej liści *Sorbus domestica* L.

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Promotor:
dr hab. n. farm. Monika A. Olszewska, prof. UM

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Poniżej przedstawiono spis publikacji stanowiących podstawę rozprawy doktorskiej pt. „Analiza profilu polifenolowego i aktywności biologicznej liści *Sorbus domestica* L.". Sumaryczny **IF** (*ISI Journal Citation Reports*) cyklu zgodny z rokiem publikacji jest równy **8.706**, zaś punktacja **MNiSW** wynosi **110 (MNiSW$_{2018}$)/310 (MNiSW$_{2019}$)**.

Publikacja I: **Matczak M.**, Marchelak A., Michel P., Owczarek A., Piszczan A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A. (2018). *Sorbus domestica* L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Journal of Functional Foods*, 40, 207-218, doi: 10.1016/j.jff.2017.10.046.

IF$_{2018}$ = 3.197, MNiSW$_{2018}$ = 45, MNiSW$_{2019}$ = 100

Publikacja II: **Rutkowska M.**, Owczarek A., Kolodziejczyk-Czepas J., Michel P., Piotrowska D.G., Kapusta P., Nowak P., Olszewska M.A. (2019). Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control. *Phytochemistry Letters*, 30, 278-287, doi: 10.1016/j.phytol.2019.02.004.

IF$_{2018}$ = 1.338, MNiSW$_{2018}$ = 20, MNiSW$_{2019}$ = 70

Publikacja III: **Rutkowska M.**, Olszewska M.A., Kolodziejczyk-Czepas J., Nowak P., Owczarek A. (2019). *Sorbus domestica* leaf extracts and their activity markers: antioxidant potential and synergy effects in scavenging assays of multiple oxidants. *Molecules*, 24, 2289, doi: 10.3390/molecules24122289.

IF$_{2018}$ = 3.060, MNiSW$_{2018}$ = 30, MNiSW$_{2019}$ = 100

Publikacja IV: **Rutkowska M.**, Dubicka M., Olszewska M.A. (2019). Comparison of the phenolic profile and *in vitro* antioxidant capacity of *Sorbus domestica* L. leaf samples from Poland and Croatia. *Acta Biologica Cracoviensia Series Botanica*, 61(1), 63-73, doi: 10.24425/118059.

IF$_{2018}$ = 1.111, MNiSW$_{2018}$ = 15, MNiSW$_{2019}$ = 40

Wyniki badań prezentowane w ramach niniejszej pracy zostały również przedstawione w formie 6 komunikatów zjazdowych (5 posterów i 1 referatu ustnego) na krajowych i międzynarodowych konferencjach naukowych:

- **Rutkowska M.**, Owczarek A., Michel P., Kołodziejczyk-Czepas J., Nowak P., Olszewska M.A. Isolation, identification and biological activity of two diglycosides from *Sorbus domestica* (L.) leaves. 11th International Symposium on Chromatography of Natural Products, Lublin, 04-07.06.2018, poster.
- **Matczak M.**, Olszewska M.A. Liście *Sorbus domestica* L. - profil fitochemiczny, aktywność antyoksydacyjna i przeciwzapalna. VIII Sesja Magistrantów i Doktorantów Łódzkiego Środowiska Chemików, Łódź, 22.06.2017, referat ustny.
- **Matczak M.**, Marchelak A., Piszczana A., Michel P., Owczarek A., Kołodziejczyk-Czepas J., Olszewska M.A. Badania fitochemiczne i aktywności antyoksydacyjnej liści *Sorbus domestica* L. (Rosaceae). Konferencja Naukowa Studentów Wydziału Farmaceutycznego z Oddziałem Kosmetologii i Oddziałem Analityki Medycznej Uniwersytetu Medycznego w Łodzi "150 rocznica urodzin Marii Skłodowskiej-Curie", Łódź, 22.04.2017, poster.
- **Matczak M.**, Marchelak A., Owczarek A., Olszewska M.A. Phytochemical profiling of extracts from the leaves of *Sorbus domestica* L. 2nd International Young Scientists Symposium "Plants in Pharmacy and Nutrition", Wrocław, 15-17.09.2016, poster.
- **Matczak M.**, Marchelak A., Michel P., Piszczan A., Kołodziejczyk-Czepas J., Olszewska M.A. Evaluation of antioxidant activity of *Sorbus domestica* L. leaves using complementary *in vitro* and *ex vivo* methods. 2nd International Young Scientists Symposium "Plants in Pharmacy and Nutrition", Wrocław, 15-17.09.2016, poster.
- **Matczak M.**, Marchelak A., Piszczan A., Michel P., Olszewska M.A. Analiza profilu chemicznego oraz aktywności antyoksydacyjnej liści *Sorbus domestica* L. X Kopernikańskie Seminarium Doktoranckie, Bachotek, 21-24.06.2016, poster.

Wykaz skrótów

AA	kwas askorbinowy (*ang. ascorbic acid*)
BF	frakcja *n*-butanolu (*ang. n-butanol fraction*)
CHA	kwas chlorogenowy (*ang. chlorogenic acid*)
COSY	spektroskopia korelacyjna (*ang. correlation spectroscopy*)
CYE	ekwiwalenty chlorku cyjanidyny (*ang. cyanidin chloride equivalents*)
1D/2D NMR	jedno-/dwuwymiarowa spektroskopia magnetycznego rezonansu jądrowego (*ang. 1-dimensional/2-dimensional nuclear magnetic resonance*)
DEF	frakcja eteru dietylowego (*ang. diethylether fraction*)
DPPH	2,2-difenylo-1-pikrylohydrazyl (*ang. 2,2-diphenyl-1-picrylhydrazyl*)
EAF	frakcja octanu etylu (*ang. ethyl acetate fraction*)
$EC_{50}/SC_{50}/IC_{50}$	stężenie analitu wywołujące 50% badanego efektu/ stężenie analitu „zmiatające" ROS/RNS w 50%/ stężenie inhibitora hamujące badany czynnik w 50% (*ang. half maximal effective/scavenging/inhibitory concentration*)
ECA	(-)-epikatechina (*ang. (-)-epicatechin*)
ELISA	test immunoenzymosorpcyjny (*ang. enzyme-linked immunosorbent assay*)
ESI-MS	spektrometria mas z jonizacją typu elektrosprej (*ang. electrospray ionization mass spectrometry*)
FOX-1	test poziomu wodoronadtlenków z użyciem oranżu ksylenolowego (*ang. ferric-xylenol orange hydroperoxide assay*)
FRAP	test zdolności do redukcji jonów żelaza Fe^{3+} (*ang. ferric reducing antioxidant power assay*)
GAE	ekwiwalenty kwasu galusowego (*ang. gallic acid equivalents*)
HMBC	heterojądrowa korelacja dalekiego zasięgu (*ang. heteronuclear multiple bond coherence*)
HMQC	heterojądrowa korelacja z detekcją przejść wielokwantowych (*ang. heteronuclear multiple quantum coherence*)
HRQ	3-*O*-(2''-*O*-β-D-glukopiranozylo)-α-L-ramnopiranozyd kwercetyny (*ang. quercetin 3-O-(2''-O-β-D-glucopyranosyl)-α-L-rhamnopyranoside*)
HYAL	hialuronidaza (*ang. hyaluronidase*)
LOOH	wodoronadtlenki lipidów (*ang. lipid hydroperoxides*)
LOX	lipooksygenaza (*ang. lipoxygenase*)
MED	odtłuszczony ekstrakt metanolowo-wodny (7:3, *v*/*v*) (*ang. defatted methanol-water (7:3, v/v) extract*)
3-NT	3-nitrotyrozyna (*ang. 3-nitrotyrosine*)
NCDs	choroby niezakaźne (*ang. non-communicable diseases*)
NEAC	nieenzymatyczna pojemność antyoksydacyjna osocza (*ang. non-enzymatic antioxidant capacity of plasma*)

PB2	procyjanidyna B2 (*ang. procyanidin B2*)
PBMCs	jednojądrzaste komórki krwi obwodowej (*ang. peripheral blood mononuclear cells*)
PC1	procyjanidyna C1 (*ang. procyanidin C1*)
PRQ	3-*O*-(2''-*O*-β-D-ksylopiranozylo)-α-L-ramnopiranozyd kwercetyny (*ang. quercetin 3-O-(2''-O-β-D-xylopyranosyl)-α-L-rhamnopyranoside*)
QCT	kwercytryna, 3-*O*-α-L-ramnopiranozyd kwercetyny (*ang. quercitrin, quercetin 3-O-α-L-rhamnopyranoside*)
QU	kwercetyna (*ang. quercetin*)
ROS/RNS	reaktywne formy tlenu/ reaktywne formy azotu (*ang. reactive oxygen species/reactive nitrogen species*)
RT	rutyna, 3-*O*-(6''-*O*-α-L-ramnopiranozylo)-β-D-glukopiranozyd kwercetyny (*ang. rutin, quercetin 3-O-(6''-O-α-L-rhamnopiranosyl)-β-D-glucopyranoside*)
s.m.	sucha masa (*ang. dw, dry weight*)
TBARS	substancje reagujące z kwasem tiobarbiturowym (*ang. thiobarbituric acid-reactive substances*)
TPA	całkowita zawartość proantocyjanidyn (*ang. total proanthocyanidin content*) oznaczona metodą Portera (*ang. n-butanol/HCl assay*)
TPC	całkowita zawartość polifenoli (*ang. total phenolic content*) oznaczona metodą Folina-Ciocalteu'a (*ang. Folin-Ciocalteu assay*)
TPH	całkowita zawartość polifenoli (*ang. total contents of phenolic compounds*) oznaczona metodą HPLC-PDA
TX	Troloks (*ang. Trolox®*)
(U)HPLC-PDA	(ultra)wysokosprawna chromatografia cieczowa z detekcją fotodiodową (*ang. (ultra)-high-performance liquid chromatography with photodiode array detection*)
WR	pozostałość wodna (*ang. water residue*)

Uzasadnienie podjętej tematyki badawczej

Istnienie związku pomiędzy rozwojem przewlekłych chorób niezakaźnych (NCDs) a połączeniem czynników genetycznych, środowiskowych i behawioralnych stanowi podstawę strategii prewencyjnych, wdrażanych w społeczeństwach. Według raportów Światowej Organizacji Zdrowia, NCDs, w szczególności choroby układu sercowo-naczyniowego, oddechowego, nowotwory i cukrzyca, są obecnie przyczyną ponad 70% zgonów globalnie, powodując równocześnie milionowe straty ekonomiczne (WHO, 2018). Mając na uwadze badania ostatnich dwóch dekad, stres oksydacyjny, definiowany jako stan zaburzonej równowagi redoks organizmu, objawiający się wysokim stężeniem reaktywnych form tlenu i azotu (ROS/RNS), a także przewlekły stan zapalny, uznaje się za jedne z głównych przyczyn rozwoju schorzeń kardiologicznych, insulino-oporności, oraz innych NCDs (Koch, 2019; Hussain i wsp., 2016; Tresserra-Rimbau i wsp., 2018). Pośród proponowanych nisko-budżetowych strategii ograniczenia postępu chorób przewlekłych znajduje się redukcja modyfikowalnych czynników ryzyka, poprzez m.in. promocję zdrowego stylu życia, w tym właściwej diety. Wyjątkowo dobre rezultaty uzyskuje się przez zwiększenie spożycia produktów roślinnych bogatych w tzw. naturalne antyoksydanty, w szczególności polifenole, czego dowodem są prowadzone w ostatnich latach badania kliniczne oraz epidemiologiczne (Bonaccio i wsp., 2017; Cory i wsp., 2018; Koch, 2019; de Melo Ribeiro i wsp., 2019).

Polifenole stanowią bogatą i zróżnicowaną grupę wyspecjalizowanych metabolitów roślinnych, w skład której wchodzi ponad 8 (12) tysięcy różnych struktur (zależnie od stopnia kalkulacji), z czego około 800 wykrytych zostało w produktach spożywczych (Koch, 2019; Tresserra-Rimbau i wsp., 2018). Są to zarówno proste związki o stosunkowo małej masie cząsteczkowej, jak np. kwasy fenolowe czy flawonoidy, jak również wielkocząsteczkowe polimery o masie ponad 30 kDa, jak np. garbniki. W naturze występują najczęściej w formie połączeń z różnymi cukrami, przede wszystkim glukozą, ale też galaktozą, ramnozą, ksylozą, arabinozą i innymi, co dodatkowo potęguje liczbę poznanych oraz jeszcze nieodkrytych związków (Koch, 2019). Konsekwencją różnorodności strukturalnej jest zdolność do reagowania z rozmaitymi mikro- i makrocząsteczkami, a przez to wielokierunkowy wpływ na organizm ludzki (Bonaccio i wsp., 2017; Cory i wsp., 2018; Koch, 2019; de Melo Ribeiro i wsp., 2019; Tresserra-Rimbau i wsp., 2018).

Efekty biologiczne działania polifenoli wynikają w przeważającej mierze z ich regulującego wpływu na procesy oksydoredukcyjne i zapalne zachodzące w komórkach i tkankach. Według ostatnich badań, bezpośrednia redukcja ROS/RNS (tzw. „zmiatanie”); inhibicja enzymów pro-oksydacyjnych, w tym cyklooksygenazy-2, lipooksygenaz, oksydazy ksantynowej, czy syntazy tlenku azotu; aktywacja czynników transkrypcyjnych zaangażowanych w wytwarzanie endogennych enzymów antyoksydacyjnych, np. dysmutazy ponadtlenkowej, transferazy glutationowej, oksydazy hemowej-1, czy oksydoreduktaz; a także regulacja szlaków sygnalizacji komórkowej w procesie zapalnym i procesach metabolicznych, stanowią podstawowe mechanizmy aktywności związków polifenolowych (Koch, 2019; Hussain i wsp., 2016; de Melo Ribeiro i wsp., 2019; Tresserra-Rimbau i wsp., 2018).

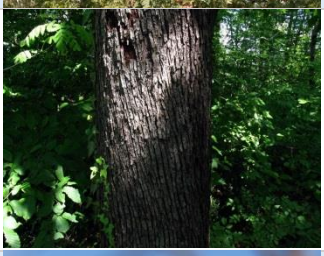
Biorąc pod uwagę złożoność procesów patologicznych odpowiedzialnych za rozwój NCDs, różnorodność mechanizmów działania polifenoli stanowi prawdopodobną przyczynę ich skuteczności w terapii wspomagającej i profilaktyce chorób (Tresserra-Rimbau i wsp., 2018). Ponadto, poza aktywnościami charakterystycznymi dla poszczególnych związków, równie istotne są możliwe efekty

synergistyczne obserwowane między polifenolami w wieloskładnikowych mieszaninach, jak np. w produktach spożywczych, czy ekstraktach roślinnych (Cory i wsp., 2018; Tresserra-Rimbau i wsp., 2018). Liczne badania pokazują, iż zastosowanie substancji naturalnych w formie mieszanin obecnych w produktach pochodzenia roślinnego, pozwala na zmniejszenie spożytej dawki czystych związków i uzyskanie efektów klinicznych, które z uwagi na zbyt niską biodostępność lub potencjalne skutki uboczne, są niemożliwe do osiągnięcia przy użyciu pojedynczych substancji (Cory i wsp., 2018; Sonam i Guleria, 2017; Tresserra-Rimbau i wsp., 2018).

Dowody wskazujące na skuteczność polifenolowych antyoksydantów w profilaktyce i terapii NCDs, stały się przyczyną wzrostu zainteresowania problematyką produktów roślinnych, źródłami ich pozyskiwania, metodami wydajnej ekstrakcji, analizą losów w organizmie oraz zasadami ewentualnej suplementacji (Cory i wsp., 2018; Xu i wsp., 2017). Jednym z wiodących kierunków badawczych stała się analiza surowców roślinnych stosowanych w medycynie tradycyjnej, które po odpowiedniej weryfikacji fitochemicznej, farmakologicznej i toksykologicznej miałby szanse znaleźć zastosowanie w produkcji preparatów leczniczych, suplementów diety, czy żywności funkcjonalnej.

Pragnąc wpisać się w ten kontekst badawczy, w niniejszej dysertacji zwrócono uwagę na gatunek pochodzący z rejonów Morza Śródziemnego, uprawiany jako roślina owocowa i ozdobna, o wysokiej wartości odżywczej i potwierdzonym zastosowaniu w medycynie ludowej, lecz o słabo rozpoznanym składzie chemicznym i mechanizmach aktywności biologicznej, tj. jarząb domowy (*Sorbus domestica*).

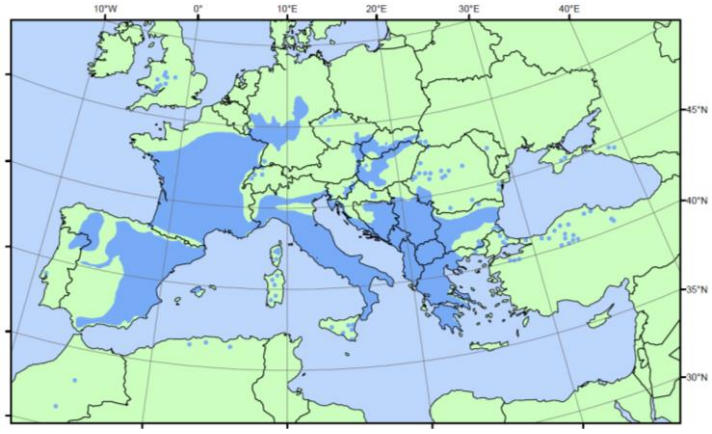
Tabela 1. Charakterystyka botaniczna gatunku *Sorbus domestica* L.

CECHY MORFOLOGICZNE		
POKRÓJ	wysokość średnio 15-20 m; korona silnie sklepiona, w zarysie okrągława	
KORA	brązowa, początkowo gładka, później spękana	
PĘDY/PĄKI	pędy początkowo owłosione, później nagie; pąki oliwkowozielone, owalne, długości ok. 1 cm, nagie lub lekko owłosione, pokryte woskowatym nalotem	
LIŚCIE	ułożone skrętolegle, nieparzystopierzastozłożone, długości 12-25 cm i szerokości do 10 cm, złożone z 13-21 listków (3-5 cm dł., 1-2 cm szer., podłużne, eliptyczne/jajowate, piłkowane, symetryczne, bezogonkowe, nasada zaokrąglona, strona grzbietowa nieowłosiona, ciemnozielona lub żółtozielona, strona brzuszna lekko owłosiona, jaśniejsza), przylistki na długopędach wcześnie odpadają, ogonek liściowy 3-5 cm długi	

KWIATY	szypułkowe, średnica ok. 1.5 cm, zebrane w owłosione podbaldachy (6-10 cm szerokości), obupłciowe; działki kielicha trwałe, trójkątne, całobrzegie; płatki korony (5) białe lub kremowobiałe; pręciki kremowobiałe (15-25); słupków 5-6; okres kwitnienia maj-czerwiec	
OWOCE	duże (średnica 1.5-2 cm, długość 2-3 cm), gruszko-/jabłkokształtne, zielone/żółte/brązowe, zaczerwienione od strony słonecznej, z licznymi przetchlinkami i komórkami kamiennymi, okres owocowania wrzesień-październik	
NASIONA	czerwono-brązowe, 6-7 mm długości, 4-4.5 mm szerokości, kształtu odwrotnie-jajowatego, zakrzywione, po obu stronach spłaszczone, z ostrymi krawędziami	

Opis: Rotach, 2003; Kremer, 1996; Lim, 2012; Seneta i Dolatowski, 2008; Kamm i wsp., 2009; Bojnňansky i Fargasowa, 2007. Fotografie: https://www.actaplantarum.org/galleria_flora/galleria1.php?aid=674, pokrój - Luciano Capasso, kora - Graziano Propetto, pąk - Vito Buono, liść - Ionut Carjan, kwiaty - Franco Rossi, owoce - Maria Grazia Lobba, nasiona - Vito Buono.

S. domestica L. jest jedynym przedstawicielem podrodzaju *Cormus* (Spach) Duch., należącym do rodzaju *Sorbus sensu lato* (jarząb), podrodziny *Maloideae* (jabłoniowate), rodziny Rosaceae (różowate) (McAllister, 2005). Jarząb domowy to drzewo liściaste, ciepło- i światłolubne, preferujące gleby suche i wapienne (Seneta i Dolatowski, 2008) oraz klimat ciepły i łagodny (Rotach, 2003), występujące w rejonach Południowej i Środkowej Europy, Północno-Zachodniej Afryki i Południowo-Zachodniej Azji. W Europie obszar jego naturalnego występowania obejmuje Półwysep Bałkański, Włochy, Francję oraz Północno-Wschodnią Hiszpanię, w Polsce gatunek ten jest uprawiany (Rotach, 2003).



Rycina 1. Obszar naturalnego występowania (kolor niebieski) gatunku *Sorbus domstica* L. EUFORGEN 2014, www.euforgen.org.

W lecznictwie tradycyjnym wykorzystywane są zarówno liście, jak i owoce oraz kora, gatunku *S. domestica* L., zaś głównym źródłem informacji o ich zastosowaniu są wywiady zbierane wśród ludności lokalnej (osoby starsze, pasterze, zielarze, uzdrowiciele) różnych prowincji na terenie Turcji, połączone z analizą taksonomiczną próbek surowców (Tulzaci i Tolon, 2000; Tulzaci i Aymaz, 2001; Kültür, 2007) (Tabela 2).

Tabela 2. Zastosowanie lecznicze surowców z gatunku *S. domestica* L. (Tulzaci i Tolon, 2000; Tulzaci i Aymaz, 2001; Kültür, 2007).

	PRZYGOTOWANIE	ZASTOSOWANIE
KORA	odwar	owrzodzenie przewodu pokarmowego, ból brzucha
LIŚCIE	napar	zapalenie nerek, cukrzyca
	odwar	zapalenie nerek, zapalenie gruczołu krokowego, kamica nerkowa, schorzenia pęcherzyka żółciowego, biegunka, cukrzyca, hipercholesterolemia, osłabiona diureza
OWOCE	świeże	biegunka
	marynowane	zapalenie nerek, cukrzyca
	syrop	kaszel

Mimo szerokiego zastosowania w lecznictwie tradycyjnym, w szczególności liści *S. domestica*, brak badań wspomnianego surowca uniemożliwia jego szersze wykorzystanie medyczne. Dotychczasowe badania jarzębu domowego skupiały się na owocach, które choć uznawane za bogate źródło bioaktywnych polifenoli (całkowita zawartość: 2.27-341 µg kwasu galusowego/mg suchej masy ekstraktu, w zależności od stopnia dojrzałości owoców i użytego ekstrahenta) (Termentzi i wsp., 2006), są surowcem o ograniczonej dostępności na dużej części terytoriów europejskich z uwagi na osłabione kwitnienie i owocowanie omawianego gatunku w warunkach klimatu umiarkowanego. Ponadto, ostatnie doniesienia wskazują na wyższą aktywność frakcji fenolowych ekstrahowanych z liści roślin owocowych niż z odpowiednich owoców (Teleszko i Wojdyło, 2015), co znajduje odzwierciedlenie w licznych badaniach i wprowadzaniu na rynek coraz większej liczby preparatów na bazie liści (np. winorośli, czy czarnej porzeczki), skutecznych w ochronie przed schorzeniami związanymi ze stresem oksydacyjnym (Franz i wsp., 2011).

Spośród wielu badanych surowców, liście gatunków z rodzaju *Sorbus* wydają się być obiecującym materiałem roślinnym, z uwagi na zarówno czynniki ekonomiczne (dostępność w całym sezonie wegetacyjnym), jak i chemotaksonomiczne (wysoka zawartość polifenolowych antyoksydantów) (Olszewska i wsp. 2010; Olszewska i wsp. 2012). Dowodzą tego badania różnych surowców pozyskanych m.in. z gatunków *S. aucuparia, S. aria, S. torminalis, S.intermedia,* według których zawartość polifenoli w liściach osiąga poziom 2-4 razy wyższy niż w przypadku owoców, co przekłada się na odpowiednio wyższą aktywność antyoksydacyjną (Olszewska, 2011; Olszewska i Michel, 2009).

Biorąc pod uwagę wysoką zawartość oraz różnorodność związków polifenolowych obserwowaną dla owoców *S. domestica* (około 50 związków z grupy flawonoidów, kwasów fenolowych, proantocyjanidyn oraz bifenyli) (Forino i wsp., 2015; Ölschläger i Dieter, 2004; Ritter i wsp., 1994; Termentzi i wsp., 2008a; Termentzi i wsp., 2009), profil ich aktywności biologicznej (Tabela 3) (Termentzi i wsp., 2006; Termentzi i wsp., 2008b), a także dane dotyczące gatunków pokrewnych (Olszewska i wsp., 2012; Olszewska, 2011), można przypuszczać, iż ekstrakty z liści jarzębu domowego mogą być cennym surowcem leczniczym, o walorach przekraczających te obserwowane dla owoców ww. gatunku. W związku z tym, jednym z głównych celów niniejszej rozprawy stała się ocena aktywności biologicznej, w szczególności właściwości antyoksydacyjnych, liści *S. domestica* pochodzących z uprawy krajowej.

Tabela 3. Aktywność biologiczna owoców *Sorbus domestica* L. (Termentzi i wsp., 2006; Termentzi i wsp., 2008b).

	MATERIAŁ BADAWCZY	METODA *IN VITRO*	PARAMETRY	KONTROLA (STANDARD)
ANTYOKSYDACYJNA	ekstrakty roślinne: ekstrakcja frakcjonowana owoców w różnych stadiach dojrzałości; rozpuszczalniki: metanol, dichlorometan, eter dietylowy, octan etylu, *n*-butanol	DPPH	EC_{50} = 0.34-39.50 mg s.m./mg DPPH; najwyższa aktywność: frakcja *n*-butanolu z miąższu owoców dojrzałych	Troloks: EC_{50} = 0.18 mg s.m./mg DPPH; kwercetyna: EC_{50} = 0.07 mg s.m/mg DPPH
		chemiluminescencja indukowana Co(II)/EDTA	IC_{50} = 0.68-352.0 µg s.m./ml; najwyższa aktywność: frakcja eteru dietylowego z owoców dojrzewających po zebraniu przez tydzień	Troloks: IC_{50} = 2.5 µg/ml; kwercetyna: IC_{50} = 0.32 µg/ml
PRZECIWCUKRZYCOWA	ekstrakty roślinne: ekstrakcja frakcjonowana owoców w różnych stadiach dojrzałości; rozpuszczalniki: metanol, dichlorometan, eter dietylowy, octan etylu, *n*-butanol	test hamowania reduktazy aldozowej	72-93% hamowania dla frakcji eteru dietylowego i 80-86% dla frakcji octanu etylu, przy stężeniu 50 µg/ml	sorbinyl: 45% hamowania przy stężeniu 0.25 µM (0.059 µg/ml)

Aktywność antyoksydacyjna produktów roślinnych zależy od ich składu jakościowego i ilościowego, w tym przede wszystkim zawartości związków polifenolowych (de Melo Ribeiro i wsp., 2019; Tresserra-Rimbau i wsp., 2018). Na profil fitochemiczny preparatów roślinnych wpływa szereg czynników, począwszy od czasu i miejsca zbioru surowca, co wiąże się z sezonową i środowiskową zmiennością syntezy i akumulacji metabolitów roślinnych (Aninbon i wsp., 2016; Chen i wsp., 2018; Ncube i wsp., 2012; Zhang, Wu i wsp., 2017), skończywszy na rodzaju przygotowanego ekstraktu (Kneifel i wsp., 2002; Yau i wsp., 2015). We współczesnej fitoterapii coraz częściej stosowane są ekstrakty suche, z uwagi na wyższe stężenie substancji aktywnych biologicznie, łatwość ich przechowywania i dawkowania, a także większą stabilność fizyko-chemiczną i mikrobiologiczną w porównaniu do ekstraktów płynnych, czy wyjściowego materiału roślinnego (Kneifel i wsp., 2002).

Newralgiczny punktem badania produktów naturalnych jest z kolei dobór adekwatnych metod analitycznych, mających na celu ocenę składu oraz aktywności surowca roślinnego (Xu i wsp., 2017). W celu zapewnienia odpowiedniej skuteczności i bezpieczeństwa stosowania preparatów roślinnych o złożonym składzie konieczna jest ich odpowiednia standaryzacja i kontrola jakości (Yau i wsp., 2015). Mnogość związków wchodzących w skład ekstraktów roślinnych, będąca z jednej strony przyczyną potencjalnie wyższej skuteczności w profilaktyce i terapii chorób niż ma to miejsce w przypadku pojedynczych substancji, wymaga często co najmniej dostosowania znanych metod do analizy nowych surowców. Według Europejskiej Agencji Leków (European Medicines Agency) jakość ekstraktów powinna być oceniana poprzez zastosowanie palety metod umożliwiających potwierdzenie tożsamości, wykrycie ewentualnych zafałszowań, ocenę stabilności oraz analizę ilościową związków aktywnych (EMA, 2018). W przypadku nowych, słabo przebadanych surowców, konieczne jest uprzednie wytypowanie adekwatnych markerów aktywnych do kontroli jakości, co z kolei wiąże się z ustaleniem profilu działania biologicznego, zależności pomiędzy składem ekstraktu, a jego efektywnością, oraz mechanizmów aktywności odpowiedzialnych za skuteczność *in vivo* (EMA,

2009; Granato i wsp., 2018). W przypadku surowców polifenolowych, wstępna analiza działania biologicznego polega w pierwszej kolejności na badaniu właściwości antyoksydacyjnych w modelach *in vitro* opartych o różne mechanizmy, co pozwala obrać właściwy kierunek dla poszerzonych dociekań, w tym badań *in vivo*. Ponadto, odnosząc się do coraz częstszych doniesień wskazujących na ścisły związek stresu oksydacyjnego oraz przewlekłego stanu zapalnego (Biswas, 2016), badania aktywności antyoksydacyjnej łączy się z analizą wpływu na procesy zapalne. Do metod oceny aktywności antyoksydacyjnej *in vitro* należą, m.in. testy „zmiatania" ROS/RNS, w tym obecnych w układach biologicznych $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, HClO, $HO^{\bullet}$, $ONOO^{-}$; ocena zdolności do inhibicji utleniania/nitracji makrocząsteczek (białek, lipidów, kwasów nukleinowych); analiza właściwości redukujących; ocena wpływu na komórkową sekrecję ROS/RNS; czy analiza wpływu na endogenne enzymy zaangażowane w procesy oksydoredukcyjne (Prior i wsp., 2016; Tresserra-Rimbau i wsp., 2018; Xu i wsp., 2017). Analogicznie wśród metod badania aktywności przeciwzapalnej znajdują się analizy w modelach bezkomórkowych, w tym testy inhibicji enzymów prozapalnych (np. cyklooksygenazy-2, lipooksygenazy), jak również badania w modelach komórkowych, m.in. testy uwalniania mediatorów stanu zapalnego (cytokin i enzymów) przez komórki układu odpornościowego (Nile i Park, 2013; Biswas, 2016). Dodatkowo, w przypadku analizy ekstraktów roślinnych o skoncentrowanej zawartości różnorodnych związków naturalnych, podobnie jak dla każdej nowej substancji leczniczej, konieczna jest ocena bezpieczeństwa ich stosowania w modelach *in vitro*, a następnie *in vivo* (Parasuraman, 2011; Roggen, 2011).

Biorąc pod uwagę fakt, iż niniejsza rozprawa stanowi pierwsze przedsięwzięcie badawcze zmierzające do charakterystyki liści *S. domestica* jako roślinnej substancji leczniczej i źródła związków aktywnych biologicznie, prowadzone badania zostały zaplanowane dwutorowo i stanowiły połączenie pogłębionej analizy fitochemicznej ze wskazaniem markerów analitycznych i opracowaniem metodyki kontroli jakości oraz wstępnej oceny aktywności antyoksydacyjnej i przeciwzapalnej suchych ekstraktów w komplementarnych chemicznych i biologicznych modelach *in vitro*.

Cele naukowe pracy

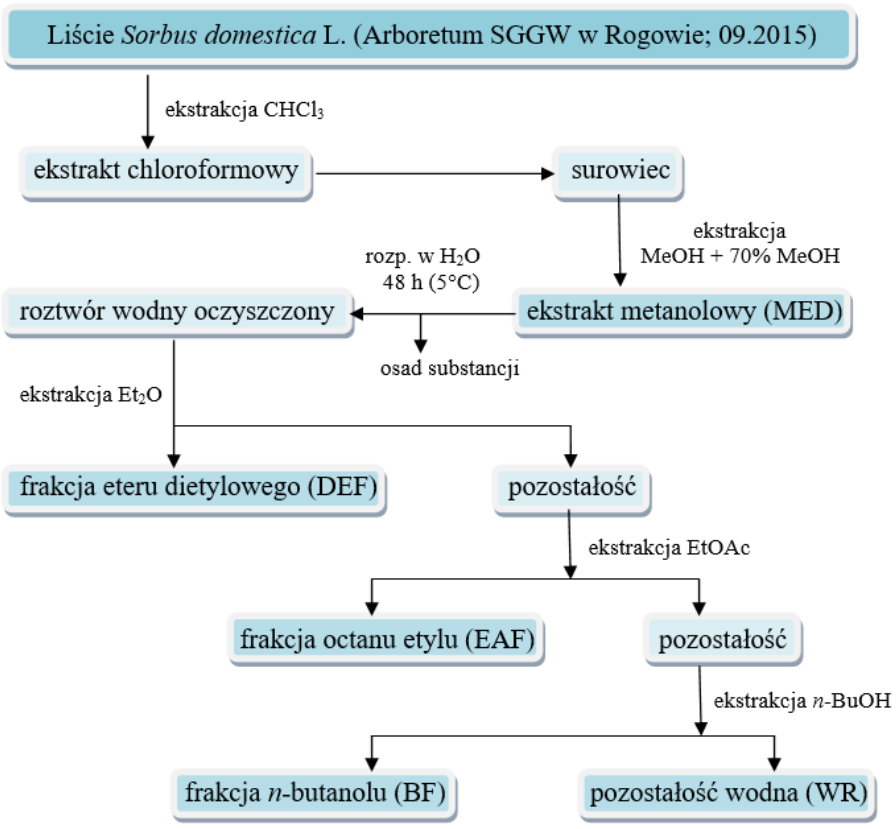
Celem naukowym rozprawy doktorskiej była ocena wartości liści *Sorbus domestica*, pochodzących z uprawy krajowej, jako źródła naturalnych, biologicznie aktywnych polifenoli, co przeprowadzono w oparciu o następujące badania:

1. Analizę profilu polifenolowego suchych ekstraktów uzyskanych z liści *S. domestica* w funkcji ekstrakcji frakcjonowanej, w tym:
   - ❖ standaryzację jakościową i ilościową ekstraktów (**Publikacja I**);
   - ❖ izolację i określenie struktury wybranych składników polifenolowych (**Publikacja II**);
   - ❖ identyfikację markerów analitycznych, w tym markerów aktywnych odpowiednich dla kontroli jakości ww. ekstraktów (**Publikacja II**)
   - ❖ optymalizację i walidację metody HPLC-PDA analizy związków polifenolowych w materiale roślinnym i ekstraktach pochodnych (**Publikacja II**);
2. Analizę *in vitro* aktywności biologicznej suchych ekstraktów z liści *S. domestica* oraz związków modelowych, w tym:
   - ❖ analizę aktywności antyoksydacyjnej w testach chemicznych (DPPH, FRAP, TBARS) (**Publikacja I**), w tym wobec reaktywnych form tlenu i azotu operujących *in vivo*, tj. $O_2^{\bullet -}$, $HO^{\bullet}$, H_2O_2, $NO^{\bullet}$, $ONOO^-$ i HClO (**Publikacja III**);
   - ❖ ocenę działania ochronnego w stosunku do białkowych i lipidowych składników ludzkiego osocza (**Publikacja I i II**);
   - ❖ ocenę zdolności do hamowania enzymów prozapalnych: lipooksygenazy i hialuronidazy (**Publikacja I i II**);
   - ❖ ocenę efektów synergicznych pomiędzy cząsteczkami reprezentatywnymi dla każdej z głównych grup związków warunkujących aktywność badanych ekstraktów (**Publikacja III**);
   - ❖ wstępną ocenę bezpieczeństwa stosowania ekstraktów poprzez analizę ich wpływu na przeżywalność jednojądrzastych komórek krwi obwodowej (**Publikacja I**);
3. Porównawcza analiza fitochemiczna i aktywności antyoksydacyjnej liści z uprawy krajowej i z terenu Bałkanów (**Publikacja IV**).

Część doświadczalna

1. Materiał roślinny i ekstrakty

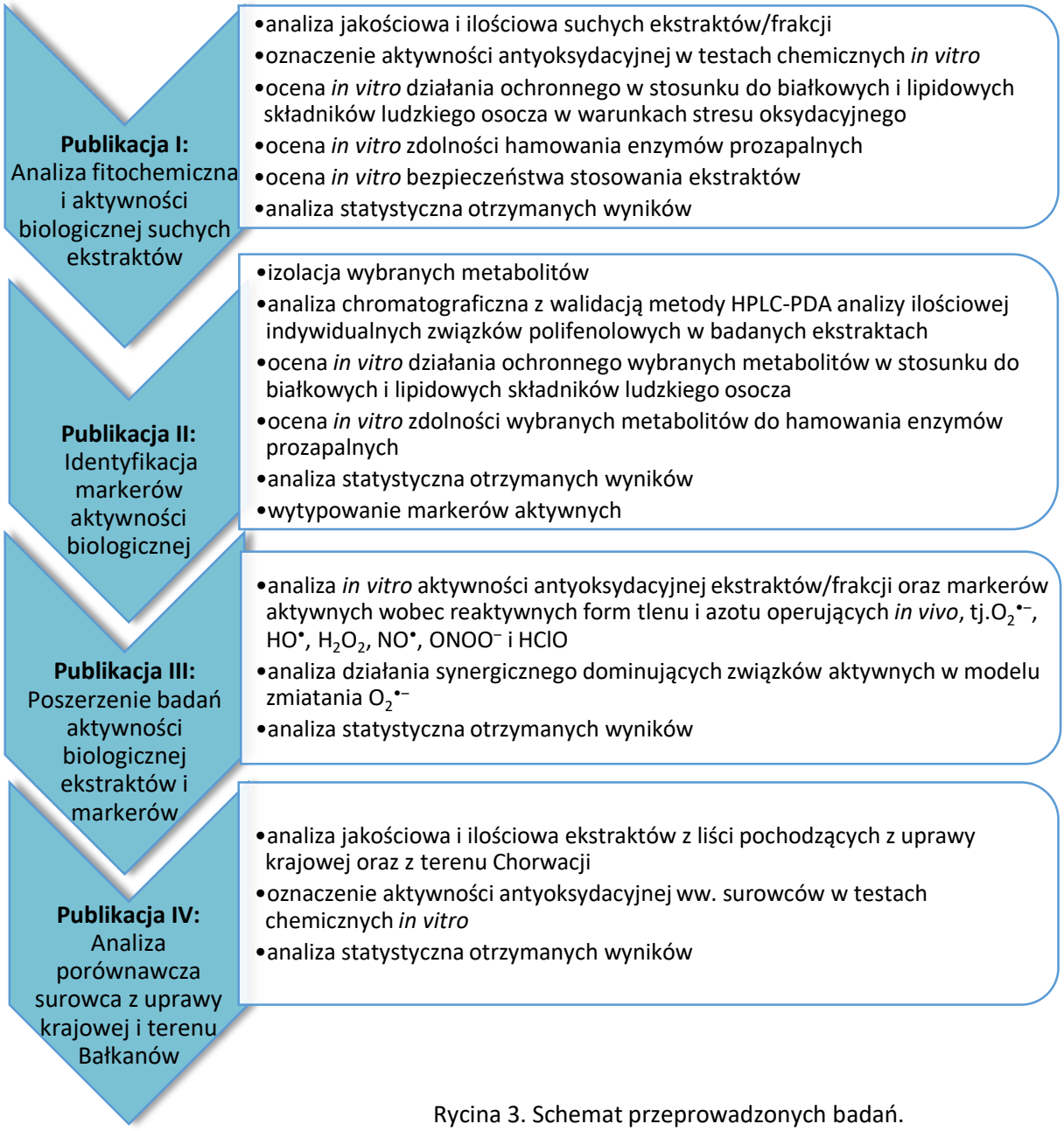
Materiał do badań izolacyjno-identyfikacyjnych i analiz aktywności biologicznej stanowiły liście gatunku *Sorbus domestica* L. zebrane we wrześniu 2015 roku w Arboretum Szkoły Głównej Gospodarstwa Wiejskiego (SGGW) w Rogowie, natomiast surowiec przeznaczony do porównawczej analizy fitochemicznej liści z uprawy krajowej i z terenu Bałkanów zebrano w sierpniu 2014 i 2016 roku w Arboretum SGGW w Rogowie oraz w miejscowości Risika na wyspie Krk (Chorwacja). Tożsamość gatunkowa została potwierdzona przez mgr inż. Piotra Banaszczaka, kierownika Arboretum w Rogowie (dla surowca krajowego) oraz dr hab. n. farm. Monikę A. Olszewską, prof. UM (dla surowca zagranicznego). Optymalny czas zbioru surowca został wytypowany na podstawie wstępnych badań zmienności sezonowej (analiza całkowitej zawartości polifenoli) dla surowca zbieranego w miesięcznych odstępach w Arboretum SGGW, według których okres późnego lata i jesieni sprzyja akumulacji związków polifenolowych (dane nieopublikowane). Przedmiotem analiz były ekstrakty suche otrzymane metodą ekstrakcji frakcjonowanej (Rycina 2) z ww. materiału roślinnego, po jego uprzednim wysuszeniu i zmieleniu (**Publikacje I-III**). Dodatkowo, w badaniu zmienności geograficznej zastosowano metanolowo-wodne ekstrakty płynne (**Publikacja IV**).



Rycina 2. Schemat ekstrakcji liści *Sorbus domestica* L.

2. Koncepcja i plan badań

Schemat przeprowadzonych badań przedstawiono na Rycinie 3.



Rycina 3. Schemat przeprowadzonych badań.

3. Metodyka badań

3.1. Analiza profilu polifenolowego

Standaryzację jakościową ekstraktów z liści *Sorbus domestica* L. przeprowadzono metodą UHPLC-PDA-ESI-MS^3 z wykorzystaniem chromatografu sprzężonego ze spektrometrem mas typu pułapki jonowej. Anality identyfikowano strukturalnie z wykorzystaniem bazy literaturowej oraz kolekcji wzorców Katedry Farmakognozji (**Publikacja I, II i IV**).

Izolację wytypowanych analitów przeprowadzono z wykorzystaniem grawitacyjnej chromatografii kolumnowej (Sephadex LH-20) i preparatywnej chromatografii HPLC, zaś struktury związków oznaczono głównie metodami spektroskopowymi (1D i 2D NMR: COSY, HMBC, HMQC) (**Publikacja II**).

Standaryzacja ilościowa obejmowała oznaczenia zawartości polifenoli i proantocyjanidyn fotometrycznymi metodami Folina-Ciocalteu'a i Portera (wg Olszewska i wsp., 2012), a także profilu indywidualnych polifenoli metodą HPLC-PDA-fingerprint po jej optymalizacji i walidacji wg ICH Q2R1 (2005) i Olszewska (2012) (**Publikacja I, II i IV**).

Szczegółowy opis zastosowanych metod, aparatury, odczynników, przygotowania próbek i warunków analizy opisano we właściwych publikacjach.

