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Standaryzowane preparaty z różnych organów rokitnika zwyczajnego (*Elaeagnus rhamnoides* (L.) A. Nelson) jako modulatory stresu oksydacyjnego i hemostazy

Standardized preparations from various organs of the sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) as modulators of oxidative stress and hemostasis

Praca doktorska

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Streszczenie

Rokitnik zwyczajny (*Elaeagnus rhamnoides* (L.) A. Nelson) jest krzewem, którego naturalnym siedliskiem występowania są obszary nadmorskie. Rokitnik zwyczajny należy do rodziny oliwnikowatych (*Elaegnaceae*). Jego cechą charakterystyczną jest obecność cierni. Posiada on lancetowate liście oraz intensywnie pomarańczowe owoce-jagody. Rokitnik zwyczajny jest rośliną dwupienną.

Liczne badania nad składem chemicznym różnych organów rokitnika zwyczajnego pokazują, że roślina ta jest bogatym źródłem witamin, związków fenolowych, tokoferoli, karotenoidów, aminokwasów, kwasów tłuszczowych, mikro- oraz makroelementów. Szeroki zakres doświadczeń prowadzonych na preparatach z różnych organów rokitnika zwyczajnego udowodnił działanie przeciwnowotworowe, przeciwwrzodowe oraz hepatoochronne tej rośliny. Ponadto, rokitnik posiada właściwości przeciwbakteryjne i przeciwwirusowe.

Nadrzędnym celem niniejszej rozprawy doktorskiej była ocena wpływu preparatów (ekstraktów, frakcji fenolowych oraz bogatych w związki niepolarne) z rokitnika zwyczajnego na stres oksydacyjny oraz hemostazę w układzie *in vitro*. Materiał badany stanowiły ekstrakty oraz frakcje z różnych organów rokitnika zwyczajnego (owoce, liście, gałązki). Materiałem biologicznym było osocze ubogo- oraz bogatopłytkowe, płytki krwi oraz krew pełna ludzka. Badania aktywności biologicznej preparatów z rokitnika zwyczajnego przeprowadzane były z zastosowaniem różnych metod, w tym metod kolorymetrycznych, metody turbidymetrycznej, koagulometrycznej oraz cytometrii przepływowej. Analizie poddano parametry oceny stresu oksydacyjnego (oznaczenie stężenia produktów peroksydacji lipidów z kwasem tiobarbiturowym (TBA), oznaczenie stężenia grup karbonylowych oraz tiolowych w białkach oraz metoda opierająca się na redukcji cytochromu c) i hemostazy (pomiar czasów krzepnięcia, pomiar adhezji oraz agregacji płytek krwi, pomiar peroksydacji lipidów w płytkach krwi, pomiar ekspresji selektyny P i receptora GPIIb/IIIa na powierzchni płytek krwi oraz pomiar fosforylacji VASP).

Wykonane badania pokazują istotny wpływ ekstraktów oraz frakcji wyizolowanych z różnych organów rokitnika zwyczajnego na badane parametry. Przypuszcza się, że wyjątkowy skład chemiczny stanowi o istotnej roli rokitnika zwyczajnego na hamowanie stresu oksydacyjnego w osoczu oraz płytkach krwi np. badane preparaty istotnie hamowały peroksydację lipidów osocza traktowanego induktorami stresu oksydacyjnego. Zaobserwowano również, że związki obecne w badanej roślinie wpływają na hemostazę

osoczową – obserwowano istotny wpływ testowanych ekstraktów na wydłużenie czasu kaolinowo-kefalinowego. Przypuszcza się, że aktywność antykoagulacyjna badanych preparatów zależna jest od ich składu chemicznego. Ponadto, obserwuje się również wpływ licznych związków bioaktywnych obecnych w różnych organach tej rośliny, na hamowanie aktywacji płytek krwi. Zaobserwowano zmniejszoną adhezję oraz agregację płytek krwi traktowanych badanymi preparatami. Badania wykonane z wykorzystaniem cytometrii przepływowej wskazały istotne zahamowanie aktywacji płytek krwi inkubowanych z badanymi preparatami. Największe działanie biologiczne wykazuje frakcja fenolowa wyizolowana z gałązek rokitnika zwyczajnego. Frakcja ta jest bogata w proantocyjanidyny oraz katechiny.

Podsumowując, różne organy rokitnika zwyczajnego są źródłem licznych związków biologicznie czynnych. Związki te mogą w przyszłości zostać wykorzystane jako suplementy czy nowe leki w terapii chorób układu sercowo-naczyniowego.

Summary

Sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) is a shrub whose natural habitat is coastal areas. The sea buckthorn belongs to the olive family (*Elaegnaceae*). Its characteristic feature is the presence of thorns. It has lanceolate leaves and intense orange berries. The sea buckthorn is a dioecious plant.

Numerous studies on the chemical composition of various organs of the sea buckthorn show that this plant is a rich source of vitamins, phenolic compounds, tocopherols, carotenoids, amino acids, fatty acids, micro- and macroelements. A wide range of experiments carried out on preparations from various organs of the sea buckthorn have proved the anti-cancer, anti-ulcer and hepatoprotective properties of this plant. In addition, sea buckthorn has antibacterial and antiviral properties.

The main goal of this doctoral dissertation was to evaluate the effect of sea buckthorn preparations (extracts, phenolic fraction and rich in non-polar compounds) on oxidative stress and hemostasis in the *in vitro* system. The tested material consisted of extracts and fractions from various organs of the sea buckthorn (fruit, leaves, twigs). The biological material was platelet-poor and platelet-rich plasma, blood platelets and human whole blood. The biological activity of sea buckthorn preparations was tested using various methods, including colorimetric, turbidimetric, coagulometric and flow cytometry. The parameters of oxidative stress assessment (determination of the concentration of lipid peroxidation products with thiobarbituric acid (TBA), determination of the concentration of carbonyl and thiol groups in proteins and the method based on the reduction of cytochrome c) and hemostasis (measurement of coagulation times, measurement of adhesion and aggregation of platelets, measurement of lipid peroxidation in platelets, measurement of P-selectin and GPIIb/IIIa receptor expression on the platelet surface and measurement of VASP phosphorylation).

The performed tests show a significant influence of extracts and isolated fractions from various organs of the sea buckthorn on the tested parameters. It is assumed that the unique chemical composition constitutes a significant role of sea buckthorn in inhibiting oxidative stress in plasma and platelets, e.g. the tested preparations significantly inhibited the peroxidation of plasma lipids treated with inducers of oxidative stress. It was also observed that the compounds present in the tested plant affect plasma hemostasis - a significant effect of the tested extracts on the extension of activated partial thromboplastin time was observed. It is

presumed that the anticoagulant activity of the tested preparations depends on their chemical composition. Moreover, the effect of numerous bioactive compounds present in various organs of this plant on inhibiting the activation of platelets is also observed. Reduced adhesion and aggregation of platelets treated with the test preparations was observed. The tests performed with the use of flow cytometry showed a significant inhibition of the activation of platelets incubated with the tested preparations. The isolated phenol fraction from sea buckthorn twigs shows the greatest biological effect. This fraction is rich in proanthocyanidins and catechins.

In summary, the various organs of the sea buckthorn are the source of numerous biologically active compounds. These compounds may be used as suplements or new drugs in the futurein the treatment of diseases of the cardiovascular system.

Lista publikacji wchodzących w skład rozprawy doktorskiej:

1. „Biological properties of *Elaeagnus rhamnoides* (L.) A. Nelson twig and leaf extracts"
   B. Skalski, B. Kontek, B. Lis, B. Olas, Ł. Grabarczyk, A. Stochmal, J. Żuchowski. BMC Complementary and Alternative Medicine. 2019, 19(148), 1-12.
   [IF = 2,833; MNiSW = 100 pkt]
2. „Phenolic fraction and nonpolar fraction from sea buckthorn leaves and twigs: chemical profile and biological activity"
   B. Skalski, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Future Medicinal Chemistry. 2018, 10(20), 2381-2394.
   [IF = 3,617; MNiSW = 40 pkt]
3. „Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – induced oxidative stress and changes in hemostasis"
   B. Skalski, B. Lis, Ł. Pecio, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Food and Chemical Toxicology. 2019, 125, 614-620.
   [IF = 3,977; MNiSW = 100 pkt]
4. „Anti-platelet properties of phenolic extracts from the leaves and twigs of *Elaeagnus rhamnoides* (L.) A. Nelson"
   B. Skalski, B. Kontek, A. Rolnik, B. Olas, A. Stochmal, J. Żuchowski. Molecules. 2019, 24(3620), 1-17.
   [IF = 3,060; MNiSW = 100 pkt]
5. „Response of blood platelets to phenolic faction and non-polar fraction from the leaves and twigs of *Elaeagnus rhamnoides* (L.) A. Nelson *in vitro*"
   B. Skalski, A. Stochmal, J. Żuchowski, Ł. Grabarczyk, B. Olas. Biomedicine & Pharmacotherapy. 2019, 124(109897), 1-11.
   [IF = 3,743; MNiSW = 100 pkt]
6. „Anti-platelet properties of phenolic and nonpolar fractions isolated from from various organs of *Elaeagnus rhamnoides* (L.) A . Nelson in whole blood"
   B. Skalski. J. Rywaniak, A. Szustka, J. Żuchowski, A. Stochmal, B. Olas. International Journal of Molecular Sciences. 2021, 22(3282) 1- 13.
   [IF = 4,556; MNiSW = 140 pkt]

Współczynnik oddziaływania IF publikacji wchodzących w skład rozprawy doktorskiej wynosi 17,23. Całkowita liczba punktów za publikacje stanowiące rozprawę doktorską, według listy czasopism punktowanych MNiSW (w latach 2018-2020), wynosi 440.

Lista pozostałych publikacji wchodzących w skład dorobku naukowego:

1. „Comparative chemical composition, antioxidant and anticoagulant properties of phenolic fraction (a rich in non-acylated and acylated flavonoids and non-polar compounds) and non-polar fraction from *Elaeagnus rhamnoides* (L.) A. Nelson fruits”
   Beata Olas, Jerzy Żuchowski, Bernadetta Lis, **Bartosz Skalski**, Bogdan Kontek, Łukasz Grabarczyk, Anna Stochmal. Food Chemistry. 2018, 247: 39-45.
   [IF = 5,399; MNiSW = 40 pkt]
2. „Oxidative stress and hemostatic parameters in patients with nephrolithiasis before and after ureteroscopic lithotripsy”
   Beata Olas, Paweł Woźniak, Bogdan Kontek, **Bartosz Skalski**, Anna Król, Waldemr Rozański. Frontiers in Physiology. 2019, 10(799): 1-8.
   [IF 3,394; MNiSW= 100 pkt]
3. „Bioactive compounds and antiradical activity of the Rosa canina L. leaf and twig extracts”
   Małgorzata Kubczak, Ainur B. Khassenova, **Bartosz Skalski**, Sylwia Michalewska, Marzena Wielanek, Araylim N. Aralbayeva, Maira K. Murzakhmetova, Maria Zamaraeva, Maria Skłodowska, Maria Bryszewska, Maxim Ionov. Agronomy. 2020, 10(1897): 1-20.
   [IF = 2,603; MNiSW = 40 pkt]
4. „Multidirectional effects of saponin fraction isolated from the leaves of sea buckthorn *Elaeagnus rhamnoides* (L.) A. Nelson”
   Michał Juszczak, Magdalena Kluska, **Bartosz Skalski**, Jerzy Żuchowski, Anna Stochmal, Beata Olas, Katarzyna Woźniak. Biomedicine & Pharmacotherapy. 2021, 137(111395): 1-9.
   [IF = 4,545 ;MNiSW = 100]
5. „The anticancer activity of sea buckthorn [*Elaeagnus rhamnoides* (L.) A. Nelson]”
   Beata Olas, **Bartosz Skalski**, Karolina Ulanowska. Frontiers in Pharmacology. 2018, 9(232): 1-8.
   [IF = 3,845; MNiSW = 40 pkt]
6. „Rokitnik zwyczajny (*Hippophae rhamnoides* L.) jako źródło związków o aktywności przeciwnowotworowej i radioprotekcyjnej”
   Karolina Ulanowska, **Bartosz Skalski**, Beata Olas. Postępy Higieny i Medycyny Doświadczalnej. 2017, 72: 240-252.
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8. „Rokitnik zwyczajny – źródło zdrowia i młodości”
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Udział w konferencjach międzynarodowych:

1. Konferencja międzynarodowa: New & Old Phytochemicals: Their role in ecology, veterinary & welfare; PREZENTACJA POSTERU pt. „*Hippophae rhamnoides* L. fruit as a modulator of hemostatic parameters of human plasma *in vitro*". Beata Olas, Bernadetta Lis, Bartosz Skalski, Anna Stochmal, Jerzy Żuchowski. Włochy, Francavilla al Mare, 2017.
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3. Konferencja międzynarodowa: I EIAMNP Euroindoamerican Natural Product Meeting; PREZENTACJA POSTERU pt. „Isorhamnetin and its derivatives isolated from *Elaeagnus rhamnoides* (L.) A. Nelson berries as modulators of hemostasis". Bartosz Skalski, Beata Olas, Bogdan Kontek, Jerzy Żuchowski, Anna Stochmal. Hiszpania, Madryt, 2018.
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4. Konferencja krajowa: IV Krajowa Konferencja – Naturalne Substancje Roślinne: aspekty strukturalne i aplikacyjne; PREZENTACJA USTNA pt. „Porównanie efektu działania ekstraktów z rokitnika, z

ekstraktami innych wybranych roślin na proces peroksydacji lipidów w osoczu ludzkim *in vitro*". Bogdan Kontek, **Bartosz Skalski**, Beata Olas, Izabela Grzegorczyk-Karolak, Ewelina Piątczak, Anna Stochmal, Jerzy Żuchowski. Polska, Puławy, 2017.

