Charakterystyka fitochemiczna i aktywności biologicznej ekstraktów z kwiatów *Prunus spinosa* L. w kontekście chorób układu krążenia

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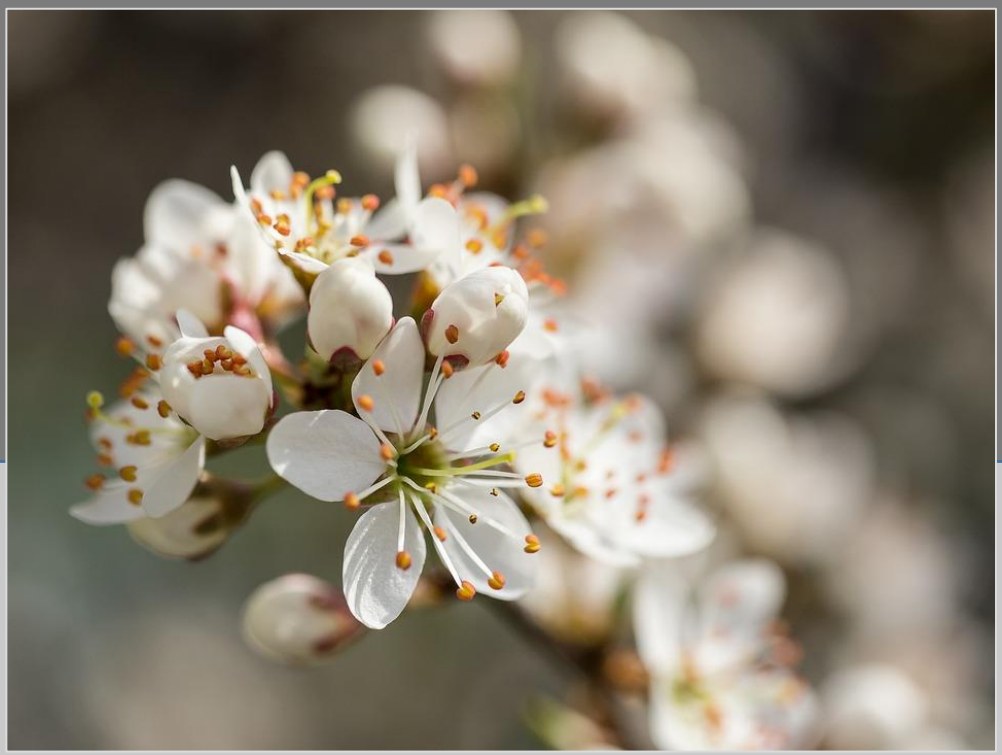

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UNIWERSYTET MEDYCZNY W ŁODZI

WYDZIAŁ FARMACEUTYCZNY

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(nazwisko rodowe Kwapisz)

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Publikacja I

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IF_{2017} = 1.575, $MNiSW_{2017}$ = 20, $MNiSW_{2019}$ = 70

Publikacja II

Marchelak A., Owczarek A., Matczak M., Pawlak A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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,* 2017, 8, *e*680, 1-15, https://doi.org/10.3389/fphar.2017.00680.

IF_{2017} = 3.831, $MNiSW_{2017}$ = 40, $MNiSW_{2019}$ = 100

Publikacja III

Marchelak A., Owczarek A., Rutkowska M., Michel P., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2019, 30, 288-295, https://doi.org/10.1016/j.phytol.2019.02.011.

IF_{2018} = 1.338, $MNiSW_{2018}$ = 20, $MNiSW_{2019}$ = 70

Publikacja IV

A) Marchelak A., Olszewska M.A., Owczarek A.: Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control. *Journal of Pharmaceutical and Biomedical Analysis*, 2020, 184, 113121, https://doi.org/10.1016/j.jpba.2020.113121.

IF_{2018} = 2.983, $MNiSW_{2018}$ = 35, $MNiSW_{2019}$ = 100

B) Marchelak A., Olszewska M.A., Owczarek A.: Data on the optimization and validation of HPLC-PDA method for quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof. *Data in Brief*, 2020, 29, 105319, http://doi.org/10.1016/j.dib.2020.105319.

$MNiSW_{2019}$ = 40

Wyniki badań prezentowane w ramach niniejszej pracy zostały również przedstawione w formie 8 komunikatów zjazdowych na krajowych i międzynarodowych konferencjach naukowych:

- **Marchelak A.**, Matczak M., Pawlak A., Owczarek A., Olszewska M. A.: Analiza zawartości polifenoli i aktywności antyoksydacyjnej suchych ekstraktów z kwiatów *Prunus spinosa* L. X Kopernikańskie Seminarium Doktoranckie, Wydział Chemii UMK w Toruniu, 21-24 czerwca 2016, Bachotek.
- Owczarek A., Magiera A., **Marchelak A.**, Olszewska M. A.: Application of response surface methodology in optimization of separation of four isomeric flavonoid diglycosides from *Prunus spinosa* leaf. 10th International Symposium On Chromatography Of Natural Products, Medical University of Lublin, 6-9 June 2016, Lublin Poland.
- **Marchelak A.**, Matczak M., Owczarek A., Pawlak A., Olszewska M. A.: Profile of antioxidant polyphenols in dry extracts of *Prunus spinosa* L. flowers. Plants in Pharmacy and Nutrition, 2nd International Young Scientists Symposium, Wroclaw Medical University, 15-17 September 2016, Wroclaw Poland.
- **Marchelak A.**, Matczak M., Owczarek A., Pawlak A., Kołodziejczyk-Czepas J., Olszewska M. A.: Antioxidative effects of *Prunus spinosa* L. flower extracts in chemical and human plasma models. Plants in Pharmacy and Nutrition, 2nd International Young Scientists Symposium, Wroclaw Medical University, 15-17 September 2016, Wroclaw Poland.
- **Marchelak A**., Owczarek A., Matczak M., Pawlak A., Kołodziejczyk-Czepas J., Olszewska M.A.: Ocena kwiatów *Prunus spinosa* L. jako źródła polifenoli o aktywności antyoksydacyjnej. Konferencja Naukowa Studentów Wydziału Farmaceutycznego z Oddziałem Kosmetologii i Oddziałem Analityki Medycznej Uniwersytetu Medycznego w Łodzi „150 rocznica urodzin Marii Skłodowskiej-Curie", 22 kwietnia 2017, Łódź.
- **Marchelak A.**, Matczak M., Owczarek A., Kołodziejczyk-Czepas J., Markowicz-Piasecka M., Olszewska M.A.: Wstępne badania aktywności biologicznej suchych ekstraktów z kwiatów *Prunus spinosa* L. w kontekście chorób układu krążenia. XXIII Naukowy Zjazd Polskiego Towarzystwa Farmaceutycznego „Farmacja w Polsce perspektywy nauki I zawodu", 19-22.09.2017, Kraków.
- **Marchelak A.**, Rutkowska M., Michel P., Owczarek A., Kołodziejczyk-Czepas J., Nowak P., Olszewska M.A.: *In vitro* evaluation of the antioxidant activity of *Prunus spinosa* flower extracts towards the most common *in vivo*-relevant oxidants. 11th International Symposium On Chromatography Of Natural Products, Medical University of Lublin, 4-7.06.2018, Lublin Poland.
- **Marchelak A.**, Owczarek A., Olszewska M.A.: A validated HPLC-PDA method for quantification of bioactive constituents in blackthorn flowers and dry extracts prepared thereof. 4th International Conference on Natural Products Utilization From Plants to Pharmacy Shelf, 29.05-2.06.2019, Albena Bulgaria.

Wykaz skrótów

1D/2D NMR	jedno-/dwuwymiarowa spektroskopia magnetycznego rezonansu jądrowego (*ang. 1-dimension/2-dimension nuclear magnetic resonance*)
3-NT	3-nitrotyrozyna (*ang. 3-nitrotyrosine*)
BF	frakcja *n*-butanolu (*ang. n-butanol fraction*)
ChUK	choroby układu krążenia (*ang. cardiovascular diseases*)
COSY	spektroskopia korelacyjna (*ang. correlation spectroscopy*)
CYE	ekwiwalenty chlorku cyjanidyny (*ang. cyanidin chloride equivalents*)
DEF	frakcja eteru dielylowego (*ang. diethyl ether fraction*)
DPPH	test zmiatania rodnika 2,2-difenylo-1-pikrylohydrazylowego (*ang. 2,2-diphenyl-1-picrylhydrazyl free-radical scavenging assay*)
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*)
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/g	ekwiwalenty kwasu galusowego (*ang. gallic acid equivalents*)
(U)HPLC-PDA	(ultra)wysokosprawna chromatografia cieczowa z detekcją fotodiodową (*ang. (ultra)-high-performance liquid chromatography with photodiode array detection*)
H_2O_2	nadtlenek wodoru (*ang. hydrogen peroxide*)
HClO	kwas podchlorawy (*ang. hypochlorous acid*)
HMBC	heterojądrowa korelacja dalekiego zasięgu (ang. heteronuclear multiple bond coherence)
HMQC	heterojądrowa korelacja z detekcją przejść wielokwantowych (*ang. heteronuclear multiple quantum coherence*)
ICH	Międzynarodowa Rada Harmonizacji (*ang. International Council for Harmonisation*)
IL	interleukina (*ang. interleukin*)

MED	odtłuszczony ekstrakt metanolowo-wodny (7:3, *v/v*) (*ang. defatted methanol-water (7:3, v/v) extract*)
NEAC	nieenzymatyczna pojemność antyoksydacyjna osocza (*ang. non-enzymatic antioxidant capacity of blood plasma*) oznaczona metodą FRAP
$NO^{\bullet}$	tlenek azotu (*ang. nitric oxide*)
$O_2^{\bullet -}$	anionorodnik ponadtlenkowy (*ang. superoxide anion*)
$OH^{\bullet}$	rodnik wodorotlenowy (*ang. hydroxyl radical*)
$ONOO^{-}$	nadtlenoazotyn (*ang. peroxynitrite*)
ORAC	test oznaczania zdolności absorpcji rodników tlenowych (*ang. Oxygen Radical Absorbance Capacity)*
PBMCs	jednojądrzaste komórki krwi obwodowej (*ang. peripheral blood mononuclear cells*)
ROS/RNS	reaktywne formy tlenu/ reaktywne formy azotu (*ang. reactive oxygen species/reactive nitrogen species*)
RSM	metoda płaszczyzny odpowiedzi (*ang. response surface methodology*)
s.m.	sucha masa (*ang. dry matter*)
SDS-PAGE	elektroforeza w żelu poliakrylamidowym w warunkach denaturujących (*ang. sodium dodecyl sulfate–polyacrylamide gel electrophoresis*)
TBARS	substancje reagujące z kwasem tiobarbiturowym (*ang. thiobarbituric acid-reactive substances*)
TPC	całkowita zawartość polifenoli (*ang. total phenolic content*) oznaczona metodą Folina-Ciocalteu'a (*ang. Folina-Ciocalteu assay*)
WR	pozostałość wodna (*ang. water residue*)

Uzasadnienie podjętej tematyki badawczej

Choroby układu krążenia (ChUK) – najczęściej występujące na świecie choroby niezakaźne – są odpowiedzialne za około 30% zgonów globalnie, stanowiąc tym samym jeden z najpoważniejszych problemów zdrowotnych współczesnych społeczeństw (WHO, 2019). Niewystarczająca skuteczność i jednocześnie wysoki koszt stosowanych obecnie terapii sprawia, że opracowanie bardziej efektywnych strategii prewencji i leczenia ChUK jest istotnym celem dla medycyny zarówno ze względów epidemiologicznych jak i ekonomicznych. Badania prowadzone w ostatnich dwóch dekadach wykazują, iż kluczową rolę w patomechanizmie wielu ChUK, w tym m.in. miażdżycy, choroby niedokrwiennej serca, nadciśnienia, kardiomiopatiach, przeroście mięśnia sercowego i zastoinowej niewydolności serca, odgrywa stres oksydacyjny (Valko i wsp., 2007; Santilli i wsp., 2015; Gracia i wsp., 2017). Stres oksydacyjny, rozwijający się w sytuacji, gdy generacja reaktywnych form tlenu i azotu (ROS/RNS) przewyższa możliwości obrony antyoksydacyjnej organizmu, prowadzi do uszkodzeń ważnych składników komórkowych, takich jak białka, lipidy czy DNA, oraz przyczynia się do rozwoju stanu zapalnego. Z drugiej strony w procesie zapalnym dochodzi do wzmożonej produkcji ROS/RNS m.in. przez komórki fagocytyjące, takie jak makrofagi i neutrofile, co może skutkować powstaniem lokalnego stresu oksydacyjnego i uszkodzeniem tkanek (Biswas, 2016; Hussain i wsp., 2016; Pashkow, 2011). Odkrycie powiązania pomiędzy współistniejącymi procesami stresu oksydacyjnego i stanu zapalnego a rozwojem ChUK wzbudziło znaczne zainteresowanie tematyką antyoksydantów egzogennych, w tym polifenoli roślinnych, jako związków mogących potencjalnie znaleźć zastosowanie w profilaktyce i terapii ChUK.

Polifenole stanowią obszerną i bardzo zróżnicowaną pod względem zarówno struktury, masy cząsteczkowej, jak i właściwości fizyko-chemicznych oraz biologicznych grupę związków naturalnych. Są to wyspecjalizowane metabolity roślin, biosyntezowane najczęściej szlakiem kwasu szikimowego i posiadające w swojej strukturze przynajmniej jedną grupę fenolową, warunkującą ich właściwości redukujące. Do polifenoli należą zarówno proste związki o stosunkowo niskiej masie cząsteczkowej (kwasy fenolowe, flawonole, dimery procyjanidynowe), jak i wielkocząsteczkowe polimery o masie cząsteczkowej powyżej 30 kDa (garbniki). Największą grupę związków polifenolowych stanowią flawonoidy, zbudowane z dwóch pierścieni fenylowych połączonych mostkiem propylowym, w oparciu o który najczęściej wytwarza się dodatkowy pierścień heterocykliczny. Stopień utlenienia pierścienia heterocyklicznego jest podstawą podziału flawonoidów na poszczególne klasy, tj. m.in. chalkony, aurony, flawony, izoflawony, flawany, antocyjanidyny, flawonole, a także di-, tri-, oligo- i polimeryczne proantocyjanidyny, które oprócz odmiennej budowy strukturalnej wykazują również zróżnicowany wpływ na organizm ludzki. Spośród wymienionych związków plejotropową aktywność biologiczną w obrębie układu krążenia, udokumentowaną licznymi badaniami, w tym również klinicznymi, przypisuje się głównie flawonolom, flawonom, antocyjanidynom i proantocyjanidynom (Pandey i wsp., 2009; Tresserra-Rimbau i wsp., 2018; Koch, 2019; Perez-Vizcaino i Duarte, 2010; Schroeter i wsp., 2010).

Wielokierunkowe działanie związków polifenolowych, w tym flawonoidów, na organizm ludzki jest w znacznej części związane z ich regulującym wpływem na procesy oksydacyjno-redukcyjne oraz zapalne zachodzące w żywych komórkach (Hussain, 2016; Koch, 2019). Mechanizmy działania antyoksydacyjnego polifenoli są zróżnicowane i obejmują zdolność do bezpośredniego

„zmiatania" ROS/RNS, w tym anionorodnika ponadtlenkowego ($O_2^{\bullet-}$), nadtlenku wodoru (H_2O_2), rodnika hydroksylowego ($HO^{\bullet}$), kwasu podchlorawego (HClO), tlenku azotu ($NO^{\bullet}$) i nadtleno-azotynu ($ONOO^-$); chelatowania jonów metali (głównie żelaza i miedzi) katalizujących produkcję ROS/RNS; inhibicji enzymów prooksydacyjnych (m.in. oksydazy ksantynowej, indukowalnej syntazy tlenku azotu, kinazy proteinowej C, cyklooksygenaz, lipooksygenaz, mitochondrialnej dehydrogenazy bursztynianowej, mieloperoksydaz i metaloproteinaz) oraz stymulacji produkcji enzymatycznych i nieenzymatycznych antyoksydantów endogennych (m.in. dysmutazy ponadtlenkowej, glutationu) (Quiñones i wsp., 2013; Tresserra-Rimbau i wsp., 2018; Koch, 2019). Aktywność antyoksydacyjna polifenoli może potencjalnie warunkować efekty wazodylatacyjne, przeciwpłytkowe, hipolipemiczne, antiaterogenne i antyapoptotyczne, ściśle kojarzone z obniżonym ryzykiem sercowo-naczyniowym (Quiñones i wsp., 2013). W związku z oksydacyjno-zapalnym patomechanizmem rozwoju ChUK, nie mniej istotna od aktywności antyoksydacyjnej wydaje się być aktywność przeciwzapalna polifenoli (Biswas, 2016; Hussain i wsp., 2016; Koch, 2019). Wykazano, że związki polifenolowe mają zdolność do modyfikacji funkcji komórek układu immunologicznego, w tym hamowania sekrecji prozapalnych cytokin i chemokin, m.in. interleukin (IL-1β, IL-2, IL-6 i IL-8), czynnika martwicy nowotworów (TNF-α), interferonu γ (IFN-γ), białka chemotaktycznego monocytów (MCP-1); zwiększania sekrecji przeciwzapalnych cytokin, m.in. IL-10; oraz hamowania wydzielania prozapalnych enzymów remodelujących składniki macierzy zewnątrzkomórkowej, m.in. elastazy-2 i metaloproteinaz. Obserwowane efekty mogą być wynikiem modulowania ekspresji jądrowego czynnika transkrypcyjnego NF-κB, kontrolującego ekspresję wielu genów zaangażowanych w proces zapalny, w tym genów dla cytokin, chemokin i enzymów prozapalnych, cząsteczek adhezyjnych, immunoreceptorów i czynników wzrostu. Ponadto polifenole wykazują zdolność do bezpośredniej inhibicji enzymów o działaniu prozapalnym, w tym cyklooksygenaz (głównie cyklooksygenazy 2), fosfolipaz A2, 5-lipooksygenazy i hialuronidaz (Hussain i wsp., 2016; Cory i wsp., 2018; Yahfoufi i wsp., 2018).

Liczne badania kliniczne i epidemiologiczne prowadzone w ostatnich dekadach sugerują istnienie ścisłego związku pomiędzy dietą bogatą w polifenole roślinne a obniżonym ryzykiem zapadalności na ChUK (Cory i wsp., 2018, Scalbert i wsp., 2005; Koch, 2019; Bonaccio i wsp., 2017; de Melo Ribeiro i wsp., 2019). O aktywności tej grupy związków w obrębie układu krążenia świadczy też fakt, że substancje roślinne zawierające polifenole są od wieków wykorzystywane z dobrymi rezultatami w medycynie tradycyjnej w schorzeniach sercowo-naczyniowych, a potencjał terapeutyczny wielu z nich (m.in. kwiatostanów/owoców głogu, nasion/liści winorośli, owoców aronii) został potwierdzony we współczesnych badaniach, w tym również klinicznych (Zhao i wsp., 2017; Nassiri-Asl i wsp., 2016; Parandoosh i wsp., 2019; Jurikova i wsp., 2017). Dowody wskazujące na skuteczność polifenolowych antyoksydantów w profilaktyce i terapii ChUK zaowocowały wzrostem zainteresowania tematyką i w rezultacie licznymi badaniami, dotyczącymi m.in. aktywności biologicznej, mechanizmów działania, efektów synergistycznych czy losów polifenoli w ustroju (wchłanianie, metabolizm). Równocześnie trwają poszukiwania nowych, wydajnych źródeł polifenoli, oraz analizy fitochemiczne, farmakologiczne i toksykologiczne surowców polifenolowych stosowanych w medycynie tradycyjnej, co w dalszej perspektywie może doprowadzić do wprowadzenia na rynek nowych produktów leczniczych, suplementów diety, dietetycznych środków spożywczych specjalnego przeznaczenia do stosowania w profilaktyce i w terapii wspomagającej ChUK. Przywracanie do lecznictwa częściowo zapomnianych surowców

medycyny tradycyjnej wydaje się być kierunkiem szczególnie obiecującym ze względu na fakt, że badania weryfikujące aktywność substancji roślinnych stosowanych przez medycynę tradycyjną wskazują, że udowodnione współcześnie kierunki aktywności są nawet w 80% zgodne z tradycyjnymi wskazaniami terapeutycznymi (Fabricant i Farnsworth, 2001).

Pragnąc wpisać się w ten kontekst badawczy w niniejszej dysertacji zwrócono uwagę na gatunek znany w Europie od ponad 7000 lat, początkowo jako źródło jadalnych owoców, później również jako roślina lecznicza, o szeroko dokumentowanym zastosowaniu w medycynie tradycyjnej, jednakże niewystarczająco poznanym składzie oraz nielicznych danych naukowych na temat kierunków i mechanizmów aktywności biologicznej, tj. na śliwę tarninę (*Prunus spinosa* L.).

Śliwa tarnina to krzew z rodziny *Rosaceae*, charakterystyczny głównie dla flory Europy, gdzie występuje pospolicie (poza jej północną częścią). Spotykany jest również na terenie zachodniej Azji (Kaukaz), północnej Afryki (Tunezja, Algieria) oraz Ameryki Północnej (tu jako gatunek wtórnie zdziczały). W Polsce występuje dość często na całym niżu i w niższych położeniach górskich. Typowymi siedliskami są słoneczne zbocza, skarpy, obrzeża pól uprawnych, miedze, pastwiska, rumowiska skalne, doliny rzeczne i wąwozy, a także obrzeża lasów czy widne lasy liściaste, rzadziej iglaste. Śliwa tarnina nie ma dużych wymagań glebowych, jest odporna na mróz i suszę, preferuje duże ilości światła, ale znosi też półcień. Rozmnaża się za pomocą odrostów korzeniowych, co powoduje, że często tworzy rozległe jednogatunkowe agregacje i jest uznawana za gatunek ekspansywny (Rumińska i Ożarowski, 1990; Wichtl, 2004; Seneta i Dolatowski, 2000).

Rycina 1 Obszar naturalnego występowania *P. spinosa* L. Źródło: https://www.cwrnl.nl/en/CWRnl-1/CWRbybotanicalname/Prunus-spinosa-L.-1.htm.

Współcześnie gatunek dostarcza kilku rodzajów substancji roślinnych, takich jak kwiaty, owoce, liście, młode pędy oraz rzadziej kora, wykorzystywanych głównie w medycynie tradycyjnej (Ożarowski i Jaroniewski, 1987; Wichtl, 2004; Pinacho i wsp., 2015). Według danych etnofarmakologicznych, substancje pozyskiwane z *P. spinosa* posiadają szerokie spektrum aktywności biologicznej, w tym m.in. działanie wazoprotekcyjne, przeciwzapalne, hipotensyjne, detoksykujące, moczopędne, spazmolityczne, łagodnie przeczyszczające, napotne i wykrztuśne. Tradycyjne wskazania do stosowania obejmują schorzenia układu pokarmowego, oddechowego

i moczowego, stany zapalne jamy ustnej i gardła, jak również choroby układu sercowo-naczyniowego (Berger, 1949; Hoppe, 1981; Wichtl, 2004; Blumenthal i Busse, 1998). Działanie wspomagające pracę układu krążenia jest raportowane niemalże dla wszystkich rodzajów substancji roślinnych dostarczanych przez śliwę tarninę. Gałązki, cieszące się największą popularnością na południu Europy, są uznawane za środki obniżające ciśnienie krwi (Calvo i Cavero, 2014). Aktywność hipotensyjną przypisuje się również owocom, stosowanym ponadto w celu wzmocnienia serca (Kültür, 2007; Jarić i wsp., 2015). Kwiaty są natomiast składnikiem wielu tradycyjnych mieszanek ziołowych, polecanych w takich dolegliwościach sercowych jak zapalenie mięśnia sercowego, nerwica serca czy miażdżyca (Wawrzyniak, 1992).

Aktywnymi składnikami śliwy tarniny są polifenole, w tym flawonoidy, procyjanidyny typu A, antocyjany, kumaryny i kwasy fenolowe, tworzące zróżnicowane profile w poszczególnych organach. W ostatnich latach owoce i gałązki *P. spinosa* zostały poddane dokładniejszym badaniom fitochemicznym z wykorzystaniem technik chromatograficznych wysokiej rozdzielczości sprzężonych ze spektrometrią mas (Guimarães i wsp., 2013; Pinacho i wsp., 2015). W przypadku kwiatów badania koncentrowały się głównie na izolacji i zaowocowały objaśnieniem struktury 16 glikozydów flawonoidowych i 5 procyjanidyn (Olszewska i Wolbiś, 2001, 2002a,b; Kolodziej i wsp., 1991; Sakar i Kolodziej, 1993). Zwraca się uwagę, że niektóre ze składników śliwy tarniny, tj. pentozydy flawonolowe (arabinozydy, ksylozydy, ramnozydy) oraz dimery procyjanidynowe typu A, to rzadkie w naturze związki, których występowanie jest ograniczone jedynie do wybranych rodzin i gatunków (Pinacho i wsp., 2015).

Rycina 2 Kwitnące i owocujące krzewy *P. spinosa* L.

Pomimo zachęcających doniesień literaturowych na temat składu oraz szerokiego wykorzystania w medycynie tradycyjnej przetworów z różnych organów/części śliwy tarniny, niewiele jest danych pochodzących z badań naukowych potwierdzających ich aktywność biologiczną. Przeprowadzone na przełomie lat 60-tych i 70-tych XX wieku wstępne analizy *ex vivo* i *in vivo* wykazały, że flawonoidowe frakcje otrzymane z liści i kwiatów tarniny znacząco zmniejszają przepuszczalność naczyń krwionośnych oraz wykazują działanie przeciwzapalne w narządach wewnętrznych zwierząt doświadczalnych, normalizują poziom cholesterolu i stosunek cholesterol/fosfolipidy u aterogennych królików, oraz zwiększają amplitudę skurczu w perfuzji izolowanych serc żabich (Makarov i Khadzhai, 1969; Makarov, 1968, 1972), wskazując na prawdopodobną szczególną aktywność kwiatów i liści tarniny w obrębie układu krążenia.

Współczesne badania farmakologiczne dotyczące *P. spinosa* koncentrowały się głównie na analizie aktywności antyoksydacyjnej, wskazując ją jako możliwy mechanizm działania warunkujący efekty biologiczne obserwowane w medycynie tradycyjnej. Wykazano wysoki potencjał antyoksydacyjny suchych ekstraktów z liści, owoców i gałązek śliwy tarniny, korelujący z całkowitą zawartością polifenoli, w tym flawonoidów (Pinacho i wsp., 2015; Guimarães i wsp., 2014). Jednakże analizy były prowadzone jedynie prostymi metodami chemicznymi (DPPH, FRAP, TBARS i ORAC) i nie objęły najszerzej stosowanych w lecznictwie tradycyjnym kwiatów.

Tabela 1 Charakterystyka botaniczna gatunku *P. spinosa* L.

Cechy morfologiczne	
Pokrój	krzew (1-3 m) lub niewielkie drzewo (5-8 m), bardzo silnie rozgałęzione, tworzy gęste zarośla, tzw. „czyżnie”
Pęd	pęd do 8 cm grubości; pędy boczne drugiego rzędu odstające pod kątem prostym lub rozwartym, zakończone pojedynczymi cierniami; młode pędy delikatnie omszone, starsze nagie, połyskujące; pączki kwiatowe i liściowe liczne, drobne, kulisto-jajowate, osadzone pojedynczo lub serialnie po 2-3 obok siebie, przy czym skrajne zazwyczaj kwiatowe; pąki okryte szorstko owłosionymi czerwonawo-brunatnymi łuskami; liście w pączkach tutkowato zwinięte
Kora	na starszych pniach czarnofioletowa, delikatnie spękana w wąskie pasma
Liście	ulistnienie skrętoległe; liście pojedyncze, krótkoogonkowe, opatrzone dwoma przylistkami, kształt odwrotnie jajowaty, eliptyczny lub lancetowaty, góra ciemnozielona, spód jaśniejszy, długość blaszki liściowej 2-6.5 cm, szerokość 1-2.8 cm; nasada klinowata, szczyt zaostrzony lub lekko stępiony, brzeg pojedynczo lub podwójnie karbowano-piłkowany; młode liście obustronnie owłosione, starsze nagie lub pokryte włoskami tylko wzdłuż nerwów, zwłaszcza po spodniej stronie blaszki; unerwienie pierzaste, wklęsłe, nerw główny silnie zaznaczony; ogonek liściowy dł. 0.2-1.5 cm, przeważnie owłosiony, nieogruczolony; przylistki równowąskie, ogruczolone, o brzegu ząbkowanym, zwykle nieco dłuższe od ogonka liściowego
Kwiaty	kwitnienie bardzo obfite, wyprzedzające rozwój liści, marzec-kwiecień (południowa i środkowa Europa), kwiecień-maj (Europa wschodnia, w tym Polska); kwiaty pięciokrotne, pojedyncze lub w pęczkach, drobne, o średnicy 1.2-1.8 cm, osadzone na krótkich (do 5-8 mm dł.) nagich szypułkach; kielich dzwonkowaty, o działkach trójkątnych lub jajowatych, nagich, na szczycie piłkowanych, brzegiem orzęsionych i nie odgiętych, do 2 mm dł.; płatki korony białe lub zielonkawo-białe, podłużnie lub odwrotnie jajowate, dł. 5-8 mm, osadzone na kieliszkowatym lub rurkowatym dnie kwiatowym; słupek górny, wolny, zakończony szerokim, płaskim znamieniem; zalążnia jednokomorowa, dwa zalążki; pręciki liczne (ok. 20), dł. 5 mm, pylniki barwy żółtej lub czerwonej
Owoce	pestkowce; kształt kulisty lub jajowaty; barwa fioletowa, granatowa lub czarna z modrawym, woskowym nalotem; krótkoszypułkowe, sztywno wzniesione; średnica 1-1.5 cm; miąższ cienki, brązowawy lub zielony, przyrośnięty do pestki, o kwaśnym i cierpkim smaku; pestka jajowata lub eliptyczna, o zaostrzonym szczycie, wymiary 1×0.8 cm, z boków spłaszczona, powierzchnia jasnobrunatna, brodawkowana z wyraźnymi podłużnymi żeberkami; owocowanie październik-listopad; owoc pozostaje na roślinie w czasie zimy
Nasiona	kształt migdału; barwa jasnobrązowa, wymiary 7×5 mm; smak gorzki

Szerokie zastosowanie kwiatów śliwy tarniny w medycynie tradycyjnej, obejmujące m.in. ChUK, wyniki wstępnych badań farmakologicznych, oraz unikatowy profil polifenolowy sprawiają, że surowiec wydaje się prezentować obiecujący potencjał terapeutyczny, szczególnie w kontekście schorzeń sercowo-naczyniowych. Do szerszego wykorzystania w lecznictwie konieczne są jednak dalsze badania fitochemiczne i aktywności biologicznej ze wskazaniem możliwych mechanizmów działania, badania toksykologiczne, czy wreszcie opracowanie adekwatnej metodyki standaryzacji substancji roślinnej i pochodnych ekstraktów.

Dobór odpowiednich metod badawczych umożliwiających ocenę substancji roślinnych w zakresie jakościowego i/lub ilościowego składu chemicznego oraz aktywności biologicznej jest uznawany za newralgiczny punkt badań. Skład chemiczny substancji/preparatów roślinnych, znacząco wpływający na ich skuteczność i bezpieczeństwo stosowania, jest zmienny i zależy od wielu czynników, w tym m.in. czynników środowiskowych czy sposobu przetwarzania surowców, w tym ekstrakcji (Ncube i wsp., 2012; Xu i wsp., 2017). Obecnie w terapii rekomenduje się stosowanie standaryzowanych suchych ekstraktów, ze względu na wyższą zawartość substancji aktywnych, ułatwione przechowywanie i dozowanie, a także większą stabilność fizyko-chemiczną i mikrobiologiczną w porównaniu do ekstraktów płynnych i nieprzetworzonych substancji roślinnych (Kneifel i wsp., 2002). Wobec zmienności sezonowej i środowiskowej składu chemicznego roślin, standaryzacja jakościowa i ilościowa materiału/preparatów roślinnych zapewnia stałość składników aktywnych i potencjalnie niebezpiecznych, gwarantując odpowiednią skuteczność i bezpieczeństwo stosowania. Ze względu na złożoność i specyficzność gatunkową składu chemicznego substancji roślinnej opracowanie adekwatnej metodyki kontroli jakości jest nierzadko procesem trudnym i czasochłonnym, wymagającym co najmniej adaptacji znanych metod analitycznych, a zwykle opracowania całkowicie nowych procedur (Wolfender, 2015). Niezależnie od tego, standaryzacja jest podstawowym narzędziem w zapewnieniu jakości substancji/preparatów roślinnych, współcześnie stanowiąc ponadto obowiązkowy etap poprzedzający badania ich aktywności biologicznej. W przypadku substancji polifenolowych wstępna ocena aktywności biologicznej obejmuje badania aktywności antyoksydacyjnej i przeciwzapalnej na różnych modelach *in vitro*, dające podstawy naukowe do rozszerzenia badań we właściwych kierunkach, w tym również o badania *in vivo*. Analizy *in vitro* aktywności biologicznej polifenoli uwzględniają różne mechanizmy ich działania, np. w przypadku badań aktywności antyoksydacyjnej ocenia się m.in. zdolność polifenoli do bezpośredniego „zmiatania" ROS/RNS; do zapobiegania uszkodzeniom oksydacyjno/nitracyjnym makromolekuł, takich jak białka, lipidy i kwasy nukleinowe; do hamowania aktywności enzymów biorących udział w tworzeniu ROS/RNS; oraz do regulacji aktywności enzymatycznych i nieenzymatycznych białek zaangażowanych w procesy oksydoredukcyjne. Metody analiz *in vitro* aktywności przeciwzapalnej związków polifenolowych obejmują natomiast ocenę bezpośredniej inhibicji enzymów o działaniu prozapalnym; wpływu na sekrecję prozapalnych cytokin, chemokin i enzymów przez komórki układu odpornościowego; a także ocenę wpływu na ekspresję jądrowych czynników transkrypcyjnych zaangażowanych w ekspresję genów dla m.in. prozapalnych cytokin czy enzymów. W celu przybliżenia możliwych efektów *in vivo* analizom aktywności biologicznej *in vitro* coraz częściej poddaje się nie tylko substancje roślinne, ale również związki uznawane za ich metabolity w ustroju bądź frakcje uzyskane w wyniku symulowanego trawienia *in vitro*. Wykazano, że większość polifenoli ulega metabolizmowi w jelicie grubym przy udziale bakterii jelitowych, a produktami metabolizmu są

m.in. kwas 2-(3'4'-dihydroksyfenylo)octowy, kwas 3-(3',4'-dihydroksyfenylo)propionowy, kwas 3-(4'-hydroksyfenylo)propionowy i kwas protokatechowy (Marin i wsp., 2015; Ou i Gu, 2014; Tomas-Barberan i wsp., 2014). Kolejnym etapem niezbędnym do pełnej oceny substancji roślinnych jest określenie bezpieczeństwa stosowania – wstępnie *in vitro* na modelach komórkowych, a następnie *in vivo* na modelach zwierzęcych.

Wobec niepełnych danych na temat składu chemicznego i aktywności biologicznej kwiatów śliwy tarniny, niniejsza rozprawa stanowi część projektu badawczego zmierzającego do kompleksowej oceny materiału roślinnego i pochodnych ekstraktów suchych jako substancji leczniczych o potencjalnym zastosowaniu w profilaktyce i wspomagającym leczeniu ChUK. Badania w ramach rozprawy były prowadzone dwutorowo i obejmowały pogłębioną analizę fitochemiczną suchych ekstraktów z kwiatów *P. spinosa* z opracowaniem metodyki kontroli jakości oraz wstępną ocenę aktywności antyoksydacyjnej i przeciwzapalnej na komplementarnych chemicznych i biologicznych modelach *in vitro*.

Cele naukowe pracy

Celem naukowym rozprawy doktorskiej była pełna charakterystyka fitochemiczna kwiatów *P. spinosa* i pochodnych ekstraktów suchych z opracowaniem metodyki kontroli jakości/ standaryzacji w zakresie składników aktywnych (polifenolowych) oraz analiza ich aktywności przeciwutleniającej i przeciwzapalnej, co uzyskano w oparciu o następujące badania:

1. Analizę profilu fitochemicznego suchych ekstraktów z kwiatów *P. spinosa* w funkcji ekstrakcji frakcjonowanej, w tym:

- izolację i określenie struktury wybranych związków polifenolowych – pozyskanie substancji wzorcowych (**Publikacja I**);
- analizę jakościową i ilościową ekstraktów – wskazanie związków modelowych (**Publikacja II**);
- optymalizację i walidację metody HPLC-PDA-fingerprint analizy związków polifenolowych w materiale roślinnym i pochodnych ekstraktach (**Publikacja IVA, IVB**).

2. Analizę zmienności zawartości indywidualnych polifenoli w materiale roślinnym w zależności od producenta i roku zbioru (**Publikacja IVA, IVB**).

3. Analizę *in vitro* aktywności biologicznej suchych ekstraktów z kwiatów *P. spinosa* w funkcji ekstrakcji frakcjonowanej, związków polifenolowych uznanych za składniki modelowe kwiatów oraz ich głównych metabolitów *in vivo*, w tym:

- analizę aktywności antyoksydacyjnej w prostych testach chemicznych (DPPH, FRAP, TBARS) (**Publikacja II**);
- ocenę efektu ochronnego wobec białkowych i lipidowych składników ludzkiego osocza poddanego działaniu stresu oksydacyjnego indukowanego *in vitro* (**Publikacja II**);
- analizę aktywności antyoksydacyjnej względem oksydantów generowanych w układzie krążenia *in vivo*, tj. $O_2^{\bullet-}$, $HO^{\bullet}$, H_2O_2, $NO^{\bullet}$, $ONOO^-$ i HClO (**Publikacja III**);
- ocenę zdolności do inhibicji enzymów prozapalnych: lipooksygenazy i hialuronidazy (**Publikacja II**);
- wstępną ocenę bezpieczeństwa komórkowego ekstraktów w testach cytotoksyczności/przeżywalności jednojądrzastych komórek krwi obwodowej (PBMCs) (**Publikacja II**).

Część doświadczalna

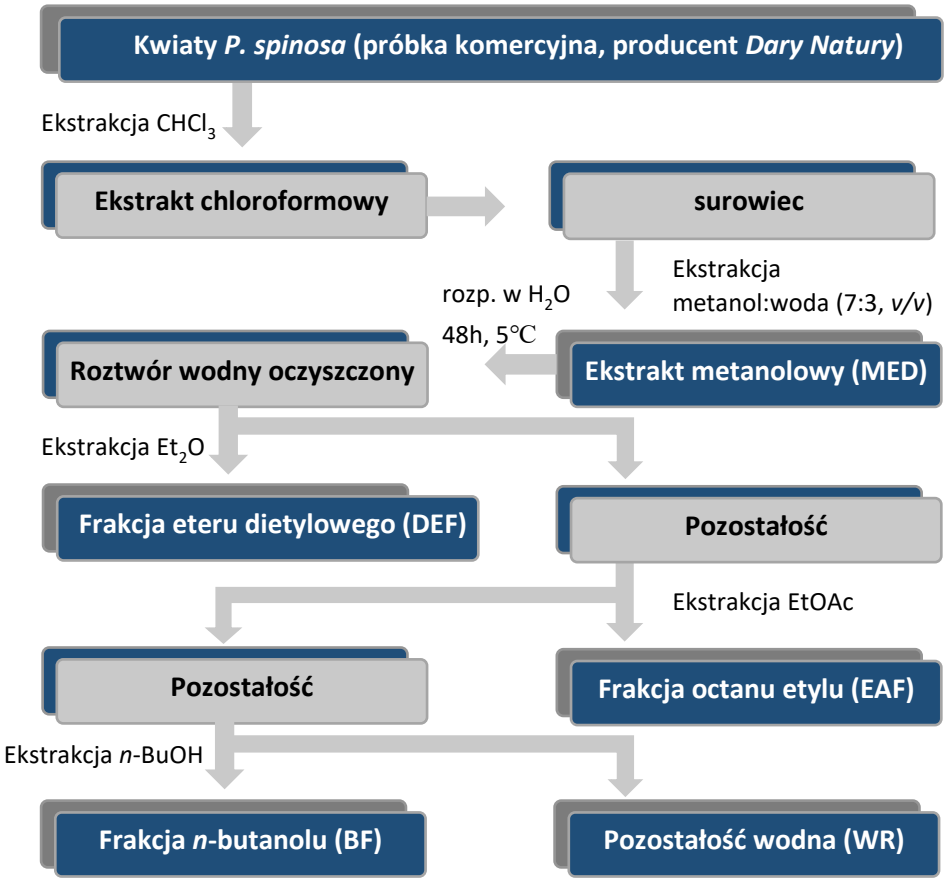
Materiał roślinny

Materiał do badań fitochemicznych i analiz aktywności biologicznej stanowiły suche ekstrakty otrzymane z kwiatów *P. spinosa* metodą frakcjonowanej ekstrakcji, w tym suchy ekstrakt metanolowo-wodny (7:3, *v/v*) (MED), jego frakcje eteru dietylowego (DEF), octanu etylu (EAF) i *n*-butanolu (BF), oraz pozostałość wodna (WR). Do przygotowania ekstraktów użyto surowca handlowego producenta *Dary Natury* (Koryciny, Polska) zakupionego w 2015 roku (zbiór kwiecień 2015). Proces ekstrakcji został przedstawiony na rycinie 3.

Analiza zmienności zawartości indywidualnych polifenoli w materiale roślinnym objęła próbki komercyjne kwiatów *P. spinosa* L. zakupione od trzech europejskich producentów: *Dary Natury* (Koryciny, Polska), *Flos* (Mokrsko, Polska) and *Kräuter Kühne* (Berlin, Niemcy) w latach 2015-2018.

Do izolacji części substancji wzorcowych (wybranych glikozydów flawonoidowych) wykorzystano liście *P. spinosa* zebrane ze stanu naturalnego w miejscowości Rzeczyca (Polska) w lipcu 2015 r.

Tożsamość gatunkowa surowców została potwierdzona w badaniach makroskopowych i mikroskopowych przez dr hab. n. farm. Monikę Annę Olszewską, prof. UM zgodnie ze standardami farmakognostycznymi (Wichtl, 2004).



Rycina 3 Schemat ekstrakcji kwiatów *P. spinosa* L.

Koncepcja i plan badań

Koncepcja badań oraz zestawienie przeprowadzonych analiz zostały przedstawione w Tabeli 2.

Tabela 2 Koncepcja badań oraz zestawienie przeprowadzonych analiz.

Publikacja I	**Izolacja wybranych substancji wzorcowych z liści *P. spinosa***
	• izolacja i określenie struktury wybranych glikozydów flawonoidowych
Publikacja II	**Analiza fitochemiczna i wstępna ocena aktywności biologicznej suchych ekstraktów z kwiatów *P. spinosa***
	• analiza profilu jakościowego (LC-MS/MS) i ilościowego • analiza aktywności antyoksydacyjnej w chemicznych testach *in vitro* (DPPH, FRAP, TBARS), • analiza aktywności antyoksydacyjnej na biologicznym modelu *in vitro* – ocena efektu ochronnego wobec białkowych i lipidowych składników ludzkiego osocza poddanego działaniu stresu oksydacyjnego • analiza *in vitro* aktywności przeciwzapalnej w testach inhibicji enzymów prozapalnych • ocena bezpieczeństwa komórkowego ekstraktów • analiza statystyczna wyników
Publikacja III	**Rozszerzone badania *in vitro* aktywności biologicznej suchych ekstraktów z kwiatów *P. spinosa*, polifenolowych związków modelowych i ich potencjalnych metabolitów *in vivo***
	• ocena *in vitro* aktywności antyoksydacyjnej suchych ekstraktów z kwiatów *P. spinosa*, związków polifenolowych (uznanych za składniki modelowe kwiatów *P. spinosa*) oraz ich potencjalnych metabolitów *in vivo* względem oksydantów generowanych w układzie krążenia *in vivo*, tj. $O_2^{\bullet-}$, $HO^{\bullet}$, H_2O_2, $NO^{\bullet}$, $ONOO^-$ i HClO • analiza statystyczna wyników
Publikacja IVA, IVB	**Opracowanie metodyki kontroli jakości kwiatów *P. spinosa* i pochodnych ekstraktów**
	• optymalizacja i walidacja metody HPLC-PDA-fingerprint analizy związków polifenolowych w kwiatach *P. spinosa* i ekstraktach pochodnych • analiza zawartości indywidualnych polifenoli w suchych ekstraktach z kwiatów *P. spinosa* • analiza zmienności zawartości indywidualnych polifenoli w materiale roślinnym w zależności od producenta i roku zbioru surowca • analiza statystyczna wyników

Metodyka badań

Analiza profilu fitochemicznego suchych ekstraktów z kwiatów P. spinosa oraz analiza zmienności profilu polifenolowego w komercyjnym materiale roślinnym

Analiza profilu jakościowego ekstraktów została przeprowadzona metodą UHPLC-PDA-ESI-MS^3 z wykorzystaniem ultra-wysokosprawnego chromatografu cieczowego sprzężonego z detektorem fotodiodowym oraz ze spektrometrem masowym, zaopatrzonym w przystawki umożliwiające jonizację próbek metodą elektrorozpylania (ESI) oraz analizator typu pułapka jonowa. Anality identyfikowano na podstawie porównania danych fragmentacyjnych ESI-MS^3 (w trybie jonów ujemnych i dodatnich) oraz widm UV-Vis z danymi literaturowymi oraz danymi uzyskanymi dla substancji wzorcowych.

Analiza profilu ilościowego ekstraktów objęła oznaczenie całkowitej zawartości polifenoli i procyjanidyn spektrofotometrycznymi metodami Folina-Ciocalteu'a i Portera wg Olszewskiej i wsp. (2012), oraz oznaczenie zawartości aglikonów flawonoidowych po hydrolizie metodą HPLC-PDA wg Olszewskiej (2012) i całkowitej zawartości kwasów fenolowych metodą HPLC-PDA wg Olszewskiej i wsp. (2012). Ponadto oznaczono zawartość indywidualnych składników polifenolowych przy użyciu metody HPLC-PDA-fingerprint, po jej wcześniejszej optymalizacji i walidacji według wytycznych International Council for Harmonisation (ICH) Guidance for Industry (ICH, 2005) i odpowiednich doniesień literaturowych (Olszewska, 2012), z wykorzystaniem chromatografu HPLC i kolumny chromatograficznej typu fused-core. Metodę tę wykorzystano także do analizy zawartości składników polifenolowych w materiale roślinnym zakupionym od trzech europejskich producentów (*Flos*, *Dary Natury*, *Kräuter Kühne*) w latach 2015-2018.

W analizie UHPLC-MS oraz HPLC-PDA-fingerprint wykorzystano paletę 14 substancji wzorcowych uzyskanych podczas wcześniejszych prac izolacyjnych Promotora (Olszewska i Wolbiś, 2001; Olszewska i Wolbiś, 2002a; Olszewska i Wolbiś, 2002b) oraz cztery diglikozydy flawonoidowe wyizolowane z liści *P. spinosa* w ramach niniejszej rozprawy doktorskiej (ze względu na ich wyższą zawartość niż w kwiatach). Izolację przeprowadzono metodą preparatywnej wysokosprawnej chromatografii cieczowej (HPLC-PDA), opracowaną i zoptymalizowaną metodą płaszczyzny odpowiedzi (RSM), a struktura wyodrębnionych związków została potwierdzona głównie metodami spektroskopowymi (1D i 2D NMR: COSY, HMBC, HMQC).

Szczegółowy opis odczynników, aparatury, przygotowania próbek oraz procedur badawczych znajduje się w poszczególnych publikacjach, będących podstawą rozprawy doktorskiej.

Analiza in vitro aktywności biologicznej suchych ekstraktów z kwiatów P. spinosa

Analizy *in vitro* aktywności antyoksydacyjnej ekstraktów objęły eksperymenty z użyciem metod chemicznych opartych na mechanizmie SET (przeniesieniu pojedynczego elektronu, ang. single electron transfer), tj. DPPH i FRAP, oraz na mechanizmie HAT (przeniesienia atomu wodoru, ang. hydrogen atom transfer), tj. TBARS. Oznaczenia zdolności ekstraktów do zmiatania rodnika DPPH oraz redukowania jonów żelaza (III) zostały przeprowadzone metodami spektrofotometrycznymi według zoptymalizowanych wcześniej procedur (Olszewska i wsp., 2012). Oznaczanie aktywności antyoksydacyjnej ekstraktów w teście inhibicji oksydacji kwasu linolenowego (TBARS) zostało przeprowadzone według procedury opisanej przez Kljak i Grbeša (2015) zmodyfikowanej w ramach niniejszej rozprawy.

Komplementarne analizy *in vitro* aktywności antyoksydacyjnej ekstraktów na modelu biologicznym objęły ocenę efektu ochronnego ekstraktów wobec białkowych i lipidowych składników ludzkiego osocza w warunkach stresu oksydacyjnego wywołanego $ONOO^-$. Oznaczono poziom markerów stresu oksydacyjnego, w tym markerów nitrowania białek: 3-nitrotyrozyny (3-NT), immunoenzymatyczną metodą ELISA (wg Kolodziejczyk-Czepas i wsp., 2013), oraz markerów peroksydacji lipidów: wodoronadtlenków lipidów (FOX-1) (wg Kolodziejczyk-Czepas i wsp., 2013), oraz substancji reagujących z kwasem tiobarbiturowym TBARS (wg Kolodziejczyk i wsp., 2011). Ponadto oznaczono nieenzymatyczną pojemność antyoksydacyjną osocza (NEAC) metodą FRAP wg Kolodziejczyk-Czepas i wsp. (2014).