3.2. Analiza *in vitro* aktywności biologicznej

Oznaczenia aktywności antyoksydacyjnej ekstraktów/frakcji przeprowadzono *in vitro* w testach chemicznych opartych o mechanizmy typu SET (*single electron transfer*), tj. metodami DPPH (analiza zdolności do zmiatania rodnika DPPH) i FRAP (analiza zdolności do redukcji jonów Fe^{3+}) oraz HAT (*hydrogen electron transfer*), tj. metodą inhibicji oksydacji kwasu linolenowego (TBARS), wg wcześniej zoptymalizowanych procedur (Olszewska i wsp., 2012) (**Publikacja I i IV**).

Efekt ochronny ekstraktów/frakcji oraz związków modelowych wobec składników ludzkiego osocza w warunkach stresu oksydacyjnego (indukowanego $ONOO^-$) analizowano spektrofotometrycznie/immunoenzymatycznie, przez oznaczenie markerów nitrowania białek (3-nitrotyrozyny, 3-NT) wg Olas i wsp. (2006), peroksydacji lipidów (wodoronadtlenków lipidów, LOOH, i substancji reagujących z kwasem tiobarbiturowym, TBARS) wg Gay i Gebicki (2000) oraz Kołodziejczyk-Czepas i wsp. (2011, 2016), a także ocenę nieenzymatycznej pojemności antyoksydacyjnej osocza (NEAC) metodą FRAP wg Kołodziejczyk-Czepas i wsp. (2014) (**Publikacja I i II**). Oznaczenie 3-NT w białkach osocza przeprowadzono za pomocą immunoenzymatycznej metody ELISA. Stężenie LOOH w próbkach osocza oznaczono spektrofotometrycznie przy użyciu odczynnika żelazowo-ksylenolowego (FOX-1). Oznaczenia TBARS oraz NEAC w próbkach osocza przeprowadzono za pomocą rutynowych metod fotometrycznych.

Ponadto, oznaczono aktywność antyoksydacyjną ekstraktów/frakcji oraz ich markerów aktywnych wobec oksydantów operujących *in vivo*, tj. $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, HClO, $HO^{\bullet}$, $ONOO^-$ z wykorzystaniem spektro/fluorymetrycznych metod *in vitro* po ich wcześniejszej optymalizacji (**Publikacja III**). Zdolność do zmiatania $O_2^{\bullet-}$ analizowano poprzez monitorowanie poziomu zredukowanych form błękitu nitrotetrazoliowego (NBT) powstałych w reakcji NBT z $O_2^{\bullet-}$, wytworzonym w reakcji przekształcenia ksantyny w kwas moczowy pod wpływem oksydazy ksantynowej (wg. Granica i wsp. 2013). Zdolność do redukcji H_2O_2 oceniano poprzez monitorowanie poziomu chinoinoiminy wytwarzanej w reakcji 4-aminoantipiryny, fenolu i H_2O_2, katalizowanej przez

peroksydazę chrzanową (wg. Fernando i Soysa, 2015). Zdolność do zmiatania $NO^{\bullet}$ mierzono poprzez monitorowanie poziomu triazofluoresceiny wytwarzanej w reakcji 4,5-diamino-fluoresceiny z $NO^{\bullet}$ (wg. Czerwińska i wsp., 2012). Aktywność wobec HClO analizowano poprzez monitorowanie poziomu kwasu 5,5'-ditiobis(2-nitrobenzoesowego) wytwarzanego w reakcji kwasu 5-tio-2-nitrobenzoesowego z HClO (wg. Czerwińska i wsp., 2012). Zdolność do zmiatania $HO^{\bullet}$ badano przez pomiar poziomu dihydroksypochodnych kwasu benzoesowego, powstałych w reakcji kwasu salicylowego z $HO^{\bullet}$, wytworzonym w reakcji Fentona (wg. Fu i wsp., 2014). Zdolność do zmiatania $ONOO^{-}$ (otrzymanego syntetycznie) została oznaczona poprzez pomiar stężenia barwnika Evans Blue, który w reakcji z $ONOO^{-}$ ulega utlenieniu i odbarwieniu (wg. Krzyzanowska-Kowalczyk i wsp., 2017).

Ocena bezpieczeństwa komórkowego ekstraktów/frakcji objęła badania przeżywalności jednojądrzastych komórek krwi obwodowej (PBMCs) metodą fluorymetryczną (wg. Krzyzanowska-Kowalczyk i wsp., 2017) (**Publikacja I**). Zmianę integralności błony komórkowej PBMCs oceniano poprzez wybarwienie martwych komórek (kwasów nukleinowych) jodkiem propidyny.

Potencjalne działanie przeciwzapalne analizowano w testach inhibicji enzymów prozapalnych: lipooksygenazy i hialuronidazy (wg. Michel i wsp., 2017) (**Publikacja I i II**). Zdolność do hamowania hialuronidazy oceniano za pomocą metody turbidymetrycznej opartej na spektrofotometrycznym pomiarze zmętnienia powstałego na skutek wytworzenia kompleksów hialuronan-albumina bydlęca w środowisku kwasowym. Zdolność do hamowania lipooksygenazy analizowano w oparciu o spektrofotometryczny pomiar poziomu dienów sprzężonych wytworzonych jako produkty uboczne w procesie utleniania kwasu linolowego katalizowanym przez enzym.

Wszystkie ww. analizy zostały przeprowadzone w stosunku do kontroli pozytywnej (odpowiednich dla danego testu związków o udowodnionym działaniu). Badania w modelach biologicznych wykonane zostały na materiale odzyskiwanym (osocze, PBMCs) z kożuszków leukocytarno-płytkowych dostępnych komercyjnie w Regionalnym Centrum Krwiodawstwa i Krwiolecznictwa (RCKiK) w Łodzi (niewymagana zgodna Komisji Bioetycznej). Badania w modelach biologicznych prowadzono w min. 3 stężeniach, obejmujących zarówno zakresy fizjologiczne (1-5 µg/mL) dla polifenoli, jak i wyższe (50 µg/mL), w celu uchwycenia wszystkich możliwych efektów działania. Wyniki testów chemicznych oraz hamowania aktywności enzymów wyrażono jako wartości $EC_{50}/SC_{50}/IC_{50}$ oraz w ekwiwalentach odpowiednich wzorców pozytywnych. Szczegółowy opis zastosowanych metod, aparatury, odczynników, przygotowania próbek i warunków analizy zamieszczono we właściwych publikacjach.

3.3. Ocena statystyczna wyników

Do oceny uzyskanych wyników przedstawionych we wszystkich publikacjach użyto programu Statistica 12/13 Pl (StatSoft). Wyniki weryfikowano pod kątem istotności statystycznej różnic i korelacji na poziomie istotności min. $\alpha = 0.05$. Wszystkie szczegóły dotyczące zastosowanych testów podano w załączonych publikacjach.

Realizacja celów naukowych – wyniki i dyskusja

1. Analiza profilu polifenolowego suchych ekstraktów uzyskanych z liści *S. domestica* w funkcji ekstrakcji frakcjonowanej

Ekstrakty analizowane w ramach niniejszej pracy zostały przygotowane z uwzględnieniem wyników wcześniejszych badań rodzaju *Sorbus* (Olszewska i wsp., 2012), podczas których ustalone zostało, iż ekstrakcja mieszaniną metanol-woda (7:3, *v*/*v*), a następnie oczyszczenie i rozdział składników analizowanych surowców metodą ekstrakcji frakcjonowanej, są optymalne dla koncentracji związków aktywnych liści przedstawicieli ww. rodzaju. Analiza ekstraktu metanolowo-wodnego (MED) z liści *Sorbus domestica* L. oraz jego frakcji, stanowiąca element prezentowanej rozprawy, wykazała znaczącą wartość badanego materiału jako źródła związków polifenolowych – całkowita zawartość polifenoli, mierzona zarówno spektrofotometryczną metodą Folina-Ciocalteu'a (TPC) jak i metodą wysokosprawnej chromatografii cieczowej (TPH), oznaczona w MED (TPC, 283 mg GAE/g s.m.; TPH, 73 mg/g s.m.) oraz frakcjach octanu etylu, eteru dietylowego i *n*-butanolu (TPC, 464-700 mg GAE/g s.m.; TPH, 113-558 mg/g s.m.) była porównywalna lub wyższa w stosunku do wartości dostępnych literaturowo dla ekstraktów cenionych w prewencji chorób związanych ze stresem oksydacyjnym i stanem zapalnym, jak np. z nasion winorośli (TPC, 630-790 mg GAE/g s.m.) (Baydara i wsp., 2004; Peschel i wsp., 2007), liści winorośli (TPC, 60-260 mg GAE/g s.m.) (Lacerda i wsp., 2016), zielonej herbaty (TPC, 447 mg GAE/g s.m.) (Peschel i wsp., 2007), czy liści czarnej porzeczki (TPC, 249 mg GAE/g s.m; TPH, 132 mg/g s.m.) (Bonarska-Kujawa i wsp., 2014; Luzak i wsp., 2014). Ponadto, uzyskane wartości wskazują na wyższą zawartość polifenoli w badanych ekstraktach, w porównaniu z analogicznymi ekstraktami z liści i kwiatów innych gatunków z rodzaju *Sorbus* (Olszewska i wsp., 2012), jak również ekstraktami z owoców *S. domestica* (Termentzi i wsp., 2006) (**Publikacja I**).

Szczegółowa analiza jakościowa i ilościowa ekstraktów metodami chromatograficznymi (UHPLC-PDA-ESI-MS^3; HPLC-PDA-fingerprint) pozwoliła na wykrycie 44 małocząsteczkowych związków polifenolowych, w tym pochodnych flawan-3-olu (16 związków z grupy katechin i proantocyjanidyn o sumarycznej zawartości do 228 mg/g s.m. we frakcji EAF), flawonoidów (13 związków z grupy flawonoli o sumarycznej zawartości do 195 mg/g s.m. we frakcji DEF) oraz kwasów fenolowych (12 związków o sumarycznej zawartości do 93 mg/g s.m. we frakcji DEF). Ponadto, w ekstraktach polarnych (MED, BF, WR) pośrednio wykazano wysoką zawartość proantocyjanidyn o wysokim stopniu polimeryzacji (zbudowanych z więcej niż czterech monomerów flawan-3-olu), niemożliwych do wykrycia za pomocą metody RP-HPLC, natomiast oznaczonych jako suma oligo- i polimerycznych pochodnych flawan-3-olu przy zastosowaniu spektrofotometrycznej metody Portera (TPA do 183 mg CYE/g s.m. we frakcji BF). Wysoka zawartość pochodnych flawanolowych, zarówno monomerycznych katechin jak i oligo- i polimerycznych proantocyjanidyn, obecnych w ekstraktach z liści *S. domestica,* zaś niewykrytych w dojrzałych owocach, stanowi istotną przewagę liści jako potencjalnego surowca leczniczego, z uwagi na dobrze udokumentowaną aktywność przeciwutleniającą i przeciwzapalną *in vivo* tego rodzaju połączeń (Manach i wsp., 2005; Middleton i wsp., 2000). Standaryzacji jakościowej i ilościowej badanych ekstraktów została poświęcona **Publikacja I**.

Biorąc pod uwagę wytyczne Europejskiej Agencji Leków (EMA, 2018), kolejnym etapem niniejszej rozprawy stała się identyfikacja markerów analitycznych, w tym markerów aktywnych, odpowiednich dla kontroli jakości ekstraktów z liści *S. domestica* (**Publikacja II**). W tym celu konieczna okazała się

izolacja oraz określenie struktury dwóch glikozydów flawonoidowych, charakterystycznych dla badanego surowca oraz do tej pory niewykrytych zarówno w rodzaju *Sorbus,* jak i rodzinie Rosaceae, tj. 3-*O*-(2''-*O*-β-D-glukopiranozylo)-α-L-ramnopiranozydu kwercetyny (HRQ) oraz 3-*O*-(2''-*O*-β-D-ksylopiranozylo)-α-L-ramnopiranozydu kwercetyny (PRQ). Ostatecznie, przy użyciu metod chromatograficznych, spektroskopowych i testów aktywności biologicznej, wytypowano dziewięć markerów mogących służyć do oceny jakości badanych ekstraktów, tj. (-)-epikatechinę (ECA), procyjanidynę B2 (PB2), procyjanidynę C1 (PC1), kwercetynę (QU), kwercytrynę (QCT), rutynę (RT), wyizolowane diglikozydy kwercetyny (HRQ i PRQ) oraz kwas chlorogenowy (CHA). Związki te zostały zastosowane jako standardy kalibracyjne w procesie walidacji metody RP-HPLC-PDA analizy ekstraktów z liści *S. domestica* (**Publikacja II**). W efekcie opracowana precyzyjna, czuła, dokładna i powtarzalna metoda, umożliwiająca stosunkowo szybką (30 min) analizę jakościową i ilościową zespołu polifenoli *S. domestica*, została zarekomendowana jako efektywne narzędzie kontroli jakości badanych ekstraktów.

2. Analiza *in vitro* aktywności biologicznej suchych ekstraktów z liści *S. domestica* oraz związków modelowych

Biorąc pod uwagę profil związków polifenolowych obecnych w badanych ekstraktach, dane literaturowe dotyczące aktywności polifenoli roślinnych (Manach i wsp., 2005; Middleton i wsp., 2000; Williamson i Manach, 2005) oraz surowców z rodzaju *Sorbus* (Olszewska i wsp., 2012; Termentzi i wsp., 2006), a także wzmianki o tradycyjnym zastosowaniu liści *S. domestica* w schorzeniach o podłożu antyoksydacyjnym i przeciwzapalnym (Kültür, 2007), celem niniejszej rozprawy stała się analiza *in vitro* ww. typów aktywności w modelach chemicznych i biologicznych.

W pierwszym etapie badań ekstrakty analizowano przy pomocy popularnych testów chemicznych, tj. metodami DPPH, FRAP i TBARS, umożliwiających szybką i prostą ocenę aktywności antyoksydacyjnej oraz porównanie uzyskanych danych ze źródłami literaturowymi. Uzyskane wyniki, wskazujące na wysoki potencjał antyoksydacyjny badanych ekstraktów oraz korelujące z zawartością polifenoli (szczególnie proantocyjanidyn i flawonoidów), stanowiły podstawę do poszerzenia badań o testy w modelu biologicznym (**Publikacja I**).

Wykazano, że badane ekstrakty/frakcje skutecznie zwiększały nieenzymatyczną pojemność antyoksydacyjną ludzkiego osocza oraz wykazywały efekt ochronny wobec składników osocza, tj. zmniejszały stężenie biomarkerów stresu oksydacyjnego (produktów nitrowania białek oraz peroksydacji lipidów) w warunkach stresu oksydacyjnego indukowanego $ONOO^-$ (**Publikacja I**). Podobną aktywność wykazywały związki modelowe dla badanych ekstraktów, zaś wśród nich epikatechina (ECA) i procyjanidyny (PB2, PC1) okazały się najefektywniejsze (**Publikacja II**). Znajduje to potwierdzenie w doniesieniach literaturowych wskazujących na flawanole jako jedne z najsilniejszych antyoksydantów obecnych w produktach spożywczych, wpływających korzystnie na funkcjonowanie m.in. układu sercowo-naczyniowego, w tym zmniejszających kruchość i przepuszczalność naczyń włosowatych, wpływających korzystnie na stosunek HDL/LDL, a także obniżających agregację płytek i ciśnienie krwi (Williamson i Manach, 2005). Przypuszcza się, że jednym z mechanizmów osoczowej aktywności polifenoli jest bezpośrednia reakcja z $ONOO^-$, wysoce reaktywnym i szkodliwym czynnikiem, modyfikującym funkcję biocząsteczek (białek, lipidów, kwasów nukleinowych), a przez to zaangażowanym w patofizjologię szeregu tzw. chorób cywilizacyjnych, w tym schorzeń kardiologicznych i neurodegeneracyjnych (Szabo i wsp., 2007). Ponadto, nie mniej

ważna jest zdolność polifenoli do zmiatania rodników pokrewnych, zaangażowanych w tworzenie $ONOO^-$ lub powstających na skutek reakcji $ONOO^-$ z innymi cząsteczkami (Heijnen i wsp., 2001).

W związku z tym, w celu dokładniejszego określenia aktywności badanych ekstraktów oraz prawdopodobnych mechanizmów stojących za ich ochronnym działaniem w stosunku do białkowych i lipidowych składników ludzkiego osocza, elementem niniejszej rozprawy stała się analiza aktywności antyoksydacyjnej ekstraktów/frakcji oraz ich markerów aktywnych wobec oksydantów operujących *in vivo*, tj. $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, HClO, $HO^{\bullet}$, $ONOO^-$ (**Publikacja III**). Podobnie jak w przypadku wcześniejszych testów, frakcje EAF, DEF, a następnie BF, okazały się najaktywniejsze w stosunku do wszystkich badanych oksydantów. Ekstrakty okazały się być najefektywniejsze w reakcji z $HO^{\bullet}$ (do 3-4 razy wyższa aktywność w porównaniu do kwasu askorbinowego, AA i Troloksu, TX), choć aktywność w stosunku do innych reaktywnych form tlenu/azotu (ROS/RNS) była również znacząca. Świadczy to o szerokim spektrum działania badanych ekstraktów i częściowo wyjaśnia ich efektywność w układach biologicznych. Podobnie analizowane związki modelowe charakteryzowały się wysoką sumaryczną zdolnością do zmiatania ROS/RNS, mieszczącą się w przedziale 15.49–47.69 mol AA/mol oraz 34.91–135.58 mol TX/mol, w porównaniu do substancji wzorcowych (6 mol AA(TX)/mol), choć w zależności od budowy strukturalnej, dla różnych grup związków przeważała aktywność w stosunku do innych oksydantów.

Kolejną kwestią wartą poruszenia jest konkurencyjna, a nawet niekiedy wyższa aktywność ekstraktów/frakcji w porównaniu do czystych markerów ich działania biologicznego, zarówno w przypadku testów na osoczu, jak i aktywności wobec konkretnych ROS/RNS. Przyczyną tego zjawiska może być z jednej strony obecność niepolifenolowych składników aktywnych (co z uwagi na przeprowadzone wcześniej analizy i proces ekstrakcji frakcjonowanej jest w przypadku badanych ekstraktów mniej prawdopodobne), zaś z drugiej możliwość występowania efektów synergistycznych pomiędzy poszczególnymi składnikami ekstraktów. W przypadku aktywności antyoksydacyjnej działanie synergiczne obserwowane jest szczególnie często w przypadku cząsteczek posiadających w swojej budowie układ katecholowy (Hajimehdipoor i wsp., 2014; Sak, 2017). Biorąc pod uwagę fakt, iż ekstrakty z liści *S. domestica* są szczególnie bogate w polifenole o ww. strukturze, kolejnym etapem niniejszej pracy stała się analiza synergii pomiędzy cząsteczkami reprezentatywnymi dla trzech głównych grup związków polifenolowych warunkujących aktywność badanych ekstraktów, tj., ECA, QCT i CHA (**Publikacja III**). W rzeczy samej, w teście zmiatania $O_2^{\bullet-}$, zaobserwowano istotną synergię między badanymi związkami przy różnych kombinacjach stężeń, z czego najsilniejszy efekt widoczny był dla mieszaniny CHA-QCT (niezależnie od stężenia) oraz CHA-ECA (w stężeniu 1:1 µg/ml). W świetle otrzymanych wyników, ekstrakt MED oraz frakcja BF charakteryzują się optymalnymi proporcjami kwasów chlorogenowych, flawonoli i flawanoli, co może tłumaczyć ich wysoką aktywność, mimo niższej w porównaniu do innych frakcji (DEF, EAF) zawartości polifenoli. Działanie synergistyczne między poszczególnymi składnikami ekstraktów stanowi o wyższości omawianych produktów naturalnych nad pojedynczymi związkami, umożliwiając osiągnięcie efektów biologicznych przy niższych stężeniach, co m.in. może minimalizować ewentualne działania niepożądane wynikające ze stosowania wysokich dawek związków chemicznych.

Uzyskane wyniki świadczą o wysokiej wartości liści *S. domestica* jako źródła biologicznie czynnych ekstraktów mogących potencjalnie znaleźć zastosowanie w prewencji czy terapii wspomagającej chorób o podłożu oksydacyjnym. Jednakże *in vivo* stres oksydacyjny jest ściśle związany ze stanem zapalnym – nasilenie jednego procesu pociąga za sobą rozwój drugiego, który jednocześnie pogłębia

ten pierwszy, powodując przyśpieszony rozwój stanów patologicznych (Biswas, 2016). W związku z tym, coraz częściej mówi się, iż antyoksydanty wpływające jednocześnie na procesy zapalne, na drodze mechanizmów innych niż bezpośrednie zmiatanie ROS/RNS, są znacznie skuteczniejsze w terapii chorób o podłożu oksydacyjno-zapalnym. Biorąc pod uwagę powyższe przesłanki, w celu oceny potencjału przeciwzapalnego liści *S. domestica,* badane ekstrakty/frakcje (**Publikacja I**) oraz związki modelowe (**Publikacja II**) poddano analizie *in vitro* zdolności do inhibicji dwóch enzymów zaangażowanych w procesy zapalne *in vivo*, tj. lipooksygenazy (LOX), biorącej udział m.in. w produkcji chemokin i ROS, oraz hialuronidazy (HYAL), zwiększającej przepuszczalność tkanek dla czynników prozapalnych poprzez degradację hialuronianu w macierzy zewnątrzkomórkowej (Girish i wsp., 2009; Mashima i Okuyama, 2015). Efektywność najaktywniejszych frakcji była porównywalna z wynikami uzyskanymi dla substancji wzorcowych, w tym indometacyny (niesteroidowego leku przeciwzapalnego). Frakcja EAF okazała się najsilniejszym inhibitorem LOX, co korelowało z całkowitą zawartością polifenoli, a także sumą proantocyjanidyn i flawonoidów, natomiast frakcja BF wykazywała najwyższą aktywność w stosunku do HYAL, korelującą z całkowitą zawartością proantocyjanidyn oraz sumą proantocyjanidyn i kwasów chlorogenowych. Podobnie jak w przypadku aktywności antyoksydacyjnej, dzięki synergicznemu działaniu polifenolowych składników aktywnych, zdolność do inhibicji enzymów prozapalnych wyjściowego ekstraktu metanolowego była jedynie nieco niższa niż w przypadku frakcji o skoncentrowanej zawartości polifenoli.

Analiza *in vitro* aktywności biologicznej suchych ekstraktów z liści *S. domestica* oraz związków modelowych, przeprowadzona w ramach niniejszej pracy, częściowo potwierdza zasadność tradycyjnych zastosowań omawianego surowca, wskazując na korzystny wpływ badanych ekstraktów zarówno na zahamowanie rozwoju stresu oksydacyjnego, jak i prawdopodobnie procesów zapalnych. Jednak dla przyszłego wykorzystania w lecznictwie, poza pogłębionym badaniem aktywności biologicznej, w tym poszerzoną analizą wpływu na procesy zapalne, równie ważna jest ocena bezpieczeństwa stosowania otrzymanych produktów. Brak wpływu na przeżywalność jednojądrzastych komórek krwi obwodowej (PBMCs) ekstraktów w zakresie stężeń 1-50 µg/ml, zaobserwowany w ramach niniejszej pracy, pozwala wstępnie zaklasyfikować badane ekstrakty jako bezpieczne (**Publikacja I**). Obiecujące wyniki analiz *in vitro*, stanowić mogą zatem podstawę do rozszerzenia badań w bardziej złożonych układach biologicznych, w tym modelach komórkowych, oraz testów *in vivo*.

3. Porównawcza analiza fitochemiczna i aktywności antyoksydacyjnej liści *S. domestica* z uprawy krajowej i z terenu Bałkanów

Polifenole, jako wyspecjalizowane metabolity roślinne, poza właściwościami prozdrowotnymi dla organizmów ludzkich, w pierwszej kolejności stanowią podstawę mechanizmów adaptacyjnych roślin macierzystych, będąc odpowiedzialnymi za obronę przed czynnikami stresowymi, jak niska lub wysoka temperatura, promieniowanie słoneczne, susza lub nadmierna wilgotność (Ncube i wsp., 2012). Wpływ warunków klimatycznych na biosyntezę i akumulację polifenoli, a tym samym jakość produktów roślinnych, znajduje liczne potwierdzenia w literaturze (Aninbon i wsp., 2016; Chen i wsp., 2018; Zhang, Wu i wsp., 2017), szczególnie w przypadku roślin uprawianych poza miejscem ich naturalnego występowania. Mając na uwadze fakt, iż surowce z różnych rejonów geograficznych mogą różnić się właściwościami, kolejnym etapem niniejszej pracy stała się analiza porównawcza liści *S. domestica* z uprawy krajowej i z terenu Chorwacji (**Publikacja IV**). Ocenie poddano zarówno profil jakościowy i ilościowy (UHPLC-PDA-ESI-MS^3, HPLC-PDA-fingerprint, metody spektrofotometryczne),

jak i aktywność antyoksydacyjną (testy DPPH i FRAP) liści zebranych w trakcie dwóch sezonów wegetacyjnych.

Wyniki uzyskane dla próbek z Polski (lata 2014 i 2016) były porównywalne z wcześniejszymi oznaczeniami (próbka z 2015 roku), co świadczy o stałości składu polifenolowego pomiędzy latami. Podobnie w przypadku próbek z Chorwacji, zmiany w składzie w zależności od roku zbioru były znacznie mniejsze, niż różnice pomiędzy krajami. Podstawową cechą różnicującą badane próbki okazała się proporcja pomiędzy flawonoidami i proantocyjanidynami – mimo iż te drugie były dominującą grupą związków we wszystkich próbkach, w liściach z Polski stanowiły 59-64% całkowitej zawartości polifenoli, zaś w liściach z Chorwacji 45-52%, z odpowiednio wyższym udziałem flawonoidów, w szczególności monoglikozydów (głównie hiperozydu). Wyniki te są zgodne z danymi literaturowymi, sugerującymi wzrost biosyntezy flawonoidów w tkankach roślinnych w odpowiedzi na promieniowanie UV-B (Agati i Tattini, 2010; Harborne i Williams, 2000; Tattini i wsp., 2004; Zhang, Chen i wsp., 2017; Zhang, Wu i wsp., 2017), oraz wskazującymi na stężenie hiperozydu jako jeden z czynników najbardziej podatnych na wpływ promieniowania słonecznego (Chen i wsp., 2018; Zhang, Chen i wsp., 2017; Zhang, Wu i wsp., 2017). Jednak pomimo wyższej całkowitej zawartości polifenoli i w konsekwencji aktywności antyoksydacyjnej dla surowca z Chorwacji w przeliczeniu na suchą masę liści, ekstrakty otrzymane zarówno z próbek krajowych jak i zagranicznych okazały się porównywalne pod względem potencjalnego działania biologicznego, przy niższej, co zrozumiałe, wydajności ekstrakcji dla liści z Polski. W związku z tym, według wstępnych badań surowiec z obu stanowisk wydaje się być cennym materiałem do produkcji aktywnych ekstraktów.

Podsumowanie osiągniętych wyników

W ramach niniejszej pracy po raz pierwszy przeprowadzono **analizę profilu polifenolowego i aktywności biologicznej liści *Sorbus domestica* L.**, na podstawie której:

- wykazano zależność pomiędzy składem otrzymanych ekstraktów, a rodzajem użytego ekstrahenta oraz wytypowano ekstrakty najbardziej zasobne w polifenole (**Publikacja I**);
- w ekstraktach udokumentowano występowanie ponad 40 związków polifenolowych, a także wskazano poziomy zawartości dominujących grup polifenoli (proantocyjanidyn, flawonoidów, kwasów fenolowych) oraz pojedynczych substancji (**Publikacja I**);
- po raz pierwszy wyizolowano z rodzaju *Sorbus* dwa diglikozydy flawonoidowe, tj. 3-*O*-(2''-*O*-β-D-glukopiranozylo)-α-L-ramnopiranozyd kwercetyny (HRQ) oraz 3-*O*-(2''-*O*-β-D-ksylopiranolzylo)-α-L-ramnopiranozyd kwercetyny (PRQ) (**Publikacja II**);
- wytypowano dziewięć markerów aktywności biologicznej liści *S. domestica,* tj. ECA, PB2, PC1, CHA, QU, QCT, RT, HRQ, PRQ (**Publikacja II**);
- opracowano i poddano walidacji metodę RP-HPLC-PDA analizy polifenoli, która może służyć jako narzędzie do kontroli jakości badanych liści i pochodnych ekstraktów, z zastosowaniem ww. związków jako standardów kalibracyjnych (**Publikacja II**);
- wykazano ochronne działanie badanych ekstraktów oraz markerów ich aktywności biologicznej wobec składników osocza (białek i lipidow) w warunkach stresu oksydacyjnego indukowanego $ONOO^-$, a także zdolność do zwiększania nieenzymatycznej pojemności antyoksydacyjnej ludzkiego osocza (**Publikacja I i II**);
- wykazano szerokie spektrum działania *in vitro* ekstraktów oraz ich markerów aktywnych wobec oksydantów operujących *in vivo*, tj. $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, HClO, $HO^{\bullet}$, $ONOO^-$, wskazując na zmiatanie ROS/RNS jako jeden z możliwych mechanizmów aktywności obserwowanej w zastosowanym modelu biologicznym (**Publikacja III**);
- potwierdzono występowanie efektów synergicznych pomiędzy cząsteczkami reprezentatywnymi dla każdej z trzech głównych grup związków warunkujących aktywność badanych ekstraktów, tj., ECA, QCT i CHA, co może tłumaczyć relatywnie wyższą aktywność ekstraktów w porównaniu do czystych związków (**Publikacja III**).
- wykazano potencjał przeciwzapalny badanych ekstraktów oraz markerów ich aktywności biologicznej w testach hamowania enzymów: lipooksygenazy i hialuronidazy (**Publikacja I i II**).
- wykazano brak wpływu ekstraktów na przeżywalność jednojądrzastych komórek krwi obwodowej w badanym zakresie stężeń, co pozwoliło wstępnie zaklasyfikować testowane ekstrakty jako potencjalnie bezpieczne (**Publikacja I**).
- wykazano, iż pomimo różnic w składzie liści pochodzących z uprawy krajowej i terenu Bałkanów, spowodowanych odmiennymi warunkami klimatycznymi, surowiec z obu źródeł może być cennym materiałem do produkcji biologicznie aktywnych ekstraktów (**Publikacja IV**).

Wnioski końcowe

Wyniki uzyskane w ramach niniejszej pracy wskazują na zróżnicowane strukturalnie polifenole jako składniki czynne liści *S. domestica*, istotną rolę mechanizmów antyoksydacyjnych i przeciwzapalnych dla ich efektu biologicznego, a także wyższość liści ww. gatunku nad jego owocami, zarówno pod względem składu, jak i aktywności. Wielokierunkowość działania, obejmująca zmiatanie różnorodnych ROS/RNS operujących *in vivo*, efekt ochronny wobec składników ludzkiego osocza w warunkach stresu oksydacyjnego oraz wpływ na aktywność enzymów prozapalnych, świadczy o wysokiej wartości ekstraktów z liści *S. domestica* jako potencjalnego składnika preparatów stosowanych w prewencji czy terapii wspomagającej chorób o podłożu oksydacyjno-zapalnym. Ponadto, nie bez znaczenia pozostaje działanie synergiczne związków polifenolowych obecnych w badanym materiale, które wskazuje na wyższość wieloskładnikowych ekstraktów z liści *Sorbus* nad pojedynczymi, izolowanymi substancjami. Liście jarzębu domowego, stosowane dotychczas jedynie w medycynie tradycyjnej, wydają się być zatem obiecującym materiałem do dalszych badań fitochemicznych, farmakologicznych i toksykologicznych, umożliwiających ich pełną charakterystykę i ewentualne wprowadzenie do oficjalnego lecznictwa.

Perspektywy i plany na przyszłość

Uwzględniając wyniki zaprezentowane w niniejszej rozprawie, liście *S. domestica* zasługują na większe zainteresowanie i szersze lecznicze zastosowanie. Jednak do pełnej charakterystyki badanego surowca, niezbędne są kolejne badania, które już rozpoczęłam lub planuję podjąć w najbliższej przyszłości, umożliwiające dokładniejszą ocenę fitochemiczną, farmakologiczną i toksykologiczną. Biorąc pod uwagę złożoność metabolizmu polifenoli w organizmach żywych, dalsze badania uwzględniać będą, poza ekstraktami pierwotnymi, analogiczne próbki poddane procesowi symulowanego trawienia *in vitro*. W ramach przyszłych analiz, zbadane zostaną inne potencjalne mechanizmy aktywności antyoksydacyjnej ekstraktów, w tym wpływ na ekspresję i aktywność endogennych enzymów przeciwutleniających, a także komórkową sekrecję ROS/RNS. Z kolei ocena działania przeciwzapalnego, wymaga poszerzenia o bezkomórkowe testy aktywności wobec innych enzymów prozapalnych, jak np. fosfolipazy A2 i cyklooksygenazy-2, jak również o badania w modelach komórkowych, w tym testy uwalniania mediatorów stanu zapalnego (cytokin i enzymów) przez komórki układu odpornościowego (neutrofile, monocyty i limfocyty). Dodatkowo, biorąc pod uwagę złożoność układów biologicznych i wzajemne powiązania między stresem oksydacyjnym, stanem zapalnym i rozwojem szeregu stanów patologicznych, inne potencjalne kierunki działania, jak np. aktywność przeciwcukrzycowa, sugerowana przez tradycyjne zastosowania, czy wpływ na procesy hemostazy osoczowej i płytkowej, mogą okazać się wartymi uwagi. Niemniej istotne są również szersze badania toksykologiczne, uwzględniające różne linie komórkowe, a także testy *in vivo*. Dodatkowo, mając na uwadze zaobserwowaną zmienność geograficzną składu liści, podobna analiza z wykorzystaniem większej ilości próbek jest niezbędna w celu pełnego zrozumienia zróżnicowania omawianego surowca.

Streszczenie

Wysokie stężenie reaktywnych form tlenu i azotu (ROS/RNS) generowane w konsekwencji zaburzonej równowagi redoks organizmu, prowadzące do rozwoju stresu oksydacyjnego oraz przewlekły stan zapalny, uznaje się za istotne przyczyny rozwoju schorzeń kardiologicznych, insulino-oporności, oraz innych przewlekłych chorób niezakaźnych (NCDs). Pośród proponowanych strategii ograniczenia postępu NCDs znajduje się promocja zdrowego stylu życia, w tym właściwej diety. Dobre rezultaty uzyskuje się przez zwiększenie spożycia produktów roślinnych bogatych w zróżnicowane strukturalnie polifenole – związki o wielokierunkowym działaniu na organizm człowieka, wynikającym w przeważającej mierze z ich regulującego wpływu na procesy oksydoredukcyjne i zapalne zachodzące w komórkach i tkankach. Dowody wskazujące na skuteczność polifenolowych antyoksydantów w profilaktyce i terapii NCDs stały się przyczyną wzrostu zainteresowania problematyką produktów roślinnych, źródłami ich pozyskiwania, oraz zasadami ewentualnej suplementacji. Jednym z wiodących kierunków badawczych stała się analiza surowców roślinnych stosowanych w medycynie tradycyjnej, które po odpowiedniej weryfikacji fitochemicznej, farmakologicznej i toksykologicznej, miałyby szanse znaleźć zastosowanie w produkcji preparatów leczniczych, suplementów diety, czy żywności funkcjonalnej. Pragnąc wpisać się w ten kontekst badawczy, zwrócono uwagę na gatunek pochodzący z rejonów Morza Śródziemnego, tj. jarząb domowy (*Sorbus domestica*). Mimo udokumentowanego zastosowania w lecznictwie tradycyjnym liści *S. domestica*, brak badań wspomnianego surowca uniemożliwia jego szersze wykorzystanie medyczne. Dotychczasowe badania jarzębu domowego skupiały się na owocach, które choć uznawane za bogate źródło bioaktywnych polifenoli są surowcem o dostępności jedynie regionalnej, z uwagi na osłabione kwitnienie i owocowanie omawianego gatunku w warunkach klimatu umiarkowanego. Ponadto, ostatnie doniesienia wskazują na wyższą aktywność frakcji fenolowych ekstrahowanych z liści roślin owocowych niż z odpowiednich owoców, co znajduje odzwierciedlenie w licznych badaniach i wprowadzaniu na rynek coraz większej liczby preparatów na bazie liści (np. winorośli, czy czarnej porzeczki).

Biorąc pod uwagę powyższe przesłanki, celem naukowym rozprawy doktorskiej stała się ocena wartości liści *Sorbus domestica* pochodzących z uprawy krajowej jako źródła naturalnych, biologicznie aktywnych polifenoli. Badania prowadzono na dwóch współzależnych płaszczyznach, obejmujących analizy fitochemiczne i badania aktywności biologicznej. W ramach części fitochemicznej przeprowadzono szczegółową analizę profilu polifenolowego suchych ekstraktów/frakcji otrzymanych z liści *S. domestica*, uwzględniającą badania jakościowe składu ekstraktów techniką UHPLC-PDA-ESI-MS^3, optymalizację i walidację metody HPLC-PDA analizy ilościowej związków polifenolowych w badanym materiale, a także badania izolacyjne oraz identyfikację markerów aktywnych, odpowiednich dla kontroli jakości ww. materiału roślinnego i pochodnych ekstraktów. Równolegle ekstrakty i modelowe polifenole (markery aktywne) poddane zostały analizie *in vitro* aktywności antyoksydacyjnej i przeciwzapalnej w komplementarnych testach w modelach chemicznych oraz biologicznych, z uwzględnieniem oceny bezpieczeństwa komórkowego. Ponadto, mając na uwadze wpływ warunków klimatycznych na jakość produktów roślinnych, zaprezentowana rozprawa uwzględnia analizę porównawczą składu i aktywności antyoksydacyjnej surowców pochodzących z Polski (uprawa) oraz z Chorwacji (stan naturalny).

Całkowita zawartość polifenoli oznaczona w ekstrakcie metanolowo-wodnym z liści *S. domestica* L. (283 mg GAE/g s.m.) oraz jego najbogatszych w polifenole frakcjach (464-700 mg GAE/g s.m.) była

porównywalna lub wyższa w stosunku do wartości dostępnych literaturowo dla ekstraktów cenionych w prewencji chorób związanych ze stresem oksydacyjnym i stanem zapalnym, jak np. z nasion winorośli (630-790 mg GAE/g s.m.), liści winorośli (60-260 mg GAE/g s.m.), zielonej herbaty (447 mg GAE/g s.m.), czy liści czarnej porzeczki (249 mg GAE/g s.m). Szczegółowa analiza jakościowa i ilościowa ekstraktów/frakcji przeprowadzona metodami chromatograficznymi (UHPLC-PDA-ESI-MS^3; HPLC-PDA-fingerprint), pozwoliła na wykrycie ponad 40 związków polifenolowych oraz wskazanie poziomów zawartości dominujących grup polifenoli (proantocyjanidyn, flawonoidów, kwasów fenolowych) i pojedynczych substancji. Ponadto, po raz pierwszy wyizolowano z rodzaju *Sorbus* dwa diglikozydy flawonoidowe, tj. 3-*O*-(2''-*O*-β-D-glukopiranozylo)-α-L-ramnopiranozyd kwercetyny (HRQ) oraz 3-*O*-(2''-*O*-β-D-ksylopiranolzylo)-α-L-ramnopiranozyd kwercetyny (PRQ). Ostatecznie, badania fitochemiczne doprowadziły do opracowania i walidacji metody HPLC-PDA analizy polifenoli w badanych ekstraktach oraz wytypowania dziewięciu markerów aktywności biologicznej liści *S. domestica,* tj. (-)-epikatechiny (ECA), procyjanidyny B2 (PB2), procyjanidyny C1 (PC1), kwasu chlorogenowego (CHA), kwercetyny (QU), kwercytryny (QCT), rutyny (RT), HRQ i PRQ.

W toku analiz aktywności biologicznej, wykazano ochronne działanie badanych ekstraktów oraz ich markerów aktywnych wobec składników ludzkiego osocza (białek i lipidow) w warunkach stresu oksydacyjnego indukowanego $ONOO^-$, a także zdolność do zwiększenia nieenzymatycznej pojemności antyoksydacyjnej osocza. Udokumentowano również szerokie spektrum działania *in vitro* ekstraktów oraz ich markerów aktywnych wobec oksydantów operujących *in vivo*, tj. $O_2^{\bullet -}$, H_2O_2, $NO^{\bullet}$, HClO, $HO^{\bullet}$, $ONOO^-$, wskazując na zmiatanie ROS/RNS jako jeden z możliwych mechanizmów aktywności obserwowanej w zastosowanym modelu biologicznym. Ponadto, w analizie synergii potwierdzono interakcje pomiędzy cząsteczkami reprezentatywnymi dla każdej z grup związków warunkujących aktywność badanych ekstraktów, tj., ECA, QCT i CHA, co może tłumaczyć konkurencyjną, a nawet niekiedy wyższą aktywność ekstraktów/frakcji w porównaniu do czystych związków, zarówno w przypadku testów na osoczu, jak i aktywności wobec konkretnych ROS/RNS. Brak wpływu ekstraktów na przeżywalność jednojądrzastych komórek krwi obwodowej (PBMCs), oznaczony w ramach niniejszej pracy, pozwolił wstępnie zaklasyfikować badane ekstrakty jako bezpieczne. Wykazano również potencjał przeciwzapalny ekstraktów oraz markerów ich aktywności biologicznej w testach hamowania enzymów lipooksygenazy i hialuronidazy.

Z kolei porównawcze analizy składu i aktywności antyoksydacyjnej liści pochodzących z uprawy krajowej i terenu Bałkanów wykazały, iż pomimo różnic w składzie, spowodowanych odmiennymi warunkami klimatycznymi, surowiec z obu źródeł może być cennym materiałem do produkcji biologicznie aktywnych ekstraktów. Podstawową cechą różnicującą badane próbki okazała się proporcja pomiędzy flawonoidami i proantocyjanidynami – mimo iż te drugie były dominującą grupą związków we wszystkich próbkach, w liściach z Polski stanowiły 59-64% całkowitej zawartości polifenoli, zaś w liściach z Chorwacji 45-52%, z odpowiednio wyższym udziałem flawonoidów.

Wyniki uzyskane w ramach niniejszej pracy, wskazują na zróżnicowane strukturalnie polifenole jako składniki czynne liści *S. domestica* oraz istotną rolę mechanizmów antyoksydacyjnych i przeciwzapalnych dla ich efektu biologicznego. Wielokierunkowość działania świadczy o wysokiej wartości ekstraktów z liści *S. domestica* jako potencjalnego składnika preparatów stosowanych w prewencji czy terapii wspomagającej chorób o podłożu oksydacyjno-zapalnym. Liście jarzębu domowego, stosowane dotychczas jedynie w medycynie tradycyjnej, wydają się być zatem obiecującym materiałem do dalszych badań fitochemicznych, farmakologicznych i toksykologicznych, umożliwiających ich pełną charakterystykę i ewentualne wprowadzenie do oficjalnego lecznictwa.

Summary

High levels of reactive oxygen/nitrogen species (ROS/RNS), generated as a consequence of disturbed redox balance, are the cause of oxidative stress, which together with chronic inflammation is considered to be among the major causes of cardiovascular disorders, insulin resistance, and other chronic non-communicable diseases (NCDs). Healthy lifestyle and, in particular, proper diet are one of the suggested strategies for limiting NCDs progression. Positive results are obtained by the increased consumption of plant products rich in structurally diverse polyphenols, which exert multidirectional effects on human body by the regulation of redox homeostasis and inflammation processes in cells and tissues. The proven efficacy of polyphenolic antioxidants in prevention and therapy of NCDs has generated interest in plant products, their sources, and the rules of potential supplementation. As a result, a large amount of research has focused on the analysis of plants raw materials that, after proper phytochemical, pharmacological and toxicological evaluation, could be used as herbal medicines, dietary supplements or functional food ingredients. In that context, the attention has been paid to one of the species from the Mediterranean region, i.e. service tree (*Sorbus domestica*). Despite the documented traditional usage of *S. domestica* leaves, the lack of research limits their boarder medical application. Previous studies have focused on fruits of service tree. However, despite being rich source of bioactive polyphenols, the fruits are available only regionally due to the weakened flowering and fruiting of *S. domestica* in the temperate climate. Moreover, in case of many fruit plants, the leaves are actually a better source of active phenolics than fruits, which was confirmed by numerous studies and is reflected in the increasing number of leaf-based products placed on the market (e.g. grapevine, or blackcurrant).