5. Konferencja krajowa: IV Ogólnopolska Konferencja Doktorantów Nauk o Życiu BIOOPEN; PREZENTACJA POSTERU pt. „Izoramnetyna jako inhibitor stresu oksydacyjnego w osoczu". **Bartosz Skalski**, Bogdan Kontek, Jerzy Żuchowski, Anna Stochmal, Beata Olas. Polska, Łódź, 2018.
6. Konferencja krajowa: National Scientific Conference: Knowledge – Key to Success 2019; PREZENTACJA USTNA pt. „Effect of leaf and twig extracts of sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson on the adhesion of platelets treated with thrombin and ADP to fibrynogen". **Bartosz Skalski**, Beata Olas, Jerzy Żuchowski, Anna Stochmal. Polska, Toruń, 2019.
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9. Konferencja krajowa: V Krajowa Konferencja – Naturalne Substancje Roślinne: aspekty strukturalne i aplikacyjne; PREZENTACJA USTNA pt. „Wpływ związków aktywnych roślin z rodziny zarazowatych (*Orobanche ramosa*, *Orobanche arenaria* oraz *Orobanche caryophyllacea*) na stres oksydacyjny i hemostazę *in vitro*". **Bartosz Skalski**, Rostyslav Pietukhov, Dariusz Jędrejek, Sylwia Pawelec, Anna Stochmal, Beata Olas. Polska, Puławy, 2019.

Wstęp

Rokitnik zwyczajny (*Elaeagnus rhamnoides* (L.) A. Nelson) jest krzewem, ale może też osiągać rozmiary niewielkiego drzewa. Należy do rodziny oliwnikowatych (*Elaeagnaceae*). Nazywany jest również rosyjskim ananasem. Naturalnie roślina ta występuje w Rosji, Chinach, Kaukazie oraz na Syberii, głównie wzdłuż wybrzeży morskich, natomiast w Polsce występuje na wybrzeżu Morza Bałtyckiego, a jego niewielkie areały uprawne powstały na Suwalszczyźnie [Li i Schroeder, 2016; Malinowska i Olas, 2016; Olas i wsp., 2018].

Liczne badania *in vitro* oraz *in vivo* nad aktywnością biologiczną preparatów otrzymanych z różnych organów rokitnika zwyczajnego wskazują, że roślina ta może być źródłem związków chemicznych o aktywności przeciwnowotworowej, działaniu przeciwwrzodowym i hepatoochronnym oraz właściwościach antybakteryjnych i antywirusowych [Zeb, 2004; Malinowska i Olas, 2016; Olas i wsp., 2018]. Aktywność biologiczna tych preparatów może wynikać z obecności witamin, związków fenolowych, tokoferoli, karotenoidów, aminokwasów, nienasyconych kwasów tłuszczowych, mikro- oraz makroelementów [Upadhyay i wsp., 2010; Syruakumar i Gupta, 2011; Christaki, 2012;]. Nie ma natomiast dokładnych danych na temat roli preparatów na bazie rokitnika w profilaktyce czy leczeniu chorób układu krążenia, które mogą wynikać z zaburzeń hemostazy czy stresu oksydacyjnego.

Choroby układu krążenia, w wyniku postępującego wzrostu zachorowalności, zostały zaliczone do chorób cywilizacyjnych. W Polsce szacuje się, że około połowę wszystkich zgonów stanowią choroby układu sercowo-naczyniowego. Do najczęściej występujących chorób układu krążenia można zaliczyć: zawał serca, udar mózgu, nadciśnienie tętnicze, miażdżycę czy niewydolność serca (NS) [Majewicz i Marcinkowski, 2008].

Czynnikami, które istotnie wpływają na powstawanie chorób układu krwionośnego są np. palenie wyrobów tytoniowych, które skutkuje zjawiskiem stresu oksydacyjnego. Liczne badania wskazują, że reaktywne formy tlenu (RFT) mogą modyfikować poszczególne komponenty układu hemostazy (płytki krwi, komórki śródbłonka naczyń krwionośnych, białka osoczowe układu krzepnięcia i fibrynolizy) [Nowak i wsp., 2010; Eitan i Mutaz, 2018]. Dodatkowym problemem występującym w chorobach układu sercowo-naczyniowego jest nadreaktywność płytek krwi. W przypadku osób chorych na NS dochodzi do wzmożonej aktywności płytek krwi i układu krzepnięcia. W krwi obserwuje się m. in. wzrost stężenia fibrynogenu oraz czynnika von Willebranda. Ponadto, następuje wzrost selektyny P na powierzchni aktywowanych

komórek śródbłonka naczyniowego [Gębalska, 2010; Nowak i wsp., 2010]. Do aktualnie stosowanych leków przeciwpłytkowych, które stosuje się w terapii oraz zapobieganiu chorobom układu sercowo-naczyniowego można zaliczyć m. in. kwas acetylosalicylowy, który hamuje aktywność cyklooksygenazy. Enzym ten odpowiedzialny jest za tworzenie w płytkach krwi tromboksanu i prostaglandyny, które są aktywatorami agregacji płytek krwi [Karaźniewicz-Łada i wsp., 2013; Zuniga-Ceron i wsp., 2015]. Niestety kwas acetylosalicylowy, jak i inne leki z grupy przeciwkrzepliwych niosą ze sobą różne skutki uboczne np. generując zwiększone ryzyko krwawień. Naukowcy ciągle poszukują nowych preparatów przeciwpłytkowych, które nie powodują skutków ubocznych. Duże nadzieje mogą budzić preparaty na bazie substancji pozyskiwanych z roślin [Karaźniewicz-Łada i wsp., 2013; Iqbal, 2015; Broncel, 2019]. Przykładem związków, które istotnie wpływają na układ krążenia i stres oksydacyjny są związki fenolowe występujące w dużych ilościach np. w owocach aronii czy winogronach [Tolić i wsp., 2015]. Badania wskazują, że związki te również hamują aktywność cyklooksygenazy. Ponadto, blokują one powierzchniowe receptory dla białek adhezyjnych (kolagen, fibrynogen). Związki fenolowe wykazują również właściwości przeciwutleniające m. in.: posiadają własności redukujące, wiążą wolne rodniki, pełnią rolę inhibitorów oksydaz oraz terminatorów, które przerywają łańcuchowe reakcje rodnikowe. Dodatkowym atutem stosowania suplementacji związkami fenolowymi jest brak skutków ubocznych [Parus, 2013; Balsam i Grabowski, 2014; Luo i wsp., 2017].

Celem niniejszej pracy doktorskiej było określenie wpływu preparatów (ekstraktów oraz frakcji) wyizolowanych z różnych organów rokitnika zwyczajnego (*Elaeagnus rhamnoides* (L.) A. Nelson) na wybrane parametry oceny stresu oksydacyjnego oraz hemostazy w modelu *in vitro*.

Analiza składu chemicznego testowanych przeze mnie preparatów z organów rokitnika zwyczajnego wykonana została w Instytucie Uprawy, Nawożenia i Gleboznawstwa w Puławach. Wykazano, że badane ekstrakty są bogatym źródłem związków fenolowych [praca 1]. W kolejnej fazie badań określano skład chemiczny frakcji z rokitnika zwyczajnego. Zaobserwowano np., że frakcja fenolowa z liści rokitnika zwyczajnego jest bogatym źródłem tanin ulegających hydrolizie (przede wszystkim są to elagotaniny). Analiza frakcji związków niepolarnych wykazała, że jest ona źródłem triterpenów oraz niezidentyfikowanych związków niepolarnych. Frakcja fenolowa z gałązek badanej rośliny jest źródłem proantocyjanidyn oraz katechin, z kolei frakcja niepolarna jest bogata w triterpeny (Tab. 1) [praca 2].

Interesujący skład chemiczny badanych preparatów stał się podstawą do realizacji badań dotyczących ich wpływu na hemostazę i stres oksydacyjny w modelu *in vitro*. W tabeli 1 przedstawiono preparaty z różnych organów rokitnika zwyczajnego, które były przedmiotem moich badań.

Tab. 1 Preparaty z różnych organów rokitnika zwyczajnego, które zostały poddane analizie w niniejszej pracy doktorskiej

OWOCE
frakcja fenolowa
frakcja zawierająca związki niepolarne
izoramnetyna 3-O-beta-glukozyd-7-O-alfa-ramnozyd
izoramnetyna 3-O-beta-glukozyd-7-O-alfa-(3”’- izowalerylo)-ramnozyd
LIŚCIE
ekstrakt
frakcja fenolowa
frakcja zawierająca związki niepolarne
GAŁĄZKI
ekstrakt
frakcja fenolowa
frakcja zawierająca związki niepolarne

Badanie stresu oksydacyjnego w osoczu i w płytkach krwi w obecności preparatów z różnych organów rokitnika zwyczajnego

Ocenę wpływu preparatów z różnych organów rokitnika zwyczajnego na poziom stresu oksydacyjnego prowadzono z wykorzystaniem następujących metod kolorymetrycznych: oznaczenie stężenie produktów peroksydacji lipidów z kwasem tiobarbiturowym (TBA), oznaczenie stężenia grup karbonylowych oraz tiolowych w białkach. Jako induktory stresu oksydacyjnego wykorzystano nadtlenek wodoru (H_2O_2) oraz mieszaninę reakcyjną H_2O_2/Fe. W pierwszym etapie pracy badaniom poddano ekstrakty z liści oraz gałązek rokitnika zwyczajnego w pięciu stężeniach (0,5-50 µg/ml). Dodatkowo, w celu porównania działania preparatów z rokitnika zwyczajnego prowadzono analizę wpływu komercyjnego ekstraktu z owoców aronii – Aronox® (*Aronia melancorapa*) oraz ekstraktu z pestek winogron (Binorica, Niemcy) na poziom stresu oksydacyjnego w osoczu.

Na podstawie przeprowadzonych analiz zaobserwowano, że badane ekstrakty z liści i gałązek rokitnika zwyczajnego hamują peroksydację lipidów osocza traktowanego H_2O_2 w przypadku 15 minutowej inkubacji. Ekstrakt z gałązek w dawkach 5, 10 i 50 µg/ml w czasie 15 i 60 minutowej inkubacji obniżył także poziom peroksydacji lipidów osocza indukowanej H_2O_2/Fe, w tym przypadku aktywność ekstraktu była zależna od stężenia. Ekstrakt z liści również wykazał właściwości przeciwutleniające w tych samych stężeniach, ale tylko przy zastosowaniu 60 minutowej inkubacji. Ponadto, w tym modelu badawczym zaobserwowano, że ekstrakt z gałązek (50 µg/ml, 60 minut) posiada silniejsze działania antyoksydacyjne niż ekstrakt z liści (50 µg/ml, 60 minut) [praca 1].

Ekstrakty natomiast nie miały wpływu na proces karbonylacji białek w czasie krótkiej inkubacji (15 minut). Nie mniej jednak karbonylacja białek indukowana H_2O_2/Fe została istotnie zahamowana w obecności ekstraktów z liści i gałązek przy dłuższym czasie inkubacji (60 minut). Również i w tym modelu badawczym zaobserwowano, że ekstrakt z gałązek (50 µg/ml, 60 minut) posiada silniejsze działanie hamujące na karbonylację białek osocza ludzkiego niż ekstrakt z liści (50 µg/ml, 60 minut) [praca 1].

W niniejszych doświadczeniach dodatek mieszaniny reakcyjnej H_2O_2/Fe do osocza ludzkiego spowodował znaczy wzrost markerów stresu oksydacyjnego. Wyniki moich eksperymentów wskazują, że badane ekstrakty z liści oraz gałązek rokitnika zwyczajnego istotnie zmniejszały poziom stresu oksydacyjnego. Zaobserwowano również silniejszą aktywność antyoksydacyjną ekstraktu z gałązek w porównaniu z ekstraktem z liści. Przypuszcza się, że różnice w chemicznym profilu badanych ekstraktów wpływały na intensywność ich działania. Na przykład, ekstrakt z gałązek posiada wysokie stężenie proantocyjanidyn. Prawdopodobnie właśnie te związki odpowiadają za tak silną aktywność antyoksydacyjną tego ekstraktu [praca 1].

W kolejnej fazie badań analizie poddano frakcję fenolową oraz zawierającą związki niepolarne z liści oraz gałązek rokitnika zwyczajnego [praca 2]. Badania nad określeniem wpływu testowanych frakcji na parametry stresu oksydacyjnego pokazały, że tylko frakcja fenolowa wyizolowana z gałązek istotnie zmniejszała peroksydację lipidów w osoczu stymulowaną H_2O_2/Fe. Co więcej, niepolarna frakcja z gałązek (w najwyższym testowanym stężeniu – 50 µg/ml) hamowała peroksydację lipidów osocza. W tym modelu badawczym frakcja bogata w związki niepolarne wyizolowana z liści hamowała też proces peroksydacji lipidów we wszystkich badanych stężeniach (0,5 – 50 µg/ml) [praca 2].

Stwierdzono również, że frakcja fenolowa oraz frakcja niepolarna wyizolowana z gałązek rokitnika zwyczajnego chronią przed karbonylacją białek osocza ludzkiego indukowaną przed H_2O_2/Fe. Dodatkowo zaobserwowano, że frakcja fenolowa z liści oraz gałązek chronią przed utlenianiem grup tiolowych białek osocza [praca 2].

Na podstawie wykonanych doświadczeń zaobserwowano, że wysoki potencjał antyoksydacyjny frakcji fenolowej wyizolowanej z gałązek rokitnika zwyczajnego skorelowany jest z obecnością proantocyjanidyn oraz katechin. Proantocyjanidyny są uważane za najsilniejszy, naturalny przeciwutleniacz [praca 2].

Dodatkowo zauważono, że w obecności izoramnetyny zakupionej komercyjnie oraz jej dwóch pochodnych wyizolowanych z jagód rokitnika zwyczajnego (izoramnetyna 3-O-beta-glukozyd-7-O-alfa-ramnozyd oraz izoramnetyna 3-O-beta-glukozyd-7-O-alfa-(3'''-izowalerylo)-ramnozyd) peroksydacja lipidów osocza indukowana H_2O_2/Fe była istotnie zmniejszona po 30 minutowej inkubacji. Natomiast frakcja fenolowa z owoców nie miała istotnego wpływu na ten proces. Podobne wyniki uzyskano dla oznaczania poziomu grup karbonylowych w osoczu ludzkim: w tym układzie izoramnetyna, jej obie pochodne oraz frakcja fenolowa z jagód rokitnika istotnie hamowały ten proces. W przypadku oznaczenia grup tiolowych zaobserwowano ochronne właściwości dwóch testowanych pochodnych izoramnetyny oraz frakcji fenolowej, natomiast izoramnetyna nie wpływała na ten proces. W tym przypadku przypuszcza się, że flawonoidy (izoramnetyna oraz jej dwie pochodne) działają jako zmiatacze wolnych rodników [praca 3].