Analizy *in vitro* aktywności antyoksydacyjnej ekstraktów wobec oksydantów generowanych *in vivo* w układzie krążenia ($O_2^{\bullet-}$, $HO^\bullet$, H_2O_2, $NO^\bullet$, $ONOO^-$ i HClO) były przeprowadzone metodami spektro- i fluorymetrycznymi według zoptymalizowanych wcześniej procedur. Zdolność do zmiatania $O_2^{\bullet-}$ została określona w układzie ksantyna/oksydaza ksantynowa z użyciem błękitu nitrotetrazoliowego do detekcji wg Granicy i wsp. (2013). W teście zmiatania $HO^\bullet$ spektrofotometrycznie monitorowano poziom dihydroksypochodnych kwasu benzoesowego, powstających w wyniku reakcji rodnika $HO^\bullet$, generowanego w reakcji Fentona, z kwasem salicylowym wg Fu i wsp. (2014). Aktywność antyoksydacyjna wobec $NO^\bullet$ była określana fluorymetrycznie wg Czerwińskiej i wsp. (2012) poprzez monitorowanie poziomu triazolofluoresceiny, powstającej z 4,5-diaminofluoresceiny w obecności $NO^\bullet$. Zdolność do zmiatania HClO była oceniana z użyciem kwasu 5-tio-2-nitrobenzoesowego, utlenianego w obecności HClO do kwasu 5,5-ditiobis-2-nitrobenzoesowego, którego poziom mierzono spektrofotometrycznie wg Czerwińskiej i wsp. (2012). Zdolność do zmiatania $ONOO^-$ (otrzymanego syntetycznie) była określana spektrofotometrycznie wg Krzyzanowskiej-Kowalczyk i wsp. (2017) poprzez określenie hamowania utleniania azowego błękitu Evansa. Zdolność do redukcji H_2O_2 oceniano wg. Fernando i Soysa (2015) poprzez monitorowanie poziomu chinoinoiminy wytwarzanej w reakcji 4-aminoantipiryny, fenolu i H_2O_2, katalizowanej przez peroksydazę chrzanową. Ww. analizy aktywności antyoksydacyjnej względem ROS/RNS generowanych *in vivo* objęły również wyselekcjonowane w badaniach wstępnych modelowe polifenole, tj. kemferol, kwercetynę i ich glikozydy: juglaninę, kemferytrynę i awikularynę, ponadto kwas chlorogenowy, procyjanidynę A2 oraz kwas *p*-kumarowy . Ponadto, dla pełniejszej oceny efektów spodziewanych *in vivo*, analizy na ww. modelu chemicznym były prowadzone wobec związków uznawanych za główne produkty metabolizmu polifenoli w ustroju, odpowiednich dla profilu polifenolowego surowca, tj. 3-glukuronidu kwercetyny, kwasu 2-(3'4'-dihydroksyfenylo)octowego, kwasu 3-(3',4'-dihydroksyfenylo)propionowego, kwasu 3-(4'-hydroksyfenylo)propionowego oraz kwasu protokatechowego; a także wzorcowych przeciwutleniaczy osocza, tj. kwasu askorbinowego oraz syntetycznego analogu witaminy E, tj. Troloxu.

Potencjalna aktywność przeciwzapalna ekstraktów była badana w testach hamowania enzymów prozapalnych – lipooksygenazy i hialuronidazy. Hamowanie aktywności hialuronidazy określono metodą turbidymetryczną opartą na spektrofotometrycznym pomiarze zmętnienia powstałego na skutek wytworzenia kompleksów hialuronan-albumina bydlęca w środowisku kwasowym wg Michela i wsp. (2017). 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 wg Michela i wsp. (2017).

Ocena bezpieczeństwa komórkowego (potencjalnej cytotoksyczności) ekstraktów objęła badania z użyciem jednojądrzastych komórek krwi obwodowej – PBMCs. Pomiary wpływu analitów na przeżywalność PBMCs wykonane zostały metodą spektrofluorymetryczną z zastosowaniem jodku propidyny do wybarwiania martwych komórek, zgodnie z procedurą opisaną przez producenta zastosowanego testu komercyjnego.

Badania analitów na modelach biologicznych (3 steżenia) objęły zarówno zakresy fizjologiczne dla polifenoli (1-5 µg/ml; obserwowane w osoczu po podaniu doustnym), jak i wyższe (50 µg/ml), w celu uwidocznienia wszystkich możliwych efektów działania. We wszystkich testach stosowana była właściwa kontrola pozytywna z wykorzystaniem związków o udowodnionym działaniu, odpowiednio do danego testu. Dla zwiększenia przepustowości analiz i ograniczenia kosztów, badania aktywności były w większości prowadzone z wykorzystaniem czytników płytek. Materiał biologiczny do badań (osocze, PBMCs) został odzyskany z kożuszków leukocytarno-płytkowych dostępnych komercyjnie w Regionalnym Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi (niewymagana zgoda Komisji Bioetycznej). 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.

Ocena statystyczna wyników

Wszystkie wyniki były weryfikowane pod kątem istotności statystycznej różnic i korelacji na poziomie istotności α min. 0.05, z wykorzystaniem programu Statistica12Pl (StatSoft). Szczegółowe informacje na temat zastosowanych testów znajdują się w poszczególnych publikacjach, będących podstawą rozprawy doktorskiej.

Realizacja celów naukowych – wyniki i dyskusja

Analiza profilu fitochemicznego suchych ekstraktów z kwiatów *P. spinosa*

Zważywszy na fakt, iż standaryzowane ekstrakty roślinne, a w szczególności ekstrakty suche, są preferowane we współczesnej fitoterapii ze względu na wyższą efektywność od nieprzetworzonych substancji roślinnych, głównym przedmiotem zainteresowania niniejszej rozprawy doktorskiej były suche ekstrakty z kwiatów *P. spinosa*. Na podstawie dostępnej literatury i wyników badań wstępnych ustalono, że optymalnym ekstrahentem dla niskocząsteczkowych polifenoli (o potencjalnie najwyższej biodostępności) występujących w surowcu jest mieszanina metanol-woda (7:3, *v/v*), a frakcjonowana ekstrakcja rozpuszczalnikami o wzrastającej polarności (eterem dietylowym, octanem etylu, *n*-butanolem) otrzymanego ekstraktu alkoholowo-wodnego pozwala na jego oczyszczenie i koncentrację związków aktywnych (Manach i wsp., 2005; Olszewska i wsp., 2012). Analiza całkowitej zawartości polifenoli (TPC) przeprowadzona metodą Folina-Ciocalteu'a potwierdziła, że zarówno wyjściowy suchy ekstrakt metanolowo-wodny (MED), jak i jego poszczególne frakcje stanowią bogate źródło związków polifenolowych. Dla MED wartość TPC wyniosła 206.1 mg GAE/g s.m., natomiast w przypadku najbogatszych w polifenole frakcji EAF i DEF wartości TPC wyniosły odpowiednio 584.1 i 464.6 mg GAE/g s.m. i były porównywalne z wartościami literaturowymi dla ekstraktów otrzymanych z substancji roślinnych cenionych w prewencji ChUK, jak np. ekstrakt etanolowy z nasion winorośli (TPC, 630-670 mg GAE/g s.m.) (Baydara i wsp., 2004) i frakcja octanu etylu z zielonej herbaty (TPC, 480-580 mg GAE/g s.m.) (Erol i wsp., 2009). Porównanie otrzymanych wyników z danymi literaturowymi dla ekstraktów z innych części rośliny pokazało, że pod względem zawartości polifenoli kwiaty plasują się na drugim miejscu po gałązkach, a przed owocami i liśćmi, stanowiąc obiecujący materiał do dalszych badań (Pinacho i wsp., 2015) (**Publikacja II**).

Analiza profilu jakościowego metodą UHPLC-PDA-ESI-MS^3, przeprowadzona w ramach niniejszej rozprawy po raz pierwszy dla kwiatów śliwy tarniny, znacząco poszerzyła wiedzę o ich składzie chemicznym. W badanych ekstraktach całkowicie lub częściowo zidentyfikowano ponad 50 związków polifenolowych (36 nowych dla kwiatów), z czego 37 sklasyfikowano jako flawonoidy (głównie mono- i diglikozydy kemferolu i kwercetyny), pozostałe zaś jako pochodne flawan-3-olu (katechiny i procyjanidyny typu A), pseudodepsydy kwasu kawowego i proste kwasy fenolowe (**Publikacja II**). Analogiczne analizy przeprowadzone dla liści *P. spinosa* wykazały obecność 25 związków (**Publikacja I**), co w połączeniu z danymi literaturowymi dla gałązek i owoców tarniny (26 i 29 wykrytych związków, odpowiednio) (Pinacho i wsp., 2015; Guimarães i wsp., 2013), pokazuje jak bardzo złożoną matrycą polifenolową charakteryzują się kwiaty. Wartość surowca jako źródła związków polifenolowych podnosi ponadto fakt, że część zidentyfikowanych składników, takich jak pentozydy flawonoidowe (arabinozydy, ksylozydy, ramnozydy) oraz dimery procyjanidynowe typu A, to rzadko występujące w naturze związki (**Publikacja II**).

Wstępna analiza ilościowa ekstraktów przeprowadzona metodami spekrofotometrycznymi i chromatograficznymi pozwoliła na określenie zawartości głównych grup związków czynnych (flawonoidów, procyjanidyn i kwasów fenolowych) oraz wykazała, że dominującą grupą związków czynnych we wszystkich ekstraktach i frakcjach za wyjątkiem pozostałości wodnej stanowią flawonoidy. Najwyższą zawartością aglikonów flawonoidowych po hydrolizie oznaczoną metodą

HPLC odznaczał się DEF (459.6 mg/g s.m. w przeliczeniu na glikozydy). Zawartość procyjanidyn oznaczona spektrofotometryczną metodą Portera była najwyższa dla EAF (109.4 mg CYE/g s.m.), natomiast najwyższą zawartość kwasów fenolowych oznaczoną metodą HPLC zaobserwowano dla BF (66.8 mg/g s.m.) (**Publikacja II**).

Opisana powyżej wstępna analiza ilościowa potwierdziła hipotezę o wartości ekstraktów/frakcji z kwiatów tarniny jako bogatego źródła polifenoli oraz dała wgląd we wzajemne proporcje pomiędzy głównymi grupami składników czynnych, nie pozwalając jednak na ocenę udziałów poszczególnych związków w zespole. Ponadto proces nie był optymalny jako potencjalne narzędzie kontroli jakości ze względu na konieczność wykorzystania czterech metod analitycznych. Do celów kontroli jakości najbardziej adekwatna byłaby metoda uniwersalna, pozwalająca na określenie zawartości wszystkich, bądź większości grup związków czynnych substancji roślinnej, równolegle z jednoczesną oceną/potwierdzeniem profilu jakościowego. Metoda UHPLC-PDA-ESI-MS^3, wykorzystana do celów analizy jakościowej, nie spełniała tych kryteriów ze względu na typ zastosowanego detektora selektywności masy (pułapka jonowa) i jego ograniczeń w kontekście analiz ilościowych (zależna od związku odpowiedź, mały zakres dynamiczny i stosunkowo słaba odtwarzalność). Biorąc pod uwagę powyższe przesłanki, kolejnym etapem prezentowanej rozprawy stała się optymalizacja i walidacja metody HPLC-PDA-fingerprint analizy indywidualnych polifenoli, mogąca znaleźć zastosowanie w kontroli jakości kwiatów *P. spinosa* oraz przygotowanych z nich ekstraktów. Ze względu na wysoką złożoność matrycy polifenolowej surowca oraz podobieństwo strukturalne związków, opracowanie odpowiedniej metodyki standaryzacji nie było łatwym zadaniem, szczególnie, że postawiono sobie za cel uzyskanie rozdziału analitów w czasie krótszym niż stosowany w jakościowej analizie UHPLC. Proces optymalizacji rozdziału przebiegał z wykorzystaniem 30 związków polifenolowych charakterystycznych dla surowca, wytypowanych na podstawie analizy LC-MS, i objął wiele czynników, w tym skład fazy ruchomej, profil elucji, szybkość przepływu i temperaturę. Analizy prowadzono z wykorzystaniem kolumny C18 typu fused-core, wybranej na podstawie doniesień literaturowych i wstępnych testów, oraz umożliwiającej uzyskanie wysokiej rozdzielczości przy zachowaniu poziomu ciśnienia akceptowalnego dla konwencjonalnych aparatów HPLC (Olszewska, 2012). Najlepsze wyniki zostały uzyskane przy zastosowaniu trójskładnikowego eluentu (acetonitryl-tetrahydrofuran-0.5% kwas ortofosforowy) w układzie elucji gradientowej, dla przepływu fazy ruchomej 1.09 ml/min i w temperaturze 28°C. Zoptymalizowana metoda pozwoliła na rozdział analitów z satysfakcjonującą rozdzielczością w czasie 35 minut. Walidacja przeprowadzona wg wytycznych ICH pokazała dobrą precyzję (RSD < 5%), dokładność (93.5-102.1%), liniowość (r > 0.9998), i czułość (LODs 0.51-2.05 ng) metody. Opracowana metoda umożliwiła równoległe oznaczenie zawartości kluczowych analitów kwiatów *P. spinosa* z wykorzystaniem 30 wzorców kalibracyjnych. Ponadto wprowadzono współczynniki korekcyjne, umożliwiające oznaczenie ww. analitów z wykorzystaniem palety substancji wzorcowych zredukowanej do 5 związków dostępnych komercyjnie (**Publikacja IVA, IVB**).

Opracowana metoda HPLC-PDA-fingerprint została z powodzeniem wykorzystana do analizy zawartości indywidualnych polifenoli w suchych ekstraktach i frakcjach z kwiatów *P. spinosa*. Obserwowano znaczące różnice w profilu ilościowym składników, a całkowita zawartość analitów w ekstraktach/frakcjach wahała się w zakresie 15.9-491.7 mg/g s.m. Należy podkreślić, że skład ekstraktów był ściśle skorelowany z polarnością rozpuszczalnika użytego do ekstrakcji. Na przykład

dominującymi składnikami DEF były pentozydy flawonolowe i aglikony, ponadto obserwowano względnie wysoką zawartość kwasu kawowego, kwasu *p*-kumarowego i katechiny (związków, które w pozostałych ekstraktach występowały w śladowych ilościach). Z kolei EAF był bogaty w diglikozydy i monoglikozydy flawonolowe, w tym heksozydy, a także procyjanidyny, natomiast w BF przeważały diglikozydy flawonolowe i izomeryczne kwasy chlorogenowe (**Publikacja IVA, IVB**).

Przeprowadzenie powyżej opisanych analiz LC-MS oraz HPLC-fingerprint wymagało izolacji wybranych diglikozydów flawonoidowych, charakterystycznych dla gatunku *P. spinosa*. Większość z flawonoidów typowych dla gatunku (19 związków) została wyizolowana we wcześniejszych badaniach kwiatów i liści tarniny prowadzonych w Zakładzie Farmakognozji Uniwersytetu Medycznego w Łodzi (Olszewska i Wolbiś, 2001; Olszewska i Wolbiś, 2002a, 2002b). Wstępne analizy LC-MS wykonane w ramach niniejszej pracy wykazały, że dwa diglikozydy kemferolu pozostają nieznane, oraz, że najbardziej wydajnym źródłem tych związków są liście tarniny, co dało przesłanki do zastosowania tego właśnie surowca w badaniach izolacyjnych. Materiał badawczy do prac izolacyjnych stanowiła frakcja diglikozydów kemferolu z liści tarniny, zawierająca oprócz dwóch ww. przedmiotowych związków również dwa inne, znane wcześniej związki o podobnych właściwościach chromatograficznych (czasach retencji), których efektywny rozdział przedstawiał istotny problem analityczny. Izolację przeprowadzono metodą preparatywnej chromatografii HPLC stosując procedurę rozdziału zoptymalizowaną z wykorzystaniem statystycznych metod optymalizacyjnych. Rozdzielone związki wyodrębniono z wysoką wydajnością (84.8–94.5%) i czystością (92.5–99.8%), i zidentyfikowano strukturalnie jako 3-*O*-*β*-D-ksylopiranozydo-7-*O*-*α*-L-ramnopiranozyd kemferolu (lepidozyd) i 3-*O*-*α*-L-arabinopiranozydo-7-*O*-*α*-L-ramnopiranozyd kemferolu (po raz pierwszy opisane w gatunku), oraz 3-*O*-*α*-L-arabinofuranozydo-7-*O*-*α*-L-ramnopiranozyd kemferolu i 3,7-di-*O*-*α*-L-ramnopiranozyd kemferolu (kemferytryna). Wszystkie cztery związki wykorzystano jako substancje wzorcowe w ww. analizach fitochemicznych oraz w późniejszych testach aktywności biologicznej (**Publikacja I**).

Analiza zmienności profilu polifenolowego w komercyjnym materiale roślinnym

Biorąc pod uwagę, że kwiaty śliwy tarniny są pozyskiwane ze stanu naturalnego i niekontrolowane czynniki środowiskowe, tj. nasłonecznienie, temperatura, wilgotność, zasolenie gleby, mogą wpływać na profil polifenolowy surowca, w kolejnym etapie pracy dokonano analizy zmienności zawartości indywidualnych polifenoli metodą HPLC-PDA-fingerprint w materiale roślinnym zakupionym od trzech producentów europejskich w latach 2015-2018. Wszystkie związki uznane na podstawie wcześniejszej analizy LC-MS za modelowe dla kwiatu tarniny zostały wykryte w każdej próbce, co świadczy o względnej stabilności profilu jakościowego surowca. Jednakże analizowane próbki różniły się pod względem profilu ilościowego, co potwierdza konieczność kontroli jakości surowca. Całkowita zawartość polifenoli wahała się w zakresie 32.0-49.0 mg/g s.m., natomiast zawartość poszczególnych składników różniła się nawet czterokrotnie, np. dla kwasu chlorogenowego wynosiła od 0.5 do 2.2 mg/g s.m. Kemferytryna i awikularyna dominowały w większości próbek, a ich zawartość wynosiła odpowiednio 3.1-6.5 mg/g s.m. i 2.5-6.0 mg/g s.m. Inne związki, których zawartość w większości próbek przekraczała 3 mg/g s.m. to kwas neochlorogenowy, afzelina, 3-*O*-*α*-L-arabinofuranozydo-7-*O*-*α*-L-ramnopiranozyd kemferolu i juglanina. Zawartość ww. sześciu związków stanowiła 51.4-60.4% całkowitej zawartości

polifenoli, a ich oznaczenie uznano za absolutne minimum w kontroli jakości surowca (**Publikacja IVA, IVB**).

Analiza *in vitro* aktywności biologicznej suchych ekstraktów z kwiatów *P. spinosa*

Mając na względzie profil fitochemiczny ekstraktów, wielokierunkową aktywność związków polifenolowych, w tym m.in. antyoksydacyjną i przeciwzapalną, tradycyjne zastosowania kwiatów śliwy tarniny w ChUK oraz rolę stresu oksydacyjnego i współistniejącego stanu zapalnego w patomechanizmie ChUK, celem niniejszej pracy stała się ocena aktywności antyoksydacyjnej i przeciwzapalnej ekstraktów na komplementarnych chemicznych i biologicznych modelach *in vitro*, pozwalających na wskazanie bezpośrednich mechanizmów ww. typów aktywności.

Analizy aktywności antyoksydacyjnej w chemicznych testach *in vitro* (DPPH, FRAP, TBARS) wykazały wysoki potencjał przeciwutleniający ekstraktów, korelujący z zawartością polifenoli (szczególnie flawonoidów), dla najbardziej aktywnych frakcji EAF i DEF porównywalny z aktywnością szeregu wzorcowych przeciwutleniaczy, zarówno naturalnych jak i syntetycznych (kwercetyna, Trolox, BHA – butylowany hydroksyanizol, BHT – butylowany hydroksytoluen). Przeprowadzenie ww. prostych testów chemicznych nie tylko pozwoliło na szybką ocenę aktywności ekstraktów i porównanie z danymi literaturowymi, ale również wskazało potencjalne mechanizmy ich aktywności antyoksydacyjnej („zmiatanie" wolnych rodników, redukowanie jonów metali, inhibicja oksydacji lipidów), dając podstawy do rozszerzenia badań aktywności antyoksydacyjnej o testy na modelu biologicznym ludzkiego osocza (**Publikacja II**).

Analizy aktywności antyoksydacyjnej na modelu ludzkiego osocza były prowadzone *in vitro* w warunkach stresu oksydacyjnego wywołanego $ONOO^-$, jednym z głównych czynników utleniających i nitrujących *in vivo*, generowanym m.in. w układzie krążenia w ostrych i przewlekłych stanach zapalnych oraz w przypadkach niedokrwienia i reperfuzji. Wykazano, że ekstrakty już w niskich stężeniach odnoszących się do osoczowych poziomów osiągalnych dla polifenoli *in vivo* po podaniu doustnym (1-5 µg/ml) chronią białka i lipidy komórkowe przed zmianami oksydacyjno-nitracyjnymi. Obserwowano obniżony poziom markerów nitrowania białek (3-NT) i peroksydacji lipidów (FOX-1 i TBARS), oraz wzrost całkowitej nieenzymatycznej pojemności antyoksydacyjnej osocza (NEAC). W przypadku oznaczenia 3-NT dla wszystkich ekstraktów spadek nitrowania reszt tyrozynowych był istotny statystycznie ($p < 0.001$) i wynosił do 50% już przy stężeniu 1 µg/ml. 3-NT jest ważnym markerem zmian zachodzących w organizmie pod wpływem stresu oksydacyjnego, a jej podwyższony poziom, obserwowany m.in. u pacjentów cierpiących na chorobę wieńcową, miażdżycę, nadciśnienie i kardiomiopatie, jest uznawany za predyktor wystąpienia incydentów sercowo-naczyniowych (**Publikacja II**).

Wysoki potencjał antyoksydacyjny ekstraktów przekładający się na istotne efekty ochronne w stosunku do składników ludzkiego osocza w warunkach stresu oksydacyjnego indukowanego *in vitro* potwierdza hipotezę, że aktywność przeciwutleniająca może częściowo warunkować korzystne działanie kwiatów śliwy tarniny w obrębie układu krążenia, sugerowane przez medycynę tradycyjną. Efekty biologiczne obserwowane na modelu ludzkiego osocza wynikały najprawdopodobniej z bezpośredniego zmiatania/redukcji $ONOO^-$ i/lub rodników potomnych $ONOO^-$, tj. m.in. $CO_3^{\bullet-}$, $^{\bullet}NO_2$, $OH^{\bullet}$, mogących podobnie jak $ONOO^-$ reagować z biomolekułami osocza, powodując oksydacyjne i nitracyjne uszkodzenia ich struktury (Szabo i wsp., 2007; Pacher i wsp.,

2008). $ONOO^-$ nie jest jedynym ROS/RNS generowanym w układzie krążenia *in vivo*, a inne potencjalne źródła stresu oksydacyjnego obejmują m.in. bezpośrednich prekursorów $ONOO^-$, tj. $O_2^{\bullet-}$ i $NO^\bullet$, oraz $HO^\bullet$, H_2O_2 i HClO, wydzielane przez komórki układu immunologicznego (neutrofile, monocyty) w procesie zapalnym (Yang i wsp., 2013; Mittal i wsp., 2014). Wymienione powyżej ROS/RNS tworzą razem złożony zespół czynników oksydacyjnych, a efektywna neutralizacja ich negatywnego wpływu na układy biologiczne wymaga oddziaływania przynajmniej z kilkoma z tych czynników. W kolejnym etapie rozprawy doktorskiej oceniono zatem aktywność ekstraktów/frakcji kwiatów tarniny wobec szeregu rodnikowych i nierodnikowych oksydantów generowanych *in vivo* ($O_2^{\bullet-}$, $HO^\bullet$, H_2O_2, $NO^\bullet$, $ONOO^-$, HClO). Badania poszerzono ponadto o związki polifenolowe uznane za składniki modelowe kwiatów *P. spinosa* oraz ich główne metabolity *in vivo*, co umożliwiło wskazanie głównych determinantów testowanej aktywności oraz bliższą ocenę spodziewanych efektów *in vivo*. Wykazano wysoki, zależny od dawki i korelujący z zawartością polifenoli, potencjał antyoksydacyjny ekstraktów/frakcji względem wszystkich badanych oksydantów, przy czym najwyższą efektywność obserwowano w stosunku do $O_2^{\bullet-}$, $HO^\bullet$, $ONOO^-$ i HClO. Zdolność do neutralizacji ROS/RNS w przypadku najbardziej aktywnych frakcji DEF i EAF była porównywalna lub wyższa niż antyoksydantów wzorcowych, w tym kwasu askorbinowego. Szczególnie istotny wydaje się wysoki potencjał antyoksydacyjny frakcji śliwy tarniny względem $HO^\bullet$ i $ONOO^-$, wysoce destrukcyjnych, nieselektywnych czynników oksydacyjnych, które ze względu na znaczną reaktywność nie mogą być efektywnie neutralizowane przez antyoksydanty endogenne (Niki, 2014; Niki, 2016). Rezultaty przeprowadzonych analiz wskazują, że mechanizm efektów ochronnych ekstraktów wykazanych na modelu ludzkiego osocza może być związany zarówno z bezpośrednią reakcją z $ONOO^-$, jak również ze zmiataniem rodników pokrewnych, zaangażowanych w tworzenie $ONOO^-$ lub powstających na skutek reakcji $ONOO^-$ z innymi cząsteczkami. Wśród ośmiu modelowych polifenoli kwiatów śliwy tarniny najbardziej aktywne były kwercetyna, awikularyna, procyjanidyna A2 i kwas chlorogenowy, wykazując podobnie jak ekstrakty najwyższy potencjał względem $O_2^{\bullet-}$, $HO^\bullet$, $ONOO^-$ i HClO, co może sugerować znaczący udział ww. czterech związków w aktywności ekstraktów i uzasadniać ich potencjalne zastosowanie jako markerów aktywności w badaniach standaryzacyjnych. Natomiast dominujące w ekstraktach pochodne kemferolu, tj. juglanina i kemferytryna, wykazywały znacząco niższą zdolność do neutralizacji badanych oksydantów. Jednakże, biorąc pod uwagę doniesienia literaturowe na temat możliwych efektów addycyjnych i synergistycznych pomiędzy składnikami ekstraktów, w tym również pomiędzy kemferolem a kwercetyną i jej glikozydami (Hildago i wsp., 2010), wydaje się, że wysoka zawartość pochodnych kemferolu jest również nie bez znaczenia dla aktywności ekstraktów. Spośród pięciu analizowanych metabolitów polifenolowych kwas dihydrokawowy i kwas 2-(3′,4′-dihydroksyfenylo)octowy charakteryzowały się najwyższą aktywnością względem oksydantów, w niektórych testach działając nawet silniej niż związki macierzyste. Rezultaty badań wskazują zatem na znaczącą aktywność metabolitów polifenolowych i potwierdzają, że związki te powinny być brane pod uwagę podczas oceny polifenolowych substancji roślinnych w zakresie aktywności biologicznej (**Publikacja III**).

Uwzględniając związek pomiędzy stresem oksydacyjnym, stanem zapalnym a rozwojem ChUK, dla pełniejszej oceny substancji roślinnej w kolejnym etapie przeprowadzono analizy *in vitro* potencjalnej aktywności przeciwzapalnej. Wykazano, że ekstrakty/frakcje hamują działanie enzymów prozapalnych: lipooksygenazy i hialuronidazy, a aktywność najbardziej aktywnych frakcji

DEF i EAF plasuje się pomiędzy aktywnością indometacyny, będącej niesteroidowym lekiem przeciwzapalnym, a rutyny, flawonoidu o potwierdzonym działaniu wazoprotekcyjnym (**Publikacja II**). Porównanie aktywności ekstraktów z aktywnością modelowych polifenoli, w tym badania prowadzone równolegle w naszym zespole (Michel i wsp., 2019) sugerują, że wszystkie główne frakcje związków czynnych tarniny (flawonole, kwasy chlorogenowe i procyjanidyny) mogą mieć istotny, synergiczny udział w tej aktywności.

Rezultaty analiz *in vitro* aktywności antyoksydacyjnej i przeciwzapalnej potwierdzają potencjalną wartość kwiatów śliwy tarniny jako źródła substancji leczniczych mogących znaleźć zastosowanie w profilaktyce i leczeniu wspomagającym ChUK, jak sugerują tradycyjne wskazania. Jak wykazano powyżej, ekstrakty/frakcje mogą wpływać na obydwa współistniejące procesy, zarówno stresu oksydacyjnego jak i stanu zapalnego, co wydaje się być szczególnie istotne w obliczu dowodów doświadczalnych i klinicznych pokazujących, że patologie, takie jak przerost lewej komory serca, zwłóknienie mięśnia sercowego, zaburzenia kurczliwości, niewydolność serca i uszkodzenia niedokrwienno-reperfuzyjne, są uwarunkowane zaburzeniem mechanizmów kontrolujących procesy stresu oksydacyjnego/nitrozacyjnego i stanu zapalnego (Gracia i wsp., 2017).

Oprócz badań aktywności biologicznej, kluczowa dla szerszego wykorzystania kwiatów śliwy tarniny w lecznictwie wydaje się ocena bezpieczeństwa stosowania ekstraktów. Wstępny test cytotoksyczności z użyciem jednojądrzastych komórek krwi obwodowej PBMCs przeprowadzony w ramach niniejszej rozprawy dla ekstraktów w stężeniu fizjologicznym (5 µg/ml) wykazał brak różnic w przeżywalności komórek po 60 i 120 minutach inkubacji w roztworze soli fizjologicznej buforowanej fosforanami, co pozwoliło wstępnie zaklasyfikować ekstrakty jako bezpieczne (**Publikacja II**).

Podsumowanie osiągniętych wyników

Badania przeprowadzone w ramach prezentowanej rozprawy doktorskiej pozwoliły na poszerzenie stanu wiedzy o składzie chemicznym oraz aktywności biologicznej ekstraktów z kwiatów *P. spinosa* w funkcji ekstrakcji frakcjonowanej, w szczególności:

- wykazano zależność pomiędzy profilem polifenolowym ekstraktu a rodzajem rozpuszczalnika użytego do ekstrakcji oraz wytypowano ekstrakty/frakcje o najwyższej zawartości związków polifenolowych (**Publikacja II, IVA, IVB**)
- potwierdzono wysoką złożoność matrycy polifenolowej surowca, w tym dokonano całkowitej lub częściowej identyfikacji 57 związków (36 nowych dla kwiatów) (**Publikacja II**)
- obecność dwóch z ww. związków, tj. 3-*O*-*β*-D-ksylopiranozydo-7-*O*-*α*-L-ramnopiranozydu kemferolu (lepidozydu) i 3-*O*-*α*-L-arabinopiranozydo-7-*O*-*α*-L-ramnopiranozydu kemferolu, stwierdzono po raz pierwszy w gatunku *P. spinosa* po izolacji w skali preparatywnej (HPLC) i oznaczeniu struktury (1D i 2 D NMR) (**Publikacja I)**
- wykazano, że ekstrakty z kwiatów stanowią bogate źródło związków polifenolowych, w tym oznaczono zawartość dominujących grup związków (flawonoidów, proantocyjanidyn i kwasów fenolowych) (**Publikacja II**) oraz zawartość indywidualnych związków polifenolowych (**Publikacja IVA, IVB**)
- zoptymalizowano i poddano walidacji metodę HPLC-PDA-fingerprint analizy polifenoli z wykorzystaniem 30 wzorców kalibracyjnych, która może znaleźć zastosowanie w kontroli jakości kwiatów *P. spinosa* i pochodnych ekstraktów (**Publikacja IVA, IVB**)
- wykazano, że surowiec zakupiony od różnych europejskich producentów w różnych latach charakteryzuje się względnie stabilnym profilem jakościowym, natomiast różnice w zawartości poszczególnych związków mogą być znaczne, co wskazuje na konieczność kontroli jakości surowca (**Publikacja IVA, IVB**)
- wykazano wysoką aktywność antyoksydacyjną ekstraktów na komplementarnych chemicznych i biologicznych modelach *in vitro*, w tym efekty ochronne w stosunku do białkowych i lipidowych składników ludzkiego osocza w warunkach stresu oksydacyjnego indukowanego $ONOO^-$ oraz zdolność do zwiększania całkowitej nieenzymatycznej pojemności antyoksydacyjnej osocza (NEAC) (**Publikacja II**)
- wykazano wysoki potencjał antyoksydacyjny ekstraktów, wybranych związków polifenolowych (uznanych za składniki modelowe kwiatów *P. spinosa*) oraz ich głównych metabolitów *in vivo* względem oksydantów generowanych w układzie krążenia *in vivo*: $O_2^{\bullet-}$, $HO^\bullet$, H_2O_2, $NO^\bullet$, $ONOO^-$, HClO (**Publikacja III**)
- wykazano aktywność przeciwzapalną ekstraktów w testach hamowania enzymów prozapalnych: hialuronidazy i lipooksygenazy (**Publikacja II**)
- wykazano, że ekstrakty w osoczowych stężeniach fizjologicznych dla polifenoli *in vivo* (osiągalnych po podaniu doustnym) nie wpływają na przeżywalność jednojądrzastych komórek krwi obwodowej, co pozwoliło je wstępnie zaklasyfikować jako bezpieczne (**Publikacja II**)

Wnioski końcowe

Realizacja prezentowanej rozprawy doktorskiej doprowadziła do szczegółowego scharakteryzowania frakcji polifenolowej kwiatów *P. spinosa* i pochodnych ekstraktów suchych, oraz opracowania metodyki standaryzacji ww. substancji roślinnej w zakresie polifenolowych składników aktywnych, mogącej stanowić potencjalne narzędzie kontroli jakości. Wstępne analizy *in vitro* aktywności antyoksydacyjnej i przeciwzapalnej analitów w prostych układach chemicznych, enzymatycznych i biologicznych pozwoliły na wskazanie podstawowych mechanizmów ich działania, obejmujących bezpośrednią neutralizację zespołu ROS/RNS generowanych *in vivo*, ochronę biomolekuł ludzkiego osocza przed oksydacyjnymi i nitracyjnymi uszkodzeniami spowodowanymi stresem oksydacyjnym, oraz bezpośrednią inhibicję enzymów prozapalnych. Rezultaty zarówno badań fitochemicznych, jak i analiz aktywności biologicznej sugerują znaczący potencjał ekstraktów/frakcji z kwiatów *P. spinosa* w profilaktyce i terapii wspomagającej chorób o podłożu oksydacyjno-zapalnym, w tym ChUK, częściowo potwierdzając tradycyjne wskazania. Do kompleksowej oceny substancji roślinnej konieczne są jednak dalsze badania, w tym badania farmakologiczne uwzględniające kierunki aktywności kluczowe dla efektu ochronnego na układ sercowo-naczyniowy, takie jak m.in. aktywność przeciwpłytkowa, przeciwzakrzepowa i hipotensyjna. Niewątpliwie uzyskane w ramach niniejszej rozprawy wyniki dają podstawy naukowe do rozszerzonych badań nad substancją roślinną stosowaną jak dotąd jedynie w medycynie tradycyjnej, co w dalszej perspektywie może zaowocować wprowadzeniem jej do oficjalnego lecznictwa.

Perspektywy i plany na przyszłość

Tradycyjne zastosowanie kwiatów śliwy tarniny w ChUK oraz obiecujące rezultaty realizowanych w ramach niniejszej rozprawy doktorskiej badań (wysoki potencjał antyoksydacyjny ekstraktów w komplementarnych chemicznych i biologicznych testach *in vitro*) stały się punktem wyjścia dla projektu „Standaryzacja i aktywność biologiczna kwiatów śliwy tarniny (*Prunus spinosa* L.) w kontekście chorób układu krążenia", który w 2018 roku uzyskał finansowanie Narodowego Centrum Nauki (grant NCN Preludium 2017/27/N/NZ7/02074, kierownik grantu mgr farm. Anna Marchelak, opiekun prof. UM Monika Anna Olszewska, we współpracy z prof. UŁ Joanną Kołodziejczyk-Czepas, realizacja w latach 2018-2021, zgoda Komisji Bioetycznej przy UM w Łodzi RNN/213/18/KE). Biorąc pod uwagę, że główne kierunki aktywności biologicznej substancji naturalnych, kluczowe dla efektu ochronnego na układ krążenia, obejmują m.in. działanie przeciwutleniające, hamowanie aktywacji płytek krwi oraz działanie antykoagulacyjne, celem projektu stała się charakterystyka kwiatów *P. spinosa* jako źródła substancji aktywnych w obrębie układu krążenia, w tym w szczególności pogłębiona ocena aktywności antyoksydacyjnej ekstraktów oraz badanie wpływu na układ hemostazy.

Do chwili obecnej w ramach realizacji ww. projektu udało się m.in. wykazać ochronny wpływ ekstraktów, wybranych modelowych polifenoli oraz związków uznawanych za ich główne metabolity w ustroju na fibrynogen (białko uczestniczące w procesie agregacji płytek krwi i tworzące nierozpuszczalną sieć włóknika, stanowiącą zasadniczy zrąb czopu hemostatycznego) w warunkach stresu oksydacyjnego wywołanego $ONOO^-$. Badania prowadzono metodą elektroforezy typu SDS-PAGE i techniką Western blot (ocena zmian strukturalnych fibrynogenu/tworzenia agregatów białkowych) oraz ilościowo immunoenzymatyczną metodą ELISA (oznaczenie 3-NT). Obserwowane efekty ochronne mogą mieć istotne znaczenie ze względu na fakt, że oksydacyjne uszkodzenia struktury fibrynogenu skutkują zmianami w jego fizjologicznej aktywności, przyczyniając się do wzrostu potencjału prokoagulacyjnego krwi i powstawania zakrzepów. Ponadto dokonano analizy wpływu ekstraktów z kwiatów *P. spinosa* na procesy hemostazy osoczowej, w tym na aktywność trombiny, będącej osoczowym czynnikiem krzepnięcia, umożliwiającym powstanie skrzepu fibryny. Trombina dzięki centralnej pozycji w kaskadzie krzepnięcia wydaje się być obiecującym celem dla nowoczesnej terapii przeciwzakrzepowej, dlatego też trwają poszukiwania nowych substancji naturalnych, które mogą hamować jej aktywność. Rezultaty przeprowadzonych badań wskazują, że ekstrakty z kwiatów śliwy tarniny prezentują wysoki potencjał w tym kierunku. Obserwowano hamowanie zarówno aktywności amidolitycznej trombiny (badania z użyciem niskocząsteczkowego substratu chromogennego), jak i aktywności proteolitycznej trombiny (polimeryzacja fibrynogenu – badania na wyizolowanym białku oraz na osoczu krwi), co wskazuje na ich istotny potencjał antykoagulacyjny i zachęca do dalszych badań mających na celu wskazanie składników warunkujących ten kierunek aktywności. Takie badania z zastosowaniem m.in. metod bioinformatycznych planowane są w najbliższej przyszłości. W ramach oceny wpływu ekstraktów na hemostazę osoczową, przeprowadzono również komplementarne analizy wpływu ekstraktów na czasy krzepnięcia. Wykazano, że ekstrakty w badanym zakresie stężeń nie wpływały na czas protrombinowy (PT) i czas częściowej tromboplastyny po aktywacji (APTT), obserwowano natomiast nieznaczne, aczkolwiek istotne statystycznie, wydłużenie czasu trombinowego (TT).

Ponadto przeprowadzono rozszerzone analizy cytotoksyczności ekstraktów z wykorzystaniem komórek PBMCs i erytrocytów, potwierdzające wyniki niniejszej rozprawy sugerujące ich bezpieczeństwo stosowania. Kolejny etap realizacji projektu obejmie ocenę wpływu ekstraktów z kwiatów śliwy tarniny na hemostazę płytkową. Planuje się pomiar agregacji płytek krwi w osoczu bogatopłytkowym z zastosowaniem dwóch agonistów (ADP i kolagenu), co umożliwi analizę różnych szlaków aktywacji płytek krwi.

Rezultaty przeprowadzonych dotychczas analiz wydają się potwierdzać potencjał kwiatów śliwy tarniny w kontekście ChUK, dlatego po zakończeniu realizacji grantu planuję kontynuować badania nad tą substancją roślinną i pochodnymi ekstraktami. Kolejne analizy obejmą m.in. ocenę aktywności hipotensyjnej ekstraktów w teście *in vitro* inhibicji konwertazy angiotensynowej. Ponadto celowe wydają się również rozszerzone badania *in vitro* aktywności przeciwzapalnej, w tym bezkomórkowe testy bezpośredniego hamowania innych enzymów prozapalnych (cyklooksygenazy-2, fosfolipazy A2) oraz badania na modelach komórkowych, obejmujące testy uwalniania mediatorów stanu zapalnego (cytokin i enzymów) przez komórki układu odpornościowego (neutrofile, monocyty i limfocyty). Istotna dla pełnej charakterystyki surowca w kontekście układu krążenia wydaje się również ocena potencjalnego wpływu ekstraktów na wybrane markery dysfunkcji śródbłonka naczyniowego, dlatego w dalszej perspektywie planuję również badania w tym kierunku.

Streszczenie

Istotnym celem dla współczesnej medycyny jest opracowanie bardziej efektywnych strategii prewencji i leczenia chorób układu krążenia (ChUK), stanowiących jeden z najpoważniejszych problemów zdrowotnych współczesnych społeczeństw. Rezultaty badań prowadzonych w ostatnich dwóch dekadach wskazują, iż kluczową rolę w etiologii wielu ChUK odgrywają współistniejące procesy stresu oksydacyjnego, czyli zaburzonej równowagi pomiędzy generacją reaktywnych form tlenu i azotu (ROS/RNS) a możliwościami obrony antyoksydacyjnej organizmu, oraz stanu zapalnego. Doniesienia te spowodowały wzrost zainteresowania tematyką antyoksydantów egzogennych, w tym szczególnie polifenoli roślinnych jako związków posiadających wielokierunkowe działanie na organizm ludzki, wynikające głównie z regulującego wpływu na procesy oksydacyjno-redukcyjne i zapalne zachodzące w żywych komórkach. O aktywności związków polifenolowych w obrębie układu krążenia świadczą wyniki badań klinicznych i epidemiologicznych, wskazujące na ścisły związek między dietą bogatą w polifenole a obniżonym ryzykiem zapadalności na ChUK, oraz fakt, że surowce polifenolowe są od wieków wykorzystywane z dobrymi rezultatami w medycynie tradycyjnej w schorzeniach sercowo-naczyniowych. Poszukiwania nowych źródeł polifenoli oraz analizy fitochemiczne, farmakologiczne i toksykologiczne tradycyjnie stosowanych surowców polifenolowych wydają się zatem w pełni uzasadnione, gdyż w dalszej perspektywie mogą doprowadzić do wprowadzenia na rynek nowych preparatów leczniczych do stosowania w profilaktyce i w terapii ChUK.

Pragnąc wpisać się w ten kontekst badawczy, w niniejszej rozprawie zwrócono uwagę na kwiat śliwy tarniny (*Pruni spinosae flos*) ceniony surowiec polifenolowy tradycyjnej medycyny środkowo- i wschodnioeuropejskiej, którego wskazania do stosowania obejmują m.in. schorzenia układu sercowo-naczyniowego. Kwiaty tarniny wchodzą w skład wielu tradycyjnych mieszanek ziołowych, rekomendowanych m.in. w dolegliwościach sercowych, takich jak zapalenie mięśnia sercowego, nerwica serca czy miażdżyca. Przeprowadzone na przełomie lat 60-tych i 70-tych XX wieku analizy *ex vivo* i *in vivo* wykazały, że flawonoidowe frakcje otrzymane z kwiatów i liści *P. spinosa* znacząco zmniejszają przepuszczalność naczyń krwionośnych oraz wykazują działanie przeciwzapalne w narządach wewnętrznych zwierząt doświadczalnych, normalizują poziom cholesterolu i stosunek cholesterol/fosfolipidy u aterogennych królików, oraz zwiększają amplitudę skurczu w perfuzji izolowanych serc żabich. Surowiec wydaje się zatem prezentować obiecujący potencjał terapeutyczny, szczególnie w kontekście schorzeń układu krążenia, jednakże ze względu na brak podstaw naukowych potwierdzających jego wartość biologiczną wykorzystywany jest obecnie w niewielkim stopniu. Do szerszego wykorzystania w lecznictwie konieczne są dalsze badania fitochemiczne, aktywności biologicznej, badania toksykologiczne, czy wreszcie opracowanie metodyki kontroli jakości substancji roślinnej. Biorąc pod uwagę powyższe przesłanki zdecydowano, że niniejsza dysertacja stanowić będzie część projektu badawczego zmierzającego do kompleksowej oceny kwiatów *P. spinosa* i pochodnych ekstraktów suchych jako substancji leczniczych o potencjalnej aktywności w obrębie układu krążenia, a jej celem będzie pełna charakterystyka fitochemiczna substancji roślinnej z opracowaniem metodyki kontroli jakości w zakresie polifenolowych składników aktywnych oraz analiza aktywności przeciwutleniającej i przeciwzapalnej.

Badaniom poddano suche ekstrakty/frakcje z kwiatów śliwy tarniny, tj. formę preparatów rekomendowaną we współczesnej fitoterapii, m.in. ze względu na wyższą efektywność od nieprzetworzonej substancji roślinnej. Analizy fitochemiczne objęły opracowanie adekwatnej metodyki kontroli jakości materiału roślinnego i pochodnych ekstraktów/frakcji, analizę jakościową i ilościową profilu polifenolowego ekstraktów/frakcji ze wskazaniem związków modelowych, oraz ocenę zmienności zawartości indywidualnych związków polifenolowych w komercyjnym materiale roślinnym. Analiza profilu jakościowego ekstraktów przeprowadzona metodą UHPLC-PDA-ESI-MS^3 wykazała istnienie złożonej matrycy polifenolowej w badanym materiale – całkowicie lub częściowo zidentyfikowano ponad 50 związków polifenolowych (36 nowych dla kwiatów), z czego 37 sklasyfikowano jako flawonoidy (głównie mono- i diglikozydy kemferolu i kwercetyny), pozostałe jako pochodne flawan-3-olu (katechiny i procyjanidyny typu A), pseudodepsydy kwasu kawowego i proste kwasy fenolowe. Analiza całkowitej zawartości polifenoli (TPC) potwierdziła, że ekstrakty/frakcje z kwiatów *P. spinosa* stanowią bogate ich źródło – dla MED wartość TPC wyniosła 206.1 mg GAE/g s.m., natomiast w przypadku najbogatszych w polifenole frakcji octanu etylu (EAF) i eteru dietylowego (DEF) wartości TPC wyniosły odpowiednio 584.1 i 464.6 mg GAE/g s.m. Dalsze analizy profilu ilościowego ekstraktów objęły oznaczenia spektrofotometryczne i chromatograficzne zawartości głównych grup związków aktywnych (flawonoidów, kwasów fenolowych, procyjanidyn). Wykazano, że dominującą grupę związków stanowią flawonoidy (najwyższą ich zawartością charakteryzował się DEF – 459.6 mg/g s.m. w przeliczeniu na glikozydy). W kolejnym etapie dokonano optymalizacji i walidacji metody HPLC-PDA-fingerprint analizy związków polifenolowych, która pozwoliła na dokonanie efektywnego rozdziału matrycy polifenolowej kwiatów śliwy tarniny oraz na równoległe oznaczenie zawartości kluczowych analitów z wykorzystaniem 30 wzorców kalibracyjnych, typowych dla analizowanego gatunku. Wprowadzono ponadto współczynniki korekcyjne, umożliwiające oznaczenie ww. analitów z wykorzystaniem palety substancji wzorcowych zredukowanej jedynie do pięciu związków dostępnych komercyjnie. Opracowana metoda została z powodzeniem użyta do oceny zmienności zawartości indywidualnych polifenoli w komercyjnym materiale roślinnym oraz analizy zawartości indywidualnych polifenoli w ekstraktach/frakcjach, co potwierdziło jej aplikacyjność w kontroli jakości zarówno surowca, jak i ekstraktów pochodnych. Jako wzorce kalibracyjne w wyżej opisanej metodzie LC-MS oraz HPLC-fingerprint zastosowano m.in. diglikozydy flawonoidowe wyizolowane z liści *P. spinosa* metodą preparatywnej wysokosprawnej chromatografii cieczowej po jej uprzedniej optymalizacji metodą płaszczyzny odpowiedzi (RSM).