Taking into account the above mentioned premises, the evaluation of the leaves of *Sorbus domestica* cultivated in Poland as the source of natural, bioactive polyphenols become the objective of the presented dissertation. The studies consisted of two interdependent parts, i.e. phytochemical analysis and bioactivity assessment. The phytochemical part aimed for the detailed characterization of polyphenolic profile of dry extracts/fractions from *S. domestica* leaves and included qualitative UHPLC-PDA-ESI-MS^3 assays, optimization and validation of the quantitative HPLC-PDA method, as well as isolation and identification of active markers for quality control of the plant material and extracts. In the bioactivity studies, the extracts and model polyphenols (active markers) have been subjected to *in vitro* tests of antioxidant and anti-inflammatory activity in complementary chemical and biological models, including evaluation of their cellular safety. Furthermore, taking into account the impact of the climatic conditions on the quality of plant products, the comparative analysis of the composition and antioxidant activity of the plant material from Poland (cultivation) and Croatia (natural habitat) was carried out.

The total phenolic content in the methanol-water extract from *S. domestica* leaves (283 mg GAE/g dw) and its polyphenol-richest fractions (464-700 mg GAE/g s.m.) was comparable to or higher than that found in the extracts recognized in prevention of oxidative stress-related ailments, such as extracts from grape seeds (630-790 mg GAE/g dw), grape leaves (60-260 mg GAE/g dw), green tea (447 mg GAE/g dw) or blackcurrant leaves (249 mg GAE/g dw). In the course of detailed qualitative and quantitative chromatographic analysis of the extracts/fractions (UHPLC-PDA-ESI-MS^3; HPLC-PDA-fingerprint) over 40 polyphenolic compounds were detected, and the contents of the dominant groups of polyphenols (flavan-3-ols and proanthocyanidins, flavonoids and phenolic acids) and

individual analytes were determined. Moreover, two flavonoid diglycosides, i.e. quercetin 3-*O*-(2''-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside (HRQ) and quercetin 3-*O*-(2''-*O*-β-D-xylopyranosyl)-α-L-rhamnopyranoside (PRQ) were isolated for the first time from genus *Sorbus*. The final step of the phytochemical studies was the development and validation of the HPLC-PDA method for quantitative analysis of polyphenols in the tested extracts, as well as the selection of nine active markers of *S. domestica* leaves, i.e. (–)-epicatechin (ECA), procyanidin B2 (PB2), procyanidin C1 (PC1), chlorogenic acid (CHA), quercetin (QU), quercitrin (QCT), rutin (RT), HRQ and PRQ.

During the biological activity studies, the extracts and their active markers were proven to protect the components of human plasma (proteins and lipids) from the oxidative/nitrative damage induced by $ONOO^-$ as well as to increase the non-enzymatic antioxidant capacity of blood plasma. The ROS/RNS scavenging potential of the extracts and markers was suggested as one of the possible mechanisms of their activity in the applied model, after they were demonstrated *in vitro* as wide-spectrum antioxidants, effective against *in vivo*-operating reactive species, i.e. $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, HClO, $HO^{\bullet}$, $ONOO^-$. Moreover, the study of the interactions between the representatives of the main group of polyphenols responsible for the extracts activity (ECA, QCT and CHA) implied some synergistic effects, which might explain the similar or even higher activity of the extracts/fractions in comparison to pure compounds, both in the biological model and towards particular ROS/RNS. Furthermore, the safety of the extracts was preliminarily confirmed after demonstrating, that they have no impact on the viability of peripheral blood mononuclear cells (PBMCs). The anti-inflammatory potential of the extracts and their activity markers was established in the lipoxygenase and hyaluronidase inhibition assays.

Additionally, comparative analysis of the composition and antioxidant activity of the leaves from Polish cultivation and Balkans area showed, that despite the differences in phytochemical profile, caused by climatic factors, samples from both sources may be valuable materials for production of bioactive extracts. The tested samples differed mainly in terms of the proportion between flavonoids and proanthocyanidins. Even though the latest were the dominant group in all samples, in leaves from Poland they accounted for 59-64% of total polyphenols, while in the samples from Croatia their percentage share was lower (45-52% of total polyphenols) with accordingly higher share of flavonoids.

The results obtained in the present dissertation imply that structurally diverse polyphenols are the active compounds of *S. domestica* leaves and emphasize the importance of the antioxidant and anti-inflammatory mechanisms for their biological effects. The observed multidirectional activity of the investigated *S. domestica* leaf extracts indicates their high value as potential components of herbal medicines for the prevention or adjunctive therapy of oxidative stress/inflammation-related diseases. Leaves of service tree, used so far only in the traditional medicine, seem to be a promising material for further phytochemical, pharmacological and toxicological studies, that would evaluate their properties more thoroughly and, possibly, enable their introduction to the official medicine.

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Sorbus domestica L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*

Magdalena Matczak[a], Anna Marchelak[a], Piotr Michel[a], Aleksandra Owczarek[a], Anna Piszczan[a], Joanna Kolodziejczyk-Czepas[b], Paweł Nowak[b], Monika A. Olszewska[a,*]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Lodz, 90-151 Lodz, Poland
[b] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland

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ABSTRACT

The work combines detailed chemical profiling (UHPLC-PDA-ESI-MS^2, HPLC-PDA, UV-spectrophotometric) and *in vitro* activity testing to provide the first comprehensive characteristics of leaves of the fruit plant *S. domestica* as a source of bioactive extracts used for the treatment of oxidative stress/inflammation-related pathologies. Polyphenol-rich (44 analytes, total content up to 700.0 ± 19.7 mg/g) extracts, obtained by fractionated extraction, were highly-efficient antioxidants (tests: DPPH, FRAP, TBARS) and anti-inflammatory agents (lipoxygenase and hyaluronidase inhibitors). Moreover, at *in vivo*-relevant levels (1–5 μg/mL) they effectively protected human plasma components against peroxynitrite-induced oxidative damage (reduced the levels of 3-nitrotyrosine, lipid hydroperoxides and thiobarbituric acid-reactive substances) and normalised/enhanced the total antioxidant status of plasma. Concentration- and phenolic-dependence was clearly evidenced for all models. Furthermore, the extracts were found safe in cytotoxicity test on human PBMCs. The defatted methanol-water (7:3, *v/v*) extract and its organic solvents fractions were demonstrated as the most advantageous for future applications.

1. Introduction

Sorbus domestica L. (service tree) is a wild rosaceous species native to the Mediterranean Basin and Central Europe which has been cultivated in these regions since Roman times as a fruit and ornamental plant. Its various organs have been reported to be of high nutritional value, as containing substantial quantities of proteins, vitamins, and minerals (Lim, 2012; Majić, Šola, Likić, Juranović Cindrić, & Rusak, 2015). The most popular dietary product obtained from the plant are fruits, which might be consumed directly or processed into jams, jellies, marmalades or juices (Lim, 2012). Medicinal application is also indicated, especially for berries and leaves that are reported to possess diuretic, anti-inflammatory, anti-diabetic, anti-atherogenic, and anti-diarrheal properties (Kültür, 2007). In particular, the fruits of the service tree have recently been recommended as strong antioxidants for various food and pharmacological applications, and a growing body of research links their biological effects with the content of polyphenols, such as flavonols, quinic acid esters, and proanthocyanidins (Majić et al., 2015; Termentzi, Alexiou, Demopoulos, & Kokkalou, 2008; Termentzi, Kefalas, & Kokkalou, 2006, 2008; Ölschläger, Milde, Schempp, & Treutter, 2004). Although berries in general are recognised as rich sources of bioactive polyphenols and good candidates for functional food production (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010), recent studies underline superior activity of phenolic fractions extracted from the leaves of fruit plants, which constitute more cost-effective plant materials available throughout the whole growing season (Teleszko & Wojdyło, 2015). Indeed, the leaf extracts of grapevine, blackberry, blackcurrant and olive have already been introduced worldwide as antioxidant nutraceuticals, and extensive ongoing research is being conducted to broaden the spectrum of leaf-based products effective in protecting against oxidative stress-related pathologies (Franz, Chizzola, Novak, & Sponza, 2011). Among numerous plants under study, *S. domestica* appears to be a promising target for closer investigation, as the leaves of some related species (*S. aucuparia*, *S. aria*, *S. intermedia*, *S. torminalis*) are distinguished by high content of polyphenolic antioxidants, sometimes exceeding four times the value observed for the corresponding fruits (Olszewska, 2011; Olszewska & Michel, 2009; Olszewska, Presler, & Michel, 2012).

* Corresponding author.
E-mail address: monika.olszewska@umed.lodz.pl (M.A. Olszewska).

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Oxidative stress, closely linked with inflammation, is believed to be involved in pathogenesis of numerous chronic diseases, including neurodegenerative and autoimmune disorders, cancers, diabetes, and particularly, cardiovascular disorders: the leading cause of mortality in the world today (Biswas, 2016). As suggested by epidemiological studies, the development of oxidative stress-related ailments might be effectively prevented by a diet rich in polyphenols (Pandey & Rizvi, 2009). As free radical scavengers, metal chelators, inhibitors of pro-inflammatory enzymes, and modifiers of cell signalling pathways, these specialised plant metabolites may protect cells against lipid peroxidation, protein nitration, chronic inflammation and oxidative damage to DNA; they may thus exhibit i.a. vasodilatory, vasoprotective, anti-atherogenic, antithrombotic, and anti-apoptotic effects (Hassan et al., 2017; Pandey & Rizvi, 2009; Quiñones, Miguel, & Aleixandre, 2013). Moreover, the reducing polyphenols and their metabolites can increase the total antioxidant capacity of blood plasma and thus the tolerance of body tissues against ischemic/reperfusion injuries (Pandey & Rizvi, 2009). As the typical diet of modern societies, which bear an increasingly heavy burden of civilization diseases, is usually poor in these constituents, polyphenolic supplementation is regarded as an important aspect of the prevention and adjunctive therapy of oxidative stress-related ailments (Franz et al., 2011). Considering the common occurrence and wide structural diversity of natural polyphenols, careful phytochemical investigations together with *in vivo*-relevant studies of biological activity and safety are essential in the search for novel functional food ingredients to document their possible health benefits.

Therefore, the aim of the study was to investigate the phenolic profile and biological activity of the leaves of *S. domestica* and evaluate their value as a potential functional food ingredient. As the modern recommendations in food supplementation favour the use of standardised dry extracts containing purified and concentrated active components, the study used fractionated extraction as its starting point. Phenolic profiles of the extracts were investigated by UHPLC-PDA-ESI-MS^3, HPLC-PDA, and UV-spectrophotometric methods, while their biological effects were studied *in vitro* by nine complementary tests (both chemical and human plasma models) covering some of the key mechanisms of beneficial action of polyphenols in chronic diseases: direct scavenging of free radicals, inhibition of pro-inflammatory enzymes, protection against oxidative and nitrative damage of lipid and protein components of human plasma, as well as their influence on the total antioxidant capacity of blood plasma. The relationship between activity of the extracts and the presence and structure of their different polyphenolic constituents was explored statistically. Furthermore, cellular safety of the extracts was evaluated *in vitro* in cytotoxicity tests against human peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. General

HPLC grade reagents and standards, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH); 2,4,6-tris-(2-pyridyl)-*s*-triazine (TPTZ); (±)-6-hydroxy-2,2,7,8-tetramethylchroman-2-carboxylic acid (Trolox®); gallic acid monohydrate; chlorogenic acid hemihydrate; *p*-coumaric acid; protocatechuic acid; caffeic acid, (–)-epicatechin; kaempferol; quercetin trihydrate; isorhamnetin, rutin trihydrate, quercitrin, cyanidin chloride; indomethacin; bovine testis hyaluronidase; lipoxygenase from soybean; linoleic acid; 2-thiobarbituric acid; and phenolic standards used for qualitative LC-MS studies were purchased from Sigma-Aldrich (Seelze, Germany/St. Louis, MO, USA), as were analytical grade butylated hydroxyanisole (BHA); 2,6-di-*tert*-butyl-4-methylphenol (BHT); Tween® 40; xylenol orange disodium salt; Histopaque®-1077 medium; and RPMI-1640 medium. HPLC grade solvents (acetonitrile and methanol) were obtained from Avantor Performance Materials (Gliwice, Poland). A (Ca^{2+})-free phosphate buffered saline (PBS) was purchased from Biomed (Lublin, Poland). Peroxynitrite was synthesised according to Pryor et al. (1995). All immunoreagents for 3-nitrotyrosine detection were purchased from Abcam (Cambridge, UK). Pierce BCA Protein Assay Kit was obtained from Thermo Scientific (Waltham, MA, USA). All other chemicals and solvents were of analytical grade and obtained from Avantor (Poland). In all analyses, redistilled water was used. For chemical tests, samples were incubated at a constant temperature using a BD 23 incubator (Binder, Tuttlingen, Germany) and measured using a UV-1601 Rayleigh spectrophotometer (Beijing, China) in 10 mm quartz cuvettes. Activity tests in blood plasma models and enzyme inhibitory assays were conducted using 96-well plates and a microplate reader, SPECTROStar Nano (BMG LabTech, Ortenberg, Germany).

2.2. Plant material and preparation of dry extracts

Leaves of *Sorbus domestica* L. were collected and authenticated in September 2015 in the Arboretum (51°49′N, 19°53′E), Forestry Experimental Station of Warsaw University of Life Science (SGGW) in Rogow (Poland). The raw material was powdered with an electric grinder, and sieved through a 0.315-mm sieve. A portion (220 g) was extracted with chloroform (1 L, 30 h) in a Soxhlet apparatus and the pellet was next refluxed three times with methanol-water (7:3, *v/v*; 3 × 1 L × 6 h) to give the defatted methanol extract (MED, 39.7 g dw). The MED (35.4 g) was suspended in water (0.5 L) and subjected to sequential liquid-liquid extraction with organic solvents (8 × 100 mL each) to yield diethyl ether fraction (DEF, 0.63 g dw), ethyl acetate fraction (EAF, 2.43 g dw), *n*-butanol fraction (BF, 5.73 g dw) and water residue (WR, 14.50 g dw). The organic solvent extracts were evaporated *in vacuo*, and the water-containing fractions were lyophilised using an Alpha 1–2/LD Plus freeze dryer (Christ, Osterode am Harz, Germany) before weighing. In further analyses, freshly prepared solutions of the extracts in methanol-water (7:3, *v/v*) were used. All quantitative results were calculated per dry weight (dw) of the extracts.

2.3. Phytochemical profiling

2.3.1. Qualitative LC-MS study

The UHPLC-PDA-ESI-MS^3 analysis was performed on UHPLC-3000 RS system (Dionex, Dreieich, Germany) equipped with a dual low-pressure gradient pump, an autosampler, a column compartment, a diode array detector, and an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik, Bremen, Germany). Separations were carried out on a Kinetex XB-C18 column (1.7 µm, 150 mm × 2.1 mm i.d.; Phenomenex, Torrance, CA, USA) at 25 °C. The mobile phase consisted of solvent A (water/acetonitrile/formic acid, 95:5:0.1, *v/v/v*), and solvent B (acetonitrile/formic acid, 100:0.1, *v/v*) with the elution profile as follows: 0–45 min, 6–26% B (*v/v*); 45–55 min, 26–95% B; 55–63 min, 95% B; 63–70 min, 95%–6% B; 70–80 min, 6% B (equilibration). The flow rate was 0.3 mL/min. Before injection, sample solutions of the extracts (3.0 mg/mL) were filtered through a PTFE syringe filter (13 mm, 0.2 µm, Whatman, Pittsburgh, PA, USA). UV–vis spectra were recorded over the range of 200–600 nm. The LC eluate was introduced directly into the ESI interface without splitting and analysed in a negative ion mode. The ESI parameters were as follows: the nebulizer pressure was 40 psi, dry gas flow 9 L/min, dry temperature 300 °C and capillary voltage 4.5 kV. MS^2 and MS^3 fragmentations were obtained in Auto MS/MS mode for the most abundant ions at the time. Analysis was carried out using scan from *m/z* 200 to 2200.

2.3.2. Quantitative standardisation

The total phenolic content (TPC) and total proanthocyanidin content (TPA) were quantified by the Folin-Ciocalteu and *n*-butanol-HCl methods, respectively, as described previously (Olszewska et al., 2012). Results were expressed as equivalents of gallic acid (GAE) and cyanidin chloride (CYE), respectively.

The HPLC-PDA-fingerprint assays were performed according to Olszewska et al. (2012) using the same equipment and procedure. The phenolic analytes were quantified as equivalents of external standards: (–)-epicatechin (flavan-3-ols and proanthocyanidins), protocatechuic and *p*-hydroxybenzoic acids (hydroxybenzoic acids), chlorogenic acid (caffeoylquinic acid isomers), caffeic and *p*-coumaric acids (hydroxycinnamic acid derivatives), quercitrin (flavonoid monoglycosides), rutin (flavonoid diglycosides), and quercetin (flavonoid aglycones), depending on the PDA spectra.

The total flavonoid contents were determined by HPLC-PDA as the total content of flavonoid aglycones after acid hydrolysis (TFA). Samples of the extracts (1–5 mg) were heated under reflux for 30 min with methanol-water (9:1, *v/v*; 30 mL) and 25% (*w/v*) hydrochloric acid (9 mL). The hydrolysates were diluted with methanol-water (7:3, *v/v*) to 50 mL, filtered through a PTFE syringe filter (as above) and injected (5 µL) into the HPLC system. The HPLC-PDA assays were carried out using the equipment and procedure described previously (Olszewska, 2012), and with quercetin, kaempferol and isorhamnetin used as external calibration standards.

2.4. Antioxidant activity in chemical models

The DPPH free-radical scavenging activity was determined according to the method optimised previously (Olszewska et al., 2012) and expressed as normalised EC_{50} values calculated from concentration-inhibition curves.

The FRAP (Ferric Reducing Antioxidant Power) was determined according to Olszewska et al. (2012) and expressed in µmol of ferrous ions (Fe^{2+}) produced by 1 g of the dry extract or standard, which was calculated from the calibration curve of ferrous sulphate.

The ability of the extracts to inhibit AAPH-induced peroxidation of linoleic acid (LA) was assayed as described previously (Olszewska et al., 2012) with peroxidation monitored by quantitation of thiobarbituric acid-reactive substances (TBARS) according to the method of Kljak and Grbeša (2015) with some changes. Briefly, for the peroxidation test the samples were incubated for three hours at 50.0 ± 0.1 °C in the dark, and the TBARS levels were measured in the samples, both before and after incubation. For colour development, the reaction solution (0.5 mL) was mixed with 0.67% (*w/w*) aqueous thiobarbituric acid (1 mL), 0.05 M HCl (0.5 mL), Tween®40 (1 mL), and then heated for 30 min in the water bath at 95 °C. The absorbance was measured at 535 nm versus the control with methanol-water (7:3, *v/v*) instead of the reaction mixture. The inhibition ratio (I%) of the LA-peroxidation was calculated as follows: $I\% = (\Delta A_{control} - \Delta A_{sample})/\Delta A_{control}$, where ΔA is the difference between the absorbance measured before and after the three-hour incubation. Finally, antioxidant activity was expressed as IC_{50} values calculated from concentration-inhibition curves.

2.5. Antioxidant activity in human plasma models

2.5.1. Isolation of blood plasma and preparation of samples

Blood from five healthy, non-smoking volunteers was obtained from the Regional Centre of Blood Donation and Blood Treatment in Lodz (Poland), collected on CPD (citrate/phosphate/dextrose) solution in the Fresenius-Kabi Compoflex bags; the plasma was then isolated by differential centrifugation of blood (Kolodziejczyk-Czepas et al., 2013). All experiments were approved by the committee on the Ethics of Research at the University of Lodz 8/KBBN-UŁ/II/2015. For the FRAP assay and measurements of 3-nitrotyrosine, plasma samples were diluted with a (Ca^{2+})-free PBS buffer (1:4, *v/v*), whereas for hydroperoxide and TBARS assays plasma was diluted with (Ca^{2+})-free PBS in a volume ratio 1:1. For all tests, samples were pre-incubated for five minutes at 37 °C with the examined extracts added to the final concentration range of 1–50 µg/mL, and then exposed to 100 µM peroxynitrite ($ONOO^-$). Control samples were prepared with plasma untreated with the extracts and/or peroxynitrite. In the experiments with blood plasma and the extracts only (without adding $ONOO^-$) no pro-oxidative effect was found. Protein concentration in blood plasma was estimated using bicinchoninic acid (BCA) assay (according to protocol provided by the manufacturer).

2.5.2. Determination of 3-nitrotyrosine in human plasma proteins

Detection of nitrotyrosine-containing proteins by the competitive ELISA (C-ELISA) method in plasma samples (control or antioxidants and $ONOO^-$-treated plasma) was performed according to Kolodziejczyk-Czepas et al. (2013). The concentrations of nitrated proteins were estimated from the standard curve and are expressed as 3NT-Fg equivalents (in nmol/mg of plasma protein).

2.5.3. Ferric-xylenol orange hydroperoxide (FOX-1) assay

Concentration of hydroperoxides in plasma samples (control or antioxidants and $ONOO^-$-treated plasma) was determined by a FOX-1 protocol (Kolodziejczyk-Czepas et al., 2013). The FOX-1 reagent contained 125 µM xylenol orange and 100 mM sorbitol in 25 mM sulfuric acid, and was freshly prepared each time before use by the addition of ammonium ferrous sulphate to the final concentration of 250 µM. To perform FOX-1 assay, blood plasma samples were mixed with the reagent in a volume ratio 1:9 and incubated for 30 min in the dark (25 °C). Absorbance of the sample was measured at 560 nm against blank (water instead of plasma). The amount of lipid hydroperoxides was calculated from the standard curve of hydrogen peroxide and expressed in nmol/mg of plasma proteins.

2.5.4. TBARS test

Determination of TBARS in plasma samples (control or antioxidants and $ONOO^-$-treated plasma) was performed according to Kolodziejczyk-Czepas et al. (2011). Results were expressed in µmol TBARS/mL of plasma.

2.5.5. Ferric reducing ability of plasma (FRAP) assay

The influence of the extracts on the total antioxidant activity of plasma (dependent on non-enzymatic antioxidants) was determined according to Kolodziejczyk-Czepas, Nowak, Kowalska, and Stochmal (2014) with some modifications. The freshly collected plasma samples were diluted with a (Ca^{2+})-free PBS buffer in a volume ratio of 1:4, prepared as described above (Section 2.5.1), and then added to the reagent mixture in a volume ratio of 1:10:1:1 for plasma, acetate buffer (300 mM, pH 3.6), TPTZ (10 mM, in 0.04 M hydrochloric acid), and ferric chloride (20 mM), respectively. After incubation for 15 min at 37 °C, the measured FRAPs of plasma samples (control or antioxidants and $ONOO^-$-treated plasma) were expressed in mM of Fe^{2+} equivalents.

2.6. Inhibition of pro-inflammatory enzymes

The ability of the extracts to inhibit lipoxygenase (LOX) and hyaluronidase (HYAL) was examined as described by Michel et al. (2017) with some changes. Briefly, in the LOX tests the reagents were used in a volume ratio of 1:2:1 for working solutions of the tested analyte, linoleic acid, and enzyme, respectively. The results of both assays were expressed as IC_{50} values calculated from concentration-inhibition curves.

2.7. Cellular safety

Cytotoxicity of the examined extracts was determined in an experimental model of PBMCs, according to the previously described protocol (Krzyzanowska-Kowalczyk et al., 2017). The cells were isolated from human blood (obtained and collected according to Section 2.5.1) using the Histopaque®-1077 medium: a sterile solution of 57 g/L polysucrose and 90 g/L sodium diatrizoate, with a density of 1.077 g/mL. From each of nine donors, two independent PBMC isolations and incubations with the extracts were performed. Blood was carefully

layered (in a volume ratio of 1:1) onto the medium, and centrifuged for 30 min (400 x *g*, at room temperature). The pellet was then washed two times with 0.02 M PBS buffer. The obtained fraction of PBMCs was suspended in PBS. Cell suspensions (1×10^6 PBMCs/mL) were incubated with plant extracts, added to the final concentration of 1, 5, 25 and 50 μg/mL for 1, 2, 4 and 6 h (at 37 °C). Cell viability (%) was determined during a spectrofluorimetric analysis, involving the use of propidium iodide as a fluorescent dye. Measurements were conducted using an Adam-MC DigitalBio microchip-type automatic cell counter (NanoEnTek Inc., Seoul, Korea) according to the manufacturer's protocol. The initial cell viability was at the level of 94.5 ± 5.3%. After 1 h the viability attained 93.9 ± 4.6%, after 2 h it was 90.9 ± 4.8%, after 4 h it attained 87.0 ± 7.3%, and after 6 h it was 67.8 ± 7.9% in control (untreated) cells suspensions. Additionally, the PBMCs, isolated as described above, were suspended in the RPMI-1640 medium (3×10^6 PBMCs/mL) and incubated with the extracts (1–50 μg/mL) for 24 h (in 96-well microplates, at 37 °C, in a humidified atmosphere). Measurements were carried out analogously as described above.

2.8. Data analysis

The results are reported as means ± SD (standard deviation) or ± SE (standard error) for the indicated number of experiments. The significance of differences between samples and controls was determined with one-way ANOVA (for chemical tests) or one-way ANOVA for repeated measures (for human plasma model), followed by the *post hoc* Tukey's test for multiple comparisons. The correlations were evaluated using *F*-test. All calculations were performed using Statistica 12Pl software for Windows (StatSoft Inc., Krakow, Poland) with *p* values less than 0.05 regarded as significant.

3. Results

3.1. Metabolite profiling of leaf extracts

Phytochemical profiling of the dry extracts revealed significant differences in their chemical composition depending on the extraction solvent (Fig. 1, Table 1). The UHPLC-PDA-ESI-MS^3 analysis led to full or partial identification of over forty phenolic constituents (UHPLC peaks **1–44**) belonging to three main classes of phytochemicals: flavan-3-ol derivatives (catechins and proanthocyanidins, sixteen peaks), flavonols (thirteen analytes) and phenolic acids (twelve compounds). The analytes were structurally identified based on their chromatographic behaviour, UV–vis spectra, and ESI-MS^3 fragmentation patterns (in a negative ionisation mode) compared with the reference standards and literature data (Clifford, Knight, & Kuhnert, 2005; Raudonė et al., 2014; Termentzi, Kefalas, et al., 2008; Ölschläger et al., 2004). In comparison to the basic defatted methanol-water (7:3, *v/v*) extract (MED), the fractions of diethyl ether (DEF), ethyl acetate (EAF), *n*-butanol (BF) and water residue (WR) obtained after sequential liquid-liquid partitioning of MED were enriched in selected analytes, depending on the fractionation solvent. Among the analytes, only chlorogenic acid (**4**, 5-*O*-caffeoylquinic acid) was found in all extracts.

The levels of total phenolics (TPC) in the extracts, determined by the Folin-Ciocalteu assay and expressed as gallic acid equivalents (GAE), varied across a wide range of 76.7–700.0 mg GAE/g dw, with the highest values observed for EAF (700.0 mg GAE/g dw) and DEF (637.6 mg GAE/g dw) (Fig. 2). Subsequently, the phenolic contents were verified by RP-HPLC-PDA-fingerprinting, and the total phenolic levels (TPH) were calculated as a sum of individual analytes quantified with authentic standards. The obtained TPH values were in the range of 13.1–557.9 mg/g dw (Fig. 2), still with the highest concentration observed for DEF (557.9 mg/g dw) and EAF (377.3 mg/g dw). Except for DEF, the TPH levels were about two to six times lower than the TPCs, which suggested that some polyphenols in the extracts cannot be detected by RP-HPLC.

This observation may be attributed to the proanthocyanidins: compounds occurring in plants at various degrees of polymerisation which are quantifiable by RP-HPLC only in the form of oligomers built from less than four flavan-3-ol monomers. Indeed, the total proanthocyanidin contents determined in the leaf extracts by *n*-butanol/HCl assay (TPA; expressed in cyanidin chloride equivalents, CYE) and by RP-HPLC (TLPA) differ significantly (Fig. 3A). In the polar extracts MED, BF and WR, the TPA levels were three to six times higher than the TLPA values. This difference could be explained by the presence of highly polymerised proanthocyanidins with known affinity for polar solvents (Santos-Buelga & Scalbert, 2000). For less hydrophilic extracts (DEF and EAF), the opposite trend was observed: the TLPAs considerably exceeded the TPAs, and were related to the abundant presence of monomeric flavan-3-ols and low-molecular-weight proanthocyanidins, i.e.(–)-epicatechin, and procyanidins B2 and C1 (Figs. 1 and 3A). As (–)-epicatechin gives no response in the *n*-butanol/HCl assay, it was equally responsible for the underestimated TPA levels of DEF and EAF. Summarizing the results of both assays, the greatest proanthocyanidin content was found in DEF, EAF and BF. Moreover, with the levels constituting about 25–60% of the TPC and TPH contents, these compounds were the dominant phenolics of all extracts.

The second relevant group of constituents was formed by flavonoids. As a result of fractionation, they were concentrated in DEF, EAF and BF (constituted 25.8–34.9% of their TPH levels), but virtually absent from WR. The total flavonoid content (TFL, Fig. 3B), determined by HPLC-PDA as a sum of native glycosides, ranged in these fractions from 21.9 to 194.6 mg/g dw, with the highest level found for DEF. Generally, the dominant glycoside was quercitrin (**39**) with the levels constituting 58.0–85.3% of the TFL values. The exception was BF, in which a quercetin hexoside-rhamnoside (**37**) was the prevailing constituent (24.7% of the TFL). As regards individual aglycones (Fig. 3C), quercetin was the predominant one, representing 84.2–100.0% of the total aglycones released after acid hydrolysis. The contents of kaempferol and isorhamnetin were much lower and formed at most 14.2% and 1.6% of the sum of aglycones, respectively.

Phenolic acids constituted the third fraction of the analysed extracts (Fig. 3D). As assayed by HPLC-PDA, the total content of phenolic acids (TPHA) was in the range of 13.1–92.6 mg/g dw with the highest level found once again for DEF. In comparison to other extracts, DEF was selectively enriched in simple hydroxycinnamic and hydroxybenzoic acids (SPHA; 87.5 mg/g dw) with the predominant *p*-hydroxybenzoic acid (**3**; 42.2 mg/g dw). In all other extracts, isomeric chlorogenic acids prevailed (TCHA; 71.7–85.5% of TPHA) with the peak contents in BF (51.3 mg/g dw). The major isomer was chlorogenic acid (**4**; 5-*O*-caffeoylquinic acid) forming not less than 72% of the TCHA contents. Two further isomers were also detected in some of the tested extracts but at much lower levels (3-*O*-caffeoylquinic acid: 2.0–3.6 mg/g dw; 4-*O*-caffeoylquinic acid: 0.9–2.7 mg/g dw).

3.2. Antioxidative effects of leaf extracts in chemical models

All of the investigated extracts showed significant and concentration-dependent ability to scavenge free radicals (DPPH, a stable synthetic radical), directly reduce transition metal ions (Fe^{3+}, FRAP), and inhibit AAPH-induced linoleic acid peroxidation (chemical model of lipid peroxidation) (Table 2). Regardless of the test, the activity of the extracts decreased in a similar order, i.e. EAF ≥ DEF > BF > MED > WR, and correlated with the amounts of polyphenols (TPC) (Table 3). The strongest relationship was recorded for the FRAP test ($r = 0.9850$, $p = .002$), whereas for the DPPH and TBARS assays, the correlation coefficients were negatively influenced by WR, the extract exhibiting the lowest levels of phenols. When WR was excluded from the data matrix, the impact of TPC ($|r| > 0.87$, $p < .05$) on the antioxidant parameters was evident for all assays (data not shown). Moreover, the presence of strong and statistically significant correlations ($r > 0.87$, $p < .05$) between the FRAP values and the TPH,

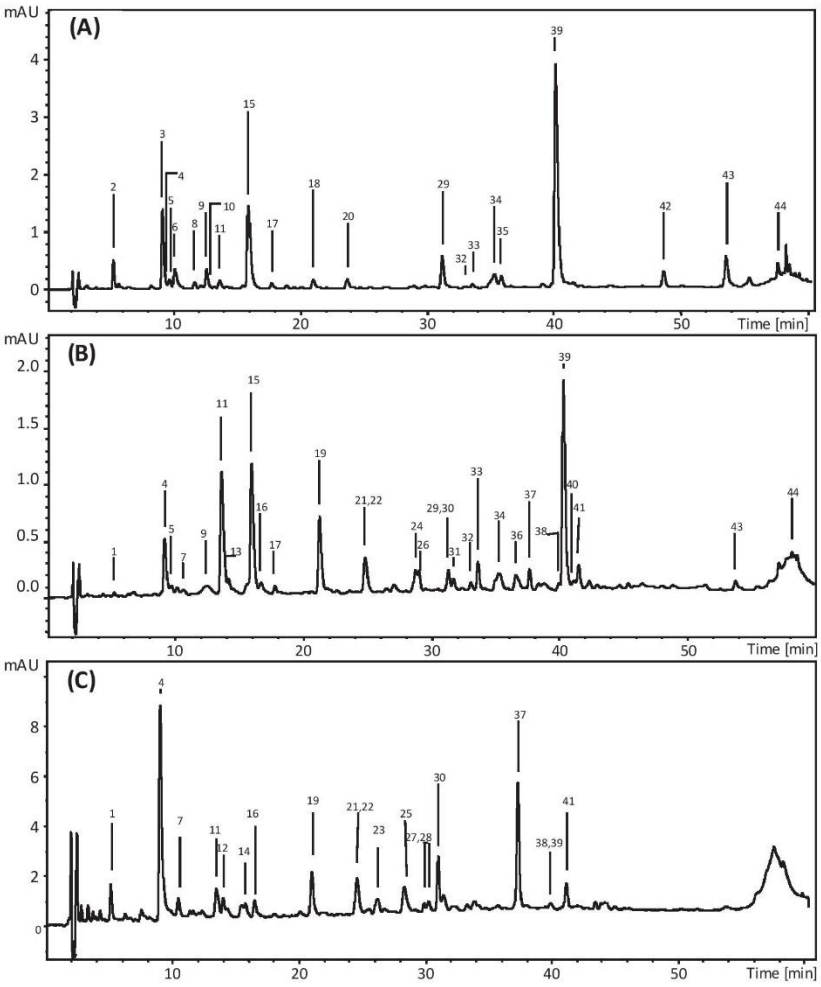


Fig. 1. Representative UHPLC chromatograms of the *S. domestica* leaf extracts at 280 nm: DEF, diethyl ether fraction (A); EAF, ethyl acetate fraction (B); BF, *n*-butanol fraction (C). Peak numbers refer to those implemented in Table 1.

TLPA, (–)-epicatechin, TFA, and TFL levels suggested that the ferric reducing ability of the tested extracts is influenced primarily by low-molecular-weight polyphenols detectable by RP-HPLC, especially proanthocyanidins and flavonoids (Table 3). In the DPPH and TBARS tests, the effects of individual groups of low-molecular-weight phenols were less evident ($|r| > 0.55, p > .05$), but simultaneously the impact of higher proanthocyanidins (represented by the TPA levels) was equally apparent ($|r| > 0.53$, $p > .05$). However, some synergic effects, for instance for combined TPA and TFL levels (TFL + TPA/DPPH: $r = –0.9229$, $p = .025$; TFL + TPA/TBARS: $r = –0.9228$, $p = .025$), suggested that free-radical scavenging activity and ability to inhibit lipid peroxidation of the tested extracts is in chemical *in vitro* models influenced by both low- and high-molecular-weight polyphenols.

The antioxidant capacity of the most active extracts EAF and DEF was not significantly different ($p > .05$) or higher ($p < .05$) than that of industrial antioxidants – BHT (all three tests) and BHA (FRAP tests), as well as Trolox, a synthetic analogue of vitamin E (FRAP and TBARS tests) (Table 2). The third organic solvent fraction, BF, exhibited activity not lower than that of BHT (DPPH and TBARS) or Trolox (FRAP and TBARS). Moreover, if activity parameters of the native extracts were recalculated to GAE (Table 2) using the TPC values, the obtained antioxidant capacities of the phenolic fractions constituting the dry extracts were at least comparable to all of the positive standards except quercetin and (–)-epicatechin.

3.3. Protective effects of the extracts on human plasma components

After demonstrating the promising antioxidant capacity in *in vitro* chemical assays, the extracts were examined in terms of their effects on human plasma exposed to oxidative stress induced by peroxynitrite ($ONOO^-$; 100 μM). In comparison to the control (untreated) samples, the $ONOO^-$-stimulated plasma exhibited a markedly raised level (Fig. 4A; $p < .001$) of 3-nitrotyrosine in plasma proteins (a marker of protein nitration), a considerable increase of lipid hydroperoxides (FOX-1 assay) and TBARS – markers of lipid peroxidation (Fig. 4B and C; $p < .001$), as well as a modest decrease (Fig. 4D; $p > .05$) in ferric reducing ability (FRAP, a marker of total antioxidant status of plasma). In the presence of the extracts (at 1–50 μg/mL), the rate of oxidative/nitrative damage was significantly reduced (Fig. 4A–C; $p < .05$). All tested extracts effectively reduced the nitration of tyrosine residues (Fig. 4A; $p < .001$) by about 15–40% and 51–63% at 1 μg/mL and 50 μg/mL, respectively. The impact of the extraction solvent was, however, different than that observed in chemical tests. This time, at the levels of 1 and 5 μg/mL, MED was more effective than all its fractions ($p < .05$) and only at 50 μg/mL did its activity not differ significantly from those of DEF and EAF ($p > .05$). Nevertheless, as a consequence of the strong concentration-dependency observed for activity of all extracts, significant correlations were found between percentage inhibition of tyrosine nitration and phenolic contents (Table 3) ($p < .05$), with the strongest effect found for the TPC levels ($r = 0.7173$). All of the extracts

Table 1
UHPLC-PDA-ESI-MS^3 data of identified polyphenols in the *S. domestica* leaf extracts.

No	Analyte	R_t (min)	Formula	UV λ_{max} (nm)	$[M-H]^-$	MS^2 (% relative abundance)	MS^3 (% relative abundance)	Extract
1	Neochlorogenic acid (3-*O*-caffeoylquinic acid)[a]	5.2	$C_{16}H_{18}O_9$	325	353	191(100), 179(47)		MED, EAF, BF, WR
2	Protocatechuic acid[a]	5.2	$C_7H_6O_4$	260, 293	153	153(100)		DEF
3	*p*-Hydroxybenzoic acid[a]	9.0	$C_7H_6O_3$	254	137	93(35)		DEF
4	Chlorogenic acid (5-*O*-caffeoylquinic acid)[a]	9.2	$C_{16}H_{18}O_9$	325	353	191(100), 179(4)		All
5	(+)-Catechin[a]	9.5	$C_{15}H_{14}O_6$	280	289	245(87), 205(38)		DEF, EAF
6	Dihydroxybenzoic acid derivative	9.9		325	329	153(100)		DEF
7	Cryptochlorogenic acid (4-*O*-caffeoylquinic acid)[a]	10.6	$C_{16}H_{18}O_9$	325	353	173(80), 191(50), 179(50)		MED, EAF, BF
8	Protocatechuic acid derivative	11.5		260, 293	305	183(100), 153(2)		DEF
9	Procyanidin dimer B-type	12.4	$C_{30}H_{26}O_{12}$	280	577	451(34), **425(100)**, 407(52), 289(23)	407(100), 273(10)	DEF, EAF
10	Caffeic acid[a]	12.7	$C_9H_8O_4$	325	179	179(100), 135(17)		DEF
11	Procyanidin B2[a]	13.7	$C_{30}H_{26}O_{12}$	280	577	451(17), **425(100)**, 407(32), 289(11)	407(100), 273(4)	MED, DEF, EAF, BF
12	Procyanidin dimer B-type	13.9	$C_{30}H_{26}O_{12}$	280	577	451(12), **425(100)**, 407(34), 289(8)	407(100), 273(11)	BF
13	Procyanidin trimer B-type	14.0	$C_{45}H_{38}O_{18}$	280	865	**713(38)**, 695(100), 577(10), 289(4)	695(100), 543(82), 425(74),407(53)	EAF
14	Unidentified compound	15.6		315	431	385(100), 179(23)		BF
15	(−)-Epicatechin[a]	16.0	$C_{15}H_{14}O_6$	280	289	245(100), 205(22)		MED, DEF, EAF
16	Proanthocyanidin dimer B-type	16.8	$C_{30}H_{26}O_{12}$	280	577	425(60), 407(25), 289(30)		EAF, BF
17	Caffeoyl shikimic acid isomer	17.8	$C_{16}H_{16}O_8$	325	335	179(100), 161(3), 135(19)		MED, DEF, EAF
18	*p*-Coumaric acid derivative	21.0		310	353	163(92), 119(4)		DEF
19	Procyanidin trimer B-type (C1)	21.4	$C_{45}H_{38}O_{18}$	280	865	**713(15)**, 695(96), 577(19)	695(100), 425(28),407(33),289(3)	MED, EAF, BF
20	*p*-Coumaric acid[a]	23.7	$C_9H_8O_3$	310	163	163(100), 119(39)		DEF
21	Procyanidin dimer B-type	24.6	$C_{30}H_{26}O_{12}$	280	577	451(15), 425(14), 407(5), 245(15), 289(100)		EAF, BF
22	Procyanidin trimer B-type	24.6	$C_{45}H_{38}O_{18}$	280	865	**713(100)**, 695(94), 577(21)	695(100), 425(59),407(61),287(34)	EAF, BF
23	Proanthocyanidin derivative	26.0		280	720	635(97), 575(68), 451(16), 289(41)		BF
24	Procyanidin dimer B-type	28.2	$C_{30}H_{26}O_{12}$	280	577	451(62), **425(49)**, 407(21), 289(92), 245(81)	407(93), 289(42)	EAF
25	Proanthocyanidin derivative	28.3		280	720	**635(100)**, 575(53), 425(14), 289(37), 245(18)		BF
26	Procyanidin dimer hexoside	29.0	$C_{36}H_{36}O_{17}$	280	739	**587(91)**, 451(21), 339(17), 289(10)	435(100), 339(74),287(4)	MED, EAF
27	Quercetin rhamnoside-hexoside	29.7	$C_{27}H_{30}O_{16}$	255, 353	609	**463(8)**, 301(100)	301(100)	BF
28	Quercetin rhamnoside-hexoside	30.2	$C_{27}H_{30}O_{16}$	275, 350	609	**463(12)**, 301(100)	301(100)	BF
29	Unidentified compound	31.1		280	451	341(100), 217(3)		DEF, EAF
30	Rutin (quercetin 3-*O*-β-D-(6″-*O*-α-L-rhamnosyl)-glucoside)[a]	31.2	$C_{27}H_{30}O_{16}$	256, 355	609	463(11), 301(94), 179(7)		MED, EAF BF
31	Hyperoside (quercetin 3-*O*-β-D-galactoside)[a]	31.9	$C_{21}H_{20}O_{12}$	254, 353	463	**301(100)**	179(60)	MED, DEF, EAF
32	Isoquercitrin (quercetin 3-*O*-β-D-glucoside)[a]	32.8	$C_{21}H_{20}O_{12}$	256, 353	463	**301(90)**	179(51)	DEF, EAF
33	Procyanidin dimer B-type	33.7	$C_{30}H_{26}O_{12}$	280	577	451(18), **425(100)**, 407(31), 289(13)	407(56), 473(14)	DEF, EAF
34	Reinutrin (quercetin 3-*O*-β-D-xylopyranoside)[a]	35.3	$C_{20}H_{18}O_{11}$	275, 350	433	301(100)		DEF, EAF
35	Proanthocyanidin derivative	35.7		280	483	451(31), 341(29), 289(100)		DEF
36	Proanthocyanidin derivative	36.4		280	591	439(100), 289(56), 245(3)		EAF
37	Quercetin hexoside-rhamnoside	37.7	$C_{27}H_{30}O_{16}$	256, 350	609	**447(13)**, 301(51), 179(6)	301(100)	MED, EAF, BF
38	Isorhamnetin diglycoside	39.8	$C_{28}H_{32}O_{16}$	275, 350	623	315(100)		EAF, BF
39	Quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside)[a]	40.0	$C_{21}H_{20}O_{11}$	276, 350	447	301(100), 179(3)		MED, DEF, EAF, BF
40	3,5-Dicaffeoylquinic acid	40.9	$C_{25}H_{24}O_{12}$	280, 325	515	**353(89)**, 191(4),179(2)	191(100), 179(48), 135(6)	EAF
41	Quercetin pentoside-rhamnoside	41.5	$C_{26}H_{28}O_{15}$	256, 353	579	**447(9)**, 301(100)	301(100)	MED, EAF, BF
42	Afzelin (kaempferol 3-*O*-α-L-rhamnoside)[a]	48.6	$C_{21}H_{20}O_{10}$	263, 346	431	285(100), 255(4)		DEF
43	Unidentified compound	53.5		280	451	341(73), **299(3)**	189(71)	DEF, EAF
44	Quercetin[a]	57.8	$C_{15}H_{10}O_7$	255, 364	301	301(100)		MED, DEF, EAF

[a] Identified with authentic standards. R_t, retention times. UV λ_{max}, absorbance maxima in PDA spectra. $[M-H]^-$, pseudomolecular ions in MS spectra recorded in a negative mode. MS^2/MS^3, secondary ions (MS^2 ions in bold were subjected to MS^3 fragmentation). MED, defatted methanol-water (7:3, *v/v*) extract. DEF, diethyl-ether fraction. EAF, ethyl acetate fraction. BF, *n*-butanol fraction. WR, water residue.