Badanie właściwości antyoksydacyjnych ekstraktów, frakcji oraz czystych związków wyizolowanych z różnych organów rokitnika zwyczajnego w płytkach krwi

Następstwem badań nad aktywnością antyoksydacyjną badanych preparatów w osoczu w niniejszej pracy doktorskiej była analiza ich działania przeciwutleniającego w płytkach krwi [praca 4; praca 5]. Wiadomo, że RFT, mogą działać jako wtórne cząsteczki sygnalizacyjne. Są one generowane w płytkach krwi niestymulowanych oraz stymulowanych trombiną [praca 4]. Na przykład, powstawanie anionorodnika ponadtlenkowego ($O_2^{\bullet-}$) skorelowane jest z enzymatycznym szlakiem metabolizmu kwasu arachidonowego. W celu stwierdzenia, jak preparaty z rokitnika wpływają na poziom $O_2^{\bullet-}$ w płytkach krwi zastosowano metodę opierającą się na redukcji cytochromu c. Stwierdzono, że jedynie ekstrakt z gałązek rokitnika zwyczajnego istotnie ogranicza produkcję anionorodnika ponadtlenkowego w płytkach krwi niestymulowanych oraz stymulowanych trombiną [praca 4]. Identyczny układ badań

zastosowano dla frakcji fenolowej oraz bogatej w niepolarne związki z liści oraz gałązek rokitnika zwyczajnego. Wszystkie użyte frakcje (1 i 10 μg/ml) znacznie zmniejszyły ilość $O_2^{\bullet-}$ w płytkach krwi niestymulowanych oraz aktywowanych trombiną [praca 5]. Można wnioskować, że badany ekstrakt z gałązek oraz frakcja fenolowa i frakcja bogata w związki niepolarne z liści i gałązek rokitnika zwyczajnego są źródłem związków, które mogą modulować aktywność płytek krwi poprzez zakłócanie matabolizmu kwasu arachidonowego [praca 4; praca 5].

Wpływ preparatów z rokitnika zwyczajnego na hemostazę osoczową

Jednym z etapów niniejszej rozprawy doktorskiej była ocena wpływu różnych preparatów (ekstraktów oraz frakcji) wyizolowanych z liści oraz gałązek rokitnika zwyczajnego na hemostazę osoczową. W tym celu wykonano pomiar czasów krzepnięcia krwi: czasu trombinowego (TT), protrombinowego (PT) oraz kaolinowo-kefalinowego (APTT). Wykonanie powyższych doświadczeń pozwoliło odpowiedzieć na pytanie: czy i na jakim etapie hemostazy osoczowej następuje interakcja testowanych preparatów oraz czy badane preparaty wpływają na wydłużenie lub skrócenie powstawania skrzepu. Ekstrakty z gałązek i liści rokitnika zwyczajnego (w najwyższym badanym stężeniu – 50 μg/ml) inkubowane z osoczem ludzkim przez 30 minut w temperaturze 37°C znacząco wydłużały czas APTT. Parametr ten jest miarą skuteczności wewnętrzpochodnego mechanizmu aktywacji protrombiny bez udziału płytek krwi. Wykazano również, że ekstrakt z gałązek ma silniejsze działanie przeciwzakrzepowe niż ekstrakt z liści. Dodatkowo, wyciąg z gałązek miał silniejsze działanie przeciwzakrzepowe niż ekstrakt z jagód rokitnika zwyczajnego. Badane preparaty nie zmieniły istotnie czasów TT i PT [praca 1].

Ponadto, analiza wpływu frakcji fenolowej i niepolarnej z liści i gałązek rokitnika zwyczajnego (w zakresie dawek 0,5-50 μg/ml; czas inkubacji 30 minut) na właściwości koagulacyjne osocza wykazały, że frakcja fenolowa z liści znacząco wydłużyła czas protrombinowy. Przypuszcza się, że antykoagulacyjne własności tej frakcji mogą być związane z modulacją aktywności protrombiny lub czynników krzepnięcia V, VII oraz X przez związki bioaktywne w niej obecne. Co więcej, niepolarna frakcja z gałązek również istotnie wydłużyła czas kaolinowo-kefalinowy. Prawdopodobnie aktywność ta związana jest z obecnością triterpenów, acylowanych triterpenów oraz niezidentyfikowanych związków polarnych. Żadna z badanych frakcji nie zmieniła czasu TT [praca 2].

Analiza wpływu izoramnetyny, jej dwóch pochodnych (wyizolowanych z frakcji fenolowej z owoców rokitnika zwyczajnego) oraz frakcji bogatej w związki fenolowe z owoców rokitnika pokazała, że wyłącznie izoramnetyna 3-O-beta-glukozyd-7-O-alfa-(3”’-izowalerylo)-ramnozyd istotnie wydłużyła czas trombinowy. Przypuszcza się, że aktywność ta nie zależy od modulacji aktywności trombiny. Z drugiej strony wyniki uzyskane przez Choi i wsp., ukazują, że flawonoidy mogą hamować enzymatyczną aktywność trombiny. Trombina jako proteaza serynowa pełni nie tylko istotne funkcje w procesach krzepnięcia, ale jest ona również aktywatorem płytek krwi [Choi i wsp., 2016]. Nie odnotowano istotnego pływu badanych preparatów na inne czasy krzepnięcia [praca 3]. Z drugiej strony po raz pierwszy w swojej pracy obserwowałem hamujący wpływ izoramnetyny oraz izoramnetyny 3-O-beta-glukozyd-7-O-alfa-(3”’- izowalerylo)-ramnozyd na agregację płytek krwi stymulowanych trombiną. Co więcej, nie odnotowano wpływu badanych związków na agregację płytek krwi stymulowanych ADP oraz kolagenem, Może to oznaczać, że izoramnetyna oraz jej pochodna (izoramnetyna 3-O-beta-glukozyd-7-O-alfa-(3”’- izowalerylo)-ramnozyd) mogą modulować aktywację płytek krwi poprzez zakłócanie działania receptorów trombiny na płytkach krwi [praca 3].

Zmiany aktywacji płytek krwi w obecności ekstraktów, frakcji oraz czystych związków wyizolowanych z różnych organów rokitnika zwyczajnego

Na tym etapie pracy analizie poddano wpływ ekstraktów oraz frakcji z rokitnika zwyczajnego na wybrane markery aktywacji płytek krwi (stosowano wyizolowane płytki krwi, osocze bogatopłytkowe (PRP) oraz krew pełną) [praca 4, 5, 6]:

- pomiar adhezji płytek krwi do kolagenu i fibrynogenu metodą kolorymetryczną,
- pomiar agregacji płytek krwi metodą turbidymetryczną,
- pomiar peroksydacji lipidów (nieenzymatycznej i enzymatycznej peroksydacji lipidów – przemiana kwasu arachidonowego) z kwasem tiobarbiturowym (TBA) w płytkach krwi metodą kolorymetryczną,
- pomiar ekspresji selektyny P i receptora GPIIb/IIIa na powierzchni płytek krwi z wykorzystaniem metody cytometrii przepływowej we krwi pełnej,
- pomiar fosforylacji VASP (vasodilator-stimulated phoshoprotein).

Uzyskane wyniki pokazują znacznie niższą adhezję niestymulowanych i stymulowanych trombiną płytek krwi do kolagenu w obecności ekstraktów z liści oraz gałązek rokitnika zwyczajnego (0,5-50 µg/ml). W najwyższym użytym stężeniu ekstrakt z gałązek rokitnika zwyczajnego powodował większe hamowanie adhezji płytek krwi

aktywowanych trombiną do kolagenu i fibrynogenu niż ekstrakt z liści. W kolejnej części badań zbadano potencjał antyagregacyjny testowanych ekstraktów [praca 4]. Agregację płytek krwi wywoływano różnymi agonistami: ADP, kolagenem oraz trombiną. Nie stwierdzono, aby badane ekstrakty wykazywały jakiekolwiek właściwości antyagregacyjne, gdy stosowano ADP i kolagen. Jednak ekstrakt z liści (10 i 50 μg/ml) istotnie hamował agregację płytek krwi traktowanych trombiną, podobnie jak ekstrakt z gałązek przy najwyższym testowanym stężeniu, może to wynikać z interakcji związków chemicznych obecnych w badanych preparatach z białkami osocza bogatopłytkowego (PRP), co zapobiega aktywności agregacyjnej [praca 4].

Aktywacja płytek krwi jest związana z metabolizmem kwasu arachidonowego, któremu towarzyszy powstawanie tromboksanu A_2. W niniejszych doświadczeniach wykorzystano stężenie TBARS jako wskaźnik enzymatycznej peroksydacji kwasu arachidonowego w płytkach krwi. Nie zaobserwowano wpływu ekstraktu z liści na poziom TBARS w płytkach krwi stymulowanych oraz niestymulowanych trombiną. Z drugiej strony, wszystkie użyte stężenia ekstraktu z gałązek znacząco zmniejszały peroksydację lipidów zarówno w płytkach krwi niestymulowanyh oraz traktowanych trombiną. Działanie to może wynikać z różnic w składzie chemicznym testowanych ekstraktów. Przypuszcza się też, że związki fenolowe obecne w ekstrakcie z gałązek rokitnika zwyczajnego wpływają na zahamowanie aktywacji szlaku kwasu arachidonowego [praca 4].

Niewiele wiadomo na temat wpływu liści i gałązek rokitnika zwyczajnego oraz ich związków składowych na aktywację i procesy biochemiczne płytek krwi, dlatego podobny układ badawczy zastosowano dla frakcji fenolowej oraz bogatej w związki niepolarne z liści oraz gałązek rokitnika zwyczajnego. Analizie poddano dwa rodzaje frakcji w zakresie stężeń od 1 do 50 μg/ml. Zbadano ich wpływ na wybrane etapy aktywacji płytek krwi oraz na wybrane procesy biochemiczne (nieenzymatyczną oraz enzymatyczną peroksydację lipidów) [praca 5]. Zaobserwowano, że testowane frakcje nie wpływają na poziom TBARS w niestymulowanych płytkach krwi. Natomiast obie badane frakcje z liści oraz gałązek istotnie hamowały enzymatyczną peroksydację lipidów płytek krwi stymulowanych trombiną we wszystkich badanych stężeniach. Może to sugerować, że związki bioaktywne obecne w testowanych frakcjach są w stanie modulować aktywność płytek krwi poprzez zakłócanie metabolizmu kwasu arachidonowego oraz mogą wpływać na reaktywność płytek krwi poprzez modyfikację poziomu RFT i modulację ekspresji receptorów płytkowych. [praca 5].

Ponadto, zaobserwowano istotne zahamowanie adhezji płytek krwi niestymulowanych do kolagenu preinkubowanych z badanymi preparatami tj. frakcją fenolową z gałązek (1 μg/ml) oraz liści (1; 10 μg/ml), frakcją bogatą w związki niepolarne z gałązek (1; 10 μg/ml) oraz z liści we wszystkich testowanych stężeniach (1-50 μg/ml). W przypadku adhezji płytek krwi stymulowanych trombiną do kolagenu odnotowano, że frakcja fenolowa z gałązek hamowała ten proces we wszystkich badanych stężeniach (1, 10 i 50 μg/ml), podczas gdy frakcja fenolowa z liści istotnie hamowała adhezję w stężeniach 1 i 50 μg/ml. Ponadto, zaobserwowano, że adhezja płytek krwi stymulowanych trombiną i ADP do fibrynogenu była istotnie zahamowana po preinkubacji płytek krwi ze wszystkimi badanymi frakcjami. Stwierdzono także, że agregację płytek krwi stymulowaną przez ADP istotnie hamowały obie badane frakcje z liści i gałązek rokitnika zwyczajnego. Nie zaobserwowano natomiast istotnego wpływu testowanych frakcji na agregację płytek krwi traktowanych kolagenem czy trombiną. Odnotowano również, że jedynie frakcja fenolowa z liści przy najwyższym stężeniu istotnie hamowała agregację płytek wywołaną ADP. Na podstawie otrzymanych wyników można zasugerować, że testowane frakcje wyizolowane z liści oraz gałązek rokitnika zwyczajnego mają właściwości antypłytkowe [praca 5]. Różne badania potwierdzają, że proantocyjanidyny oraz antocyjanidyny charakteryzują się pozytywnym wpływem na układ sercowo-naczyniowy poprzez hamowanie aktywacji płytek krwi [Chong i wsp., 2010; Olas, 2017]. Jednakże, mechanizmy przeciwpłytkowego działania triterpenoidów i ich pochodnym nie są do końca poznane i wymagają dalszych badań.

Do pomiaru aktywacji płytek krwi i ich reaktywności w obecności dwóch fizjologicznych agonistów (10 i 20 μM ADP i 10 μg/ml kolagenu) w pełnej krwi traktowanej sześcioma różnymi frakcjami z rokitnik zwyczajnego zastosowano trójkolorową cytometrię przepływową. Analizie poddano ekspresję selektyny P (CD62P) oraz aktywację kompleksu GPIIb/IIIa (wiązanie PAC-1). Wyniki wskazują na zmieniony poziom aktywacji płytek krwi we wszystkich próbkach traktowanych badanymi frakcjami roślinnymi w porównaniu z nietraktowanymi kontrolami, dotyczyło to zarówno próbek stymulowanych agonistą (ADP lub kolagen), jak i tych niestymulowanych. Testowane frakcje fenolowe i zawierające związki niepolarne z owoców i liści zmniejszały wiązanie PAC-1 w płytkach krwi aktywowanych 10 μM ADP, podczas gdy frakcja związków niepolarnych z gałązek zwiększyła wiązanie PAC-1 w płytkach krwi aktywowanych przez 20 μM ADP. W niniejszym badaniu oceniano również stopień odpowiedzi płytek krwi na kolagen (10 μg/ml) w obecności sześciu użytych frakcji roślinnych w dwóch stężeniach (5 i 50 μg/ml) poprzez pomiar ekspresji CD62P i wiązania PAC-1. Stwierdzono, że frakcja fenolowa (5 i 50 μg/ml) z owoców zmniejszała wiązanie PAC-

1 w płytkach krwi aktywowanych kolagenem. Można przypuszczać, że zahamowanie agregacji płytek krwi, potwierdzone we wcześniejszych badaniach [praca 4; praca 5] skorelowane jest z niską ekspresją receptora GPIIb/IIIa [praca 6].