Równolegle ekstrakty/frakcje z kwiatów śliwy tarniny zostały poddane analizom aktywności antyoksydacyjnej i przeciwzapalnej na komplementarnych modelach *in vitro*. Wykazano wysoki, korelujący z zawartością polifenoli potencjał antyoksydacyjny ekstraktów/frakcji w prostych testach chemicznych (DPPH, FRAP,TBARS), co pozwoliło wskazać potencjalne mechanizmy aktywności analitów („zmiatanie" wolnych rodników, redukowanie jonów metali, inhibicja oksydacji lipidów), dając podstawy do rozszerzenia badań o testy na modelu biologicznym ludzkiego osocza. Ekstrakty/frakcje już w niskich stężeniach odnoszących się do osoczowych poziomów osiągalnych dla polifenoli *in vivo* po podaniu doustnym (1-5 µg/ml) wykazywały znaczące efekty ochronne w stosunku do białkowych i lipidowych składników ludzkiego osocza w warunkach stresu oksydacyjnego indukowanego $ONOO^-$, jednym z głównych czynników utleniających i nitrujących *in vivo*, generowanym m.in. w układzie krążenia w ostrych i przewle-

kłych stanach zapalnych oraz w przypadkach niedokrwienia i reperfuzji. Obserwowano obniżony poziom markerów nitrowania białek, w tym 3-NT, ważnego markera zmian zachodzących w organizmie pod wpływem stresu oksydacyjnego, uznawanego za predyktor wystąpienia incydentów sercowo-naczyniowych; peroksydacji lipidów (FOX-1 oraz TBARS), oraz wzrost całkowitej nieenzymatycznej pojemności antyoksydacyjnej osocza (NEAC). Biorąc pod uwagę, że potencjalne źródła stresu oksydacyjnego w układzie krążenia *in vivo* obejmują oprócz $ONOO^-$ także jego bezpośrednich prekursorów, tj. $O_2^{\bullet-}$ i $NO^\bullet$, oraz $HO^\bullet$, H_2O_2 i HClO, wydzielane przez komórki układu immunologicznego (neutrofile, monocyty) w procesie zapalnym, w kolejnym etapie rozprawy doktorskiej oceniono aktywność ekstraktów/frakcji śliwy tarniny wobec szeregu ROS/RNS ($O_2^{\bullet-}$, $HO^\bullet$, H_2O_2, $NO^\bullet$, $ONOO^-$, HClO). W celu wskazania determinantów testowanej aktywności oraz przybliżenia możliwych efektów *in vivo*, analizom poddano również wybrane związki polifenolowe (uznane za składniki modelowe kwiatów *P. spinosa*) oraz ich główne metabolity *in vivo*. Wykazano wysoki, zależny od dawki i korelujący z zawartością polifenoli, potencjał antyoksydacyjny ekstraktów/frakcji względem wszystkich badanych oksydantów, przy czym najwyższą efektywność obserwowano w stosunku do $O_2^{\bullet-}$, $HO^\bullet$, $ONOO^-$ i HClO. Zdolność do neutralizacji ROS/RNS w przypadku najbardziej aktywnych frakcji DEF i EAF była porównywalna lub wyższa niż antyoksydantów wzorcowych, w tym kwasu askorbinowego. Wśród ośmiu modelowych polifenoli kwiatów śliwy tarniny najbardziej aktywne były kwercetyna, awikularyna, procyjanidyna A2 i kwas chlorogenowy, co może sugerować znaczący udział ww. czterech związków w aktywności ekstraktów i uzasadniać ich potencjalne zastosowanie jako markerów aktywności w badaniach standaryzacyjnych. Spośród pięciu analizowanych metabolitów polifenolowych kwas dihydrokawowy i kwas 2-(3′,4′-dihydroksyfenylo)octowy charakteryzowały się najwyższą aktywnością względem badanych oksydantów, w niektórych testach działając nawet silniej niż związki macierzyste, co potwierdza, że powinny być brane pod uwagę podczas oceny polifenolowych substancji roślinnych w zakresie aktywności biologicznej. Analizy aktywności przeciwzapalnej wykazały, że ekstrakty/frakcje mogą hamować działanie enzymów prozapalnych: lipooksygenazy i hialuronidazy, a aktywność najbardziej aktywnych frakcji DEF i EAF plasuje się pomiędzy aktywnością indometacyny (niesteroidowy lek przeciwzapalny), a rutyny (flawonoid o potwierdzonym działaniu wazoprotekcyjnym). Porównanie aktywności ekstraktów z aktywnością modelowych polifenoli sugeruje, że wszystkie główne frakcje związków czynnych kwiatów tarniny (flawonole, kwasy chlorogenowe i procyjanidyny) mogą mieć istotny, synergiczny udział w tej aktywności. Ponadto wstępna ocena bezpieczeństwa komórkowego w testach z użyciem PBMCs wykazała, że ekstrakty/frakcje w stężeniu fizjologicznym nie wpływają na przeżywalność komórek PBMCs, co pozwoliło je wstępnie zaklasyfikować jako bezpieczne.

Rezultaty badań przeprowadzonych w ramach prezentowanej rozprawy doktorskiej pokazują znaczący potencjał kwiatów *P. spinosa*, będących bogatym źródłem naturalnych polifenoli o aktywności antyoksydacyjnej i przeciwzapalnej, w profilaktyce i terapii wspomagającej chorób o podłożu oksydacyjno-zapalnym, w tym ChUK, częściowo potwierdzając tradycyjne wskazania. Stosowane jak dotąd jedynie w medycynie ludowej, kwiaty śliwy tarniny wydają się być zatem obiecującym materiałem do dalszych badań, w tym głównie farmakologicznych uwzględniających kierunki aktywności kluczowe dla efektu ochronnego na układ krążenia, umożliwiających ich pełną charakterystykę, co w dalszej perspektywie może zaowocować ich wprowadzeniem do oficjalnego lecznictwa.

Summary

The major goal of contemporary medicine is the development of more effective strategies for prevention and therapy of cardiovascular diseases (CVDs), one of the primary health problems in modern societies. The studies carried out in the last two decades have revealed that pathogenesis of CVDs is related to two closely intertwined processes of oxidative stress and inflammation. As a result, a large amount of research has focused on the exogenous antioxidants, in particular plant polyphenols, which may exert multidirectional effects on human body primarily by the regulation of redox homeostasis and inflammation processes in living cells. The activity of the polyphenolic compounds within the circulatory system has been confirmed by successful centuries-old traditional use of polyphenol-rich plant materials in the treatment of CVDs, as well as by the results of the clinical and epidemiological studies, which indicated the direct relationship between the polyphenol-rich diet and decreased incidence of CVDs. Thus, the search for novel source of polyphenols as well as phytochemical, pharmacological and toxicological investigations of traditional polyphenolic plant materials seem justified, as in further perspective they may lead to development of new medicinal plant preparations relevant for prophylaxis and treatment of CVDs.

In an attempt to follow this scientific trend, the presented dissertation focuses on blackthorn flowers (*Pruni spinosae flos*), a valued traditional polyphenolic plant remedy from Central and Eastern Europe, recommended among others in various circulatory disorders. Ethno-pharmacological sources document the use of the flowers as ingredients of compound herbal prescriptions applied, e.g. to treat numerous cardiac complaints, such as myocarditis, cardiac neurosis and atherosclerosis. The biological studies conducted in the 60s and 70s of the 20th century suggested that the flavonoid fraction of blackthorn flowers significantly reduces capillary permeability and shows anti-inflammatory effects in internal organs of experimental animals, normalizes the blood cholesterol and cholesterol/phospholipid ratio in atherogenic rabbits, and increases the amplitude of heart contractions in perfusion of isolated frog hearts. Thus, *P. spinosa* flowers seem to present promising therapeutic potential, in particular within the circulatory system; that however, due to the scarcity of data confirming their biological value, cannot be fully utilized. For wider recommendation of the plant material, further phytochemical, pharmacological and toxicological studies are required, as is the development of standardization methodology. Taking into account the above mentioned premises, the presented dissertation became the initial part of a wider project aiming at comprehensive assessment of *P. spinosa* flowers as a source of natural, bioactive polyphenols with potential influence on the circulatory system. The particular objectives of the dissertation were the detailed characterization of the phytochemical profile, development of quality control procedure in terms of polyphenolic compounds, as well as the preliminary evaluation of the antioxidant and anti-inflammatory activity.

The studies were carried out using dry extracts/fractions from blackthorn flowers, which are preparations recommended by modern phytotherapy due to their higher effectiveness in comparison to the unprocessed plant materials. The phytochemical part of the studies included comprehensive profiling of the extracts/fractions composition (qualitative and quantitative analyses), development of an adequate quality control methodology for the flower and extracts prepared thereof, and evaluation of the variation in the individual polyphenols contents in commercial plant materials. The qualitative UHPLC-PDA-ESI-MS^3 analysis showed huge complexity

of the phenolic matrix and led to full or partial identification of over 50 constituents (36 new for the flowers); among them, 37 were classified as flavonoids (mostly kaempferol and quercetin mono- and diglycosides), while the other as flavan-3-ols derivatives (catechins and A-type procyanidins), caffeic acid pseudodepsides and simple phenolic acids. The results of the quantitative studies showed that extracts are rich sources of phenolic compounds as the total phenolic content (TPC) in the methanol-water extract amounted to 206.1 mg GAE/g dw, whereas in the polyphenol-richest ethyl acetate (EAF) and diethyl ether fractions (DEF) – to 584.1 and 464.6 mg GAE/ g dw, respectively. Moreover, the spectroscopic and chromatographic quantification of the main groups of polyphenols (flavonoids, procyanidins, phenolic acids) revealed that flavonoids are the dominant phenolic components and the highest total flavonoid contents was observed for DEF (459.6 mg/g dw, counted as glycosides). In the next step, the HPLC-PDA-fingerprint method for determination of individual blackthorn flowers constituents was optimized and validated. The developed method allowed for efficient separation of the matrix peaks and simultaneous quantification of crucial analytes with the use of 30 calibration standards typical of the analyzed species. Moreover, the relative response factors (RRFs) were established, that would enable an absolute quantification of the abovementioned compounds using only 5 commercially available reference standards. The real sample analyses demonstrated the applicability of the developed procedure for quality control, as it was successfully employed for quantification of the phenolics both in commercial samples of *P. spinosa* flowers, as well as in extracts prepared thereof. Among the standards used in the LC-MS and HPLC-fingerprint studies were flavonoid diglycosides isolated from the leaves of *P. spinosa* by preparative HPLC-PDA method optimized by application of the response surface methodology (RSM).

The bioactivity studies, conducted alongside the phytochemical investigations, included an *in vitro* assessment of the antioxidant and anti-inflammatory activity in complementary chemical and biological models. The simple chemical *in vitro* tests (DPPH, FRAP, TBARS) revealed significant and phenolic-dependent antioxidant effects of the blackthorn flowers extracts/fractions and indicated that scavenging of free radicals, reduction of metal ions and inhibition of lipid peroxidation might be among potential mechanisms of the antioxidant activity of the analytes. These analyses encouraged further studies in the biological model of human plasma, in which it was demonstrated that the extracts/fractions at *in vivo*-relevant levels available for polyphenols after oral administration (1–5 µg/mL) effectively protected components of human plasma against damage induced by $ONOO^-$, one of the most powerful secondary oxidative/nitrative species, generated among others in the circulatory system during acute and chronic inflammation, as well as in ischemia and reperfusion. Not only did the analyzed extracts/fractions effectively reduce the levels of oxidative stress biomarkers, including 3-NT (important marker of protein nitration, considered as predictor of cardiovascular incidents), FOX-1 and TBARS (lipid peroxidation markers), but they also enhanced the total antioxidant status (NEAC) of the $ONOO^-$-treated plasma. Taking into account that the potential sources of oxidative stress in the circulatory system *in vivo* include, besides $ONOO^-$, its direct precursors, i.e. $O_2^{\bullet-}$ and $NO^\bullet$, as well as $HO^\bullet$, H_2O_2 and HClO generated by the immune system cells (neutrophils and monocytes) during the inflammation, the next step involved the assessment of the antioxidant activity of the extracts/fractions towards multiple ROS/RNS ($O_2^{\bullet-}$, $HO^\bullet$, H_2O_2, $NO^\bullet$, $ONOO^-$, HClO). To identify the compounds that are crucial for the activity of the extracts and for more thorough evaluation of

expected *in vivo* effects, the analyses included also selected polyphenolic compounds (considered as model constituents of the blackthorn flowers) and their main metabolites *in vivo*. All extracts exhibited significant, concentration- and phenolic-dependent scavenging potential towards the tested oxidants, and the highest responses were observed for $O_2^{\bullet-}$, $HO^{\bullet}$, $ONOO^{-}$, and HClO. Importantly, the scavenging capacity of the most active fractions DEF and EAF was comparable or superior to the activity of positive standards, including ascorbic acid. The most active model polyphenols were quercetin, avicularin, procyanidin A2 and chlorogenic acid, which suggested decisive impact of these compounds on the activity of the blackthorn flowers and indicated them as valuable activity markers for standardization study. Among the metabolites, dihydrocaffeic acid and 2-(3′,4′-dihydroxyphenyl)acetic acid were particularly active towards the investigated oxidants, in some tests exhibiting even stronger activity than their parent compounds. These results confirmed that phenolic metabolites deserve more attention during the assessment of the bioactivity of plant derived products. The anti-inflammatory activity study proved that the extracts/fractions might inhibit the activity of pro-inflammatory enzymes: lipoxygenase and hyaluronidase. The efficacies of the most active fractions DEF and EAF were between those of rutin (a flavonoid with proved vasoprotective activity) and indomethacin (a nonsteroidal anti-inflammatory drug). The juxtaposition of the activity parameters for extracts and model compounds suggested that all of the main groups of blackthorn phenolics (flavonols, chlorogenic acids, and procyanidins) might be responsible for this effect, with possible synergistic action. Furthermore, the preliminary assessment of the cellular safety with the use of human peripheral blood mononuclear cells (PBMCs) revealed that the blackthorn flowers extracts/fractions at the physiological concentration did not affect the viability of the cells, and might be regarded as safe.

The results of the studies described in the present dissertation demonstrated that *P. spinosa* flowers are a rich source of natural polyphenols with antioxidant and anti-inflammatory activity, and possess a significant potential in the prevention or adjunctive therapy of oxidative stress/inflammation-related diseases, including CVDs, partly confirming their traditional application. The blackthorn flowers, used so far only in the traditional medicine, seem therefore to be a promising material for wider investigations, which should include pharmacological studies focused on the protective effects within the circulatory system. A more thorough assessment of the plant material in this context may lead in further perspective to their introduction to the official medicine.

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Publikacja I

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Optimisation of preparative HPLC separation of four isomeric kaempferol diglycosides from *Prunus spinosa* L. by application of the response surface methodology

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ABSTRACT

A fast and efficient preparative HPLC-PDA method was developed for the separation and isolation of four rare isomeric kaempferol diglycosides from leaves of *Prunus spinosa* L. The separation procedure of the enriched diglycoside fraction of the 70% (*v/v*) aqueous methanolic leaf extract was first optimised on analytical XBridge C18 column (100 mm × 4.6 mm i.d., 5 μm) and central composite design combined with response surface methodology was utilized to establish the optimal separation conditions. The developed method was directly transferred to preparative XBridge Prep C18 column (100 mm × 19 mm i.d., 5 μm) and the final separation was accomplished by isocratic elution with 0.5% acetic acid-methanol-tetrahydrofuran (75.2:16.6:8.2, *v/v/v*) as the mobile phase, at a flow rate of 13.6 mL/min, in less than 12 min for a single run. Under these conditions, four flavonoid diglycosides: kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside, kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (kaempferitrin), and reported for the first time for *P. spinosa* kaempferol 3-*O*-β-D-xylopyranoside-7-*O*-α-L-rhamnopyranoside (lepidoside) and kaempferol 3-*O*-α-L-arabinopyranoside-7-*O*-α-L-rhamnopyranoside, were isolated in high separation yield (84.8–94.5%) and purity (92.45–99.79%). Their structures were confirmed by extensive 1D and 2D NMR studies. Additionally, the UHPLC-PDA-ESI–MS^3 qualitative profiling led to the identification of twenty-one phenolic compounds and confirmed that the isolates were the major components of the leaf material.

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1. Introduction

Prunus spinosa L. (blackthorn or sloe) is a large thorny shrub or small tree widespread in the temperate regions of the northern hemisphere. It has been known since ancient times as medicinal and dietary plant. Its leaves has been used traditionally for various purposes, *e.g.* for the treatment of urinary tract inflammations and in blood cleansing therapies (Berger, 1950). The plant material is a rich source of flavonoid glycosides (up to 4.27% dry weight), comparable to other widely applied flavonoid herbal remedies such as birch leaves (*Betulae folium*) and elder flower (*Sambuci flos*) (Olszewska et al., 2001). Nine different flavonoids have been isolated to date from the leaves of *P. spinosa* including kaempferol, quercetin, their 3-*O*-α-L-arabinofuranosides, kaempferol 3- and 7-*O*-α-L-rhamnopyranosides, kaempferol 3,7-di-*O*-α-L-rhamnopyranoside, kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside, quercetin 3-*O*-(2″-*O*-β-D-glucopyranoside)-α-L-arabinofuranoside and kaempferol 3-*O*-(2″-*E*-*p*-coumaroyl)-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside (Olszewska and Wolbiś, 2002a). Some of these compounds, especially mono- and dipentosides, which are characteristic of *P. spinosa*, and relatively rare in nature, could probably be applied as analytical markers in standardisation of plant materials derived from the taxon (Olszewska and Wolbiś, 2001, 2002a,b). However, the typical flavonoid pattern of the leaves still requires full characterisation, as no comprehensive studies on their phenolic profile has been published so far.

The flavonoid fraction of blackthorn leaves was proven in *in vivo* studies to significantly reduce capillary permeability, exhibit anti-inflammatory effects in the animal skin and internal organs, normalize the blood cholesterol and a cholesterol/phospholipid ratio in atherogenic rabbits, display spasmolytic effects on isolated

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uterine and intestinal segments from different animals, increase the amplitude of heart contractions in perfusion of isolated frog hearts, and demonstrate significant diuretic and natriuretic activity in rats (Lisevitskaya et al., 1970; Makarov, 1972, 1970; Makarov and Khadzhai, 1969). These effects were especially pronounced for kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside and kaempferol 3,7-di-*O*-α-L-rhamnopyranoside – activity of which was higher than that of rutin – suggesting that the 3,7-disubstituted kaempferol derivatives might be primary determinants of the activity of *P. spinosa* leaves (Makarov and Khadzhai, 1969). However, despite these promising results, published in the early 70's of the 20th century, further *in vivo* studies on the target flavonoids were hindered by their commercial unavailability and lack of effective methods for isolation from natural sources.

According to our previous studies on *P. spinosa*, the blackthorn leaves appear rich in kaempferol dipentosides, the fraction of which is, on one hand, easy to isolate from the plant due to unique solubility, but extremely difficult to separate into components (due to their considerable structural similarities) on the other. The target fraction was composed of at least four analytes, two of which remain structurally unidentified to date. Moreover, two major constituents of the fraction (kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside and kaempferol 3,7-di-*O*-α-L-rhamnopyranoside) were isolated from the leaves but with unsatisfactory yield resulting from low resolution of the applied conventional open column chromatography techniques (Olszewska and Wolbiś, 2002a).

Therefore, the aim of the present study was to develop and optimise the first preparative HPLC procedure for fast and efficient isolation of four isomeric kaempferol dipentosides from the leaves of blackthorn. Since the optimisation of HPLC separations by a trial-and-error method can be inefficient and time-consuming, and does not guarantee the optimal results, it was facilitated by the application of the response surface methodology (RSM) – a systematic experimental design and statistically-assisted construction of a response function describing relationships between the process variables and performance parameters of the chromatographic separation. The optimal conditions, based on isocratic elution with ternary mobile phase, established in an analytical scale, were directly transferred to a preparative one, and successful isolation was then achieved with a run time of 12 min. The purity of isolates and their structures were thoroughly analysed by the spectroscopic (ESI–MS, ^{1}H NMR, ^{13}C NMR, COSY, HMQC, HMBC) studies and hydrolytic experiments. Finally, the qualitative composition of the raw leaf extracts and the target dipentoside fraction was fully characterised by an UHPLC-PDA-ESI–MS^{3} assay with comparison to the currently and previously isolated standards.

2. Results and discussion

2.1. Optimisation of the separation procedure

The target kaempferol diglycosidic fraction was isolated from the leaves of *P. spinosa* according to the simplified procedure described previously (Olszewska and Wolbiś, 2002a), *i.e.* from *n*-butanolic fraction of the defatted 70% (*v*/*v*) aqueous methanolic extract, separated from accompanying polymeric proanthocyanidins and quercetin diglycosides by gel permeation chromatography on Sephadex LH-20. Thus, 2.1 g of the dipentoside fraction was obtained from 100 g of the dried plant material (2.1% yield, *w*/*w*). Before optimisation of the HPLC separation, the qualitative profiles of the flavonoid fraction and mother 70% methanolic extract were evaluated by UHPLC-PDA-ESI–MS^{3} (Fig. 1, Table 1; for detailed description see Section 2.4), which confirmed purity of the fraction and predominant character of its constituents in the plant material.

Although the development of a chromatographic separation procedure can be carried out in any scale, in order to minimize the solvent use, it is often beneficial to optimise the separation on an analytical column and then transfer the established conditions to a preparative one. In such cases, the crucial factor is to use columns with identical chemistry, particle size and, preferably, of the same length. This approach ensures the similar separation power of the columns and allows for maintaining similar resolution between crucial peak pairs (Aubin and Cleary, 2009; Huber and Majors, 2007). In this study, two matching Waters XBrigde C18 columns (10 cm × 4.6 mm i.d. and 10 cm × 19 mm i.d., 5 μm particle size each) were used. Octadecyl silica is a clear choice, as a vast majority of polyphenol separations has been performed using this column chemistry (Stalikas, 2007; Stefova et al., 2003). The selection of column length of 100 mm allowed to shorten the analysis time.

Apart from the column chemistry, the most important factor influencing the effectiveness of the separation is the composition of the mobile phase. In flavonoid separations, binary mobile phases consisting of water with acetic or formic acid and methanol or

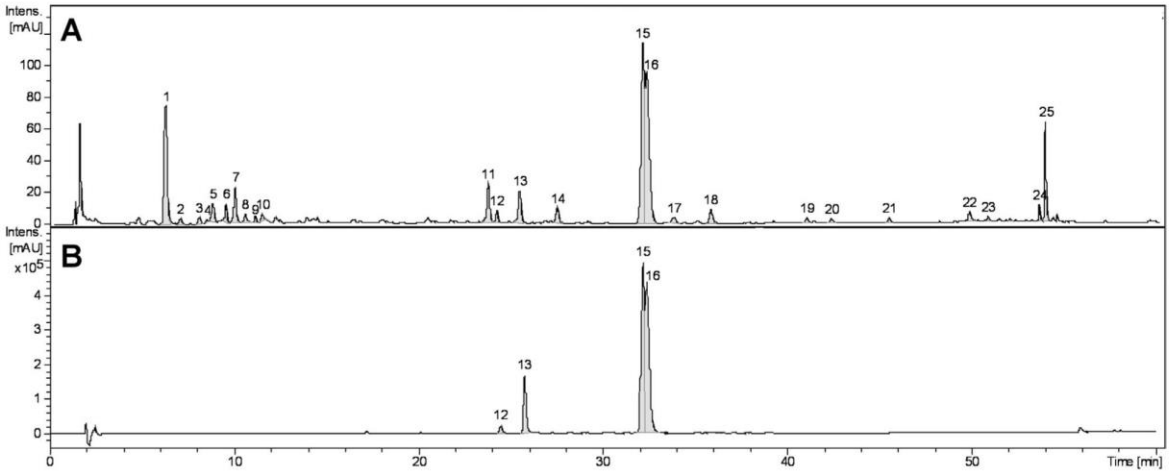

Fig. 1. Representative UHPLC chromatograms at λ = 350 nm of: (**A**) dry 70% (*v*/*v*) aqueous methanolic extract from the leaves of *P. spinosa* (5 mg/mL); (**B**) fraction of kaempferol dipentosides from the leaves of *P. spinosa* (1 mg/mL).

Table 1
UHPLC-PDA-ESI–MS^3 data of polyphenols detected in the leaves of *P. spinosa*.

Peak	Analyte	R_t (min)	UV λ_{max} (nm)	$[M-H]^-$ (*m/z*)	Fragmentary ions (*m/z*)
1	3-*O*-caffeoylquinic acid (neochlorogenic acid)[a]	6.2	325	353	191, 179
2	unknown compound	7.0	312	341	195, 163
3	unknown compound	8.0	312	341	195, 163
4	3-*O*-*p*-coumaroylquinic acid	8.5	312	337	163, 191
5	procyanidin type-B dimer	8.8	280	577	425, 407, 451, 289
6	procyanidin type-B dimer	9.5	280	577	425, 407, 451, 289
7	(+)-catechin[a]	10.0	280	289	245
8	3-*O*-feruloylquinic acid	10.5	325	367	193
9	4-*O*-caffeoylquinic acid (cryptochlorogenic acid)[a]	11.0	325	353	173, 191, 135
10	unknown compound	11.5	280	499	417
11	unknown compound	23.7	280	433	287
12	kaempferol 3-*O*-α-L-arabinopyranoside–7-*O*-α-L-rhamnopyranoside[a]	24.3	268, 356	593	447, 285
13	kaempferol 3-*O*-β-D-xylopyranoside–7-*O*-α-L-rhamnopyranoside (lepidoside)[a]	25.5	265, 354	563	431, 417, 285
14	quercetin 3-*O*-(2″-*O*-β-D-glucopyranoside)-α-L-arabinofuranoside[a]	27.4	258, 354	595	577, 505, 415, 433, 301, 179
15	kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (kaempferitrin) [a]	32.1	254, 356	577	431, 285
16	kaempferol 3-*O*-α-L-arabinofuranoside–7-*O*-α-L-rhamnopyranoside[a]	32.4	254, 356	563	431, 285
17	quercetin 3-*O*-α-L-arabinofuranoside (avicularin)[a]	33.7	255, 355	433	301
18	kaempferol hexoside-pentoside	36.0	254, 355	579	417, 285
19	kaempferol 3-*O*-α-L-arabinofuranoside (juglanin)[a]	41.0	254, 356	417	285
20	kaempferol 3-*O*-α-L-rhamnopyranoside (afzelin)[a]	42.3	256, 356	431	285
21	quercetin acetyl-hexoside-rhamnoside	45.4	255, 356	651	609, 301
22	kaempferol acetyl-hexoside-rhamnoside	49.8	255, 355	635	593, 285
23	kaempferol 7-*O*-α-L-rhamnopyranoside[a]	50.0	254, 356	431	285
24	kaempferol[a]	53.8	255, 356	285	–
25	kaempferol 3-*O*-(2″-*E*-*p*-coumaroyl)-α-L-arabinofuranoside–7-*O*-α-L-rhamnopyranoside[a]	53.9	254, 356	709	563, 285

[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.

acetonitrile as organic modifiers are most commonly applied. While in analytical experiments gradient elution is generally performed, allowing for separation of a wide range of compounds in a reasonable time, in preparative applications where separation is limited to only a couple of compounds, isocratic mode is preferable, as it eliminates the need of column equilibration and, thus, greatly reduces the costs of the analysis (Marston and Hostettmann, 2006; Stalikas, 2007; Stefova et al., 2003). In the first stage of development of the present method, a series of binary phases in the isocratic elution mode were tested, specifically, 0.5% acetic acid with methanol in concentrations 10–60% (*v/v*) and with acetonitrile in concentrations 10–50% (*v/v*). Unfortunately, none of these phases provided satisfactory separation of the analysed mixture of diglycosides, with peaks **15** and **16** being the critical peak pair.

To improve the mobile phase selectivity, a second organic modifier might be introduced. In particular, in several chromatographic studies including one of our own, it was reported that the addition of tetrahydrofuran (THF), whose selectivity is usually significantly different from that of methanol or acetonitrile, might substantially improve the separation parameters for flavonoid analytes (Fang et al., 2006; Olszewska, 2012; Wang et al., 2011; Wang and Huang, 2004). Despite good results, this approach is not often used, as the optimisation of ternary mobile phase is much more complicated. However, in the case of the investigated diglycosides fraction, the addition of THF indeed greatly enhanced the separation process by improving the peak shapes, increasing the resolutions, and shortening retention times. Therefore, the ternary mobile phase consisting of methanol, THF, and 0.5% acetic acid in the isocratic elution mode was selected to develop the current separation method.

For complete optimisation, two additional factors must be considered – temperature and flow rate. As the temperature control of HPLC preparative columns in a standard column oven is virtually impossible (due to high flow rates used), the separation temperature was not included into the present optimisation process, and thus analytical experiments were run at room temperature.

The central composite design (CCD) combined with RSM is a statistical approach generally accepted in chromatographic studies on various analytical stages from extraction to chromatographic separation (Ferreira et al., 2007), but, to the best of our knowledge, it has not so far been used for optimisation of preparative HPLC separations. CCD is a solution suitable for optimisation of two to three factors; it combines full factorial design with axial or star points and at least one point at the center of the experimental region (Lundstedt et al., 1998). The design used in the present study was three-factor CCD comprising eight factorial and six axial runs, as well as three duplicates of the central point. Three factors selected for optimisation purposes included concentration of methanol (C_{MeOH}) and concentration of tetrahydrofuran (C_{THF}) in the mobile phase, as well as the flow rate (F_R). After preliminary experiments setting the central point (C_{MeOH}: 20%; C_{THF}: 6%; F_R: 1 mL/min), the independent variables were tested on five different levels as shown in Table 2. The experiments were run in random order to minimize the impact of interfering factors (*e.g.* temperature).

The efficiency of the experimental separations was measured in terms of resolution – specifically, the resolution between two crucial peak pairs **12** and **13** (R_{s1}) and **15** and **16** (R_{s2}) as well as the separation time (T_S, *i.e.* the retention time of the last peak). As the selected responses have distinct targets, *e.g.* improving resolution usually means elongating analysis time, in order to establish optimal conditions a compromise is required. Therefore, a fourth response (D) was calculated using Derringer's desirability function, which is defined as a geometric mean of the individual desirability functions (for detailed equations see Section 4.4). The ranges for the calculations (Table 3) were set based on the preliminary experimental data and the desirable results of the separation.

The results were fitted into a second-order polynomial models (see Section 4.4) and the insignificant coefficients ($p > 0.05$) were removed using backwards elimination process. The reduced

Table 2
Design approach and experimental results.

Run Order	Factors (Levels)						Responses			
	C_{MeOH} (%)		C_{THF} (%)		F_R (mL/min)		T_S (min)	R_1	R_2	D
1	20.0	(0)	6.0	(0)	1.00	(0)	9.45	2.56	1.59	0.74
2	25.0	(+1)	4.0	(−1)	1.20	(+1)	7.55	1.97	0.89	0.47
3	25.0	(+1)	8.0	(+1)	1.20	(+1)	3.49	1.06	0.68	0.16
4	20.0	(0)	6.0	(0)	1.00	(0)	9.33	2.56	1.52	0.72
5	15.0	(−1)	4.0	(−1)	1.20	(+1)	24.77	3.76	1.49	0.00
6	20.0	(0)	9.4	(+1.68)	1.00	(0)	6.12	1.98	1.47	0.66
7	25.0	(+1)	4.0	(−1)	0.80	(−1)	11.41	2.51	1.20	0.59
8	20.0	(0)	2.6	(−1.68)	1.00	(0)	25.65	3.31	1.11	0.00
9	15.0	(−1)	8.0	(+1)	0.80	(−1)	13.22	2.98	2.26	0.76
10	20.0	(0)	6.0	(0)	1.33	(+1.68)	6.95	2.46	1.49	0.75
11	20.0	(0)	6.0	(0)	1.00	(0)	9.37	2.53	1.61	0.74
12	20.0	(0)	6.0	(0)	0.67	(−1.68)	14.37	3.11	1.88	0.70
13	25.0	(+1)	8.0	(+1)	0.80	(−1)	3.76	1.25	0.90	0.32
14	15.0	(−1)	8.0	(+1)	1.20	(+1)	8.97	2.50	1.76	0.77
15	15.0	(−1)	4.0	(−1)	0.80	(−1)	38.31	4.35	1.46	0.00
16	11.6	(−1.68)	6.0	(0)	1.00	(0)	25.03	3.76	2.29	0.00
17	28.6	(+1.68)	6.0	(0)	1.00	(0)	4.42	1.93	0.94	0.51

C_{MeOH}, methanol concentration; C_{THF}, tetrahydrofuran concentration; F_R, flow rate; T_S, separation time (retention time of the last peak); R_1, resolution between peaks **12** and **13**; R_2, resolution between peaks **15** and **16**; resolutions calculated acc. to Eq. (5); D, Derringer desirability function acc. to Eq. (2).

equations and statistical parameters obtained from ANOVA are presented in Table 4, and the corresponding response surface for the separation process is shown in Fig. 2. All models were found to be significant in the F-test with p-values <0.0001. The adjusted R^2 values (R^2 adjusted for the number of terms in the model) were in the range of 0.8492-0.9817 and indicated generally good fitting of the experimental data with the calculated equations (Lundstedt et al., 1998). The adequate precision, that is the measure of the response to deviation ratio, was in the range of 11.55–23.21, over two to four times above the desirable value of 4 (Giriraj and Sivakkumar, 2014). The relatively high (9.64–24.01%) coefficients of variation (CV) were probably caused by the lack of temperature control, still as other ANOVA parameters were acceptable, the models were regarded as suitable for final calculations.

Using the calculated models, the optimal separation conditions were established. The overall desirability function D reached its maximum for a mobile phase containing 16.6% of methanol and 8.2% of tetrahydrofuran. Interestingly, the function proved to be independent of flow rate (in the tested range of 0.67-1.23 mL/min), as all F_R-related coefficients were found statistically insignificant ($p > 0.05$, Fig. 3) and were eliminated from the model. This provided some elasticity, as by adjusting the flow rate, the preferable resolutions could be reached, while keeping the optimal mobile phase composition ensured the optimal separation time. Finally, the flow rate of 0.8 mL/min was selected, that provided at least base-line separation of both critical peak pairs, which means resolution factors (R_s) of minimum 2.0.

Subsequently, the analysis was conducted in the optimised conditions (Fig. 4A). The close consistency between the calculated and experimental responses (Table 5) demonstrates the predictive power of the model and its usefulness in describing the chromatographic behaviour of the analytes.

Table 3
Criteria for calculation of individual desirability functions d_1-d_3.

Response	L	U	Goal	Equation
T_S **(min)**	5	20	minimize	(3)
R_1	1	3	maximize	(4)
R_2	0.5	2	maximize	(4)

T_S, separation time (retention time of the last peak); R_1, resolution between peaks **12** and **13**; R_2, resolution between peaks **15** and **16**; resolutions calculated acc. to Eq. (5); L – lower limit; U – upper limit.

2.2. Application of the optimised method to the real sample

In the next step, the optimised analytical method was transferred to preparative conditions. Considering identical column chemistries and their length, to maintain the same linear velocity of the mobile phase and, thus, to achieve similar separation quality, the flow rate (analytical) must be multiplied by the factor equal to the ratio of squared columns diameters (17 in our case). Therefore, the flow rate applied for the preparative isolation was 13.6 mL/min.

Finally, the column loading requires consideration. In preparative applications, the peak shape and symmetry are of secondary importance, since the aim is usually to obtain the maximal yield. Therefore, for the purification of larger samples column overload is often applied. As the concentration of the sample is usually limited by its solubility, the combination of concentration and volume overload is used (Huber and Majors, 2007). To avoid the solvent incompatibility issues, the investigated sample was dissolved in the mobile phase, in which its solubility amounted to about 15 mg/mL as estimated in the present study. When the subsequent injections of increasing sample volume into the chromatographic system were carried out the gradual deterioration of peak shapes and resolution was noticed, especially for the two dominating peaks of **15** and **16**. Finally, the injection volume of 250 μL was selected, that provided the largest column load and still satisfactory separation.

The isolation procedure was repeated several times, resulting in isolation of 1.2 mg of **12**, 9.2 mg of **13**, 24.9 mg of **15**, and 29.5 mg of **16** from 75 mg of the sample (86% total isolation yield, w/w). The representative chromatogram of the preparative separation is presented in Fig. 4B. The final efficiency parameters of the separation process are presented in Table 6.

2.3. Structure elucidation of the isolated flavonoids

Compounds **12**, **13**, **15** and **16** (Fig. 5) were isolated as pale yellow needles soluble in methanol, aqueous methanol and water. Their UV–vis (220–450 nm) spectra showed two maxima at 264–266 nm and 345–349 nm, and were consistent with glycoside derivatives of kaempferol.

The ESI–MS spectrum of compound **12** exhibited parent $[M-H]^-$ ion at 563 m/z, corresponding to the molecular formula of $C_{26}H_{28}O_{14}$. Its fragmentation pattern with ions at 417 $[M-146-H]^-$, 431 $[M-132-H]^-$, and 285 $[M-146-132-H]^-$ m/z

Table 4
Response models and statistical parameters obtained from ANOVA.

Response[a]	Reduced response model	R^2	Adjusted R^2[b]	F-Value[c]	p-Value[d]	CV (%)[e]	Adequate precision
T_S	$9.93-6.88C_{MeOH}+1.75C_{MeOH}^2-6.27C_{THF}+2.17C_{THF}^2-2.52FR+3.64C_{MeOH}C_{THF}+1.71C_{MeOH}FR+1.61C_{THF}FR$	0.9909	0.9817	112.74	<0.0001	11.73	23.21
R_1	$2.62-0.73C_{MeOH}-0.52C_{THF}-0.21FR$	0.9395	0.9256	69.83	<0.0001	9.64	23.39
R_2	$1.55-0.41C_{MeOH}-0.13C_{THF}^2-0.12FR-0.20C_{MeOH}C_{THF}$	0.9059	0.8746	29.25	<0.0001	11.71	17.89
D	$0.72-0.17C_{MeOH}^2+0.15C_{THF}-0.15C_{THF}^2-0.27C_{MeOH}C_{THF}$	0.8869	0.8492	24.17	<0.0001	24.01	11.55

[a] T_S, separation time (retention time of the last peak); R_1, resolution between peaks **12** and **13**; R_2, resolution between peaks **15** and **16**; resolutions calculated acc. to Eq. (5); D, Derringer desirability function acc. to Eq. (2).
[b] R^2 adjusted for number of terms in the model.
[c] Test for comparing model variance with residual (error) variance.
[d] Probability of seeing F-value if the null hypothesis is true.
[e] Coefficient of variance.

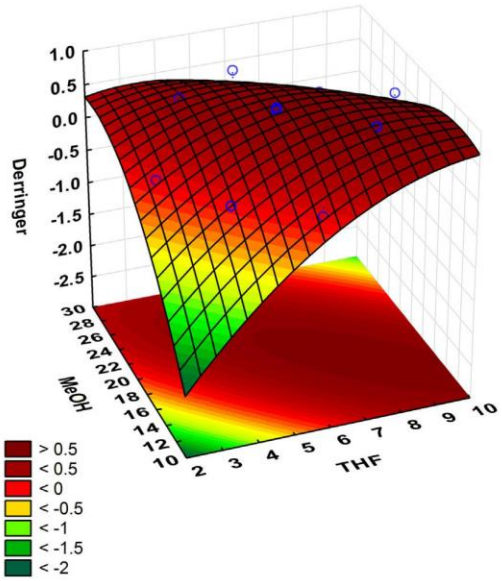

Fig. 2. Response surface of the overall Derringer's desirability function (D) estimated from the central composite design as a function of mobile phase composition (concentrations of tetrahydrofuran and methanol).

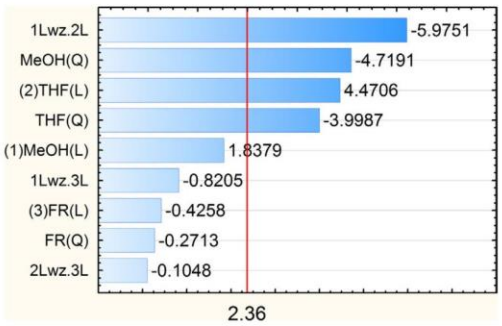

Fig. 3. Pareto chart of standardised effects of optimised variables on overall Derringer's desirability function (D). Effects that extend beyond the reference line are statistically significant ($p < 0.05$).

indicated a kaempferol rhamnoside pentoside derivative. The acid hydrolysis of compound **12** gave an aglycone, identical with kaempferol (based on HPLC-PDA analysis according to Olszewska (2008)), and two carbohydrates identified after conversion to the 1-[(*S*)-*N*-acetyl-methylbenzylamino]-1-deoxy-alditol pentaacetate derivatives (Olszewska and Roj, 2011) as L-rhamnose and D-arabinose. In ^{1}H NMR four characteristic aromatic proton resonances of kaempferol were present, as well as two doublets from the anomeric protons. The anomeric proton resonance at δ 5.23 ppm (1H, *d*) with diaxial coupling constant J_{1-2} = 6.2 Hz suggested the presence of α-L-arabinopyranoside moiety. The pyranoside form of L-arabinose was further confirmed by two distinctive signals from C-5″ protons at δ 3.78-3.84 (2H, *m*, proton signal of H-5$_a$″ partially overlapped by that of H-4″) and δ 3.43 ppm (1H, *dd*, J = 1.9 and J = 11.2 Hz, proton signal of H-5$_b$″) (Harborne, 1994; Zapesochnaya, 1979). The second anomeric signal at δ 5.60 ppm (1H, *d*, J_{1-2} = 1.3 Hz) as well as the high-field three-proton doublet (J = 6.2 Hz) from a methyl group were characteristic of α-L-rhamnopyranose (Harborne, 1994). The ^{13}C NMR spectrum exhibited thirteen kaempferol resonances as well as eleven signals belonging to the carbohydrate units. Their assignments were established based on the 2D spectra (COSY, HMBC, HMQC). The glycosylation positions were established based on long-range correlations of the anomeric protons visible in the HMBC spectrum. Eventually, compound **12** was identified as kaempferol 3-*O*-α-L-arabinopyranoside-7-*O*-α-L-rhamnopyranoside. This is the first report about the presence of this rare compound in *Prunus* species. It was previously isolated from several taxa, including *Asplenium trichomanes*, *Delphinium hybridum*, *Rapistrum rugosum*, and *Hippophae rhamnoides* (Al-Taweel et al., 2012; Imperato, 1979; Yoshimitsu et al., 2007; Zhang et al., 2012), but none from Rosaceae family.

The ESI–MS spectrum of compound **13** exhibited parent [M−H]$^-$ ion at 563 *m/z* identical as for the compound **12**, and both compounds showed very similar fragmentation patterns. The acidic hydrolysis of **13** gave kaempferol and two carbohydrates identified as L-rhamnose and D-xylose. Its NMR spectra were similar to those of the compound **12** with regard to resonances of kaempferol and α-L-rhamnopyranoside moieties. The presence of β-D-xylopyranose moiety was confirmed by the anomeric proton resonance at δ 5.27 ppm (1H, *d*) with diaxial coupling constant (J_{1-2} = 7.2 Hz) (Harborne, 1994), and the correlation of that signal with the C-7 resonance in the HMBC spectrum indicated the glycosylation position. Thus, the compound **13** was identified as kaempferol 3-*O*-β-D-xylopyranoside-7-*O*-α-L-rhamnopyranoside (lepidoside). Lepidoside was isolated for the first time from *Lepidium perfoliatum* (Fursa and Litvinenko, 1970) and since then it was found in only five other species (Dhasmana and Garg, 1991; Jain and Gupta, 1986; Jaiswal et al., 2014; Li et al., 2010; Zhao et al., 2016). This is the first report on the presence of this compound in the genus *Prunus* and Rosaceae family.

The structures of compounds **15** and **16** were confirmed as kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (kaempferitrin) and 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside,

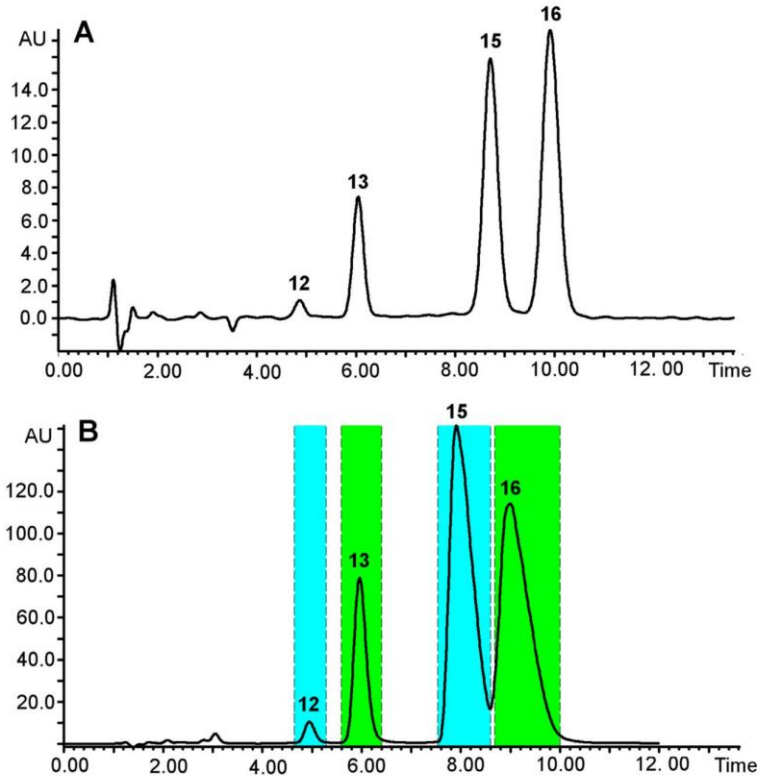

Fig. 4. Representatives HPLC chromatograms at λ = 350 nm of kaempferol diglycoside fraction from the leaves of *P. spinosa*: (**A**) separation in the optimised analytical conditions; (**B**) final preparative separation.

Table 5
Experimental and predicted responses under optimal conditions.

Response[a]	Predicted values	Experimental values	Error[b] (%)
$T_{S(min)}$	10.27	9.92	3.11
R_1	2.64	2.50	5.30
R_2	2.08	2.01	3.36
D	0.786	0.795	1.15

[a] T_S, separation time (retention time of the last peak); R_1, resolution between peaks **12** and **13**; R_2, resolution between peaks **15** and **16**; resolutions calculated acc. to Eq. (5); D, Derringer desirability function acc. to Eq. (2).
[b] Percent error between predicted and experimental values.

Table 6
Efficacy parameters of the separation procedure.

Compound	Purity (%)[a]	Recovery (%)[b]	Efficiency (mg/h)
12	92.45	93.8	0.3
13	99.79	94.5	2.3
15	97.62	85.2	6.2
16	99.44	84.8	7.3

[a] Assessed by HPLC-PDA.
[b] Based on the content estimated from peak areas (λ = 350 nm) and the yield of the total fraction.

respectively. Both compounds were previously isolated from the leaves of *P. spinosa* (Olszewska and Wolbiś, 2002a). While kaempferitrin is rather a ubiquitous plant constituent, 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside has been found in only few other species. Just five taxa have been reported to contain both compounds together – *i.e. Cinnamomum sieboldi*, *Alianthus altissima*, *Phymatopteris hastate*, *Polypodium hastatum*, and *Cissus sicyoides* (Duan et al., 2014, 2012; Nakano et al., 1983; Xu et al., 2009; Zhao et al., 2009). The latter species contains also kaempferol 3-*O*-α-L-arabinopyranoside-7-*O*-α-L-rhamnopyranoside (Xu et al., 2009). However, the presence of all four isolated flavonoids with high abundance of kaempferitrin and 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside appears a unique characteristic of *P. spinosa*. The isolates might be, therefore, considered as analytical and chemotaxonomic markers of the investigated plant material.

2.4. UHPLC-PDA-ESI–MS profile

The qualitative UHPLC-PDA-ESI–MS^3 profile of the 70% aqueous methanolic extract from the leaves of *P. spinosa* revealed the presence of twenty five phenolic compounds, twenty one of which were fully or tentatively identified by comparing their retention times, UV–vis spectra and MS profiles with those of the reference compounds and literature data (Fig. 1, Table 1).

Compounds **1** and **9** displayed absorption maxima at 325–328 nm and UV–vis spectra characteristic of caffeic acid derivatives. Based on their parent $[M-H]^-$ ions at *m/z* 353 and the further MS fragmentation, they were classified as monocaffeoylquinic acid isomers. Compound **1** exhibited the base ion at *m/z* 191 and the secondary ion at *m/z* 179 with 55% intensity, whereas compound **9** displayed distinctive base peak at *m/z* 173. Consequently, based on the elution order, hierarchical discrimination keys (Clifford et al., 2003) and comparison with commercial standards, compounds **1** and **9** were identified as 3-*O*-caffeoylquinic acid (NCHA, neochlorogenic acid) and 4-*O*-caffeoylquinic acid (CCHA, cryptochlorogenic acid). Compound **4**, with UV-maximum at 312 nm, parent $[M-H]^-$ ion at *m/z* 337, and base peak

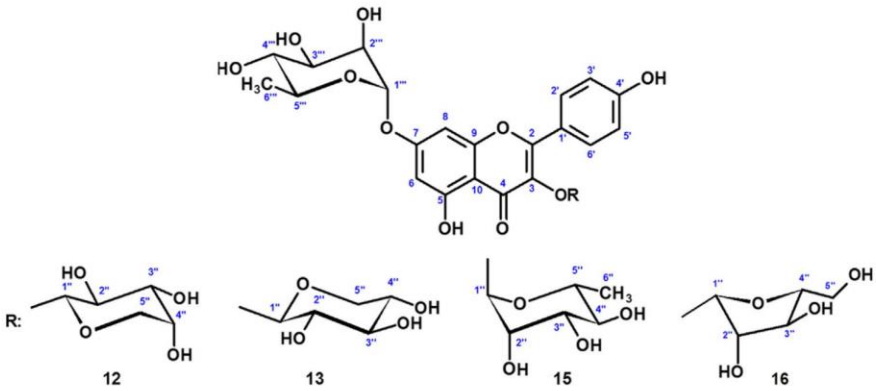

Fig. 5. Structures of the isolated kaempferol diglycosides.

in MS^2 spectrum at *m/z* 163, was identified as 3-*O*-*p*-coumaroylquinic acid while compound **8,** with parent $[M-H]^-$ ion at *m/z* 367 and MS^2 base fragment ion at n/z 193, was identified as 3-*O*-feruloylquinic acid (Clifford et al., 2003).

Compounds **5**, **6**, and **7** with UV-maxima at 280 nm were characterised as proanthocyanidins. Compound **7** exhibited parent $[M-H]^-$ ion at *m/z* 289, and by comparison with the reference substance it was identified as (+)-catechin. Compounds **5** and **6** revealed the same pseudomolecular ions at *m/z* 577 and secondary peaks at *m/z* 451, 425, 407 and 289 and, thus, were classified as procyanidin B-type dimers (Hamed et al., 2014).