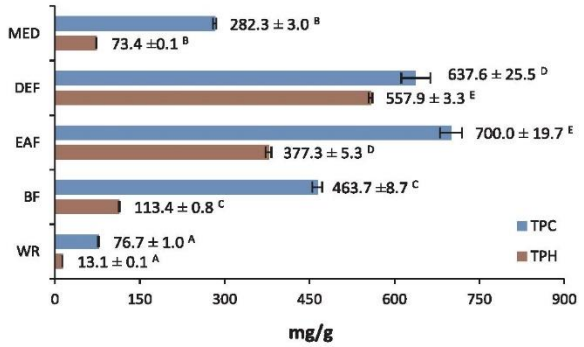


Fig. 2. Total contents of phenolic compounds in the *S. domestica* leaf extracts determined by the Folin-Ciocalteu assay (TPC) and RP-HPLC-PDA (TPH). Data expressed as mean values ± SD ($n = 3$). For each parameter different letters indicate significant differences ($p < .05$) by Tukey's test.

protected also the plasma lipids against peroxynitrite-induced peroxidation (Fig. 4B and C; $p < .05$), regardless of the concentration levels. For example, in the samples pre-incubated with 5 μg/mL of the extracts, the levels of hydroperoxides and TBARS decreased by about 46–59% and 15–24%, respectively. The solvent effects were less pronounced but similar to those revealed in chemical tests, with DEF found to be the most active. At 50 μg/mL, DEF reduced the peroxidation parameters by about 67% in FOX-1 and 46% in TBARS assays. In general, the observed capacities were concentration-dependent; however, some of the dose-effects were not significant, e.g. those of MED at the levels of 5 and 50 μg/mL in the FOX-1 test (Fig. 4B; $p < .05$). In consequence, strong and significant correlations were observed (Table 3) between the percentage of lipid peroxidation assayed by TBARS and the levels of phenolics, including the TPC, TPH, TFA, TFL and TPHA values ($|r| > 0.87$, $p < .001$), whereas the analogous relationships for FOX-1 were weaker ($|r| > 0.52$, $p < .05$). The analysed extracts were also able to normalize and/or enhance the total antioxidant status of $ONOO^-$-treated plasma (FRAP), depending on the concentration; however, MED, DEF, and EAF at the lowest level (1 μg/mL) did not change significantly the FRAP value of the oxidised plasma (Fig. 4D; $p > .05$). For all other extracts and levels, a significant improvement in the plasma reducing ability was observed with the increase up to about 360% compared to the $ONOO^-$-stimulated plasma and 280% compared to the control (untreated) sample for EAF at 50 μg/mL (Fig. 4D; $p < .001$). A strong concentration-dependency of the analytical response was found, and thus strong and statistically significant relationships between the percentage increase in the FRAP values of the oxidised plasma and all parameters of the phenolic contents (Table 3).

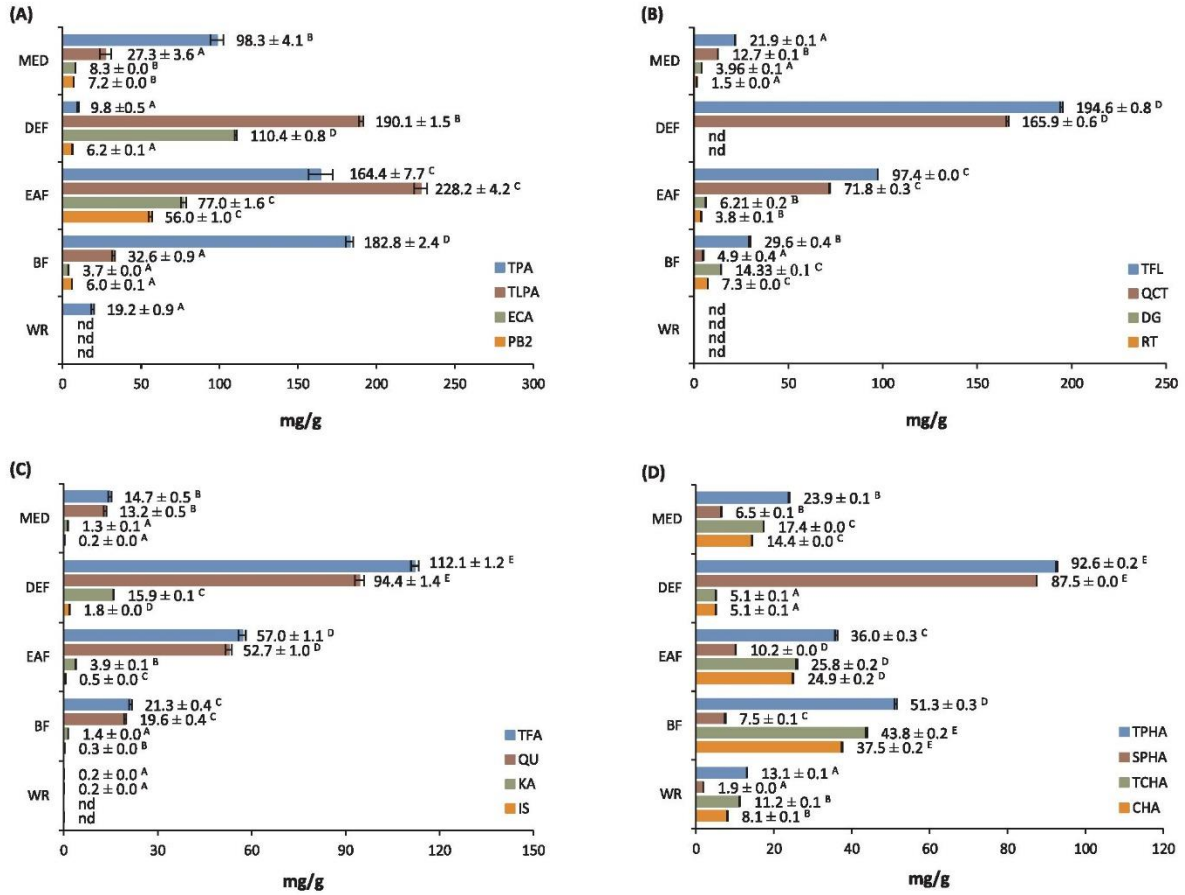


Fig. 3. Quantitative profile of *S. domestica* leaf extracts: flavan-3-ols and proanthocyanidins (A); native flavonoids (B); flavonoid aglycones after acid hydrolysis (C); phenolic acids (D). Data expressed as mean values ± SD ($n = 3$). For each parameter, different capitals indicate significant differences ($p < .05$). TPA, total proanthocyanidin content determined by the *n*-butanol/HCl assay; TLPA, total content of individual proanthocyanidins assayed by RP-HPLC; ECA, (−)-epicatechin; B2, procyanidin B2; TFL, total content of native flavonoids; QCT, quercitrin; RT, rutin; DG, quercetin hexoside-rhamnoside (peak 37 acc. to Table 1); TFA, total content of flavonoid aglycones released after acid hydrolysis; QU, quercetin; KA, kaempferol; IS, isorhamnetin; TPHA, total content of phenolic acids; SPHA, total content of simple hydroxybenzoic and hydroxycinnamic acids; TCHA, total content of chlorogenic acid isomers; CHA, chlorogenic acid.

Table 2
Antioxidant activity of *S. domestica* leaf dry extracts and standard antioxidants in DPPH, FRAP and TBARS tests.

Analyte	DPPH-radical scavenging activity		Ferric reducing antioxidant power		LA-peroxidation TBARS	
	EC_{50} (μg/mL)[a]	EC_{50} (μg GAE/mL)[b]	FRAP (mmol Fe^{2+}/g)[c]	FRAP (mmol Fe^{2+}/g GAE)[d]	IC_{50} (μg/mL)[e]	IC_{50} (μg GAE/mL)[f]
MED	10.16 ± 0.29 ^{H}	2.87	7.35 ± 0.21 ^{B}	26.04	11.87 ± 0.62 ^{G}	3.35
DEF	5.15 ± 0.21 ^{F}	3.28	18.92 ± 0.70 ^{E}	29.67	5.14 ± 0.13 ^{D}	3.28
EAF	3.61 ± 0.15 ^{D}	2.53	18.77 ± 0.05 ^{E}	26.81	3.51 ± 0.16 ^{C}	2.46
BF	5.46 ± 0.27 ^{F}	2.53	10.57 ± 0.28 ^{C}	22.79	6.65 ± 0.22 ^{E}	3.08
WR	37.72 ± 1.23 ^{I}	2.89	2.07 ± 0.06 ^{A}	26.99	46.46 ± 1.85 ^{H}	3.56
QU	1.63 ± 0.07 ^{A}	–	36.02 ± 1.10 ^{G}	–	1.85 ± 0.12 ^{A}	–
RT	4.19 ± 0.10 ^{E}	–	15.17 ± 0.25 ^{D}	–	6.01 ± 0.46 ^{D,E}	–
ECA	2.35 ± 0.15 ^{B}	–	35.79 ± 0.93 ^{G}	–	2.25 ± 0.19 ^{A,B}	–
CHA	4.42 ± 0.13 ^{E}	–	25.58 ± 0.52 ^{F}	–	2.49 ± 0.15 ^{A,B}	–
TX	4.34 ± 0.22 ^{E}	–	10.83 ± 0.32 ^{C}	–	8.47 ± 0.45 ^{F}	–
BHA	2.90 ± 0.14 ^{C}	–	16.13 ± 0.83 ^{D}	–	3.16 ± 0.22 ^{B,C}	–
BHT	6.54 ± 0.28 ^{G}	–	18.89 ± 0.42 ^{E}	–	9.31 ± 0.16 ^{F}	–

Results are presented as mean values (± SD for replicates, $n = 3$) calculated per dry weight of the extract or standard (QU, quercetin; RT, rutin; ECA, (–)-epicatechin; CHA, chlorogenic acid; TX, Trolox). For extract codes see Table 1. Different superscripts (capitals) in each column indicate significant differences in the means at $p < .05$. [a,b]Scavenging efficiency (amount of antioxidant needed to decrease the initial DPPH concentration by 50%) expressed as follows: [a]in μg of the dry extract or standard/mL of the DPPH solution; [b]in μg of phenolics/mL of the DPPH solution (values obtained by converting the original EC_{50} values using the TPC levels). [c]Values expressed per g of the dry extract or standard. [d]Values expressed per g of the phenolics (obtained by converting the original FRAP values using the TPC levels). [e,f]Linoleic Acid (LA) Peroxidation test; inhibition concentration (amount of antioxidant needed to decrease the LA-peroxidation by 50%) expressed as follows: [e]in μg of the dry extract or standard/mL of the LA solution; [f]in μg of phenolics/mL of the LA solution (values obtained by converting the original IC_{50} values using the TPC levels).

Like in the chemical tests, the activity of the extracts was comparable or higher than that of positive standards: rutin, quercetin, Trolox and chlorogenic acid (Fig. 2A–D). For instance, the range of percentage inhibition of tyrosine nitration achieved for the standards (about 13–42%) was lower than for the extracts (about 34–54%) applied at the same concentration of 5 μg/mL (Fig. 4A). Moreover, the activity of MED at 5 μg/mL was significantly higher ($p < .001$) than that of all standards. Furthermore quercetin, the strongest antioxidant in chemical models, was found to counteract the plasma nitration more strongly than all extracts except MED, but the observed differences in activity were relatively small (Fig. 4A; $p < .05$). A similar trend was observed for $ONOO^{-}$-induced lipid peroxidation with TBARS detection (Fig. 4C) and for the FRAP test (Fig. 4D). Only in the FOX-1 assay did quercetin reduce formation of hydroperoxides more effectively than all of the tested leaf extracts at the corresponding concentration; still activity of the extracts was not lower than that of three other standards (Fig. 4B; $p < .001$).

3.4. Inhibitory effects of the extracts on pro-inflammatory enzymes

The extracts inhibited the activity of lipoxygenase (LOX) and hyaluronidase (HYAL) in a concentration-dependent manner, but with different responses towards particular enzyme (Table 4). Considering the IC_{50} values expressed in μg/U, the extracts were stronger inhibitors of lipoxygenase than hyaluronidase. A slightly different order of potency was also observed in both tests, with EAF the most active towards lipoxygenase, and BF towards hyaluronidase. Still, inhibitory effects of the most potent extracts against both enzymes were comparable to those of positive standards, including indomethacin, a strong non-steroidal anti-inflammatory drug. The responses in the LOX test were strongly TPC-dependent ($r = -0.8969$, $p < .05$) with significant synergic effects demonstrated for the main groups of phenolics, e.g. TPA + TFL ($r = -0.9446$, $p < .05$) and TPA + TPHA ($r = -0.9032$, $p < .05$). The activity of extracts in the HYAL test was only slightly related to the TPC levels ($r = -0.3832$, $p > .05$), but strongly determined by proanthocyanidins (TPA; $r = -0.8843$, $p < .05$) and their synergic action with chlorogenic acids (TPA + TCHA; $r = -0.9324$, $p < .05$).

3.5. Influence of the extracts on cells viability

The potential cytotoxicity of the dry leaf extracts of *S. domestica* was evaluated in a model of PBMCs after 1–24 h of incubation with plant extracts at 1–50 μg/mL. Regardless of the incubation time and extract concentration, the viability of the PBMCs treated with the extracts constituted 93.5–99.9% of that of the control (untreated) samples. Cellular safety of the extracts was evidenced by the lack of significant differences ($p > .05$) between the respective results (Fig. S1).

4. Discussion

Modern phytotherapy recommends the use of plant medicines in the form of standardised dry extracts containing purified and concentrated active components, which are more effective than unprocessed plant materials. Extraction from natural matrices is thus a crucial step in the utilisation of phenolic compounds as it affects the composition, activity

Table 3
Correlation (*r*) coefficients and probability (*p*) values of linear relationships between antioxidant activity parameters and phenolic contents of *S. domestica* leaf dry extracts.

r (p) for:	Antioxidant activity (chemical models)			Antioxidant activity (human plasma model)			
	DPPH	FRAP	TBARS	3 NT-Fg	TBARS	FOX-1	FRAP
TPC	−0.8633 (0.059)	0.9850 (0.002)**	−0.8690 (0.056)	0.7173 (0.003)**	0.8812 (0.000)***	0.5676 (0.027)*	0.8760 (0.000)***
TPH	−0.6023 (0.282)	0.9295 (0.022)*	−0.6169 (0.268)	0.5966 (0.019)*	0.9122 (0.000)***	0.5266 (0.044)*	0.7039 (0.003)**
TPA	−0.5511 (0.336)	0.2099 (0.735)	−0.5359 (0.352)	0.5809 (0.023)*	0.4496 (0.093)	0.3657 (0.018)*	0.7560 (0.001)***
TLPA	−0.6093 (0.275)	0.9428 (0.016)*	−0.6229 (0.262)	0.5857 (0.022)*	0.8850 (0.000)***	0.4593 (0.085)	0.6679 (0.007)**
TFA	−0.5797 (0.306)	0.8786 (0.050)*	−0.5942 (0.291)	0.5686 (0.027)*	0.9034 (0.000)***	0.5348 (0.040)*	0.6812 (0.005)**
TFL	−0.5558 (0.331)	0.8701 (0.055)	−0.5709 (0.315)	0.5545 (0.032)*	0.8971 (0.000)***	0.5229 (0.045)*	0.6592 (0.008)**
TPHA	−0.6022 (0.283)	0.7390 (0.154)	−0.6091 (0.276)	0.6163 (0.014)*	0.8732 (0.000)***	0.6682 (0.006)**	0.7956 (0.000)***

Activity and concentration parameters according to Table 2 and Figs. 2–4. Asterisks mean statistical significance of the estimated linear relationships ($^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$).

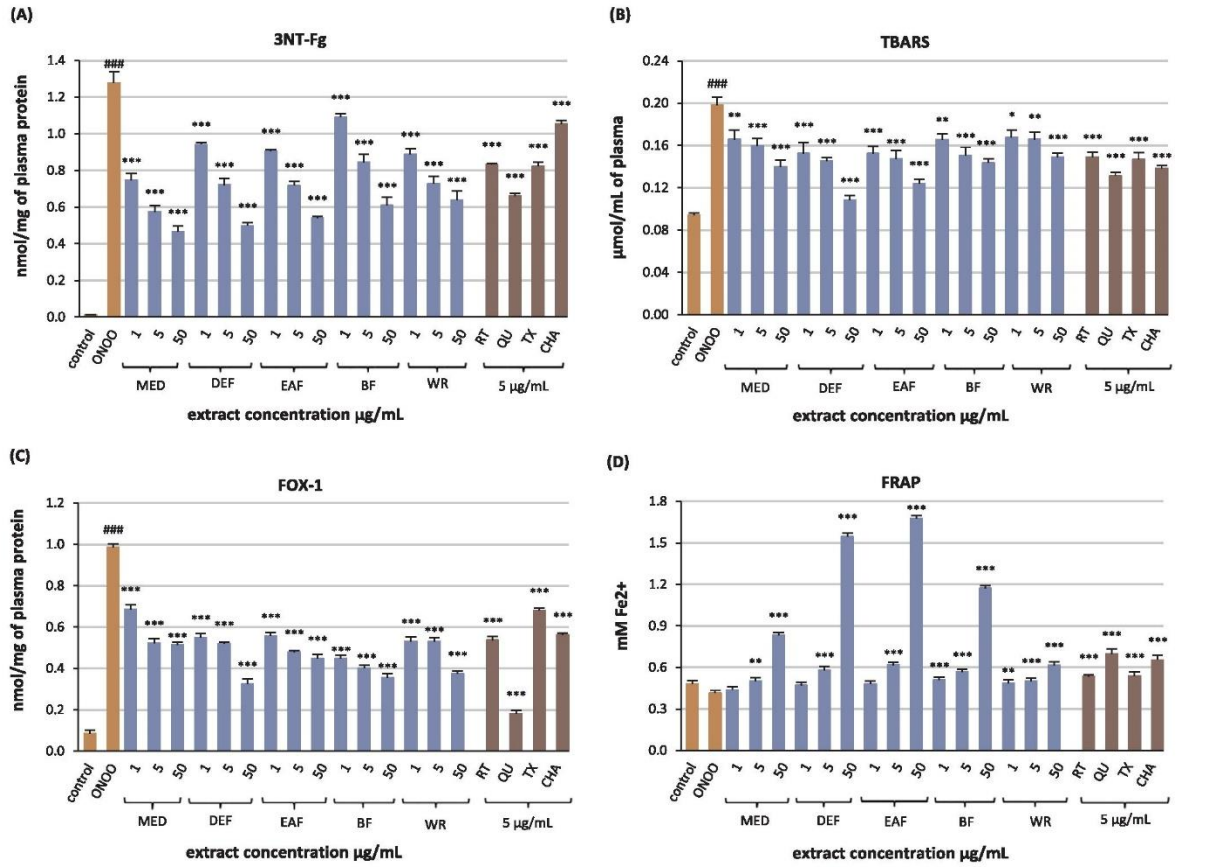


Fig. 4. Effects of *S. domestica* leaf extracts on human plasma exposed to oxidative stress: effects on the nitration of tyrosine residues in plasma proteins and formation of 3-nirotyrosine, 3-NT-Fg (A); effects on the peroxidation of plasma lipids including formation of lipid hydroperoxides, LOOH (B), and thiobarbituric acid-reactive substances, TBARS (C); effects on ferric reducing ability of plasma, FRAP (D). Results are presented as means ± SE ($n = 10$) for repeated measures: ###$p < .001$ for control plasma versus $ONOO^-$-treated plasma (without the extracts); *$p < .05$, **$p < .01$, ***$p < .001$ for $ONOO^-$-treated plasma in the presence of the extracts (1, 5, or 50 µg/mL) or standards (5 µg/mL) versus $ONOO^-$-treated plasma in the absence of the extracts. Standards: RT, rutin; QU, quercetin; TX, Trolox; CHA, chlorogenic acid.

Table 4
Inhibitory activity of *S. domestica* leaf extracts on lipoxygenase (LOX) and hyaluronidase (HYAL).

Analyte	LOX		HYAL	
	IC_{50} (µg/mL)[a]	IC_{50} (µg/U)[b]	IC_{50} (µg/mL)[a]	IC_{50} (µg/U)[b]
MED	264.69 ± 12.67 ^{E}	6.34	24.66 ± 0.10 ^{B}	10.94
DEF	134.23 ± 3.63 ^{C}	3.22	39.36 ± 0.68 ^{E}	17.45
EAF	115.54 ± 4.99 ^{B}	2.77	26.97 ± 0.51 $^{B, C}$	11.96
BF	155.51 ± 3.70 ^{D}	3.72	11.06 ± 0.30 ^{A}	4.90
WR	681.76 ± 21.93 ^{F}	16.33	50.24 ± 2.01 ^{F}	22.28
QU	89.23 ± 2.13 ^{A}	2.14	30.78 ± 1.84 ^{D}	13.65
RT	162.70 ± 3.70 ^{D}	3.90	54.63 ± 2.61 ^{G}	24.23
ECA	161.84 ± 8.05 ^{D}	3.88	23.76 ± 1.15 ^{B}	10.56
CHA	166.83 ± 7.15 ^{D}	4.00	28.59 ± 1.21 $^{C, D}$	12.68
IND	92.60 ± 3.71 ^{A}	2.22	12.77 ± 0.91 ^{A}	5.66

Results are presented as mean values ± SD ($n = 3$) calculated per dry weight of the extract or standard (IND, indomethacin). For other standards and extracts codes see Table 1. Different superscripts (capitals) in each column indicate significant differences in the means at $p < .05$. [a,b]Inhibition concentration (amount of analyte needed for 50% inhibition of enzyme activity) expressed as follows: [a]in µg of the dry extract or standard/mL of the enzyme solution; [b]in µg of the extracts/enzyme unit (U).

and yield of the extract. Our previous studies have established that the active phenolic fractions of the leaves of *Sorbus* may be effectively extracted with methanol-water (7:3, *v/v*) (Olszewska & Michel, 2009), and further purified by liquid-liquid partitioning (Olszewska et al., 2012). The results of the present study confirm these findings (Fig. 2). The total phenolic contents (both TPC and TPH levels) in the richest fractions, EAF and DEF, were comparable to those found in the extracts of plants considered important in the prevention of oxidative stress-related ailments, for instance in commercial extracts of grape seed (about 790–630 mg GAE/g dw) (Baydara, Özkanb, & Sağdiçç, 2004; Peschel, Dieckmanna, Sonnenschein, & Plescher, 2007) or green tea (about 450 mg GAE/g dw) (Peschel et al., 2007). Moreover, the obtained TPC values put the investigated leaf extracts in a good position compared to the leaf and flower extracts of other *Sorbus* plants (82.1–581.7 mg GAE/g dw; Olszewska et al., 2012) and demonstrate their superiority in comparison to those obtained from fruits of *S. domestica*. The advantage is most apparent in case of the initial alcoholic extracts, i.e. those exhibiting the highest extraction yields and thus the best industrial potential: 282.3 mg GAE/g dw for the leaf MED vs. 5.6–32.5 mg GAE/g dw for methanolic extracts from fruits of various maturity stages (Termentzi et al., 2006).

The LC-MS metabolite profile of the leaf extracts of *S. domestica* (Figs. 1–3; Table 1), presented here for the first time, is relatively simple in comparison to the composition of the fruits, with 44 constituents

found in leaves and 62 in the fruits, but is distinguished by the abundancy of flavan-3-ols, both monomeric catechins and oligomeric proanthocyanidins, which are absent from the ripe fruits utilised for dietary purposes (Termentzi, Kefalas, et al., 2008; Ölschläger et al., 2004). Flavanols, including (-)-epicatechin and procyanidin B2 dominating in the analysed extracts, are among the dietary antioxidants with the strongest effects *in vivo*. Intervention studies indicate that they act predominantly within the vascular system and may substantially increase the plasma antioxidant activity and plasma ascorbate concentrations, decrease platelet aggregation and LDL-oxidation, improve HDL/LDL ratio, reduce serum concentrations of pro-inflammatory cytokines and related inflammation of blood vessel walls, diminish capillary fragility and permeability, and decrease blood pressure (Williamson & Manach, 2005). Similar activity profiles are also known for quercetin glycosides and caffeoylquinic acids (Manach, Williamson, Morand, Scalbert, & Remesy, 2005; Middleton, Kandaswami, & Theoharides, 2000), found in the present study as accompanying constituents. The composition of the leaf extracts appears to offer promise with regard to their potential application as antioxidant and anti-inflammatory agents, especially in the prevention of cardiovascular disorders (CVD).

The antioxidant activity of the extracts was verified in complementary chemical and biological models. Although simple chemical *in vitro* assays are hardly relevant to *in vivo* conditions, they enable preliminary screening for potential mechanisms and comparison with the literature data. The present study employs the three most frequently-used tests of both single electron transfer (DPPH, FRAP) and hydrogen atom transfer basic mechanisms (TBARS) to explore direct interactions of the extracts with free-radicals and transition metal ions, as well as to study some of these effects in a model of lipid peroxidation. The results demonstrate that the leaf extracts are potent antioxidants, and that their concentration-dependent activity is determined by the content of phenolics, primarily proanthocyanidins and flavonoids, and is strongly influenced by the extraction solvent (Tables 2 and 3). The extracts from the fruits of *S. domestica* have also been reported as DPPH scavengers (Termentzi et al., 2006); however, after comparing those results to ours in terms of Trolox equivalent antiradical activity (TEAA), it is evident that leaf extracts are superior to those from fruit. For instance, the TEAA value for leaf MED was 0.427 g Trolox/g dw (recalculated from Table 2), while the fruit methanolic extracts exhibited the values of 0.009–0.122 g Trolox/g dw, depending on the fruit maturity (Termentzi et al., 2006).

To give a more accurate approximation of the possible *in vivo* effects of the *S. domestica* leaf extracts, we extended the study to the model of human plasma exposed to oxidative stress. From multiple oxidants known to be operating in plasma *in vivo* (Morita, Naito, Yoshikawa, & Niki, 2017), peroxynitrite ($ONOO^-$) was selected to induce the stress conditions. This powerful oxidative and nitrative species is generated *in vivo* by the reaction of nitric oxide (NO) and superoxide anion ($O_2^{\bullet-}$) and involved in the pathophysiology of various inflammatory, neurodegenerative, and especially cardiovascular disorders including atherosclerosis, myocardial infarction and chronic heart failure (Ronson, Nakamura, & Vinten-Johansen, 1999). The concentration of $ONOO^-$ used in our study (100 μM) enables quantitative measurements of the changes induced in plasma components and corresponds to its levels that can be reached *in vivo* in local compartments under the conditions of accelerated production of NO and $O_2^{\bullet-}$, e.g. during a serious inflammation of blood vessels, when its synthesis can increase up to 50–100 μM/min (Szabo, Ischiropoulos, & Radi, 2007). During the study, the plasma samples were pre-incubated with the leaf extracts of *S. domestica* in a wide concentration range (1–50 μg/mL), equivalent to 0.08–35.0 μg GAE/mL, depending on the extract. From the physiological point of view, the concentrations of phenolic substances that are likely to occur in blood plasma *in vivo* after oral supplementation can reach up to 5–7 μM, depending on the food matrix. For example, it has been reported that a 100 mg dose of quercetin from onions, a 150 mg dose of pure isoquercitrin (quercetin-3-glucoside), and a 323 mg dose of catechins from cocoa resulted in the concentrations of up to 7.6 μM, 5 μM, and 5.9 μM in plasma, respectively, which is an equivalent of 1.5–2.3 μg/mL of quercetin, and 1.7 μg/mL of catechin (Manach et al., 2005). On the other hand, Farah, Monteiro, Donangelo, and Lafay (2008) demonstrate that the total plasma levels of caffeoylquinic acids can reach even 14.8 μM (5.2 μg/mL) after ingestion of green coffee bean extract (200 mg). Therefore, the lower levels of the extracts (1–5 μg/mL) used in the present study appear to closely correspond to the range of physiological level of plant-derived phenolic compounds available after oral administration. In accordance with the common practice of *in vitro* studies (Kolodziejczyk-Czepas et al., 2011, 2013, 2014), the extracts were studied also at 50 μg/mL to enable observation of all possible effects of their interaction with peroxynitrite.

The results demonstrated that the leaf extracts of the service tree effectively enhanced the total antioxidant status of the $ONOO^-$-treated plasma, and diminished the levels of well-known oxidative stress biomarkers: the products of protein nitration (3-nitrotyrosine) and lipid peroxidation (hydroperoxides and TBARS) (Fig. 4). The mechanism of action was probably a direct and polyphenol-related scavenging of peroxynitrite or/and secondary radicals formed in the induced chain reactions. It is consistent with the noticeable scavenging potential of the extracts towards the model radical (DPPH), and with the accumulating evidence of anti-radical effects of polyphenols against $ONOO^-$ and $ONOO^-$-related radicals operating in plasma, such as $O_2^{\bullet-}$, NO, $^{\bullet}NO_2$, $^{\bullet}OH$, etc. (Heijnen, Haenen, Vekemans, & Bast, 2001). Moreover, all of the activity parameters in plasma were significantly correlated with phenolic contents and in the first line by both flavanols and flavonols (Table 3). The activity differences between the examined extracts were, however, not as evident as in the case of chemical tests. This may be an effect of the complexity and ontogenetic diversity of blood plasma and their influence on the interactions between endogenous plasma constituents and *S. domestica* phenolics. Numerous studies indicated that binding of polyphenols to proteins may suppress their antioxidant properties, and that these masking effects are dependent both on the proteins and polyphenols (Ozdal, Capanoglu, & Altay, 2013). On the other hand, the complexity of the extracts and synergic effects of their individual components are probably behind the superior activity profile revealed by the extracts compared to the pure standards.

Accumulating evidence indicates that the increased levels of the investigated oxidative stress biomarkers and low plasma antioxidant capacity are good predictors of various pathologies including cardiovascular events, diabetes, rheumatoid arthritis, chronic hepatitis and even multiple sclerosis and Alzheimer disease (Ahsan, 2013). For instance, in CVD patients, they are usually correlated with prothrombotic, proatherogenic, and pro-inflammatory intravascular effects, plaque instability, and endothelial dysfunctions (Lee, Margaritis, Channon, & Antoniades, 2012; Thomson, 2015). As the influence of exogenous non-enzymatic antioxidants on the oxidizability of plasma components *in vitro* has been linked with the corresponding *in vivo* effects (Atkin, Gasper, Ullegaddi, & Powers, 2005), the observed activity of the *S. domestica* leaf extracts might be an argument for their use in prophylaxis or adjunctive therapy of oxidative stress-related diseases.

The question remains, however, as to what extent our results might be relevant to *in vivo* conditions. Specifically, it is well documented that dietary polyphenols undergo extensive metabolisation in humans, which may include hydrolysis, methylation, glucuronidation, sulfonation, and oxidative degradation into phenolic acids (Chen, Zheng, Li, & Jiang, 2014; Ou & Gu, 2014). Still, also the main metabolites of flavonols and flavanols, such as glucuronides and/or sulphates of quercetin, isorhamnetin, and (–)-epicatechin, as well as phenolic artefacts of proanthocyanidins exhibit substantial protective effects against lipoprotein oxidation, although the magnitude of these effects is about 50% of that measured for free aglycones and monomers (Morand et al., 1998; Natsume et al., 2004; Ou & Gu, 2014). The leaf extracts analysed in the present study were composed in part from the compounds likely

to be converted *in vivo* into metabolites of lower activity (flavan-3-ols), but in part from those already conjugated (flavonol glycosides) and therefore likely to exert activity comparable to their metabolites. Considering that the extracts were found effective at the level of 1 μg/mL – an equivalent of 0.08–0.70 μg GAE/mL, which is markedly lower than the concentrations of phenolic metabolites (1.5–5.2 μg/mL) achievable in plasma after oral supplementation (Manach et al., 2005), they might be expected to act as antioxidants also in *in vivo* conditions at the appropriate oral doses. Indeed, in an earlier study of Lee, Lee, Lee, and Im (2006), a closely related phenolic extract of *Sorbus commixta* effectively reduced hepatic lipid peroxidation *in vivo*. In the context of future applications, it is of note that in a wide range of concentrations (1–50 μg/mL) including the physiologically-relevant levels the analysed extracts did not deteriorate the viability of peripheral blood mononuclear cells, and may be regarded as safe.

Oxidative stress *in vivo* is closely linked with inflammation, and thus, it is crucial that antioxidant therapies exert a simultaneous influence on both processes (Biswas, 2016). The present study investigates the anti-inflammatory potential of the extracts of *S. domestica* by determining their inhibitory activity towards two model enzymes involved in inflammation. The first belongs to the family of lipoxygenases, enzymes catalysing the dioxygenation of polyenoic fatty acids in biological membranes and in lipoproteins, and the production of key chemokines and reactive oxygen species, such as leukotrienes and $O_2^{\bullet-}$, associated with the development of oxidative stress-related inflammatory pathologies, e.g. atherosclerosis, myocardial infarction/reperfusion injury, rheumatoid arthritis, and cancer (Schneider & Bucar, 2005). The second enzyme, hyaluronidase, is a spreading factor increasing the permeability of tissues (i.a. vascular) during the inflammatory processes by degrading hyaluronan, an anti-inflammatory extracellular matrix component (Girish, Kemparaju, Nagaraju, & Vishwanath, 2009). Both enzymes are targets of many synthetic drugs used in anti-inflammatory therapies, but plant extracts, especially those containing phenolics, can also significantly inhibit their activity. Indeed, the investigated extracts, rich in polyphenols, were found to be potent inhibitors of both model enzymes (Table 4) and some synergy effects between phenolic constituents were evidenced. The activity of the most active organic solvent fractions was at least comparable to that of the commercial agents, i.e. indomethacin (nonsteroidal anti-inflammatory drug) and rutin (flavonoid vasoprotective agent), while the basic methanol-water extract was only slightly less active. These results might be a starting point of more extensive *in vitro* investigations of anti-inflammatory activity of the *S. domestica* extracts involving, i.a., a release of pro-inflammatory cytokines from stimulated immune cells. Moreover, together with the results of antioxidant activity tests, they suggest that the extracts might influence both interdependent processes of oxidative stress and inflammation.

5. Conclusions

This work is the first study of phytochemical composition, antioxidant activity, pro-inflammatory enzyme inhibition, and cellular safety of the leaf extracts of the service tree, and also the first evaluation of *S. domestica* antioxidants in complementary chemical and biological models. The results revealed that the leaves accumulate a vast diversity of bioactive polyphenols at the levels exceeding those reported previously for fruits of the same species (Termentzi et al., 2006). The leaf extracts appear to be superior to those from fruit also in terms of the activity, and thus have a great potential for the use as functional food, nutraceuticals or dietary supplements. Considering both the yield and the activity, the defatted methanol-water (7:3, *v/v*) extract and its organic solvent fractions are the most advantageous for biological applications. However, further research is required to specify in detail the activity profile of the leaf extracts, including other possible molecular mechanisms and *in vivo* effects. First of all, as oxidation of biological molecules *in vivo* is a complex phenomenon mediated by multiple oxidants (Morita et al., 2017; Prior, Sintara, & Chang, 2016), the activity of the extract against different biologically-relevant oxidants should be investigated. Moreover, as the observed protective effects of the extracts against $ONOO^-$-induced changes in plasma components and the inhibitory effect on pro-inflammatory enzymes might be related to their possible anti-atherogenic, anticoagulant and antiplatelet functions or ability to influence the endothelium, these issues should also be addressed in the future studies. Nowadays, when the interest in nutritional and health properties of dietary products has grown, and plant polyphenols are recommended both in the prevention and adjunctive therapy of oxidative stress-related diseases, especially CVD, the leaves of *S. domestica* represent promising herbal raw material that might be used for these purposes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.10.046.

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Sorbus domestica L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*

Magdalena Matczak[a], Anna Marchelak[a], Piotr Michel[a], Aleksandra Owczarek[a], Anna Piszczan[a], Joanna Kolodziejczyk-Czepas[b], Paweł Nowak[b], and Monika A. Olszewska[a].

[a]*Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Lodz, 90-151 Lodz, Poland*
[b]*Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland*

SUPPLEMENTARY MATERIAL: Figure S1

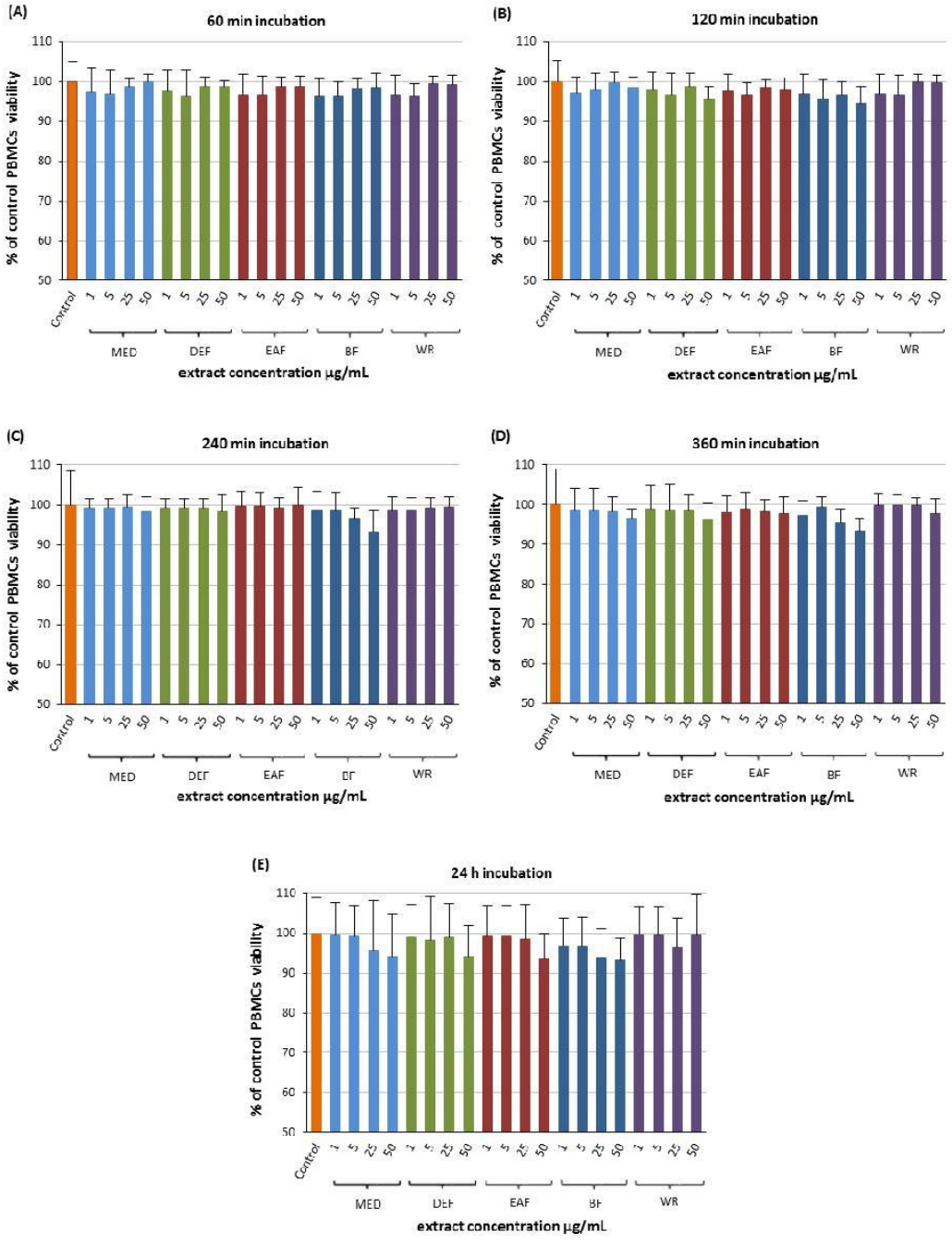


Fig. S1. Viability of peripheral blood mononuclear cells (PMBCs) after 1, 2, 4, 6 and 24 h of incubation with *S. domestica* dry leaf extracts at 1, 5, 25 and 50 µg/mL. MED, defatted methanol extract. DEF, diethyl-ether fraction. EAF, ethyl acetate fraction. BF, *n*-butanol fraction. WR, water residue. Results are presented as means ± SD (n = 18).