Wiadomo, że w aktywacji płytek krwi stymulowanych ADP uczestniczą dwa receptory purynergiczne P_2Y_1 i P_2Y_{12}. Fosforylacja VASP (vasodilator-stimulated phoshoprotein) koreluje z hamowaniem receptora P_2Y_{12}, podczas gdy stan braku fosforylacji koreluje z aktywacją tego receptora. Test śledzący fosforylację VASP jest wykorzystywany do badania interakcji leków przeciwpłytkowych (na przykład klopidogrelu) z receptorem P_2Y_{12}. W tym etapie badań monitorowałem specyficzny receptor ADP dla płytek krwi (P_2Y_{12}) za pomocą zestawu do cytometrii przepływowej (PLT VASP/P_2Y_{12}). Test przeprowadzono zgodnie z instrukcjami producenta, a wyniki przedstawiono w postaci PRI (wskaźnik reaktywności płytek). Nie zaobserwowano żadnych różnic między wartościami PRI próbek traktowanych testowanymi frakcjami roślinnymi (50 μg/ml) i próbek kontrolnych, co może wskazywać, że potencjał przeciwpłytkowy badanych frakcji nie jest zależny od receptora P_2Y_{12} [praca 6].

Analiza procesu tworzenia skrzepliny w warunkach pół-fizjologicznych, w przepływie krwi traktowanej preparatami z rokitnika zwyczajnego

W dalszym etapie prac analizowano proces tworzenia skrzepu krwi traktowanej frakcjami z rokitnika zwyczajnego w modelu hydrodynamicznego przepływu krwi w czasie rzeczywistym. Badanie to służy do oceny trombogenności w krwi pełnej. W tym doświadczeniu wykorzystano chip pokryty kolagenem do wizualizacji powstawania skrzepliny płytek krwi. Krew pełną inkubowano z badanymi preparatami (frakcje: fenolowa oraz związków niepolarnych z owoców, liści oraz gałązek rokitnika zwyczajnego) (37°C; 30 minut). Odnotowano, że frakcja fenolowa z owoców, liści i gałązek badanej rośliny istotnie spowalnia proces powstawania skrzepliny. Dodatkowo zaobserwowano, że frakcja bogata w związki niepolarne z liści również hamuje ten proces [praca 6].

Analiza elektroforetyczna białek płytkowych traktowanych frakcjami z rokitnika zwyczajnego

W tym etapie badań wykonano analizę elektroforetyczną w warunkach redukujących oraz nieredukujących (+/- 2-merkaptoetanol) i nie zaobserwowano różnic między elektroforogramami płytek krwi traktowanych frakcjami roślinnymi, a próbkami kontrolnymi [praca 6].

Ocena toksyczności testowanych preparatów z rokitnika zwyczajnego

Ocenę toksyczności badanych preparatów z rokitnika zwyczajnego przeprowadzono z wykorzystaniem pomiaru aktywności dehydrogenazy mleczanowej (LDH). Żaden z badanych preparatów nie był toksyczny w stosunku do płytek krwi [praca 4, 5].

Wnioski:

1) Preparaty wyizolowane z różnych organów rokitnika zwyczajnego wykazują hamujący wpływ na proces aktywacji płytek krwi.
2) Izoramnetyna zakupiona komercyjnie oraz jej dwie pochodne wyizolowane z frakcji fenolowej z jagód rokitnika zwyczajnego charakteryzują się aktywnością antypłytkową.
3) Zahamowanie aktywacji płytek krwi przez testowane preparaty wiąże się z zaburzeniami przemian biochemicznych zachodzących w płytkach krwi.
4) Badane preparaty z rokitnika zwyczajnego nie są toksyczne w stosunku do płytek krwi.
5) Rokitnik zwyczajny jest źródłem związków o aktywności antyoksydacyjnej w osoczu oraz płytkach krwi.
6) Właściwości antypłytkowe i antyoksydacyjne preparatów wyizolowanych z różnych organów rokitnika zwyczajnego skorelowane są z ich składem chemicznym.
7) Największe działanie antypłytkowe i antyoksydacyjne wykazuje frakcja fenolowa wyizolowana z gałązek rokitnika zwyczajnego. Frakcja ta jest bogatym źródłem proantocyjanidyn i katechin.

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Biological properties of *Elaeagnus rhamnoides* (L.) A. Nelson twig and leaf extracts

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Abstract

Background: Sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson, SBT) is a valuable plant because of its medical and therapeutic potential. Different bioactive compounds in SBT berries are of special interest to various researchers. However, not only sea buckthorn berries, but also leaves of this plant (both fresh and dried) contain a lot of nutrients and bioactive compounds, including phenolic compounds. The present study was carried out in order to investigate antioxidant and anticoagulant properties of sea buckthorn twig and leaf extracts (0.5–50 μg/mL) by using various in vitro models. Moreover, the aim of present experiments was to compare the biological activity of SBT leaf extract and SBT twig extract with selected berry extracts (a rich source of phenolic compounds): SBT berry extract (flavonoids being the dominant components), a commercial extract from the berries of *Aronia melanocarpa* (Aronox®), and a grape seed extract.

Methods: We determined the effect of plant extracts on the oxidative stress using selected markers of this process, i.e. the level of carbonyl groups in proteins. Additionally, we analysed the potential mechanism of modulation of hemostatic properties of human plasma (using selected coagulation times).

Results: SBT twig and leaf extracts were observed to exhibit an antioxidant activity against two strong biological oxidants: hydrogen peroxide (H_2O_2) and H_2O_2/Fe (the donor of hydroxyl radicals), which induced human plasma lipid peroxidation and protein carbonylation. Both extracts also showed anticoagulant properties.

Conclusions: Our present results have demonstrated that extracts from different parts of SBT, especially berries and twigs, in comparison to well-known berries (aronia and grape), may also be viewed as a good source of active substances – antioxidants for pharmacological or cosmetic applications. Moreover, it is very important from an economic point of view to know that there is a possibility of obtaining phenolic compounds not only from the berries or leaves, but also from twigs, which constitute a production waste.

Keywords: Oxidative stress, *Elaeagnus rhamnoides* (L.) a. Nelson, Twig, Leaf, Berry, Phenolic compounds, Hemostasis

Background

Sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson, SBT) is an important plant because of its immense medical and therapeutic potential [1–4]. Different bioactive compounds in SBT berries are of special interest to various researchers [1, 5, 6]. However, not only sea buckthorn berries, but also leaves of this plant (both fresh and dried) contain large amounts of nutrients and bioactive compounds, including phenolic compounds [7]. Over the recent years, SBT leaf extracts have been scientifically investigated and various biological properties, i.e. radioprotective, anti-inflammatory and immunomodulatory, have been reported [1, 7, 8]. Results of Lee et al. [9] and Pichiah et al. [10] demonstrated that SBT leaves (used in the form of teas and extracts) possess anti-obesity properties. Recently, Sadowska et al. [11] have shown that not only SBT leaf extract, but also its twig extract, have anti-virulence action in vitro. However, lack

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of interest in the potential value of these extracts, especially SBT twig extract as the source of antioxidants and anticoagulants, is surely a significant hindrance for the development of alternative substances for prevention and treatment of cardiovascular diseases, which are frequently associated with oxidative stress and changes in hemostasis.

The aim of present experiments was to determinate the potential of SBT twig extract components and SBT leaf extract components for: (I) modulation of oxidative stress in human plasma treated with a strong biological oxidant: hydrogen peroxide (H_2O_2) and H_2O_2/Fe (the donor of hydroxyl radicals) (using selected markers of oxidative stress, i.e. the level of carbonyl groups in proteins); (II) modulation of hemostatic properties of human plasma (using selected coagulation times). It should be also emphasized that a novel aspect of our study focused on the comparison of biological activity of SBT leaf extract and SBT twig extract with selected berry extracts (rich in phenolic compounds): SBT berry extract (flavonoids were the dominant components [3, 4]), a commercial extract from the berries of *Aronia melanocarpa* (black chokeberry or aronia berry; Aronox®), and a grape seed extract, which displays not only antioxidative, but also anticoagulant and antiplatelet properties [2, 4, 12–14].

Methods

Reagents

Dimethylsulfoxide (DMSO), thiobarbituric acid (TBA), H_2O_2, and formic acid (LC-MS grade) were acquired from Sigma-Aldrich (St. Louis, MO., USA). Methanol (isocratic grade) and acetonitrile (LC-MS grade) were purchased from Merck (Darmstadt, Germany). All remaining reagents represented analytical grade and were provided by commercial suppliers.

A stock solution of *A. melanocarpa* berry extract (commercial product – Aronox® by Agropharm Ltd., Poland; batch No. 020/2007 k) was prepared in H_2O at a concentration of 5 mg/mL, then kept frozen and subsequently used for experiments. The total content of phenolics in the phenolic-rich powder used in this study amounted to 309.6 mg/g of extract, including phenolic acids (isomers of chlorogenic acid) – 149.2 mg/g of extract, anthocyanins (anthocyanin glycosides: cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, cyanidin 3-xyloside) – 110.7 mg/g, and flavonoids (quercetin glycosides) – 49.7 mg/g of extract. The HPLC determination of the phenolic-rich extract from *A. melanocarpa* berries had been previously described [12–14].

The grape seed extract was supplied by Bionorica (Germany) and was characterized by a total content of phenolics equalling 500 mg/g of extract [13]. A stock solution of grape seed extract was prepared in 50% DMSO.

Plant material

Sea buckthorn berries, twigs and leaves were harvested from a horticultural farm in Sokółka, Podlaskie Voivodeship, Poland (53°24′N, 23°30′E), the greatest Polish producer of sea buckthorn fruits. The plant material was identified by Mr. Stanislaw Trzonkowski, the owner of the farm. A voucher specimens have been deported at the Institute of Soil Science and Plant Cultivation – Sate Research Institute, Pulawy, Poland (IUNG/HRH/2015/2) .

Chemical characteristics of the extract of phenolic compounds from sea buckthorn berries, twigs and leaf

Extracts from the fruit, leaves and twigs of sea buckthorn were prepared as previously described [3, 11]. Their composition was determined by reverse-phase UHPLC-MS/MS, using ACQUITY UPLC™ system (Waters, Milford, MA, USA), coupled with an ACQUITY TQD (Waters) triple quadrupole mass detector. Chromatographic separations were performed on an ACQUITY HSS C18 (100 × 2.1 mm, 1.8 μm; Waters) column (the fruit extract) and an ACQUITY BEH C18 (100 mm × 2.1 mm, 1.7 μm; Waters) column (leaf and twig extracts). Components of the extracts were identified on the basis of their MS and UV spectra, as well as literature data [15–17].

Stock solutions of the SBT berry extract, SBT twig extract and SBT leaf extract were made in 50% DMSO. The final concentration of DMSO in tested samples was lower than 0.05% and its effects were determined in all experiments.

Plasma isolation

Fresh human plasma and blood were obtained from healthy and medication-free donors of a blood bank at a Medical Center (Lodz, Poland). Moreover, blood was obtained from non-smoking men and women (collected into CPD solution (citrate/phosphate/dextrose; 9:1; v/v blood/CPD) or CPDA solution (citrate/phosphate/dextrose/adenine; 8.5:1; v/v; blood/CPDA)). Our analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research, and approved by the Committee on the Ethics of Research in Human Experimentation at the University of Lodz (resolution No. 3/KBBN-UŁ/II/2016). Plasma was incubated (15, 30 or 60 min, at 37 °C) with:

- SBT extracts at the final concentrations of 0.5–50 μg/mL
- SBT extracts at the final concentrations of 0.5–50 μg/mL plus 2 mM H_2O_2

- SBT extracts at the final concentrations of 0.5–50 μg/mL plus 4.7 mM H_2O_2/3.8 mM Fe_2SO_4/2.5 mM EDTA
- Aronia berry extract or grape seed extract at the final concentration of 50 μg/mL
- Aronia berry extract or grape seed extract at the final concentration of 50 μg/mL plus 2 mM H_2O_2
- Aronia berry extract or grape seed extract at the final concentration of 50 μg/mL plus 4.7 mM H_2O_2/3.8 mM Fe_2SO_4/2.5 mM EDTA.

The protein concentration, determined by measuring absorbance at 280 nm (in tested samples), was calculated according to the procedure of Whitaker and Granum [18].

Markers of oxidative stress

Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring the concentration of TBARS. Absorbance was measured at 535 nm (the SPECTROstar Nano Microplate Reader- BMG LABTECH Germany) [19, 20]. The TBARS concentration was calculated using the molar extinction coefficient ($\varepsilon = 156{,}000\ M^{-1}\ cm^{-1}$). More details were described in Skalski et al. [21].

Carbonyl group measurement

The detection of carbonyl groups in proteins was carried out according to Levine et al. [22] and Bartosz [20]. The carbonyl group concentration was calculated using a molar extinction coefficient ($\varepsilon = 22{,}000\ M^{-1}\ cm^{-1}$). The level of carbonyl groups was presented as nmol carbonyl groups/mg of protein. More details were described in Olas et al. [23].

Thiol group determination

The level of thiol group was measured spectrophotometrically (the SPECTROstar Nano Microplate Reader-BMG LABTECH Germany) by absorbance at 412 nm with Ellman's reagent: 5,5′-dithio-bis-(2-nitrobenzoic acid). The level of thiol groups was expressed as nmol thiol groups/mg of plasma protein [24, 25]. More details were described in Olas et al. [23].

Parameters of hemostasis

The measurement of prothrombin time (PT)

Human plasma was incubated at 37 °C on a block heater. After incubation, the cuvette was transferred to measuring holes. Then 100 μL of Dia-PT liquid (commercial preparation) was added. The PT was determined coagulometrically using Optic Coagulation Analyser model K-3002 [26].

The measurement of thrombin time (TT)

Human plasma was added to a coagulometric cuvette and incubated at 37 °C on a block heater. Then the cuvette was transferred to measuring holes and 100 μL of thrombin (final concentration - 5 U/mL) was added. The TT was determined coagulometrically using Optic Coagulation Analyser model K-3002 [26].

The measurement of activated partial thromboplastin time (APTT)

Human plasma was added to a coagulometric cuvette. Then the incubation was conducted at 37 °C on a block heater with 50 μL of Dia-PTT liquid (commercial preparation). The cuvette was transferred to measuring holes. Then 50 μL of 25 mM $CaCl_2$ was added. The APTT was determined coagulometrically (Optic Coagulation Analyser model K-3002) [26].