Compounds **12-25** based on their UV–vis spectra exhibiting two absorption maxima, first at 260–280 and second at 345–355 nm, were classified as flavonoids. Based on their MS^2 spectra which revealed signals assignable to the aglycone moieties at *m/z* 301 or 285, they were identified as quercetin or kaempferol derivatives, respectively. The sugar moieties were identified due to neutral losses in the MS spectra of the mother glycosides (−132 for pentose, −162 for hexose and −146 for rhamnose). The structural identity of most of these analytes was confirmed by the comparison of their retention times, UV–vis spectra and MS-profiles with those of the authentic standards (Table 1) isolated in our laboratory from the leaves of *P. spinosa* during the present study and previously (Olszewska and Wolbiś, 2002a).

3. Conclusion

The present paper describes the application of the central composite design in combination with the response surface methodology for optimisation of preparative HPLC separation of four isomeric kaempferol diglycosides. The applied statistical approach, used to the best of our knowledge for the first time for optimisation of preparative HPLC separation, proved highly effective in finding the most suitable analysis conditions for closely structurally related flavonoid isomers. The crucial factor for the satisfactory resolution between the co-eluting flavonoid bands was the use of tetrahydrofuran, which, therefore, could be recommended for separation of other complicated flavonoid mixtures. The developed procedure may be applied for the isolation of rare kaempferol diglycosides for further pharmacological or analytical studies. The co-occurrence of all four glycosides in the leaves of *P. spinosa*, with the dominant kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (kaempferitrin) and 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside, was found to be specific feature of the analysed plant material, and the isolated flavonoids might be used as its chemotaxonomic and analytical markers.

4. Materials and methods

4.1. General experimental procedures

HPLC-PDA experiments (both analytical and preparative) were performed on preparative Waters 2545 binary system (Waters, Milton, MA, USA) equipped with Waters 2767 autosampler and Waters 2998 PDA detector.

UHPLC-PDA-ESI–MS^n analyses were performed on UHPLC-3000 RS system (Dionex, Dereieich, Germany) with a diode array detector with multiple wavelength (Thermo Fisher Scientific Inc., Waltham, MA, USA), and an ultrahigh resolution hybrid quadrupole/time-of-flight mass spectrometer (UHR-Q-TOF–MS, Bruker Daltonics GmbH, Bremen, Germany) using an electrospray ionization (ESI) source operating in a negative mode. Instrument control, data acquisition, and evaluation were done with the QTOFControl 3.2, HyStar 3.2, and Chromeleon 6.8.1 Chromatography Data System software, respectively.

All chemicals and standards used in UHPLC and HPLC analyses including methanol, tetrahydrofuran (Avantor Performance Materials, Gliwice, Poland), water (Merck, Darmstadt, Germany), acetic acid, formic acid, (+)-catechin, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, kaempferol, L-rhamnose, L-arabinose, and D-xylose (Sigma-Aldrich, Seelze, Germany/St. Louis, MO, USA) were of HPLC-grade. Solvents used for extraction of the plant material and purification of the diglycoside fraction (Chempur, Piekary Śląskie, Poland), as well as Sephadex LH-20 (Sigma-Aldrich) used for gel permeation chromatography, were of analytical grade.

4.2. Plant material

Leaves of *P. spinosa* were collected in June 2015 in Rzeczyca (51°36′N, 20°17′E; Poland) from plants growing in their natural state and authenticated by prof. M.A. Olszewska (Department of Pharmacognosy, Medical University of Lodz, Poland). A voucher specimen (KFG/HB/06015-PSP) was deposited in the Department of Pharmacognosy, Medical University of Lodz, Poland. Prior to the analysis the plant samples were powdered using an electric grinder, sieved through a 0.315-mm sieve, and stored in airtight containers at ambient temperature and in darkness until used.

The diglycosidic fraction was isolated from the plant material as described previously (Olszewska and Wolbiś, 2002a) with slight modifications. Briefly, the powdered leaves (100 g) were pre-extracted with chloroform in a Soxhlet apparatus (200 mL, 48 h) to remove lipoidal components, and then extracted exhaustively with boiling 70% (*v/v*) aqueous methanol (3 × 500 mL). The combined

methanol extracts were evaporated *in vacuo*, and the residue (18 g) was dissolved in hot water (500 mL) and left at 4 °C for spontaneous precipitation. After filtering of the ballast precipitate (3 g), the water solution was extracted with ethyl acetate (6 × 100 mL) to remove less polar constituents (phenolic acids, flavonoid aglycones, monoglycosides, and their acylated derivatives), and the diglycosides were extracted with *n*-butanol (3 × 100 mL). The *n*-butanol extract was evaporated to dryness, and the dry residue (8.7 g) was subjected to gel permeation chromatography with methanol over Sephadex LH-20 (100 g) to obtain the fraction of kaempferol dipentosides (2.1 g) depleted, among others, of polymeric proanthocyanidins (2.8 g) and quercetin diglycosides (0.5 g).

4.3. Qualitative UHPLC-PDA-ESI–MS^n profiling

Samples (5 mg) of the 70% (*v/v*) aqueous methanol extract and the fraction of kaempferol diglycosides were dissolved in 1 mL of the mixture of methanol and 0.1% formic acid (1:1, *v/v*), filtered through a syringe filter PTFE (25 mm, 0.2 μm, Ahlstrom, Helsinki, Finland), and directly injected (2 μL) into the UHPLC system. Separations were carried out on a Kinetex XB-C18 column (1.7 μm, 150 mm × 2.1 mm i.d.; Phenomenex, Torrance, CA, USA). The mobile phase consisted of solvent A (water-formic acid, 100:0.1, *v/v*) and solvent B (acetonitrile-formic acid, 100:0.1, *v/v*) with the following elution profile: 0–1 min, 6% (*v/v*) B; 1–10 min, 6–13% (*v/v*) B; 10–15 min, 13% (*v/v*) B; 15–19 min, 13–15% (*v/v*) B; 19–24 min, 15% (*v/v*) B; 24–40 min, 15–23% (*v/v*) B; 40–60 min, 23–40% (*v/v*) B; 60–68 min, 5% (*v/v*) B (equilibration). The flow rate was 0.3 mL/min. The column temperature was 25 °C. The LC eluate was introduced directly into the ESI interface without splitting and analysed in the negative ion mode using a scan from *m/z* 70 to 2200. The MS^2 and MS^3 fragmentations were obtained for the most abundant ions at the time. The nebuliser pressure was 40 psi; dry gas flow 9 L/min; dry temperature 300 °C; 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.

4.4. Optimisation of the HPLC separation of the kaempferol diglycosides fraction

The optimisation was carried out on an analytical XBridge C18 column (100 mm × 4.6 mm i.d., 5 μm, Waters) at room temperature. A central composite design was used to investigate the effects of three independent variables – methanol concentration (C_{MeOH}), tetrahydrofuran concentration (C_{THF}) and flow rate (F_R) – on the separation process. The independent variables were coded on five different levels (−1.68, −1, 0, 1, 1.68), preliminary experimental data being used as a guidance to set the ranges of the factors. The complete design consisted of 17 experimental points including three replications of the central point (all variables coded as zero). The behaviour of the system was described by the following second-degree polynomial equation:

$$Y = \beta_0 + \beta_i C_{MeOH} + \beta_j C_{THF} + \beta_k F_R + \beta_{ii} C_{MeOH}^2 + \beta_{jj} C_{THF}^2 + \beta_{kk} F_R{}^2 + \beta_{ij} C_{MeOH} C_{THF} + \beta_{ik} C_{MeHO} F_R + \beta_{jk} C_{THF} F_R \quad (1)$$

where Y is a response, β_0 is a constant coefficient, β_i, β_j and β_k are linear coefficients, β_{ii}, β_{jj} and β_{kk} are quadratic coefficients, β_{ij}, β_{ik} and β_{jk} are interaction coefficients (Myers et al., 2009).

Four responses $Y_{1\text{-}4}$ were considered including the separation time (Y_1, T_S) understood as the retention time of the latest peak (peak **16**), the resolution between peaks **12** and **13** (Y_2, R_{s1}) the resolution between peaks **15** and **16** (Y_3, R_{s2}), and a combined response (Y_4, D) described by the overall desirability function according to Derringer and Suich (Derringer and Suich, 1980):

$$D = (d_1 d_2 d_3)^{\frac{1}{3}} \quad (2)$$

where d_1, d_2, and d_3 correspond to individual desirability functions for each response calculated according to Table 3 and the following equations:

$$d_1 = \begin{cases} 0 & Y > U \\ \frac{U-Y}{U-L} & L < Y < U \\ 1 & Y < L \end{cases} \quad (3)$$

$$d_{2,3} = \begin{cases} 0 & Y < L \\ \frac{Y-L}{U-L} & L < Y < U \\ 1 & Y > U \end{cases} \quad (4)$$

where Y is a response, U is its upper limit, and L – is its lower limit.

The resolution (R_S) was calculated using the following equation:

$$R_s = 2.0 \times \frac{t_1 - t_2}{w_2 - w_1} \quad (5)$$

in which t_1 and t_2 are retention times, and ($w_2 + w_1$) is a sum of peak widths at baseline between tangents lines drawn at 50% peak height.

The statistical analysis of the data and calculation of regression equations between the process variables and analytical responses were performed using the Statistica 12.0 PL software (Statsoft, Kraków, Poland). According to the experimental data, the fitting models were constructed and the statistical significance of the model terms were examined by regression analysis and analysis of variance (ANOVA, α = 0.05).

4.5. Isolation of kaempferol diglycosides

The isolation process was carried out using a preparative XBridge Prep C18 column (100 mm × 19 mm i.d., 5 μm, Waters) in an isocratic elution mode. The mobile phase was composed of 0.5% acetic acid, methanol, and tetrahydrofuran (75.2:16.6:8.2, *v/v/v*). Prior to the isolation, the diglycoside fraction (75 mg) was dissolved in the mobile phase (5 mL). The separations were carried out at room temperature (20–25 °C). The flow rate was 13.6 mL/min, and the injection volume was 250 μL. The fraction collection was triggered automatically by the signal from UV–vis detector at λ = 350 nm. The separation process was repeated 20 times, and fractions containing respective analytes were combined and purified by gel permeation chromatography over Sephadex LH-20. Eventually, 1.2 mg of **12**, 9.2 mg of **13**, 24.9 mg of **15**, and 29.5 mg of **16** was obtained from 75 mg of the sample (86% total isolation yield, *w/w*) which corresponds to 0.06 mg of **12**, 0.46 mg of **13**, 1.24 of **15**, and 1.47 mg of **16** per injection.

4.6. Structure elucidation

The acid hydrolysis was performed as described before (Olszewska and Roj, 2011). Free kaempferol in the diethyl ether fraction was identified with an authentic standard by HPLC-PDA (Olszewska, 2008). Free monosaccharides in the water layer were characterised qualitatively and in respect to the absolute configuration following conversion to their 1-[(*S*)-*N*-acetyl-amethylbenzylamino]-1-deoxy-alditol pentaacetate derivatives according to the method described by Oshima et al. (1982) with some modifications (Olszewska and Roj, 2011). The derivatised monosaccharides were analysed by HPLC-PDA, and identified by comparison with the retention times of derivatives of the standard samples obtained in the same manner: derivative of L-arabinose (R_t 15.54 min), derivative of D-xylose (R_t 14.65 min), derivative of

L-rhamnose (R_t 22.08 min). As the result, L-rhamnose was identified in the hydrolysates of **12**, **13**, **15**, and **16**; L-arabinose in the hydrolysates of **12** and **16**; and D-xylose in the hydrolysate of **13**.

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

4.7. Kaempferol 3-O-α-L-arabinopyranoside-7-O-α-L-rhamnopyranoside (12)

Pale yellow needles; m.p. 195–199 °C; UV (methanol) λ_{max} nm: 266, 323sh, 350; ESI–MS *m/z*: 563 [M−H]$^-$; ESI–MS^2 *m/z* (intensity): 417 (100) [M−H−rhamnose]$^-$, 431 (52.2) [M−H−arabinose]$^-$; 285 (16.2) [M−H−rhamnose−arabinose]$^-$; ^{1}H and ^{13}C NMR data (methanol-d_4): see Table 7.

4.8. Kaempferol 3-O-β-D-xylopyranoside-7-O-α-L-rhamnopyranoside (13)

Pale yellow needles; m.p. 194–197 °C; UV (methanol) λ_{max} nm: 266, 322sh, 349; ESI–MS *m/z*: 563 [M−H]$^-$; ESI–MS^2 *m/z* (intensity): 431 (100) [M−H−xylose]$^-$, 417 (24.8) [M−H−rhamnose]$^-$, 285 (84.2) [M−H−rhamnose−xylose]$^-$; ^{1}H and ^{13}C NMR data (methanol-d_4): see Table 7.

Table 7
NMR spectral data of compounds 12 and 13 in methanol-d_4 (600 MHz for ^{1}H and 150.9 MHz for ^{13}C)[a].

Pos.[b]	12		13	
	δ_H	δ_C	δ_H	δ_C
2		158.05		158.11
3		134.42		134.20
4		178.38		178.25
5		161.51		161.52
6	6.51 (1H, *d*, *J* = 2.1)	99.22	6.51 (1H, *d*, *J* = 2.1 Hz)	99.24
7		162.27		162.28
8	6.79 (1H, *d*, *J* = 2.1)	94.18	6.79 (1H, *d*, *J* = 2.1 Hz)	94.23
9		156.65		156.64
10		106.00		105.97
1′		121.07		121.11
2′	8.12 (2H, *d*, *J* = 8.8)	131.47	8.09 (2H, *d*, *J* = 8.8)	130.90
3′	6.94 (2H, *d*, *J* = 8.8)	114.94	6.92 (2H, *d*, *J* = 8.8)	114.83
4′		160.44		160.44
5′	6.94 (2H, *d*, *J* = 8.8)	114.51	6.92 (2H, *d*, *J* = 8.8)	114.83
6′	8.12 (2H, *d*, *J* = 8.8)	131.00	8.09 (2H, *d*, *J* = 8.8)	130.90
	3-α-L-arabinopyranosyl:		3-β-D-xylopyranosyl:	
1″	5.23 (1H, *d*, *J* = 6.2)	102.72	5.27 (1H, *d*, *J* = 7.2)	103.00
2″	3.93 (1H, *dd*, *J* = 6.2, 8.1)	71.35	3.49–3.54 (3H, *m*)	73.90
3″	3.67 (1H, *dd*, *J* = 8.1, 3.2)	72.54	3.43 (1H, *t*, *J* = 8.5)	76.03
4″	3.78–3.84 (2H, *m*)	67.44	3.49–3.54 (3H, *m*)	69.63
5$_a$″	3.78–3.84 (2H, *m*)	65.27	3.79 (1H, *dd*, *J* = 5.1, 11.7)	65.79
5$_b$″	3.43 (1H, *dd*, *J* = 1.9, 11.2)		3.13 (1H, *dd*, *J* = 9.4, 11.7)	
	7-α-L-rhamnopyranosyl:		7-α-L-rhamnopyranosyl:	
1‴	5.60 (1H, *d*, *J* = 1.3)	98.53	5.59 (1H, *brs*)	98.54
2‴	4.05 (1H, *dd*, *J* = 1.3, 3.4)	70.31	4.04 (1H, *d*, *J* = 3.4)	70.32
3‴	3.86 (1H, *dd*, *J* = 3.4, 9.4)	70.71	3.86 (1H, *dd*, *J* = 3.4, 9.4)	70.72
4‴	3.51 (1H, *t*, *J* = 9.4)	72.22	3.49–3.54 (3H, *m*)	72.22
5‴	3.62 (1H, *dd*, *J* = 6.2, 9.4)	69.90	3.61 (1H, *m*)	69.91
6‴	1.28 (3H, *d*, *J* = 6.2)	16.65	1.29 (3H, *d*, *J* = 6.2)	16.65

[a] 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.
[b] For trivial atom numbering, see chemical formulas of **12** and **13** (Fig. 5).

4.9. Kaempferol 3,7-di-O-α-L-rhamnopyranoside (15)

Pale yellow needles; m.p. 193–197 °C; UV (methanol) λ_{max} nm: 264, 320sh, 349; ESI–MS *m/z*: 577 [M−H]$^-$; ESI–MS^2 *m/z* (intensity): 431 (100) [M−H−rhamnose]$^-$, 285 (53.4) [M−H−rhamnose−rhamnose]$^-$; ^{1}H NMR (methanol-d_4) δ ppm: 7.82 (2H, *d*, *J* = 8.82 Hz, H-2′ and H-6′), 6.97 (2H, *d*, *J* = 8.82 Hz, H-3′ and H-5′), 6.75 (1H, *d*, *J* = 2.16 Hz, H-8), 6.50 (1H, *d*, *J* = 2.16 Hz, H-6), 5.59 (1H, *d*, *J* = 1.68 Hz, H-1‴), 5.43 (1H, *d*, *J* = 1.62 Hz, H-1″), 4.25 (1H, *dd*, *J* = 1.62, 3.35 Hz, H-2″), 4.04 (1H, *dd*, *J* = 1.68, 3.45 Hz, H-2‴), 3.86 (1H, *dd*, *J* = 3.45, 9.48 Hz, H-3‴), 3.74 (1H, *dd*, *J* = 3.35, 8.97 Hz, H-3″), 3.63 (1H, *dd*, *J* = 9.48, 6.18 Hz, H-5‴), 3.51 (1H, *t*, *J* = 9.48 Hz, H-4‴), 3.34-3.40 (2H, *m*, H-4″ and H-5″), 1.29 (3H, *d*, *J* = 6.18 Hz, Me-6‴), 0.96 (3H, *d*, *J* = 5.82 Hz, Me-6″); ^{13}C NMR (methanol-d_4) δ ppm: 178.44 (C-4), 162.20 (C-7), 161.64 (C-5), 160.37 (C-4′), 158.45 (C-2), 156.74 (C-9), 135.13 (C-3), 130.60 (C-2′ and C-6′), 121.07 (C-1′), 115.20 (C-3′ and C-5′), 106.21 (C-10), 102.15 (C-1″), 99.18 (C-6), 98.53 (C-1‴), 94.25 (C-8), 72.24 (C-4‴), 71.83 (C-4″), 70.78 (C-5″), 70.73 (C-3″), 70.69 (C-3‴), 70.53 (C-2″), 70.32 (C-2‴), 69.91 (C-5‴), 16.66 (C-6‴), 16.26 (C-6″).

4.10. Kaempferol 3-O-α-L-arabinofuranoside-7-O-α-L-rhamnopyranoside (16)

Pale yellow needles; m.p. 196–198 °C; UV (methanol) λ_{max} nm: 265, 320sh, 347; ESI–MS *m/z*: 563 [M−H]$^-$; ESI–MS^2 *m/z* (intensity): 417 (1.4) [M−H−rhamnose]$^-$, 431 (100) [M−H−arabinose]$^-$; 285 (28.6) [M−H−rhamnose−arabinose]$^-$; ^{1}H NMR (methanol-d_4) δ ppm: 8.00 (2H, *d*, *J* = 8.82 Hz, H-2′ and H-6′), 6.94 (2H, *d*, *J* = 8.82 Hz, H-3′ and H-5′), 6.76 (1H, *d*, *J* = 2.10 Hz, H-8), 6.47 (1H, *d*, *J* = 2.10 Hz, H-6), 5.59 (1H, *d*, *J* = 1.74 Hz, H-1‴), 5.54 (1H, *brs*, H-1″), 4.36 (1H, *dd*, *J* = 0,72, 2.92 Hz, H-2″), 4.06 (1H, *dd*, *J* = 1.74, 3.45 Hz, H-2‴), 3.94 (1H, *dd*, *J* = 2.94, 5.13 Hz, H-3″), 3.86 (1H, *dd*, *J* = 3.45, 9.48 Hz, H-3‴), 3.79–3.85 (1H, *m*, H-4″), 3.63 (1H, *dd*, *J* = 9.48, 6.18 Hz, H-5‴), 3.49–3.60 (3H, *m*, H-5″, H-4‴), 1.29 (3H, *d*, *J* = 6.18 Hz, Me-6‴); ^{13}C NMR (methanol-d_4) δ ppm: 178.66 (C-4), 162.22 (C-7), 161.45 (C-5), 160.35 (C-4′), 158.44 (C-2), 156.67 (C-9), 133.93 (C-3), 130.72 (C-2′ and C-6′), 121.18 (C-1′), 115.20 (C-3′ and C-5′), 105.95 (C-10), 108.36 (C-1″), 99.22 (C-6), 98.48 (C-1‴), 94.25 (C-8), 86.67 (C-4″), 82.03 (C-2″), 77.31 (C-3″), 72.24 (C-4‴), 70.71 (C-3‴), 70.31 (C-2‴), 69.90 (C-5‴), 61.18 (C-5″), 16.69 (C-6‴).

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Bioactivity Potential of *Prunus spinosa* L. Flower Extracts: Phytochemical Profiling, Cellular Safety, Pro-inflammatory Enzymes Inhibition and Protective Effects Against Oxidative Stress *In Vitro*

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Flower extracts of *Prunus spinosa* L. (blackthorn)—a traditional medicinal plant of Central and Eastern Europe indicated for the treatment of urinary tract disorders, inflammation, and adjunctive therapy of cardiovascular diseases—were evaluated in terms of chemical composition, antioxidant activity, potential anti-inflammatory effects, and cellular safety in function of fractionated extraction. The UHPLC-PDA-ESI-MS^3 fingerprinting led to full or partial identification of 57 marker constituents (36 new for the flowers), mostly flavonoids, A-type proanthocyanidins, and phenolic acids, and provided the basis for authentication and standardization of the flower extracts. With the contents up to 584.07 mg/g dry weight (dw), 490.63, 109.43, and 66.77 mg/g dw of total phenolics (TPC), flavonoids, proanthocyanidins, and phenolic acids, respectively, the extracts were proven to be rich sources of polyphenols. In chemical *in vitro* tests of antioxidant (DPPH, FRAP, TBARS) and enzyme (lipoxygenase and hyaluronidase) inhibitory activity, the extracts effects were profound, dose-, phenolic-, and extraction solvent-dependent. Moreover, at *in vivo*-relevant levels (1–5 μg/mL) the extracts effectively protected the human plasma components against peroxynitrite-induced damage (reduced the levels of oxidative stress biomarkers: 3-nitrotyrosine, lipid hydroperoxides, and thiobarbituric acid-reactive substances) and enhanced the total antioxidant status of plasma. The effects observed in biological models were in general dose- and TPC-dependent; only for protein nitration the relationships were not significant. Furthermore, in cytotoxicity tests, the extracts did not affect the viability of human peripheral blood mononuclear cells (PBMC), and might be regarded as safe. Among extracts, the defatted methanol-water (7:3, v/v) extract and its diethyl ether and ethyl acetate fractions appear to be the most advantageous for biological applications. As compared to the positive controls, activity of the extracts was favorable, which might be attributed to some synergic effects of their constituents. In conclusion, this research proves that the antioxidant and enzyme inhibitory capacity of phenolic fractions should be counted as one of the mechanisms behind the activity

of the flowers reported by traditional medicine and demonstrates the potential of the extracts as alternative ingredients for functional products supporting the treatment of oxidative stress-related pathologies cross-linked with inflammatory changes, especially in cardiovascular protection.

Keywords: *Prunus spinosa*, oxidative stress, antioxidants, human plasma, lipoxygenase, hyaluronidase, polyphenols, LC-MS

INTRODUCTION

Medicinal plants as primary sources of natural bioactive compounds are attracting growing interest as constituents of functional products active in prevention and adjunctive therapy of numerous chronic diseases, including cardiovascular disorders (CVD), the leading cause of mortality in the world today. In the search of new candidates for closer investigation, ethnobotanical knowledge constitutes an important guideline indicating species with the relevant activity (Franz et al., 2011).

One of such plants might be *Prunus spinosa* L. (blackthorn or sloe)—a wild plume tree native to Europe, western Asia, north-western Africa, and naturalized in New Zealand and North America (Tutin et al., 1968). In European tradition it has been known for over 7,000 years, at first as a source of edible fruit and then also as a medicinal plant (Poonam et al., 2011; Zohary et al., 2012), used i.a. in the treatment of various circulatory system disorders. For medicinal applications the plant has been used throughout Europe with the flowers being the most popular in central and eastern parts of the continent (Hoppe, 1981). Ethnopharmacological sources indicate vasoprotective, anti-inflammatory, diuretic, detoxifying (blood purifying), and spasmolytic activities for the flowers, and document their use as ingredients of compound herbal prescriptions traditionally applied, e.g., to treat intestinal and respiratory tract disorders, but also various cardiac complaints, such as myocarditis, cardiac neurosis and atherosclerosis (Berger, 1949; Hoppe, 1981; Wawrzyniak, 1992; Blumenthal and Busse, 1998). The fruits, according to German Commission E, have been indicated mainly in mild inflammation of the oral and pharyngeal mucosa, as well as an astringent (Blumenthal and Busse, 1998); however, local European sources report their use also as a heart-strengthening remedy (Kültür, 2007; Jarić et al., 2015). Branches, on the other hand, have been more popular in the south of Europe and suggested to possess anti-hypertensive properties (Calvo and Cavero, 2014).

Active components of the plant are believed to be polyphenols, including flavonoids, A-type proanthocyanidins, anthocyanins, coumarins, and phenolic acids, forming unique and diversified profiles in particular organs, among which the flowers are the least characterized (Kolodziej et al., 1991; Sakar and Kolodziej, 1993; Olszewska and Wolbiś, 2001, 2002a,b; Guimarães et al., 2013; Pinacho et al., 2015; Owczarek et al., 2017). Some blackthorn constituents, such as flavonoid pentosides (arabinosides, xylosides, rhamnosides) and A-type procyanidin dimers with twice-bonded structures are quite rare in nature and their distribution is generally limited to selected species and plant families (Pinacho et al., 2015). This unique composition may correspond to the distinctive activity profile of *P. spinosa* reported by traditional medicine. Indeed, the earliest studies suggested that the flavonoid fraction of blackthorn flowers significantly reduces capillary permeability and shows anti-inflammatory effects in animal internal organs, normalizes the blood cholesterol and cholesterol/phospholipid ratio in atherogenic rabbits, exhibits spasmolytic effects on isolated intestinal segments from different animals, and increases the amplitude of heart contractions in perfusion of isolated frog hearts (Lisevitskaya et al., 1968; Makarov, 1968, 1972; Makarov and Khadzhaĭ, 1969). However, despite these promising *in vivo* and *ex vivo* results, the potential of blackthorn, especially the blackthorn flowers, as a source of biologically active extracts (that means standardized dry extracts—more effective than unprocessed plant materials and recommended in modern phytotherapy) remains unexplored, and the plant materials are still used mostly in the form of traditional herbal teas, partly due to the lacking molecular background for their activity and safety. Recently, special attention has been given to the antioxidant activity of the extracts from the branches, leaves, and fruits, as one of the possible mechanism of action of the blackthorn polyphenols (Barros et al., 2010; Guimarães et al., 2014; Pinacho et al., 2015). Nevertheless, as these studies were based only on simple, mostly chemical tests, and did not cover the flower extracts, the subject requires more detailed investigations.

Polyphenols, including flavonoids, are specialized plant metabolites, the beneficial effects of which in CVD is commonly linked with their ability to influence two interdependent pathological processes of oxidative/nitrative stress and inflammation (Biswas, 2016). As free radical scavengers, metal chelators, inhibitors of pro-inflammatory enzymes, and modifiers of cell signaling pathways, polyphenols may protect cellular and functional elements of the circulatory system against lipid peroxidation, protein nitration, chronic inflammation, and oxidative damage to DNA, which results i.a. in vasodilatory, vasoprotective, anti-atherogenic, antithrombotic, and anti-apoptotic effects (Alissa and Ferns, 2012; Quiñones et al., 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 and Rizvi, 2009).

Therefore, the aim of this project was a comprehensive characteristic of the flowers of *P. spinosa* in a function of fractionated extraction with respect to the chemical composition and biological activity of the dry extracts obtained with solvents of different polarity. 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 main mechanisms of the beneficial action of polyphenols in CVD, including direct scavenging of free radicals, inhibition of pro-inflammatory enzymes, enhancement of the total antioxidant capacity of blood plasma and protection against oxidative and nitrative damage of its lipid and protein components. Moreover, the relationship between activity of the extracts and the presence of different groups of polyphenolic constituents was explored statistically. Additionally, cellular safety of the extracts was evaluated *in vitro* in cytotoxicity tests against human peripheral blood mononuclear cells (PBMCs).

MATERIALS AND METHODS

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; caffeic acid; kaempferol; quercetin trihydrate; isorhamnetin; rutin trihydrate; cyanidin chloride; indomethacin; bovine testis hyaluronidase; lipoxygenase from soybean; linoleic acid; 2-thiobarbituric acid; 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; and Histopaque®-1077 medium. HPLC grade solvents (acetonitrile and methanol) used for UHPLC and HPLC analyses were obtained from Avantor Performance Materials (Gliwice, Poland). A (Ca^{2+})-free phosphate buffered saline (PBS) was purchased from Biomed (Lublin, Poland). Peroxynitrite was synthesized 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 in 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 done using 96-well plates and a microplate reader, SPECTROStar Nano (BMG LabTech, Ortenberg, Germany).

Plant Material and Extracts Preparation

Commercial samples of *Prunus spinosa* L. flowers were purchased in 2015 (harvest in April 2015) from Dary Natury (Koryciny, Poland). According to the manufacturer, the raw material was collected in the district Rudka, Malopolska province (50°02′N, 20°52′E). The raw material was powdered with an electric grinder, and sieved through a 0.315-mm sieve. A portion (100 g) was extracted with chloroform (3 L, 30 h) in a Soxhlet apparatus and the pellet was next four times refluxed with methanol-water (7:3, v/v; 4 × 1 L) to give the defatted methanol extract (MED, 27.3 g dw). The extraction solvent was selected from methanol-water mixtures of different alcohol concentration after optimization performed in terms of extracts yield and total phenolic content (results not shown). The MED (25.0 g) was suspended in water (1 L) and subjected to sequential liquid-liquid extraction with organic solvents (8 × 100 mL each) to yield diethyl ether fraction (DEF, 1.23 g dw), ethyl acetate fraction (EAF, 4.00 g dw), *n*-butanol fraction (BF, 4.86 g dw) and water residue (WR, 13.08 g dw). The organic solvent extracts were evaporated *in vacuo*, and the water-containing fractions were lyophilized 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.

Phytochemical Profiling

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/formic acid, 100:0.1, v/v), and solvent B (acetonitrile/formic acid, 100:0.1, v/v) with the elution profile as follows: 0–10 min, 6–13% B (v/v); 10–15 min, 13% B (v/v); 15–19 min, 13–15% B (v/v); 19–24 min, 15% B (v/v); 24–40 min, 15–23% B (v/v); 40–55 min, 23–40% B (v/v); 55–60 min, 40% B (v/v); 60–63 min, 40–6% B (v/v); 63-70 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 a range of 200–600 nm, and chromatograms were acquired at 254, 280, and 350 nm. The LC eluate was introduced directly into the ESI interface without splitting and analyzed in both negative and positive ion modes. ESI parameters: 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 200–2,200.

Quantitative Standardization

The total phenolic contents (TPC) and total proanthocyanidin contents (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 total flavonoid contents (TFA) were determined by HPLC-PDA as the total content of flavonoid aglycones after acid hydrolysis. 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 according to Olszewska (2012) with quercetin, kaempferol and isorhamnetin used for external calibration. Results were re-calculated for total contents of glycosides with the molecular masses of avicularin, juglanin, and isorhamnetin diglucoside, correspondingly. The total contents of phenolic acids (TAC) were assayed by HPLC-PDA according to Olszewska et al. (2012) with caffeic acid and chlorogenic acid used as calibration standards for quantification of simple hydroxycinnamic acids and quinic acid pseudodepsides, respectively.

Antioxidant Activity in Chemical Models

The DPPH free-radical scavenging activity was determined according to the method optimized previously (Olszewska et al., 2012) and expressed as normalized 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 sulfate. 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 quantification of thiobarbituric acid-reactive substances (TBARS) according to the method of Kljak and Grbeša (2015) with some changes. Briefly, before and after the samples were incubated for 3 h at 50.0 ± 0.1°C in the dark, 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 vs. 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 incubation. Finally, antioxidant activity was expressed as IC_{50} values calculated from concentration-inhibition curves.

Antioxidant Activity in Human Plasma Models

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, and next plasma was 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 5 min 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).

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 the 3NT-Fg equivalents (in nmol/mg of plasma protein).

Ferric-Xylenol Orange Hydroperoxide Assay

Concentration of hydroperoxides in plasma samples (control or antioxidants and $ONOO^-$-treated plasma) was determined by a ferric-xylenol orange (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 sulfate 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.

TBARS Test

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

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 et al. (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, 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 Fe^{2+} in plasma.

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. Results

of the both assays were expressed as IC_{50} values calculated from concentration-inhibition curves.

Cell Viability Assay

PBMCs were isolated from human blood (obtained and collected as described above) using the Histopaque®-1077 medium (a sterile solution of polysucrose, 57 g/L, and sodium diatrizoate, 90 g/L, with a density of 1.077 g/mL). From each of eight donors, two independent PBMCs 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 × g, at room temperature). Then, the pellet was 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 5 μg/mL. Cell viability (%) was determined during a spectrofluorimetric analysis, involving the use of propidium iodide as a fluorescent dye. Measurements were conducted using a microchip-type automatic cell counter Adam-MC DigitalBio (NanoEnTek Inc., Seoul, Korea), after 60 and 120 min of incubation of PBMCs with the examined substances (at 37°C). The procedure was carried out according to the manufacturer's protocol.

Statistical Analysis

Normality of the distribution of the results was verified using the Shapiro-Wilk test, and the homogeneity of variances using the Levene's test. 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 the Satistica12Pl software for Windows (StatSoft Inc., Krakow, Poland) with *p*-values less than 0.05 regarded as significant.

RESULTS

LC-MS Metabolite Profiling

Phytochemical profiling of the dry extracts revealed significant differences in their chemical composition depending on the extraction solvent (**Figure 1**, **Tables 1**, **2**). The qualitative UHPLC-PDA-ESI-MS^3 analysis resulted in full or partial identification of over fifty phenolic constituents (**Figure 1**, **Table 1**, peaks **1–59**) belonging to three main classes of phytochemicals—flavonols (thirty eight analytes), flavan-3-ol derivatives (catechins and proanthocyanidins, seven peaks) and phenolic acids (twelve compounds). The analytes were structurally characterized based on the comparison of their chromatographic behavior and ESI-MS^3 fragmentation patterns (in positive and negative ionization modes) with the literature data or reference standards, both commercial and isolated

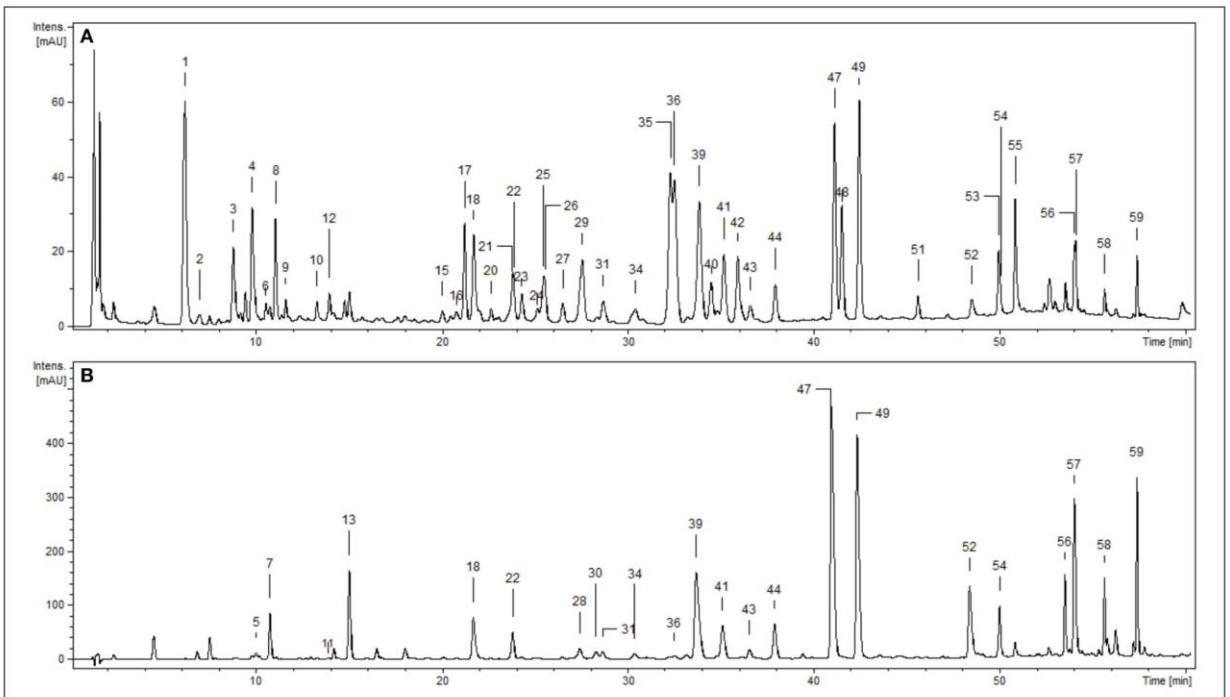

FIGURE 1 | Representative UHPLC chromatograms of the *P. spinosa* flower dry extracts at 280 nm: **(A)** MED, defatted methanol-water (7:3, v/v) extract; **(B)** DEF, diethyl ether fraction. Peak numbers refer to those implemented in **Table 1**.

TABLE 1 | UHPLC-PDA-ESI-MS^3 data of polyphenols detected in *P. spinosa* flower dry extracts.

Peak	Analyte	R_t (min)	UV λ_{max} (nm)	$[M–H]^-$ (m/z)	Fragmentary ions	$[M+H]^+$ (m/z)	Fragmentary ions	Formula	Extract	References
1	3-*O*-Caffeoylquinic acid (neochlorogenic acid)[a,b]	6.2	325	353	191, 179	355	163	$C_{16}H_{18}O_9$	BF, EAF, MED, WR	[1]
2	Caffeic acid hexoside[b]	7.0	325	341	179	365	185	$C_{15}H_{18}O_9$	BF, MED	
3	3-*O*-*p*-Coumaroylquinic acid[b]	8.8	310	337	163, 191	339	147	$C_{16}H_{18}O_8$	BF, EAF, MED, WR	[1]
4	5-*O*-Caffeoylquinic acid (chlorogenic acid)[a,b]	9.8	325	353	191, 179	355	163	$C_{16}H_{18}O_9$	BF, EAF, MED, WR	[1]
5	(+)-Catechin (CA)[a,b]	10.0	280	289	245	291	139	$C_{15}H_{14}O_6$	DEF	[5]
6	3-*O*-Feruloylquinic acid[b]	10.5	325	367	193	369	177	$C_{17}H_{20}O_9$	BF, EAF, MED, WR	[1]
7	Caffeic acid[a,b]	10.7	325	179	–	181	–	$C_9H_8O_4$	DEF	[5]
8	4-*O*-Caffeoylquinic acid (cryptochlorogenic acid)[a,b]	11.0	325	353	173, 191, 135	355	163	$C_{16}H_{18}O_9$	BF, EAF, MED, WR	[1]
9	Ferulic acid hexoside[b]	11.5	325	355	193	379	217	$C_{16}H_{20}O_9$	BF, EAF, MED	
10	5-*O*-*p*-Comaroylquinic acid[b]	13.3	310	337	191, 163	339	177	$C_{16}H_{18}O_8$	BF, EAF, MED	[1]
11	(–)-Epicatechin[a,b]	13.7	280	289	245	291	139	$C_{15}H_{14}O_6$	DEF	[5]
12	4-*O*-*p*-Comaroylquinic acid[b]	13.9	310	337	173, 163	339	177	$C_{16}H_{18}O_8$	BF, EAF, MED	[1]
13	*p*-Coumaric acid[a,b]	15.1	310	163	–	165	–	$C_9H_8O_3$	DEF	
14	4-*O*-Feruloylquinic acid[b]	15.7	325	367	173, 191	369	177	$C_{17}H_{20}O_9$	BF	[1]
15	Kaempferol hexoside[b]	20.0	264, 355, 285	447	357, 327, 287	449	431, 329	$C_{21}H_{20}O_{11}$	BF, EAF, MED	
16	(Epi)catechin-A-(epi)catechin	20.8	280	575	423, 289	577	559, 425, 287	$C_{30}H_{24}O_{12}$	EAF, MED	[2], [3], [4], [5]
17	Isorhamnetin dihexoside[b]	21.1	265, 353	639	459, 315	641	479, 317	$C_{28}H_{32}O_{17}$	BF, MED	
18	(Epi)catechin-A-(epi)catechin	21.7	280	575	539, 423, 289	577	559, 425, 287	$C_{30}H_{24}O_{12}$	DEF, MED	[2], [3], [4], [5]
19	Kaempferol rhamnoside-hexoside[b]	22.0	260, 358	593	447, 285	595	449, 287	$C_{27}H_{30}O_{15}$	BF	
20	Kaempferol dihexoside[b]	22.6	264, 355	609	447, 429, 285	611	449, 287	$C_{27}H_{30}O_{16}$	BF, MED	
21	Quercetin hexoside-pentoside[b]	23.6	265, 358	595	505, 433, 301	597	435, 303	$C_{26}H_{28}O_{16}$	BF, MED	
22	Unknown compound	23.7	280	433	287	435	289		EAF, DEF, MED	
23	Kaempferol 3-*O*-α-L-arabinopyranoside-7-*O*-α-L-rhamnopyranoside[a,b]	24.3	268, 356	563	417, 285	565	419, 287	$C_{26}H_{28}O_{14}$	BF, MED	[5], [9]
24	Quercetin 3-*O*-β-D-galactoside (hyperoside)[a,b]	25.1	264, 355	463	301	465	303	$C_{21}H_{20}O_{12}$	EAF, MED	
25	Quercetin 3-*O*-(6″-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (rutin)[a,b]	25.4	265, 354	609	301	611	303	$C_{27}H_{30}O_{16}$	BF, MED	[5]
26	Kaemferol 3-O-β-D-xylopyranoside-7-O-α-L-rhamnopyranoside (lepidoside)[a,b]	25.5	265, 354	563	431, 417, 285	565	433, 287	$C_{26}H_{28}O_{14}$	EAF, MED	[9]
27	Quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin)[a]	26.4	265, 356	463	301	465	303	$C_{21}H_{20}O_{12}$	EAF, MED	[7]
28	(Epi)catechin-A-(epi)catechin	27.3	280	575	539, 449, 289	577	559, 425, 287	$C_{30}H_{24}O_{12}$	EAF, DEF	[2], [3], [4], [5]
29	Quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl)-α-L-arabinofuranoside[a,b]	27.4	258, 354	595	433, 301	597	435, 303	$C_{26}H_{28}O_{16}$	BF, MED	[8]
30	(Epi)afzalechin-A-(epi)catechin	28.2	280	559	523, 407, 289	561	543, 409, 271	$C_{30}H_{24}O_{11}$	EAF, DEF	[2], [3], [4], [5]
31	Quercetin 3-*O*-α-D-xylopyranoside (reinutrin)[a]	28.5	256, 356	433	301	435	303	$C_{20}H_{18}O_{11}$	EAF, DEF, MED	[7]
32	Kaempferol rhamnoside-hexoside[b]	28.7	256, 356	593	447, 285	595	449, 287	$C_{27}H_{30}O_{15}$	BF	

(Continued)

TABLE 1 | Continued

Peak	Analyte	R_t (min)	UV λ_{max} (nm)	$[M–H]^-$ (m/z)	Fragmentary ions	$[M+H]^+$ (m/z)	Fragmentary ions	Formula	Extract	References
33	Kaempferol hexoside-pentoside[b]	30.2	254, 358	579	417, 285	581	419, 287	$C_{26}H_{28}O_{15}$	BF	
34	Quercetin 3-*O*-α-L-arabinopyranoside (guaiaverin)[a]	30.4	255, 355	433	301	435	303	$C_{20}H_{18}O_{11}$	EAF, DEF, MED	[7]
35	Kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (kaempferitrin)[a]	32.1	254, 356	577	431, 285	579	433, 287	$C_{27}H_{30}O_{14}$	BF, EAF, MED	[7], [9]
36	Kaempferol 3-*O*-α-L-arabinofuranoside-7-O-α-L-rhamnopyranoside[a]	32.4	254, 356	563	431, 285	565	433, 287	$C_{26}H_{28}O_{14}$	BF, EAF, DEF, MED	[9]
37	(Epi)afzalechin-A-(epi)catechin	33.2	280	559	523, 407, 289	561	543, 409, 271	$C_{30}H_{24}O_{11}$	EAF	[2], [3], [4], [5]
38	Kaempferol hexoside-rhamnoside[b]	33.6	255, 356	593	285	595	449, 287	$C_{27}H_{30}O_{15}$	BF	
39	Quercetin 3-*O*-α-L-arabinofuranoside (avicularin)[a]	33.7	255, 355	433	301	435	303	$C_{20}H_{18}0_{11}$	EAF, DEF, MED	[5], [6]
40	Quercetin 3-*O*-(4″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside (multinoside A)[a]	34.3	254, 356	609	447, 301	611	449, 303	$C_{27}H_{30}O_{16}$	BF, EAF, MED	[7]
41	Quercetin 3-*O*-α-L-rhamnopyranoside (quercitrin)[a]	35.1	255, 355	447	301	449	303	$C_{21}H_{20}O_{11}$	EAF, DEF, MED	[7]
42	Kaempferol hexoside-pentoside[b]	36.0	254, 355	579	417, 285	581	419, 287	$C_{26}H_{28}O_{15}$	BF, EAF, MED	
43	Kaempferol pentoside[b]	36.3	254, 355	417	285	419	287	$C_{20}H_{18}O_{10}$	EAF, DEF, MED	
44	Kaempferol 3-*O*-β-D-xylopyranoside[a]	37.8	255, 356	417	285	419	287	$C_{20}H_{18}O_{10}$	EAF, DEF, MED	[6]
45	Kaempferol hexoside[b]	39.9	255, 356	447	285	449	287	$C_{21}H_{20}O_{11}$	EAF	
46	Kaempferol hexoside-rhamnoside[b]	40.4	254, 356	593	431, 285	595	433, 287	$C_{27}H_{30}O_{15}$	BF	
47	Kaempferol 3-*O*-α-L-arabinofuranoside (juglanin)[a]	41.0	254, 356	417	285	419	287	$C_{20}H_{18}O_{10}$	EAF, DEF, MED	[6]
48	Kaempferol 3-*O*-(4″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside (multiflorin B)[a]	41.4	256, 354	593	285	595	433, 287	$C_{27}H_{30}O_{15}$	BF, EAF, MED	[7]
49	Kaempferol 3-*O*-α-L-rhamnopyranoside (afzelin)[a]	42.3	256, 356	431	285	433	287	$C_{21}H_{20}O_{10}$	EAF, DEF, MED	[6]
50	Quercetin 7-*O*-α-L-ramnopyranoside[b]	43.5	254, 356	447	301	449	303	$C_{21}H_{20}O_{11}$	EAF	
51	Quercetin acetyl-hexoside-rhamnoside[b]	45.4	255, 356	651	609, 447, 301	653	449, 413, 303	$C_{29}H_{32}O_{17}$	EAF, MED	
52	Quercetin[a]	48.3	255, 356	301	–	303	–	$C_{15}H_{10}O_7$	EAF, DEF, MED	[5], [6]
53	Kaempferol acetyl-hexoside-rhamnoside[b]	49.8	255, 355	635	593, 285	637	619, 415, 353, 287	$C_{29}H_{32}O_{16}$	EAF, MED	
54	Kaempferol 7-*O*-α-L-ramnopyranoside[a]	50.0	254, 356	431	285	433	287	$C_{21}H_{20}O_{10}$	BF, EAF, DEF, MED	[6]
55	Unknown compound	50.8	310	614	452, 358, 316	616	454, 436		EAF, MED	
56	Kaempferol[a]	53.8	255, 356	285	–	287	–	$C_{15}H_{10}O_6$	EAF, DEF, MED	[5], [6]
57	Kaempferol 3-*O*-(2″-*O*-*E*-*p*-coumaroyl)-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside[a]	53.9	267, 316, 355	709	563, 285	711	565, 279	$C_{35}H_{34}O_{16}$	EAF, DEF, MED	[8]
58	Quercetin *p*-coumaroyl-pentoside[b]	55.4	267, 316, 355	579	433, 301	581	279	$C_{29}H_{24}O_{13}$	DEF, MED	
59	Kaempferol 3-*O*-(2″-*O*-*E*-*p*-coumaroyl)-α-L-arabinofuranoside[a]	57.3	267, 316, 355	563	285	565	279	$C_{29}H_{24}O_{12}$	EAF, DEF, MED	[6]

[a]*Identified with authentic standards.*

[b]*Detected in P. spinosa flowers for the first time. R_t, retention times. UV λ_{max}, absorbance maxima in PDA spectra. $[M–H]^-$, pseudomolecular ions in MS spectra recorded in a negative mode. $[M+H]^+$, pseudomolecular ions in MS spectra recorded in a positive mode. MED, defatted methanol-water (7:3, v/v) extract. DEF, diethyl-ether fraction. EAF, ethyl acetate fraction. BF, n-butanol fraction. WR, water residue. References: [1] Clifford et al. (2003); [2] Hamed et al. (2014); [3] Li and Deinzer (2008); [4] Kolodziej et al. (1991); [5] Pinacho et al. (2015); [6] Olszewska and Wolbiś (2001); [7] Olszewska and Wolbiś (2002a); [8] Olszewska and Wolbiś (2002b); [9] Owczarek et al. (2017).*

TABLE 2 | Quantitative standardization data for *P. spinosa* flower dry extracts.