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Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control

Magdalena Rutkowska[a,*], Aleksandra Owczarek[a], Joanna Kolodziejczyk-Czepas[b], Piotr Michel[a], Dorota Gabriela Piotrowska[c], Paulina Kapusta[b], Pawel Nowak[b], Monika Anna Olszewska[a]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Lodz, 1 Muszynskiego St., 90-151 Lodz, Poland
[b] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 141/143 Pomorska, 90-236 Lodz, Poland
[c] Department of Bioorganic Chemistry, Faculty of Pharmacy, Medical University of Lodz, 1 Muszynskiego St., 90-151 Lodz, Poland

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ABSTRACT

This work is aimed at establishing analytical and bioactivity markers for the quality control of polyphenol-rich extracts of *Sorbus domestica* leaves – an antioxidant and anti-inflammatory agent. The potential marker analytes, representative of the main groups of *S. domestica* polyphenols, were pre-selected by LC–MS/MS analysis of the methanol-water (7:3, *v/v*) leaf extract (MED). For structural identification, two of them (rare flavonoids) were isolated from MED by preparative HPLC and analysed by 1D and 2D NMR (COSY, HMBC, HMQC) spectroscopy. The *in vitro* tests revealed that all analytes increase the non-enzymatic antioxidant capacity of plasma, effectively protect human plasma components against oxidative/nitrative damage induced by peroxynitrite (reduce the levels of thiobarbituric acid-reactive substances and 3-nitrotyrosine), and inhibit lipoxygenase and hyaluronidase. Considering the activity parameters and concentration levels, the highest contribution (70–80%) to the activity of the total phenolic fraction of MED was attributed to flavanols represented by (–)-epicatechin, procyanidin B2 and procyanidin C1. However, taking into account the observed synergistic effects, flavonoid and phenolic acid fractions (represented by chlorogenic acid, quercitrin, rutin, quercetin and its 3-*O*-(2″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside and 3-*O*-(2″-*O*-β-D-xylopyranosyl)-α-L-rhamnopyranoside) had also a substantial impact. All nine markers were thus applied as calibration standards, and a simple, accurate, reproducible and fully validated RP-HPLC-PDA method for the standardisation of the target extracts was proposed.

1. Introduction

Medicinal plants are a substantial source of active extracts that can be used in therapy and/or prevention of numerous complaints and may serve as a valuable starting point for drug development. However, herbal extracts are usually complex and contain different chemical constituents that contribute to their biological effects. The composition of the final medicinal product is affected by multiple factors that influence plants at the stage of growth, and then the extract during manufacturing process. Thus, a careful standardisation is required to assure their therapeutic effectiveness and safety (Yau et al., 2015). According to the European Medicines Agency, the quality of an herbal product/extract should be defined based on chromatographic fingerprinting (for purpose of identity and/or stability testing) and quantitative assays of selected phytochemicals that confer therapeutic properties (EMA, 2018). The selection of bioactivity markers suitable for quality control purposes of plant products is thus essential and should be established in integrated phytochemical and pharmacological studies.

Sorbus domestica L. (service tree) is a rosaceous deciduous tree, native to the south of Europe, where it is cultivated for its fruits and as a medicinal and decorative plant. The species is easy to acclimatise to temperate conditions of Central Europe with generally good results, except for diminished flowering and fruit production (Poljak et al., 2015). The fruits and leaves of service tree are traditional herbal medicines used for anti-inflammatory, anti-diabetic, diuretic and anti-atherogenic properties (Kültür, 2007; Termentzi et al., 2008). Due to high content of bioactive molecules (polyphenols) and because of their availability throughout the whole growing season and in different cultivation regions, leaves of the plant appear to be a favourable source

* Corresponding author.
E-mail address: magdalena.rutkowska@umed.lodz.pl (M. Rutkowska).

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material for development of advanced medicinal and functional food products. In our previous work, we reported on strong antioxidant and anti-inflammatory activity of leaf dry extracts of *S. domestica* that support their application in oxidative stress/inflammation-related pathologies (Matczak et al., 2018). In particular, we demonstrated the protective effects of the extracts against nitrative and oxidative damage to human plasma components under oxidative stress conditions and their inhibitory action towards pro-inflammatory enzymes, which mechanisms might be behind the activity of the leaves reported by traditional medicine. Among the molecules considered to influence the plant activity are flavonoids (flavonols and their glycosides), quinic acid pseudodepsides and proanthocyanidins composed of (–)-epicatechin (Ölschläger et al., 2004; Matczak et al., 2018; Termentzi et al., 2008, 2009). However, the impact of these groups of phenolics was concluded only on the basis of the linear correlations between their total contents and selected activity parameters (Termentzi et al., 2006; Matczak et al., 2018). No data is available on the contribution of individual polyphenols to biological effects of *S. domestica* extracts, which is crucial for the establishing of markers suitable for their quality control. There is also an information gap on the molecular structures of the leaf components. The leaf extracts are complex mixtures containing up to 44 phenolic constituents, depending on the extraction/fractionation solvent (Matczak et al., 2018). The UHPLC-PDA-ESI-MS^3 method, developed previously for qualitative fingerprinting of the extracts (Matczak et al., 2018), demonstrated good suitability for the authentication of extracts and plant material, but was of moderate usefulness for the structural identification of some leading constituents, especially flavonoid diglycosides. An unequivocal elucidation of their structures requires isolation of pure compounds and subsequent spectroscopic studies.

Therefore, the objectives of the present work were the selection and characterisation of the bioactivity markers suitable for the quality control of *S. domestica* leaves. Nine model leaf polyphenols were preselected based on the LC–MS/MS analysis of the defatted methanol-water (7:3, *v/v*) extract (MED). For full structural identification, two flavonoid diglycosides were isolated from the leaves by preparative HPLC and analysed by 1D and 2D NMR spectroscopy, hydrolytic experiments and absolute configuration studies of their sugar components. All compounds were subjected to *in vitro* evaluation of their anti-inflammatory and antioxidant effects in the studies of pro-inflammatory enzymes inhibition and in a model of human plasma exposed to oxidative stress (induced by peroxynitrite, $ONOO^-$), respectively. The ability of the compounds to reduce nitration/oxidation of plasma lipids and proteins, to enhance the total non-enzymatic antioxidant status of plasma (NEAC), and to inhibit hyaluronidase and lipoxygenase was assessed, and their contribution to the activity of MED was determined. Finally, an HPLC-PDA method for the quality control of the extract was proposed and successfully validated.

2. Results and discussion

2.1. Selection and identification of model S. domestica leaf polyphenols

Our previous work on the dry extracts from *S. domestica* leaves revealed that MED represents the best compromise between extraction yield and activity parameters (Matczak et al., 2018) and is thus the most advantageous for wider applications and closer investigation. For the purposes of this study, which required the isolation and identification of two primary flavonoid diglycosides, MED was freshly obtained in a preparative scale. The routine UHPLC-PDA-ESI-MS^3 analysis under previously described conditions (Matczak et al., 2018) allowed for detection of twenty five main polyphenols (UHPLC peaks **1–25**; Fig. 1), twenty three of which were fully or tentatively identified (Table 1) by comparing their retention times, UV–vis and MS spectra with those of the available reference compounds and literature data (Clifford et al., 2005; Fang et al., 2002; Ncube et al., 2014). The analysis revealed some qualitative differences in comparison to the semi-preparative scale MED examined earlier (Matczak et al., 2018). In particular, apart from fourteen compounds found previously, eleven more were discovered now (Table 1), however, at relatively low levels. These differences might be due to extraction scale-up and/or slight changes in the plant material during storage – the preparative MED was obtained after six month of storage of the leaf material in normal conditions. Also in the quantitative profile of the extract (Table 2) some slight but statistically significant ($p < 0.05$) differences from that of the semi-preparative MED (Matczak et al., 2018) were observed. Nevertheless, regardless of the extraction scale, nine compounds (coloured peaks in Fig. 1) dominated in the extracts and may be considered as model structures for primary groups of *S. domestica* leaf polyphenols, i.e. flavan-3-ols, proanthocyanidins, flavonol glycosides, and caffeoylquinic acids. These compounds were identified as (–)-epicatechin (**ECA**), procyanidin B2 (**PB2**) and procyanidin C1 (**PC1**) from the flavanols group (which accounted for about 75% of the total content of individual proanthocyanidins, TLPA, and about 26% of the total content of phenolic compounds, TPH, assayed by HPLC-PDA); rutin (**RT**), quercitrin (**QCT**), quercetin (**QU**) and two tentatively identified quercetin diglycosides (**HRQ** and **PRQ**) from flavonols group (which accounted for about 92% of the total flavonoid content, TFL, and about 31% of TPH); and chlorogenic acid (**CHA**) from caffeoylquinic acids (which accounted for about 63% of total phenolic acids, TPHA, and about 20% of TPH).

Numerous studies indicated that flavanols, including **ECA** and **PB2**, are among the dietary antioxidants with the strongest effects *in vivo*. Daily consumption of cocoa, grapes/wine or fruits rich in procyanidins has a positive effect especially on the cardiovascular system, and might substantially increase the ascorbate levels and plasma antioxidant capacity, improve HDL/LDL ratio, diminish capillary permeability and fragility, and reduce levels of pro-inflammatory cytokines in plasma, among others (Williamson and Manach, 2005). Similar activities have also been reported for caffeoylquinic acids and quercetin glycosides (Manach et al., 2005; Middleton et al., 2000), which indicates that the pre-selected nine model polyphenols might be good candidates for bioactivity markers of the *S. domestica* leaf extracts.

Before examining their influence on the biological effects of MED, two flavonoid analytes, **HRQ** and **PRQ** (Fig. 2), had to be isolated. Both compounds were separated from the *n*-butanol fraction of MED (obtained by liquid-liquid partitioning) using combination of gel permeation on Sephadex LH-20 and preparative RP-HPLC. Their structures were established based on spectroscopic methods, including ESI-MS, 1D and 2D NMR (^{1}H NMR, ^{13}C NMR, COSY, HMBC, HMQC) (Table 3) and literature data (Harborne, 1994; Francielli de Oliveira et al., 2014; Nielsen et al., 2005). The identity of aglycone and sugar components (including absolute configuration) was additionally confirmed/determined by HPLC analyses of acidic hydrolysates (Olszewska, 2008; Olszewska and Roj, 2011). Eventually, **HRQ** and **PRQ** were identified as quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside and quercetin 3-*O*-(2″-*O*-β-D-xylopyranosyl)-α-L-rhamnopyranoside, respectively. **PRQ** was previously isolated from several taxa, e.g. *Eugenia jambos* (L.) Alston, *Kalanchoë daigremontiana* Raym.-Hamet & H.Perrier, *Kalanchoë blossfeldiana* Poelln., *Fagopyrum tataricum* (L.) Gaertn., and *Persicaria mitis* (Schrank) Holub. (Slowing et al., 1996; Ürményi et al., 2016; Nielsen et al., 2005; Ren et al., 2013; Granica and Hink, 2015), while **HRQ** was obtained for the first time from *Ginkgo biloba* L. leaves (Hasler et al., 1992) and since then it was found only in eight other species, i.e. *Kalanchoë blossfeldiana* Poelln., *Roupala montana* Aubl., *Sabia japonica* Maxim., *Urceola rosea* (Hook. & Arn.) D.J.Middleton, *Saururus chinensis* (Lour.) Baill., *Botrychium ternatum* (Thunb.) Sw., *Alphitonia neocaledonica* (Schltr.) Guillaumin., and *Cheilanthes grisea* Blanf. (Francielli de Oliveira et al., 2014; Lee et al., 2012; Mishra and Verma, 2013; Muhammad et al., 2015; Nguyen Ngoc et al., 2018; Nielsen et al., 2005; Warashina et al., 2012; Yu et al., 2009). To our knowledge, this is the first report on these compounds in *Sorbus* species and Rosaceae family. Since the both compounds are rare, they might be

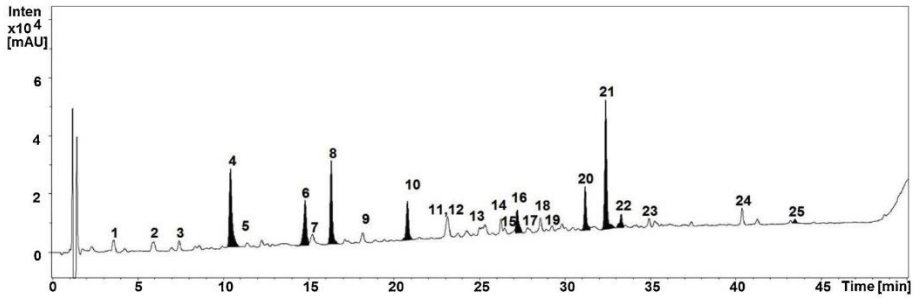


Fig. 1. UHPLC sample chromatogram of the MED extract at 280 nm. Peak numbers refer to those implemented in Table 1. Nine model compounds (painted) are: **CHA** (4), **PB2** (6), **ECA** (8), **PC1** (10), **RT** (16), **HRQ** (20), **QCT** (21), **PRQ** (22), and **QU** (25).

interesting from the chemotaxonomic point of view. Especially characteristic of *Sorbus domestica* seems to be the glucorhamnose substitution as the molecule similar to **HRQ**, i.e. quercetin 3-*O*-(3″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside, was previously separated from the fruits (Termentzi et al., 2009).

2.2. Biological activity of model S. domestica leaf polyphenols

Our previous research pointed to strong antioxidant and anti-inflammatory effects of *S. domestica* leaf extracts in both chemical and biological models (Matczak et al., 2018). In the present work we studied the antioxidant activity of the nine selected polyphenols and juxtaposed this activity with that of MED using the most complex and *in vivo*-relevant of the previously applied methods – the model of human plasma exposed to $ONOO^-$-induced oxidative stress. *In vivo*, $ONOO^-$ is involved in the pathophysiology of serious oxidative stress/inflammation-related disorders, such as cardiovascular, neuro-inflammatory and neurodegenerative diseases, diabetes and diabetic complications, rheumatoid arthritis, colitis, *etc*. (Luo et al., 2010; Pacher et al., 2007).

As shown in Fig. 3, in comparison to the control plasma, the $ONOO^-$-treated serum samples exhibited a visibly raised level ($p < 0.001$; Fig. 3A-B) of well-known oxidative stress biomarkers: the

Table 2
Quantitative profile of the source leaf extract (MED).

Compound	Content (mg/g dw)	Fraction	Content (mg/g dw)
CHA	13.66 ± 0.61	TPC (GAE)	253.4 ± 9.6*
ECA	7.62 ± 0.22*	TPA (CYE)	82.8 ± 2.7*
PB2	5.87 ± 0.22*	TPH	68.6 ± 3.3*
PC1	4.32 ± 0.51	TPHA	21.8 ± 1.2*
QCT	12.35 ± 0.57	TLPA	23.9 ± 1.2*
RT	2.10 ± 0.05*	TFL	22.9 ± 1.1
HRQ	4.92 ± 0.17*		
PRQ	1.49 ± 0.06		
QU	0.16 ± 0.01		

Results are presented as means ± SD ($n = 3$), calculated per dw of the compound or MED (for fractions of compounds). The values marked with an asterisk differ significantly ($p < 0.05$) from those obtained earlier for the semi-preparative scale extract (Matczak et al., 2018). Abbreviations: TPC, total phenolic content (Folin-Ciocalteu assay) expressed in GAE, gallic acid equivalents. TPH, total phenolic content (HPLC-PDA-peaks). TFL, total flavonoid content (HPLC-PDA-peaks). TPHA, total content of phenolic acids (HPLC-PDA-peaks). TPA, total proanthocyanidin content (*n*-butanol/HCl assay) expressed in CYE, cyanidin chloride equivalents. TLPA, total content of low-molecular-mass flavanols and proanthocyanidins (HPLC-PDA-peaks). For analytes codes see Table 1.

Table 1
UHPLC-PDA-ESI-MS^3 data of polyphenols identified in the *S. domestica* leaf MED extract.

No	Analyte	R_t (min)	UV λ_{max} (nm)	$[M\text{-}H]^-$	MS^2	MS^3
1	protocatechuic acid hexoside	3.6	235,315	315	153, 217,109	
2	3-*O*-caffeoylquinic acid[a]	5.9	325	353	191, 179	
3	*p*-hydroxybenzoic acid derivative	7.4	254	195	137, 97, 93	
4	5-*O*-caffeoylquinic acid (**CHA**)[a]	10.5	325	353	191, 179	
5	4-*O*-caffeoylquinic acid[a]	11.3	325	353	173, 191, 179	
6	procyanidin B2 (**PB2**)[a]	14.8	280	577	451, **425**, 407, 289	407, 273
7	procyanidin dimer B-type	15.1	280	577	451, **425**, 407, 289	407, 273
8	(-)-epicatechin (**ECA**)[a]	16.3	280	289	245, 205	
9	3-*O*-caffeoyl shikimic acid	18.1	325	335	179, 161, 135	
10	procyanidin C1 (**PC1**)[a]	20.8	280	865	**713**, 695, 577	695, 425, 407, 289
11	proantocyanidin dimer B-type	23.0	280	577	425, 407, 289	
12	procyanidin trimer B-type	23.1	280	865	**713**, 695, 577	695, 425, 407, 289
13	procyanidin dimer hexoside	25.1	280	739	**587**, 451, 339, 289	477, 417, 339, 255
14	unidentified compound	26.2	280	451	341, 217	
15	hyperoside[a]	26.5	254, 353	463	**301**	179
16	rutin (**RT**)[a]	27.2	256, 355	609	463, 301, 179	
17	isoquercitrin[a]	28.0	256, 353	463	**301**	179
18	procyanidin dimer B-type	28.7	280	577	451, **425**, 407, 289	289, 341, 245
19	reinutrin[a]	29.2	275, 350	433	301	
20	quercetin hexoside-rhamnoside (**HRQ**)	31.2	256, 350	609	**447**, 301, 179	301
21	quercitrin (**QCT**)[a]	32.5	276, 350	447	301, 179	
22	quercetin pentoside-rhamnoside (**PRQ**)	33.2	256, 353	579	**447**, 301	301
23	quercitrin derivative	35.0	276,350	573	**447**, 301	301
24	unidentified compound	40.3	280	451	341, **299**	189
25	quercetin (**QU**)[a]	43.5	255, 364	301	301	

[a] Identified with the authentic standards. R_t, retention times. UV λ_{max}, absorbance maxima in PDA spectra. $[M-H]^-$, pseudomolecular ions in MS spectra recorded in a negative mode. MS^2/MS^3, secondary ions (MS^2 ions in bold were subjected to MS^3 fragmentation). MED, defatted methanol-water (7:3, *v/v*) extract.

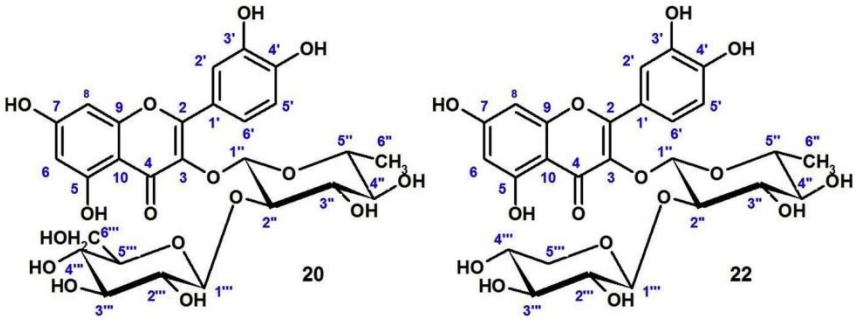


Fig. 2. Structures of the isolated quercetin diglycosides.

Table 3
NMR spectral data of **HRQ** and **PRQ** in methanol-d_4 (600 MHz for ^{1}H and 150.9 MHz for ^{13}C).

Pos.	HRQ		PRQ	
	δ_H	δ_C	δ_H	δ_C
2		157.9		157.8
3		135.1		135.2
4		178.2		178.4
5		161.7		162.7
6	6.11 (1H, *d*, *J* = 2.2)	98.6	6.13 (1H, *d*, *J* = 1.9)	98.5
7		164.6		164.7
8	6.28 (1H, *d*, *J* = 1.9)	93.4	6.39 (1H, *d*, *J* = 1.5)	93.4
9		157.2		157.2
10		104.5		104.4
1'		121.5		121.3
2'	7.26 (1H, *d*, *J* = 2.2)	115.6	7.37 (1H, *d*, *J* = 1.9)	115.5
3'		145.1		145.1
4'		148.5		148.2
5'	6.84 (1H, d, *J* = 8.3)	115.1	6.96 (1H, *d*, *J* = 8.3)	115.1
6'	7.22 (1H, *dd*, J_1 = 2.2, J_2 = 8.3)	121.5	7.32 (1H, *dd*, J_1 = 1.9, J_2 = 8.3)	121.3
	3-α-L-rhamnopyranosyl:		3-α-L-rhamnopyranosyl:	
1"	5.54 (1H, *d*, *J* = 0.8)	101.2	5.38 (1H, *d*, *J* = 0.8)	101.9
2"	4.18 (1H, *dd*, J_1 = 1.5, J_2 = 3.4)	81.4	4.21 (1H, *dd*, J_1 = 1.9, J_2 = 3.4)	81.3
3"	3.76 (1H, *dd*, J_1 = 3.4, J_2 = 9.8)	70.5	3.88 (1H, *dd*, J_1 = 3.4, J_2 = 9.8)	70.6
4"	3.25 (1H, *t*, *J* = 9.8)	72.2	3.37 (1H, *t*, *J* = 9.8)	72.4
5"	3.48 (1H, *dq*, J_1 = 6.4, J_2 = 9.8)	70.5	3.85 (1H, *dq*, J_1 = 6.4, J_2 = 9.8)	70.4
6"	0.88 (3H, *d*, *J* = 6.4)	16.3	1.05 (3H, *d*, *J* = 6.4)	16.3
	2"-β-D-glucopyranosyl:		2"-β-D-xylopyranosyl:	
1"'	4.28 (1H, *d*, *J* = 7.9)	105.7	4.29 (1H, *d*, *J* = 7.5)	106.4
2"'	3.13 (1H, *t*, *J* = 7.9)	74.0	3.21 (1H, *t*, *J* = 7.9)	73.9
3"'	3.24 (1H, *t*, *J* = 8.7)	76.5	3.31 (1H, *t*, *J* = 9.0)	76.4
4"'	3.28 (1H, *t*, *J* = 9.3)	69.5	3.41 (1H, *dt*, J_1 = 5.3, J_2 = 10.2)	69.6
5"'	3.08 (1H, *dt*, J_1 = 9.3, J_2 = 3.4)	76.7	3.09 (1H, *dd*, J_1 = 10.2, J_2 = 11.7, H-5$_a$"') 3.68 (1H, *dd*, J_1 = 5.3, J_2 = 11.7, H-5$_e$"')	65.7
6"'	3.57 (2H, *d*, *J* = 3.4)	60.9		

Data acquired with TMS as the internal standard, δ in ppm. Multiplicities and coupling constants (in Hz) are given in parentheses. Assignments confirmed by ^{1}H–^{1}H COSY, HMQC and HMBC experiments. For trivial atom numbering, see chemical formulas of **HRQ** and **PRQ** (Fig. 1).

products of lipid peroxidation (thiobarbituric acid reactive substances, TBARS) and protein nitration (3-nitrotyrosine, 3NT), as well as a moderate decrease ($p < 0.01$; Fig. 3C) in the NEAC value measured by the ferric reducing ability of plasma (FRAP) assay. In the presence of the tested compounds or MED extract, the rate of oxidative/nitrative damage was significantly reduced ($p < 0.05$). All tested polyphenols efficiently reduced the nitration of tyrosine residues ($p < 0.001$; Fig. 3A) by about 46–75% and 49–90% at 1 μg/mL and 50 μg/mL, respectively. Among analytes, **ECA** displayed the strongest activity at all concentration levels (75–90% decrease in tyrosine nitration) and was one of the molecules (besides **PB2**, **CHA**) showing significant dose-dependent effect ($p < 0.05$). All tested compounds exhibited also protective properties against lipid peroxidation induced by $ONOO^-$ ($p < 0.05$; Fig. 3B), regardless of the concentration levels. **ECA**, **PB2** and **PC1** were the most effective compounds, i.e. at 1 μg/mL they decreased the levels of TBARS by about 20–23%. Interestingly, for the large number of compounds (i.e. **CHA**, **ECA**, **PB2**, **PC1**, **QCT** and **PRQ**) the higher concentration corresponded to the lower activity. While no pro-oxidative effect was found in the experiments with the control plasma, this phenomenon may be due to interactions of polyphenols with serum components, which may result in protein binding or some solubility issues, among others (Ozdal et al., 2013). Nevertheless, depending on the concentration, the analysed compounds were able to normalize and/or enhance the NEAC status of the $ONOO^-$-treated plasma; however, **RT**, **HRQ** and **PRQ** at the 1–5 μg/mL did not modify significantly the FRAP value of the oxidised plasma ($p > 0.05$; Fig. 3C). For all other analytes, a significant improvement in the plasma reducing ability was observed, with the increase up to about 212% compared to the $ONOO^-$-stimulated plasma and 190% compared to the control (untreated) sample for **PC1** at 50 μg/mL ($p < 0.001$). Eventually, the protective effects of the tested nine *S. domestica* polyphenols on human plasma components were similar or more pronounced than those of positive standard – ascorbic acid (AA), the main antioxidant of human plasma *in vivo*. Catechins and proanthocyanidins (**ECA**, **PB2**, **PC1**) were the most effective antioxidants in all tests and might be thus considered as the main bioactivity markers of the source extract (MED), observed in this and previous (Matczak et al., 2018) experiments.

However, considering that phenolic fraction constitutes only a part of the dry weight of MED (its total phenolic content, TPC, determined by the Folin-Ciocalteu assay was 224.5 ± 9.6 mg/g dw), its protective effect on human plasma components was higher than expected, i.e. comparable to pure polyphenols. This gap may be assigned to synergy between the individual constituents. To evaluate these effects and approximate the contribution of individual compounds to the activity of MED, we expressed the observed activity parameters of all analytes in equivalents of AA and, taking into account the content of particular constituents and groups of constituents in MED (Table 2), we calculated their additive contribution to the extract capacity (Table 4). According to the results, the additive effects of total polyphenols differ between tests being the highest for the FRAP assay (72.5% of the MED activity) and noticeably lower for the 3NT (17.8%) and TBARS (12.8%) tests. In consequence, the possible synergy might be behind ca. 27%, 82%, and 87% of the antioxidant capacity of MED, respectively. However, to get more accurate data regarding this issue further research is necessary. Nevertheless, the presented calculations enabled to conclude that the prevailing influence on the MED activity might be ascribed to the total proanthocyanidins (TPA) and low molecular weight catechins (TLPA),

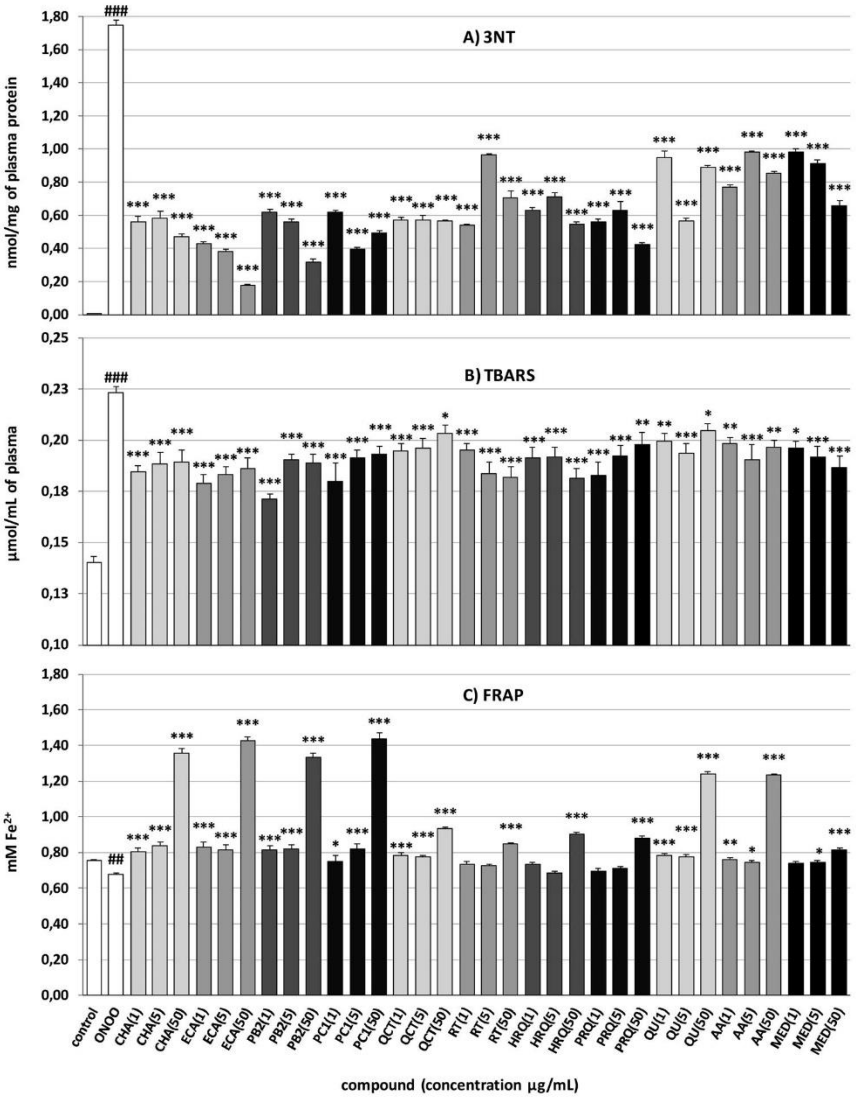


Fig. 3. Effects of *S. domestica* model compounds and MED on human plasma exposed to oxidative stress: (A) effects on the nitration of tyrosine residues in plasma proteins and formation of 3NT; (B) effects on the peroxidation of plasma lipids including formation of TBARS; (C) effects on NEAC of plasma (measured as the ferric-reducing ability of plasma - FRAP). Results are presented as means ± SE ($n = 10$) for repeated measures: $###p < 0.001$, $##p < 0.01$ for control plasma versus $ONOO^-$-treated plasma (without the analytes); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ for $ONOO^-$-treated plasma in the presence of the analytes (1, 5, or 50 µg/mL) versus $ONOO^-$-treated plasma in the absence of the analytes. For compounds codes see Table 1. Standard: AA, ascorbic acid.

the contents of which explained 70–80% of the activity of total phenolic fraction (TPH + TPA) of MED (Table 4). Although the relative contribution of phenolic acids (TPHA) and flavonoids (TFL) was lower, they cannot be disregarded as bioactivity markers because together their contents explained 20–29% of the activity of TPH + TPA, and **CHA** exhibited the highest contribution to the MED activity among all individual polyphenols tested (Table 4).

The valuable quality control markers ought to contribute in a significant manner to the activity of the plant product *in vivo*. It is well established that the biological effects of phenolics *in vivo* might concentrate in the enteron or be systemic, depending on their absorption and bioavailability (Manach et al., 2005). Depending on their physicochemical properties and behaviour in the intestine, the plasma phenolic levels are reported to be up to 2.5 µg/mL, 2.3 µg/mL and 5.2 µg/mL, for flavonoids, procyanidins and caffeoylquinic acids, respectively (Farah et al., 2008; Hollman, 2004; Ou and Gu, 2014; Thilakarathna and Rupasinghe, 2013; Viskupičová et al., 2008). Considering that, the lower levels (1–5 µg/mL) of the tested compounds used in the present work appear to well correspond to the range of physiological levels of plant-derived polyphenols achievable in the plasma after oral administration. On the other hand, polyphenols, besides revealing systemic action, may have some local effects. It is estimated that about 90–95% of phenolics reach the large intestine and accumulate at the millimolar levels in the digestive tract (Gracia-Sancho and Salvadó, 2017). Therefore, the antioxidant properties observed *in vitro* at concentrations 50 µg/mL or higher may be relevant to *in vivo* conditions in the gastrointestinal compartment. The gastrointestinal effects of polyphenols *via* redox mechanisms have recently been updated to include the modulation of nitrite-dependent oxidation and/or nitration of both lipids and proteins (Gracia-Sancho and Salvadó, 2017; Rocha et al., 2014). Reactive oxygen/nitrogen species (ROS) induce intestinal barrier dysfunction and increase gut permeability by modulating the epithelial cell cytoskeleton and microcirculation, which leads to a variety of diseases, including irritable bowel syndrome, inflammatory bowel disease, gastritis, gastroduodenal ulcers or cancers (Gracia-Sancho and Salvadó, 2017). However, polyphenols can alleviate symptoms of these disorders not only by antioxidant action but also by various anti-inflammatory mechanisms.

Table 4
Contribution of *S. domestica* model compounds to the activity of MED in the model of human plasma exposed to oxidative stress.

Analyte	3NT			TBARS			FRAP		
	AAE (g/g)	CT (%)	RCT (%)	AAE (g/g)	CT (%)	RCT (%)	AAE (g/g)	CT (%)	RCT (%)
CHA	1.42	1.60	8.98	1.27	1.27	9.92	1.21	6.69	9.23
ECA	1.75	1.10	6.17	1.38	0.77	6.02	1.35	4.15	5.73
PB2	1.59	0.77	4.32	1.28	0.55	4.30	1.18	2.79	3.85
PC1	1.40	0.50	2.81	1.12	0.36	2.81	1.36	2.38	3.28
QCT	1.32	1.35	7.58	0.75	0.68	5.31	0.46	2.27	3.13
RT	1.16	0.20	1.12	1.55	0.24	1.88	0.30	0.25	0.35
HRQ	1.34	0.55	3.09	1.56	0.56	4.38	0.41	0.81	1.12
PRQ	1.48	0.18	1.01	0.95	0.10	0.78	0.36	0.22	0.30
QU	0.96	0.013	0.07	0.70	0.008	0.06	1.01	0.07	0.10
TPHA	–	2.56	14.36	–	2.03	15.86	–	10.67	14.72
TLPA	–	3.18	17.85	–	2.25	17.58	–	12.21	16.85
TPA	–	9.57	53.70	–	6.82	53.28	–	45.60	62.92
TFL	–	2.51	14.09	–	1.70	13.28	–	3.99	5.51
TPH + TPA	–	17.82	100.0	–	12.80	100.0	–	72.47	100.0
MED	1.21	100.0	–	1.36	100.0	–	0.25	100.0	–

For analytes codes see Tables 1 and 2. AAE, ascorbic acid equivalents calculated from the experimental values in 3NT, TBARS, and FRAP tests obtained at 50 μg/mL (Fig. 3). CT, contribution to the activity of MED based on the theoretical additive values calculated acc. to Eq. 1. RCT, contribution to the activity of total phenolic fraction (TPH + TPA) calculated acc. to Eq. 2.

Table 5
Inhibitory activity of *S. domestica* model compounds on lipoxygenase (LOX) and hyaluronidase (HYAL), and their contribution to the activity of MED.

Analyte	LOX					HYAL				
	IC_{50} (μg/mL)	IC_{50} (μg/U)	IE (g/g)	CT (%)	RCT (%)	IC_{50} (μg/mL)	IC_{50} (μg/U)	IE (g/g)	CT (%)	RCT (%)
CHA	166.83 ± 7.66 ^{C}	4.00	0.55	2.15	6.35	28.59 ± 1.21 ^{B,C}	12.68	0.45	1.18	9.83
ECA	161.84 ± 8.05 ^{C}	3.88	0.57	1.24	3.66	23.76 ± 1.15 ^{B}	10.56	0.54	0.79	6.58
PB2	121.20 ± 6.01 ^{B}	2.91	0.76	1.27	3.75	24.63 ± 1.26 ^{B}	10.95	0.52	0.59	4.92
PC1	102.77 ± 4.95 ^{A}	2.46	0.90	1.11	3.28	30.23 ± 1.36 ^{C}	13.44	0.42	0.35	2.92
QCT	131.21 ± 4.25 ^{B}	3.15	0.71	2.51	7.41	52.11 ± 1.68 ^{D}	23.16	0.25	0.59	4.92
RT	162.70 ± 3.70 ^{C}	3.90	0.57	0.34	1.00	54.63 ± 2.61 $^{D, E}$	24.28	0.23	0.09	0.75
HRQ	190.51 ± 6.40 ^{D}	4.57	0.49	0.69	2.04	58.88 ± 1.97 ^{E}	26.17	0.22	0.21	1.75
PRQ	174.87 ± 5.36 $^{C, D}$	4.19	0.53	0.23	0.68	57.80 ± 2.60 ^{E}	25.69	0.22	0.06	0.50
QU	89.23 ± 2.13 ^{A}	2.14	1.04	0.01	0.03	30.78 ± 1.84 ^{C}	13.65	0.94	0.03	0.25
IND	92.60 ± 4.71 ^{A}	2.22	–	–	–	12.77 ± 0.91 ^{A}	5.66	–		–
TPHA	–	–	–	3.43	10.13	–	–	–	1.89	15.75
TLPA	–	–	–	4.95	14.62	–	–	–	2.34	19.50
TPA	–	–	–	21.29	62.88	–	–	–	6.69	55.75
TFL	–	–	–	4.19	12.37	–	–	–	1.08	9.00
TPH + TPA	–	–	–	33.86	100	–	–	–	12.00	100
MED	264.69 ± 12.67 ^{E}	6.34	0.35	100	–	24.66 ± 0.50 ^{B}	10.94	0.52	100	–

For analytes codes see Tables 1 and 2. The IC_{50} values are presented as means ± SD ($n = 3$), calculated per dw of the analyte, and expressed in μg/mL of the enzyme solution and in μg/enzyme unit (U). Different superscripts (capitals) in each column indicate significant differences in the means at $p < 0.05$. IE, indomethacin equivalents calculated from the IC_{50} values. CT, contribution to the activity of MED based on the theoretical additive values calculated acc. to Eq. (1). RCT, contribution to the activity of total phenolic fraction (TPH + TPA) calculated acc. to Eq. (2).

Therefore, the anti-inflammatory potential of nine selected *S. domestica* polyphenols was investigated in the present study by the evaluation of their inhibitory potential toward two model pro-inflammatory enzymes, i.e. lipoxygenase (LOX) – the enzyme involved in the production of key chemokines and ROS, and hyaluronidase (HYAL) – a spreading factor increasing tissues permeability and involved in the progression of inflammation (Mashima and Okuyama, 2015; Girish et al., 2009). Similarly to fractionated leaf extracts analysed previously (Matczak et al., 2018), the pure phenols were found to be potent inhibitors of both enzymes (Table 5) and exhibited stronger effects towards LOX than HYAL (considering the IC_{50} values expressed in μg/U). The inhibitory activity of the most effective compounds, i.e. **QU** and **PC1** against LOX was not significantly different ($p < 0.05$) from the activity of positive standard – indomethacin (IND), a strong non-steroidal anti-inflammatory drug. On the other hand, the most active against HYAL were: **ECA**, **PB2**, **PC1**, and **CHA**. The relative contribution of the analytes to the activity of MED was calculated as described above for human plasma models, after conversion of the IC_{50} values to equivalents of IND (Table 5). The additive effects of total polyphenols explained only 12.0% and 33.9% of the MED activity towards HYAL and LOX, respectively, suggesting possible synergic interactions of individual components. As in the case of antioxidant activity, the prime contributors to the anti-inflammatory activity of MED were catechins and proanthocyanidins, which contents explained 75–78% of the activity of total phenols (TPH + TPA), depending on the target enzyme. Although the impact of flavonoids and phenolic acids was lower, their representatives such as **QCT** and **CHA** exhibited the highest contribution to the MED activity towards LOX and HYAL, respectively, among all individual polyphenols tested (Table 5).

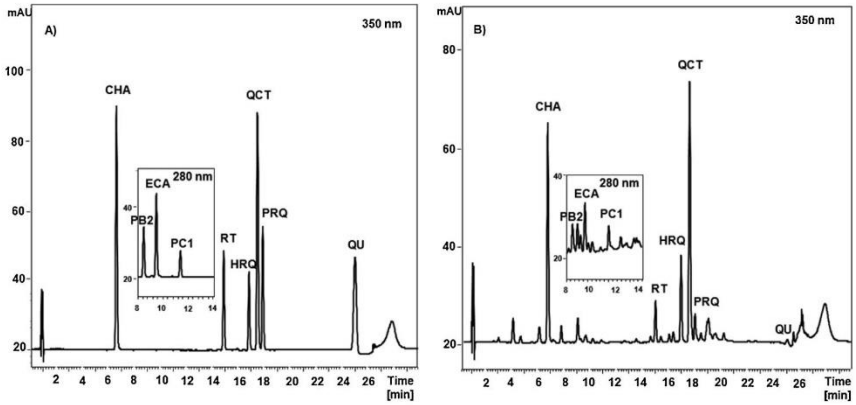


Fig. 4. HPLC-UV representative chromatograms at 350 nm and 280 nm of: (A) the standards mixture (111.4 µg/mL CHA, 44.2 µg/mL PB2, 74.1 µg/mL ECA, 23.0 µg/mL PC1, 45.1 µg/mL RT, 32.9 µg/mL HRQ, 81.4 µg/mL QCT, 51.2 µg/mL PRQ, 39.0 µg/mL QU); (B) the real sample of MED. For analytes codes see Table 1.

2.3. Validation of HPLC-PDA assay

Based on the obtained results we proposed an HPLC-PDA method for the quality control of the *S. domestica* leaf extracts. The procedure was optimised to achieve acceptable separation quality of all main extracts constituents (Fig. 4) at shorter time (26 min) than used in the qualitative UHPLC profiling (50 min), which was possible with the application of a RP-18 fused-core column technology (Olszewska, 2012). The method was successfully applied for quantification of polyphenols in the *S. domestica* extracts in this and previous work (Matczak et al., 2018). The present work allowed us to confirm that nine selected polyphenols are appropriate to serve as analytical markers. Despite low concentration of **PRQ** and **QU** in MED (Fig. 1), we decided not to remove these compounds from the set as our previous work revealed that some concentrated leaf fractions may contain higher levels of both constituents (Matczak et al., 2018). Moreover, **PRQ**, being relatively rare in the nature, might be considered as analytical and chemotaxonomic marker of the investigated plan material, while **QU**, being the main flavonoid aglycone, it may be an indicator of the extract stability (hydrolysis of the native glycosides). The quantitative method was validated by determination of linearity, selectivity, accuracy and precision, according to the ICH Guidance for Industry (2005). The results (Table 6) indicated that the proposed method is sensitive, accurate, reproducible, and might be recommended as effective standardisation tool for the target extracts.

3. Materials and methods

3.1. Chemicals

All reagents and standards used in UHPLC and HPLC analyses including acetonitrile (Avantor Performance Materials, Gliwice, Poland), acetic acid, formic acid, (–)-epicatechin, chlorogenic acid hemihydrate, quercetin trihydrate, rutin trihydrate, quercitrin (Sigma-Aldrich, St. Louis, MO, USA/Seelze, Germany), as well as procyanidin B2 and procyanidin C1 (PhytoLab, Vestenbergsgreuth, Germany) were of HPLC-grade. Solvents used for extraction (Chempur, Piekary Slaskie, Poland) and Sephadex LH-20 (Sigma-Aldrich) were of analytical grade. Peroxynitrite was synthesised according to Pryor et al. (1995). Immunoreagents for 3-nitrotyrosine determination were obtained from Abcam (Cambridge, UK); Pierce BCA Protein Assay Kit from Thermo Scientific (Waltham, MA, USA); and a (Ca^{2+})-free phosphate buffered saline (PBS) from Biomed (Lublin, Poland). 2,4,6-Tris-(2-pyridyl)-s-triazine (TPTZ), 2-thiobarbituric acid, ascorbic acid, linoleic acid, lipoxygenase from soybean, bovine testis hyaluronidase, indomethacin and all other chemicals (Sigma-Aldrich) used in activity studies were of analytical grade. In all analyses, ultra-pure water was used.

3.2. Plant material

Leaves of *Sorbus domestica* L. were collected in September 2015 in the Arboretum (51°49′N, 19°53′E), Forestry Experimental Station of

Table 6
Validation parameters for the proposed HPLC-PDA quantitative method.