Data analysis

Several tests were used to carry out statistical analysis. All the values in this study were expressed as mean ± SD. Obtained results were analysed under the account of normality with Shapiro-Wilk test and equality of variance with Levine test. Statistical significance of differences among experimental variants was assessed by ANOVA (the significance level was $p < 0.05$), followed by Tukey multiple comparison test or Kruskal-Wallis test.

Results

The UHPLC-MS analyses demonstrated that different glycosides of isorhamnetin and quercetin (with isorhamnetin 3-*O*-Hex-dHex; isorhamnetin 3-*O*-Hex, and isorhamnetin 3-*O*-Hex-7-*O*-dHex as dominant compounds) were main constituents of the phenolic extract of sea buckthorn berries and their total amount, expressed as isorhamnetin 3-O-β-glucosyl-(1 → 2)-β-galactoside equivalent (214.04 mg/g). Other phenolic compounds were difficult to identify and most of them were present in small amounts. Their total content was 28.65 mg/g of the extract (expressed as isorhamnetin 3-O-β-glucosyl-(1 → 2)-β-galactoside equivalent) [3]. Ellagitannins (259.6 ± 3.1 mg/g) were identified as principal phenolic constituents of the SBT leaf extract. Flavonoids (74.7 ± 0.7 mg/g) were represented by glycosides of isorhamnetin (the dominant aglycone), quercetin, and kaempferol. The SBT twig extract consisted mainly of B –type proanthocyanidins and catechin (the total content 597.1 ± 10.2 mg/g). More details can be found in the original literature [3, 11]. The total content of phenolics in SBT berry extract, SBT twig extract and SBT leaf extract is demonstrated in Table 1.

Antioxidant properties of plant extracts cannot be evaluated by a single method, due to the complex nature of phytochemicals. Therefore, in the present study

Table 1 Total content of phenolics in the extracts used in this experiment [3, 4, 11–14, 23]

Tested extract	Total content of phenolics
Aronia berry extract (commercial product, Aronox®, by Agropharm Ltd. Poland)	309.8 mg/g of extract
Grape seed extract (by Bionorica, Germany)	500 mg/g of extract
SBT berry extract (phenolic extract)	242.7 mg/g of fraction
SBT leaf extract (butanolic extract)	341.5 mg/g of extract
SBT twig extract (butanolic extract)	621.2 mg/g of extract

antioxidant activity of tested plant extracts was evaluated on the basis of their influence on levels of lipid peroxidation, carbonyl groups and thiol groups in human plasma. The antioxidant activity of SBT twig and leaf extracts (at the concentrations: 0.5–50 μg/mL; incubation time: 15 and 60 min) were studied in vitro. As demonstrated in Fig. 1a, two tested extracts inhibited lipid peroxidation in human plasma treated with H_2O_2, but this inhibition was not concentration-dependent for 15 min of incubation time. However, we observed that the two tested extracts (at all concentrations) did not change plasma lipid peroxidation (induced by H_2O_2)

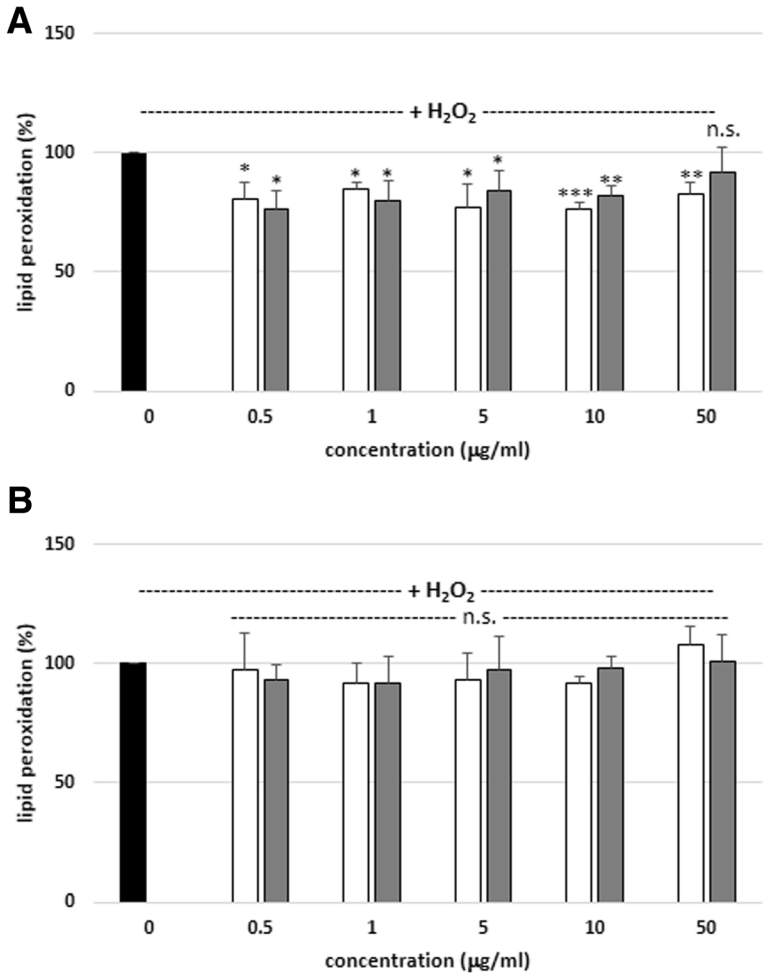

Fig. 1 Effects of SBT twig and leaf extracts (0.5–50 μg/mL; 15 min (**a**) and 60 min (**b**)) on plasma lipid peroxidation induced by H_2O_2. In these experiments, the TBARS level (marker of lipid peroxidation) in control samples (plasma treated with only H_2O_2) was 0.254 ± 0.046 nmol/mL of plasma. Data represent means ± SD of 5–10. The effect of five different concentrations of two tested extracts (0.5, 1, 5, 10 and 50 μg/mL; for 15 min) was statistically significant (*$p < 0.05$, **$p < 0.005$; ***$p < 0.001$) in comparison to control. The effect of five different concentrations of two tested extracts (0.5, 1, 5, 10 and 50 μg/mL; for 60 min) was not statistically significant ($p > 0.05$ (n.s.)) in comparison to control. The effects were not statistically significant: SBT twig extract-treated plasma vs. SBT leaf extract-treated plasma ($p > 0.05$ (n.s.); for all tested concentrations- 0.5 - 50 μg/mL; for 15 and 60 min). black diagram – control, white diagram – twig, grey diagram - leaf

when longer incubation time (60 min) was applied (Fig. 1b). On the other hand, SBT twig extract (at doses: 5, 10 and 50 μg/mL, for 15 and 60 min of incubation time) reduced the level of plasma lipid peroxidation induced by H_2O_2/Fe; additionally, activity of the extract was concentration-dependent (Fig. 2a and b). SBT leaf extract revealed antioxidant properties at the same concentrations, but only for 60 min of incubation time (Fig. 2b). Moreover, in this model (with H_2O_2/Fe as the inducer of oxidative stress), SBT twig extract (at the highest concentration – 50 μg/mL; 60 min) demonstrated stronger antioxidant properties than SBT leaf extract (at the same concentration) (Tab. 2). SBT twig extract reduced lipid peroxidation by about 40%, and SBT leaf extract by about 30% (Tab. 2).

Another set of experiments focused on plasma protein carbonylation levels; the tested extracts had no effect on this process during short incubation – 15 min (Figs. 3a and 4a). Nevertheless,, the protein carbonylation (induced by H_2O_2 and H_2O_2/Fe) was reduced in the presence of SBT twig and leaf extracts, when a longer incubation time was applied – 60 min (Fig. 3b and 4b). In addition, SBT twig extract (at 50 μg/mL, incubation time – 60 min) had stronger inhibitory effect on plasma

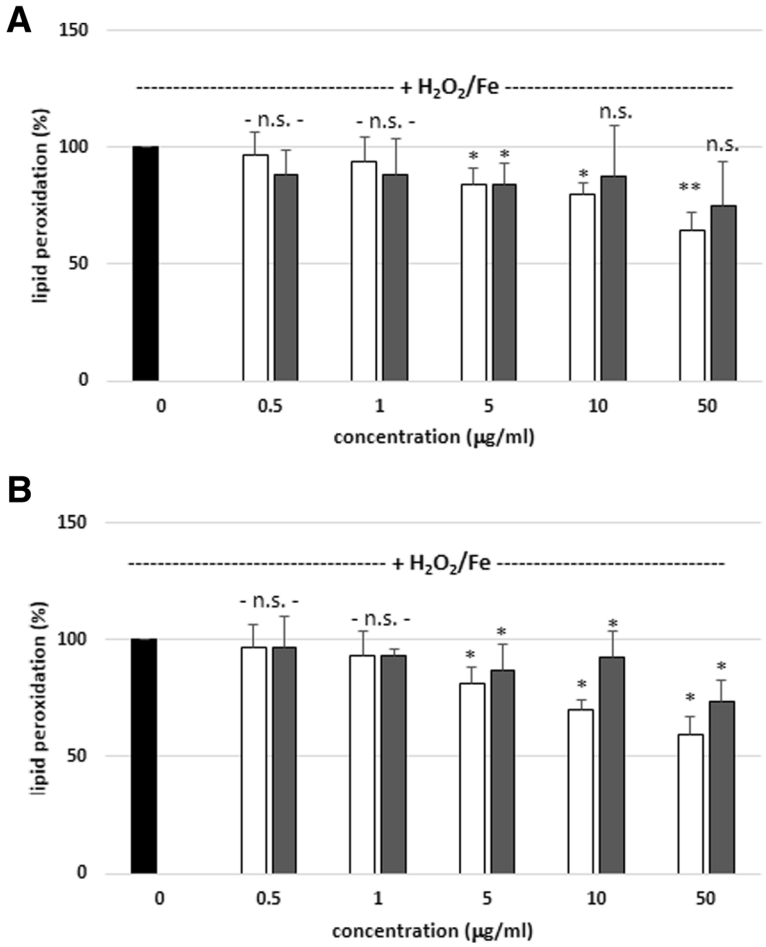

Fig. 2 Effects of SBT twig and leaf extracts (0.5–50 μg/mL; 15 min (**a**) and 60 min (**b**)) on plasma lipid peroxidation induced by H_2O_2/Fe. In these experiments, the TBARS level (marker of lipid peroxidation) in control samples (plasma treated with only H_2O_2/Fe) was 0.341 ± 0.078 nmol/mL of plasma. Data represent means ± SD of 5–10. The effect of two different concentrations of two tested extracts (0.5 and 1 μg/mL; for 15 and 60 min) was not statistically significant ($p > 0.05$ (n.s.)) in comparison to control. The effect of two different concentrations of SBT leaf extract (10 and 50 μg/mL; for 15 min) was not statistically significant ($p > 0.05$ (n.s.)) in comparison to control. The effect of three different concentrations of SBT twig extract (5, 10 and 50 μg/mL; for 15 min) was statistically significant (*$p < 0.05$, **$p < 0.005$) in comparison to control. The effect of one concentration of SBT leaf extract (5 μg/mL; for 15 min) was statistically significant (*$p < 0.05$) in comparison to control. The effect of three different concentrations of two tested extracts (5, 10 and 50 μg/mL; for 60 min) was statistically significant (*$p < 0.05$) in comparison to control. The effects were not statistically significant: SBT twig extract-treated plasma vs. SBT leaf extract-treated plasma (for 15 min: $p > 0.05$ (n.s.), for all tested concentrations- 0.5 - 50 μg/mL); for 60 min ($p > 0.05$ (n.s.), for tested concentrations: 0.5, 1 and 10 μg/mL). The effects were statistically significant: SBT twig extract-treated plasma vs. SBT leaf extract-treated plasma, (for 60 min, $p < 0.05$ for tested concentrations: 10 and 50 μg/mL). black diagram – control, white diagram – twig, grey diagram - leaf

Table 2 Comparison of antioxidant properties of SBT twig and leaf extracts with properties of selected berry extracts (50 μg/mL; 15 and 60 min) in human plasma. Data represent means ± SD of 5–12. The level of marker of oxidative stress in control sample (plasma treated with H_2O_2 or H_2O_2/Fe) was expressed as 100%