Extract	Total phenolics[a] TPC (mg GAE/g dw)	Total flavonoids (mg/g dw)[b]				Total proanthocyanidins TPA (mg CYE/g dw)[c]	Total phenolic acids TAC (mg/g dw)[d]
		Kaempferol	Quercetin	Isorhamnetin	TFC		
MED	206.07 ± 10.86^{B}	62.47 ± 0.17^{B}	19.30 ± 0.39^{B}	1.47 ± 0.13^{B}	125.12 ± 0.55^{B}	45.13 ± 2.38^{B}	29.24 ± 0.76^{C}
DEF	464.57 ± 20.57^{D}	259.68 ± 3.30^{E}	61.14 ± 2.22^{E}	5.16 ± 0.01^{D}	490.63 ± 8.16^{E}	49.5 ± 2.23^{B}	8.76 ± 0.27^{A}
EAF	584.07 ± 12.98^{E}	158.69 ± 1.32^{D}	53.21 ± 1.15^{D}	4.57 ± 0.29^{C}	325.53 ± 4.23^{D}	109.43 ± 3.71^{C}	17.20 ± 0.47^{B}
BF	296.57 ± 3.28^{C}	123.05 ± 1.99^{C}	32.06 ± 1.26^{C}	4.58 ± 0.03^{C}	241.27 ± 4.74^{C}	46.6 ± 1.14^{B}	66.77 ± 2.86^{D}
WR	64.6 ± 1.93^{A}	0.80 ± 0.01^{A}	0.38 ± 0.02^{A}	0.06 ± 0.01^{A}	1.88 ± 0.04^{A}	12.43 ± 0.25^{A}	17.71 ± 0.30^{B}

Results are presented as mean values ± SD (n = 3) calculated per dry weight (dw) of the extract. For extract codes see ***Table 1****. Different superscripts (capitals) in each column indicate significant differences in the means at $p < 0.05$.*

[a]*Values expressed in gallic acid equivalents.*

[b]*Values expressed as the levels of individual aglycones released after acid hydrolysis, and TFC, total glycosides.*

[c]*Values expressed in cyanidine chloride equivalents.*

[d]*Values calculated as a sum of caffeic acid and chlorogenic acid equivalents.*

previously in our laboratory from flowers and leaves of *P. spinosa* (Olszewska and Wolbiś, 2001, 2002a,b; Owczarek et al., 2017). The greatest chemical diversity was observed for the defatted methanol-water (7:3, v/v) extract (MED), while its fractions of diethyl ether (DEF), ethyl acetate (EAF), *n*-butanol (BF), and water residue (WR) obtained after sequential liquid-liquid partitioning were enriched in selected analytes, depending on the fractionation solvent.

Compounds **1–4**, **6–10**, and **12–14** displayed absorption maxima at 325 or 310 nm and UV-Vis spectra characteristic of caffeic acid or *p*-coumaric acid derivatives, respectively. According to the spectral profiles and hierarchical discrimination key proposed by Clifford et al. (2003), the peaks showing parent $[M–H]^-$ ions at m/z 353, 337, and 367 were identified as isomeric caffeoylquinic acids (chlorogenic acids; **1**, **4**, **8**), *p*-coumaroylquinic acids (**3**, **10**, **12**), and feruloylquinic acids (**6**, **14**), correspondingly. The identity of chlorogenic acids was additionally confirmed by experiments with standards, as was the presence of simple caffeic (**7**) and *p*-coumaric acids (**13**) giving the typical $[M–H]^-$ ions at m/z 179 and 163, respectively. The same ions were found in MS^2 spectra of compounds **2** and **9** after neutral losses of sugar moieties (−162 Da each), which led to the tentative identification of hexosides of caffeic acid and ferulic acid, respectively.

Peaks **5**, **11**, **16**, **18**, **28**, **30,** and **37** with UV maxima at 280 nm were classified as flavan-3-ols and proanthocyanidins. Based on standard spiking and literature data, compounds **5** and **11** were characterized as (+)-catechin and (−)-epicatechin, respectively. Compounds **16**, **18,** and **28** with parent ions $[M+H]^+$ at m/z 577 were classified as dimeric A-type procyanidins. These compounds gave secondary ions in MS^2 spectra at m/z 559, 425, and 287. The ions at m/z 425, arising from retro-Diels-Alder (RDA) fission of the $[M+H]^+$ (577–152 Da), and the ions at m/z 287 (577–290 Da) resulting from quinone methide (QM) fission, confirmed the presence of two (epi)catechin units in the structures (Li and Deinzer, 2008; Hamed et al., 2014). Compounds **16**, **18,** and **28** were thus tentatively identified as (epi)catechin-A-(epi)catechins. Two A-type proanthocyanidins of that kind have already been isolated from the flowers of *P. spinosa* and identified as *ent*-epicatechin-(4α→8;2α→O→7)-catechin and *ent*-epicatechin-(4α→8;2α→O→7)-epicatechin (Kolodziej et al., 1991). Compounds **30** and **37** with pseudomolecular $[M+H]^+$ ions at m/z 561 and ions in MS^2 spectra at m/z 543, 409 and 271 were classified as dimeric A-type proanthocyanidins. The mass difference between compounds **16**, **18**, **28,** and **30**, **37** was 16 Da, which indicated that in the latter molecules one (epi)catechin unit is probably replaced by (epi)afzelechin, the flavan-3-ol reported to occur in blackthorn flowers (Kolodziej et al., 1991) and branches (Pinacho et al., 2015). m/z 409 (561–152 Da) arising from RDA fission of the $[M+H]^+$ ion, confirmed the presence of (epi)catechin in both compounds. On the other hand, the product ions at m/z 271 (561–290 Da) resulting from QM fission of the $[M+H]^+$ ions, showed that the upper unit in both cases was (epi)afzelechin, and hence the terminal unit must be (epi)catechin, m/z 287 (Li and Deinzer, 2008; Hamed et al., 2014). The structures of compounds **30** and **37** were thus tentatively identified as (epi)afzelechin-A-(epi)catechin. Two proanthocyanidins of that type, i.e., *ent*-epiafzelechin-(4α→8;2α→O→7)-epicatechin and *ent*-epiafzelechin-(4α→8;2α→O→7)-catechin have previously been isolated from blackthorn flowers (Kolodziej et al., 1991).

Peaks **15**, **17**, **19–21**, **23–27**, **29**, **31–36**, **38–54**, and **56–59** with two UV-Vis maxima at 250–268 and 355–365 nm were classified as flavonoids. Compounds **52** and **56** showed parent $[M–H]^-$ ions at m/z 301 and 285, respectively, and were identified with standards as free aglycones quercetin and kaempferol. Peaks **23**, **26**, **35**, **36**, **44**, **47–49**, **54**, **57,** and **59** with fragment ions in MS^2 m/z 285 (typical for kaempferol) were identified with authentic standards of kaempferol glycosides (**Table 1**). Compounds **15**, **32**, **33**, **38**, **42**, **43**, **45,** and **46** were tentatively characterized based on their fragmentation pattern as kaempferol mono- and diglycosides. Peak **53** with parent $[M–H]^-$ ion at m/z 635 and fragment ions at m/z 593, 285 in MS spectra was tentatively identified as kaempferol acetyl hexoside-rhamnoside due to the neutral loss of an acetyl moiety (−42 Da), hexose (−162 Da) and rhamnose (−146 Da). Peaks **24**, **25**, **27**, **31**, **34**, **39–41**, and **50** with fragment ions in MS^3 spectra at m/z 301 (typical for

quercetin) were identified with standards of quercetin glycosides (**Table 1**). Compound **21** with pseudomolecular $[M–H]^-$ ion at m/z 595 and fragment ion at m/z 301 was tentatively assigned to quercetin hexoside-pentoside. Peak **51** with parent ion at m/z 651 and fragment ions at m/z 609, 447, and 301 was determined as a quercetin analog of **53**, i.e., quercetin acetyl hexoside-rhamnoside. Similarly, compound **58** was tentatively identified as an analog of **59**, i.e., quercetin *p*-coumaroyl-pentoside, probably quercetin 3-*O*-(2″-*E*-*p*-coumaroyl)-α-L-arabinofuranoside, due to the additional UV-Vis absorption maximum at 316 nm (typical for an aromatic acyl unit), and the neutral loss of *p*-coumaroyl moiety (−146 Da) and a pentose (−132 Da). Peak **17** gave the parent ion at m/z 639 and its MS spectra revealed the cleavage of two hexoses (−162 amu twice) and the aglycone signal consistent with isorhamnetin, which enabled tentative identification of isorhamnetin dihexoside.

Quantitative Standardization

According to the LC-MS findings, total contents of polyphenols (TPC), flavonoids (TFC), proanthocyanidins (TPA), and phenolic acids (TAC) were selected as standardization targets (**Table 2**). The TPC levels assayed by the standard Folin-Ciocalteu method and expressed in gallic acid equivalents (GAE) varied in the range of 64.6–584.07 mg GAE/g dw of the extracts with the highest values found for EAF (584.07 mg GAE/g dw) and DEF (464.57 mg GAE/g dw). The total flavonoid content (TFC) was determined by HPLC-PDA as a sum of flavonoid aglycones released after acid hydrolysis of native extracts, and recalculated on the dominant glycosides. With the levels constituting 55.7–105.5% of the TPC values, flavonoids were the dominant phenolic components of all extracts except WR, in which phenolic acids prevailed. The highest TFC contents were observed for DEF (490.63 mg/g dw) and EAF (325.53 mg/g dw). As regards individual aglycones, kaempferol was the dominant one and constituted 64.4–79.7% of the total aglycones. The contents of quercetin and isorhamnetin were much lower and constituted 18.8–30.9% and 1.6–4.7% of the sum of aglycones, respectively. The total TPA, determined by the *n*-butanol-HCl assay and expressed as cyanidin chloride equivalents (CYE), ranged from 12.43 to 109.43 mg CYE/g dw, with the peak value found again for EAF. Contrastingly, the highest level of phenolic acids (TAC), assayed by HPLC-PDA, was observed for BF (66.77 mg/g dw), while in other extracts the TAC contents varied in the range of 8.76–29.24 mg/g dw.

Antioxidative Effects in Chemical Models

All of the extracts showed significant and dose-dependent antioxidant activity and 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) significantly diminishing levels of thiobarbituric acid-reactive substances (TBARS), with the final parameters strongly influenced by the extraction solvent (**Table 3**). Regardless of the test, activity of the extracts decreased in similar order, i.e., EAF $\geq$ DEF $>$ BF $>$ MED $>$ WR, and correlated with the amounts of polyphenols (TPC) and flavonoids (TFC) in the samples (**Table 4**). The strongest relationships were observed for the FRAP test, whereas for the DPPH and TBARS assays, the correlation coefficients were negatively influenced by WR, a virtually flavonoid-free extract. If WR was excluded from the data matrix, the impact of TPC ($|r| > 0.91$, $p < 0.05$) on the antioxidant parameters of the other extracts was evident for all tests (results not shown). In a comparison to the positive standards, the antioxidant capacity of the most active extracts EAF and DEF was higher ($p < 0.05$) or not statistically different ($p > 0.05$) than that of an industrial antioxidant BHT (DPPH and TBARS tests), and Trolox, a synthetic analog of vitamin E (FRAP and TBARS tests). Moreover, if activity parameters of the native extracts were recalculated to GAE (**Table 3**) using the TPC values, the obtained antioxidant capacities of the phenolic fractions constituting the dry extracts were comparable to all of the positive standards except quercetin.

Protective Effects on Human Plasma Components

After demonstrating the promising antioxidant capacity in 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 peroxynitrite-stimulated plasma exhibited a considerably enhanced level (**Figure 2A**; $p < 0.001$) of 3-nitrotyrosine in plasma proteins (3-NT-Fg, a marker of protein nitration), a strong increase of lipid hydroperoxides (ferric-xylenol orange assay, FOX-1) and thiobarbituric acid-reactive substances (TBARS)—markers of lipid peroxidation (**Figures 2B,C**; $p < 0.001$), as well as a noticeable decrease (**Figure 2D**; $p < 0.001$) in ferric reducing ability (FRAP, a marker of total antioxidant status of plasma). In plasma samples incubated with $ONOO^-$ in the presence of the extracts (at 1–50 μg/mL), the rate of oxidative/nitrative damage was significantly reduced (**Figures 2A–C**; $p < 0.05$). All tested extracts effectively diminished the nitration of tyrosine residues (**Figure 2A**; $p < 0.001$)—by about 15–50% and 36–54% at 1 and 50 μg/mL, respectively. The impact of extraction solvent on the anti-nitrative activity was less pronounced than that observed in chemical tests; still, WR was again the least effective at each concentration level ($p < 0.05$). Among the other extracts, MED displayed the strongest activity (50–58% decrease in tyrosine nitration); however, only the effects of EAF, BF, and WR were dose-dependent (**Figure 2A**; $p < 0.05$). In consequence, the correlation between the percentage inhibition of tyrosine nitration and the phenolic contents (TPC, TFC, TPA, TAC) was not significant (**Table 4**). All of the tested extracts exhibited also protective properties against peroxynitrite-induced lipid peroxidation (**Figures 2B,C**; $p < 0.001$), regardless of the concentration level. In samples pre-incubated with the medium extracts concentration (5 μg/mL), the levels of hydroperoxides and TBARS decreased by about 47–53% and 21–34%, respectively. The solvent effects were negligible, although EAF at 50 μg/mL was the most effective antioxidant, able to reduce the peroxidation parameters by

TABLE 3 | Antioxidant activity of *P. spinosa* flower 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)[d, f]
MED	15.46 ± 0.38[F]	3.19	4.40 ± 0.10[B]	21.35	20.02 ± 1.95[F]	4.13
DEF	6.91 ± 0.22[D]	3.42	9.46 ± 0.04[D,E]	19.12	6.87 ± 0.59[C,D]	3.40
EAF	6.04 ± 0.07[D]	3.53	9.02 ± 0.11[D]	15.44	5.81 ± 0.40[B,C]	3.40
BF	11.79 ± 0.50[E]	3.50	6.15 ± 0.23[C]	20.75	11.34 ± 0.76[E]	3.36
WR	51.32 ± 0.93[G]	3.32	1.31 ± 0.04[A]	20.33	49.73 ± 2.05[G]	3.21
QU	1.63 ± 0.07[A]	–	36.02 ± 1.1[H]	–	1.85 ± 0.12[A]	–
TX	4.34 ± 0.22[C]	–	10.83 ± 0.32[E]	–	8.47 ± 0.45[C,D,E]	–
BHA	2.90 ± 0.14[B]	–	16.13 ± 0.83[F]	–	3.16 ± 0.22[A,B]	–
BHT	6.54 ± 0.28[D]	–	18.89 ± 0.42[G]	–	9.31 ± 0.16[D,E]	–

Results are presented as mean values (± SD for replicates, n = 3) calculated per dry weight of the extract or positive control (QU, quercetin; TX, Trolox; BHA; BHT). For extract codes see ***Table 1****. Different superscripts (capitals) in each column indicate significant differences in the means at $p < 0.05$. [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).*

TABLE 4 | Correlation coefficients (*r*) and probability (*p*) values of linear relationships between antioxidant activity parameters and phenolic contents.

r (p) for:	Antioxidant activity (chemical models)			Antioxidant activity (human plasma model)			
	DPPH	FRAP	TBARS	3NT-Fg	TBARS	FOX-1	FRAP
TPC	−0.8277 (0.084)	0.9721 (0.006)**	−0.8696 (0.055)	0.5094 (0.052)	0.8657 (0.000)***	0.7567 (0.001)***	0.9877 (0.000)***
TFC	−0.8122 (0.095)	0.9606 (0.009)**	−0.8536 (0.066)	0.4733 (0.075)	0.7335 (0.002)**	0.6273 (0.012)*	0.9284 (0.000)***
TPA	−0.7273 (0.164)	0.7586 (0.137)	−0.7453 (0.148)	0.5084 (0.053)	0.9018 (0.000)***	0.8001 (0.000)***	0.9535 (0.000)***
TAC	−0.1415 (0.820)	−0.1517 (0.808)	−0.1417 (0.820)	0.2173 (0.437)	0.4021 (0.134)	0.2125 (0.447)	0.5691 (0.021)*

Activity and concentration parameters according to ***Tables 2****,* ***3****, and* ***Figure 2****. Asterisks mean statistical significance of the estimated linear relationships (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).*

about 70% in FOX-1 and 45% in TBARS tests. The inhibitory activity on lipid peroxidation was dose-dependent for all extracts if measured by TBARS (**Figure 2C**; $p < 0.05$); however, in the FOX-1 assay only some dose-effects for MED, DEF and EAF were significant, i.e., differences observed between the two lowest concentration levels (1–5 μg/mL) and the highest dose of 50 μg/mL (**Figure 2B**; $p < 0.05$). Consequently, relatively strong correlations ($|r| > 0.73$, $p < 0.01$) were observed between the percentage of lipid peroxidation assayed by TBARS and the phenolic contents (TPC, TFC, TPA), whereas the analog relationships for FOX-1 test were weaker ($|r| > 0.62$, $p < 0.01$) (**Table 4**). All of the extracts were also able to normalize and/or enhance the total antioxidant status of $ONOO^-$-treated plasma (FRAP), depending on the concentration used, and only MED at 1 μg/mL did not change significantly the FRAP value of the oxidized plasma (**Figure 2D**; $p > 0.05$). For all other extracts, a significant improvement in the plasma reducing ability was observed with the increase up to about 190% vs. the $ONOO^-$-stimulated plasma and 140% vs. the control sample for EAF at 50 μg/mL (**Figure 2D**; $p < 0.001$). Strong dose-dependency of the analytical response was found, and thus strong relationships ($|r| > 0.92$, $p < 0.001$) between the percentage increase in the FRAP values of the oxidized plasma and the TPC, TFC, and TPA levels (**Table 4**).

Simultaneously, the $ONOO^-$-treated plasma was incubated with 5 μg/mL of standard phenolics (**Figures 2A–D**). In contrast to results of the chemical tests, in the plasma model the pure compounds were merely comparable or inferior to the blackthorn extracts in terms of the antioxidant activity. For instance, the range of percentage inhibition of tyrosine nitration achieved for the standards (ca. 11–34%) was lower than for the extracts (ca. 19–58%) applied at the same concentration of 5 μg/mL (**Figure 2A**). Moreover, the activity of MED and EAF at 5 μg/mL was significantly higher ($p < 0.001$) than that of all standards. Furthermore, quercetin—the strongest antioxidant in chemical models—counteracted the plasma nitration significantly less effectively than all extracts (**Figure 2A**; $p < 0.001$). Similar trend was observed for $ONOO^-$-induced lipid peroxidation, both with FOX-1 detection—only rutin exhibited protective activity comparable to that of the extracts at 5 μg/mL (**Figure 2B**; $p > 0.05$), and with TBARS test—overlapped ranges of percentage inhibition of peroxidation were found for the standards (ca. 26–32%) and the extracts (21–34%) at 5 μg/mL (**Figure 2C**; $p > 0.05$). Only in the FRAP assay, two of the standards—quercetin and chlorogenic acid—enhanced the

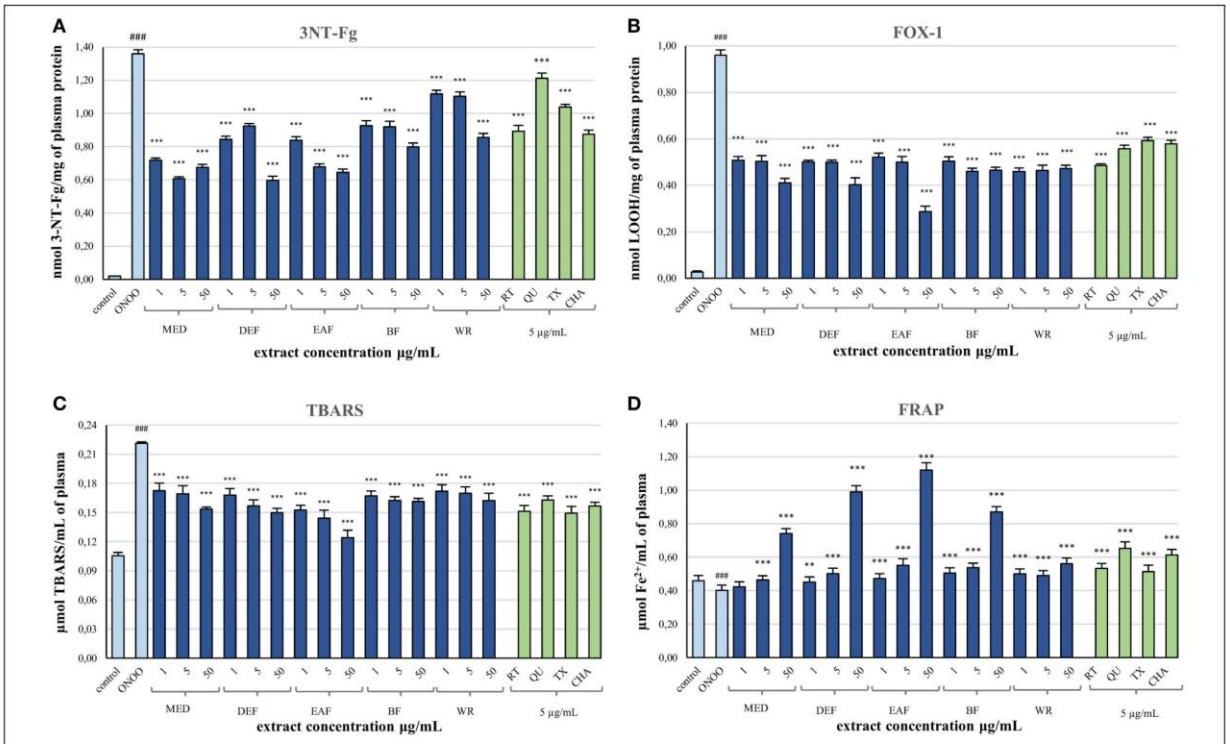

FIGURE 2 | Effects of *P. spinosa* flower dry extracts on human plasma exposed to oxidative stress: **(A)** effects on the nitration of tyrosine residues in plasma proteins and formation of 3-nirotyrosine, 3-NT-Fg; effects on the peroxidation of plasma lipids including formation of lipid hydroperoxides, LOOH **(B)**, and thiobarbituric acid-reactive substances, TBARS **(C)**; **(D)** effects on ferric reducing ability of plasma, FRAP. Results are presented as means ± SE ($n = 10$) for repeated measures: ###$p < 0.001$ for control plasma vs. $ONOO^-$-treated plasma (without the extracts); **$p < 0.01$, ***$p < 0.001$ for $ONOO^-$-treated plasma in the presence of the extracts (1, 5, or 50 µg/mL) or standards (5 µg/mL) vs. $ONOO^-$-treated plasma in the absence of the extracts. Standards: RT, rutin; QU, quercetin; TX, Trolox; CHA, chlorogenic acid.

reducing ability of plasma more strongly than the extracts at the corresponding concentration (**Figure 2D**; $p < 0.001$).

Inhibitory Effects on Pro-Inflammatory Enzymes

The extracts inhibited the activity of lipoxygenase (LOX) and hyaluronidase (HYAL) in a dose-dependent manner, but with different responses toward particular enzyme (**Table 5**). Considering the IC_{50} values expressed in µg/U, the extracts were stronger inhibitors of LOX than HYAL. In both tests the strongest effects observed for EAF and DEF were intermediate between those of phenolic standards and indomethacin, a strong nonsteroidal anti-inflammatory drug. The inhibitory activity toward both enzymes was strongly correlated with the TPC levels ($|r| > 0.88$, $p < 0.05$). The responses in the LOX test were also strongly TFC-dependent ($r = -0.8868$, $p < 0.05$), while in the HYAL assay this relationship was noticeable ($r = -0.8691$) but not significant ($p = 0.056$).

Influence on Cells Viability

The potential cytotoxicity of the extracts was evaluated in a model of PBMCs after 60 and 120 min of incubation with the extracts at 5 µg/mL. Cellular safety of the extracts was evidenced by the lack of significant differences ($p > 0.05$) in cell viability observed between PBMCs incubated with the extracts (85.6–90.5% viability) and the control (untreated) samples (87.85–90.85 viability), regardless of the incubation time and extraction solvent (**Figure 3**).

DISCUSSION

As beneficial for human health, including prevention of CVD, polyphenol-rich extracts are widely used in the preparation of dietary supplements and pharmaceuticals (Franz et al., 2011). Extraction from natural matrices is thus the crucial step in their utilization as it affects extract composition, activity, and yield. Generally, alcohol and alcohol-water mixtures are the best extractants for low-molecular-weight polyphenols of high antioxidant potential and good bioavailability (Manach et al., 2005). The crude extracts are often fractionated to remove fat-soluble ballast substances (chlorophylls and waxes) and thereby purify and concentrate the phenolic fractions (Rana et al., 2016). The results from the present study confirmed these observations: the defatted methanol-water (7:3, v/v) extract from the blackthorn flowers (MED), demonstrated to be a rich source

TABLE 5 | Inhibitory activity of *P. spinosa* flower dry 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	327.36 ± 5.93[F]	7.85	51.74 ± 2.16[E]	23.00
DEF	150.36 ± 4.47[C]	3.60	21.40 ± 0.76[B]	9.51
EAF	135.36 ± 5.55[B]	3.25	21.27 ± 0.16[B]	9.45
BF	171.10 ± 1.36[E]	4.11	42.23 ± 0.99[D]	18.77
WR	479.50 ± 3.38[G]	11.50	121.72 ± 5.73[F]	54.10
QU	89.23 ± 2.13[A]	2.14	30.78 ± 1.84[C]	13.65
RT	162.70 ± 3.70[D]	3.90	54.63 ± 2.61[E]	24.23
CHA	166.83 ± 7.15[D,E]	4.00	28.59 ± 1.21[C]	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 positive control (QU, quercetin; RT, rutin; CHA, chlorogenic acid; IND, indomethacin). For extract codes see ***Table 1****. Different superscripts (capitals) in each column indicate significant differences in the means at $p < 0.05$. [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).*

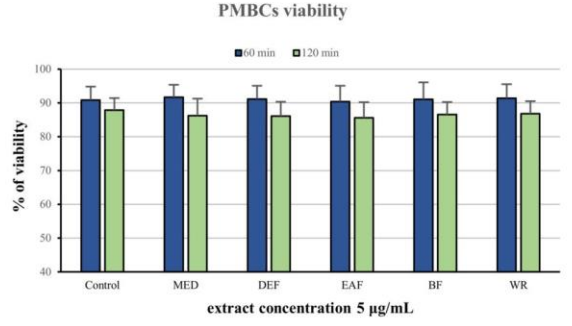

FIGURE 3 | Viability of peripheral blood mononuclear cells (PMBCs) after 60 and 120 min of incubation with *P. spinosa* flower dry extracts at 5 μg/mL.

of phenolic compounds, could be further enriched by liquid-liquid partitioning with organic solvents (**Table 2**). The TPC in the richest fractions EAF and DEF, as determined by the Folin-Ciocalteu (FC) assay, were comparable to those found in the extracts considered important in CVD prevention, for instance in commercial ethanol extracts of grape seed (ca. 630–670 mg GAE/g dw) (Baydara et al., 2004) or in ethyl acetate fractions of green tea and green mate (ca. 480–580 mg GAE/g dw) (Erol et al., 2009). The previous literature data referring to *P. spinosa* are limited to studies on fruits, branches, and leaves. According to Pinacho et al. (2015), the highest TPC level quantified by the Prussian Blue method (PB) was found for ethanol fraction from branches (732.34 mg GAE/g dw), followed by those from fruits (359.11 mg GAE/g dw) and leaves (228.56 mg GAE/g dw). Despite some differences in chemistry of the PB assay in comparison to the standardized FC test, in the preliminary studies focused on selection of the plant material for the present work, we have observed the magnitude of phenolic levels to some extent similar, which enables ordering of the blackthorn tissues in respect to the TPC values as follows: branches > flowers > fruits ≥ leaves. Therefore, the flowers were found promising for further studies.

The present paper is the first presenting comprehensive LC-MS profile of *P. spinosa* flowers (**Figure 1**, **Table 1**). The previous studies aimed at isolation and resulted in structure elucidation of 16 flavonoids (Sakar and Kolodziej, 1993; Olszewska and Wolbiś, 2001, 2002a) and five proanthocyanidins (Kolodziej et al., 1991), the presence of which was now confirmed in the flower dry extracts. In addition, 36 constituents, mainly flavonoids, and phenolic acids, were reported here for the first time for the blackthorn flowers (**Table 1**). With nearly 60 components detected, in contrast to only 25 observed in the leaves (Owczarek et al., 2017), 26 in branches (Pinacho et al., 2015), and 29 in fruits (Guimarães et al., 2013), the phenolic matrix of the flowers is obviously the most complex. A distinctive qualitative feature of the flower extracts is a vast diversity of the flavonoid fraction (thirty seven peaks). While according to Owczarek et al. (2017) flavonoids prevail also in the leaf samples, only 14 structures have been detected in this organ. The branches and fruits of *P. spinosa* may be in turn distinguished by the presence of numerous proanthocyanidins (twelve peaks in branches) and anthocyanins (eight peaks in fruits), respectively (Guimarães et al., 2013; Pinacho et al., 2015). In phytotherapy, the blackthorn flowers are indeed recognized as a flavonoid herbal product (Hoppe, 1981; Poonam et al., 2011). High content of flavonoids found in the present study and their prevalence in flower dry extracts (**Table 2**), were in agreement with these findings. In addition to flavonoids, the analyzed extracts contained moderate levels of proanthocyanidins and phenolic acids (**Table 2**). The TPA level of MED is in accordance with the results reported recently by Ropiak et al. (2016) for aqueous acetone extracts of blackthorn flowers (40 mg/g dw). As indicated in the latter paper, the average degree of polymerization of flower procyanidins is 2.9, which means that the majority of TPA fraction is within the bioavailable range of molecular masses (Manach et al., 2005). Thus, the composition of the flower extracts of blackthorn appears promising in the context of CVD and potential industrial application. Regular intake of flavonoids with the diet or supplements is associated with reduced risk of cardiovascular episodes and mortality (Alissa and Ferns, 2012). In *in vivo* studies, they have been shown to improve antioxidant status, exert anti-atherosclerotic and anti-atherothrombotic effects in early stages of atherosclerosis development (e.g., decrease LDL oxidation), modulate lipid metabolism (e.g., normalize LDL/HDL profile), improve capillary permeability (vasoprotective effects) and endothelial function, and increase nitric oxide release (vasodilatory effects) (Middleton et al., 2000; Alissa and Ferns, 2012). As the most common group of polyphenols in the human diet, flavonoids are also regarded as safe in internal applications. Like flavonoids, both low-molecular-mass proanthocyanidins and phenolic acids have been shown to modulate lipid metabolism, increase plasma antioxidant capacity, improve vascular functions, and reduce platelet activity in humans (Manach et al., 2005).

As antioxidant activity of polyphenols is one of the most important aspects of their beneficial effects in CVD (Middleton et al., 2000; Alissa and Ferns, 2012; Quiñones et al., 2013;

Santilli et al., 2015) we decided to verify it in complementary chemical and biological models that reflect various direct and complex mechanisms. 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. In the present study, we employed three the 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 demonstrated that the flower extracts are potent and universal antioxidants, and that their dose-dependent activity is determined by the contents of phenolics, primarily flavonoids, and strongly influenced by the fractionation solvent (**Tables 3**, **4**). Also the extracts produced from other organs of *P. spinosa* have recently been reported effective DPPH scavengers, as well as inhibitors of lipid peroxidation (Guimarães et al., 2014; Pinacho et al., 2015). Comparing our results to those of Pinacho et al. (2015) and in terms of capacity of BHA after 60 min of incubation, it seems that the activity of flower extracts (EAF, DEF) is comparable to that of branches, and superior to those of leaves and fruits. Moreover, the comparison of our results from TBARS assay and the results obtained by Guimarães et al. (2014) in terms of activity of Trolox, shows that the lipid peroxidation inhibitory activity of flower extracts (EAF, DEF) is higher than that of phenolic-enriched extracts from wild fruits. On the other hand, the outcomes from DPPH test suggest similar scavenging activity for flower and fruit extracts.

To give a more accurate approximation of the possible *in vivo* effects of the flower extracts, we extended the *in vitro* study to the model of human plasma exposed to oxidative stress. The stress conditions were induced by peroxynitrite ($ONOO^-$), a powerful oxidative and nitrative species, 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 et al., 1999). The concentration of $ONOO^-$ used in our *in vitro* study (100 μM) enables quantitative measurements of the changes induced in plasma components and corresponds to its levels *in vivo*, that can be reached in local compartments in 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 et al., 2007). During the study, the plasma samples were pre-incubated with the extracts at the levels of 1–50 μg/mL, equivalent to 0.06–29.2 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 (Manach et al., 2005). For example, it has been reported that 100-mg dose of quercetin from onions and 150-mg dose of pure isoquercitrin (quercetin-3-glucoside) resulted in the concentrations up to 7.6 and 5 μM in plasma, respectively, which is an equivalent of 1.5–2.3 μg/mL of quercetin (Hollmann, 2004; Manach et al., 2005). It has also been suggested that the bioavailability of kaempferol and its glycosides, the dominant flavonoids of blackthorn flowers according to our results, is higher than that of quercetin (DuPont et al., 2004). Therefore, the lower levels of the extracts (1–5 μg/mL) used in the 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 et al., 2011; Kolodziejczyk-Czepas et al., 2013, 2014), the extracts were studied also at 50 μg/mL to enable that of observation of all possible effects of their interaction with peroxynitrite.

The results from biological model confirmed our hypothesis that antioxidant activity of the *P. spinosa* flower extracts may be crucial in understanding their beneficial effects in CVD *in vivo*. The analyzed extracts not only enhanced the total antioxidant status of the $ONOO^-$-treated plasma but also effectively reduced the levels of well-known oxidative stress biomarkers—products of protein nitration (3-nitrotyrosine) and lipid peroxidation (hydroperoxides and TBARS) (**Figure 3**). In CVD patients, the increased levels of these biomarkers are good predictors of cardiovascular events and are correlated with prothrombotic, proatherogenic, and pro-inflammatory intravascular effects, plaque instability, and endothelial dysfunctions (Lee et al., 2012; Thomson, 2015). The mechanism of action of the blackthorn extracts in the protection of plasma proteins and lipids probably involves a direct scavenging of peroxynitrite or/and secondary radicals formed in the induced chain reactions. It is consistent with the noticeable scavenging potential of the extracts toward 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 et al., 2001). Despite some arguments being presented for the impact of blackthorn polyphenols on the target activity in plasma, the differences between the examined extracts were not as evident as in the case of chemical tests. The discrepancies may be an effect of complexity and ontogenetic diversity of blood plasma, as well as differences in the sensitivity to $ONOO^-$ between the plasma samples. Some interactions between endogenous plasma constituents and blackthorn phenolics could also be the reason. 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 comparing to the pure standards. Finally, it is of note that all beneficial effects of the extracts on plasma were measurable at physiological concentrations (1–5 μg/mL), which do not deteriorate the viability of PBMCs and may be regarded as safe.

Pathogenesis of CVD covers, apart from oxidative stress, also a closely related process of inflammation (Biswas, 2016). To evaluate the potential anti-inflammatory effects of the blackthorn extracts, we decided to study their impact on two model pro-inflammatory enzymes. The first belongs to the family of lipoxygenases, enzymes catalyzing dioxygenation of polyenoic fatty acids in biological membranes and lipoproteins, and producing key chemokines and ROS, such as leukotrienes and $O_2^{\bullet -}$, associated with the development of oxidative stress-related inflammatory pathologies of CVD, e.g., myocardial infarction/reperfusion injury (Schneider and Bucar, 2005). The second one—hyaluronidase—is a spreading factor increasing

tissues (i.a. vascular) permeability during the inflammatory processes by degrading hyaluronan, an anti-inflammatory extracellular matrix component (Girish et al., 2009). Both enzymes are targets of many synthetic drugs used in anti-inflammatory therapies, but it has been shown that plant extracts, especially those containing phenolics, can also significantly inhibit their activity (Schneider and Bucar, 2005; Piwowarski et al., 2011). Indeed, the investigated extracts, rich in polyphenols, were found to be inhibitors of both enzymes (**Table 5**). The activity of the most active fractions was between those of the commercial agents—indomethacin (nonsteroidal anti-inflammatory drug) and rutin (flavonoid vasoprotective agent), while the basic methanol-water extract was slightly less active. However, with the results of antioxidant activity tests, these findings suggest that the extracts might influence both interdependent processes of oxidative stress and inflammation.

CONCLUSIONS

This work is the first comprehensive phytochemical and activity study of the flower extracts of *P. spinosa*. The detailed LC-MS patterns presented here can be recommended as reference fingerprints useful in authentication and standardization studies of blackthorn flowers and flower extracts. Distinct phenolic profiles, significant antioxidant effects comparable to or surpassing the activity of pure phenolics in both chemical and biological models, noticeable inhibitory effects on pro-inflammatory enzymes, and cellular safety suggest that the investigated extracts may be promising candidates for the production of pharma- and nutraceuticals effective in cardiovascular protection. Considering both the yield and the activity, the defatted methanol-water (7:3, v/v) extract and its diethyl ether and ethyl acetate fractions appear to be the most advantageous for biological applications. As the observed capacities might be considered as some of the mechanisms behind the activity of blackthorn phenolics within the circulatory system, the present study forms a basis for their further studies in the context of CVD. For instance, the demonstrated protective effects of the extracts against $ONOO^-$-induced changes in plasma components and ability to inhibit pro-inflammatory enzymes might be cross-linked with their possible anti-atherogenic, anticoagulant, and antiplatelet functions or ability to influence endothelium, which should be addressed in future research.

AUTHOR CONTRIBUTIONS

AM, AO, and MO planned the experiments and wrote the manuscript. AM, AO, MM, AP, and JK-C performed the experiments. AM and AO carried out data analysis. PN acted as the consultant on biological methods. MO supervised all work. All authors critically revised and approved the final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publikacja III

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New insights into antioxidant activity of *Prunus spinosa* flowers: Extracts, model polyphenols and their phenolic metabolites in plasma towards multiple *in vivo*-relevant oxidants

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ABSTRACT

Prunus spinosa L. flowers are a traditional herbal medicine recommended for the adjunctive treatment of oxidative stress-related diseases. In search of the main bioactive constituents of the flowers, the antioxidant activity of dry extracts obtained thereof by fractionated extraction was tested *in vitro* towards primary *in vivo*-relevant reactive oxygen species ($O_2^{\cdot-}$, $OH^{\cdot}$, $NO^{\cdot}$, H_2O_2, $ONOO^-$, HOCl) and juxtaposed with the activity of eight model native polyphenols and five compounds considered to be their main metabolites in human plasma. All extracts exhibited concentration- and phenolic-dependent scavenging potential towards the tested oxidants, and the highest responses were observed for $O_2^{\cdot-}$, $OH^{\cdot}$, $ONOO^-$, and HOCl. The total antioxidant capacity of the extracts towards the six oxidants, expressed as sum of ascorbic acid (AA) equivalents, was in the range of 4.8–29.2 µmol AA/mg dw. The most active model polyphenols were quercetin, avicularin, procyanidin A2 and chlorogenic acid (44.5–72.5 µmol AA/mg), while the prevalent in the extracts kaempferol derivatives exhibited relatively weak capacity (11.8–27.0 µmol AA/mg). Among the metabolites, dihydrocaffeic acid (68.8 µmol AA/mg) and 2-(3′,4′-dihydroxyphenyl)acetic acid (63.2 µmol AA/mg) were particularly active – in some tests exhibited stronger activity than their parent compounds. The work gives an insight into the role of native and metabolised phenols as contributory factors to systemic activity of flower extracts and proposes the analytical markers for their standardisation.

1. Introduction

Reactive oxygen/nitrogen species (ROS/RNS) are important physiological molecules involved, among others, in cell signalling and host defence (Dröge, 2002). However, an uncontrolled and excessive ROS/RNS production may overwhelm the antioxidant capacity of the cell and lead to deleterious oxidative stress, which is implicated in the pathogenesis of numerous chronic diseases (Niki, 2016a). As ROS production is related to oxygen consumption, the circulatory system is especially prone to oxidative stress that contributes to the development and/or progression of cardiovascular disorders (CVD) – the leading cause of death and disabilities in the Western societies (Taverne et al., 2013). Polyphenols and polyphenol-rich plant extracts, as natural antioxidants, are often investigated as a potential treatment option in prophylaxis and adjunctive therapy of CVD, and their mechanisms of action, including possible interactions with ROS/RNS, are closely looked into (Pandey and Rizvi, 2009). The *in vivo*-operating ROS/RNS comprise molecules of various chemical behaviour, including free radicals such as $O_2^{\cdot-}$, $OH^{\cdot}$, and $NO^{\cdot}$, the non-radical oxidants such as H_2O_2, $ONOO^-$ and HOCl, as well as products of their downstream reaction cascade with cellular components. All these species differ in function, reactivity and reaction mechanisms, forming an aggressive

Abbreviations: AA, ascorbic acid; AV, avicularin (quercetin 3-*O*-α-L-arabinofuranoside); BF, *n*-butanol fraction; CHA, chlorogenic acid; DCA, 3-(3′4′-dihydroxyphenyl)propionic acid (dihydrocaffeic acid); DEF, diethyl ether fraction; EAF, ethyl acetate fraction; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; JU, juglanin (kaempferol 3-*O*-α-L-arabinofuranoside); KA, kaempferol; KT, kaempferitrin (kaempferol 3,7-di-*O*-α-L-rhamnopyranoside); MED, defatted methanol-water 7:3 *v/v*) extract; MQ, miquelianin (quercetin 3-*O*-β-D-glucuronopyranoside); $NO^{\cdot}$, nitric oxide; $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical; $ONOO^-$, peroxynitrite; PA2, proanthocyanidin A2; PAA, 2-(3′4′-dihydroxyphenyl)acetic acid; *p*-CA, *p*-coumaric acid; PCA, protocatechuic acid; PPA, 3-(4′-hydroxyphenyl)propionic acid; QU, quercetin; ROS/RNS, reactive oxygen/nitrogen species; TX, ± -6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); WR, water residue

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cocktail of oxidants able to interact with the most critical biomolecules including proteins, lipids and nucleic acids (Biswas, 2016). On the other side of the equation, there is the huge chemical variability of the polyphenols themselves, with different selectivity and specificity towards different ROS/RNS. Thus, to fully understand the interactions between the two groups, simultaneous investigation of the activity of natural substances towards multiple oxidants is required (Niki, 2016b; Prior et al., 2016).

Blackthorn (*Prunus spinosa* L., Rosaceae) flower is a polyphenol-rich plant material valued in the medicinal tradition of Central and Eastern Europe as a vasoprotective, anti-inflammatory, diuretic, detoxifying (blood purifying), and spasmolytic agent (Blumenthal and Busse, 1998; Hoppe, 1981; Wawrzyniak, 1992). Its application in compound recipes, recommended, among others, in various cardiac complaints such as inflammation of the heart muscle, cardiac neurosis or atherosclerosis (Blumenthal and Busse, 1998; Hoppe, 1981; Wawrzyniak, 1992) as well as the results of the preliminary *ex vivo* and *in vivo* animal studies indicating beneficial cardiovascular effects of the blackthorn extracts (Lisevitskaya et al., 1968; Makarov, 1968, 1972; Makarov and Khadzhai, 1969) prompted us to investigate more closely this plant material and its possible molecular effects in the context of CVD. Our previous study demonstrated that the antioxidant capacity of *P. spinosa* flower extracts might be counted as one of the mechanisms behind their activity within the circulatory system reported by traditional medicine (Marchelak et al., 2017). In particular, we have demonstrated the protective effects of the extracts against oxidative and nitrative damage to human plasma components under oxidative stress conditions induced *in vitro* by $ONOO^-$, one of the strongest oxidants operating *in vivo*. The beneficial action of the extracts at *in vivo*-relevant concentrations (1–5 μg/mL of plasma) was reflected in the reduction of the levels of main oxidative stress biomarkers (3-nitrotyrosine, lipid hydroperoxides, and thiobarbituric acid-reactive substances) and in the enhancement of the total non-enzymatic antioxidant status of plasma (NEAC) (Marchelak et al., 2017). However, the molecular mechanisms of these effects remain unexplored. In particular, there is no information on the interactions of the *P. spinosa* extracts and constituents with $ONOO^-$ as well as other ROS/RNS which might be implicated in the generation of oxidative stress in biological systems.

Moreover, our studies on phytochemical profile of the flower extracts proved the huge complexity of the phenolic matrix (nearly 60 components detected in the qualitative LC–MS/MS analysis), which comprised constituents from various chemical groups such as flavonoids with rare in nature flavonol pentosides (arabinosides, xylosides, rhamnosides), flavan-3-ol derivatives including A-type procyanidin dimers, as well as simple phenolic acids and quinic acid pseudodepsides (Marchelak et al., 2017) with potentially various impact on the activity of the plant material. Closer insight into these differences might provide valuable clues for the understanding of activity mechanisms of the extracts and for the establishing of bioactivity markers for the purposes of future standardisation and quality control.

One other important aspect of the activity of plant derived products, often omitted during routine studies, is their metabolism. During intestinal transit, plant polyphenols undergo transformations due to the conditions in the digestive tract and microbiota activity and often enter the bloodstream in a modified form, and further changes are introduced after absorption (Manach et al., 2004). Thus, the overall biological effects of polyphenols *in vivo* can be both due to the native compounds (if absorbed unmodified) as well as their metabolites.

Therefore, the aim of the present work was to provide a closer insight into the antioxidant capacity of *P. spinosa* flower extracts and its primary constituents towards multiple ROS/RNS of physiological significance ($O_2^{\cdot-}$, $HO^{\cdot}$, H_2O_2, $NO^{\cdot}$, $ONOO^-$, HOCl) and to juxtapose this activity with that of a series of phenolic compounds considered to be the main metabolites of polyphenols in human body and corresponding to the phenolic profile of the examined extracts.

2. Results and discussion

2.1. Antioxidant activity of P. spinosa flower extracts against multiple oxidants

Oxygen metabolism that gives rise to various ROS/RNS is a multistep process, the critical point of which is formation of $O_2^{\cdot-}$, the primary reactive species produced in human cells (Dröge, 2002). The endogenous sources of $O_2^{\cdot-}$ include NADPH oxidases, xanthine oxidases, cyclooxygenases, lipoxygenases, NO synthases, the mitochondrial electron-transport chain, and cytochrome P450s. Importantly, $O_2^{\cdot-}$ serves as a progenitor of many other oxidizing agents operating, among others, in the circulatory system, including $OH^{\cdot}$, H_2O_2, $ONOO^-$ and HOCl (Biswas, 2016; Taverne et al., 2013). For instance, superoxide dismutase (SOD) converts $O_2^{\cdot-}$ to H_2O_2, which is the most stable and diffusible form of ROS, selectively reactive towards cysteine residues of proteins. In the presence of various *in vivo* reductants H_2O_2 can generate $OH^{\cdot}$, the most reactive species known to chemistry, able to attack and damage almost every molecule in the living cell (Biswas, 2016). HOCl, generated in stimulated neutrophils *via* the myeloperoxidase-catalysed reaction of H_2O_2 with chloride ions, is a powerful antimicrobial agent but at the same time, it is a highly destructive and non-selective oxidant involved in CVD progression (Ford, 2010). The good example of a dual nature of ROS/RNS is also $NO^{\cdot}$, formed in biological tissues through the oxidation of L-arginine to citrulline by distinct izoformes of NO synthases. When produced by endothelial NO synthase (eNOS) in the vessel endothelium it plays an important role in vessel dilatation and inflammatory protection (inhibition of leukocytes adhesion) but if synthesised in stimulated macrophages by inducible NO synthase (iNOS), it comes into contact with $O_2^{\cdot-}$ and rapidly forms the toxic $ONOO^-$ (Förstermann and Sessa, 2012; Szabo et al., 2007). The $ONOO^-$-mediated oxidative damage to biomolecules *in vivo*, especially protein nitration, is one of the most dangerous processes in the vascular system, and may result from direct interactions with this highly reactive oxidative and nitrative agent or from the secondary oxidants derived from $ONOO^-$, such as $NO^{\cdot}$ or $^{\cdot}NO_2$ (Szabo et al., 2007; Niki 2016a, b).

Considering the critical importance of the above mentioned low-molecular ROS/RNS for the generation of oxidative stress *in vivo*, we selected six of them ($O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$, HOCl, $NO^{\cdot}$, $ONOO^-$) to continue and extend our study on the antioxidant properties of *P. spinosa* flower extracts (Marchelak et al., 2017). The starting point for the present investigation were the fractionated dry extracts, *i.e.* the source extract MED and its fractions of different polarity (DEF, EAF, BF, WR), obtained during the earlier study (Marchelak et al., 2017) by liquid-liquid partitioning. The phytochemical profiles of the extracts were characterised in details (Marchelak et al., 2017) and their total phenolic contents (TPC) varied in the range of 64.6–584.07 mg gallic acid equivalents (GAE)/g dw (Fig. 1). To enable the comparison of the extracts efficiency towards different ROS/RNS, the results of each test were expressed as SC_{50} values (Fig. 2) and as equivalents of known antioxidant standards (Table 1) – AA, the primary antioxidant of human plasma *in vivo*, and TX, a synthetic analogue of vitamin E. In the latter case, the sum of TX equivalents and AA equivalents from the individual tests (TE_{MR6} and AAE_{MR6}, respectively) was applied as a measure of the total antioxidant effectiveness.