Analyte	λ (nm)	Regression (linear model)		*r*	Linear range (µg/mL)	*F*-test	LOD (µg/mL)	Precision		Recovery (%)
		equation	*n*					Intra-day RSD (%)	Inter-day RSD (%)	
CHA	325	$y = 44{,}634.84x$	9	0.9994	0.28–111.4	6306.4	0.139	1.45	2.33	100.2
ECA	280	$y = 10{,}462.19x$	7	0.9996	0.74–74.1	7936.6	0.370	2.05	2.68	98.3
PB2	280	$y = 10{,}124.49x$	7	0.9990	0.88–55.3	2600.0	0.442	2.54	3.15	97.4
PC1	280	$y = 9704.06x$	7	0.9998	0.92–46.0	11335.1	0.345	2.33	3.09	99.1
QCT	350	$y = 27{,}820.05x$	8	0.9995	0.41–81.4	6753.2	0.203	1.97	2.05	101.3
RT	350	$y = 18{,}288.45x$	7	0.9994	0.45–45.1	4648.4	0.226	1.25	1.55	99.7
HRQ	350	$y = 20{,}126.56x$	8	0.9994	0.66–65.8	3977.5	0.247	1.77	1.94	100.7
PRQ	350	$y = 21{,}400.93x$	7	0.9995	0.51–51.2	5636.3	0.256	2.29	2.07	98.5
QU	370	$y = 53{,}774.41x$	6	0.9991	0.78–39.0	1310.4	0.190	1.79	2.44	97.7

Abbreviations: λ, detection wavelength; *y*, peak area; *x*, concentration of standard in (µg/mL); *n*, number of concentration levels (data points) used for construction of the regression equation; *F*-test, value of the statistical Fisher variance ratio for the experimental data (the critical values at $\alpha = 0.01$ are 13.745 for $n = 6$, 12.246 for $n = 7$, 11.259 for $n = 8$, 10.561 for $n = 9$ calibration data points). LOD, limit of detection. Limits of quantification (LOQ) are identical with the lowest concentrations of the standard curves. Precision data are presented for the lowest level tested. Recovery data are mean values for both levels tested with RSDs less than 5%. For analytes codes see Table 1.

Warsaw University of Life Science (SGGW) in Rogow (Poland) and authenticated by Piotr Banaszczak (Head of the Arboretum). A voucher specimen (KFG/HB/15001-SDOM) was deposited in the Department of Pharmacognosy, Medical University of Lodz, Poland. The powdered and sieved (0.315-mm sieve) leaves were stored in airtight containers, in darkness, under ambient conditions.

3.3. Extract preparation and initial characterisation

The powdered leaf sample (470 g) was extracted in a Soxhlet apparatus with chloroform (1 L; 30 h); and then the pellet was refluxed with methanol-water (7:3, *v/v*; 3 × 1 L × 6 h). A part of the defatted methanol extract (MED) was left for phytochemical and biological activity studies, and the rest, after suspension in water (0.5 L), was sequentially extracted with diethyl ether, ethyl acetate and *n*-butanol (8 × 100 mL each). The *n*-butanol fraction (BF) was then used for isolation of diglycosides. Both, MED and BF were evaporated *in vacuo*, and lyophilised (Alpha 1–2/LD Plus freeze dryer, Christ, Osterode am Harz, Germany).

The qualitative UHPLC-PDA-ESI-MS^3 profiling of MED was carried out on UHPLC-3000 RS system (Dionex, Dreieich, Germany) equipped with a dual low-pressure gradient pump, a column thermostat, a PDA detector, and an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik, Bremen, Germany). A Kinetex XB-C18 column (1.7 µm, 150 mm × 2.1 mm i.d.; Phenomenex, Torrance, CA, USA) was used for separations. The mobile phase was as follows: solvent A (water/formic acid, 100:0.1, *v/v*); solvent B (acetonitrile/formic acid, 100:0.1, *v/v*); with the elution profile: 0–45 min, 6–26% B (*v/v*); 45–55 min, 26–95% B; 55–63 min, 95% B; 63–70 min, 95%–6% B; 70–80 min, 6% B (equilibration); the temperature was set to 25 °C and the flow rate was 0.3 mL/min. UV–Vis spectra were recorded over the range of 200–600 nm. The LC eluate was introduced directly into the ESI interface without splitting and analysed in a negative ion mode in the range from *m/z* 200 to 2200. The nebuliser pressure was 40 psi; dry temperature 300 °C; dry gas flow 9 L/min; and capillary voltage 4.5 kV. The MS/MS fragmentation amplitude was set to 1.0 V and automatically adjusted by the system in the range of 60–300% of this value. Before the analyses, samples of the extracts were dissolved (3.0 mg/mL) in methanol-water (7:3, *v/v*) and filtered through syringe PTFE filters (25 mm, 0.22 µm; Alchem, Toruń, Poland).

The total content of polyphenols (TPC) and proanthocyanidins (TPA) in MED were assessed, as described previously, using Folin-Ciocalteu and *n*-butanol-HCl methods (Matczak et al., 2018) and expressed in gallic acid (GAE) and cyanidin chloride (CYE) equivalents, respectively.

3.4. Isolation of flavonoid diglycosides

BF (8 g) was separated by gel permeation chromatography over Sephadex LH-20 using methanol as eluent, to yield the fraction of quercetin diglycosides (0.6 g), quercetin monoglycosides (0.1 g), proanthocyanidins (1.4 g), and caffeoylquinic acids (2.2 g). Next, the fraction of diglycosides was subjected to preparative HPLC using an XBridge Prep C18 column (5 µm, 100 mm × 19 mm i.d.; Waters, Milton, MA, USA) and a Waters 2545 binary system equipped with PDA detector, autosampler and fraction collector. The mobile phase for isocratic elution was composed of acetonitrile and 0.5% acetic acid (13:87, *v/v*). The separations were carried out at room temperature (20–25 °C), and the flow rate was 14 mL/min. The diglycoside fraction was dissolved (50 mg/mL) in the mobile phase and injected into the system in portions (300 µL/injection). The fractions were collected automatically using the UV–vis signal at λ = 350 nm as the trigger. For further purification, the combined fractions containing respective analytes subjected to gel permeation over Sephadex LH-20. Finally, 149 mg of **HRQ** and 35 mg of **PRQ** was obtained.

3.5. Structure elucidation

The samples of isolated compounds were hydrolysed according to Olszewska and Roj (2011) and a free aglycone was identified by HPLC-PDA analysis of the diethyl ether fraction of the hydrolysate using an authentic standard for comparison (Olszewska, 2008). The identity and absolute configuration of the sugars were determined by HPLC-PDA analysis of the aqueous layer after the conversion of the sugars to their 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxy-alditol pentaacetate derivatives, as described earlier (Olszewska and Roj, 2011). For reference, the derivatives of the standards obtained analogously were used: derivative of D-xylose (R_t 15.54 min), derivative of D-glucose (R_t 20.16 min), and derivative of L-rhamnose (R_t 22.08 min). Finally, L-rhamnose and D-glucose were identified in the hydrolysate of **HRQ**, while D-xylose and L-rhamnose were found in the hydrolysate of **PRQ**.

^{1}H NMR, ^{13}C NMR, COSY, HMBC, and HMQC spectra (600 MHz for ^{1}H and 150.9 MHz for ^{13}C) were recorded on a Bruker Avance III 600 spectrometer (Bruker BioSpin, Billerica, MA, USA) in methanol-d_4, at 25 °C, using TMS as the internal standard.

3.5.1. Quercetin 3-O-(2′′-O-β-D-glucopyranosyl)-α-L-rhamnopyranoside (HRQ)

Yellow powder; LC-ESI–MS *m/z*: 609 $[M-H]^-$; ESI–MS^2 *m/z* (intensity): 447 (13) $[M-H–rhamnose]^-$, 301 (51) $[M-H–rhamnose-glucose]^-$; ^{1}H and ^{13}C NMR data (methanol-d_4): see Table 3.

3.5.2. Quercetin 3-O-(2′′-O-β-D-xylopyranosyl)-α-L-rhamnopyranoside (PRQ)

Yellow powder; LC-ESI–MS *m/z*: 579 $[M-H]^-$; ESI–MS^2 *m/z* (intensity): 447 (9) $[M-H–rhamnose]^-$, 301 (100) $[M-H–rhamnose-glucose]^-$; ^{1}H and ^{13}C NMR data (methanol-d_4): see Table 3.

3.6. HPLC-PDA quantitative assay and its validation

The HPLC-PDA quantifications were carried out on a Waters 600E Multisolvent Delivery System (Waters, Milford, MA, USA) equipped with a PDA detector, a column thermostat and a sample injection valve with a 5 µL injection loop. An Ascentis Express C18 fused-core column (2.7 µm, 150 mm × 4.6 mm id; Supelco, Bellefonte, PA, USA) with an Ascentis C18 Supelguard guard column (3 µm, 20 mm × 4 mm id; Supelco) was used for separation. The mobile phase was as follows: solvent A (0.5% orthophosphoric acid in water, *w/v*) and solvent B (acetonitrile); with the elution profile: 0–1 min, 5% B (*v/v*); 1–17 min, 5–19% B; 17–23 min, 19% B; 23–24 min, 19–30% B; 24–25 min, 30–50% B; 25–26 min, 50-5% B; 26–30 min, 5% B (equilibration). The temperature was set to 30 °C, and the flow rate was 1.4 mL/min. Before the analyses, samples of the extracts were dissolved (5.0 mg/mL) in methanol-water (7:3 *v/v*), and filtered through a syringe PTFE filter (25 mm, 0.22 µm; Alchem, Toruń, Poland). The UV–vis data were collected over the range of 220–450 nm and the polyphenols were classified according to their spectra. The λ for detection and quantification was set to 280 nm for flavanols, 325 nm for caffeic acid derivatives including caffeoylquinic acid isomers, 350 nm for flavonoid glycosides, and 370 nm for flavonoid aglycones. Nine external standards (**ECA**, **PB2**, **PC1**, **CHA**, **QCT**, **RT**, **HRQ**, **PRQ**, **QU**) were used for calibration. The tentatively identified peaks were quantified as equivalents of **PB2** (flavanols), **CHA** (phenolic acids), and **QCT** (flavonoid glycosides).

Method validation was performed according to the ICH Guidance for Industry (2005) and previous works (Olszewska, 2012) and included the assessment of linearity, sensitivity, precision and accuracy. The standard stock solution containing nine reference compounds was prepared and serially diluted (in triplicates) with methanol-water (7:3, *v/v*) to at least six concentration levels (including LOQs); each concentration was injected three times into the HPLC system. Mean peak areas were plotted versus the concentrations to obtain the calibration graphs and their linearity was confirmed by the least squares regression

method and the *t*- and *F*-tests at the 99% confidence level. The LOQ and LOD values were established by serial dilution of the standard solutions with methanol-water (7:3, *v/v*) to obtain the smallest detectable peaks. The concentration was accepted as LOQs if the *RSDs* for peak area were smaller than 15% for both intra-day and inter-day variability with signal-to-noise (S/N) ratio larger than 10, while the criterion for LODs was 3-δ S/N ratio. The precision was tested for standard solutions at two concentration levels (at 10% and 75% of the higher linear range limits), and the *RSD* values for the peak area were considered as a measure of precision. The intra-day variability (repeatability) was measured by triplicate analysis of the samples during 24 h. The inter-day variability (reproducibility) was determined on three non-consecutive days. The accuracy was checked by the standard addition/recovery procedure. MED was diluted with methanol-water (7:3, *v/v*) in a ratio 1:2 (*v/v*), spiked in triplicate with standards at two different concentration levels (at 5% and 25% of the higher linear range limits), and each sample was analyzed in triplicate by HPLC. The mean recovery of the analytes (the spiked versus the non-spiked extract) were considered as the measure of accuracy.

3.7. Antioxidant activity in human plasma models

Blood plasma was recovered from buffy coat fractions obtained from seven healthy volunteers (non-smoking), and purchased from the Regional Centre of Blood Donation and Blood Treatment in Lodz. The fractionation of the buffy coat fractions was carried out according to Kolodziejczyk-Czepas et al. (2011). The experiments were approved by the committee on the Ethics of Research at the University of Lodz (11/KBBN-UŁ/I/2017). In all assays, samples were pre-incubated (37 °C; 5 min) with the examined analytes added to the final concentration range of 1–50 μg/mL, and then exposed to 150 μM (the FRAP assay) or 100 μM (the remaining experiments on blood plasma) of $ONOO^-$. Control samples contained plasma only (without the analytes and/or $ONOO^-$). Preliminary tests were run with the blood plasma containing only the analytes (without $ONOO^-$) and no pro-oxidative effects was observed. The level of proteins in the analysed blood plasma was determined using bicinchoninic acid (BCA) assay (according to protocol provided by the manufacturer). The levels of nitrated proteins and TBARS in the samples were determined according to Kolodziejczyk-Czepas et al. (2011) and expressed as 3NT-Fg equivalents (nmol/mg of plasma protein) and in μmol TBARS/mL of plasma, respectively. The non-enzymatic antioxidant capacity of blood plasma (NEAC) was evaluated by measuring its ferric-reducing ability (FRAP) as described by Kolodziejczyk-Czepas et al. (2014) except that plasma samples were diluted with a (Ca^{2+})-free PBS buffer (1:4, *v/v*) before analysis. The results were expressed in mM of Fe^{2+} equivalents. All measurements were performed on 96-well plates using a microplate reader (SPECTROStar Nano; BMG LabTech, Ortenberg, Germany).

3.8. Inhibition of pro-inflammatory enzymes

Hyaluronidase (HYAL) and lipoxygenase (LOX) inhibition was evaluated according to Michel et al. (2017) with one modification – in the LOX assay, the volume ratio of tested analyte, enzyme and linoleic acid was 1:1:2. In both tests, concentration-inhibition curves were prepared and IC_{50} values were determined. Measurements were carried out using a microplate reader described above.

3.9. Contribution of S. domestica model compounds to the activity of MED

Calculations of the percentage contribution of an analyte (CT_A) to the activity of MED and its relative contribution (RCT_A) to the activity of the phenolic fraction (TPH + TPA) of MED were calculated using the following equations:

$$CT_A\,(\%) = \frac{c_A \times AE_A}{AE_{MED}} \times 100\% \quad (1)$$

$$RCT_A\,(\%) = \frac{c_A \times AE_A}{CT_{TPH+TPA}} \times 100\% \quad (2)$$

where c_A is the content (in g/g dw) of the analyte in MED according to Table 2; AE_A is the activity of the analyte expressed in equivalents of standard (in g/g), i.e. AA in human plasma model and IND in LOX and HYAL tests (Tables 4 and 5); AE_{MED} is the activity of MED expressed in equivalents of standard; and $CT_{TPH+TPA}$ is the percentage contribution of the whole phenolic fraction (TPH + TPA) to the activity of MED.

The percentage contribution of a group of analytes (CT_G) to the activity of MED and its relative contribution (RCT_G) to the activity of the phenolic fraction was calculated respectively as the sum of the CT_A or RCT_A values of the individual analytes in the group. In case of analytes whose activity was not tested directly, AE_A values of the model compound for a particular group was used (**QCT** for TFL-representatives, **CHA** for TPHA-representatives, and **PB2** for TLPA-representatives). The CT value for TPA was obtained using AE value of **PC1**, and $CT_{TPH+TPA}$ was calculated as a sum of CT_{TPH} and CT_{TPA}.

3.10. Statistics

The results are presented as means ± standard deviation (SD) or ± standard error (SE) for the indicated number of experiments. For anti-inflammatory activity, the significance of differences between means was determined using one-way ANOVA, followed by the post hoc Tukey's test, while for human plasma models one-way ANOVA for repeated measures with Dunnett's tests was used. All calculations were performed using Statistica 12 Pl software for Windows (StatSoft Inc., Krakow, Poland) with *p* values less than 0.05 regarded as significant.

4. Conclusion

This work is the first integrated phytochemical and biological activity study of individual polyphenols in *S. domestica* leaves. Nine model compounds selected for the study represent all classes of polyphenols typical for service tree including catechins, proanthocyanidins, flavonols (aglycones, mono- and diglycosides) and caffeoylquinic acids. The results revealed that these molecules effectively enhance the NEAC value of human plasma exposed *in vitro* to oxidative/nitrative stress, significantly diminish the levels of well-known oxidative stress biomarkers, and inhibit two key pro-inflammatory enzymes. Catechins and proanthocyanidins (**ECA**, **PB2**, **PC1**) were the most effective compounds in all tests and their contribution to the activity of total phenolic fraction was around 70–80%, depending on the test. They might be thus considered as largely responsible for the activity of the *S. domestica* extracts observed in this and previous (Matczak et al., 2018) experiments. However, the activity of other six molecules was at least comparable or only slightly less pronounced than that of flavanols. Moreover, the superior activity profile of the source extract compared to the pure molecules suggests the existence of some synergistic effects between phenolic constituents. In consequence, all nine polyphenols are recommended as bioactivity markers for quality control of *S. domestica* leaf extracts, and the proposed HPLC-PDA method, fully validated during the present study using these markers as calibration standards, is a simple, accurate and reproducible tool suitable for that purpose.

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Sorbus domestica Leaf Extracts and Their Activity Markers: Antioxidant Potential and Synergy Effects in Scavenging Assays of Multiple Oxidants

Magdalena Rutkowska [1,*], Monika Anna Olszewska [1], Joanna Kolodziejczyk-Czepas [2], Pawel Nowak [2] and Aleksandra Owczarek [1]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Lodz, ul. Muszynskiego 1, 90-151 Lodz, Poland; monika.olszewska@umed.lodz.pl (M.A.O.); aleksandra.owczarek@umed.lodz.pl (A.O.)

[2] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, ul. Pomorska 141/143, 90-236 Lodz, Poland; joanna.kolodziejczyk@biol.uni.lodz.pl (J.K.-C.); pawel.nowak@biol.uni.lodz.pl (P.N.)

* Correspondence: magdalena.rutkowska@umed.lodz.pl

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Abstract: *Sorbus domestica* leaves are a traditionally used herbal medicine recommended for the treatment of oxidative stress-related diseases. Dry leaf extracts (standardized by LC-MS/MS and LC-PDA) and nine model activity markers (polyphenols), were tested in scavenging assays towards six in vivo-relevant oxidants ($O_2^{\bullet-}$, $OH^{\bullet}$, $NO^{\bullet}$, H_2O_2, $ONOO^-$, HClO). Ascorbic acid (AA) and Trolox (TX) were used as positive standards. The most active extracts were the diethyl ether and ethyl acetate fractions with activities in the range of 3.61–20.03 μmol AA equivalents/mg, depending on the assay. Among the model compounds, flavonoids were especially effective in $OH^{\bullet}$ scavenging, while flavan-3-ols were superior in $O_2^{\bullet-}$ quenching. The most active constituents were quercetin, (−)-epicatechin, procyanidins B2 and C1 (3.94–24.16 μmol AA/mg), but considering their content in the extracts, isoquercitrin, (−)-epicatechin and chlorogenic acid were indicated as having the greatest influence on extract activity. The analysis of the synergistic effects between those three compounds in an $O_2^{\bullet-}$ scavenging assay demonstrated that the combination of chlorogenic acid and isoquercitrin exerts the greatest influence. The results indicate that the extracts possess a strong and broad spectrum of antioxidant capacity and that their complex composition plays a key role, with various constituents acting complementarily and synergistically.

Keywords: *Sorbus domestica*; oxidative stress; free radicals; reactive oxygen species (ROS); reactive nitrogen species (RNS); polyphenols; synergy

1. Introduction

Sorbus domestica L. is a wild rosaceous tree, native to the Mediterranean Basin, and cultivated as a dietary, medicinal, and decorative plant [1,2]. The polyphenol-rich leaves of this species are traditional herbal medicines. They are valued for their diuretic, antioxidant, anti-inflammatory, anti-atherogenic, and anti-diabetic properties and have been indicated in the treatment of prostatitis, nephritis, diabetes and hypercholesterolemia, among others [1]. Our ongoing studies of the molecular mechanisms of the leaves' activity primarily focus on their influence on oxidative stress and inflammation—the two intertwined processes that have been found to play a crucial role in the development of numerous inflammatory and metabolic disorders, including those described in ethnopharmacological sources. In our previous work, we demonstrated that the leaf extracts inhibit pro-inflammatory enzymes (lipooxygenase and hyaluronidase), display protective effects against nitrative and oxidative damage

to human plasma components (lipids and proteins) under oxidative stress conditions, and enhance the total non-enzymatic antioxidant status of plasma (NEAC) [3]. A detailed phytochemical study (LC-MS) of the leaf's polyphenolic fraction revealed the occurrence of 44 individual compounds. Nine of them (Figure 1) were indicated as key markers of the extracts' activity, based on their relative contribution to the total polyphenolic content (up to 74% depending on the extract) and their activity parameters in plasma [3,4].

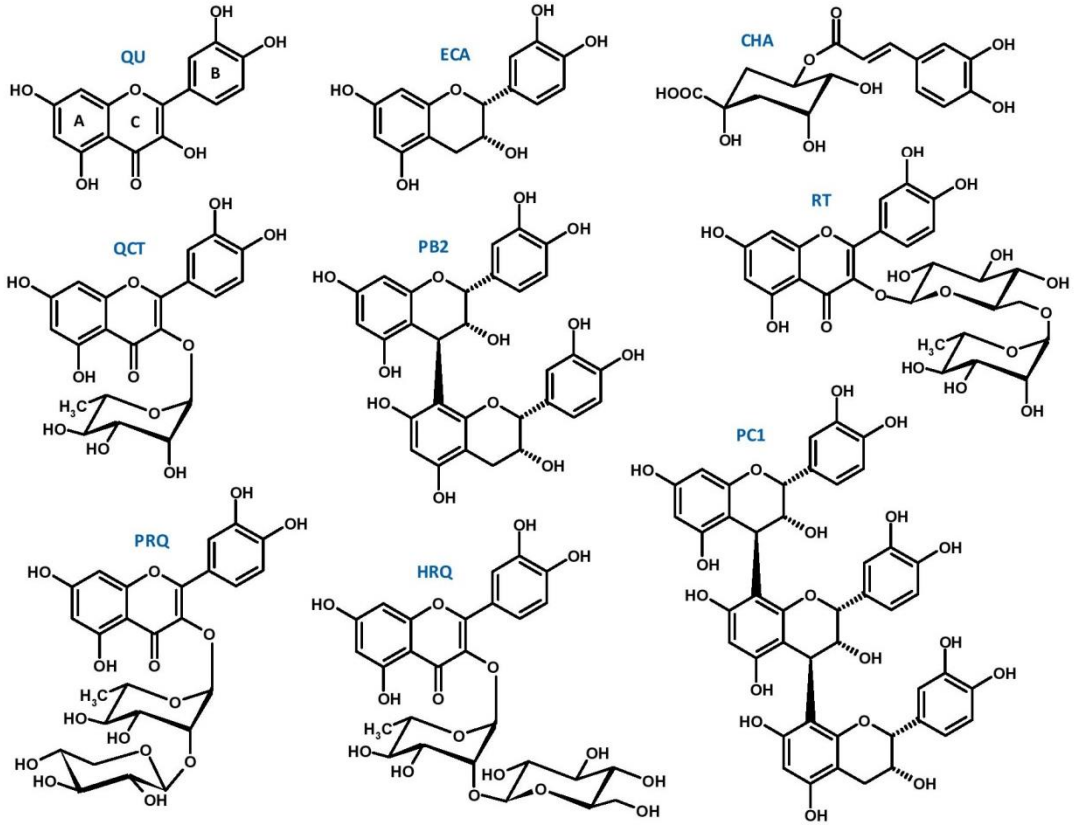


Figure 1. Structures of the activity markers of *S. domestica* leaves: ECA, (−)-epicatechin; CHA, chlorogenic acid (5-*O*-caffeoylquinic acid); PB2, procyanidin B2; PC1, procyanidin C1; QU, quercetin; QCT, quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside); RT, rutin; HRQ, quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside (HRQ); PRQ, quercetin 3-*O*-(2″-*O*-β-D-xylopyranosyl)-α-L-rhamnopyranoside; the capital letters A-C on the QU formula refer to the common nomenclature of the basic flavan skeleton.

Polyphenols are specialized (secondary) plant metabolites, believed to bestow beneficial effects on human health by their ability to counter the pathological consequences of oxidative stress [5,6]. The latter is a complex process involving multiple factors and mechanisms. However, its root cause is an excess of reactive oxygen/nitrogen species (ROS/RNS), which leads to unregulated oxidation of biological molecules, ROS/RNS-induced inflammation, and/or metabolic dysfunction [6,7]. Thus, one of the possible and most direct mechanisms of the protective effects of polyphenols and polyphenol-rich extracts against oxidative stress, and one that has not been studied so far for *S. domestica* leaves, is ROS/RNS quenching [6]. In biological systems, oxidative stress is generated by low-molecular weight ROS/RNS, such as superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), nitric oxide ($NO^{\bullet}$), and hypochlorous acid (HClO), varying in reduction potential, reaction mechanism, and selectivity towards biological molecules [8,9]. Effective protection from oxidative stress requires, therefore, antioxidants that are able to prevent different oxidative factors [8]. Due to their complex composition, plant extracts are likely to exhibit a broad reactivity

and eliminate numerous ROS/RNS [6,10]. Additionally, the presence of multiple constituents raises the possibility of some synergistic effects [11]. Indeed, in the previous research, the activity of *S. domestica* leaf extracts was higher than what might be expected based only on the content of individual compounds [3,4]. However, no systematic evaluation of synergy for *S. domestica* leaves has been conducted so far.

Therefore, the objective of the present work, is to provide a clearer insight into the antioxidant potential of *S. domestica* leaves and its constituents by evaluating their scavenging potential towards multiple ROS/RNS of physiological importance, i.e., $O_2^{\bullet-}$, $OH^{\bullet}$, H_2O_2, $ONOO^-$, $NO^{\bullet}$ and HClO. Moreover, the synergistic effects between the representatives of the three main groups of *S. domestica* polyphenols (flavonols, flavanols and caffeoylquinic acid pseudodepsides) were tested in a selected model of $O_2^{\bullet-}$ quenching.

2. Results and Discussion

2.1. Scavenging of Multiple Oxidants

The primary reactive species in human cells is $O_2^{\bullet-}$, which is constantly generated during mitochondrial electron-transport chain, as well as via other endogenous reactions, e.g., those involving lipoxygenases, cyclooxygenases, xanthine oxidases, NADPH oxidases, or NO synthases. It also plays an important role in the generation of other oxidizing agents, including H_2O_2, $OH^{\bullet}$, HClO, and $ONOO^-$ [8,9]. For instance, through the activity of superoxide dismutase (SOD), $O_2^{\bullet-}$ is converted to H_2O_2. The latter is a rather stable form of ROS that, nevertheless, might be further converted to extremely reactive $OH^{\bullet}$ in the presence of various in vivo reductants [9]. Other non-selective and highly destructive oxidants include HClO, formed via the myeloperoxidase-catalyzed transformation of H_2O_2, and $ONOO^-$, produced in the reaction between $O_2^{\bullet-}$ and $NO^{\bullet}$ [12,13]. Interestingly, only $NO^{\bullet}$ generated by inducible NO synthase (iNOS) in stimulated immune cells is a causative factor of $ONOO^-$ synthesis [14,15], whereas $NO^{\bullet}$ formed by endothelial NO synthase (eNOS) in the vessel endothelium is a beneficial antiatherogenic agent [12,15].

The analytes investigated in the present study were dry extracts from *S. domestica* leaves that have demonstrated effectiveness against $ONOO^-$–generated oxidative damage of human plasma components, as well as the nine polyphenols selected previously [3,4] as the markers of the extracts activity (Figure 1). The fractionated dry extracts, i.e., the hydro-methanolic (7:3, *v*/*v*) extract (MED) and its fractions of different polarity (diethyl ether fraction, DEF; ethyl acetate fraction, EAF; *n*-butanol fraction, BF; and water residue, WR) were obtained and thoroughly standardized previously (LC-MS/MS, LC-PDA, and spectrophotometric profiling) [3]. The extracts varied in terms of total polyphenolic content and the total content and profile of the indicated markers (Figure 2), thereby comprising a good model for evaluating the contribution of individual components to the ROS/RNS scavenging potential of the leaves, as well as for screening for potential synergy effects.

To enable the direct comparison of the analytes' capacities towards different ROS/RNS, the primary results (effective scavenging concentrations, SC_{50}, Figure S1) were expressed as equivalents of positive standards—ascorbic acid (AA), the main water-soluble antioxidant of human plasma in vivo, and Trolox (TX), a synthetic analogue of vitamin E, which is the primary lipophilic (lipoprotein-bound) plasma antioxidant (Figure 3). Additionally, total activity scores AAE_{MR6} and TE_{MR6} for each analyte were calculated, understood as the sums of the analyte activities in all assays expressed in equivalents of AA and TX, respectively (Table 1).

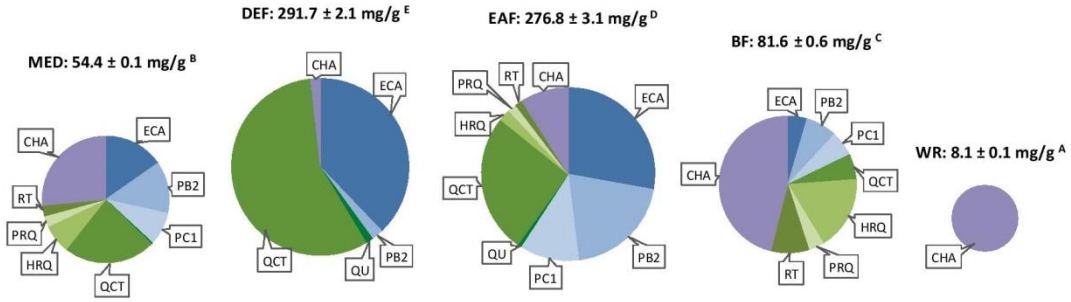


Figure 2. Total contents per g of dry weight of the *S. domestica* leaf extracts (LC-PDA-peaks; means ± SD, $n = 3$) and the relative proportions of the activity markers according to Matczak et al. [3]. The different letters A–E indicate significant differences ($p < 0.05$). MED, methanol-water (7:3, *v*/*v*) extract; DEF, diethyl ether fraction; EAF, ethyl acetate fraction; BF, *n*-butanol fraction; WR, water residue. For compound codes and structures, see Figure 1.

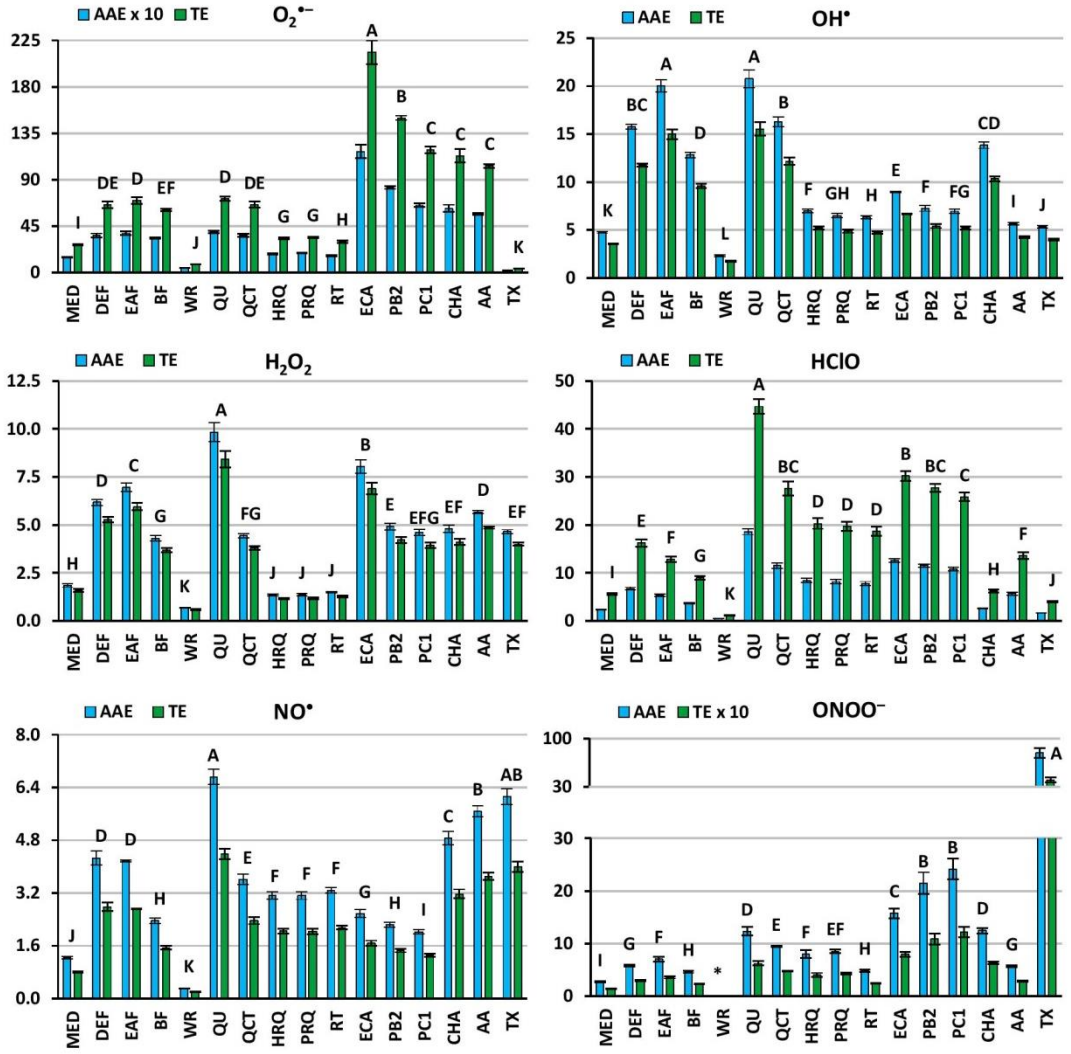


Figure 3. Antioxidant activity of *S. domestica* leaf extracts, their activity markers and standards towards in vivo-relevant oxidants. Results are expressed in micromolar equivalents of ascorbic acid (AA) and Trolox (TX) per mg of extract/compound and presented as mean values ± SD ($n = 3$). For each assay, the different letters A–K indicate significant differences ($p < 0.05$). The analyte marked with an asterisk was inactive in concentrations up to 200 µg/mL. For extracts and analyte codes, see Figures 1 and 2.

Table 1. Scavenging capacity towards multiple reactive oxygen/nitrogen species (ROS/RNS) in equivalents of standard antioxidants.

	AAE_{MR6}		TE_{MR6}			AAE_{MR6}		TE_{MR6}	
	µmol/mg	mol/mol	µmol/mg	mol/mol		µmol/mg	mol/mol	µmol/mg	mol/mol
Extracts					**Markers**				
MED	14.31	-	38.79	-	QU	72.24	21.82	145.33	43.89
DEF	42.37	-	102.16	-	QCT	49.06	21.98	116.09	52.01
EAF	47.36	-	106.71	-	HRQ	29.77	18.16	62.45	38.09
BF	31.20	-	84.82	-	PRQ	29.62	17.18	62.40	36.19
WR	4.21	-	11.62	-	RT	25.39	15.49	57.23	34.91
					ECA	59.27	17.19	253.26	73.45
Standards					PB2	55.68	32.18	189.75	109.68
AA	34.07	6.00	130.06	22.89	PC1	55.07	47.69	156.56	135.58
TX	97.10	24.28	23.97	6.0	CHA	45.69	16.17	154.12	54.56

AAE_{MR6} and TE_{MR6}, total activity scores of the analytes—the sums of the activity parameters in all assays, expressed in micromolar equivalents of ascorbic acid (AA) and Trolox (TX) per mg of dry matter (values from Figure 3) and in molar equivalents of AA and TX per mol of the pure compounds.

All of the tested extracts showed a concentration-dependent ability to scavenge all of the target ROS/RNS (Figure 3), with total activity scores varying widely: 4.2–47.4 µmol AA/mg dw and 11.6–106.7 µmol TX/mg dw (Table 1). The highest AAE_{MR6} and TE_{MR6} were obtained for EAF and DEF, and then for BF, i.e., the fractions highly enriched in polyphenols (Figure 2). These findings are consistent with those obtained previously from simple chemical tests (TBARS, DPPH, FRAP) and from a model of human plasma exposed to oxidative stress [3]. The extracts were especially effective in $OH^{\bullet}$ scavenging, in which the most active fractions were 3–4 times more efficient than both of the standards (Figure 3). This observation may be of some physiological importance, as $OH^{\bullet}$ is a highly reactive, unselective radical able to oxidize almost every cellular component [9]. Other harmful species are also HClO and $ONOO^{-}$. Both are strong oxidants and additionally may cause chlorination and nitration disrupting the structure and function of proteins and other macromolecules [14,16]. Contrary to $OH^{\bullet}$, which reacts almost instantly at the place of origin, they can diffuse through membranes, causing a wider spread of oxidative and inflammatory processes [14,16]. Moreover, as HClO is not subjected to any endogenous antioxidant enzyme system, the low molecular-weight plasma antioxidants, both endo- and exogenous, become the primary line of defense [16]. In the case of both species, the most active fractions displayed comparable or higher activity to that of AA (Figure 3), and with regard to HClO, their efficiency was visibly superior to TX (Figure 3), suggesting that the extracts' constituents may be able to support the antioxidants present in the biological system. In comparison to TX, the extracts were also highly effective towards $O_2^{\bullet-}$. Despite not being very reactive, this species can still disrupt homeostasis, e.g., by targeting Fe-S centers of proteins and causing cluster disruption [17]. Moreover, scavenging of $O_2^{\bullet-}$ may prevent the generation of the derived more harmful ROS/RNS. Only in the $NO^{\bullet}$-scavenging assay were the capacities of the extracts weaker ($p < 0.05$) than those of both reference antioxidants. Since $NO^{\bullet}$ has a positive function in intercellular signaling, vessel dilatation, the inhibition of inflammatory cell adhesion, and the promotion of fibrinolysis, targeting $ONOO^{-}$ and $O_2^{\bullet-}$, which gives $ONOO^{-}$ a reaction with $NO^{\bullet}$, might actually be more beneficial [7,12,15].

Generally, the results suggest that the most active extracts obtained from *S. domestica* leaves may be considered broad-spectrum, strong antioxidants. Their scavenging activity may have positive effects in biological systems and, e.g., it might have been one of the protective mechanisms observed in the plasma model under the conditions of oxidative stress induced by $ONOO^{-}$ [3].

The analysis of the quenching capacities of the *S. domestica* model constituents indicates their influence on the antioxidant capacity of the extracts. All of the tested compounds scavenged ROS/RNS dose-dependently (Figure 3), with total activity scores in the range of 25.4–72.2 µmol AA/mg (15.49–47.69 mol AA/mol) and 57.2–253.3 µmol TX/mg (34.91–135.58 mol TX/mol) (Table 1). The

potential of the investigated polyphenols is especially apparent when compared in molar units (mol/mol) to the corresponding AAE_{MR6} of AA (6 mol AA/mol) and TE_{MR6} of TX (6 mol TX/mol).

Among the flavonoids, the highest activity parameters in all tests were observed for quercetin (QU) (Figure 3). It was also the most active analyte among the tested compounds, with regard to its AAE_{MR6} value (Table 1). Its glycosidic derivatives were less active, which is consistent with the common observation that glycosylation of the hydroxyl group in the C3 position lowers the antioxidant potential of flavonoids [18,19]. However, when the total antioxidant scores were expressed in mol/mol, the differences were much less pronounced and in the case of quercitrin (QCT), the AAE_{MR6} and TE_{MR6} values were equal to and higher than that of the QU, respectively (Table 1). This suggests that the lower activity of glycosides (when expressed in units of weight) is mainly due to their higher molecular mass, and that the free hydroxyl group at C3 position plays a less important role in quenching of the tested ROS/RNS than the catechol moiety and phenol groups in ring A of a flavonoid skeleton (Figure 1). Consequently, taking into account the content of the flavonoids in the extracts (Figure 2), QCT should be considered as having the greatest influence on the extracts' activity.

The investigated flavan-3-ol derivatives gave the highest TE_{MR6} values, and their AAE_{MR6} scores were lower only than those of QU (Table 1). In the majority of tests, (−)-epicatechin (ECA) was found to be superior when the results were expressed per unit of weight (Figure 3), while the higher oligomers performed best with regard to molar activity (Table 1). The latter fact is in agreement with previous findings and is connected with the polyhydroxylated structures of oligomeric proanthocyanidins [20]. However, as ECA predominated in most of the examined extracts, particularly in DEF (Figure 2), it might be regarded as a good representative of the active flavanols of *S. domestica* leaves.

Some interesting differences between the assays were also observed regarding the relative activity of flavonols and flavanols. For example, flavonoids QU and QCT were particularly effective in $OH^{\bullet}$ scavenging, while flavan-3-ol derivatives were superior in terms of $O_2^{\bullet-}$ quenching (Figure 3). This observation might be connected with the decisive role of the C2–C3 double bond of flavone derivatives in $OH^{\bullet}$ radical-antioxidant interactions [21]. Conversely, a saturated C2–C3 bond was reported to be favorable for $O_2^{\bullet-}$ scavenging [22].

Chlorogenic acid (CHA), the sole representative of caffeoyl quinic derivatives, was also a highly active compound in most of the tests. However, it was found to be visibly inferior to the other tested compounds in the HClO quenching assay (Figure 3). This may be connected with the fact that in case of flavonoids and flavan-3-ol derivatives, the HClO scavenging occurs mainly via electrophilic reactions on the A and C rings of a flavonoid skeleton (Figure 1), whereas the *ortho*-dihydroxyphenyl group (catechol moiety) present in the investigated flavan (ring B) and caffeic acid derivatives does not undergo chlorination [23]. Nevertheless, the relatively high AAE_{MR6} and TE_{MR6} scores of CHA, comparable to those of QCT and procyanidin C1 (PC1), respectively (Table 1), as well as its abundant occurrence, especially in BF, WR and MED (Figure 2), make CHA an important determinant for the activity of *S. domestica* extracts.

2.2. Synergistic Effects in $O_2^{\bullet-}$ Scavenging

The complex composition of natural products represents one of their main advantage in comparison to synthetic drugs. It enables, among other effects, possible synergistic effects between components that lower the concentration of the drug required for the desired outcome and the risk of adverse events. In the case of antioxidant activity, considerable synergy has been found for molecules with catechol moiety [24,25]. Similar effects might thus be anticipated between the main constituents of *S. domestica* leaves, as they all are catechol-type polyphenols.

To assess the possibility of such effects, the predicted extract activity indices (PEA) were determined based on the extracts' phytochemical profile. For a given extract in a given assay, the PEA value was understood as the weighted sum of the markers' activities in the assay (in AA equivalents), with the percentage content of the markers in the extract used as weights. Assuming the effects of the markers were purely, or in large part, additive, the PEA values should be good predictors of the

relative capacities of the extracts in the assay, and the correlation coefficients between the PEA and the extract activity parameters should be high and statistically significant. Such relationships were found for most of the assays (Table 2). The exception was the $O_2^{\bullet-}$ scavenging test, thus suggesting, in this case, the presence of possible pronounced synergy/antagonism effects. For a closer investigation, representatives of the three main classes of polyphenols present in *S. domestica* leaf (flavanols, flavonols, and quinic acid pseudodepsides), i.e., ECA, QCT, and CHA, were chosen.

Table 2. Predicted extract activity indices (PEA) values for extracts in particular assays and their correlations with extracts' activity parameters (AAE).