% of lipid peroxidation induced by H_2O_2 (incubation time – 15 min)	
Control	100
SBT leaf extract (A)	91.9 ± 10.5
SBT twig extract (B)	82.5 ± 4.9; B vs A ($p > 0.05$ (n.s.))
Aronia berry extract (C)	83.1 ± 10.4; C vs A ($p > 0.05$ (n.s.)); C vs B ($p > 0.05$ (n.s.))
Grape seed extract (D)	79.4 ± 9.9; D vs A ($p > 0.05$ (n.s.)); D vs B (p > 0.05 (n.s.))
SBT berry extract (E)	60.3 ± 12.1; E vs A ($p < 0.01$); E vs B ($p < 0.01$)
% of lipid peroxidation induced by H_2O_2 (incubation time – 60 min)	
Control	100
SBT leaf extract (A)	107.9 ± 8.0
SBT twig extract (B)	101.1 ± 10.9; B vs A ($p > 0.05$ (n.s.))
Aronia berry extract (C)	91.0 ± 3.2; C vs A ($p > 0.05$ (n.s.)); C vs B ($p > 0.05$ (n.s.))
Grape seed extract (D)	97.2 ± 5.5; D vs A ($p > 0.05$ (n.s.)); D vs B ($p > 0.05$ (n.s.))
SBT berry extract (E)	39.4 ± 7.7; E vs A ($p < 0.001$); E vs B ($p < 0.001$)
% of lipid peroxidation induced by H_2O_2/Fe (incubation time – 15 min)	
Control	100
SBT leaf extract (A)	74.6 ± 19.1
SBT twig extract (B)	64.5 ± 15.6; B vs A ($p > 0.05$ (n.s.))
Aronia berry extract (C)	61.4 ± 11.2; C vs A ($p > 0.05$ (n.s.)); C vs B ($p > 0.05$ (n.s.))
Grape seed extract (D)	73.5 ± 8.8; D vs A ($p > 0.05$ (n.s.)); D vs B ($p > 0.05$ (n.s.))
SBT berry extract (E)	30.4 ± 9.7; E vs A ($p < 0.01$); E vs B ($p < 0.01$)
% of lipid peroxidation induced by H_2O_2/Fe (incubation time – 60 min)	
Control	100
SBT leaf extract (A)	73.2 ± 9.7
SBT twig extract (B)	59.7 ± 7.5; B vs A ($p < 0.05$)
Aronia berry extract (C)	89.3 ± 9.5; C vs A ($p < 0.05$); C vs B ($p < 0.05$)
Grape seed extract (D)	85.0 ± 8.8; D vs A ($p < 0.05$); D vs B ($p < 0.05$)
SBT berry extract (E)	59.2 ± 9.5; E vs A ($p < 0.05$); E vs B ($p > 0.05$ (n.s.))
% of protein carbonylation induced by H_2O_2 (incubation time – 15 min)	
Control	100
SBT leaf extract (A)	95.4 ± 13.9
SBT twig extract (B)	80.8 ± 26.7; B vs A ($p > 0.05$ (n.s.))
Aronia berry extract (C)	99.7 ± 17.3; C vs A ($p > 0.05$ (n.s.)); C vs B ($p > 0.05$ (n.s.))
Grape seed extract (D)	95.4 ± 11.4; D vs A ($p > 0.05$ (n.s.)); D vs B ($p > 0.05$ (n.s.))
SBT berry extract (E)	66.4 ± 10.3; E vs A ($p < 0.01$); E vs B ($p > 0.05$ (n.s.))
% of protein carbonylation induced by H_2O_2 (incubation time – 60 min)	
Control	100
SBT leaf extract (A)	55.6 ± 19.7
SBT twig extract (B)	56.4 ± 18.9; B vs A ($p > 0.05$ (n.s.))
Aronia berry extract (C)	92.4 ± 11.4; C vs A ($p < 0.01$; C vs B ($p < 0.01$)
Grape seed extract (D)	74.3 ± 10.5; D vs A ($p < 0.05$); D vs B ($p < 0.05$)
SBT berry extract (E)	61.4 ± 9.7; E vs A ($p > 0.05$ (n.s.)); E vs B ($p > 0.05$ (n.s.))
% of protein carbonylation induced by H_2O_2/Fe (incubation time – 15 min)	
Control	100
SBT leaf extract (A)	96.4 ± 8.1
SBT twig extract (B)	97.7 ± 5.1; B vs A ($p > 0.05$ (n.s.))
Aronia berry extract (C)	99.0 ± 15.5; C vs A ($p > 0.05$ (n.s.)); C vs B ($p > 0.05$ (n.s.))
Grape seed extract (D)	96.7 ± 12.4 D vs A ($p > 0.05$ (n.s.)); D vs B ($p > 0.05$ (n.s.))
SBT berry extract (E)	79.4 ± 10.2; E vs A ($p < 0.02$); E vs B ($p < 0.02$)
% of protein carbonylation induced by H_2O_2/Fe (incubation time – 60 min)	
Control	100
SBT leaf extract (A)	53.3 ± 21.4
SBT twig extract (B)	75.6 ± 18.4; B vs A ($p < 0.05$)
Aronia berry extract (C)	82.6 ± 15.1; C vs A ($p < 0.05$); C vs B ($p > 0.05$ (n.s.))
Grape seed extract (D)	80.5 ± 17.3; D vs A ($p < 0.05$); D vs B ($p > 0.05$ (n.s.))
SBT berry extract (E)	69.4 ± 12.3; E vs A ($p > 0.05$ (n.s.)); E vs B ($p > 0.05$ (n.s.))

protein carbonylation (induced by H_2O_2/Fe) than SBT leaf extract (at 50 μg/mL, incubation time – 60 min); i.e. inhibition of this process was only about 53% for SBT leaf extract (50 μg/mL), and about 76% for SBT twig extract (50 μg/mL) (Fig. 4b, Table 2).

Analysis of the effect of tested extracts (50 μg/mL) on oxidation of plasma protein thiols demonstrated that SBT twig extract and leaf extract did not affect the said process (Fig. 5).

Moreover, we have demonstrated differences in antioxidant activity between SBT leaf or twig extract and selected berry extracts, i.e. SBT berry extract (butanolic extract). Table 2 shows comparative effects of SBT twig extract, SBT leaf extract and berry extracts, including

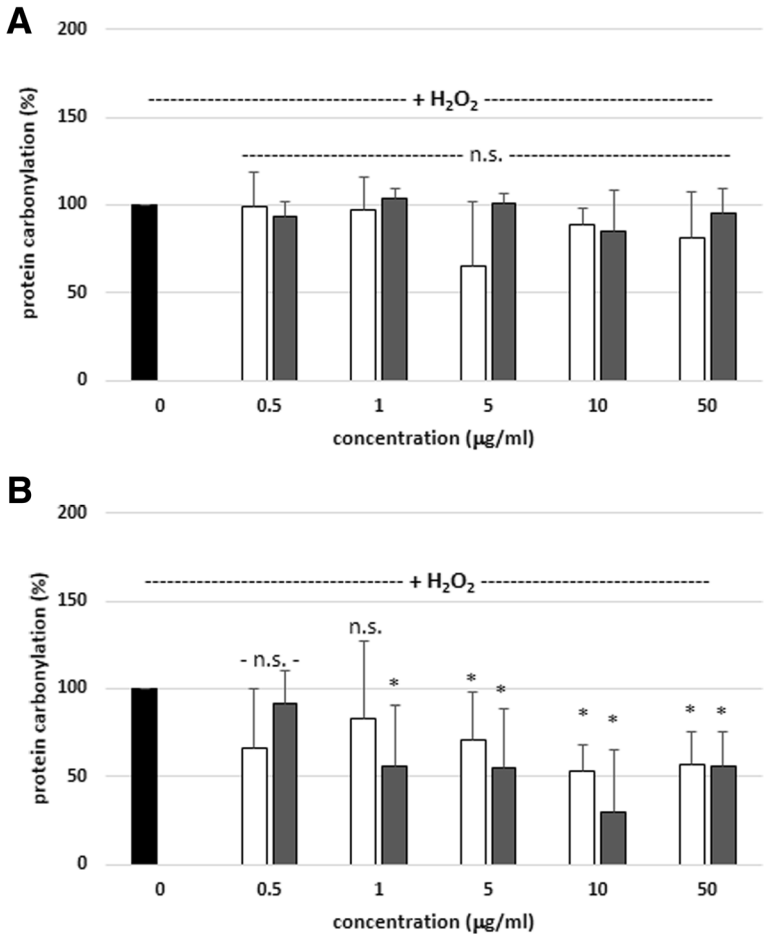

Fig. 3 Effects of SBT twig and leaf extracts (0.5–50 μg/mL; 15 min (**a**) and 60 min (**b**)) on plasma protein carbonylation induced by H_2O_2. In these experiments the carbonyl group level (marker of protein oxidation) in control samples (plasma treated only with H_2O_2) was 17.1 ± 4.3 nmol/mg of plasma protein. Data represent means ± SD of 6–12. The effect of five different concentrations of two tested extracts (0.5, 1, 5, 10 and 50 μg/mL; for 15 min) was not statistically significant ($p > 0.05$ (n.s.)) in comparison to control. The effect of two different concentrations of SBT twig extracts (0.5 and 1 μg/mL; for 60 min) was not statistically significant ($p > 0.05$ (n.s.)) in comparison to control. The effect of one concentration of SBT leaf extract (0.5 μg/mL; for 60 min) was not statistically significant ($p > 0.05$ (n.s.)) in comparison to control. The effect of three different concentrations of SBT twig extracts (5, 10 and 50 μg/mL; for 60 min) was statistically significant (*$p < 0.05$), in comparison to control. The effect of four different concentrations of SBT leaf extracts (1, 5, 10 and 50 μg/mL; for 60 min) was statistically significant (*$p < 0.05$), in comparison to control. The effects were not statistically significant: SBT twig extract-treated plasma vs. SBT leaf extract-treated plasma (for 15 and 60 min, $p > 0.05$ (n.s.); for all tested concentrations- 0.5 - 50 μg/mL). black diagram – control, white diagram – twig, grey diagram - leaf

SBT berry extract, aronia berry extract and grape seed extract (50 μg/mL) on the level of biomarkers of oxidative stress in human plasma. We observed that SBT berry extract had stronger antioxidant properties (especially for the inhibition of lipid peroxidation) than SBT twig and leaf extracts (Tab. 2), i.e. the inhibition of lipid peroxidation (induced by H_2O_2/Fe, incubation time – 15 min) reached about 70% (for SBT berry extract), about 25% (for SBT leaf extract) and about 35% (for SBT twig extract). However, antioxidant properties of SBT twig and leaf extracts were very often similar, like for aronia berry extract and grape seed extract (Tab. 2).

As shown in Table 3, SBT twig and leaf extracts (at the highest test concentration - 50 μg/mL; incubation time – 30 min) significantly prolonged the APTT time. We demonstrated that SBT twig extract had stronger activities than leaf extract. In addition, SBT twig extract had stronger anticoagulant activity than berry extracts (SBT berry extract, aronia berry extract and grape seed extract (Tab. 3). However, SBT twig and leaf extracts did not change the TT and the PT (data are not presented).

Discussion

Sea buckthorn is a wild plant that has been used for centuries as a traditional medicine for treating different diseases. Over the last two decades, researchers have demonstrated a correlation between chemical composition and biological activity of SBT [3, 4, 27]. Researchers

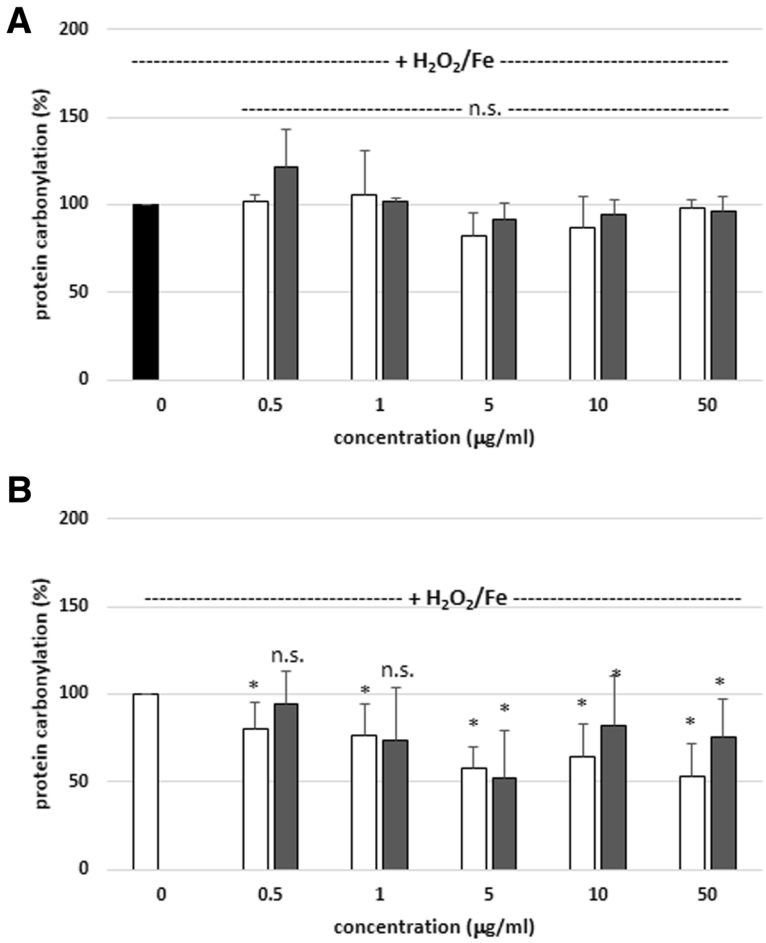

Fig. 4 Effects of SBT twig and leaf extracts (0.5–50 μg/mL; 15 min (**a**) and 60 min (**b**)) on plasma protein carbonylation induced by H_2O_2/Fe. In these experiments the carbonyl group level (marker of protein oxidation) in control samples (plasma treated only with H_2O_2/Fe) was 30.4 ± 5.1 nmol/mg of plasma protein. Data represent means ± SD of 6–12. The effect of five different concentrations of two tested extracts (0.5, 1, 5, 10 and 50 μg/mL; for 15 min) was not statistically significant ($p > 0.05$ (n.s.)) in comparison to control. The effect of five different concentrations of SBT twig extract (0.5, 1, 5, 10 and 50 μg/mL; for 60 min) was statistically significant (*$p < 0.05$) in comparison to control. The effect of three different concentrations of SBT leaf extract (5, 10 and 50 μg/mL; for 60 min) was statistically significant (*$p < 0.05$) in comparison to control. The effect of two different concentrations (0.5 and 1 μg/mL; for 60 min) was not statistically significant ($p > 0.05$ (n.s.)), in comparison to control. The effects were not statistically significant: SBT twig extract-treated plasma vs. SBT leaf extract-treated plasma (for 15 min, $p > 0.05$ (n.s.); for all tested concentrations- 0.5 - 10 μg/mL). The effects were not statistically significant: SBT twig extract-treated plasma vs. SBT leaf extract-treated plasma (for 60 min, $p > 0.05$ (n.s.); for three tested concentrations- 0.5 - 5 μg/mL). The effects were statistically significant: SBT twig extract-treated plasma vs. SBT leaf extract-treated plasma (for 60 min, $p < 0.05$; for two tested concentrations- 10 and 50 μg/mL). black diagram – control, white diagram – twig, grey diagram - leaf

have also frequently correlated the SBT action with compounds present in its extracts, especially those from berries and leaves, however, less data is available regarding compounds from SBT twigs. Results obtained by different researchers have indicated that antioxidant activities of phenolic compounds from SBT berries and leaves may be partly responsible for the beneficial effects of these compounds on human health [28–30]. Our earlier results showed that a butanolic extract from SBT berries (rich in flavonoids) exhibits antioxidant and anti-platelet properties [3, 4]. Moreover, Tian et al. [31] analysed the chemical content of extracts from berries and leaves of 13 berries and leaves of various berry plants, including sea buckthorn. They observed that sea buckthorn leaves are the richest source of phenolics (7856 mg/100 g f.w.) with ellagitannins being the dominant compound class.