Each of the investigated extracts showed significant and concentration-dependent ability to scavenge all of the abovementioned ROS/RNS (Fig. 2). The TE_{MR6} and AAE_{MR6} values for the extracts varied in a wide range of 3.8–94.1 μmol TX/mg dw and 4.8–29.2 μmol AA/mg dw, with the highest total activity parameters found for EAF and DEF. These results are consistent with our previous findings, which showed that EAF and DEF exhibit the strongest antioxidant properties, both in simple chemical tests in non-biological environments (DPPH, FRAP, TBARS) and in human plasma stimulated by $ONOO^-$ (Marchelak et al., 2017). Moreover, in most of the present tests, the activity of the extracts

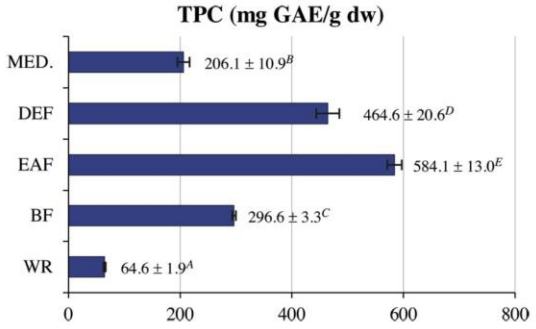

Fig. 1. Total phenolic contents (TPC) in the analysed flower extracts according to Marchelak et al. (2017). Results are presented as mean values ± SD ($n = 3$) and expressed in gallic acid equivalents (GAE). Different letters indicate significant differences in the means at $p < 0.05$. For detailed quantitative profiles see Marchelak et al. (2017).

decreased in a similar order, *i.e.* EAF ≥ DEF ≥ BF > MED > WR – the exception was the NO˙-scavenging assay, in which DEF was proved to be the most active extract. Interestingly, the activity parameters strongly correlated with the TPC levels, and these correlations were more evident than those observed in our previous study in the relevant chemical tests of DPPH scavenging and AAPH-induced linoleic acid peroxidation measured by TBARS (Marchelak et al., 2017). The correlation coefficients (r) for H_2O_2, OH˙, and $ONOO^-$ scavenging activity were 0.9595, 0.9569, 0.9807 ($p < 0.05$), respectively. The analogous correlations for $O_2^{\cdot-}$, HOCl, and NO˙ scavenging were also noticeable ($r = 0.8947$, 0.9109, 0.9111, respectively) but statistically not significant ($p > 0.05$), probably due to the relatively low number of data points ($n = 5$). Close relationships were also found for the total antioxidant capacity parameters and TPC levels, and the correlation coefficients (r) were 0.9836 and 0.9705 for TE_{MR6} and AAE_{MR6}, respectively ($p < 0.05$). These results suggest that the scavenging capacity of blackthorn flower extracts towards the *in vivo*-relevant ROS/RNS is strongly phenolic-dependent.

As compared to AA and TX, the strongest scavenging effects of the extracts were revealed towards OH˙, $O_2^{\cdot-}$, $ONOO^-$ and HClO (Fig. 2, Table 1). Importantly, the scavenging capacity of the most active extracts was comparable or superior to the activity of both positive standards. In the *in vivo* context, the activity relative to AA is of special importance. Thus, it is worth of notice that the effectiveness of EAF and DEF against OH˙, HOCl and $ONOO^-$ was higher or not significantly different ($p > 0.05$) than that of AA (Fig. 2). The strong ability of the extracts to inactivate OH˙ and $ONOO^-$ seems to be especially relevant,

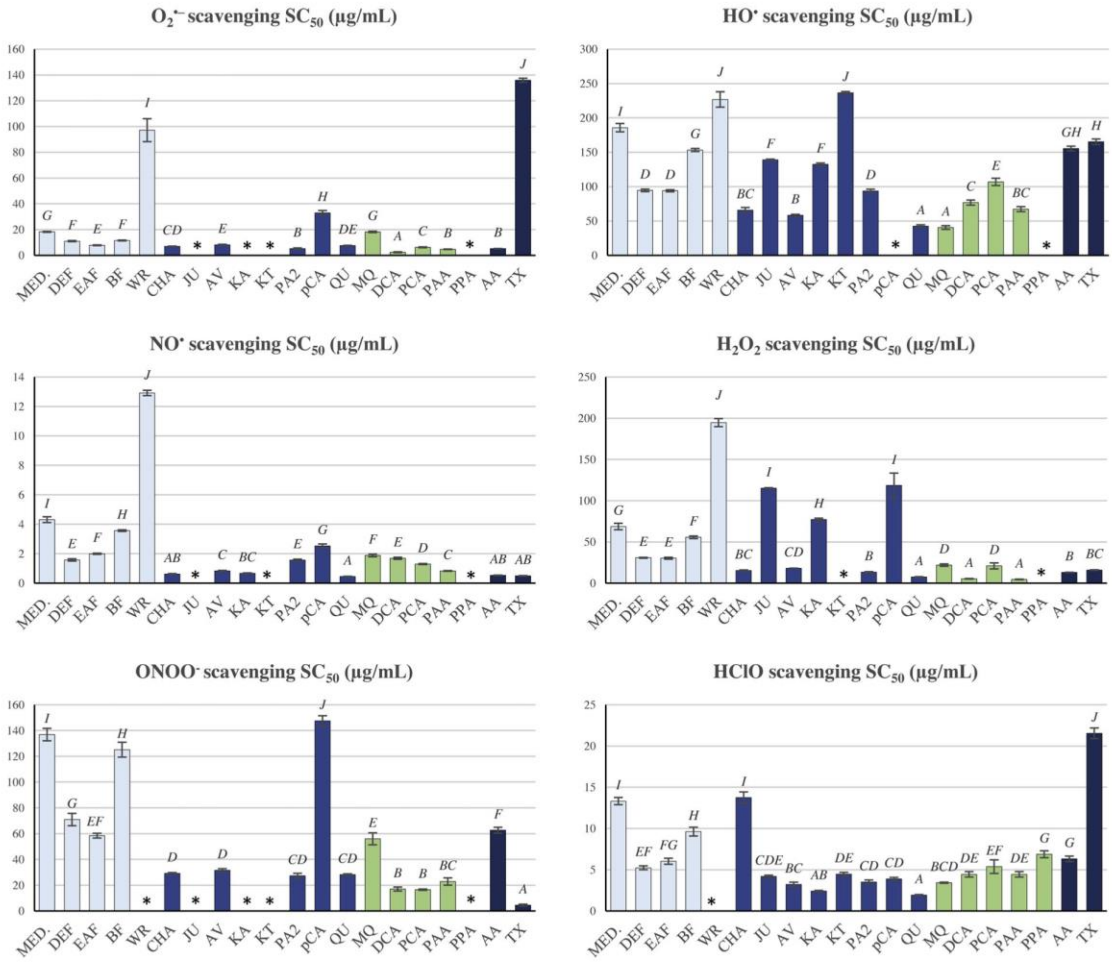

Fig. 2. Antioxidant activity of *P. spinosa* flower extracts, model polyphenols and their potential *in vivo* metabolites towards *in vivo*-relevant oxidants. Results are presented as mean values ± SD ($n = 3$). For each parameter different letters *A–J* indicate significant differences ($p < 0.05$). The analytes marked with asterisks are inactive in concentrations up to 150 µg/mL ($O_2^{\cdot-}$ scavenging test), 700 µg/mL (HO˙ scavenging test), 100 µg/mL (NO˙ scavenging test), 300 µg/mL (H_2O_2 scavenging test), and 200 µg/mL ($ONOO^-$ and HClO scavenging test). Bars colours: light blue – *P. spinosa* flower extracts; blue – model polyphenols; green – phenolic metabolites; navy blue – standards (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Scavenging capacity of the analytes towards multiple ROS/RNS in equivalents of standard antioxidants.

Analyte	Scavenging capacity (μmol AAE/mg dw)							(μmol TE/mg dw)						
	$O_2^{\cdot-}$	$HO^{\cdot}$	$NO^{\cdot}$	H_2O_2	$ONOO^-$	HClO	AAE_{MR6}	$O_2^{\cdot-}$	$HO^{\cdot}$	$NO^{\cdot}$	H_2O_2	$ONOO^-$	HClO	TE_{MR6}
Extracts:														
MED	1.63^{G}	4.75^{I}	0.70^{J}	1.08^{I}	2.59^{H}	2.69^{I}	13.5	29.6^{G}	3.55^{I}	0.46^{J}	0.92^{I}	0.13^{G}	6.46^{I}	41.3
DEF	2.70^{F}	9.34^{E}	1.92^{F}	2.41^{G}	5.02^{EF}	6.86^{F}	28.3	49.2^{F}	6.98^{E}	1.25^{F}	2.06^{G}	0.25^{F}	16.5^{F}	76.2
EAF	3.81^{E}	9.40^{E}	1.52^{G}	2.45^{G}	6.07^{E}	5.95^{FG}	29.2	69.4^{E}	7.03^{E}	0.99^{G}	2.10^{G}	0.31^{F}	14.3^{FG}	94.1
BF	2.58^{F}	5.76^{G}	0.85^{I}	1.33^{H}	3.32^{G}	3.73^{H}	17.6	47.0^{F}	4.31^{G}	0.55^{I}	1.14^{H}	0.17^{G}	8.95^{H}	62.2
WR	0.31^{I}	3.89^{J}	0.23^{K}	0.38^{K}	inact.	inact.	4.81	5.60^{I}	2.91^{J}	0.15^{K}	0.33^{K}	inact.	inact.	3.82
Model polyphenols:														
CHA	4.27^{D}	13.4^{BC}	4.87^{C}	4.81^{DE}	14.5^{C}	2.61^{I}	44.5	77.7^{D}	10.0^{BC}	3.18^{C}	4.12^{DE}	0.62^{D}	6.26^{I}	102.0
JU	inact.	6.35^{F}	inact.	0.64^{J}	inact.	8.56^{E}	15.6	inact.	4.75^{F}	inact.	0.55^{J}	inact.	20.5^{E}	25.8
AV	3.65^{E}	15.1^{B}	3.63^{D}	4.10^{E}	11.3^{D}	11.1^{C}	48.9	66.5^{E}	11.3^{B}	2.37^{D}	3.51^{E}	0.57^{DE}	26.7^{C}	110.8
KA	inact.	6.67^{F}	4.53^{C}	0.96^{I}	inact.	14.9^{B}	27.0	inact.	4.99^{F}	2.96^{C}	0.82^{I}	inact.	35.7^{B}	44.4
KT	inact.	3.73^{J}	inact.	inact.	inact.	8.03^{E}	11.8	inact.	2.79^{J}	inact.	inact.	inact.	19.3^{E}	22.1
PA2	5.67^{B}	9.43^{E}	1.93^{F}	5.55^{D}	13.0^{CD}	10.2^{D}	45.8	103.2^{B}	7.05^{E}	1.26^{F}	4.75^{D}	0.66^{D}	24.5^{D}	141.4
*p*CA	0.90^{H}	inact.	1.20^{H}	0.62^{J}	2.41^{G}	9.25^{D}	14.4	16.5^{H}	inact.	0.78^{H}	0.54^{J}	0.12^{G}	22.2^{D}	40.1
QU	3.94^{DE}	20.8^{A}	6.72^{A}	9.84^{C}	12.6^{D}	18.6^{A}	72.5	71.6^{DE}	15.5^{A}	4.39^{A}	8.43^{C}	0.64^{D}	44.7^{A}	145.3
Phenolic metabolites:														
MQ	1.64^{G}	21.8^{A}	1.61^{G}	3.38^{F}	6.35^{E}	10.5^{D}	45.3	29.8^{G}	16.3^{A}	1.05^{G}	2.90^{F}	0.32^{F}	25.1^{D}	75.5
DCA	12.2^{A}	11.5^{D}	1.79^{FG}	14.0^{B}	20.9^{B}	8.40^{E}	68.8	222.7^{A}	8.60^{D}	1.17^{FG}	12.0^{B}	1.06^{B}	20.2^{E}	265.6
PCA	4.80^{C}	8.26^{E}	2.34^{E}	3.54^{F}	21.5^{B}	6.67^{F}	47.2	87.3^{C}	6.18^{E}	1.53^{E}	3.03^{F}	1.09^{B}	16.0^{F}	115.2
PAA	6.33^{B}	13.1^{C}	3.66^{D}	16.4^{A}	15.6^{C}	8.09^{E}	63.2	115.2^{B}	9.81^{C}	2.39^{D}	14.1^{A}	0.79^{C}	19.4^{E}	161.6
PPA	inact.	inact.	inact.	inact.	inact.	5.21^{G}	5.21	inact.	inact.	inact.	inact.	inact.	12.5^{G}	12.5
Standards:														
AA	5.68^{B}	5.68^{GH}	5.68^{B}	5.68^{D}	5.68^{EF}	5.68^{FG}	34.1	103.3^{B}	4.25^{GH}	3.71^{B}	4.86^{D}	0.29^{F}	13.6^{FG}	130.1
TX	0.22^{J}	5.34^{H}	6.12^{AB}	4.67^{DE}	79.1^{A}	1.67^{J}	97.1	4.00^{J}	4.00^{H}	4.00^{AB}	4.00^{DE}	4.00^{A}	4.00^{J}	24.0

Results are mean values (n = 3, for SDs see Fig. 2) expressed as micromolar equivalents of TX (TE) and AA (AAE) per dry matter. Different superscripts (capitals) in each column indicate significant differences in the means at $p < 0.05$. TE_{MR6} and AAE_{MR6}, sums of TE and AE values from six tests, respectively. Inactivity (inact.) means lack of response in the tests at the levels up to 150 μg/mL ($O_2^{\cdot-}$ scavenging test), 700 μg/mL ($HO^{\cdot}$ scavenging test), 100 μg/mL ($NO^{\cdot}$ scavenging test), 300 μg/mL (H_2O_2 scavenging test), and 200 μg/mL ($ONOO^-$ and HClO scavenging test).

as according to the literature (Niki, 2014, 2016a) these highly destructive and non-selective oxidants appear to be too reactive for endogenous antioxidants to scavenge efficiently. The abovementioned results suggest that the flower extracts of *P. spinosa* might be able to support the endogenous non-enzymatic antioxidant system and inactivate ROS/RNS, especially when the scavenging efficiency of the plasma antioxidants or their level is insufficient. The key factors in comparisons of the reactivity of polyphenols and endogenous antioxidants are apparent second-order rate constants (*k*) of their reactions with ROS/RNS. The accumulated research (Kono et al., 1997; Niki, 2016a; Valdez et al., 2004) indicated that the *k* values for reactions of a variety of plant phenols (*e.g.* epicatechin, myricetin, caffeic acid, chlorogenic acid) with $OH^{\cdot}$ and $ONOO^-$ are at least comparable to that of endogenous antioxidants (AA, glutathione, cysteine, uric acid). Interestingly, the *k* values for reactions of $O_2^{\cdot-}$ are significantly higher for polyphenols ($1.0–1.7 \times 10^6\ M^{-1}\ s^{-1}$) than AA ($3.0 \times 10^5\ M^{-1}\ s^{-1}$) and glutathione ($1.0 \times 10^3\ M^{-1}\ s^{-1}$) (Kono et al., 1997; Niki, 2016a). It might suggest that the investigated extracts, found as effective scavengers of $O_2^{\cdot-}$, may diminish the harmful effects of $OH^{\cdot}$ and $ONOO^-$ in biological systems not only by direct interaction but also by scavenging their precursor ($O_2^{\cdot-}$). On the other hand, $ONOO^-$ is a short-lived oxidant that in biological systems quickly generates or decomposes into highly reactive ROS/RNS, such as $OH^{\cdot}$, $CO_3^{\cdot-}$, $NO^{\cdot}$, or $^{\cdot}NO_2$ (Szabo et al., 2007). It is thus possible that the protective effects of the analysed extracts against $ONOO^-$-induced oxidative damage to human plasma observed in the previous work (Marchelak et al., 2017) might result partly from the direct scavenging of $ONOO^-$ and partly from the influence of the extracts on the secondary radicals formed in the reactions of $ONOO^-$.

2.2. Antioxidant activity of model compounds

Our previous work regarding the phytochemical profile of blackthorn extracts showed huge complexity of the phenolic matrix. A distinctive feature of the flower extracts was a vast diversity of the flavonoid fraction (37 compounds, including quite rare in nature kaempferol and quercetin pentosides and their derivatives acylated with *p*-coumaroyl moiety), in addition to moderate levels of A-type proanthocyanidins and phenolic acids (Marchelak et al., 2017). To investigate which of these constituents are to the highest degree responsible for the antioxidant activity of the extracts, we selected eight model compounds (Figs. 3 and 4) and tested their scavenging efficiency towards the six ROS/RNS. Among the selected analytes were representatives of flavonoid aglycones – KA and QU; flavonoid monoglycosides – JU and AV; flavonoid diglycosides – KT; A-type proanthocyanidins – PA2; caffeoylquinic acids – CHA; and simple hydroxycinnamic acid – *p*CA.

The results demonstrated that the scavenging efficiency of the model compounds differs depending on their chemical structure (Fig. 2, Table 1). The total antioxidant activity expressed as the TE_{MR6} and AAE_{MR6} values varied in the range of 22.1–145.3 μmol TX/mg and 11.8–72.5 μmol AA/mg, with the highest parameters found for QU. This aglycone as well as its glycoside AV revealed the strongest scavenging effects towards the same oxidants as the most active extracts, *i.e.* $O_2^{\cdot-}$, $OH^{\cdot}$, HClO, and $ONOO^-$. In comparison to the both flavonoids, PA2 proved to be a better $O_2^{\cdot-}$ scavenger, while being 50–100% less effective towards $OH^{\cdot}$. The model phenolic acid CHA also exhibited high efficiency in $O_2^{\cdot-}$, $OH^{\cdot}$, and $ONOO^-$-scavenging, nevertheless its ability to inactivate HClO was only moderate. As compared to the four

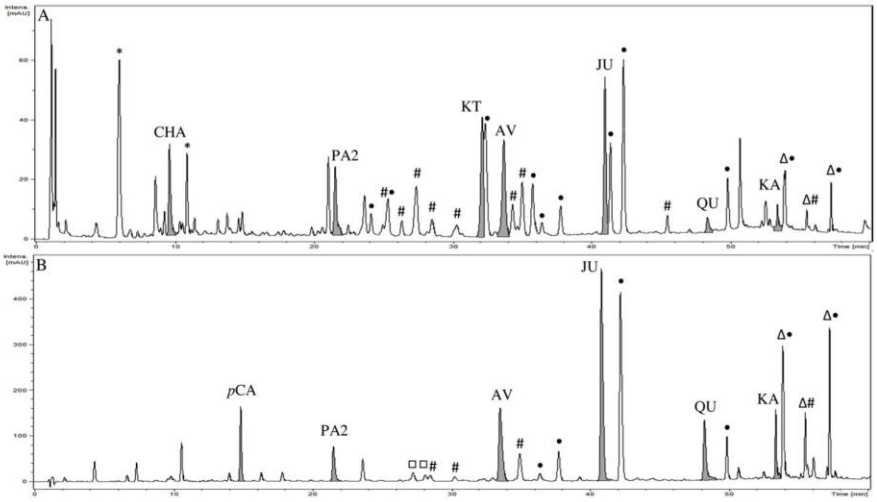

Fig. 3. Representative UHPLC-UV chromatograms of *P. spinosa* flower extracts at 280 nm: (A) MED; (B) DEF. Peaks of model compounds (CHA, PA2, KT, AV, JU, QU, KA, *p*CA) were coloured in grey. Groups of polyphenols represented by model compounds were marked with the symbols: *, CHA isomers; □, A type proanthocyanidins; •, KA glycosides; #, QU glycosides; Δ, QU and KA glycosides acetylated with *p*CA. For details of peaks identification see Marchelak et al. (2017).

abovementioned model compounds, *p*CA, KA, JU and KT, were proven to be much less effective ROS/RNS scavengers. Among this group, KA and its glycosides revealed relevant effects solely towards HClO and $OH^{\cdot}$, while *p*CA was practically inactive towards $OH^{\cdot}$, and only towards HClO its activity was comparable to the other active constituents. The present results demonstrated the strong influence of the *ortho*-diphenolic structure of polyphenols on their antioxidant activity. The flavonol aglycone (QU) with two hydroxyl group at 3′ and 4′ positions exhibited 2.5–3 times higher total antioxidant activity than KA with one hydroxyl group at 4′ position. Furthermore, on the example of QU, KA and their glycosides, it was demonstrated that the scavenging efficiency of flavonoids decrease in the order aglycone > monoglycoside > diglycoside. These findings are in accordance with the well proven fact that the glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (Rice-Evans et al., 1996).

The significant activity of QU, PA2, AV, and CHA as well as the fact that they revealed the strongest scavenging effects towards the same oxidants as the extracts, suggest their decisive impact on the activity of the blackthorn flowers. This is an interesting result, as according to our previous studies, the dominant constituents of the extracts were derivatives of KA (Marchelak et al., 2017), which showed comparatively weaker activity. These findings might indicate the active compounds as valuable activity markers for future standardisation and quality control. However, the high content of KA derivatives might still be relevant as some additive and synergistic effects may impact the final activity of the extracts. For instance, Hidalgo et al. (2010) demonstrated that when KA was paired with QU or QU 3-glucoside a statistically significant increase in antioxidant activity was observed. The increase was about 20% higher than expected theoretical values, which may be counted as the relevant synergy. It is consistent with our results demonstrating the favourable activity of the *P. spinosa* flower extracts in a biological test system (Marchelak et al., 2017), probably attributed to some synergic effects of the extracts constituents.

2.3. Antioxidant activity of phenolic metabolites

Metabolism of polyphenols in the human body is relatively well recognised (Manach et al., 2004; Marín et al., 2015; Ou and Gu, 2014 Tomas-Barberan et al., 2014). After ingestion only free phenolic acids, flavonoid aglycones and some glucosides as well as to some extent also simple flavan-3-ols or proanthocyanidin dimers can be absorbed in the small intestine. Once absorbed, these compounds are subjected to the three main types of conjugation (methylation, sulphation and glucuronidation). Nevertheless, the majority of ingested polyphenols, i.a.

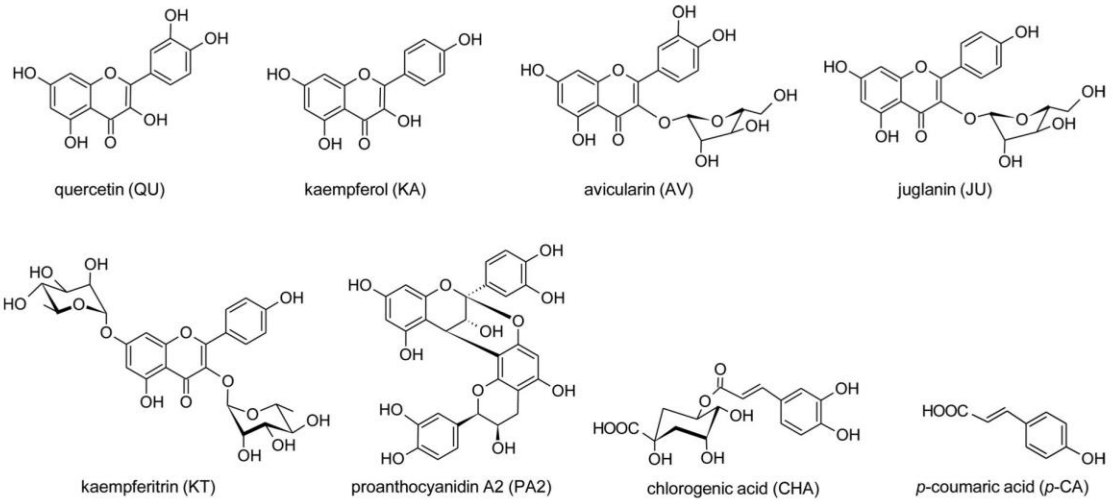

Fig. 4. Structures of the analysed model native polyphenols of *P. spinosa* flower extracts.

phenolic acids present in the esterified form, flavonoid glycosides or proanthocyanidins of higher polymerisation degree, reaches the colon and undergoes microbial degradation. *In vivo* studies showed that microbial derived phenolic acids, including mainly phenylacetic and phenylpropionic derivatives, are the predominant metabolites of polyphenols in blood and urine. For instance, it has been reported that flavonols are mainly transformed to PAA, PCA and DCA (Marín et al., 2015). The microbial-derived PPA, 3-(3′-hydroxyphenyl)propionic acid, 4-hydroxyphenylacetic acid, and phenylvalerolactone are among the primary metabolites of proanthocyanidins (Ou and Gu, 2014), while CHA is first hydrolysed by the colonic microflora to caffeic acid and next transformed to derivatives of phenylpropionic acids, such as DCA and PPA (Tomas-Barberan et al., 2014).

Despite the increasing knowledge on the metabolites of polyphenols, there is up to date not much information on their activity and influence on the efficiency of the polyphenolic extracts. Therefore, in the present study, we extended our research to compounds considered to be the main polyphenolic metabolites in human body. Based on the phenolic profile of the examined extracts and available literature data (Tomas-Barberan et al., 2014;Ou and Gu, 2014 Marín et al., 2015), five compounds were selected for the study, including MQ, the primary product of QU conjugation during the second phase of metabolism, as well as the degradation products of flavonols, flavanols and caffeoylquinic acids by the gut microbiota: DCA, PCA, PAA, and PPA (Fig. 5).

Four of the investigated compounds (MQ, DCA, PCA, and PAA) showed significant and dose-dependent ability to scavenge six the most common *in vivo*-relevant radical/oxidant agents (Fig. 2, Table 1). Their total antioxidant activity (TE_{MR6} and AAE_{MR6}) decreased in the order DCA > PAA > PCA > MQ. It should be emphasized that DCA and PAA were more efficient ROS/RNS scavengers than the most active native constituents of *P. spinosa* flowers (QU, AV, PA2, CHA). The scavenging capacity of PCA was slightly lower than that of QU and comparable to that of AV, CHA and PA2. Both DCA and PAA revealed strong antioxidant effects towards all of the tested oxidants except $NO^{\bullet}$. PCA exhibited similar efficiency towards $O_2^{\bullet-}$, $ONOO^-$, and $HO^{\bullet}$, however it appeared to be less effective in H_2O_2 quenching. The glycosidic metabolite, MQ, was also found to be an effective ROS/RNS scavenger, and its antioxidant capacity was comparable to or slightly lower than that of AV, and twice as low as that of QU. Similarly as in the case of QU and AV, MQ exhibited the highest scavenging effect towards $O_2^{\bullet-}$, HClO, $HO^{\bullet}$, and $ONOO^-$. In contrast, the scavenging capacity of PPA was negligible, which further confirmed the importance of di-*ortho* hydroxyl substitution for the antioxidant effects.

Recently, the direct antioxidant activity of polyphenols as a mechanism of their positive cardiovascular effects is put into doubt; mainly because of relatively low plasma concentrations they attain *in vivo* (Goszcz et al., 2017). However, the research show that the phenolic metabolites especially those derived from microbiota transformation display superior bioavailability parameters than their precursors. For example, in the study by Stalmach et al. (2014) on the absorption and metabolism of CHA isomers from coffee, the colon derived dihydroderivatives of phenolic acids had higher maximal plasma concentration levels and remained in the circulation longer than their parent compounds. Also, the results of bioactivity studies, although few in numbers, similarly as our findings suggest distinct biological effects of some of these analytes. For instance, in the comparative study of the antioxidant activity of wine phenolics and some of their metabolites applying ORAC assay, PAA and DCA exhibited the highest ORAC values, comparable to those of the native constituents (Villaño et al., 2005). Furthermore, PAA and DCA have been reported to possess significant anti-inflammatory activity *in vitro* as well as *in vivo*. Among 18 microbiota-derived polyphenolic metabolites found in faecal water of humans, these compounds displayed the strongest action in various models of inflammation (Larrosa et al., 2009). Moreover, PAA was suggested to be more effective inhibitor of platelet aggregation than QU and rutin (Kim et al., 1998). All these results indicate that phenolic metabolites deserve more attention in the future studies of plant derived products, both in terms of bioavailability and activity.

3. Materials and methods

3.1. General

High-purity reagents and standards for spectrophotometric and fluorometric analysis: xanthine oxidase from bovine milk; xanthine; nitrotetrazolium blue chloride; hydrogen peroxide; horseradish peroxidase; phenol; 4-aminoantipyrine; salicylic acid; iron (II) sulphate heptahydrate; 4,5-diaminofluorescein; 5-thio-2-nitrobenzoic acid; sodium borohydride; ethylenediaminetetraacetic acid; Evans blue; diethylenetriaminepentaacetic acid; PBS buffer; TX; QU dihydrate; KA; CHA; MQ; PCA; PAA; PPA; DCA; and AA were purchased from Sigma-Aldrich (Seelze, Germany/St. Louis, MO, USA), while sodium nitroprusside and NaOCl were obtained from Avantor Performance Materials (Gliwice, Poland); PA2 was purchased from Phytolab (Vestenbergsgreuth, Germany); phosphate-buffered saline (PBS) was purchased from Biomed (Lublin, Poland). AV; JU; and KT were isolated previously in our laboratory from the flowers of *P. spinosa* (Olszewska and Wolbiś, 2001, 2002). All other chemicals and solvents were of analytical grade and obtained from Avantor (Poland). In all analyses redistilled water was used. Samples were incubated in a constant temperature using a BD 23 incubator (Binder, Tuttlingen, Germany). 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).

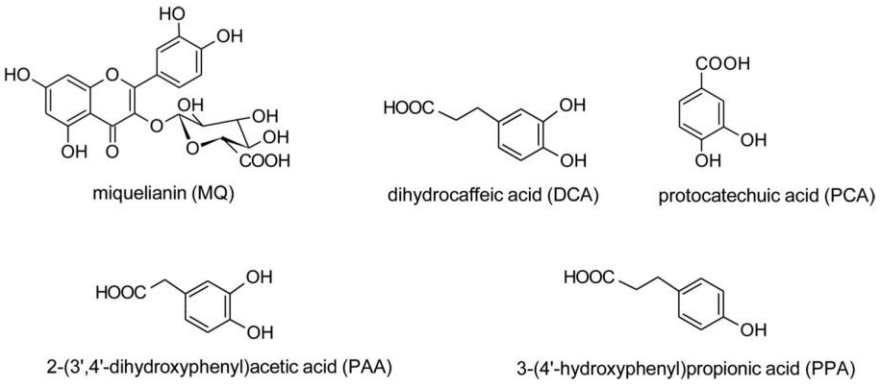

Fig. 5. Structures of the analysed phenolic metabolites.

3.2. Plant material and extracts preparation

The analyses were performed using extracts (MED, DEF, EAF, BF, WR) obtained by fractionated extraction from the flowers of *P. spinosa* L. The commercial sample of the plant material was purchased in 2015 (harvest in April 2015) from Dary Natury (Koryciny, Poland). The preparation of the extracts and their standardisation were described previously (Marchelak et al., 2017).

3.3. Antioxidant activity measurements

The scavenging activity of the analytes towards multiple oxidants was evaluated using relevant methods in 96-well microplates. The results were expressed as SC_{50} values understood as the concentration of the analyte that decreases the initial amount of the oxidant by 50% and calculated from concentration-scavenging curves. For easier comparison, the results were expressed also in equivalents of TX (TE) and AA (AAE), and for each analyte, its total antioxidant potential was calculated with respect to both standards as a sum of the results from particular tests (TE_{MR6} and AAE_{MR6}, respectively).

3.3.1. $O_2^{\cdot-}$ scavenging capacity assay

The $O_2^{\cdot-}$ scavenging activity was evaluated in xanthine/xanthine oxidase system acc. to Granica et al. (2013) with nitrotetrazolium blue chloride (NBT) used for detection of the oxidative action of $O_2^{\cdot-}$, which was monitored spectrophotometrically at 560 nm.

3.3.2. H_2O_2 scavenging capacity assay

The ability to scavenge H_2O_2 was determined spectrophotometrically acc. to Fernando and Soysa (2015) with some modifications. Briefly, 100 µL of tested analytes solutions in phosphate buffer (84 mM, pH = 7), 40 µL H_2O_2 (0.08 mM in water), 100 µL phenol (12 mM in phosphate buffer), 30 µL 4-aminoantipyrine (4-AAP; 0.5 mM in phosphate buffer), and 20 µL horseradish peroxidase (HRP; 0.6 U/mL in phosphate buffer) were added to each well. The reaction mixture was incubated in 37 °C for 30 min, and then the absorbance was measured at 504 nm. The scavenging percentage of H_2O_2 (S%) was calculated as follows:

$$S\% = 100 - \frac{(A_S - A_B)}{(A_{100} - A_0)} \times 100$$

where A_S is the absorbance of reaction mixture containing tested analytes, H_2O_2, phenol, 4-AAP, and HRP; A_B is the absorbance of reaction mixture without phenol; A_{100} is the absorbance of reaction mixture without tested analytes; A_0 is the absorbance of the reaction mixture without tested analytes and phenol.

3.3.3. $OH^{\cdot}$ scavenging capacity assay

The ability to scavenge $HO^{\cdot}$ was assayed spectrophotometrically using method by Fu et al. (2014) with some modifications. Briefly, 50 µL of $FeSO_4$ (1.5 mM, in redistilled water), 30 µL of H_2O_2 (6 mM, in redistilled water), 100 µL of tested analytes, and 20 µL of salicylic acid (20 mM, in methanol) were added to each well. After 60 min of the incubation at 37 °C, the absorbance of the hydroxylated salicylate complex was measured at 520 nm. The scavenging percentage of $OH^{\cdot}$ (S %) was calculated as follows:

$$S\% = 100 - \frac{(A_S - A_B)}{(A_{100} - A_0)} \times 100$$

where A_S is the absorbance of reaction mixture containing $FeSO_4$, H_2O_2, tested analytes, and salicylic acid; A_B is the absorbance of reaction mixture without salicylic acid; A_{100} is the absorbance of reaction mixture without tested analytes; A_0 is the absorbance of reaction mixture without tested analytes and salicylic acid.

3.3.4. $NO^{\cdot}$ scavenging capacity assay

The $NO^{\cdot}$ scavenging activity was evaluated in 96-well plates acc. to Czerwińska et al. (2012), using diaminofluorescein-2 (DAF-2) as $NO^{\cdot}$ probe. In the presence of oxygen, DAF-2 can trap $NO^{\cdot}$ generated by sodium nitroprusside (SNP), resulting in triazolofluorescein monitored fluorometrically. Changes of fluorescence ($\lambda_{excitation}$ = 495 nm, $\lambda_{emission}$ = 515 nm) were measured over a 30 min period at intervals of 5 min.

3.3.5. $ONOO^-$ scavenging capacity assay

The ability to scavenge $ONOO^-$ (obtained synthetically) was determined by spectrophotometric measurement of the inhibition of Evans Blue dye oxidation (after 30 min of incubation, λ = 611 nm) acc. to Krzyzanowska-Kowalczyk et al. (2017).

3.3.6. HOCl scavenging capacity assay

The HOCl scavenging effect was determined in 96-well microplates acc. to Czerwińska et al. (2012) with some modifications. In the method 5-thio-2-nitrobenzoic acid (TNB) was oxidized by HOCl into 5,5-dithiobis(2-nitrobenzoic acid), which was monitored spectrophotometrically at 412 nm. Briefly, 50 µL of solutions of tested analytes in PBS and 100 µL of 0.0122% NaOCl solution in PBS were added to each well. After 5 min of the incubation in the dark, 50 µL of TNB (0.1 mM) were added, and then the reaction mixture was incubated for 15 min in the dark. The scavenging percentage of HOCl (S%) was calculated as follows:

$$S\% = 100 \times \frac{(A_S - A_B)}{(A_0 - A_{100})}$$

where A_S is the absorbance of the reaction mixture containing tested analytes, NaOCl and TNB; A_B is the absorbance of the reaction mixture without TNB; A_0 is the absorbance of the reaction mixture without tested analytes and NaClO; A_{100} is the absorbance of the reaction mixture without tested analytes.

3.4. Statistical analysis

The results were reported as means ± SD (standard deviation) for the indicated number of experiments. Normality of the distribution of the results was verified using the Shapiro-Wilk test, and the homogeneity of variances using the Levene's test. The significance of differences between samples and controls was determined with 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 Satistica13Pl software for Windows (StatSoft Inc., Krakow, Poland) with *p*-values less than 0.05 regarded as significant.

4. Conclusions

This work is the first evaluation of the scavenging capacity of *P. spinosa* flower extracts and their primary polyphenolic components towards *in vivo*-relevant oxidants. It demonstrated that ROS/RNS quenching might be one of the possible molecular mechanisms of the systemic activity of the extracts. In combination with findings from our previous study on human plasma (Marchelak et al., 2017), it might support wider application of blackthorn flowers in adjunctive therapy of CVD. However, further research is required to specify in detail the activity profile of the extracts, including other possible molecular mechanisms and *in vivo* effects. Nevertheless, the results obtained for model native constituents of the flower extracts, apart from confirming their role in the activity of the investigated plant material, are especially relevant with regard to its standardisation (that should be addressed in future research), as they indicate potential candidates and activity markers for quality control. The work is also the first investigation into the bioactivity of phenolic metabolites that might be

produced in human organism after ingestion of blackthorn flowers. High activity parameters obtained for some of these compounds show that their potential impact should not be neglected in future activity studies of the title plant as well as other polyphenol-rich species.

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Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control

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ABSTRACT

The paper presents development and validation of a RP-HPLC-PDA method for quantification of 30 phenolic constituents of the blackthorn (*Prunus spinosa* L.) flower, a traditional European herbal medicine with a unique and complex composition. The target analytes were selected from over 50 active compounds present in the investigated plant material, and their separation was optimized on a C18 Ascentis Express fused-core column (2.7 μm, 150 mm × 4.6 mm), in a step-by-step process, in terms of elution solvents, gradient profile, temperature, and flow rate. The final procedure was carried out with an acetonitrile-tetrahydrofuran gradient at a flow rate of 1.09 mL/min and column temperature of 28°C. Under those conditions, the matrix peaks were satisfactorily separated within 35 min. The validation showed good precision (RSD < 5 %), accuracy (93.5–102.1 %), linearity (r > 0.9998), and sensitivity (LODs 0.51–2.05 ng) of the method. The real sample analysis demonstrated its applicability for quantification of the phenolics both in commercial samples of *P. spinosa* flowers (different manufacturers and years of collection), as well as in the extracts (of different polarity) prepared thereof. Thus, the developed procedure proved to be a useful tool in quality control, and the optimization approach might serve as a practical guideline for LC-method development in complex matrices.

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1. Introduction

The composition of plants and plant-derived medicines greatly affects their efficacy and safety. It is also very variable and depends on numerous environmental factors and processing conditions. Therefore, a careful qualitative and quantitative analysis is a prerequisite to reliable application of herbal drugs either at the stage of activity studies or as a final medicinal product. Since plant matrices are often very complex, chromatographic separation of the metabolites is required prior to detection and quantification [1]. This is often not a straightforward task, which causes the growing popularity of MS detectors that enable selective detection of the analytes without full separation [2]. While they offer excellent sensitivity and selectivity, the MS detectors are not without disadvantages when it comes to quantitative analyses. The main drawbacks include compound-dependent response, ion suppression effects in complex matrices, and a relatively narrow dynamic range [3]. Thus, for the quantification of natural constituents with strong chromophores, including phenolics, UV-based detectors, such as PDA (DAD), are still preferred.

In such cases, the efficient separation might be facilitated by developments in column technology, e.g. sub 2-μm columns suitable for use in UHPLC systems or columns packed with superficially porous particles, that, due to their design, operate with comparable efficiency but only half the operating pressure [4–6]. The latter might also be used with success in HPLC chromatographs that are less prone to clogging and have good compatibility with plant extracts [6]. Still, to achieve a satisfactory separation in the case of many herbal matrices, a careful optimization of numerous other factors is required, making method development a tedious task that needs a careful and systematic approach.

One such demanding plant material is the blackthorn (*Prunus spinosa* L.) flower, an herbal medicine valued in European tradition, with monograph by Commission E [7]. It is recommended, e.g., for the treatment of urinary tract disorders, inflammation, and various cardiac complaints such as inflammation of the heart muscle, cardiac neurosis, or atherosclerosis [7–9]. Our previous studies demonstrated that the activity of this plant material supports its traditional application and might result from the high content of diversified polyphenols [10,11]. On the other hand, in

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terms of proper quality control procedures, the *P. spinosa* flower poses a great challenge, as over 50 constituents were detected in the extracts in the UHPLC-PDA-ESI-MS^3 study, representing a wide range of phenolic classes, including flavonoids, caffeic acid pseudodepsides, flavan-3-ol derivatives (catechins and proanthocyanidins), and simple phenolic acids [10]. Moreover, many of the compounds present a close structural similarity that might impede the separation process [10].

Thus far, only one chromatographic procedure for the quantification of phenolics in blackthorn flowers has been published [12]. However, it was designed mainly for comparative purposes (mostly relative standards, narrow calibration ranges enforcing the extrapolation of the results, no full validation), and thus, is not suitable for wider application.

Therefore, the aim of this work was to optimize and validate a HPLC-PDA method suitable for quality control of the *P. spinosa* flower and extracts prepared thereof. To this end, multiple parameters were optimized during method development with the use of a standard mixture of 30 model analytes typical of the analyzed species. The developed method was finally employed for determination of individual blackthorn flower constituents in plant materials purchased from three European manufacturers between the years of 2015–2018 and dry extracts prepared thereof.

2. Experimental

2.1. Chemicals

All solvents used in the analyses, including methanol, acetonitrile, tetrahydrofuran (Avantor Performance Materials, Gliwice, Poland), and 85 % (w/w) ortho-phosphoric acid (Merck, Darmstadt, Germany), were HPLC grade. The list of reference standards of *P. spinosa* polyphenols used in the present study, as well as their source and purity, have been presented in Table 1. In regards to the reference standards isolated previously in the Department of Pharmacognosy, Medical University of Lodz, Lodz, Poland [13–16], their structures and lack of organic contaminations were established with a NMR method, and their purity was determined by HPLC (≥ 98 %). The standards purchased from Sigma-Aldrich, St. Louis, MO, USA/Seelze, Germany were of HPLC purity (≥ 95 %).

2.2. Plant material

Commercial samples of the *P. spinosa* L. flower were purchased from Dary Natury (Koryciny, Poland), Flos (Mokrsko, Poland), and Kräuter Kühne (Berlin, Germany) in the years 2015–2018. The plant material was authenticated based on the manufacturers' declaration. The identity was further confirmed in macroscopic and microscopic studies by Prof. Monika Anna Olszewska according to Wichtl [17]. The vouchers of each sample were deposited in the Department of Pharmacognosy, Medical University of Lodz, Poland. The list of samples, their origin, batch, and voucher numbers have been presented in Table 2.

2.3. Chromatographic conditions

The HPLC-PDA analyses were performed on a Shimadzu Prominence-i LC-2030C 3D chromatograph equipped with a PDA detector, a column oven, and an autosampler (Shimadzu, Kyoto, Japan). Separations were carried out on a C18 Ascentis® Express column (2.7 μm, 150 mm × 4.6 mm; Supelco, Bellefonte, PA, USA) with a C18 Ascentis® 2.7 Micron Guard Cartridge (2.7 μm, 5 mm × 4.6 mm; Supelco).

The separation conditions (mobile phase composition, elution profile, flow rate, and temperature) were optimized using a mixture of 30 model analytes (Table 1), which were selected based on a UHPLC-PDA-ESI-MS^3 assay [10]. The final elution system consisted of solvent A (0.5 % water solution of orthophosphoric acid, *w/v*), solvent B (acetonitrile) and solvent C (tetrahydrofuran). The final elution profile is shown in Table 3, as well as in Fig. 4 of the supplementary paper [18]. The flow rate was 1.09 mL/min, the column was maintained at 28 °C, and the injection volume was 5 μL.

The UV–vis data were collected over the range of 190–600 nm. The wavelength for detection and quantification was set to 280 nm for flavan-3-ols and proanthocyanidins, 310 nm for *p*-coumaric acid, 325 nm for caffeic acid derivatives, and 350 nm for flavonols. The peak purity was assessed based on the degree of similarity of the UV spectra across the peak over the whole data collection range using a LabSolutions software (Shimadzu).

2.4. Method validation

The analytical method was validated by determining the selectivity, linearity, precision, and accuracy according to the International Council for Harmonisation (ICH) Guidance for Industry [19] and a previous literature report [20].

Selectivity of the method was analyzed by comparison of retention times and UV–vis spectra with reference compounds using an automated match system.

To test linearity, the standard stock solution containing 30 reference compounds was prepared in methanol-water (7:3, *v/v*), serially diluted (in triplicate) with the same solvent to at least six concentrations levels (2 %, 10 %, 25 %, 50 %, 75 %, and 100 % of the stock concentration) within the ranges of approximately 1–55 μg/mL (Table 4), filtered through a PTFE syringe filter (13 mm, 0.2 μm, Whatman, Pittsburgh, PA, USA), and injected triply (5 μL) into the HPLC system. Calibration tests were run at the beginning, midpoint, and end of the analytical tests. Linearity of the calibration curves was tested using two linear regression models ($y = ax + b$; $y = ax$), and the *F*- and *t*-tests were applied to check the statistical significance of the regression equations, slopes, and intercepts at the 99 % confidence level. The residuals from linear regression models were tested ex post to check the validity of the assumptions of normality, independence, and homoscedasticity of the response variables. Possible matrix effects were evaluated by constructing the sensitivity plots for the standards dissolved in the real samples. Concentration ranges of the added standards were the same as those used for calibration. Statistical differences between the slopes of the matrix-matched linear regression equations and the calibration curves were tested by Tukey's HSD (honest significant difference) test at the 95 % confidence level.

The limits of detection (LODs) and limits of quantification (LOQs) were estimated from the standard solutions diluted with methanol-water (7:3, *v/v*) to provide serial dilutions with analyte concentrations decreasing to the smallest detectable peaks. The LODs were accepted with the 3-δ signal-to-noise (S/N) ratio, while the LOQs were accepted if the RSDs for peak area were less than 15 % for both intra-day and inert-day variability with S/N ratios greater than 10.

The precision (intra- and inter-day variability) tests for retention times and peak areas were performed using standard solutions at two concentration levels (10 % and 100 % of the stock concentration) and a real sample of *P. spinosa* flower extract. The RSD values were considered as a measure of precision. The repeatability (intra-day variability) was determined by triplicate analysis of each sample within 24 h, while the reproducibility (inter-day variability) was measured on three non-consecutive days within a two week span.

The accuracy was checked in the real sample of *P. spinosa* flower by the standard addition/recovery procedure at three different lev-

Table 1
Reference standards of *P. spinosa* flower polyphenols used in the optimization study.

No.	Analyte	Source of the standard	Purity of the standard
1	3-*O*-Caffeoylquinic acid (neochlorogenic acid)	A*	≥ 98
2	5-*O*-Caffeoylquinic acid (chlorogenic acid)	A*	≥ 98
3	(+)-Catechin	A*	≥ 95
4	4-*O*-Caffeoylquinic acid (cryptochlorogenic acid)	A*	≥ 98
5	Caffeic acid	A*	≥ 99
6	(−)-Epicatechin	A*	≥ 97
7	Kaempferol 3-*O*-α-L-arabinopyranoside-7-*O*-α-L-rhamnopyranoside	B	≥ 98
8	Kaempferol 3-*O*-β-D-xylopyranoside-7-*O*-α-L-rhamnopyranoside (lepidoside)	B	≥ 98
9	*p*-Coumaric acid	A*	≥ 98
10	Kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (kaempferitrin)	B	≥ 98
11	Kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside	B	≥ 98
12	Quercetin 3-*O*-(6″-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (rutin)	A*	≥ 98
13	Quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl)-α-L-arabinofuranoside	B	≥ 98
14	Quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin)	A*	≥ 97
15	Quercetin 3-*O*-β-D-galactopyranoside (hyperoside)	A*	≥ 97
16	Kaempferol 3-*O*-(6″-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside	B*	≥ 98
17	Quercetin 3-*O*-α-D-xylopyranoside (reinutrin)	B*	≥ 98
18	Quercetin 3-*O*-α-L-arabinopyranoside (guaiaverin)	B*	≥ 98
19	Quercetin 3-*O*-(4″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside (multinoside A)	B	≥ 98
20	Quercetin 3-*O*-α-L-arabinofuranoside (avicularin)	B*	≥ 98
21	Quercetin 3-*O*-α-L-rhamnopyranoside (quercitrin)	B*	≥ 98
22	Kaempferol 3-*O*-β-D-xylopyranoside	B*	≥ 98
23	Kaempferol 3-*O*-(4″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside (multiflorin B)	B	≥ 98
24	Kaempferol 3-*O*-α-L-arabinofuranoside (juglanin)	B*	≥ 98
25	Kaempferol 3-*O*-α-L-rhamnopyranoside (afzelin)	B*	≥ 98
26	Kaempferol 7-*O*-α-L-rhamnopyranoside	B	≥ 98
27	Kaempferol 3-*O*-(2″-*O*-*E*-*p*-coumaroyl)-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside	B	≥ 98
28	Quercetin	A*	≥ 95
29	Kaempferol	A*	≥ 96
30	Kaempferol 3-*O*-(2″-*O*-*E*-*p*-coumaroyl)-α-L-arabinofuranoside	B	≥ 98

A, compounds obtained from Sigma-Aldrich (Seelze, Germany/St. Louis, MO, USA); B, compounds isolated previously in our laboratory from the flowers and leaves of *P. spinosa* [13–16]; *, compounds commercially available. The nomenclature of caffeoylquinic acids isomers is according to IUPAC.

Table 2
The list of *P. spinosa* flower samples used in the current study.