	PEA					
Extracts	$O_2^{\bullet-}$	$OH^{\bullet}$	H_2O_2	HClO	$NO^{\bullet}$	$ONOO^-$
MED	0.35	0.68	0.26	0.48	0.19	0.75
DEF	1.99	3.97	1.72	3.46	0.95	3.54
EAF	2.02	3.74	1.52	2.90	0.82	4.27
BF	0.45	0.93	0.32	0.52	0.31	0.99
WR	0.06	0.13	0.04	0.02	0.04	0.10
	r (*p*)					
AAE	0.8072 (0.099)	0.8794 (0.049) *	0.9043 (0.035) *	0.9227 (0.026) *	0.9779 (0.004) *	0.9035 (0.035) *

PEA, predicted extract activity index, understood as the weighted sum of the markers' activities in AAE (ascorbic acid equivalents) with the percentage content of the markers in the extract used as weights, according to Equation (1); *r*, correlation coefficient between PEA values and the extracts' activities in AAE for a particular assay; *p*, *p*-value for statistical significance of the correlation. The relationships marked with an asterisk are statistically significant at $\alpha = 0.05$. * $p < 0.05$.

To calculate the combined effects of two or more compounds and estimate the degree of potential synergy, individual dose-response curves have to be estimated [26]. Therefore, the experimental data for each tested analyte was first fitted into different sigmoid functions, among which, the Weibull survival distribution equation offered the best fitting parameters (Figure 4, Table 3). This model has previously been found to be a good approximation of the antioxidant activity in different systems [27]. Then, because synergy depends on both the properties and the exact dose of each constituent [11,25,26], each combination of the compounds was tested in five concentration ratios. To evaluate the observed effects, the combination indices (*CI*) were calculated (Table 4). The *CI*s are indices based on the Loewe additivity model, which juxtaposes the concentrations of the analytes actually used in the assay with those theoretically required to produce the same effect (calculated from the individual dose-response curves) [28]. Consequently, a *CI* equal to 1 indicates additivity, while a *CI* less then or more than 1 indicates synergy or antagonism, respectively [28]. The concentrations and proportions in the experiments were chosen based on the individual compound scavenging efficiency (less than 60%), the relative proportions of the compounds in the extracts, as well as the range of physiological levels of plant-derived phenolic compounds in human plasma (1–5 μg/mL) available after oral administration [3,4].

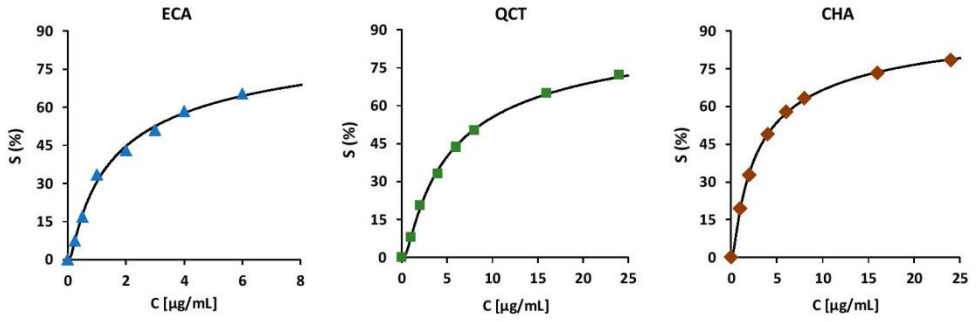


Figure 4. Observed mean data ($n = 5$) and the fitted dose-response curves for the main extracts' constituents in the $O_2^{\bullet-}$ scavenging capacity assay.

Table 3. Estimated model parameters for the dose-response curves of the main extracts' constituents in the $O_2^{\bullet-}$ scavenging capacity assay according to Equation (2).

Compound	Parameters		Model Significance			
	m	*k*	*SSE*	R^2	*F*-Test	*p*
ECA	2.622	−0.552	14.581	0.9964	2793.66	1.23×10^{-9}
QCT	7.795	−0.638	5.580	0.9988	8296.13	4.72×10^{-11}
CHA	4.192	−0.611	1.626	0.9997	41,930.01	3.66×10^{-13}

Parameters: *m*, parameter corresponding to the concentration required for 50% radical quenching; *k*, parameter related to the maximal slope of response; *SSE*, the sum of squared errors of prediction; R^2, coefficient of determination; *F*-test, value of the statistical Fisher variance ratio for the experimental data (the critical value at $\alpha = 0.01$ is 11.259 for $n = 8$); *p*, significance level.

Table 4. The synergistic effects between the main extracts' constituents.

Compound Combination	Concentration Ratio (μg/mL)	Theoretical Scavenging Efficacy (%)	Experimental Scavenging Efficacy (%)	CI ± 95% Conf.	Effect
QCT–ECA	4:1	47.75	47.71 ± 2.43	1.00 ± 0.10	additivity
	2:1	40.55	41.44 ± 2.22	0.97 ± 0.09	additivity
	1:1	35.95	39.82 ± 2.15	0.84 ± 0.08	synergy
	1:2	47.56	52.04 ± 2.86	0.81 ± 0.10	synergy
	1:4	59.12	61.57 ± 1.83	0.88 ± 0.08	synergy
QCT–CHA	4:1	43.68	54.77 ± 3.14	0.61 ± 0.08	synergy
	2:1	34.13	44.62 ± 3.02	0.64 ± 0.08	synergy
	1:1	27.46	37.44 ± 2.71	0.65 ± 0.07	synergy
	1:2	38.82	49.56 ± 3.86	0.62 ± 0.10	synergy
	1:4	51.61	61.04 ± 3.03	0.63 ± 0.09	synergy
CHA–ECA	4:1	55.91	60.39 ± 1.84	0.78 ± 0.07	synergy
	2:1	46.92	52.84 ± 2.66	0.75 ± 0.09	synergy
	1:1	40.12	51.82 ± 2.75	0.57 ± 0.07	synergy
	1:2	49.91	53.43 ± 2.63	0.84 ± 0.10	synergy
	1:4	60.26	60.33 ± 1.92	1.01 ± 0.10	additivity

Results are presented as mean values ± SD ($n = 5$) according to Equations (2) and (3). A *CI* (combination index) equal to 1 indicates additivity, while a *CI* less then or more than 1 indicates synergy and antagonism, respectively.

As shown in Table 4, with the exception of the QCT–ECA in the proportions 2:1 and 4:1 μg/mL, and CHA–ECA in proportion 1:4 μg/mL, all other combinations showed some synergy effects, although with different intensities. The best effects were observed for QCT–CHA, which presented *CI* values significantly lower than 1, regardless of the concentration ratio. For example, QCT–CHA produced an approximate 50% scavenging effect in the proportion 1:2 μg/mL (total concentration of 3 μg/mL), while up to 4.19 μg/mL of pure CHA or 7.80 μg/mL of pure QCT was needed to achieve the same effect. Consequently, while CHA and QCT were separately less effective than ECA, their combination expressed similar efficacy. In addition, the scavenging potential of ECA might also be further increased by the presence of either of the two other compounds. Especially effective was the combination ECA–CHA; e.g., a quenching effect greater than 50% was achieved by only 2 μg/mL of 1:1 μg/mL ECA–CHA in comparison to 2.62 μg/mL of pure ECA. In light of the obtained results, the disturbed correlation between PEA and the extracts activity in the $O_2^{\bullet-}$ scavenging assay might be explained by the differences in the extracts' profiles, i.e., in the proportions between different groups of constituents. For example, phenolic rich DEF and EAF exhibited activities similar to those of BF, a relatively poor fraction in polyphenols but distinguished by a well-balanced composition [3]. In particular, the largest proportion of CHA was found with the ratios CHA:flavonoids and CHA:flavan-3-ols in an optimal range of about 3:1 and 1.5:1, respectively (Figure 2).

Due to the wide occurrence of the three investigated polyphenols, the obtained results might be applicable for other plant materials with a composition similar to that of *S. domestica*. For example, the extracts from the flowers of a related *Sorbus* species, *S. aucuparia*, have been also been proven to possess a relevant antioxidant activity [29]. However, despite a higher content of total phenolics, the activity of the *S. aucuparia* extracts in the $O_2^{\bullet-}$-scavenging test was only comparable to or even lower than that of the extracts investigated in the present study. The largest difference was observed between BF fractions of the two species. Since, in contrast to *S. domestica*, *S. aucuparia* contains a very small amount of ECA and low-molecular-weight procyanidins, the discrepancies in activity might be due to the limited synergistic effects between these compounds and other polyphenols (flavonoids and caffeoylquinic acids). Apart from $O_2^{\bullet-}$-quenching, the lower relative activity of *S. aucuparia* in comparison to *S. domestica* was also noticeable in other scavenging tests, with the exception of HClO-quenching. This observation suggests that in those cases, the synergic effects might still be present and worth investigation. Indeed, the interactions between flavonoids, flavan-3-ols and caffeic acid derivatives, although relatively rarely studied so far, have been revealed in different models of antioxidant activity [25,30,31]. For example, a study of synergistic activity between phenolic acids and flavonoids, found that CHA–QU (0.6 + 0.15 µmol/mL) and CHA–RT (0.6 + 0.15 µmol/mL) exhibit enhanced ferric reducing antioxidant power (FRAP) by 17.2% and 5.5%, respectively [25]. Similarly, a study of the constituents of green tea and *Osmanthus fragrans* flowers found the highest antioxidant effects (in DPPH assay) to be associated with a combination of acteoside (caffeoyl-rhamnosyl-glucoside of hydroxytyrosol) and ECA and caffeic acid and ECA [32]. In the same model, synergistic effects were demonstrated between CHA and simple phenolic acids [33], while in ORAC assay, CHA acted synergistically in two- to four-compound combinations with flavonoids from *Citrus sinensis* [31]. The interactions between the individual compounds and groups of compounds can further translate not only to the improved efficacy of a single plant material but also to enhanced activity of plant material combinations. For example, the antioxidant capacity of tea (*Camelia sinensis*) was improved in combined formulation with the *Vitis vinifera* seed (proanthocyanidins), *Punica granatum* and *Phyllanthus emblica* fruits (hydrolysable tannins), *Ginkgo biloba* leaf (flavonoids), and *Cinnamomum cassia* bark (condensed tannins) [34].

3. Materials and Methods

3.1. General

High-purity reagents and standards used for fluorometric and spectrophotometric assays including xanthine, xanthine oxidase from bovine milk, nitrotetrazolium blue chloride (NBT), horseradish peroxidase, hydrogen peroxide, 4-aminoantipyrine, phenol, iron (II) sulfate heptahydrate, salicylic acid, 5-thio-2-nitrobenzoic acid, 4,5-diaminofluorescein, Evans blue, sodium borohydride, ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, QU dihydrate, QCT, RT, CHA, TX, and AA were from Sigma-Aldrich (Seelze, Germany/St. Louis, MO, USA), phosphate-buffered saline (PBS) was from Biomed (Lublin, Poland), sodium nitroprusside and NaOCl were from Avantor Performance Materials (Gliwice, Poland), while ECA, PB2, and PC1 were purchased from Phytolab (Vestenbergsgreuth, Germany). The species-specific flavonoids HRQ and PRQ were isolated earlier in our laboratory from the leaves of *S. domestica* [4]. All other chemicals were of analytical grade and obtained from Avantor Performance Materials (Gliwice, Poland). All activity studies were performed using 96-well plates and microplate readers: SPECTROstar Nano (BMG LabTech, Ortenberg, Germany) and Synergy HTX (BioTek, Winooski, VT, USA).

3.2. Plant Material and Extracts Preparation

The analyses were performed using extracts (MED, DEF, EAF, BF, WR) obtained by a fractionated extraction of the leaves of *S. domestica* L. The plant material was collected in September 2015 in the Arboretum (51°49'N, 19°53'E), Forestry Experimental Station of Warsaw University of Life Science

(SGGW) in Rogow (Poland) and authenticated by Piotr Banaszczak (Head of the Arboretum). The preparation of the extracts and their standardization were described previously [3].

3.3. Antioxidant Activity Measurements

The scavenging activity of the analytes towards multiple oxidants was evaluated using relevant spectrophotometric and fluorimetric methods. The $O_2^{\bullet-}$ scavenging activity was determined according to Granica et al. [35] in a xanthine/xanthine oxidase system with NBT used for detection. The ability to scavenge H_2O_2 was evaluated according to Marchelak et al. [36] by monitoring the level of quinoneimine generated in the reaction of 4-aminoantipyrine, phenol and H_2O_2, catalyzed by horse radish peroxidase. The $NO^{\bullet}$ scavenging activity was measured acc. to Czerwińska et al. [37] using 4,5-diaminofluorescein as $NO^{\bullet}$ probe. The ability to scavenge $HO^{\bullet}$ was assayed using the method by Marchelak et al. [36] with the level of $HO^{\bullet}$ monitored in the presence of salicylic acid. The HClO scavenging effect were evaluated according to Czerwińska et al. [37], with 5-thio-2-nitrobenzoic acid used for detection. The ability to scavenge $ONOO^-$ (obtained synthetically) was determined according to Krzyzanowska-Kowalczyk et al. [38], by measuring the inhibition of Evans Blue dye oxidation. The results were expressed as SC_{50} values (the concentration of the analyte that decreases the initial amount of the oxidant by 50%) and calculated from concentration-scavenging curves (5–10 calibration points). For direct comparison, the results were expressed in equivalents of AA (extract activity parameters, AAE) and TX (TE) per dry matter (μmol AA/mg dw and μmol TX/mg dw, respectively). Then the total antioxidant potential of each analyte was calculated with respect to both standards as a sum of the results from particular tests (AAE_{MR6} and TE_{MR6}, respectively).

3.4. PEA Index Calculation

PEA indices, understood as the weighted sum of the markers' activities in a particular extract, were calculated for each extract in each assay, according to Equation (1):

$$\mathrm{PEA} = \sum c_n a_n \tag{1}$$

where c_n is the relative content of compound n in the extract and a_n is the activity of compound n in a particular assay expressed in AA equivalents.

3.5. Synergistic Effect Measurement

The synergistic effects between the representatives of the main extracts' constituents (QCT, ECA, and CHA) were tested in an $O_2^{\bullet-}$ scavenging capacity assay. First, the scavenging percentage (S) of the three individual compounds was tested in different concentrations (c) – 0.25–6 μg/mL for ECA and 1–24 μg/mL for QCT and CHA, and dose-response curves (eight calibration points) were obtained by fitting the experimental data into the Weibull function (Equation (2)) [39]:

$$S(c) = Le^{-ln2(\frac{c}{m})^k} \tag{2}$$

where L is the maximum possible response and was set to 100%, the parameter m corresponds to the concentration required for 50% radical quenching (SC_{50}), and the parameter k is related to the maximal slope of the response (Figure 4, Table 3).

Then, the compound combinations (QCT–CHA, QCT–ECA, and CHA–ECA) were tested in different concentration ratios (4:1, 2:1, 1:1, 1:2, and 1:4) and the combination indices (CI) were calculated according to Equation (3):

$$CI = \frac{c_1}{c_{x,1}} + \frac{c_2}{c_{x,2}} \tag{3}$$

where c_1 and c_2 are the concentrations of the compounds actually used in the assay, and $c_{x,1}$ and $c_{x,2}$ are the concentrations theoretically required (calculated from the dose-response curves) to produce the

obtained effect x. A *CI* equal to 1 indicates additivity, while a *CI* less then or more than 1 indicates synergy and antagonism, respectively. For each dose combination, the experiment was run five times and a 95% confidence interval for each *CI* was obtained to evaluate its statistical significance.

3.6. Statistical Analysis

The results were reported as means ± *SD* (standard deviation) for the indicated number of experiments. The normality of the distribution of the results was verified using the Shapiro–Wilk test, and the homogeneity of variances was verified using Levene's test. The significance of the differences between samples and controls was determined with a one-way ANOVA, followed by the post hoc Tukey's test for multiple comparisons. The correlations were evaluated by calculating Pearson correlation coefficients. All calculations were performed using the Satistica13 Pl software for Windows (StatSoft Inc., Krakow, Poland). *p*-values less than 0.05 were regarded as significant.

4. Conclusions

This study is the first evaluation of the scavenging capacity of *S. domestica* leaf extracts and their activity markers towards in vivo-relevant oxidants. It demonstrated that both individual polyphenolic compounds and leaf extracts might be considered broad spectrum ROS/RNS scavengers and that ROS/RNS quenching might be one of the possible mechanisms of their protective effects against the oxidative/nitrative damage to human plasma noted in previous studies [3,4]. As demonstrated in the $O_2^{\bullet -}$ scavenging test, some of the activity of the tested extracts might be a result of synergy between their constituents. This may be considered an advantageous feature and serve as a prompt for further studies of similar effects in more complex biological models. The observed relations between the three investigated phenolic groups might also be used for the interpretation of antioxidant effects in other plant materials of similar composition. However, more detailed research is required to identify other possible mechanisms and in vivo effects of the tested extracts. Non-direct mechanisms of antioxidant protection should first be verified, such as the influence of the analytes on gene expression and the activity of endogenous antioxidant enzymes, as well as the impact on ROS/RNS secretion at a cellular level.

Supplementary Materials: The Supplementary Materials are available online.

Author Contributions: Conceptualization, M.A.O., A.O. and M.R.; investigation, M.R. and J.K.-C.; formal analysis, M.R. and A.O.; writing—original draft preparation, M.R. and A.O.; writing—review and editing, M.A.O., J.K.-C. and P.N.; visualization, M.R.; resources, M.A.O. and P.N.; supervision, M.A.O. All authors read and approved the final manuscript.

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Sample Availability: Plant samples are available from the authors.

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Article Supplementary Materials:

Sorbus domestica leaf extracts and their activity markers: Antioxidant potential and synergy effects in scavenging assays of multiple oxidants

Magdalena Rutkowska[1]*, Monika Anna Olszewska[1], Joanna Kolodziejczyk-Czepas[2], Pawel Nowak[2], Aleksandra Owczarek[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Lodz, ul. Muszynskiego 1, 90-151 Lodz, Poland; magdalena.rutkowska@umed.lodz.pl (M.R.); monika.olszewska@umed.lodz.pl (M.A.O.); aleksandra.owczarek@umed.lodz.pl (A.O.)

[2]Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, ul. Pomorska 141/143, 90-236 Lodz, Poland; joanna.kolodziejczyk@biol.uni.lodz.pl (J.K.-C.); pawel.nowak@biol.uni.lodz.pl (P.N.)

* Correspondence: magdalena.rutkowska@umed.lodz.pl (M.R.)

Supplementary Materials:

- **Figure S1.**

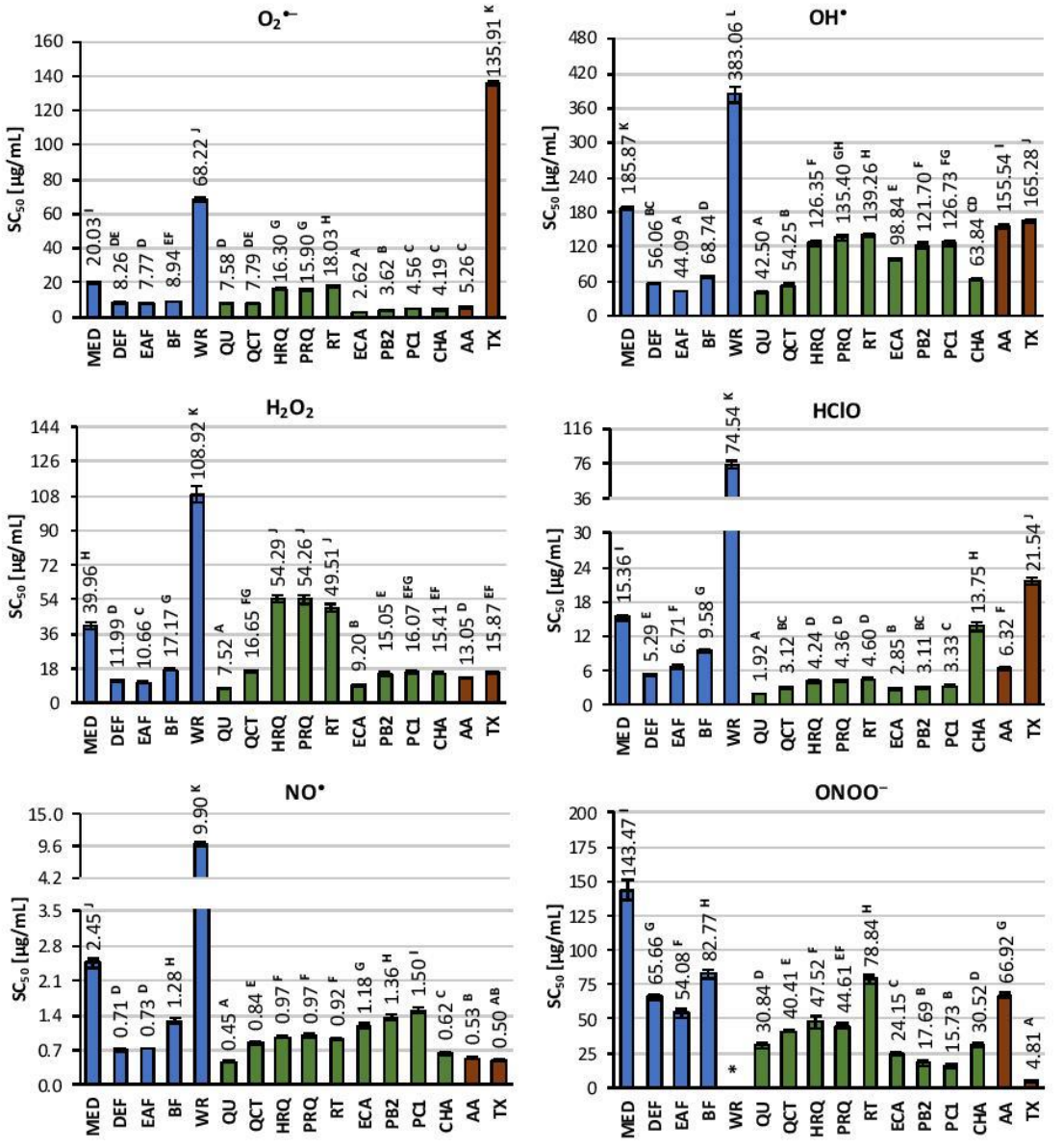


Figure S1. Antioxidant activity of *S. domestica* leaf extracts, their activity markers and standards towards *in vivo*-relevant oxidants. Results are presented as mean values of SC_{50} (amount of antioxidant needed to decrease the oxidant level by 50%) ± SD (n = 3), expressed in µg of the dry extract or standard/mL. For each parameter different letters A-K indicate significant differences ($p < 0.05$). The analyte marked with an asterisk is inactive in concentration up to 200 µg/mL. MED, methanol-water (7:3, *v/v*) extract; DEF, diethyl ether fraction; EAF, ethyl acetate fraction; BF, *n*-butanol fraction; WR, water residue; QU, quercetin; QCT, quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside); HRQ, quercetin 3-*O*-(2''-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside; PRQ, quercetin 3-*O*-(2''-*O*-β-D-xylopyranosyl) -α-L-rhamnopyranoside; RT, rutin; ECA, (–)-epicatechin; PB2, procyanidin B2; PC1, procyanidin C1; CHA, chlorogenic acid (5-*O*-caffeoylquinic acid); AA, ascorbic acid; TX, Trolox.

Publikacja IV:

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COMPARISON OF PHENOLIC PROFILE AND *IN VITRO* ANTIOXIDANT CAPACITY OF *SORBUS DOMESTICA* L. LEAF SAMPLES FROM POLAND AND CROATIA

MAGDALENA RUTKOWSKA*, MONIKA DUBICKA AND MONIKA A. OLSZEWSKA

Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, 90-151 Lodz, Poland

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This work presents a comparative analysis of the phenolic composition (UHPLC-PDA-ESI-MS^3, HPLC-PDA-fingerprint, UV-spectrophotometric methods) and antioxidant activity (DPPH, FRAP) of leaf samples from two vegetation seasons of a medicinal and dietary plant *Sorbus domestica* growing in its natural habitat (Croatia, C) and cultivated in Poland (P). The samples from both sources were rich in structurally diverse polyphenols (44 analytes: P: 73.4–76.6 and C: 98.3–106.7 mg GAE/g dry leaves) including the dominating flavan-3-ols and flavonoids. The greatest qualitative and quantitative differences were observed for flavonoids (P: 14.3–20.3%; C: 27.5–34.1% of polyphenols) – in the Polish samples flavonoid diglycosides predominated, in the Croatian samples the contents of both monoglycosides and diglycosides were similar. In the case of dry methanolic extracts, despite the higher extraction efficiency obtained for the Croatian samples (32–36% vs 23–24%), the quality of the extracts was comparable, both in terms of the total phenolic content (P: 269.4–280.0; C: 297.6–304.4; mg GAE/g dry extract) and antioxidant activity parameters (DPPH, EC_{50}, µg/mL, P: 10.5–10.9, C: 10.0–10.3; and FRAP, mmol Fe^{2+}/g, P: 6.64–7.13, C: 7.06–7.11). As a result, the study confirmed the influence of environmental conditions on the phenolic profile and antioxidant capacity of *S. domestica* leaves, as well as showed that despite some differences, plant materials from both Poland and Croatia might be suitable for production of natural health products.

Keywords: antioxidant activity, extract quality, LC-MS, leaves, polyphenolic composition, *Sorbus domestica* L.

INTRODUCTION

Sorbus domestica L. (service tree) is a deciduous tree from the Rosaceae family, which due to its edible fruits and decorative qualities has been cultivated in the Mediterranean region since Roman times. The main areas of its occurrence are the Balkan Peninsula, the Apennines, southern France and eastern Spain (Rotach, 2003). The species is easily acclimatized to various climate conditions, and its present distribution is to a large extent a result of human influence, as over the centuries it has been locally planted and sub-spontaneously spread to some parts of Central Europe. Although the plant prefers the Mediterranean climate, it can also be cultivated in temperate regions (including Poland) with generally good results, except for diminished flowering and fruit production (Poljak et al., 2015).

Service tree is a source of traditionally used plant materials (mainly fruits and leaves) with anti-inflammatory, anti-atherogenic, anti-diabetic, antidiarrheal and diuretic properties (Kültür, 2007). So far the available literature data concerning biological activity of *S. domestica* have focused mostly on the fruits from southern Europe and indicated promising antioxidant and anti-diabetic potential of the extracts and polyphenolic fractions obtained therefrom (Termentzi et al., 2006, 2008). However, although berries in general are recognized as rich sources of bioactive polyphenols, recent studies underline superiority of leaves obtained from fruit plants, which constitute cost-effective plant materials available throughout the whole growing season (Teleszko and Wojdyło, 2015). Indeed, our previous studies documented that leaves of *S. domestica* cultivated in Poland accumulate an immense diversity of polyphenols.

* Corresponding author, email: magdalena.rutkowska@umed.lodz.pl

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and thus constitute a promising herbal raw material that may be used in prevention or adjunctive therapy of oxidative stress/inflammation-related pathologies (Matczak et al., 2018). On the other hand, it is known that therapeutic efficiency of medicinal plants as a function of secondary metabolites production is influenced by numerous factors, including environmental growth conditions (Michel et al., 2017).

Plant secondary metabolites, also known as specialized or specific metabolites, are crucial components of plants adaptive mechanisms and play an important role in defence against various stress factors, such as high or low temperature, solar radiation, drought or humidity, as well as herbivory or pathogenic attack (Ncube et al., 2012). Numerous scientific reports emphasize the influence of climatic factors on the biosynthesis and accumulation of polyphenols (e.g., flavonoids) in plants tissues, depending on the species, organ or stage of plant development (Aninbon et al., 2016; Chen et al., 2018; Zhang, Wu et al., 2017). Apart from their beneficial role for the plants, some of these metabolites are of great pharmaceutical interest because of their health promoting properties (Chen et al., 2018; Ncube et al., 2012). Since the majority of medicinal species grow in wild and their metabolite pools are determined by uncontrolled environmental factors, pharmaceutical raw materials originating from plants from different regions might differ in quality (Ncube et al., 2012). Thus, for each medicinal plant, the studies of its properties in relation to the environmental and/or geographical conditions should be conducted. It is especially important for species such as service tree which are cultivated outside their typical habitat. Unfortunately, there is no previous report on *S. domestica* concerning this issue.

Therefore, the aim of this research was to determine the differences in the phenolic profile, antioxidant capacity and extraction yields of the respective hydroalcoholic extracts between *S. domestica* leaves harvested in Poland (Central Europe) and Croatia (Mediterranean region). The material for the study were dried leaves, as well as dry hydromethanolic extracts prepared thereof as the substances preferred by the pharmaceutical industry due to the improved stability, more favorable physical and biological properties, and simplicity in further processing. The raw materials were collected in two different growing seasons in late summer (August), since the period after flowering and before fruit maturity was reported to be optimal for phenolics accumulation in the leaves of fruit trees, including *Sorbus* species (Cosmulescu and Trandafir, 2011; Olszewska, 2011). The qualitative and quantitative phenolic profiles were monitored by UHPLC-PDA-ESI-MS^3. HPLC-PDA-fingerprint and spectrophotometric methods. The DPPH free radical-scavenging and FRAP (ferric reducing antioxidant power) assays were selected as *in vitro* activity models based on the earlier findings for leaf dry extracts of *S. domestica* (Matczak et al., 2018), which indicated that these two tests are good predictors of antioxidant capacities of the extracts in more complex *in vivo*-relevant models.

MATERIALS AND METHODS

PLANT MATERIAL AND EXTRACTS PREPARATION

Leaves of *S. domestica* L. were collected in August 2014 and 2016 in the Arboretum (51°49'N, 19°53'E), Forestry Experimental Station of Warsaw University of Life Science in Rogow (Poland) from long-term cultivation and authenticated by Piotr Banaszczak (Head of the Arboretum), as well as in Risika on Krk island (Croatia: 45°06'N, 14°38'E) from plants growing in their natural habitat and authenticated by prof. M.A. Olszewska (Department of Pharmacognosy, Medical University of Lodz, Poland). The voucher specimens were deposited in the herbarium of the Department of Pharmacognosy, Medical University of Lodz, Poland (KFG/HB/14001-SDOM:P/C and KFG/HB/16001-SDOM:P/C). The raw materials were dried, powdered with an electric grinder and sieved.

Extracts of raw plant materials were prepared for analysis as follows: accurately weighed samples of the leaf material (100 mg) were refluxed first for 30 min with 30 mL of methanol-water (7:3, v/v), and then twice for 15 min with 20 mL of the same solvent. The obtained extracts were combined, filtered and diluted with the extractant to 100 mL. Each plant material was extracted in triplicate to give the test extracts, which were analyzed for their total phenolic (TPC) and total proanthocyanidin contents (TPA), as well as the activity in DPPH and FRAP tests. For HPLC analysis leaf samples (1.5–2.0 g) were first defatted by pre-extraction with 100 mL chloroform for 45 min (the extracts were discarded), and then extracted with methanol-water (7:3, v/v) as described above. The results were calculated per dry weight (dw) of the leaves.

Dry extracts were prepared as follows: 2.5 g of leaf samples were pre-extracted for 18 h with 1 L chloroform in a Soxhlet apparatus and the pellet was next refluxed three times for 6 h with 100 mL of 70% (v/v) aqueous methanol to give the defatted methanol extracts. The obtained extracts were then evaporated *in vacuo* and lyophilized using an Alpha 1–2/LD Plus freeze dryer (Christ, Osterode am Harz, Germany) before weighing. The results of the TPC test were calculated per dry weight (dw) of the extract.

CHEMICALS AND INSTRUMENTATION

HPLC grade reagents and standards were purchased from Sigma-Aldrich (Seelze. Germany/ St. Louis. MO. USA). HPLC grade solvents were obtained from Avantor Performance Materials (Gliwice. Poland). In all analyses. redistilled water was used. The analytical equipment used in the study was the same or of the same origin as employed earlier (Matczak et al.. 2018).

PHYTOCHEMICAL PROFILING AND ANTIOXIDANT ACTIVITY STUDIES

The UHPLC-PDA-ESI-MS^3 was carried out according to Matczak et al. (2018) with some modifications in the elution system and sample injection. The mobile phase consisted of solvent A (water/acetonitrile/formic acid. 95:5:0.1. v/v/v). and solvent B (acetonitrile/formic acid. 100:0.1. v/v) with the elution profile as follows: 0–40 min. 5–30% B (v/v): 40–55 min. 30–95% B: 55–63 min. 95% B: 63–70 min. 95%–6% B: 70–80 min. 6% B (equilibration). The sample solutions of the extracts were prepared in methanol-water (7:3. v/v) at the concentration 5 mg/mL. The flow rate was 0.3 mL/min. UV–vis spectra were recorded over the range of 200–600 nm. The LC eluate was introduced directly into the ESI interface without splitting and analyzed in a negative ion mode. The analysis was carried out using a scan from m/z 200 to 2200.

The TPC and TPA were quantified by the Folin-Ciocalteu and *n*-butanol-HCl methods. respectively. as described previously (Olszewska et al.. 2012). The results were expressed as equivalents of gallic acid (GAE) and cyanidin chloride (CYE). respectively.

The HPLC-PDA-fingerprint assays were performed according to Olszewska et al. (2012) with some modifications in the elution system. The mobile phase consisted of solvent A (0.5% water solution of orthophosphoric acid. w/v) and solvent B (acetonitrile) with the elution profile as follows: 0–1 min. 5% B (v/v): 1–12 min. 5–19% B: 12–18 min. 19% B: 18–19 min. 19–30% B: 19–20 min. 30–50% B: 20–21 min. 50–5% B: 21–25 min. 5% B (equilibration). The flow rate was 1.4 mL/min. and the column was maintained at 30°C. The phenolic analytes were quantified as equivalents of nine external standards: (–)-epicatechin (flavan-3-ols and proanthocyanidins). protocatechuic and *p*-hydroxybenzoic acids (hydroxybenzoic acids). chlorogenic acid (caffeoylquinic acid isomers). caffeic and *p*-coumaric acids (hydroxycinnamic acid derivatives). quercitrin (flavonoid monoglycosides). rutin (flavonoid diglycosides). and quercetin (flavonoid aglycones). depending on the PDA spectra.

The DPPH and FRAP tests were performed according to the method optimized previously (Olszewska et al.. 2012) and expressed as normalized EC_{50} values (calculated from concentration-inhibition curves) and μmol of ferrous ions (Fe^{2+}) produced by 1 g of dry leaves or the dry extract (calculated from the calibration curve of ferrous sulphate). respectively.

DATA ANALYSIS

The results are reported as means ± SD (standard deviation) for the indicated number of experiments. The significance of differences between the samples and controls was determined with one-way ANOVA. followed by the post hoc Tukey's test for multiple comparisons. All calculations were performed using Statistica 12Pl software for Windows with p values less than 0.05 regarded as significant.

RESULTS

The qualitative UHPLC-PDA-ESI-MS^3 analysis of the leaf extracts from *S. domestica* revealed the presence of over forty phenolic constituents (UHPLC peaks 1–44: Fig. 1. Table 1). forty-three of which were fully or tentatively identified by comparing their retention times. UV-Vis spectral data and MS profiles with those of the reference compounds and the literature data (Clifford et al.. 2005: Fang et al.. 2002: Matczak et al.. 2018: Ncube et al.. 2014). According to the identification data. three major groups of compounds could be differentiated. including phenolic acids (peaks 1–5. 7. 8. 10. 13. 14. 17. 30). flavan-3-ols and proanthocyanidins (peaks 6. 9. 11. 12. 15. 16. 18–21. 26. 27). as well as flavonoids (peaks 22–25. 28. 29. 31–43). Direct comparison of the recorded UHPLC profiles revealed only small qualitative differences between the leaf samples from Poland and Croatia. i.e.. caffeic acid hexoside (3). one procyanidin dimer B-type (9) and quercetin rhamnoside (35) occurred only in the Polish samples. while two *p*-coumaroylquinic acid isomers (13. 14). 3.4-dicaffeoylquinic acid (17) and kaempferol hexoside (31) were found only in the Croatian samples (Table 1). However. these discrepancies may result from detection limitations. as the mentioned compounds were present in the extracts in small amounts only. and for the majority of the individual analytes and groups of analytes the substantial quantitative variations were indicated.

The levels of total phenolics (TPC). determined by the Folin-Ciocalteu assay and expressed as gallic acid equivalents (GAE). differed significantly ($p < 0.05$) between the leaf materials from Poland (73.4–76.6 mg GAE/g leaf dw) and Croatia

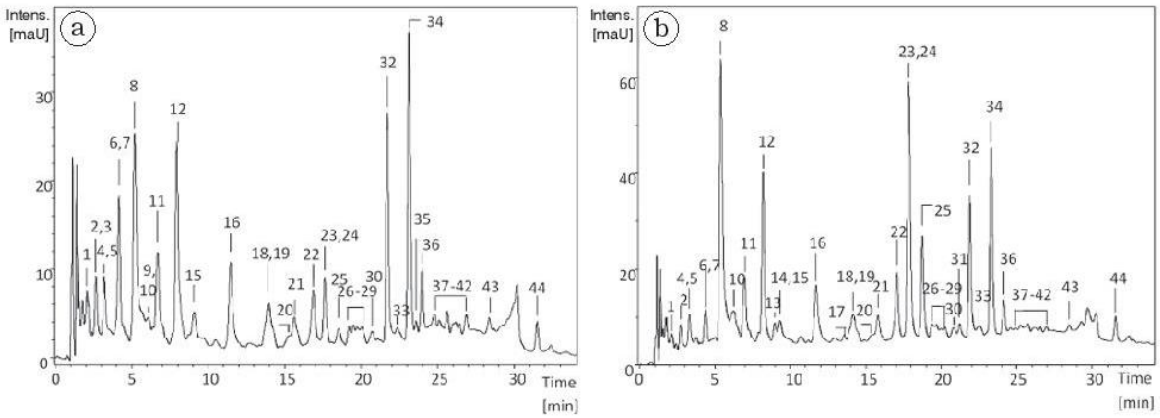


Fig. 1. Representative UHPLC-UV chromatograms of *Sorbus domestica* leaf extracts from Poland (**a**) and Croatia (**b**) at 280 nm. Peak numbers refer to those implemented in Table 1.

TABLE 1. UHPLC-PDA-ESI-MS data on polyphenols identified in *Sorbus domestica* leaf extracts.

No	Compound	R_t (min)	UV λ_{max} (nm)	[M-H]$^-$	MS^2	MS^3	Extract
1	chlorogenic acid hexoside	2.2	325	515	**353**,341,323,191	191,179	B
2	neochlorogenic acid hexoside	2.7	325	515	**353**,341,323,191,179	191,179	B
3	caffeic acid hexoside	2.7	325	341	179,161		P
4	3-*O*-caffeoylquinic acid (NCHA)*	3.3	325	353	191,179		B
5	protocatechuic acid hexoside	3.3	270	315	153,219		B
6	procyanidin dimer B-type	4.3	280	577	451,**425**,407,289	407,273	B
7	*p*-coumaric acid hexoside	4.3	310	325	163,119		B
8	5-*O*-caffeoylquinic acid (CHA)*	5.4	325	353	191,179		B
9	procyanidin dimer B-type	6.2	280	577	451,**425**,407,289	407,273	P
10	4-*O*-caffeoylquinic acid (CCHA)*	6.2	325	353	173,191,179		B
11	procyanidin B-2 (PB2)*	6.9	280	577	451,**425**,407,289	407,273	B
12	(-)-epicatechin (ECA)*	8.2	280	289	245,205		B
13	*p*-coumaroylquinic acid isomer	9.0	310	337	191,163		C
14	*p*-coumaroylquinic acid isomer	9.2	310	337	191,163		C
15	proantocyanidin dimer B-type	9.2	280	577	425,407,289		B
16	procyanidin trimer B-type (PC1)	10.5	280	865	**713**,695,577	695,425,407,289	B
17	3,4-dicaffeoylquinic acid	13.6	280,325	515	**353**,335,299,255,191	173,179,191,135	C
18	procyanidin tetramer B-type	14.1	280	1153	863,739,575,451,425		B
19	procyanidin dimer B-type	14.1	280	577	451,425,407,289		B
20	proantocyanidin derivative	15.6	280	720	635,**575**,451,289	289, 279	B
21	procyanidin dimer hexoside	15.8	280	739	**587**,451,339,289	477,417,339,255	B

No	Compound	R_t (min)	UV λ_{max} (nm)	$[M-H]^-$	MS^2	MS^3	Extract
22	quercetin hexoside-rhamnoside	17.1	275,350	609	**447**,301	301	B
23	rutin (RT)*	17.8	256,355	609	463,301,179		B
24	hyperoside (HY)*	17.8	254,353	463	**301**	179	B
25	isoquercitrin (IQ)*	18.7	256,353	463	**301**	179	B
26	procyanidin dimer B-type	19.4	280	577	451,**425**,407,289	289,341,245	B
27	proantocyanidin derivative	19.7	280	483	451,341,289		B
28	quercetin hexoside derivative	19.9	275,350	519	**357**,301	151,135	B
29	reinutrin*	20.2	275,350	433	301		B
30	4,5-dicaffeoylquinic acid	20.8	280,325	515	353,299,255,203,173	173,179,191	B
31	kaempferol hexoside	21.2	275,350	447	285		C
32	quercetin hexoside-rhamnoside (HRQ)	21.8	256,350	609	**447**,301,179	301	B
33	quercetin hexoside-rhamnoside	22.5	275,350	609	**447**,301,179	301	B
34	quercitrin (QCT)*	23.3	276,350	447	301,179		B
35	quercetin rhamnoside	23.7	280,350	447	**301**	179,151	P
36	quercetin pentoside-rhamnoside (PRQ)	41.5	256,353	579	**447**,301	301	B
37	quercetin hexoside-rhamnoside	25.0	256,350	609	**447**,301	301	B
38	quercetin hexoside-rhamnoside	25.3	256,350	609	**447**,301	301	B
39	quercetin hexoside-rhamnoside	25.9	263,350	609	**447**	301	B
40	quercetin dirhamnoside	26.2	265,347	593	**447**	301	B
41	quercetin dirhamnoside	26.6	265,335	593	**447**,301	301	B
42	quercetin derivative	27.0	255,350	591	447,301		B
43	afzelin*	28.4	263,346	431	285,255		B
44	unidentified compound	31.5	280	451	341,**299**	189	B

Peak numbers and retention times (R_t) refer to those implemented in Fig. 1. Compounds identified with authentic standards were marked with an asterisk. UV λ_{max}, absorbance maxima in PDA spectra. $[M-H]^-$, pseudomolecular ions in MS spectra recorded in a negative mode. MS^2/MS^3, secondary ions (MS^2 ions in bold were subjected to MS^3 fragmentation). P – Polish sample. C – Croatian sample. B – both extracts.