It is vital to note that SBT berries and leaves show no cytotoxicity or adverse effects upon oral administration [32–34]. Moreover, there is no report concerning the toxicity of SBT phenolic compounds. In addition, the range of tested concentrations of SBT leaf extract, SBT twig extract and selected berry extracts (0.5–50 μg/mL) in human plasma in our experiments can be achieved by way of oral supplementation with phenolics [35, 36].

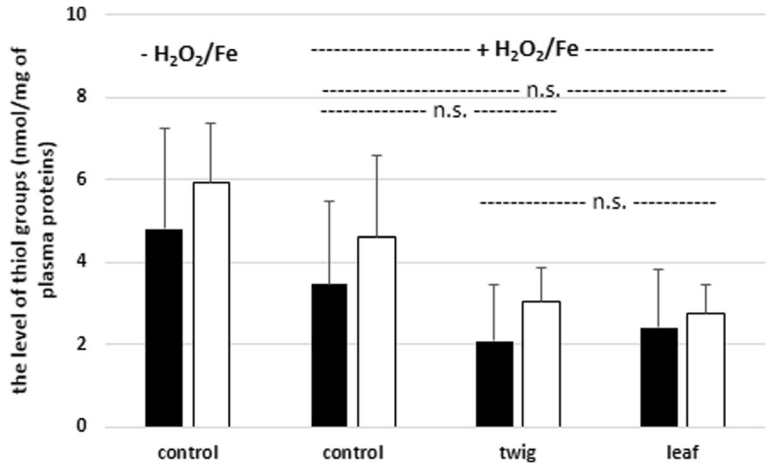

Fig. 5 Effects of SBT twig and leaf extracts (50 μg/mL; 15 min and 60 min) on oxidation of plasma protein thiols induced by H_2O_2/Fe. Data represent means ± SD of 6–9; $p > 0.05$ (n.s.). Control negative refers to plasma not treated with H_2O_2/Fe, whereas control positive to plasma treated with H_2O_2/Fe. black diagram – 15 min, white diagram – 60 min

An interesting aspect of beneficial effects of extracts from different parts of SBT on human health is its protective actions on the cardiovascular system [3, 4, 37]. The effect of various parts of SBT, especially twigs and leaves, on different components of hemostasis, including plasma, which plays a role in cardiovascular system efficiency, has not been studied yet. Thus, the main objective of our in vitro experiments was to examine the antioxidant and anticoagulant activities of SBT twig and leaf extracts in human plasma.

In the present study, the addition of H_2O_2 or H_2O_2/Fe to human plasma resulted in a significant increase in the level of different tested oxidative stress biomarkers. Two oxidative agents, namely (1) H_2O_2 and (2) H_2O_2/Fe were used, because Fe concentration in isolated plasma is low, similarly to the level of oxidative stress parameters (i.e. the level of TBARS and carbonyl groups in proteins), which is also low. However, when Fe is added to isolated human plasma, higher level of these markers can be noted. Moreover, some researchers indicate that certain complexes of iron ions (i.e. EDTA) may also react with hydrogen peroxide to form hydroxyl radicals [37, 38]. The results of our experiments indicate that SBT leaf extract exhibited an inhibitory action on H_2O_2 and H_2O_2/Fe – induced lipid peroxidation and protein carbonylation in human plasma in vitro. These results are consistent with other studies on the role of SBT leaf extract in protecting against oxidative stress [39, 40]. However, it is known that the nature and polarity of solvent may decide about biological activity of phenolic extracts from different parts of plants, i.e. Upadhyay et al. [8] have used two different leaf extracts: aqueous extract (total phenolics: 40.49 ± 2.10 mg gallic acid equivalents/g dry leaf) and hydroalcoholic extract (total phenolics: 56.28 ± 2.30 mg gallic acid equivalents/g dry leaf). They have found SBT leaf extracts to have not only antioxidant, but also cytoprotective and antibacterial effects. Both aqueous and hydroalcoholic extracts of SBT leaves (at concentration of 250 μg/mL) exhibited potent antioxidant activity when analysed by 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfoni acid) diammonium salt (ABTS) and Ferric Reducing Antioxidant Power (FRAP). However, our present results demonstrate that tested SBT leaf extract (butanolic extract), even at low concentrations (0.5–50 μg/mL), could be used as a natural source of antioxidants, i.e. inhibition of plasma protein carbonylation (induced by H_2O_2) levelled about 45% (for the concentration of 50 μg/mL, incubation time – 60 min). Results obtained by Khan et al. [40] have shown that SBT leaf extract ameliorates the gamma radiation mediated DNA damage and hepatic alterations. For this in vivo experiment, Swiss albino mice have been administered SBT (30 mg/kg body weight) for 15 consecutive days before exposing them to a single dose of 5 Gy of beta radiation. Maheshwari et al. [39] also demonstrated that the phenolic-rich

Table 3 Comparison of anticoagulant properties of SBT twig and leaf extracts with properties of selected berry extracts (50 μg/mL; 30 min). Data represent means ± SD of 12–30

APTT (s)	
Control (A)	42.3 ± 4.1
SBT leaf extract (B)	46.4 ± 4.5; B vs A ($p < 0.05$)
SBT twig extract (C)	51.9 ± 3.2; C vs A ($p < 0.05$); C vs B ($p < 0.01$)
Aronia berry extract (D)	45.3 ± 3.2; D vs A ($p < 0.05$); D vs C ($p < 0.01$)
Grape seed extract (E)	46.1 ± 2.9; E vs A ($p < 0.05$); E vs B ($p < 0.02$)
SBT berry extract (F)	40.2 ± 3.4; F vs A ($p > 0.05$ (n.s.)); F vs B ($p < 0.001$)

fraction of SBT leaves has a potent antioxidant activity, prevents oxidative damage to proteins and lipids, and affords significant protection against CCl_4-stimulated oxidative liver damage in Sprague Dawley rats. In addition, results of Bala et al. [41] indicate that standardized leaf extract from sea buckthorn (administered 12 mg/kg body weight, before irradiation) normalized brain superoxide dismutase and catalase in rats. Cho et al. [42] showed that SBT leaf extracts protect neuronal PC-12 cells from oxidative stress in vitro. Moreover, SBT leaf extract inhibits rapid proliferation of rat C6 glioma cells, possibly by inducing early events of apoptosis [43].

Few phenolic constituents of the investigated extracts can be directly absorbed in the GI tract. During their passage through the GI tract, flavonol glycosides are usually hydrolysed by microbial and intestinal enzymes. Flavonol aglycons are partly decomposed by intestinal microbes (and products of the decomposition can be absorbed by intestines), partly absorbed by intestines; the absorbed flavonol glycosides occur in the circulation system mainly as sulfates, glucuronides or diglucuronides. Catechin can be partly absorbed in the GI tract (and most the absorbed catechin is further sulfated or glucuronized), partly decomposed by intestinal microbiota (and the decomposition products also can be absorbed). It seems oligomeric proanthocyanidins cannot be directly absorbed, but products of their microbial decomposition (mainly different phenolic acids) are absorbed into the circulatory system.

Ellagitannins (from pomegranates, raspberries, strawberries, walnuts) are not directly absorbed, but products of their microbial decomposition (so called urolithins, as well as ellagic acid) can be absorbed, and occur it the circulation system mainly as sulfates or glucuronides [44].

For the first time, our findings have demonstrated antioxidant properties of SBT twig extract (the butanolic extract) in an experimental system of isolated human plasma. The tested extract significantly reduced the action on H_2O_2 and H_2O_2/Fe – induced oxidation in human plasma in vitro. However, our earlier results have shown that not only the phenolic fractions, but also the non-polar fractions (rich in triterpenes and acylated triterpenes) from sea buckthorn twigs and leaf had antioxidant and anticoagulant properties [45].

Human plasma was used in our in vitro experiments because it is an important component of hemostasis. Changes in hemostasis are often correlated with oxidative stress, and oxidative stress has been implicated in development of cardiovascular diseases. A novel finding of this study is that SBT twig and leaf extracts (at the highest tested concentration – 50 μg/mL), similarly to aronia berry extract or grape seed extract, change coagulation properties of human plasma in vitro. They prolong the clotting time – APTT, which is a measure of the efficiency of intrinsic mechanism of activation of prothrombin, without blood platelets. Thus, the obtained results indicate anticoagulant activities of SBT twig and leaf extracts in model system in vitro.

We suppose that the differences in chemical profiles of tested extracts, especially total concentration of phenolic compounds, may explain differences in their biological activities (antioxidant and anticoagulant properties), i.e. they may explain the stronger action of SBT twig extract (than SBT leaf extract), in which proanthocyanidins exhibit high concentration (about 580 mg/g). We suppose that these compounds may act not only as main antioxidants in this extract, but also as compounds with anticoagulant activity. Our previous experiments have also demonstrated strong antioxidant potential of the phenolic fractions from sea buckthorn twigs, which can be attributed to a high content of catechin and proanthocyanidins [45]. Chong et al. [46] suggest that anthocyanidins, procyanidins, flavonols and phenolic acids may have the greatest beneficial impact on cardiovascular disorders. Other authors have also demonstrated that supplementation with commercial product made from aronia berries (Aronox®) results in improved clotting and fibrinolysis in patients with metabolic syndrome, and has been shown to modify hemostasis in in vitro [47]. Our present results are consistent with other studies concerning the anticoagulant properties of aronia berry extract, a known source of anthocyanins (about 110 mg/g). On the other hand, the non-polar fraction from sea buckthorn twigs (rich in triterpenoids and acylated triterpenoids) had a greater impact on coagulation system then the phenolic fraction [45].

Conclusion

Extracts from different parts of SBT, especially berries and twigs, in comparison to well-known berries (aronia and grape) may be also a good source of active substances – anticoagulants and antioxidants for pharmacological or cosmetic applications. Moreover, it is very important from an economic point of view there is a possibility of obtaining phenolic compounds not only from berries or leaves, but also from twigs, which constitute a production waste.

Abbreviations

APTT: Activated partial thromboplastin time; DMSO: Dimethylsulfoxide; H_2O_2: Hydrogen peroxide; PT: Prothrombin time; SBT: Sea buckthorn; TBA: Thiobarbituric acid; TT: Thrombin time

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None.

Authors' contributions

BS designed the study, did experimental work (oxidative stress and parameters of hemostasis) and analyses and drafted the manuscript. BK

collaborated in analysis of results (oxidative stress). BL collaborated in did experimental work and analysis (parameters of hemostasis). LG collaborated analysis as well as performed a critical version of the manuscript. AS collaborated analysis (chemical characteristics of plant extracts) as w as well as performed a critical version of the manuscript. JZ did experimental work and analyses (chemical characteristics of plant extracts) and draft the manuscript. BO collaborated analysis as well as performed a critical version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data are presented in the manuscript. Data sets used and/or analysed in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Our analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research, and approved by the Committee on the Ethics of Research in Human Experimentation at the University of Lodz (resolution No. 3/KBBN-UŁ/II/2016). Each donor filled up and signed special questionnaire where he/she agree for commercial and scientific use of own blood by Regional Center for Transfusion Medicine.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Future Medicinal Chemistry

Phenolic fraction and nonpolar fraction from sea buckthorn leaves and twigs: chemical profile and biological activity

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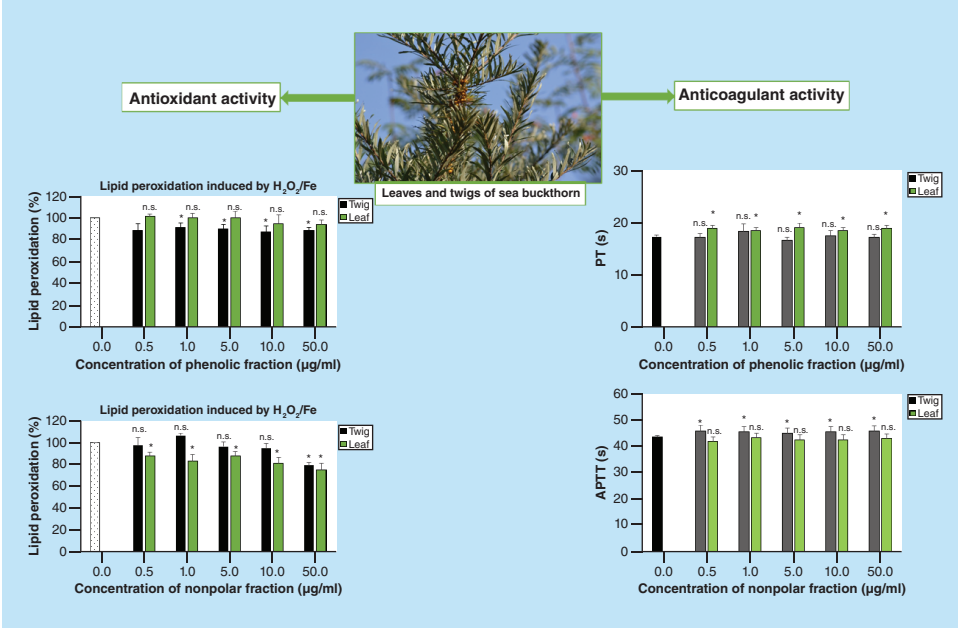
Aim: The main objective of our studies was to determine the chemical composition and biological activities (antioxidant and anticoagulant properties) of two standardized phenolic fractions from sea buckthorn twig and leaf, and two standardized nonpolar fractions from twig and leaf in human plasma *in vitro*. **Material & methods:** Appropriately prepared extracts from sea buckthorn twigs and leaves were used. Markers of oxidative stress and hemostasis were determined in this work. **Results:** The reduction of plasma lipid peroxidation induced by H_2O_2/Fe was observed for two fractions from twig. Analysis of the effect on the coagulation properties of plasma demonstrated that the nonpolar fraction from twig and the phenolic fraction from leaf, significantly prolonged the activated partial thromboplastin time and the prothrombin time, respectively. **Conclusion:** Sea buckthorn twig and leaf are new promising plant materials in the prophylaxis and treatment of cardiovascular disorders.