Sample symbol	Origin	Batch number/voucher number
Flos 2015	Flos, Mokrsko, Poland	2034/PSFL_1501_F
Flos 2016	Flos, Mokrsko, Poland	1075/PSFL_1601_F
Flos 2017	Flos, Mokrsko, Poland	1096/PSFL_1701_F
Flos 2018	Flos, Mokrsko, Poland	1068/PSFL_1801_F
Dary natury 2015	Dary Natury, Koryciny, Poland	01052015/PSFL_1501_DN
Dary natury 2016	Dary Natury, Koryciny, Poland	01112016/PSFL_1601_DN
Dary natury 2017	Dary Natury, Koryciny, Poland	01112018/PSFL_1701_DN
Dary natury 2018	Dary Natury, Koryciny, Poland	01112019/PSFL_1801_DN
Kräuter Kühne 2015	Kräuter Kühne, Berlin, Germany	210674/PSFL_1501_KK
Kräuter Kühne 2016	Kräuter Kühne, Berlin, Germany	213794/PSFL_1601_KK
Kräuter Kühne 2017	Kräuter Kühne, Berlin, Germany	213684/PSFL_1701_KK
Kräuter Kühne 2018	Kräuter Kühne, Berlin, Germany	210757/PSFL_1801_KK

Table 3
The optimized elution profile.

Solvent B (acetonitrile)		Solvent C (tetrahydrofuran)	
Time [min]	Concentration [%]	Time [min]	Concentration [%]
0-1	1 (isocratic elution)	0-11	2 (isocratic elution)
1-25	1 → 20 (linear gradient)	11-12	2 → 6 (linear gradient)
25-35	20 → 50 (linear gradient)	12-19	6 (isocratic elution)
35-37	50 (isocratic elution)	19-25	6 → 9 (linear gradient)
37-38	50 → 1 (return to initial condition)	25-37	9 (isocratic elution)
38-45	1 (equilibration)	37-38	9 → 2 (return to initial condition)
		38-45	2 (equilibration)

Solvent A: 0.5 % aqueous solution of orthophosphoric acid (*w/v*).

els of standards corresponding to the linear range limits. For each level, the samples were prepared in triplicate according to the procedure described in Section 2.5, and standards were added to the sample after pre-extraction with chloroform. Each sample was analyzed in triplicate by HPLC. The mean recovery of the analytes (the spiked versus the non-spiked extracts) was considered as the measure of accuracy.

2.5. Quantification of phenolics in raw plant material

Prior to the analysis, the plant samples were powdered using an electric grinder, sieved through a 0.315 mm sieve and stored in airtight containers at ambient temperature and in darkness until used. Each accurately weighed sample (500 mg) was first defatted by pre-extraction with chloroform (20 mL, 15 min, the chloroform

Table 4
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)
		Equation	n				
1	325	$y = 14682.25x$	7	0.99997	0.31–46.0	87754.44	0.102
2	325	$y = 14436.16x$	7	0.99997	0.36–53.8	94807.06	0.120
3	280	$y = 3097.696x$	6	0.99999	1.06–53.0	203709.60	0.353
4	325	$y = 14034.80x$	7	0.99997	0.34–51.6	72925.63	0.115
5	325	$y = 18242.80x$	6	0.99999	1.18–58.9	280754.90	0.393
6	280	$y = 3167.560x$	6	0.99997	1.08–54.2	93132.00	0.361
7	350	$y = 7986.056x$	6	0.99992	1.04–52.1	57243.99	0.347
8	350	$y = 7844.095x$	6	0.99995	0.96–48.0	52552.45	0.320
9	310	$y = 36172.14x$	7	0.99996	0.35–52.2	59486.99	0.116
10	350	$y = 6856.451x$	6	0.99994	1.02–50.9	44489.70	0.339
11	350	$y = 8565.326x$	6	0.99997	1.01–50.3	92646.12	0.335
12	350	$y = 7535.276x$	6	0.99999	1.03–51.7	188447.30	0.345
13	350	$y = 7420.369x$	6	0.99994	1.00–50.1	42845.70	0.333
14	350	$y = 10296.28x$	6	0.99999	1.18–58.9	240807.80	0.393
15	350	$y = 10435.57x$	6	0.99998	1.10–55.1	125026.90	0.367
16	350	$y = 4597.466x$	6	0.99996	1.11–55.6	57152.41	0.371
17	350	$y = 11225.47x$	6	0.99999	0.98–49.0	332086.70	0.327
18	350	$y = 10847.65x$	6	0.99997	0.95–47.6	87138.24	0.317
19	350	$y = 7256.459x$	6	0.99990	1.08–54.1	34546.49	0.360
20	350	$y = 10245.51x$	6	0.99990	1.19–59.6	25545.71	0.397
21	350	$y = 9489.342x$	6	0.99992	1.09–54.1	32135.90	0.365
22	350	$y = 10812.35x$	6	0.99994	1.05–52.5	38446.49	0.350
23	350	$y = 8498.562x$	6	0.99997	1.05–52.4	86253.50	0.350
24	350	$y = 9918.776x$	6	0.99998	1.11–55.3	110833.60	0.369
25	350	$y = 9175.311x$	6	0.99994	1.12–61.3	40093.25	0.409
26	350	$y = 9785.407x$	6	0.99997	1.12–56.1	91625.86	0.374
27	350	$y = 8073.629x$	6	0.99989	1.16–57.9	23476.30	0.390
28	350	$y = 13968.08x$	7	0.99994	0.40–59.6	39847.69	0.132
29	350	$y = 14812.50x$	7	0.99997	0.32–48.0	88253.50	0.107
30	350	$y = 10207.50x$	6	0.99990	1.16–58.0	25245.77	0.387

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 $n = 6$, and 12.246 for $n = 7$ calibration data points).

extract was discarded) and then refluxed for 30 min with 30 mL of methanol-water (7:3, *v/v*). After filtering the extract, the pellet was extracted twice for 15 min with 20 mL of the same solvent. The combined filtrates were diluted with the solvent to 100 mL, filtered through a PTFE syringe filter (13 mm, 0.2 μm, Whatman, Pittsburgh, PA, USA), and injected (5 μL) into the HPLC system.

2.6. Quantification of phenolics in dry extracts

Dry extracts for the study, i.e. the defatted methanol-water (7:3, *v/v*) extract (MED), and its diethyl ether fraction (DEF), ethyl acetate fraction (EAF), *n*-butanol fraction (BF), and water residue (WR) were obtained by fractionated extraction from the flowers of *P. spinosa* L. (sample: Dary Natury 2015) as described previously [10]. Before the analysis, samples of the extracts were dissolved in methanol-water (7:3, *v/v*), filtered through a PTFE syringe filter (13 mm, 0.2 μm, Whatman, Pittsburgh, PA, USA), and injected (5 μL) into the HPLC system.

2.7. Statistical analysis

The quantitative results were expressed as means ± standard deviations (SD) of triplicate determinations. Normality of the distribution of the results was verified using the Shapiro-Wilk test and the homogeneity of the variances using the Levene's test. The significance of differences between samples was determined with one-way ANOVA followed by the post hoc Tukey's test for multiple comparisons. All calculations were performed using the Statistica13Pl software for Windows (StatSoft Inc., Krakow, Poland), with *p* values less than 0.05 being regarded as significant.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The main challenge during the optimization process was the very complex composition of the investigated plant material. Our previous UHPLC-PDA-ESI-MS^3 study showed 44 constituents in the crude methanolic extract alone, and additional analytes were detected in the concentrated fractions, amounting in total to 59 structures [10]. Among those, 30 compounds were unambiguously identified with reference standards available commercially or obtained during our extensive isolation studies on *P. spinosa* flowers and leaves [13–16]. This set contained the dominating constituents of the plant but also some of the minor compounds that might be important from a chemotaxonomic point of view or/and for identity confirmation (Table 1). The latter group included common constituents of the plants, in general (e.g. **3**, **6**, **12**, **14**, **15**), as well as rare flavonoid glycosides, characteristic of the *P. spinosa* (e.g. **7**, **8**, **13**, **16**, **19**, **27**, **30**). Those 30 identified constituents were found to reflect the composition of the flowers with reasonable accuracy and were used to both track the quality of the separation and serve as quantitative standards.

To ensure the method's usefulness in high-throughput quality control, our aim was to obtain the separation within about 30 min and the total analysis in about 45 min (25 min shorter than a previous UHPLC assay [10]). A resolution above 1.5 allows for baseline separation; however, given the number of analytes and the time limit, we aimed to obtain a resolution over 1.0 [21] for as many compounds as possible, prioritizing the dominant constituents of the plant.

In case of HPLC studies, numerous factors, such as column type, mobile phase composition, and gradient, should be consid-

ered. A fused-core C18 Ascentis Express column was selected as it was reported to be effective for separation of phenolic mixtures, although in much simpler configurations [20]. Though other column chemistries have also been used for the separation of phenolics, C18 chemistry is the most popular, and in the case of so many constituents with close structural similarities, no column chemistry alone could provide sufficient selectivity. Thus, the main effort must be put into optimization of the gradient program.

The initial experiments proved that acetonitrile was superior to methanol in terms of peak shape and width. This phenomenon has been noticed previously by numerous authors optimizing separations of phenolics and is attributed to the higher dipole moment of acetonitrile and stronger donor-acceptor interactions between the analytes and eluent [22,23]. To keep the phenolics in an undissociated form and to improve retention, 0.5 % orthophosphoric acid (*w/v*) was used as the second phase constituent.

In some of our previous works aimed at optimization of the separation gradients, we successfully used statistical techniques to facilitate the optimization process and obtain satisfactory resolution within a short time [16,24]. Thus, in the first attempts, we tried to employ a similar approach in this work. However, this time, the number of variables which must be taken into account caused the obtained models to be highly complicated and fit the data poorly (data not shown). Therefore, we resolved to take a more traditional approach of systematic profile development by trial and error with careful analysis of the obtained results, followed by modifying the gradient accordingly.

The first phase of the optimization included simple linear gradient experiments with the initial concentration of acetonitrile varying from 1 to 7% and final concentration from 25 to 55%. These experiments demonstrated some selectivity problems that could not be resolved in a binary solvent system but helped in establishing a basic gradient (1–35 % acetonitrile in 45 min) in which 17 out of 30 constituents were separated with a resolution ≥1.1. To improve the results, tetrahydrofuran (THF) was added as a second organic modifier, and at first, constant concentrations of THF were tested (in the range of 1–7 %). The addition of THF had a generally positive impact on the separation. However, no single concentration was optimal, and a THF gradient had to be introduced. Combining the obtained results, the final gradient was developed (Table 3), in which the concentrations of THF and acetonitrile were optimized to maximize separation efficiency and minimize analysis time. The proposed gradient allowed for separation of 26 out of 30 target constituents with a resolution ≥1.1. As the final optimization step, the temperature influence was tested in the range of 20–30 °C. In comparison to the initial temperature of 25 °C, the largest improvement was noticed for the separation run at 28 °C, as the resolution of some critical pairs was improved to >1.5. In the final conditions, only one pair (**17**/**18**) still remained unresolved. As the two compounds differ only by the sugar moiety, they were quantified as a sum using the curve of **17** that, according to UHPLC-MS study [10], was somewhat more dominant. For the pair **14**/**15** separated with a resolution of 0.415, we decided to use a software feature that allowed for the deconvolution of overlapping peaks using differences in their UV spectrum. A more detailed description of the optimization process, including the influence of the optimized parameters on the separation of the critical peak pairs and the figures illustrating the subsequent stages of the method development, can be found in Section 2.3 of the supplementary paper [18].

3.2. Method validation

Good selectivity of the method was demonstrated in the real sample experiments (Fig. 1), as the investigated marker constituents were satisfactorily separated from the other matrix components. Comparison of the results with the UHPLC-MS analysis carried out previously [10] allowed (based on UV-spectra and peak proportions) for labelling of other larger peaks that could not be included in the optimization process due to a tentative identification and lack of standards. Those compounds include an isomer of *p*-coumaroylquinic acid (**CQ**), a dimeric A type proanthocyanidin (**PA**), an isorhamnetin dihexoside (**IHH**), a kaempferol rhamnoside-hexoside (**KRH**), and a spermidine derivative (**SP**). The compounds may be introduced to the analysis upon full identification, or, if necessary, quantified relatively as equivalents of chlorogenic acid (CQ), (−)-epicatechin (PA), rutin (IHH), kaempferol 3-*O*-(6"-*O*-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside (KRH), and caffeic acid (SP). Quantification data for the five tentatively identified peaks are presented in Table 4 of the supplementary paper [18].

For all investigated analytes, the linearity of the method (r > 0.9998) was confirmed over the whole concentration range tested, and the values of LODs and LOQs were in the range of 0.102–0.409 μg/mL and 0.31–1.19 μg/mL, respectively (Table 4). The method was found to be adequately precise with intra-day and inter-day variability (RSD) for the peak area of all tested compounds in the range of 0.05–3.83 % and 0.27–4.97 %, respectively. For the majority of samples, the RSD values did not exceed 3 % for all precision components. Higher RSD values were found at the lowest spiking levels for only a few analytes (Table 1 of the supplementary paper [18]). The accuracy of measurements was within the acceptable range of 93–102 % (Table 1 of the supplementary paper [18]).

3.3. Real sample analysis

3.3.1. Raw plant material

Since the majority of medicinal plants, including *P. spinosa*, grow in wild, their metabolite pools are determined by uncontrolled environmental factors. Therefore, raw materials collected at different times and from different regions might differ in the content of active compounds [25]. To ensure that the developed method is suitable for detecting those differences, we investigated commercial samples of blackthorn flowers purchased from three European manufacturers in the years 2015–2018.

As revealed by the results (Fig. 1A, 1B), all of the model compounds were detected in each sample, demonstrating a relative stability of the qualitative profile of the *P. spinosa* flower and indicating that the appropiate standards were selected. On the other hand, as expected, inter-sample variations were observed from the quantitative profile (Table 2 of the supplementary paper [18]). The total content of the investigated compounds (TPH) varied in the range of 31.95–48.96 mg/g dry weight (dw), and the contents of individual constituents showed differences up to four-fold, e.g. for **2** it was in the range of 0.51–2.22 mg/g dw and for **4**, 0.44–1.64 mg/g dw. Compounds **10** and **20** were among the most abundant, reaching levels of 3.11–6.52 mg/g dw and 2.51–5.95 mg/g dw, respectively, and were the dominant constituents in five and four out of 12 plant samples, respectively. Other major constituents, the content of which, in most samples, exceeded 3 mg/g, were **1**, **25**, **11** and **24**. Those six compounds constituted 51.39–60.41 % of the TPH values and should be considered as an absolute minimum for quantification in quality control studies. Three compounds, i.e. **6**, **9**, and **5**, were below the LOQs in all of the samples; however, all the minor, but characteristic, constituents of *P. spinosa* (e.g. **7**, **8**, **27**, **30**) were reliably quantified.

Generally, the method allowed for the collection of detailed data on the composition of *P. spinosa* flowers that might be useful in applications such as identity confirmation, quality control, as well as more advanced chemometric studies. It might also be a good starting point for bioactivity studies and an important indicator of the suitability of the plant material for a particular application. For example, our previous studies demonstrated that quercetin deriva-

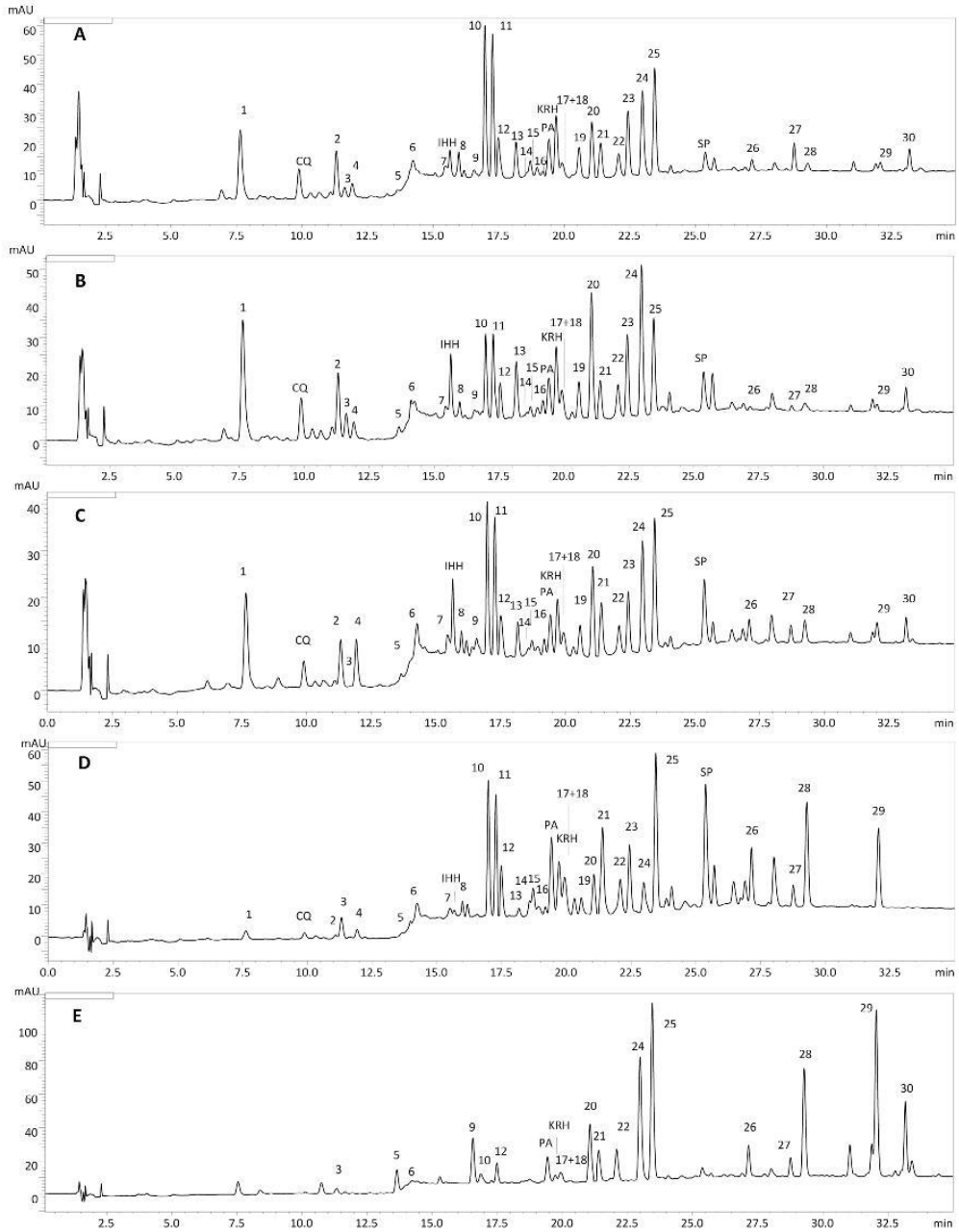

Fig. 1. Representative HPLC-UV chromatograms of the commercial samples of the *P. spinosa* flower: (A) *Flos*, 2015; (B) *Dary Natury*, 2016; and dry extracts: (C) MED; (D) EAF; (E) DEF under optimized conditions (Table 3) at 280 nm. The analytes levels per peak were in the range 0.01-0.34 μg (A), 0.01-0.28 μg (B), 0.01-0.15 μg (C), 0.01-0.21 μg (D), 0.03-0.44 μg (E). CQ, *p*-coumaroylquinic acid; PA, a dimeric A type proanthocyanidin; IHH, an isorhamnetin dihexoside; KRH, a kaempferol rhamnoside-hexoside; SP, a spermidine derivative.

tives were more effective scavengers of primary *in vivo*-relevant reactive oxygen species than the derivatives of kaempferol [11]. The presently recorded data shows that the ratio between quercetin and kaempferol derivatives in the raw material might be variable and range from about 1:1 to almost 1:3. Thus, though the blackthorn flower purchased from *Flos* in 2016 and 2015 revealed similar total contents of the analytes (40.95 and 43.71 mg/g dw, respectively), the latter sample might be preferable for therapeutic preparations aimed at diminishing the impact of oxidative stress due to a more desirable quercetin to kaempferol ratio (1:1.3 versus 1:2.5).

3.3.2. Dry extracts

The optimized RP-HPLC-PDA method was also successfully employed for determination of individual constituents in the fractionated dry extracts (Fig. 1C–E) that, in comparison to the raw materials, were much more varied both qualitatively and quanti-

Table 5
Relative response factors (RRFs).

Analyte	Reference compound	RRF
Caffeoylquinic acids		
2		1.0
4	**1**	1.0
Flavan-3-ols and proanthocyanidins		
6	**3**	1.0
Flavonoid diglycosides		
7		1.2
8		1.1
11		1.2
12		1.1
13	**10**	1.1
16		0.7
19		1.1
23		1.2
27		1.2
Flavonoid monoglycosides KA deriv.		
22		1.2
24		1.1
26	**25**	1.1
30		1.1
Flavonoid monoglycosides QU deriv.		
14		1.0
15		1.0
17	**20**	1.1
18		1.1
21		0.9

tatively (Table 3 of the supplementary data [18]). The total content of the analytes was in the range of 15.91–491.69 mg/g dw, and the content of individual analytes reached up to 115.46 mg/g (for **25** in DEF). The proportions of constituents in MED reflected, relatively well, the proportions in the raw material from which it was obtained. Further, the content of particular constituents was about four to five times higher, enabling preliminary evaluation of the extracts' quality based on the quality of the raw material. The composition of other extracts was a function of the polarity of the solvent used for extraction. For example, in DEF, the flavonol pentosides and aglycones were dominant; it also contained relevant levels of **5**, **9**, **3** – the compounds that were present in negligible amounts in other extracts. On the other hand, EAF was enriched in flavonol diglycosides (**10**, **11**) and flavonol monoglycosides (**20**, **25**, **21**), whereas in BF, diglycosides (**10**, **11**, **13**, **19**, **23**) and isomeric chlorogenic acids (**1**) prevailed. Despite the large variability of the extracts' composition, the wide range of standards allowed for reliable quantification of all the main constituents and evaluation of the proportions between different chemical groups of phenolics. In comparison to our previous work, in which four different methods were used to obtain much less detailed information [10], the newly developed method offers a huge advantage in future studies of extracts' bioactivities.

3.3.3. *Relative response factors*

One of the main issues of multi-constituent quality control of plant products is the accessibility of the reference standards. For example, many of the flavonoids used in the present work are commercially unavailable or high-priced. However, as the relative responses of different analytes in UV–vis measurements are a function of their molar absorptivity, it is possible to establish reliable correction factors that would enable an absolute quantification of one compound using another as a reference standard.

Thus, to enable reliable quantification of rare constituents and to increase the cost-efficiency of the method, we selected a couple of representative compounds that might serve as a calibration set for the investigated plant material. Using the calibration curves obtained in the study, we determined relative response factors (RRFs) that should be used to calculate the concentrations of particular compounds in the absence of actual standards (Table 5). To minimize possible errors, the standards were selected from the major constituents and, thus, are not the least expensive alternatives. Still, other RRFs might be calculated, if necessary, using calibration data from Table 4.

4. Conclusion

The developed RP-HPLC-PDA method allows the quantification of over 30 constituents of blackthorn flowers in less than 35 min. It satisfactorily compromises the time of the analysis with the amount of data obtained and their reliability. It proved suitable for both raw materials of different origin, as well as extracts obtained with various solvents. The obtained data might be relevant for quality control, identity confirmation, and chemometric studies. The procedure is also a good starting point as a standardization method before activity studies. Moreover, the presented systematic approach for method development might be useful for optimization of separation for other complex matrices.

CRediT authorship contribution statement

Anna Marchelak: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Monika Anna Olszewska:** Conceptualization, Supervision, Resources, Writing - review & editing. **Aleksandra Owczarek:** Conceptualization, Methodology, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Article

Data on the optimization and validation of HPLC-PDA method for quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof

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ABSTRACT

This paper presents data on the optimization and validation of an RP-HPLC-PDA method for quantification of 30 phenolic constituents of the blackthorn (*Prunus spinosa* L.) flower. The method development data cover detailed descriptions of the optimization process in terms of elution solvents, gradient profile, temperature, and flow rate. The validation data cover accuracy and precision (intra- and inter-day variability) for retention times and peak areas. Moreover, the quantification data for the commercial samples of blackthorn flower (different manufactures and years of collection), as well as for the extracts (of different polarity) prepared thereof, are included. The data presented here were related to the article: "Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control" [1].

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Specifications Table

Subject	Pharmaceutical science
Specific subject area	Development and validation of RP-HPLC-PDA method for quality control of blackthorn flowers
Type of data	HPLC-PDA chromatogram Figure Table
How data were acquired	Reversed phase high-performance liquid chromatography with photodiode array detector (RP-HPLC-PDA) Apparatus: Shimadzu Prominence-i LC-2030C 3D chromatograph equipped with a PDA detector, a column oven and an autosampler (Shimadzu, Kyoto, Japan) Column: C18 Ascentis® Express column (2.7 μm, 150 mm × 4.6 mm; Supelco, Bellefonte, PA, USA) with a C18 Ascentis® 2.7 Micron Guard Cartridge (2.7 μm, 5 mm × 4.6 mm; Supelco) Software: LabSolutions (Shimadzu, Kyoto, Japan)
Data format	Raw and analyzed
Parameters for data collection	The optimization process of the separation of 30 polyphenolic compounds typical for blackthorn flowers included the influence of acetonitrile, tetrahydrofuran, temperature/flow rate on the separation. The validation data of the developed method included accuracy and precision (intra- and inter-day variability) for retention times and peak areas. The quantification data were obtained using the commercial samples of blackthorn flower (different manufactures and years of collection) as well as the extracts (of different polarity) prepared thereof.
Description of data collection	LabSolutions software was employed to collect and analyze the chromatographic data delivered by PDA detector. To test precision, standard solutions of 30 reference compounds at two concentration levels (10% and 100% of the stock concentration), as well as a real sample of *P. spinosa* flower extract were used. The repeatability (intra-day variability) was determined by triplicate analysis of each sample within 24 h, while the reproducibility (inter-day variability) was measured on three non-consecutive days within a two week span. The accuracy was determined in the real sample of *P. spinosa* flower at three different levels of standards corresponding to the linear range limits. For each level, the samples were prepared in triplicate and each sample was analyzed in triplicate by HPLC. Regarding the quantitative data, the samples were prepared in triplicate and each sample was analyzed in triplicate by HPLC.
Data source location	Medical University of Lodz Lodz Poland 51°46′29.7″N 19°29′25.5″E
Data accessibility	With the article
Related research article	Marchelak, A., Olszewska, M.A., Owczarek, A., Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control, Journal of Pharmaceutical and Biomedical Analysis, 2020, 184, 113121, https://doi.org/10.1016/j.jpba.2020.113121

Value of the Data

- The systematic approach for method development presented in this paper might be useful for optimization of separation for other complex matrices.
- The optimization and validation data might serve as a reference for other laboratories working on complex plant matrices.
- The quantification data might be used for comparison by Researchers working on blackthorn flower and extracts prepared thereof.
- The presented data might be suitable for quality control and identity confirmation of blackthorn flowers.

1. Data description

Figs. 1–3 show sample chromatograms illustrating the stages of the optimization process for the separation of 30 polyphenolic compounds typical for the blackthorn (*Prunus spinosa* L.) flower, particularly the influence of acetonitrile (Fig. 1), tetrahydrofuran (Fig. 2), and temperature/flow rate (Fig. 3) on the separation. Fig. 4 presents the optimized gradient profile. Fig. 5 shows the deconvolution of overlapping peaks using the differences in their UV–Vis spectra. Table 1 summarizes the validation data of the developed method for precision and accuracy. Quantification data for the commercial samples of blackthorn flower (different manufactures and years of collection), as well as for the extracts (of different polarity) prepared thereof, are presented in Table 2 and Table 3, respectively. Moreover, the contents of five tentatively identified compounds in the commercial samples and dry extracts are shown in Table 4.

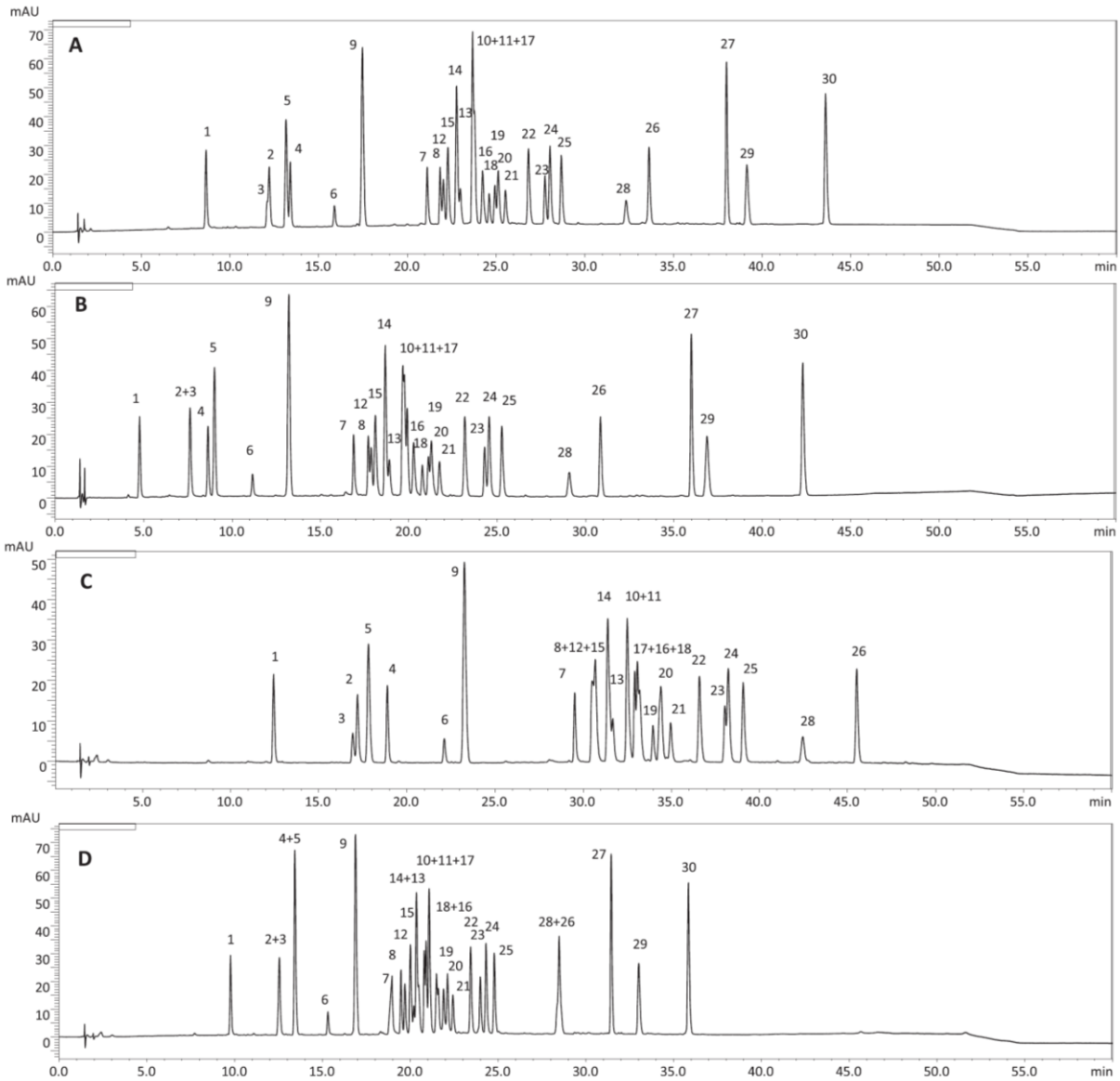

Fig. 1. The separation of *P. spinosa* flower model analytes in different profiles of acetonitrile gradient: (A) 0–45 min 3%→35%; (B) 0–45 min 7%→35%; (C) 0–45 min 1%→25%; (D) 0–45 min 1%→45%. The column temperature 25 °C, the flow rate 1 mL/min, λ = 280 nm. The analyte levels per peak 0.04–0.24 μg, eg. 0.06 μg for **1**, 0.06 μg for **3**, 0.05 μg for **9**, 0.09 μg for **24**, 0.08 μg for **29**. For details of peak identification see Table 1 of the main paper [1].

2. Experimental design, materials, and methods

2.1. Chemicals

Details regarding the chemicals are presented in the main paper [1].

2.2. HPLC analyses

The HPLC-PDA analyses were carried out on a Shimadzu Prominence-i LC-2030C 3D chromatograph equipped with a PDA detector, a column oven, and an autosampler (Shimadzu, Kyoto, Japan). Separations were performed using a C18 Ascentis® Express column (2.7 μm, 150 mm × 4.6 mm; Supelco, Bellefonte, PA, USA) with a C18 Ascentis® 2.7 Micron Guard Cartridge (2.7 μm, 5 mm × 4.6 mm; Supelco).

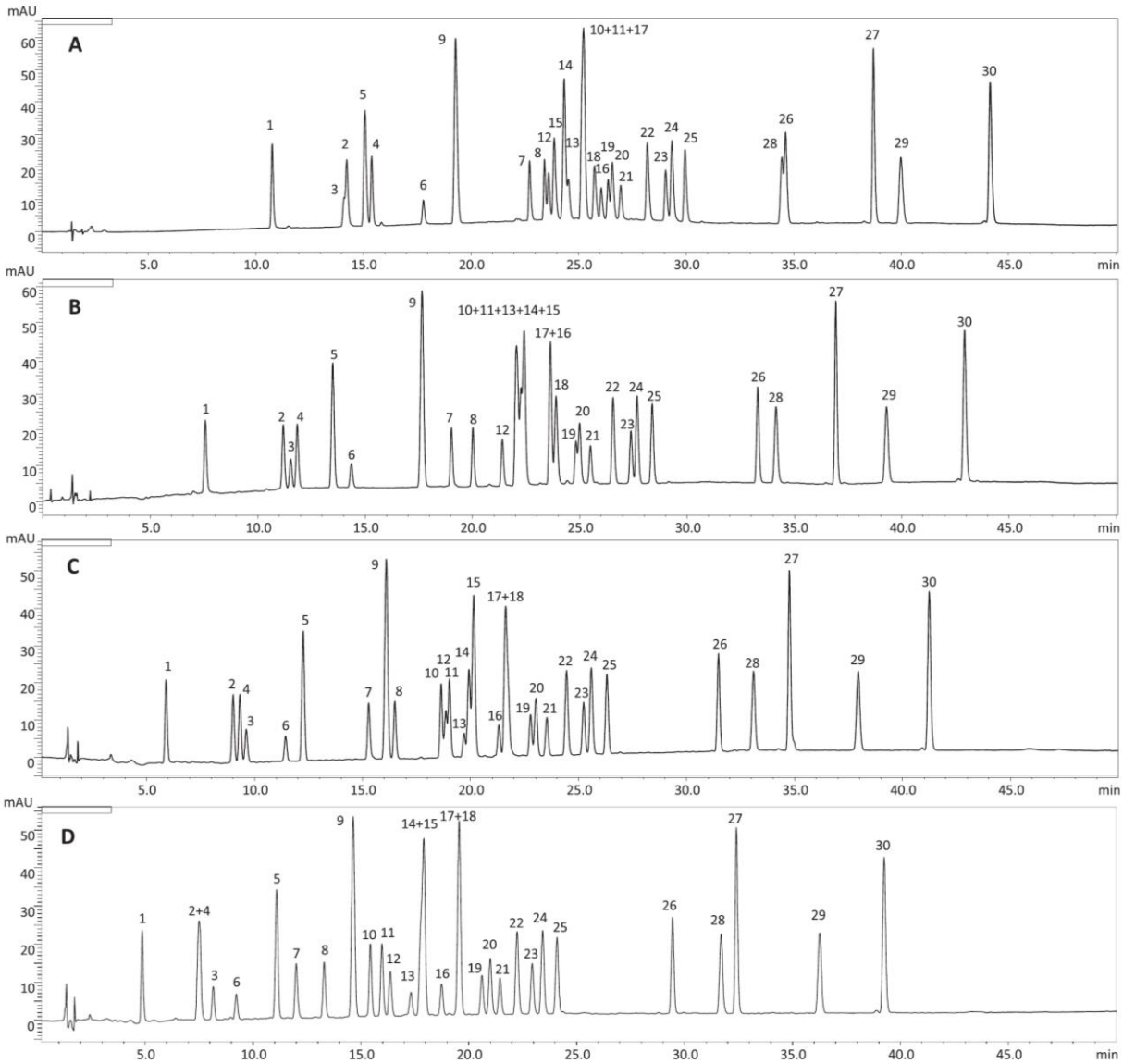

Fig. 2. The influence of tetrahydrofuran (volume percentage in the mobile phase) on the separation of *P. spinosa* flower model phenolics: (A) 0%; (B) 2% (isocratic elution); (C) 4% (isocratic elution); (D) 6% (isocratic elution). The column temperature 25 °C; the concentration of acetonitrile: 0–45 min 1%→35% (*v/v*, linear gradient); $\lambda = 280$ nm. For the analyte levels see Fig. 1. For details of peak identification see Table 1 of the main paper [1].

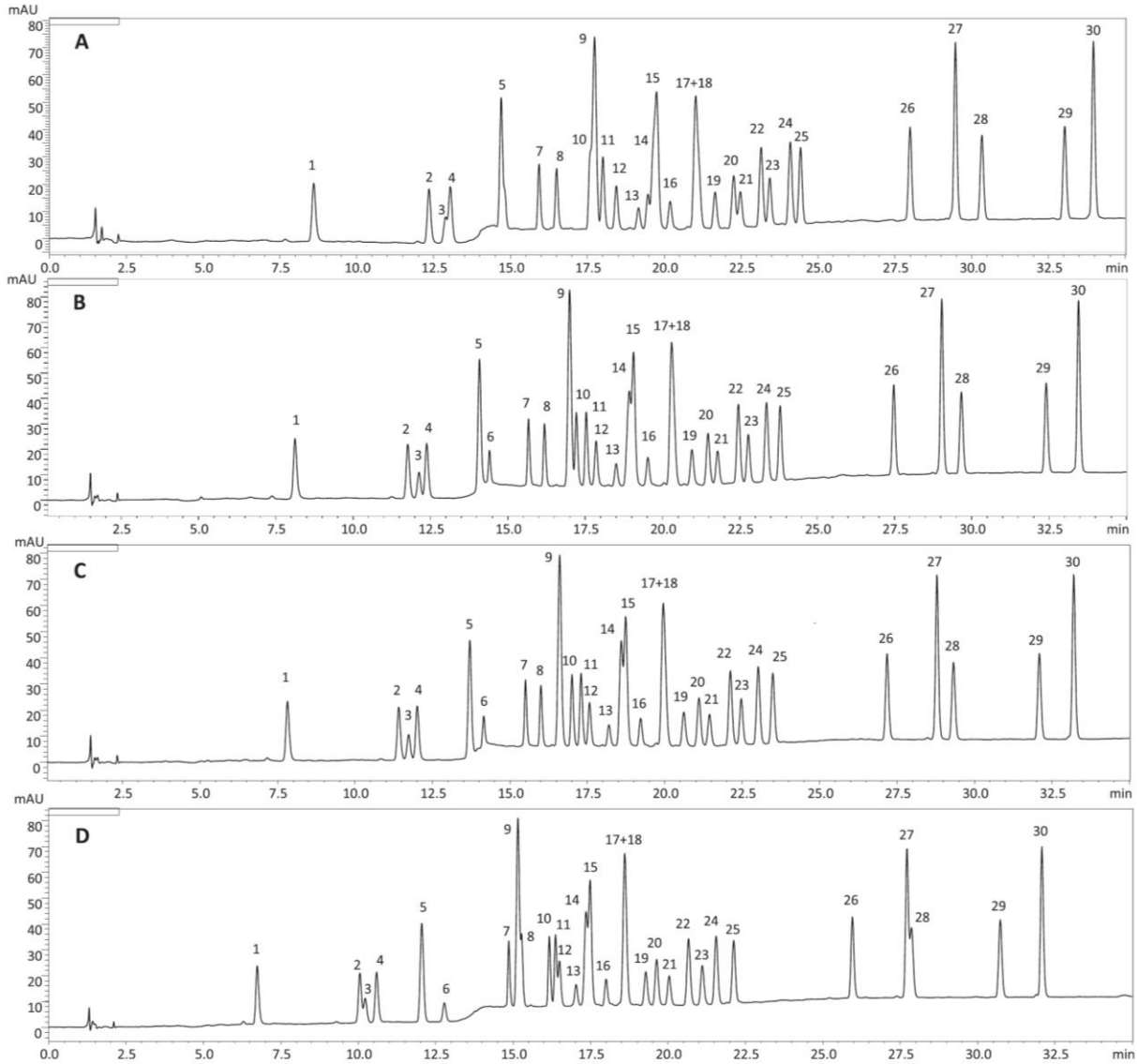

Fig. 3. The influence of temperature/flow rate on the separation of *P. spinosa* flower model phenolics: (A) 20 °C, 0.85 mL/min; (B) 25 °C, 1.0 mL/min; (C) 28 °C, 1.09 mL/min; (D) 30 °C, 1.15 mL/min under optimized gradient (Fig. 4). λ = 280 nm. For the analyte levels see Fig. 1. For details of peak identification see Table 1 of the main paper [1].

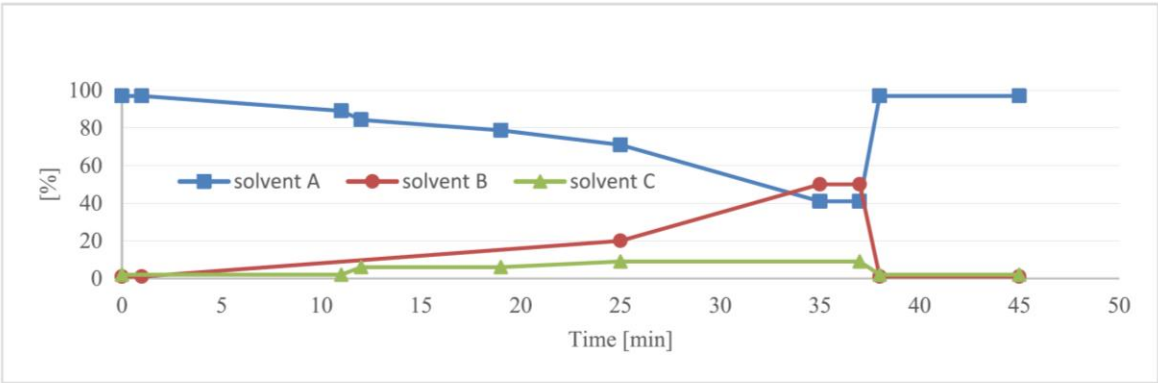

Fig. 4. The optimized elution profile. Solvent A – 0.5% water solution of orthophosphoric acid (*w*/*v*); Solvent B – acetonitrile; Solvent C – tetrahydrofuran.

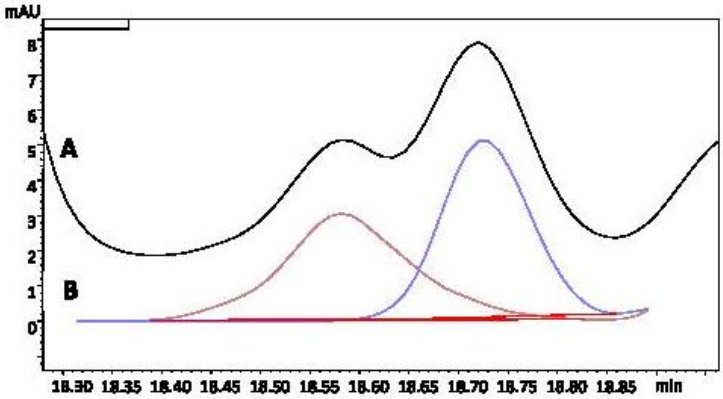

Fig. 5. The deconvolution of overlapping peaks using the differences in their UV spectrum presented on the example of peaks **14** and **15** (Dary Natury 2016); λ = 350 nm; (A) before the deconvolution; (B) after the deconvolution.

2.3. Optimization of the chromatographic conditions

The separation conditions (the mobile phase composition, elution profile, flow rate, and temperature) were optimized using a mixture of 30 model analytes typical of the analyzed species. The name, source, and purity of the standards are provided in Table 1 of the main paper [1].

In the first phase of the optimization, simple linear gradient experiments were performed with the initial concentration of acetonitrile varying in the range of 1–7% and final concentration in the range of 25–55%. The aim was to establish the elution range for the investigated constituents and identify the critical co-eluting peaks. The obtained chromatograms (examples in Fig. 1) could be divided into three regions. Simple phenolic acids (**5**, **9**), monomeric flavan-3-ols (**3**, **6**), and caffeoylquinic acid pseudo-depsides (**1**, **2**, **4**) were eluted in the front and were mostly well-separated, with the exception of **2** and **3**. In the middle part of the chromatogram, most of the flavonoid glycosides were grouped (**7**, **8**, **10**–**25**). This portion was very crowded, and the selectivity issues were particularly visible here, especially with the two main diglycosides (**10**, **11**) co-eluting in all the gradients tested. At the end of the chromatogram, the least polar compounds were eluted, i.e. a 7-*O*-monoglycoside (**26**), flavonoid aglycones (**28**, **29**), and *p*-coumaroyl esters of flavonoid glycosides (**27**, **30**) with some co-elution problems between **28** and **26**. Based on those data, a basic gradient was established for further modification. As it become clear that the addition of a second modifier would be required to improve the selectivity, the initial concentration of acetonitrile was kept at a low level of 1%, while the final concentration was set to 35%, allowing for elution of all constituents in a reasonable time frame of 45 min. In those conditions, only 17 out of 30 constituents were separated with a resolution ≥1.1 (Fig. 2A).

To improve the separation, tetrahydrofuran (THF) was added as a second organic modifier. THF proved to be efficient in the separation of natural aromatic compounds, such as flavonoids and phenolic acids [2–4]. Although, it generates relatively high back pressures that do not allow for high concentrations to be used. At first, a constant amount of THF in the range of 1–7% was added to the basic gradient, and its influence on the selectivity was observed (Fig. 2). The addition of THF at the concentration of 2% (Fig. 2B) allowed for the most efficient separation of **2**, **3** and **4** in the front section of the chromatogram. On the other hand, in the flavonoid part of the chromatogram, the best effects of THF were visible at the concentration of 6% (Fig. 2D). Importantly, in the latter variant, good resolution was obtained for peaks **10**, **11**, **20** and **24**, which were previously poorly separated; nevertheless, co-elution still occurred between pairs **14**/**15** and **17**/**18**. According to the earlier UHPLC analysis [5], those compounds were, however, only minor constituents of the *P. spinosa* flower. Based on the data from this set of experiments, the final gradient was developed, in which the concentrations of THF and acetonitrile were optimized to maximize the separation efficiency and minimize the time of the analysis (Fig. 4). The proposed gradient allowed for the separation of 26 out of 30 target constituents with a resolution ≥1.1 (Fig. 3B).

Table 1
Accuracy and precision data of the proposed method in the matrix of methanol-water (7:3, *v/v*) (standard solution, STD) and real sample of *P. spinosa* flower (*Dary Natury* 2015).