(98.3–106.7 mg GAE/g leaf dw), which was also reflected in the extraction yields (Figs. 2 and 3). However, it should be noted that the differences between years were for each country significantly smaller than those observed between countries, regardless of the harvest year. In the case of the concentrated phenolic fractions (leaf dry extracts), the differences in the TPC levels were generally less pronounced (P: 269.4–280.0 mg GAE/g extract dw; C: 297.6–304.4 mg GAE/g extract dw), and statistically significant ($p < 0.05$) only between countries (Fig. 3). Flavan-3-ols and proanthocyanidins were the dominant groups of polyphenols in all plant samples with (–)-epicatechin (12) and procyanidin B2 (11) as prevailing constituents (Figs. 1 and 2). The total proanthocyanidin content (TPA), determined by *n*-butanol/HCl assay and expressed in cyanidin chloride equivalents (CyE), in all plant materials constituted about 30–35% of their TPC levels, while the respective total contents (TLPA) determined by HPLC-PDA represented 59–64% (P) and 45–52% (C) of total phenols calculated as the sum of individual analytes (TPH, Fig. 4), depending on the harvest year. The second relevant group of constituents were flavonoids, which constituted 14–20% (P: 3.61 mg/g dw of the leaf material in 2014 and 4.18 mg/g in 2016) and 28–34% (C: 9.38 mg/g

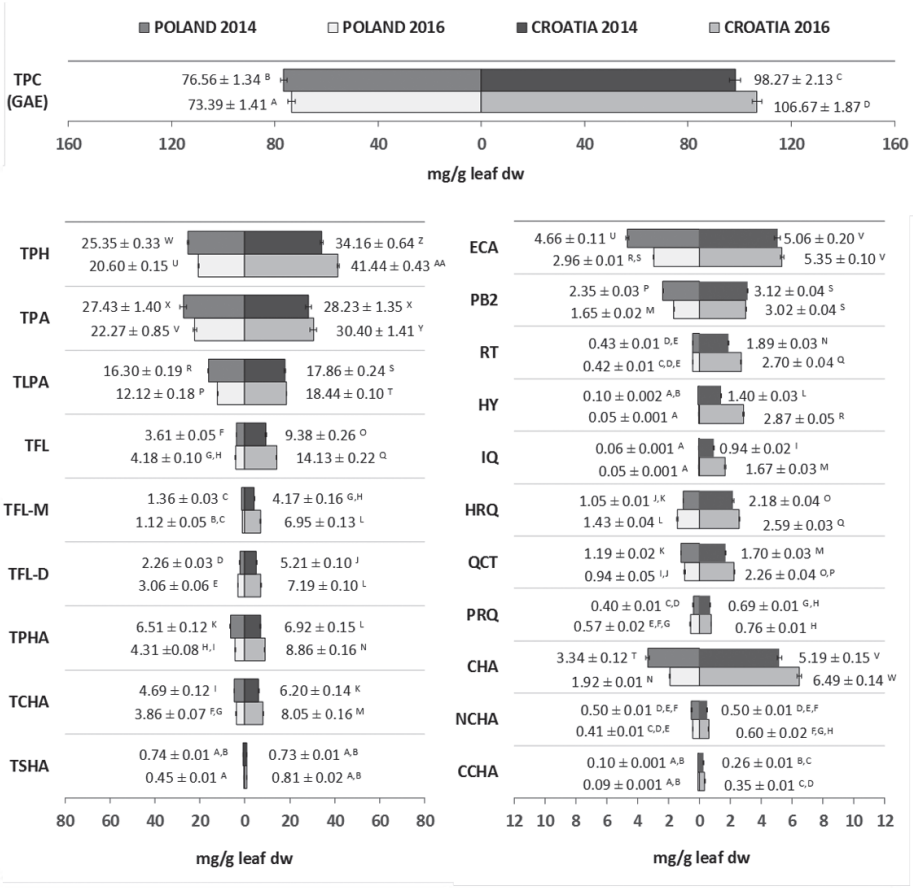


Fig. 2. Quantitative profile of *Sorbus domestica* leaves from Poland and Croatia harvested during two years (2014 and 2016). Data expressed as mean values ± SD (n = 3). For each parameter different capitals indicate significant differences ($p < 0.05$). TPC, total phenolic content in gallic acid equivalent (GAE) determined by the Folin-Ciocalteu assay; TPH, total phenolic content determined by HPLC-PDA; TPA, total proanthocyanidin content determined by the *n*-butanol/HCl assay; TLPA, total content of individual proanthocyanidins assayed by HPLC; TFL, total flavonoid content; TFL-M, total content of flavonoid monoglycosides; TFL-D, total content of flavonoid diglycosides; TPHA, total content of phenolic acids; TCHA, total content of chlorogenic acid isomers and derivatives; SPHA, total content of simple hydroxybenzoic and hydroxycinnamic acids; ECA, (−)-epicatechin; B2, procyanidin B2; RT, rutin; HY, hyperoside; IQ, isoquercitrin; HRQ, quercetin hexoside-rhamnoside; QCT, quercitrin; PRQ, quercetin pentoside-rhamnoside; CHA, chlorogenic acid; NCHA, neochlorogenic acid; CCHA, cryptochlorogenic acid.

in 2014 and 14.13 mg/g in 2016) of TPHs. respectively (Figs. 2 and 4). Interestingly. in the Croatian leaves the total contents of flavonoid monoglycosides (TFL-M) and diglycosides (TFL-D) were similar. while in the plant materials from Poland flavonoid diglycosides predominated and constituted about 62–73% of TFL (Fig. 4). Taking into account the profile of individual compounds. hyperoside (24) and isoquercitrin (25) occurred in the Polish samples in trace amounts only. while in the Croatian samples they were among the main flavonoids (Figs. 1 and 2. Table 1). The total content of phenolic acids (TPHA. Fig. 2) constituted about 20–21% of the TPH levels (Fig. 4). In this group chlorogenic acid isomers and derivatives (TCHA) were predominant and represented 86–91% of TPHA. The major isomer was chlorogenic acid (8) forming about 50–70% of the TCHA content in the Polish samples and 80–84% in the Croatian samples (Figs. 1 and 2). Chlorogenic acid was also the principal polyphenol occurring in the leaves from Croatia (5.19–6.49 mg/g) and the second main phenolic compound present in the Polish samples (1.92–3.34 mg/g) (Fig. 2).

Confirming previous results (Matczak et al.. 2018). the analyzed leaf samples exhibited

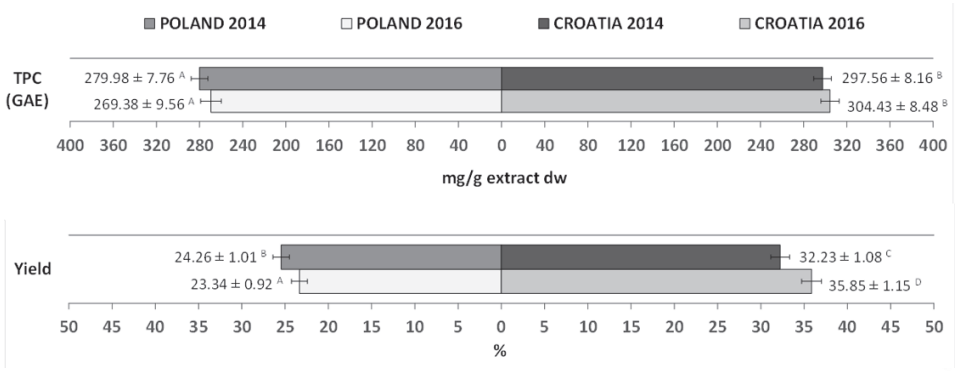


Fig. 3. Extraction yield and total phenolic contents (TPC) of *Sorbus domestica* leaf extracts determined by the Folin-Ciocalteu assay. Data expressed as means ± SD (n = 3). For each parameter different superscripts indicate significant differences ($p < 0.05$) in the Tukey's test.

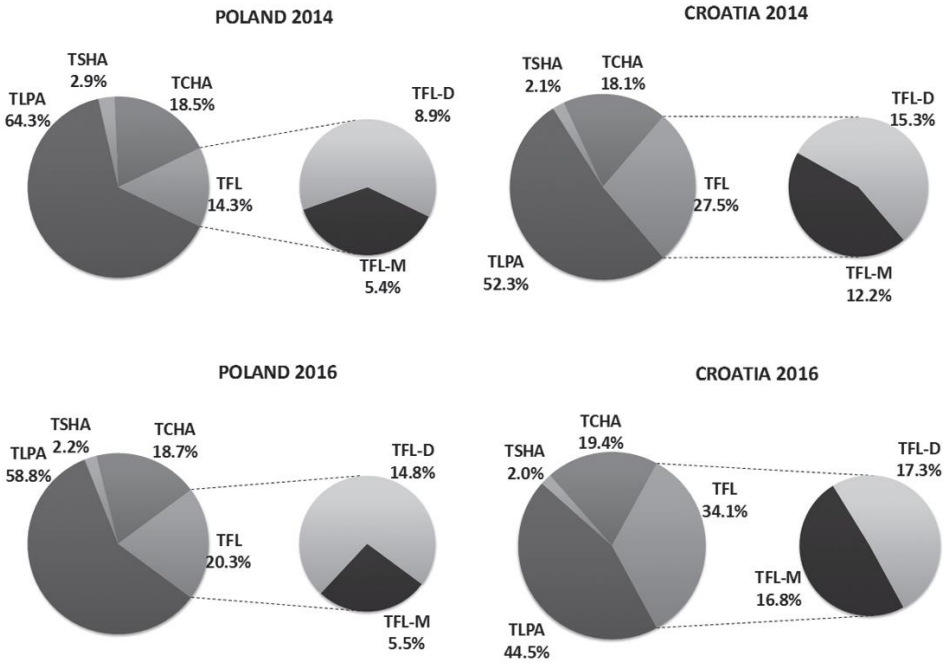


Fig. 4. Contribution of individual groups of compounds to total phenolic contents determined by HPLC-PDA and calculated as the sum of individual analytes (TPH) in *Sorbus domestica* leaves from Poland and Croatia harvested during two years (2014 and 2016). For TLPA, TFL, TFL-M, TFL-D, TCHA, SPHA codes see Fig. 2.

a concentration-dependent activity (Fig. 5). clearly corresponding to the levels of phenolics (Fig. 2). However. the differences in the TPC levels between the dry extracts of different origin (Fig. 3) were too small to be reflected in their DPPH and FRAP parameters. which were thus comparable (DPPH EC_{50}: 10.04–10.89 μg of extract dw/mL: and FRAP: 6.64–7.13 mmol Fe^{2+}/g of extract dw: $p > 0.05$). regardless of the year and country of the harvest (Fig. 5).

DISCUSSION

Variation in the phenolic composition is a common phenomenon in the plant kingdom. The presence of active substances and changes in their content are influenced by various environmental conditions. including climate. soil composition. availability of water and nutrients. as well as the stage of plant development (Ncube et al.. 2012). It is known that obtaining a raw material with the desired biological

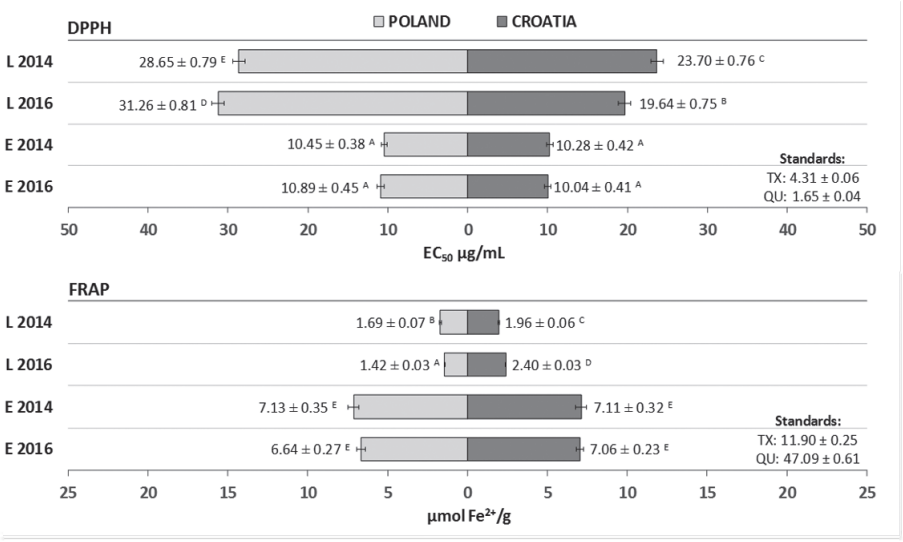


Fig. 5. Antioxidant activity of *Sorbus domestica* leaves and *S. domestica* leaf dry extracts from Poland and Croatia (2014 and 2016 year). Results are presented as mean values ± SD (n = 3) calculated per dry weight of leaves (L) or extracts (E). For each parameter (DPPH and FRAP tests) different letters indicate significant differences ($p < 0.05$) in the Tukey's test. DPPH-scavenging activity: EC_{50}, expressed in µg of dry leaves (L2014/L2016) or dry extract (E2014/E2016) per mL of the DPPH solution. FRAP activity: expressed in µmol of ferrous ions (Fe^{2+}) produced by 1 g of dry leaves (L2014/L2016) or dry extract (E2014/E2016).

properties requires its collection at a specific time of the year or. sometimes. even the day. For example. during the development of flowers. the ingredients are often allocated to generative organs. and as a result. their content decreases in other parts of the plant. such as leaves (Duda et al.. 2015: Ncube et al.. 2012). However. numerous studies showed that changes in the phenolic content over growing season in perennial trees and shrubs are relatively small in comparison to those observed for herbaceous plants (Michel et al.. 2017: Olszewska and Kwapisz. 2011). Still. the period after flowering and before fruit maturity is characterized by the strongest accumulation of phenolics in the leaves of fruit trees. including *Sorbus* species (Cosmulescu and Trandafir. 2011: Olszewska. 2011). Indeed. our preliminary investigation on seasonal variation of the phenolic content in *S. domestica* leaves in Poland (data not published) confirmed the above mentioned trends and indicated August as the most advantageous for comparison of the raw material quality from Poland and Croatia.

In general. the total phenolic contents in the tested samples were comparable to those obtained in our previous study for the *S. domestica* leaf dry extracts (Matczak et al.. 2018) and confirmed the ability of the plant to accumulate high contents of active compounds in the leaves. They put the investigated extracts in a good position compared to the leaf and flower dry extracts of other *Sorbus* plants (211.7–306.2 mg GAE/g dw). as well as to the extracts from plants considered important in the prevention of oxidative stress-related ailments. e.g.. green tea (450 mg GAE/g dw) (Matczak et al.. 2018: Olszewska et al.. 2012). Moreover. the remarkable consistency of the polyphenolic composition between the harvest years. i.e.. 2015 (Matczak et al.. 2018). 2014 and 2016 (present work) was also noticed for the samples of Polish origin.

The comparison of the samples from Poland and Croatia harvested in 2014 and 2016. leads to the conclusion. that the variations in the phenolic profile between the samples from different years were relatively small in comparison to the differences found between the countries. The discrepancies observed in the TPC levels between the leaf samples from Poland and Croatia. and thus in the extraction yields. may result from the well-known tendency of plants to accumulate higher quantities of some secondary metabolites in response to oxidative stress conditions (Ncube et al.. 2012: Roux et al.. 2017: Wei et al.. 2017). It was reported that UV-B radiation and drought stress increase the expression of genes responsible for biosynthesis of polyphenols. mainly flavonoids and hydroxycinnamates (Agati and Tattini. 2010: Ncube et al.. 2012: Zhang. Wu et al.. 2017). Indeed. higher UV Index and longer sunshine duration (lower cloud percentage and

more sunny days) in the summer months in Croatia (Table 2) resulted in a higher phenolic content in the Croatian samples. However, the differences observed between samples were not as remarkable as one would expect. The explanation might be linked to the influence of water stress on the production of some metabolites, e.g., condensed proanthocyanidins and tannins. According to literature, this correlation can be either positive or negative (Ncube et al., 2012; Zhang, Wu et al., 2017). Considering the fact that *S. domestica* naturally grows in dry to very dry conditions (Poljak et al., 2015), a higher precipitation level in Poland (rainy days: P: ~21–22 vs C: ~9–13) might influence the synthesis of some secondary metabolites.

TABLE 2. Meteorological data for the *Sorbus domestica* harvesting sites in August 2014 and 2016.

Average[a]	Poland[b]		Croatia[c]	
	2014	2016	2014	2016
Temperature (°C)	23	20	23	27
UV Index	5	5	6	7
Cloud (%)	34	39	20	23
Sunny days	7	9	11	22
Sunny hours	331	295	321	338
Humidity (%)	69	69	76	72
Rainy days	22	21	13	9

[a] Avarages based on World Weather Online forecast analysis. [b] Data for Lodz (about 25 km from Rogow Arboretum). [c] Data for Risika on Krk island.

Further determination of the main phenolic groups and individual compounds, performed using HPLC-PDA and specific UV-spectrophotometric methods, revealed that in fact the greatest qualitative and quantitative differences were observed for flavonoids. According to literature, excessive UV-B radiation increases the biosynthesis of flavonoids, mainly those with catechol group in the B-ring of the flavonoid skeleton (e.g., quercetin derivatives), which reduce photo-oxidative damage and regulate the development of plants via modulation of auxin movement (Agati and Tattini, 2010; Harborne and Williams, 2000; Tattini et al., 2004; Zhang, Chen et al., 2017). One of the molecules, whose content in plant tissues appears to be highly dependent on UV irradiation, is hyperoside (Chen et al., 2018; Zhang, Wu et al., 2017; Zhang, Chen et al., 2017). This compound, being the effective scavenger of reactive oxygen species, is suggested to play a crucial role in plant photoprotection (Agati and Tattini, 2010; Silva et al., 2008; Zhang, Chen et al., 2017). This may be the reason of the significant increase in the hyperoside content (by about 1.3 mg/g in 2014 and 2.8 mg/g in 2016) in the *S. domestica* samples from Croatia in comparison to the Polish samples (Fig. 2). Another flavonoid, the content of which substantially differed between leaf samples (by about 1.5 mg/g in 2014 and 2.3 mg/g in 2016), was rutin. According to Zhang, Wu et al. (2017), rutin accumulation depends not only on the UV-B irradiation but also on salt and drought stress, which may explain its higher content in the samples from the Mediterranean region (Fig. 2).

The next group of compounds, which seems to be highly dependent on environmental conditions, is formed by chlorogenic acid isomers and derivatives. Numerous studies indicated that the biosynthesis of hydroxycinnamic and hydroxybenzoic acid derivatives manifests an early reaction to UV exposure (Huyskens-Keil et al., 2007; Petrul'ová et al., 2014; Tattini et al. 2004). Considering its molecular structure and properties, chlorogenic acid is considered to be a potent free radical scavenger, as well as strong UV-B attenuator (Agati and Tattini, 2010; Cha et al., 2014; Tattini et al., 2004). On the other hand, it was reported that the hydroxycinnamates to flavonoids ratio decreases upon exposure to strong sunlight, suggesting the increase in the flavonoid biosynthesis at the expense of hydroxycinnamic acid derivatives under UV irradiation (Agati and Tattini, 2010). Indeed, the TCHA/TFL ratio in the Croatian samples (about 0.7 in 2014 and 0.6 in 2016) was lower than in the Polish samples (about 1.3 in 2014 and 0.9 in 2016). However, to better understand the changes in the profiles of the secondary metabolites in the investigated species depending on environmental conditions, further studies are necessary, covering samples from various parts of Europe, including both target countries.

Plant extracts are complex matrices composed of multiple molecules with various mechanisms of activity. Our previous study (Matczak et al., 2018) demonstrated that *S. domestica* leaf extracts are potent antioxidants in both chemical and biological models, and that their concentration-dependent activity is determined by the content of phenolics, primarily flavan-3-ols, proanthocyanidins and flavonoids. The DPPH free radical scavenging activity and ferric reducing ability (FRAP) turned out to be good tools for the preliminary estimation of antioxidant capacity of this plant material and for comparison of the received data with other studies. We showed that the results of DPPH and FRAP tests correlate with the TPC, TPH, TLPA and TFL levels, as well as with antioxidant activity parameters of *S. domestica* leaf extracts in a biological model of human plasma exposed to oxidative stress, and that they can be used as biological activity markers

of S. *domestica* leaf samples (Matczak et al.. 2018). Thus. these two chemical tests were selected to compare the antioxidant capacity of the leaves from Poland and Croatia. Interestingly. although the TPC values of dry extracts were slightly higher for Croatian samples. the activity parameters of all defatted leaf extracts did not differ statistically. This may be due to the proportions between the particular groups of compounds. especially flavan-3-ols. proanthocyanidins and flavonoids. and their influence on the plant antioxidant capacity. For instance. in the Polish samples flavan-3-ols and low-molecular-weight proanthocyanidins (TLPA) constituted approximately 59-64% of the TPH level. while in the Croatian samples their percentage contribution (ca. 45–52%) was significantly decreased with the increasing percentage of flavonoids (ca. 28–34%). Indeed. according to our previous research. ECA (the main flavan-3-ol in the S. *domestica* leaves) was stronger antioxidant than flavonoid glycosides in all tests. including FRAP and DPPH (Matczak et al.. 2018).

Summarising the results of the present study. it should be expected that extracts obtained from S. *domestica* leaves cultivated in various geographical regions might exhibit comparable therapeutic properties. Despite the differences in the total phenolic contents. as well as individual molecules in the plant raw materials. the defatted dry extracts prepared from the Polish and Croatian leaf samples harvested in different years (and thus exposed to various environmental conditions) were exceptionally rich in highly active polyphenolic compounds. and could be considered as biologically equal. Taking into account the fact that in our previous studies (Matczak et al.. 2018) significant differences in the content of polyphenols in various extracts from S. *domestica* resulted in comparable plasma protective activity. both samples from Poland and Croatia could be considered as promising raw materials for future applications. Nevertheless. the economic value of the particular plant material as a source of active extracts may be well defined by the extraction yield.

CONCLUSIONS

Taking into account the observed differences between the samples. as well as climatic changes between years and locations. it can be concluded that the latter have a dominant influence on the variability of the analyzed parameters. However. the present research provides only preliminary information on the variations in the phenolic profile of S. *domestica* leaves. Further research covering samples from different parts of Europe is required to better understand the influence of environmental conditions on the production of plant secondary metabolites. and thus on the quality of leaves. Nevertheless. the study demonstrated that the leaves of service tree. both from the Polish cultivation and from their natural habitat in Croatia. have a great potential as a source of active polyphenols. Considering both the content of active compounds and the extraction yields. leaves from Croatia seem to be more advantageous for industrial purposes than the Polish samples. However. although environmental conditions depending on the year and/or location had an influence on secondary metabolite production. qualitative and quantitative differences in the phenolic profile of the raw materials did not significantly affect antioxidant parameters of the products derived thereof. i.e.. dry extracts. Therefore. the leaves of S. *domestica* from both sources seem appropriate for production of extracts with health-promoting properties.

AUTHOR'S CONTRIBUTIONS

MR: research concept and design. collection and assembly of data. data analysis and interpretation. manuscript preparation. final approval of manuscript: MD: collection and assembly of data. data analysis and interpretation: MAO: research concept and design. data analysis and interpretation. critical revision of the manuscript. final approval of manuscript. The authors declare no conflict of interest.

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Łódź, dnia 04.11.2019

mgr farm. Magdalena Rutkowska
Katedra Farmakognozji
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

Ja, niżej podpisana **Magdalena Rutkowska** informuję Przewodniczącego Rady Naukowej ds. Dyscypliny Nauk Farmaceutycznych Uniwersytetu Medycznego w Łodzi, że ani badania stanowiące podstawę tez mojej pracy doktorskiej, ani sama praca pt.

„Analiza profilu polifenolowego i aktywności biologicznej liści *Sorbus domestica* L."

nie były przedmiotem postępowania o wszczęcie przewodu doktorskiego w innej jednostce uprawnionej do nadawania takiego stopnia. Jestem współwykonawcą badań stanowiących podstawę mojego doktoratu i posiadam protokoły badań dostępne do wglądu na życzenie władz Uczelni.

……
(podpis kandydata)

Łódź, dnia 04.11.2019

mgr farm. Magdalena Rutkowska
Katedra Farmakognozji
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

OŚWIADCZENIE

Oświadczam, że:

w pracy „**Matczak M.**, Marchelak A., Michel P., Owczarek A., Piszczan A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A. (2018). *Sorbus domestica* L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Journal of Functional Foods*, 40, 207-218, doi: 10.1016/j.jff.2017.10.046”,
mój udział polegał na współopracowaniu koncepcji i planu pracy, współautorstwie manuskryptu, wykonaniu analiz fitochemicznych metodami LC-MS i HPLC-PDA, oznaczeniu aktywności przeciwzapalnej suchych ekstraktów i związków modelowych, oznaczeniu aktywności antyoksydacyjnej związków modelowych metodami DPPH, FRAP i TBARS oraz nadzorze nad wykonaniem oznaczeń DPPH, FRAP i TBARS dla suchych ekstraktów, a także analizie statystycznej i interpretacji otrzymanych wyników, co określam jako **45%** wkładu w przygotowanie ww. publikacji;

w pracy „**Rutkowska M.**, Owczarek A., Kolodziejczyk-Czepas J., Michel P., Piotrowska D.G., Kapusta P., Nowak P., Olszewska M.A. (2019). Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control. *Phytochemistry Letters*, 30, 278-287, doi: 10.1016/j.phytol.2019.02.004”,
mój udział polegał na współopracowaniu koncepcji i planu pracy, izolacji i analizie strukturalnej flawonoidów, optymalizacji i walidacji metody HPLC-PDA analizy ilościowej, wykonaniu analiz fitochemicznych metodami LC-MS i HPLC-PDA, oznaczeniu aktywności przeciwzapalnej, analizie statystycznej i interpretacji wyników, współautorstwie manuskryptu oraz pełnieniu funkcji autora korespondencyjnego, w tym przygotowaniu odpowiedzi na recenzje, co określam jako **55%** wkładu w przygotowanie ww. publikacji;

w pracy „**Rutkowska M.**, Olszewska M.A., Kolodziejczyk-Czepas J., Nowak P., Owczarek A. (2019). *Sorbus domestica* leaf extracts and their activity markers: antioxidant potential and synergy effects in scavenging assays of multiple oxidants. *Molecules*, 24, 2289, doi: 10.3390/molecules24122289”,
mój udział polegał na współautorstwie koncepcji i planu pracy, współautorstwie manuskryptu, wykonaniu oznaczeń aktywności antyoksydacyjnej wobec $O_2^{\bullet-}$, $HO^{\bullet}$, H_2O_2, $NO^{\bullet}$, HClO, analizie statystycznej i interpretacji wyników oraz pełnieniu funkcji autora korespondencyjnego, w tym przygotowaniu odpowiedzi na recenzje, co określam jako **65%** wkładu w przygotowanie ww. publikacji;

w pracy „**Rutkowska M.**, Dubicka M., Olszewska M.A. (2019). Comparison of phenolic profile and *in vitro* antioxidant capacity of *Sorbus domestica* L. leaf samples from Poland and Croatia. *Acta Biologica Cracoviensia Series Botanica*, 61(1), 63-73, doi: 10.24425/118059”,
mój udział polegał na współautorstwie koncepcji i planu pracy, autorstwie manuskryptu, wykonaniu analiz fitochemicznych metodami LC-MS i HPLC-PDA dla ekstraktów suchych i płynnych oraz analiz składu i aktywności metodami spektrofotometrycznymi dla ekstraktów suchych, analizie statystycznej i interpretacji wyników oraz pełnieniu funkcji autora korespondencyjnego, w tym przygotowaniu odpowiedzi na recenzje, co określam jako **75%** wkładu w przygotowanie ww. publikacji.

…….
(podpis)

Łódź, dnia 04.11.2019

dr hab. n. farm. Monika Olszewska, prof. UM
Katedra Farmakognozji
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

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mój udział polegał na współtworzeniu koncepcji i planu pracy, współautorstwie i finalnej korekcie manuskryptu, nadzorze nad poprawnością prowadzonych eksperymentów i interpretacji wyników, konsultacji metodyki analiz fitochemicznych i aktywności antyoksydacyjnej w modelach chemicznych, rozwiązywaniu problemów naukowo-badawczych podniesionych przez recenzentów oraz pełnieniu funkcji autora korespondencyjnego;

w pracy „Rutkowska M., Owczarek A., Kolodziejczyk-Czepas J., Michel P., Piotrowska D.G., Kapusta P., Nowak P., **Olszewska M.A.** (2019). Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control. *Phytochemistry Letters*, 30, 278-287, doi: 10.1016/j.phytol.2019.02.004",
mój udział polegał na współopracowaniu koncepcji i planu pracy, nadzorze nad poprawnością prowadzonych eksperymentów i interpretacji wyników, współautorstwie i finalnej korekcie manuskryptu, a także konsultacji procesu optymalizacji i walidacji metody HPLC-PDA analizy polifenoli;

w pracy „Rutkowska M., **Olszewska M.A.**, Kolodziejczyk-Czepas J., Nowak P., Owczarek A. (2019). *Sorbus domestica* leaf extracts and their activity markers: antioxidant potential and synergy effects in scavenging assays of multiple oxidants. *Molecules*, 24, 2289, doi: 10.3390/molecules24122289",
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Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. prac wykazuje indywidualny wkład mgr Magdaleny Rutkowskiej w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

.....
(podpis współautora)

Łódź, dnia 04.11.2019

dr hab. Joanna Kołodziejczyk-Czepas, prof. UŁ
Katedra Biochemii Ogólnej
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE WSPÓŁAUTORA

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mój udział polegał na optymalizacji oraz wykonaniu oznaczeń aktywności antyoksydacyjnej w modelu ludzkiego osocza, analizie bezpieczeństwa komórkowego stosowania ekstraktów, konsultacji interpretacji wyników ww. analiz, korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów oraz rozwiązywaniu problemów podniesionych przez recenzentów w zakresie ww. analiz;

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mój udział polegał na nadzorze merytorycznym nad poprawnością wykonania analiz w modelu ludzkiego osocza, konsultacji interpretacji wyników ww. analiz oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów;

w pracy „Rutkowska M., Olszewska M.A., **Kolodziejczyk-Czepas J.**, Nowak P., Owczarek A. (2019). *Sorbus domestica* leaf extracts and their activity markers: antioxidant potential and synergy effects in scavenging assays of multiple oxidants. *Molecules*, 24, 2289, doi: 10.3390/molecules24122289",
mój udział polegał na wykonaniu analizy zmiatania $ONOO^-$ oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

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Joanna Kołodziejczyk-Czepas

.....
(podpis współautora)

Łódź, dnia 04.11.2019

dr hab. Paweł Nowak, prof. UŁ
Katedra Biochemii Ogólnej
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE WSPÓŁAUTORA

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mój udział polegał na konsultacji metodyki badań w modelu ludzkiego osocza, syntezie $ONOO^-$ oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów;

w pracy „Rutkowska M., Olszewska M.A., Kolodziejczyk-Czepas J., **Nowak P.**, Owczarek A. (2019). *Sorbus domestica* leaf extracts and their activity markers: antioxidant potential and synergy effects in scavenging assays of multiple oxidants. *Molecules*, 24, 2289, doi: 10.3390/molecules24122289",
mój udział polegał na konsultacji metodyki analizy zmiatania $ONOO^-$, syntezie $ONOO^-$ oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. prac wykazuje indywidualny wkład mgr Magdaleny Rutkowskiej w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

P. Nowak

.....

(podpis współautora)

Łódź, dnia 04.11.2019

dr n. farm. Aleksandra Owczarek
Katedra Farmakognozji
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy „Matczak M., Marchelak A., Michel P., **Owczarek A.**, Piszczan A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A. (2018). *Sorbus domestica* L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Journal of Functional Foods*, 40, 207-218, doi: 10.1016/j.jff.2017.10.046",
mój udział polegał na konsultacji zastosowanej metodyki LC-MS i metod statystycznych oraz interpretacji wyników w tym zakresie, a także korekcie merytorycznej i językowej finalnej wersji manuskryptu przygotowanego przez głównych autorów;

w pracy „Rutkowska M., **Owczarek A.**, Kolodziejczyk-Czepas J., Michel P., Piotrowska D.G., Kapusta P., Nowak P., Olszewska M.A. (2019). Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control. *Phytochemistry Letters*, 30, 278-287, doi: 10.1016/j.phytol.2019.02.004",
mój udział polegał na opracowaniu metody HPLC izolacji preparatywnej flawonoidów, konsultacji zastosowanych metod statystycznych oraz interpretacji wyników tym zakresie, a także korekcie merytorycznej i językowej finalnej wersji manuskryptu przygotowanej przez głównych autorów;

w pracy „Rutkowska M., Olszewska M.A., Kolodziejczyk-Czepas J., Nowak P., **Owczarek A.** (2019). *Sorbus domestica* leaf extracts and their activity markers: antioxidant potential and synergy effects in scavenging assays of multiple oxidants. *Molecules*, 24, 2289, doi: 10.3390/molecules24122289",
mój udział polegał na współopracowaniu koncepcji i planu pracy, opracowaniu modelu statystycznego analizy synergii, konsultacji interpretacji wyników ww. analizy, a także współautorstwie i korekcie językowej manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. prac wykazuje indywidualny wkład mgr Magdaleny Rutkowskiej w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

Aleksandra Owczarek
(podpis współautora)

Łódź, dnia 04.11.2019

mgr farm. Anna Marchelak
Katedra Farmakognozji
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy „Matczak M., **Marchelak A.**, Michel P., Owczarek A., Piszczan A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A. (2018). *Sorbus domestica* L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Journal of Functional Foods*, 40, 207-218, doi: 10.1016/j.jff.2017.10.046",
mój udział polegał na optymalizacji metody TBARS analizy aktywności antyoksydacyjnej oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. prac wykazuje indywidualny wkład mgr Magdaleny Rutkowskiej w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

Anna Marchelak
(podpis współautora)

Łódź, dnia 04.11.2019

dr n. farm. Piotr Michel
Katedra Farmakognozji
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracach:

„Matczak M., Marchelak A., Michel P., Owczarek A., Piszczan A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A. (2018). *Sorbus domestica* L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Journal of Functional Foods*, 40, 207-218, doi: 10.1016/j.jff.2017.10.046",

oraz „Rutkowska M., Owczarek A., Kolodziejczyk-Czepas J., Michel P., Piotrowska D.G., Kapusta P., Nowak P., Olszewska M.A. (2019). Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control. *Phytochemistry Letters*, 30, 278-287, doi: 10.1016/j.phytol.2019.02.004",

mój udział polegał na konsultacji metodyki analiz aktywności przeciwzapalnej oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. prac wykazuje indywidualny wkład mgr Magdaleny Rutkowskiej w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

Michel Piotr

.....

(podpis współautora)

Łódź, dnia 04.11.2019

prof. dr hab. n. farm. Dorota G. Piotrowska
Katedra Chemii Bioorganicznej i Biokoordynacyjnej
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy „Rutkowska M., Owczarek A., Kolodziejczyk-Czepas J., Michel P., **Piotrowska D.G.**, Kapusta P., Nowak P., Olszewska M.A. (2019). Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control. *Phytochemistry Letters*, 30, 278-287, doi: 10.1016/j.phytol.2019.02.004",
mój udział polegał na konsultacji interpretacji danych w analizie NMR i odpowiednich fragmentów dyskusji oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. prac wykazuje indywidualny wkład mgr Magdaleny Rutkowskiej w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

.....
(podpis współautora)

Łódź, dnia 04.11.2019

mgr Paulina Kapusta
Katedra Biologii i Ochrony Środowiska
Wydział Biochemii Ogólnej
Uniwersytet Łódzki

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy „Rutkowska M., Owczarek A., Kolodziejczyk-Czepas J., Michel P., Piotrowska D.G., **Kapusta P.**, Nowak P., Olszewska M.A. (2019). Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control. *Phytochemistry Letters*, 30, 278-287, doi: 10.1016/j.phytol.2019.02.004"
mój udział polegał na wykonaniu analiz właściwości antyoksydacyjnych w modelu ludzkiego osocza.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. prac wykazuje indywidualny wkład mgr Magdaleny Rutkowskiej w opracowanie koncepcji, wykonanie czę_ści eksperymentalnej, opracowanie i interpretację wyników ww. prac.

Paulina Kapusta
(podpis współautora)

Łódź, dnia 01.11.2019

mgr Monika Dubicka
Katedra Farmakognozji
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy „Rutkowska M., **Dubicka M.**, Olszewska M.A. (2019). Comparison of phenolic profile and *in vitro* antioxidant capacity of *Sorbus domestica* L. leaf samples from Poland and Croatia. *Acta Biologica Cracoviensia Series Botanica*, 61(1), 63-73, doi: 10.24425/118059",
mój udział polegał na wykonaniu analiz całkowitej zawartości polifenoli i proantocyjanidyn metodami spektrofotometrycznymi oraz aktywności antyoksydacyjnej metodami DPPH i FRAP w ekstraktach płynnych z surowca.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Rutkowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. prac wykazuje indywidualny wkład mgr Magdaleny Rutkowskiej w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

Monika Dubicka
(podpis współautora)

Łódź, dnia 04.11.2019

mgr farm. Magdalena Rutkowska;
dr hab. n. farm. Monika Olszewska, prof. UM
Katedra Farmakognozji
Wydział Farmaceutyczny
Uniwersytet Medyczny w Łodzi

OŚWIADCZENIE

W związku z utratą kontaktu z Panią **mgr Anną Piszczan,** byłą studentką Wydziału Farmaceutycznego Uniwersytetu Medycznego w Łodzi, członkinią studenckiego koła naukowego przy Zakładzie Farmakognozji oraz współautorką pracy:

„Matczak M., Marchelak A., Michel P., Owczarek A., **Piszczan A.**, Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A. (2018). *Sorbus domestica* L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Journal of Functional Foods*, 40, 207-218, doi: 10.1016/j.jff.2017.10.046",

oświadczamy, że w ww. pracy udział Pani mgr Anny Piszczan polegał na przygotowaniu suchych ekstraktów, a także wykonaniu analiz całkowitej zawartości polifenoli i proantocyjanidyn metodami spektrofotometrycznymi oraz aktywności antyoksydacyjnej metodami DPPH i FRAP w ww. ekstraktach.

Magdalena Rutkowska

(podpisy)

Całkowity dorobek naukowy

Autorstwo lub współautorstwo 11 oryginalnych prac naukowych o łącznym współczynniku **IF 29.859** i punktacji **MNiSW 565** (zgodnie z rokiem opublikowania), oraz 4 prac przeglądowych o łącznej punktacji **MNiSW 26**.

Prace oryginalne:

1. **Rutkowska M.**, Olszewska M.A., Kolodziejczyk-Czepas J., Nowak P., Owczarek A. (2019). *Sorbus domestica* leaf extracts and their activity markers: antioxidant potential and synergy effects in scavenging assays of multiple oxidants. *Molecules*, 24, 2289, **IF_{2018}=3.060, $MNiSW_{2018}$=30, $MNiSW_{2019}$=100**.
2. **Rutkowska M.**, Owczarek A., Kolodziejczyk-Czepas J., Michel P., Piotrowska D.G., Kapusta P., Nowak P., Olszewska M.A. (2019). Identification of bioactivity markers of *Sorbus domestica* leaves in chromatographic, spectroscopic and biological capacity tests: Application for the quality control. *Phytochemistry Letters*, 30, 278-287, IF_{2018}=1.338, $MNiSW_{2018}$=20, $MNiSW_{2019}$=70.
3. Marchelak A., Owczarek A., **Rutkowska M.**, Michel P., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A. (2019). New insights into antioxidant activity of *Prunus spinosa* flowers: Extracts, model polyphenols and their phenolic metabolites in plasma towards multiple *in vivo*-relevant oxidants. *Phytochemistry Letters*, 30, 288-295, **IF_{2018}=1.338, $MNiSW_{2018}$=20, $MNiSW_{2018}$=70**.
4. Olszewska M.A., Kolodziejczyk-Czepas J., **Rutkowska M.**, Magiera A., Michel P., Rejman M.W., Nowak P., Owczarek A. (2019). The effect of standardised flower extracts of *Sorbus aucuparia* L. on pro-inflammatory enzymes, multiple oxidants and oxidative/nitrative damage of human plasma components *in vitro*, *Oxidative Medicine and Cellular Longevity*, Article ID 9746358, 18 pages, **IF_{2018}=4.868, $MNiSW_{2018}$=30, $MNiSW_{2018}$=100**.
5. **Rutkowska M.**, Dubicka M., Olszewska M.A. (2019). Comparison of the phenolic profile and *in vitro* antioxidant capacity of *Sorbus domestica* L. leaf samples from Poland and Croatia. *Acta Biologica Cracoviensia Series Botanica*, 61(1), 63-73, **IF_{2018}=1.111, $MNiSW_{2018}$=15, $MNiSW_{2018}$=40**.
6. Kicel A., Kolodziejczyk-Czepas J., Owczarek A., **Rutkowska M.**, Wajs-Bonikowska A., Granica S., Nowak P., Olszewska M.A. (2018). Multifunctional phytocompounds in *Cotoneaster* fruits: phytochemical profiling, cellular safety, anti-inflammatory and antioxidant effects in chemical and human plasma models *in vitro*. *Oxidative Medicine and Cellular Longevity*, Article ID 3482521, 16 pages, **IF_{2018}=4.868, $MNiSW_{2018}$=30**.
7. **Matczak M.**, Marchelak A., Michel P., Owczarek A., Piszczan A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A. (2018). *Sorbus domestica* L. leaf extracts as functional products: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Journal of Functional Foods*, 45, 207-218, **IF_{2018}=3.197, $MNiSW_{2018}$=40**.
8. Marchelak A, Owczarek A, **Matczak M**, Pawlak A, Kolodziejczyk-Czepas J, Nowak P. Olszewska M.A. (2017). Bioactivity potential of *Prunus spinosa* L. flower extracts: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Frontiers in Pharmacology*, 8, 680, 1-15, **IF_{2017}=3.831, $MNiSW_{2017}$=40**.
9. Owczarek A., Magiera A., **Matczak M.**, Piotrowska D.G., Olszewska M.A., Marchelak A. (2017). Optimisation of preparative HPLC separation of four isomeric kaempferol diglycosides from

Prunus spinosa L. by application of the response surface methodology. *Phytochemistry Letters*, 20, 415-424, **IF_{2017}=1.575, $MNiSW_{2017}$=20**.

10. Michel P., Owczarek A., Kosno M., Gontarek D., **Matczak M.**, Olszewska M.A. (2017). Variation in polyphenolic profile and *in vitro* antioxidant activity of eastern teaberry (*Gaultheria procumbens* L.) leaves following foliar development. *Phytochemistry Letters*, 20, 356-364, **IF_{2017}=1.575, $MNiSW_{2017}$=20**.
11. Michel P., Owczarek A., **Matczak M**., Kosno M., Szymański P., Mikiciuk-Olasik E., Kilanowicz A., Wesołowski W., Olszewska M.A. (2017). Metabolite profiling of eastern teaberry (*Gaultheria procumbens* L.) lipophilic leaf extracts with hyaluronidase and lipoxygenase activity. *Molecules*, 22(3), 412, 1-16, **IF_{2017}=3.098, $MNiSW_{2017}$=30**.

Prace przeglądowe:

1. **Matczak M.**, Rosińska K., Michel P. (2017). *Withania somnifera* (ashwagandha, indyjski żeń szeń) – wskazania tradycyjnej ajurwedy w świetle współczesnych badań klinicznych. *Farmacja Polska*, 73(7), 410-417, **$MNiSW_{2017}$=8**.
2. **Matczak M.**, Marchelak A., Rychlińska I. (2016). Lapacho [*Tabebuia impetiginosa* (Mart. ex DC.) Standl.]- historia stosowania, skład i aktywność biologiczna, stan badań. *Farmacja Polska*, 72(5), 296-302, **$MNiSW_{2016}$=8**.
3. Marchelak A., **Matczak M.**, Owczarek A. (2016). Krokosz barwierski (*Carthamus tinctorius*) wieki tradycji a nowe badania nad składem i aktywnością farmakologiczną. *Farmacja Polska*, 72(4), 211-219, **$MNiSW_{2016}$=8**.
4. **Matczak M.**, Olszewska M.A., Michel P. (2015). Siła tkwi w naturze. Naturalne salicylany w lecznictwie. *Czasopismo Aptekarskie*, 4, 256, 39-45, **$MNiSW_{2015}$=2**.

Komunikaty zjazdowe:

Autorstwo/współautorstwo łącznie 23 komunikatów zjazdowych zaprezentowanych na 9 krajowych i międzynarodowych konferencjach naukowych.

Granty NCN:

1. „Potencjał prozdrowotny owoców jarzębu pospolitego (*Sorbus aucuparia* L.) - badania *in vitro*”; projekt w ramach konkursu PRELUDIUM 14 **(nr grantu 2017/27/N/NZ9/02134)**; realizacja projektu 2018-2021; pełniona funkcja: **kierownik projektu**.