Analyte	Precision						Accuracy	
	Matrix	Level (μg/mL)	Intra-day variability, RSD (%)		Inter-day variability, RSD (%)		Spiked level (μg/mL)	Recovery (% ± SD)
			t_R	Concentration	t_R	Concentration		
1	STD 10%	4.60	0.09	0.25	1.69	1.35	1.01	94.51 ± 1.76
	STD 100%	46.00	0.07	1.59	2.13	3.24	10.02	95.47 ± 1.49
	P. spinosa	17.00	0.05	0.14	1.89	2.99	19.89	94.17 ± 0.89
2	STD 10%	5.38	0.10	0.39	1.81	1.53	1.02	95.48 ± 3.18
	STD 100%	53.80	0.07	1.09	2.18	3.12	15.01	96.03 ± 2.81
	P. spinosa	6.36	0.06	0.27	1.69	3.46	29.98	97.65 ± 1.66
3	STD 10%	5.30	0.12	1.77	2.36	0.63	1.05	97.26 ± 2.09
	STD 100%	53.00	0.12	0.52	0.45	0.53	15.00	96.55 ± 0.46
	P. spinosa[a]	2.77	0.09	1.24	0.89	1.14	30.21	97.17 ± 0.99
4	STD 10%	5.16	0.02	0.49	2.13	4.04	1.01	97.85 ± 3.95
	STD 100%	51.60	0.06	0.06	2.36	0.63	14.98	96.50 ± 1.07
	P. spinosa	2.82	0.08	0.66	1.94	0.89	28.08	96.19 ± 0.47
5	STD 10%	5.89	0.07	1.09	0.92	4.97	1.03	95.84 ± 3.08
	STD 100%	58.90	0.05	0.10	1.11	0.27	15.03	96.11 ± 1.82
	P. spinosa[a]	2.01	0.02	1.81	0.69	1.64	30.01	97.14 ± 1.34
6	STD 10%	5.42	0.02	2.55	0.16	3.34	1.01	96.35 ± 4.80
	STD 100%	54.20	0.02	0.73	1.25	0.63	14.85	97.17 ± 3.64
	P. spinosa[a]	2.02	0.03	1.45	0.64	2.89	30.04	96.44 ± 1.75
7	STD 10%	5.21	0.03	2.89	0.29	2.64	1.05	95.58 ± 2.73
	STD 100%	52.10	0.04	1.18	1.49	0.79	15.01	95.09 ± 2.45
	P. spinosa	2.72	0.09	4.73	0.69	3.89	29.87	96.78 ± 1.26
8	STD 10%	4.80	0.04	0.94	0.15	2.71	1.01	98.94 ± 2.38
	STD 100%	48.00	0.02	0.35	1.26	1.58	15.01	97.78 ± 4.19
	P. spinosa	3.19	0.03	4.49	0.97	4.75	30.02	98.53 ± 2.81
9	STD 10%	5.22	0.02	0.09	0.26	0.78	1.00	97.62 ± 2.36
	STD 100%	52.20	0.09	0.22	1.03	0.76	14.99	96.26 ± 1.04
	P. spinosa[a]	1.99	0.02	2.15	1.04	1.69	30.02	96.55 ± 1.66
10	STD 10%	5.09	0.06	0.87	1.10	1.65	1.00	100.12 ± 0.95
	STD 100%	50.90	0.05	1.29	1.33	2.68	10.01	98.15 ± 4.85
	P. spinosa	19.28	0.05	1.05	0.98	2.74	20.03	98.51 ± 2.67
11	STD 10%	5.03	0.02	1.13	1.16	3.21	1.02	97.89 ± 0.89
	STD 100%	50.30	0.09	0.05	1.31	0.72	10.01	97.94 ± 1.30
	P. spinosa	16.75	0.08	1.42	1.46	1.46	20.02	100.01 ± 2.28
12	STD 10%	5.17	0.16	3.83	0.11	4.12	1.00	100.04 ± 1.96
	STD 100%	51.70	0.06	0.46	1.16	0.33	15.01	98.11 ± 1.65
	P. spinosa	4.23	0.02	3.99	0.43	4.51	30.01	97.97 ± 3.43
13	STD 10%	5.01	0.03	1.54	0.14	4.29	1.01	98.01 ± 2.61
	STD 100%	50.10	0.08	0.79	1.38	0.86	14.99	99.48 ± 4.99
	P. spinosa	6.02	0.04	2.48	0.16	2.69	30.10	100.21 ± 1.03
14	STD 10%	5.89	0.16	0.81	0.13	4.33	1.00	96.42 ± 2.76
	STD 100%	58.90	0.06	0.34	1.35	0.28	14.98	94.01 ± 1.85
	P. spinosa	1.87	0.10	1.37	1.12	1.57	28.99	95.47 ± 1.75
15	STD 10%	5.51	0.02	0.41	0.20	1.73	1.03	97.27 ± 2.49
	STD 100%	55.10	0.08	0.25	1.22	1.88	15.03	96.83 ± 2.28
	P. spinosa	1.27	0.06	2.75	0.65	3.71	30.01	96.50 ± 2.32
16	STD 10%	5.56	0.02	1.58	0.19	2.96	1.01	98.02 ± 1.96
	STD 100%	55.60	0.08	0.45	1.31	1.18	14.99	99.26 ± 2.44
	P. spinosa	4.19	0.02	1.08	1.12	2.45	30.01	99.55 ± 0.96
19	STD 10%	5.41	0.03	0.94	0.15	2.73	1.00	97.14 ± 1.35
	STD 100%	54.10	0.08	0.59	1.56	1.14	15.00	101.32 ± 2.86
	P. spinosa	5.22	0.04	3.79	1.63	3.65	30.02	100.17 ± 3.61
20	STD 10%	5.96	0.02	0.59	0.94	2.27	1.01	101.44 ± 2.89
	STD 100%	59.60	0.04	1.88	1.04	3.02	10.03	102.05 ± 3.88
	P. spinosa	16.21	0.05	1.12	1.03	1.45	20.04	100.27 ± 2.60
21	STD 10%	5.47	0.02	0.86	0.99	3.02	1.01	100.05 ± 2.81
	STD 100%	54.07	0.07	0.13	1.13	0.46	15.01	99.88 ± 4.73

(*continued on next page*)

Table 1 (*continued*)

Analyte	Precision						Accuracy	
	Matrix	Level (μg/mL)	Intra-day variability, RSD (%)		Inter-day variability, RSD (%)		Spiked level (μg/mL)	Recovery (% ± SD)
			t_R	Concentration	t_R	Concentration		
	P. spinosa	8.37	0.06	4.28	1.24	4.36	29.98	100.19 ± 2.26
22	STD 10%	5.25	0.03	0.51	0.96	1.25	1.00	96.58 ± 2.16
	STD 100%	52.50	0.03	1.26	1.08	2.65	14.98	96.94 ± 2.39
	P. spinosa	3.51	0.02	4.27	1.69	3.76	30.00	100.16 ± 3.26
23	STD 10%	5.24	0.02	0.76	0.69	2.29	1.01	94.24 ± 2.87
	STD 100%	52.40	0.04	0.15	1.24	1.49	14.96	95.11 ± 3.65
	P. spinosa	8.54	0.04	2.37	1.36	2.78	30.01	95.7 ± 3.33
24	STD 10%	5.53	0.01	0.70	0.89	2.24	1.02	99.91 ± 2.76
	STD 100%	55.30	0.06	0.05	1.02	0.77	10.03	100.28 ± 3.99
	P. spinosa	15.91	0.04	0.80	0.39	1.54	20.01	100.01 ± 2.38
25	STD 10%	6.13	0.03	0.35	0.82	1.76	1.00	100.42 ± 3.71
	STD 100%	61.30	0.03	1.02	0.93	2.78	10.03	98.58 ± 4.28
	P. spinosa	15.32	0.02	0.79	0.69	0.82	20.03	99.03 ± 1.88
26	STD 10%	5.61	0.02	0.51	0.64	3.01	1.02	95.02 ± 1.86
	STD 100%	56.10	0.06	0.07	0.72	0.52	14.98	96.26 ± 2.24
	P. spinosa	1.41	0.06	2.15	0.79	2.12	30.01	94.55 ± 0.77
27	STD 10%	5.79	0.11	0.36	0.08	4.82	1.00	94.17 ± 0.98
	STD 100%	57.90	0.01	0.21	0.71	0.47	15.01	94.15 ± 1.85
	P. spinosa	1.32	0.01	3.99	0.84	3.69	30.02	93.51 ± 2.64
28	STD 10%	5.96	0.02	0.27	0.47	2.60	1.02	97.29 ± 0.67
	STD 100%	59.60	0.02	0.90	0.51	4.26	14.98	97.71 ± 1.08
	P. spinosa	1.39	0.03	2.57	0.62	4.15	29.98	96.81 ± 1.72
29	STD 10%	4.80	0.02	0.28	0.46	2.64	1.01	95.48 ± 2.39
	STD 100%	48.00	0.03	0.08	0.50	0.54	15.02	96.16 ± 1.62
	P. spinosa	1.06	0.04	4.5	0.54	3.25	30.01	95.97 ± 3.83
30	STD 10%	5.80	0.03	0.43	0.43	2.82	1.01	96.90 ± 1.84
	STD 100%	58.00	0.03	0.38	0.48	0.60	15.00	96.97 ± 1.80
	P. spinosa	1.58	0.03	3.51	0.63	1.60	29.98	95.91 ± 2.21

The test levels in μg/mL refer to the analyte amount present (precision test) or added to the sample (accuracy test).[a] The contents of **3**, **5**, **6** and **9** in the real sample of *P. spinosa* flower were below LOQs: for precision tests the real sample of *P. spinosa* flower was thus spiked with 2 μg/mL of these analytes. The systematic names of the analytes are provided in Table 1 of the main paper [1].

As the final optimization step, the temperature influence was tested in the range of 20–30 °C (Fig. 3). To keep the back pressure in the range of 4000–4500 PSI (around 70%–80% of maximal operating pressure to limit wear on the equipment and leave some space for troubleshooting), the flow rate was modified accordingly in the range of 0.85–1.15 mL/min. In comparison to the initial 25 °C (Fig. 3B), the largest improvement was noticed for the separation run at 28 °C (Fig. 3C). Most importantly, it was possible to increase resolution factors for peak pairs **9/10** and **20/21** to >1.5. The pair **17**/**18** still remained unresolved. As both compounds are quercetin monoglycosides, differing only by the sugar moiety, the slope of their calibration curves were almost identical. Thus, the compounds were quantified as a sum, using the curve of **17** that, according to a UHPLC-MS analysis [5], was somewhat more dominant. On the other hand, the pair **14**/**15,** in the most optimal gradient, was separated with a resolution of 0.415. To increase the reliability of the quantification, we decided to use a software feature that allows for deconvolution of overlapping peaks using the differences in their UV–Vis spectra (Fig. 5).

Therefore, the final elution system consisted of solvent A (0.5% water solution of orthophosphoric acid, *w*/*v*), solvent B (acetonitrile), and solvent C (tetrahydrofuran). The final elution profile is shown in Fig. 4. The flow rate was 1.09 mL/min, and the column was maintained at 28 °C.

2.4. Method validation

The analytical method validation was performed according to the International Council for Harmonisation (ICH) Guidance for Industry [6] and some previous literature reports [2]. The procedure is

Table 2
Content of the investigated analytes in the commercial samples of *P. spinosa* flower (mg/g dw).

Analyte	Content (mg/g dw)											
	Flos 2015	*Flos* 2016	*Flos* 2017	*Flos* 2018	*Dary natury* 2015	*Dary natury* 2016	*Dary natury* 2017	*Dary natury* 2018	*Kräuter Kühne* 2015	*Kräuter Kühne* 2016	*Kräuter Kühne* 2017	*Kräuter Kühne* 2018
1	3.35 ± 0.02^{D}	4.78 ± 0.02^{B}	5.21 ± 0.07^{A}	5.34 ± 0.07^{A}	3.34 ± 0.08^{D}	5.22 ± 0.05^{A}	4.71 ± 0.06^{B}	3.35 ± 0.09^{D}	2.03 ± 0.02^{E}	3.78 ± 0.02^{C}	3.68 ± 0.05^{C}	3.21 ± 0.05^{D}
2	0.83 ± 0.01^{F}	0.78 ± 0.01^{F}	0.51 ± 0.01^{G}	1.06 ± 0.00^{E}	1.26 ± 0.02^{C}	2.22 ± 0.02^{A}	0.53 ± 0.03^{G}	0.83 ± 0.03^{F}	1.79 ± 0.02^{B}	1.18 ± 0.00^{D}	1.20 ± 0.01^{CD}	1.07 ± 0.04^{E}
3	< LOQ	1.15 ± 0.01^{D}	0.69 ± 0.01^{F}	1.25 ± 0.06^{C}	< LOQ	2.04 ± 0.01^{A}	0.91 ± 0.01^{E}	0.97 ± 0.02^{E}	1.49 ± 0.01^{B}	0.96 ± 0.01^{E}	0.93 ± 0.01^{E}	0.72 ± 0.02^{F}
4	0.48 ± 0.02^{G}	0.63 ± 0.01^{E}	0.88 ± 0.03^{AB}	1.02 ± 0.01^{AB}	0.56 ± 0.01^{F}	0.44 ± 0.00^{G}	0.80 ± 0.03^{C}	1.63 ± 0.02^{D}	0.69 ± 0.01^{DE}	0.87 ± 0.01^{AB}	0.92 ± 0.03^{A}	1.64 ± 0.02^{AB}
5	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
6	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
7	0.45 ± 0.01^{DE}	0.33 ± 0.01^{G}	0.38 ± 0.01^{FG}	0.44 ± 0.01^{DE}	0.55 ± 0.02^{C}	0.40 ± 0.01^{EF}	0.44 ± 0.02^{EF}	0.49 ± 0.01^{D}	0.47 ± 0.01^{D}	0.78 ± 0.01^{B}	0.90 ± 0.03^{A}	0.57 ± 0.02^{C}
8	1.06 ± 0.01^{A}	0.63 ± 0.02^{DE}	0.66 ± 0.03^{CD}	0.59 ± 0.01^{E}	0.63 ± 0.02^{DE}	0.62 ± 0.03^{DE}	0.72 ± 0.01^{C}	0.61 ± 0.01^{DE}	0.70 ± 0.03^{C}	0.93 ± 0.02^{B}	1.02 ± 0.03^{A}	0.59 ± 0.01^{E}
9	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
10	6.52 ± 0.11^{A}	3.11 ± 0.04^{H}	3.35 ± 0.03^{FG}	3.11 ± 0.02^{H}	3.80 ± 0.06^{E}	3.14 ± 0.08^{GH}	4.07 ± 0.12^{D}	3.39 ± 0.1^{F}	3.86 ± 0.05^{E}	5.56 ± 0.03^{C}	6.27 ± 0.06^{B}	3.53 ± 0.07^{F}
11	5.52 ± 0.19^{A}	3.08 ± 0.04^{E}	3.51 ± 0.03^{CD}	3.26 ± 0.05^{E}	3.27 ± 0.07^{DE}	2.76 ± 0.03^{F}	3.62 ± 0.11^{F}	3.25 ± 0.08^{E}	3.19 ± 0.05^{E}	5.02 ± 0.05^{B}	5.69 ± 0.04^{A}	3.21 ± 0.04^{E}
12	0.61 ± 0.01^{G}	1.17 ± 0.05^{BC}	1.14 ± 0.01^{C}	1.25 ± 0.02^{B}	0.81 ± 0.04^{F}	1.61 ± 0.02^{A}	1.12 ± 0.02^{C}	0.90 ± 0.01^{EF}	0.95 ± 0.04^{DE}	0.93 ± 0.02^{DE}	0.81 ± 0.01^{F}	1.02 ± 0.01^{D}
13	2.13 ± 0.01^{F}	2.60 ± 0.03^{D}	2.81 ± 0.02^{C}	3.20 ± 0.03^{A}	1.18 ± 0.02^{I}	2.85 ± 0.01^{BC}	2.95 ± 0.1^{B}	2.25 ± 0.05^{E}	1.05 ± 0.06^{I}	1.55 ± 0.02^{GH}	1.48 ± 0.02^{H}	1.61 ± 0.02^{G}
14	0.17 ± 0.00^{G}	0.29 ± 0.00^{EF}	0.28 ± 0.00^{F}	0.30 ± 0.01^{DE}	0.37 ± 0.00^{B}	0.42 ± 0.00^{A}	0.32 ± 0.00^{D}	0.33 ± 0.01^{CD}	0.32 ± 0.00^{D}	0.31 ± 0.00^{D}	0.31 ± 0.00^{D}	0.35 ± 0.01^{C}
15	0.17 ± 0.00^{G}	0.22 ± 0.00^{E}	0.20 ± 0.00^{F}	0.29 ± 0.01^{B}	0.25 ± 0.00^{C}	0.24 ± 0.00^{C}	0.23 ± 0.00^{CD}	0.23 ± 0.00^{CD}	0.35 ± 0.00^{A}	0.21 ± 0.00^{F}	0.25 ± 0.00^{C}	0.18 ± 0.00^{G}
16	0.50 ± 0.01^{H}	0.73 ± 0.02^{G}	0.89 ± 0.03^{E}	1.11 ± 0.02^{C}	0.84 ± 0.02^{EF}	1.50 ± 0.04^{A}	1.18 ± 0.05^{BC}	0.99 ± 0.02^{D}	0.78 ± 0.02^{FG}	0.80 ± 0.04^{FG}	0.78 ± 0.01^{FG}	1.27 ± 0.01^{B}
17 + 18	0.94 ± 0.01^{FG}	1.27 ± 0.03^{C}	1.36 ± 0.02^{B}	1.32 ± 0.02^{B}	0.93 ± 0.03^{G}	1.46 ± 0.02^{A}	1.31 ± 0.03^{BC}	1.00 ± 0.03^{E}	0.79 ± 0.03^{H}	1.06 ± 0.02^{E}	1.01 ± 0.02^{EF}	1.12 ± 0.00^{D}
19	2.01 ± 0.01^{B}	2.15 ± 0.02^{A}	1.99 ± 0.01^{BC}	1.89 ± 0.02^{C}	1.05 ± 0.01^{H}	2.12 ± 0.05^{A}	1.96 ± 0.07^{BC}	1.71 ± 0.04^{D}	1.09 ± 0.04^{GH}	1.33 ± 0.02^{F}	1.18 ± 0.05^{G}	1.46 ± 0.01^{E}
20	3.15 ± 0.00^{E}	4.94 ± 0.02^{C}	5.25 ± 0.04^{B}	4.92 ± 0.03^{C}	3.17 ± 0.10^{G}	5.95 ± 0.02^{A}	5.01 ± 0.16^{C}	3.19 ± 0.08^{G}	2.51 ± 0.04^{H}	3.33 ± 0.04^{FG}	3.41 ± 0.07^{F}	3.62 ± 0.03^{D}
21	1.84 ± 0.02^{CD}	1.80 ± 0.03^{CD}	1.74 ± 0.03^{DE}	2.41 ± 0.01^{A}	1.65 ± 0.02^{F}	1.77 ± 0.03^{DE}	2.36 ± 0.06^{A}	1.72 ± 0.05^{EF}	1.31 ± 0.04^{G}	1.87 ± 0.04^{C}	1.78 ± 0.05^{DE}	2.00 ± 0.02^{B}
22	0.90 ± 0.01^{CD}	0.86 ± 0.01^{DE}	0.84 ± 0.02^{DE}	1.11 ± 0.03^{A}	0.68 ± 0.02^{F}	1.14 ± 0.02^{A}	1.08 ± 0.04^{A}	0.99 ± 0.02^{B}	0.66 ± 0.02^{F}	0.90 ± 0.04^{CD}	0.80 ± 0.03^{E}	0.94 ± 0.01^{C}
23	3.42 ± 0.04^{B}	2.76 ± 0.03^{D}	2.64 ± 0.03^{D}	3.15 ± 0.05^{C}	1.67 ± 0.04^{H}	3.59 ± 0.01^{A}	3.36 ± 0.09^{B}	3.47 ± 0.05^{AB}	1.89 ± 0.05^{G}	2.11 ± 0.08^{F}	2.16 ± 0.05^{F}	2.43 ± 0.02^{E}
24	3.62 ± 0.01^{F}	3.81 ± 0.01^{E}	4.28 ± 0.06^{C}	5.02 ± 0.07^{B}	3.14 ± 0.06^{G}	5.72 ± 0.03^{A}	4.31 ± 0.09^{C}	3.82 ± 0.07^{E}	2.60 ± 0.05^{H}	3.64 ± 0.03^{F}	3.70 ± 0.05^{EF}	4.03 ± 0.05^{D}
25	4.25 ± 0.05^{A}	2.91 ± 0.04^{F}	2.96 ± 0.06^{F}	4.06 ± 0.04^{B}	3.01 ± 0.06F	3.24 ± 0.06^{E}	3.93 ± 0.11^{BC}	3.91 ± 0.07^{BC}	2.23 ± 0.02^{G}	3.50 ± 0.03^{D}	3.60 ± 0.05^{D}	3.84 ± 0.04^{C}
26	0.47 ± 0.01^{A}	0.19 ± 0.01^{D}	0.17 ± 0.01^{D}	0.23 ± 0.00^{D}	0.22 ± 0.00^{D}	< LOQ	0.20 ± 0.01^{D}	0.21 ± 0.01^{D}	0.33 ± 0.01^{C}	0.38 ± 0.02^{BC}	0.43 ± 0.01^{AB}	0.21 ± 0.01^{D}
27	0.80 ± 0.01^{A}	0.25 ± 0.01^{FG}	0.30 ± 0.01^{E}	0.23 ± 0.01^{FG}	0.27 ± 0.01^{EF}	0.14 ± 0.01^{H}	0.40 ± 0.01^{D}	0.26 ± 0.01^{EF}	0.24 ± 0.01^{FG}	0.49 ± 0.01^{C}	0.55 ± 0.01^{B}	0.22 ± 0.01^{G}
28	0.21 ± 0.01^{D}	0.27 ± 0.01^{B}	0.17 ± 0.01^{F}	0.24 ± 0.01^{BC}	0.19 ± 0.01^{DE}	0.19 ± 0.01^{DE}	0.26 ± 0.01^{B}	0.34 ± 0.01^{A}	0.33 ± 0.01^{A}	0.17 ± 0.01^{F}	0.16 ± 0.01^{FG}	0.28 ± 0.00^{B}
29	0.25 ± 0.01^{C}	0.20 ± 0.01^{D}	0.15 ± 0.01^{DE}	0.42 ± 0.01^{B}	0.17 ± 0.01^{DE}	0.14 ± 0.01^{E}	0.20 ± 0.01^{D}	0.49 ± 0.01^{A}	0.28 ± 0.01^{C}	0.17 ± 0.01^{DE}	0.17 ± 0.01^{DE}	0.41 ± 0.01^{B}
30	0.05 ± 0.01^{B}	0.05 ± 0.01^{B}	0.05 ± 0.01^{AB}	0.06 ± 0.01^{A}	0.03 ± 0.01^{CD}	0.05 ± 0.02^{BC}	0.06 ± 0.01^{A}	0.05 ± 0.02^{AB}	0.02 ± 0.01^{E}	0.04 ± 0.01^{C}	0.04 ± 0.01^{C}	0.04 ± 0.01^{C}
KA deriv.	27.81	18.91	20.19	22.80	18.30	22.44	23.59	21.93	17.24	24.32	26.11	21.30
QU deriv.	11.24	14.70	14.94	15.81	9.61	16.60	15.52	11.68	8.70	10.77	10.39	11.65
Total	**43.71**	**40.95**	**42.41**	**46.27**	**33.08**	**48.96**	**46.06**	**38.76**	**31.95**	**41.88**	**43.23**	**37.96**

The data are presented as means ± SD ($n = 3$). Different superscripts in each row indicate significant differences in the means at $p < 0.05$. KA deriv.: total content of kaempferol and its glycosides; QU deriv.: total content of quercetin and its glycosides. The systematic names of the analytes are provided in Table 1 of the main paper [1].

Table 3
Content of the investigated analytes in the dry extracts obtained from *P. spinosa* flower (mg/g dw).

Analyte	Content (mg/g dw)				
	MED	DEF	EAF	BF	WR
1	14.46 ± 0.23^{B}	nd.	3.04 ± 0.05^{D}	27.02 ± 0.37^{A}	10.83 ± 0.03^{C}
2	5.64 ± 0.11^{B}	nd.	5.69 ± 0.32^{B}	15.43 ± 0.11^{A}	2.02 ± 0.02^{C}
3	< LOQ	5.55 ± 0.14^{A}	nd.	nd.	nd.
4	4.26 ± 0.07^{B}	nd.	2.10 ± 0.08^{D}	10.56 ± 0.11^{A}	3.06 ± 0.01^{C}
5	< LOQ	7.65 ± 0.18^{A}	nd.	nd.	nd.
6	< LOQ	< LOQ	nd.	nd.	nd.
7	2.69 ± 0.10^{B}	nd.	1.91 ± 0.08^{C}	10.47 ± 0.13^{A}	nd.
8	3.17 ± 0.09^{C}	nd.	4.85 ± 0.14^{B}	10.92 ± 0.19^{A}	nd.
9	< LOQ	8.24 ± 0.22^{A}	nd.	nd.	nd.
10	17.42 ± 0.79^{C}	6.13 ± 0.30^{D}	41.46 ± 0.19^{B}	48.75 ± 0.03^{A}	nd.
11	15.13 ± 0.21^{C}	0.95 ± 0.02^{D}	41.96 ± 1.89^{A}	29.84 ± 0.07^{B}	nd.
12	4.65 ± 0.15^{B}	nd.	2.41 ± 0.06^{C}	16.56 ± 0.16^{A}	nd.
13	6.28 ± 0.25^{B}	nd.	3.71 ± 0.02^{C}	25.77 ± 0.15^{A}	nd.
14	1.33 ± 0.03^{B}	nd.	8.46 ± 0.14^{A}	nd.	nd.
15	0.92 ± 0.04^{B}	nd.	4.05 ± 0.15^{A}	nd.	nd.
16	3.67 ± 0.14^{C}	nd.	5.40 ± 0.11^{B}	14.10 ± 0.31^{A}	nd.
17 + 18	4.26 ± 0.15^{C}	8.50 ± 0.32^{B}	18.75 ± 0.05^{A}	nd.	nd.
19	5.38 ± 0.20^{C}	nd.	7.22 ± 0.34^{B}	19.56 ± 0.06^{A}	nd.
20	14.89 ± 0.65^{C}	71.04 ± 2.42^{A}	28.81 ± 1.12^{B}	nd.	nd.
21	7.41 ± 0.21^{C}	22.11 ± 0.35^{B}	34.41 ± 1.23^{A}	nd.	nd.
22	2.97 ± 0.10^{C}	16.85 ± 0.65^{A}	10.23 ± 0.29^{B}	nd.	nd.
23	8.82 ± 0.07^{C}	nd.	24.52 ± 0.80^{A}	19.57 ± 0.07^{B}	nd.
24	13.73 ± 0.43^{C}	96.14 ± 1.33^{A}	16.90 ± 0.26^{B}	nd.	nd.
25	13.33 ± 0.16^{C}	115.46 ± 3.98^{A}	43.37 ± 1.89^{B}	nd.	nd.
26	1.78 ± 0.04^{D}	16.41 ± 0.16^{A}	9.29 ± 0.21^{B}	2.41 ± 0.05^{C}	nd.
27	1.47 ± 0.02^{C}	7.37 ± 0.34^{A}	4.24 ± 0.23^{B}	nd.	nd.
28	1.32 ± 0.06^{C}	42.92 ± 1.09^{A}	20.99 ± 0.50^{B}	nd.	nd.
29	1.06 ± 0.01^{C}	41.08 ± 1.15^{A}	9.28 ± 0.34^{B}	nd.	nd.
30	1.43 ± 0.03^{B}	25.32 ± 0.64^{A}	nd.	nd.	nd.
KA deriv.	86.68	325.70	213.40	136.06	nd.
QU deriv.	46.43	144.56	128.83	61.88	nd.
Total	**157.47**	**491.69**	**353.07**	**250.95**	**15.91**

The data are presented as means ± SD ($n = 3$). Different superscripts in each row indicate significant differences in the means at $p < 0.05$. KA deriv.: total content of kaempferol and its glycosides; QU deriv.: total content of quercetin and its glycosides. The systematic names of the analytes are provided in Table 1 of the main paper [1].

described in Section 2.4 of the main paper [1]. The data on precision and accuracy are presented in Table 1, and the data on the other validation parameters are shown in Table 4 of the main paper [1].

2.5. Quantification of 30 phenolics in raw plant material

The plant materials used to obtain the data were commercial samples of the *P. spinosa* L. flower purchased from three European manufactures: *Dary Natury* (Koryciny, Poland), *Flos* (Mokrsko, Poland), and *Kräuter Kühne* (Berlin, Germany) in the years 2015–2018. The authentication of the plant material is described in Section 2.2 of the main paper [1]. Preparation of the extracts, including pre-extraction with chloroform and proper extraction with methanol-water (7:3, *v/v*), is described in detail in Section 2.5 of the main paper [1]. The contents of the investigated analytes in the commercial samples of *P. spinosa* flower are presented in Table 2.

2.6. Quantification of 30 phenolics in dry extracts

The plant material used to obtain the data were dry extracts obtained previously from the flowers of *P. spinosa* L. (sample: Dary Natury 2015) by fractionated extraction, i.e. the defatted methanol-water (7:3, *v/v*) extract (MED), and its diethyl ether fraction (DEF), ethyl acetate fraction (EAF), *n*-

Table 4
The content of compounds quantified relatively (mg/g dw).

	Analyte				
	CQ	PA	IHH	KRH	SP
Samples of *P. spinosa* flower:					
Flos 2015	0.74 ± 0.01^{B}	3.69 ± 0.08^{B}	0.71 ± 0.02^{DE}	2.23 ± 0.01^{B}	0.32 ± 0.01^{E}
Flos 2016	0.58 ± 0.02^{D}	5.01 ± 0.14^{A}	0.68 ± 0.01^{EF}	1.92 ± 0.01^{C}	0.24 ± 0.01^{F}
Flos 2017	0.66 ± 0.01^{C}	4.89 ± 0.09^{A}	0.79 ± 0.06^{CD}	1.98 ± 0.01^{C}	0.30 ± 0.01^{E}
Flos 2018	0.42 ± 0.01^{E}	1.33 ± 0.05^{F}	0.49 ± 0.02^{G}	2.85 ± 0.02^{A}	0.40 ± 0.01^{D}
Dary natury 2015	0.52 ± 0.01^{D}	3.65 ± 0.07^{B}	1.14 ± 0.01^{B}	1.10 ± 0.01^{E}	0.77 ± 0.02^{A}
Dary natury 2016	0.92 ± 0.02^{A}	3.24 ± 0.09^{C}	1.41 ± 0.04^{A}	2.14 ± 0.01^{B}	0.53 ± 0.02^{B}
Dary natury 2017	0.49 ± 0.01^{DE}	3.36 ± 0.10^{C}	0.62 ± 0.03^{EF}	2.75 ± 0.02^{A}	0.21 ± 0.01^{F}
Dary natury 2018	0.35 ± 0.01^{F}	1.63 ± 0.04^{E}	0.59 ± 0.01^{F}	2.69 ± 0.05^{A}	0.49 ± 0.01^{BC}
Kräuter Kühne 2015	0.28 ± 0.01^{G}	3.36 ± 0.15^{C}	0.84 ± 0.05^{C}	0.91 ± 0.01^{F}	0.46 ± 0.02^{C}
Kräuter Kühne 2016	0.70 ± 0.02^{B}	3.13 ± 0.01^{C}	1.11 ± 0.00^{B}	1.56 ± 0.03^{D}	0.74 ± 0.01^{A}
Kräuter Kühne 2017	0.74 ± 0.03^{B}	3.29 ± 0.06^{C}	1.09 ± 0.02^{B}	1.64 ± 0.01^{D}	0.79 ± 0.01^{A}
Kräuter Kühne 2018	0.58 ± 0.01^{D}	2.27 ± 0.01^{D}	0.82 ± 0.02^{C}	1.52 ± 0.02^{D}	0.74 ± 0.01^{A}
Extracts:					
MED	2.54 ± 0.01^{b}	9.53 ± 0.55^{c}	5.26 ± 0.11^{b}	5.98 ± 0.14^{b}	2.37 ± 0.25^{b}
DEF	nd.	29.79 ± 0.52^{b}	nd.	< LOQ	1.70 ± 0.06^{c}
EAF	1.33 ± 0.03^{c}	48.66 ± 5.04^{a}	< LOQ	38.4 ± 2.29^{a}	13.43 ± 1.15^{a}
BF	7.86 ± 0.25^{a}	nd.	21.31 ± 0.21^{a}	nd.	nd.
WR	1.21 ± 0.02^{d}	nd.	nd.	nd.	nd.

The data are presented as means ± SD (n = 3). Different superscripts (capitals and lowercase) in each row indicate significant differences in the means at $p < 0.05$. CQ, *p*-coumaroylquinic acid; PA, a dimeric A type proanthocyanidin; IHH, an isorhamnetin dihexoside; KRH, a kaempferol rhamnoside-hexoside; SP, a spermidine derivative.

butanol fraction (BF), and water residue (WR) [5]. The sample preparation is described in Section 2.6 of the main paper [1]. The contents of the investigated analytes in the dry extracts are presented in Table 3.

2.7. Quantification of other compounds in raw plant material and dry extracts

In addition to 30 phenolics that were quantified with the respect to the appropriate reference standards, five other major compounds were tentatively identified (by comparison of the present data with the UHPLC-MS analysis performed previously [5]) as an isomer of *p*-coumaroylquinic acid (**CQ**), a dimeric A type proanthocyanidin (**PA**), an isorhamnetin dihexoside (**IHH**), a kaempferol rhamnoside-hexoside (**KRH**), and a spermidine derivative (**SP**). These compounds have been quantified relatively (both in the raw plant material and in the dry extracts) as equivalents of chlorogenic acid (CQ), (−)-epicatechin (PA), rutin (IHH), kaempferol 3-O-(6″-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside (KRH), and caffeic acid (SP). The quantification data for five tentatively identified peaks are presented in Table 4.

Acknowledgments

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] A. Marchelak, M.A. Olszewska, A. Owczarek, Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control, J. Pharmaceut. Biomed. Anal. 184 (2020), 113121, https://doi.org/10.1016/j.jpba.2020.113121.

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Łódź, dnia 06.03.2020

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Ja, niżej podpisana **Anna Marchelak** 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.

„Charakterystyka fitochemiczna i aktywności biologicznej ekstraktów z kwiatów *Prunus spinosa* L. w kontekście chorób układu krążenia"

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)

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OŚWIADCZENIE

Oświadczam, że:

w pracy "Owczarek A., Magiera A., Matczak M., Piotrowska D.G., Olszewska M.A., **Marchelak A.**: Optimisation of preparative HPLC separation of four isomeric kaempferol diglycosides from *Prunus spinosa* L. by application of the response surface methodology. *Phytochemistry Letters*, 2017, 20, 415-424, doi: 10.1016/j.phytol.2017.01.010",

mój udział polegał na współopracowaniu koncepcji i planu pracy, nadzorze nad procesem ekstrakcji i frakcjonowania materiału roślinnego do celów izolacyjnych, analizie profilu fitochemicznego ekstraktów metodą LC-MS, nadzorze nad procesem izolacji analitów, a także na określeniu struktury wyizolowanych glikozydów flawonoidowych przy użyciu metod spektralnych (1D i 2D NMR), chemicznych i chromatograficznych (analizie konfiguracji absolutnej cukrów), co określam jako **40%** wkładu w przygotowanie ww. publikacji;

w pracy "**Marchelak A.**, Owczarek A., Matczak M., Pawlak A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2017, 8, e680, 1-15, doi: 10.3389/fphar.2017.00680",

mój udział polegał na współopracowaniu koncepcji i planu pracy, współautorstwie manuskryptu, analizie profilu fitochemicznego ekstraktów metodą LC-MS, oznaczeniu zawartości aglikonów flawonoidowych po hydrolizie metodą HPLC, optymalizacji metody TBARS, analizie aktywności antyoksydacyjnej ekstraktów i związków modelowych metodą TBARS, nadzorze nad procesem przygotowania i frakcjonowania ekstraktów, nadzorze nad wykonaniem oznaczeń aktywności antyoksydacyjnej ekstraktów i związków modelowych metodami DPPH i FRAP, oraz analizie statystycznej i interpretacji otrzymanych wyników, co określam jako **45%** wkładu w przygotowanie ww. publikacji;

w pracy "**Marchelak A.**, Owczarek A., Rutkowska M., Michel P., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2019, 30, 288-295, doi: 10.1016/j.phytol.2019.02.011",

mój udział polegał na współautorstwie koncepcji i planu pracy, współautorstwie manuskryptu, wykonaniu oznaczeń aktywności antyoksydacyjnej wobec $O_2^{\bullet-}$, $HO^{\bullet}$, H_2O_2, 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 pracach "**Marchelak A.,** Olszewska M.A., Owczarek A.: Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control. *Journal of Pharmaceutical and Biomedical Analysis*, 2020, 184, 113121, doi: 10.1016/j.jpba.2020.113121", oraz "**Marchelak A.,** Olszewska M.A., Owczarek A.: Data on the optimization and validation of HPLC-PDA method for quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof. *Data in Brief*, 2020, 29, 105319, doi:10.1016/j.dib.2020.105319",

mój udział polegał na współautorstwie koncepcji i planu pracy, współautorstwie manuskryptu, optymalizacji i walidacji metody HPLC-PDA-fingerprint, wykonaniu oznaczeń zawartości indywidualnych polifenoli w suchych ekstraktach i komercyjnym materiale roślinnym, analizie statystycznej i interpretacji wyników oraz pełnieniu funkcji autora korespondencyjnego, w tym przygotowaniu odpowiedzi na recenzje, co określam jako **60%** wkładu w przygotowanie ww. publikacji.

……..
(podpis)

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Oświadczam, że:

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mój udział polegał na współtworzeniu koncepcji i planu pracy, nadzorze nad poprawnością prowadzonych eksperymentów i interpretacji wyników, konsultacji metodyki analizy konfiguracji absolutnej reszt cukrowych, oraz finalnej korekcie manuskryptu;

w pracy "Marchelak A., Owczarek A., Matczak M., Pawlak A., Kolodziejczyk-Czepas J., Nowak P., **Olszewska M.A.**: 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*, 2017, 8, e680, 1-15, doi: 10.3389/fphar.2017.00680",

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

w pracy "Marchelak A., Owczarek A., Rutkowska M., Michel P., Kolodziejczyk-Czepas J., Nowak P., **Olszewska M.A.**: 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*, 2019, 30, 288-295, doi: 10.1016/j.phytol.2019.02.011",

mój udział polegał na współtworzeniu koncepcji i planu pracy, nadzorze nad poprawnością prowadzonych eksperymentów i interpretacji wyników, współautorstwie i finalnej korekcie manuskryptu;

w pracach "Marchelak A., **Olszewska M.A.**, Owczarek A.: Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control. *Journal of Pharmaceutical and Biomedical Analysis*, 2020, 184, 113121, doi: 10.1016/j.jpba.2020.113121" oraz "Marchelak A., **Olszewska M.A.**, Owczarek A.: Data on the optimization and validation of HPLC-PDA method for quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof. *Data in Brief*, 2020, 29, 105319, doi: 10.1016/j.dib.2020.105319",

mój udział polegał na współtworzeniu koncepcji i planu pracy, udostępnieniu palety substancji wzorcowych uzyskanych w ramach wcześniejszych prac izolacyjnych, konsultacji metodyki optymalizacji i walidacji procedury analitycznej, nadzorze nad poprawnością prowadzonych eksperymentów i interpretacji wyników, oraz finalnej korekcie manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr farm. Annę Marchelak 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 farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

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(podpis)

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mój udział polegał na współtworzeniu koncepcji i planu pracy, optymalizacji metody preparatywnej HPLC izolacji diglikozydów flawonoidowych z wykorzystaniem metody płaszczyzny odpowiedzi, współautorstwie manuskryptu, a także pełnieniu funkcji autora korespondencyjnego;

w pracy "Marchelak A., **Owczarek A.**, Matczak M., Pawlak A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2017, 8, e680, 1-15, doi: 10.3389/fphar.2017.00680",

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;

w pracy "Marchelak A., **Owczarek A.**, Rutkowska M., Michel P., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2019, 30, 288-295, doi: 10.1016/j.phytol.2019.02.011",

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w pracach "Marchelak A., Olszewska M.A., **Owczarek A.**: Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control. *Journal of Pharmaceutical and Biomedical Analysis*, 2020, 184, 113121, doi: 10.1016/j.jpba.2020.113121" oraz "Marchelak A., Olszewska M.A., **Owczarek A.**: Data on the optimization and validation of HPLC-PDA method for quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof. *Data in Brief*, 2020, 29, 105319, doi: 10.1016/j.dib.2020.105319",

mój udział polegał na współtworzeniu koncepcji i planu pracy, współpracy w zakresie optymalizacji rozdziału chromatograficznego, oraz współautorstwie manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr farm. Annę Marchelak 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 farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

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(podpis)

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w pracy "Marchelak A., Owczarek A., Matczak M., Pawlak A., **Kolodziejczyk-Czepas J.**, Nowak P., Olszewska M.A.: 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*, 2017, 8, e680, 1-15, doi: 10.3389/fphar.2017.00680",

mój udział polegał na optymalizacji oraz wykonaniu analiz aktywności antyoksydacyjnej na modelu ludzkiego osocza, analizie bezpieczeństwa komórkowego stosowania ekstraktów, konsultacji interpretacji wyników ww. analiz, a także korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów;

w pracy "Marchelak A., Owczarek A., Rutkowska M., Michel P., **Kolodziejczyk-Czepas J.**, Nowak P., Olszewska M.A.: 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*, 2019, 30, 288-295, doi: 10.1016/j.phytol.2019.02.011",

mój udział polegał na wykonaniu analiz aktywności antyoksydacyjnej ekstraktów w teście zmiatania $ONOO^-$, a także korekcie merytorycznej finalnej wersji manuskryptu przygotowanego przez głównych autorów.

Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr farm. Annę Marchelak 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 farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

J. Kołodziejczyk-Czepas
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(podpis)

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OŚWIADCZENIE WSPÓŁAUTORA

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mój udział polegał na wykonaniu rozdziałów preparatywnych izolowanych glikozydów flawonoidowych metodą HPLC oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

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

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. pracy.

Anna Magiera
.....
(podpis)

Łódź, dnia 06.03.2020

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

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy "Marchelak A., Owczarek A., Rutkowska M., **Michel P.**, Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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, 2019, 30, 288-295, doi: 10.1016/j.phytol.2019.02.011",

mój udział polegał na konsultacji metodyki analiz aktywności antyoksydacyjnej w testach zmiatania $O_2^{\bullet-}$, $HO^{\bullet}$ i H_2O_2 oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

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

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. pracy.

.....

(podpis)

Łódź, dnia 06.03.2020

dr hab. n. biol. Paweł Nowak, prof. UŁ
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Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy "Marchelak A., Owczarek A., Matczak M., Pawlak A., Kolodziejczyk-Czepas J., **Nowak P.**, Olszewska M.A.: 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*, 2017, 8, e680, 1-15, doi: 10.3389/fphar.2017.00680",

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

w pracy "Marchelak A., Owczarek A., Rutkowska M., Michel P., Kolodziejczyk-Czepas J., **Nowak P.**, Olszewska M.A.: 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*, 2019, 30, 288-295, doi: 10.1016/j.phytol.2019.02.011",

mój udział polegał na konsultacji metodyki analizy aktywności antyoksydacyjnej ekstraktów w teście zmiatania $ONOO^-$, syntezie $ONOO^-$, a także korekcie merytorycznej finalnej wersji manuskryptu przygotowanego przez głównych autorów.

Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr farm. Annę Marchelak 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 farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

P. Nowak
.....
(podpis)

Łódź, dnia 06.03.2020

mgr farm. Adam Pawlak
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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy "Marchelak A., Owczarek A., Matczak M., **Pawlak A.**, Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2017, 8, e680, 1-15, doi: 10.3389/fphar.2017.00680",

mój udział polegał na przygotowaniu ekstraktów, oznaczeniu całkowitej zawartości polifenoli i procyjanidyn w ekstraktach, a także wykonaniu analizy antyoksydacyjnej ekstraktów w testach DPPH i FRAP.

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

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. pracy.

Adam Pawlak
.....
(podpis)

Łódź, dnia 6.03.2020

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

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy "Owczarek A., Magiera A., Matczak M., **Piotrowska D.G.**, Olszewska M.A., Marchelak A.: Optimisation of preparative HPLC separation of four isomeric kaempferol diglycosides from *Prunus spinosa* L. by application of the response surface methodology. *Phytochemistry Letters*, 2017, 20, 415-424, doi: 10.1016/j.phytol.2017.01.010",

mój udział polegał na konsultacji interpretacji danych w analizie NMR i odpowiednich fragmentów dyskusji, a także korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

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

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. pracy.

.....
(podpis)

Łódź, dnia 06.03.2020

mgr farm. Magdalena Rutkowska
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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że:

w pracy "Owczarek A., Magiera A., **Matczak M.**, Piotrowska D.G., Olszewska M.A., Marchelak A.: Optimisation of preparative HPLC separation of four isomeric kaempferol diglycosides from *Prunus spinosa* L. by application of the response surface methodology. *Phytochemistry Letters*, 2017, 20, 415-424, doi: 10.1016/j.phytol.2017.01.010",

mój udział polegał na ekstrakcji i frakcjonowaniu materiału roślinnego do celów preparatywnych oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów;

w pracy "Marchelak A., Owczarek A., **Matczak M.**, Pawlak A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2017, 8, e680, 1-15, doi: 10.3389/fphar.2017.00680",

mój udział polegał na wykonaniu analiz aktywności przeciwzapalnej w testach inhibicji enzymów prozapalnych oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów;

w pracy "Marchelak A., Owczarek A., **Rutkowska M.**, Michel P., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2019, 30, 288-295, doi:10.1016/j.phytol.2019.02.011",

mój udział polegał na wykonaniu analiz aktywności antyoksydacyjnej ekstraktów w teście zmiatania $NO^{\bullet}$ oraz korekcie finalnej wersji manuskryptu przygotowanego przez głównych autorów.

Jednocześnie wyrażam zgodę na przedłożenie ww. prac przez mgr farm. Annę Marchelak 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 farm. Anny Marchelak w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników ww. prac.

Magdalena Rutkowska

(podpis)

Całkowity dorobek naukowy

Autorstwo lub współautorstwo oryginalnych prac naukowych o łącznym współczynniku IF 21.853 i punktacji MNiSW 465 (zgodnie z rokiem opublikowania), oraz 5 prac przeglądowych o łącznej punktacji MNiSW 40.

Prace oryginalne:

1. **Marchelak A.,** Olszewska M.A., Owczarek A.: Simultaneous quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof: HPLC-PDA method development and validation for quality control. *Journal of Pharmaceutical and Biomedical Analysis*, 2020, 184, 113121, doi: 10.1016/j.jpba.2020.113121, **IF_{2018} = 2.983, $MNiSW_{2018}$ = 35, $MNiSW_{2019}$ = 100**
2. **Marchelak A.,** Olszewska M.A., Owczarek A.: Data on the optimization and validation of HPLC-PDA method for quantification of thirty polyphenols in blackthorn flowers and dry extracts prepared thereof. *Data in Brief*, 2020, 29, 105319, doi: 10.1016/j.dib.2020.105319, **$MNiSW_{2019}$ = 40**
3. Magiera A., **Marchelak A.**, Michel P., Owczarek A., Olszewska M.A.: Lipophilic extracts from leaves, inflorescences and fruits of *Prunus padus* L. as potential sources of corosolic, ursolic and oleanolic acids with anti-inflammatory activity. *Natural Product Research*, 2019, doi: 10.1080/14786419.2019.1666385, **IF_{2018} = 1.999, $MNiSW_{2018}$ = 20, $MNiSW_{2019}$ = 70**
4. **Marchelak A.**, Owczarek A., Rutkowska M., Michel P., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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*, 2019, 30, 288-295, doi: 10.1016/j.phytol.2019.02.011, **IF_{2018} = 1.338, $MNiSW_{2018}$ = 20, $MNiSW_{2019}$ = 70**
5. Kicel A., Kolodziejczyk-Czepas J., Owczarek A., **Marchelak A.**, Sopinska M., Ciszewski P., Nowak P., Olszewska M.A.: Polyphenol-rich extracts from *Cotoneaster* leaves inhibit pro-inflammatory enzymes and protect human plasma components against oxidative *stress in vitro*, *Molecules*, 2018, 23, e.2472, doi: 10.3390/molecules23102472, **IF_{2018} = 3.060, $MNiSW_{2018}$ = 30, $MNiSW_{2019}$ = 100**
6. Matczak M., **Marchelak A.**, Michel P., Owczarek A., Piszczan A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: *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*, 2018, 40, 207-218, doi: 10.1016/j.jff.2017.10.046, **IF_{2018} = 3.197, $MNiSW_{2018}$ = 45, $MNiSW_{2019}$ = 100**
7. **Marchelak A.**, Owczarek A., Matczak M., Pawlak A., Kolodziejczyk-Czepas J., Nowak P., Olszewska M.A.: 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,* 2017, 8, *e*680, 1-15, doi: 10.3389/fphar.2017.00680, **IF_{2017} = 3.831, $MNiSW_{2017}$ = 40, $MNiSW_{2019}$ = 100**
8. Owczarek A., Magiera A., Matczak M., Piotrowska D.G., Olszewska M.A., **Marchelak A.**: Optimisation of preparative HPLC separation of four isomeric kaempferol diglycosides

from *Prunus spinosa* L. by application of the response surface methodology. *Phytochemistry Letters*, 2017, 20, 415-424, doi: 10.1016/j.phytol.2017.01.010, **IF_{2017} = 1.575, $MNiSW_{2017}$ = 20, $MNiSW_{2019}$ = 70**

9. Kicel A., Michel P., Owczarek A., **Marchelak A.**, Żyżelewicz D., Budryn G., Oracz J., Olszewska M.A.: Phenolic Profile and Antioxidant Potential of Leaves from Selected *Cotoneaster* Medik. Species. *Molecules*, 2016, 21, e. 688, doi: 10.3390/molecules21060688, **IF_{2016} = 2.861, $MNiSW_{2016}$ = 30, $MNiSW_{2019}$ = 100**
10. Olszewska M.A., **Kwapisz A.**, Metabolite profiling and antioxidant activity of *Prunus padus* L. flowers and leaves. *Natural Product Research*, 2011, 25, 1115-31, doi: 10.1080/14786410903230359. **IF_{2011} = 1.009, $MNiSW_{2011}$ = 20, $MNiSW_{2019}$ = 70**

Prace przeglądowe:

1. Magiera A., **Marchelak A.**, Owczarek A., Olszewska M.A.: Profil fitochemiczny i potencjał terapeutyczny szanty zwyczajnej (*Marrubium vulgare*) - przegląd aktualnego stanu wiedzy. Farmacja Polska, 2018, 74, 4, 210-222. **$MNiSW_{2018}$ = 8**
2. **Marchelak A.**, Magiera A., Owczarek A., Olszewska M.A.: Kora *Terminalia arjuna* - wskazania tradycyjnej medycyny ajurwedyjskiej w świetle współczesnych badań klinicznych. *Farmacja Polska*, 2017, 73, 8, 465-473. **$MNiSW_{2017}$ = 8**
3. Magiera A., Owczarek A., **Marchelak A.**: *Terminalia chebula* - wskazania tradycyjnej medycyny w świetle badań fitochemicznych i farmakologicznych. *Farmacja Polska*, 2017, 73, 5, 275-284. **$MNiSW_{2017}$ = 8**
4. **Marchelak A.**, Matczak M., Owczarek A.: Krokosz barwierski (*Carthamus tinctorius*) - wieki tradycji a nowe badania nad składem i aktywnością farmakologiczną. Farmacja Polska, 2016, 72, 4, 211-219. **$MNiSW_{2016}$ = 8**
5. Matczak M., **Marchelak A.**, Rychlińska I. Lapacho [*Tabebuia impetiginosa* (Mart. Ex DC.) Std.] - historia stosowania, skład i aktywność biologiczna, stan badań. *Farmacja Polska*, 2016, 72, 5, 296-302. **$MNiSW_{2016}$ = 8**

Komunikaty zjazdowe:

Autorstwo/współautorstwo łącznie 21 komunikatów zjazdowych zaprezentowanych na 7 krajowych i międzynarodowych konferencjach naukowych.

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