Graphical Abstract:



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Keywords: hemostasis • leaf • oxidative stress • sea buckthorn • twig

For many years, scientists have been looking for medicinal compounds among plants. The main place is currently occupied by sea buckthorn (*Elaeagnus rhamnoides* (L) A Nelson; *Elaeagnaceae* family), a shrub with characteristic

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orange berries. Sea buckthorn berries are recognized as a rich source of health-promoting substances, including phenolic compounds, numerous vitamins, minerals, omega fatty acids and phytosterols [1–5], and are very good candidates for functional food production. Recently, experiments have demonstrated that other parts of sea buckthorn – leaves and twigs, also contain phenolic compounds, which are beneficial for human health [6–8]. Results of Sadowska *et al.* [8] showed that the butanolic extracts of phenolic compounds from sea buckthorn leaves and twigs have antivirulence (blocking virulence factors) properties *in vitro*.

In this experiment, we focused on chemical composition and biological activities of four fractions obtained from sea buckthorn leaf and twig extracts: phenolic fractions and nonpolar fractions, the effect of which on human plasma, an important element of hemostasis, was not investigated. The objective was to investigate anti- or prooxidant properties of four fractions from sea buckthorn leaf and twig against the effect of a biological oxidant - H_2O_2/Fe (the donor of hydroxyl radicals) on human plasma lipids and proteins. The aim of our studies was also to determine their effect on hemostatis parameters of plasma (the activated partial thromboplastin time [APTT]), prothrombin time (PT) and thrombin time (TT)) *in vitro*. The action of the four used fractions was also compared with properties of butanolic extract of phenolic compounds from sea buckthorn leaf (which is rich in ellagitannins) and butanolic extract of phenolic compounds from sea buckthorn twig (which is rich in B-type proanthocyanidins and catechin).

Materials & methods

Chemicals

DMSO, thiobarbituric acid, formic acid (LC–MS grade) and H_2O_2 were purchased from Sigma (MO, USA). Methanol (isocratic grade) and acetonitrile (LC–MS grade) were acquired from Merck (Darmstadt, Germany). Other reagents represented analytical grade and were provided by commercial suppliers, including POCh, (Gliwice, Poland), Acros (Warsaw, Poland), and Chempur (Piekary Śląskie, Poland).

Plant material

Sea buckthorn twigs and leaves were obtained from a horticultural farm in Sokółka, Podlaskie Voivodeship, Poland (53°24′N, 23°30′E), the greatest Polish producer of sea buckthorn fruits. The plant material was identified by S Trzonkowski, the owner of the farm. Voucher specimens have been deported at the Institute of Soil Science and Plant Cultivation – State Research Institute, Pulawy, Poland (IUNG/HRH/2015/2).

Preparation & quantification of the fractions (the phenolic fraction & nonpolar fraction) from sea buckthorn twig & leaf

Extraction procedure

Lyophilized leaves of sea buckthorn were milled in a laboratory mill (Retsch ZM200, Haan, Germany). The twigs, air dried at 40°C, were ground in laboratory mills (Retsch SM300, ZM200). The milled material was stored in a freezer. The leaves (284 g) were extracted with 5 l (in three portions) of 80% methanol (v/v), at room temperature (48 h), with three cycles (10 min) of ultrasonication (3 × 10 min). The ground twigs (680 g) were extracted with 14 l of 80% methanol (in three portions), as described above. After filtration, the extracts were concentrated by rotary evaporation (40°C) and defatted with hexane. Organic solvents were removed in a rotary evaporator, the residue was subsequently resuspended in Milli-Q water (final volume ~1200 ml) and extracted with butanol (200 ml portions). The butanol extracts were rotary evaporated to remove the solvent, the residue was suspended in portions of water and 20% tert-butanol, freezed and lyophilized. The procedures yielded 12.42 g (yield 43.7 mg g^{-1} dry mass) of the dry leaf extract and 24.64 g (yield 36.2 mg g^{-1} dry mass) of the twig extract. A 12 g portion of the leaf extract was suspended in 600 ml of 50% methanol, shaken, ultrasonicated for 2 min and centrifuged. The supernatant, containing mainly phenolic compounds, was dried in a rotary evaporator, dissolved in 20% tert-butanol and lyophilized, to yield 11.37 g of the phenolic fraction. The dry residue was dissolved in a mixture of tert-butanol and water and lyophilized (0.63 g). The twig extract was fractionated in the same way (14 g was mixed with 700 ml of 50% methanol), yielding 13.07 g of the phenolic fraction and 0.83 g the nonpolar fraction.

LC-MS analysis

The composition of the sea buckthorn fractions was determined using a Thermo Ultimate 3000RS (Thermo Fischer Scientific, MS, USA) UHPLC (Ultra-High Performance Liquid Chromatography) system, equipped with a charged aerosol detector, a diode array detector. The system was hyphenated with a Bruker Impact II (Bruker Daltonics

GmbH, Bremen, Germany) Q-TOF mass spectrometer. Samples were chromatographed using an ACQUITY BEH (Ethylene Bridged Hybrid) C18 column (2.1 × 150 mm, 1.7 μm; Waters, MA, USA) maintained at 60°C, the injection volume was 2.5 μl. Chromatographic separations (500 μl min^{-1}, 30 min) were carried out using a linear gradient from 7 to 90% of solvent B (acetonitrile containing 0.1% (v/v) of formic acid) in solvent A (0.1% [v/v] formic acid in Milli-Q water). UHPLC–ESI–MS analyzes were performed in negative and positive ion mode. The scanning range was from m/z 50 to 2000. MS settings for negative ion mode: capillary voltage 3 kV; dry gas flow 6 l min^{-1}; dry gas temperature 200°C; nebulizer pressure 0.7 bar; collision RF 700 Vpp; transfer time 80 μs; prepulse storage time 10 μs. Collision energy was set automatically in the range from 15 to 140 eV, depending on the m/z of a fragmented ion. MS settings for positive mode were as follows: capillary voltage 4.5 kV; dry gas flow 6 l min^{-1}; dry gas temperature 200°C; nebulizer pressure 0.7 bar; collision RF 700 Vpp; transfer time 70 μs; prepulse storage time 7 μs. Collision energy was set automatically in the range from 9 to 85 eV, depending on the m/z value of a fragmented ion. Components of the analyzed fractions were identified on the basis of their MS and UV spectra, with the help of available literature data. The relative content of individual compounds was determined on the basis of charged aerosol detector chromatograms and expressed as a percentage of the total peak area.

Chemical characteristics of the butanolic extract of phenolic compounds from sea buckthorn twig & leaf

Extracts from the leaves and twigs of sea buckthorn were prepared as previously described [8]. Their composition was determined by reverse-phase UHPLC–MS/MS, using ACQUITY UPLC™ system (Waters), coupled with an ACQUITY TQD (Waters) triple quadrupole mass detector. Chromatographic separations were performed on an ACQUITY BEH C18 (100 mm × 2.1 mm, 1.7 μm; Waters) column (leaf and twig extracts). Components of the extracts were identified on the basis of their MS and UV spectra, as well as literature data [9–11].

Ellagitannins (259.6 ± 3.1 mg/g) were identified as principal constituents of butanolic extract from sea buckthorn leaf. The butanolic extract from sea buckthorn twigs consisted mainly of B-type proanthocyanidins and catechin (the total content 597.1 ± 10.2 mg/g) [8].

Stock solutions

Stock solutions of the phenolic fractions, nonpolar fractions and butanolic extracts from sea buckthorn twig and leaf were made in 50% DMSO. The final concentration of DMSO in samples was lower than 0.05% and its effects were determined in all experiments.

Plasma isolation

Human blood and plasma were obtained from regular donors (nonsmoking men and women) of a blood bank (Lodz, Poland) and a Medical Center (Lodz, Poland). Blood was collected into citrate/phosphate/dextrose (CPD) solution; 9:1; v/v blood/CPD or CPD/adenine (CPDA) solution; 8.5:1; v/v; blood/CPDA). They had not taken any medication or addictive substances (including tobacco, alcohol, antioxidant supplementation, aspirin or any other antiplatelet drugs). Our analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research, and approved by the Committee on the Ethics of Research in Human Experimentation at the University of Lodz (resolution number 3/KBBN-UŁ/II/2016). Plasma was incubated (30 min, at 37°C; for hemostatic parameters) with:

- The phenolic fraction from the leaves of sea buckthorn at the final concentrations of 0.5–50 μg/ml;
- The phenolic fraction from the twigs of sea buckthorn at the final concentrations of 0.5–50 μg/ml;
- The nonpolar fraction from the leaves of sea buckthorn at the final concentrations of 0.5–50 μg/ml;
- The nonpolar fraction from the twigs of sea buckthorn at the final concentrations of 0.5–50 μg/ml;
- The butanolic extract from the leaves of sea buckthorn at the final concentration of 50 μg/ml;
- The butanolic extract from the twigs of sea buckthorn at the final concentration of 50 μg/ml.

Plasma was also pre-incubated (5 min, at 37°C; for biomarkers of oxidative stress) with:

- The phenolic fraction from the leaves of sea buckthorn at the final concentrations of 0.5–50 μg/ml;
- The phenolic fraction from the twigs of sea buckthorn at the final concentrations of 0.5–50 μg/ml;
- The nonpolar fraction from the leaves of sea buckthorn at the final concentrations of 0.5–50 μg/ml;
- The nonpolar fraction from the twigs of sea buckthorn at the final concentrations of 0.5–50 μg/ml;

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- The butanolic extract from the leaves of sea buckthorn at the final concentration of 50 μg/ml;
- The butanolic extract from the twigs of sea buckthorn at the final concentration of 50 μg/ml;
- And then treated with 4.7 mM H_2O_2/3.8 mM Fe_2SO_4/2.5 mM EDTA (55 min, at 37°C).

The protein concentration, determined by measuring absorbance at 280 nm (in tested samples), was calculated according to the procedure of Whitaker and Granum [12].

Markers of oxidative stress

Lipid peroxidation measurement

Plasma lipid peroxidation was quantified by measuring the concentration of TBARS (thiobarbituric acid reactive substances). The TBARS concentration was calculated using the molar extinction coefficient ($\varepsilon = 156{,}000\ M^{-1}cm^{-1}$). More details on the method are shown in various papers [13–15].

Carbonyl group measurement

The detection of carbonyl groups in plasma proteins was carried out according to Bartosz [14], Olas *et al.* [15], and Levine *et al.* [16].

Thiol group determination

The thiol group content in plasma proteins was measured spectrophotometrically (the SPECTROstar Nano Microplate Reader, BMG LABTECH, Ortenberg, Germany) by absorbance at 412 nm with 5,5′-dithio-bis-(2-nitrobenzoic acid). More details on the method are demonstrated in various papers [14,15,17,18].

Parameters of hemostasis

The measurement of PT

The PT was determined coagulometrically using an Optic Coagulation Analyzer (model K-3002, Kselmed, Grudziadz, Poland), according to the method described by Malinowska *et al.* [19].

The measurement of TT

The TT was determined coagulometrically using an Optic Coagulation Analyser (model K-3002, Kselmed, Grudziadz, Poland), according to the method described by Malinowska *et al.* [19].

The measurement of APTT

The APTT was determined coagulometrically using an Optic Coagulation Analyser (model K-3002, Kselmed, Grudziadz, Poland), according to the method described by Malinowska *et al.* [19].

Data analysis

Several tests were used to carry out statistical analysis. In order to eliminate uncertain data, the Q-Dixon test was performed. All the values in this study were expressed as mean ± standard error. Obtained results were first analyzed under the account of normality with Shapiro–Wilk test and equality of variance with Levine test. Statistically significant differences were assessed by applying the ANOVA test (the significance level was $p < 0.05$), followed by Tukey multiple comparisons test or Kruskal–Wallis test.

Results

Chemical characterization of the tested plant fractions

Hydrolysable tannins (most of all ellagitannins) were dominant constituents of the phenolic fraction of sea buckthorn leaves (LF) (Tables 1, 2). Flavonoids were represented by different mono- and diglycosides of isorhamnetin, quercetin and kaempferol, both simple and acylated. Kaempferol hexosides acylated with p-coumaric acid and isorhamnetin diglycosides (deoxyhexoside-hexoside) acylated with rarely occurring (putative) linalool-1-oic acid were dominant acylated flavonoids. The preparation also contained significant amounts of triterpene saponins (aglycones with formulas $C_{30}H_{48}O_4$, $C_{30}H_{46}O_4$, $C_{30}H_{48}O_3$) and unidentified polar compounds, as well as smaller portions of triterpenes, acylated triterpenes and unidentified nonpolar compounds. In contrast, the nonpolar fraction (LL) was composed mostly of hydrophobic compounds, like (Tables 1, 3) triterpenes and triterpene saponins, with smaller

Table 1. The relative content of individual group of compounds in the phenolic fraction and in the non-polar fraction of sea buckthorn leaf or twig, expressed as a percentage of the total peak area (corona-charged aerosol detector).

Group of compounds	Relative peak area (%)
The phenolic fraction of sea buckthorn leaf	
Unidentified polar compounds	15.8
Gallocatechin	1.6
Hydrolysable tannins and ellagic acid	31.3
Flavonol glycosides	11.7
Acylated flavonol glycosides†	12.8
Unidentified nonpolar compounds	4.2
Triterpene saponins	15.0
Triterpenes	5.8
Acylated triterpenes‡	1.8
The nonpolar fraction of sea buckthorn leaf	
Unidentified polar compounds	1.2
Hydrolysable tannins	2.7
Flavonol glycosides	1.1
Acylated flavonol glycosides†	1.5
Unidentified nonpolar compounds	18.9
Triterpene saponins	3.5
Triterpenes	38.5
Acylated triterpenes‡	5.6
The phenolic fraction of sea buckthorn twig	
Unidentified polar compounds	35.7
Hydrolysable tannins and ellagic acid	1.9
Proanthocyanidins and catechin	49.1
Flavonol glycosides	1.3
Acylated flavonol glycosides†	1.0
Unidentified nonpolar compounds	4.3
Triterpenes	5.4
Acylated triterpenes‡	1.3
The nonpolar fraction of sea buckthorn twig	
Unidentified polar compounds	3.8
Proanthocyanidins and catechin	1.3
Unidentified nonpolar compounds	36.5
Triterpenes	33.9
Acylated triterpenes‡	24.5

†Acylated with aliphatic or phenolic acids;
‡Acylated with phenolic acids.

amounts of unidentified hydrophobic compounds and acylated triterpenes. Additionally, the preparation contained small amounts of ellagitannins, flavonoids and unidentified polar compounds.

The phenolic fraction of sea buckthorn twigs (GF) was composed mainly from proanthocyanidins (B type) and catechin. The fraction also had a high content of unidentified polar substances, and contained small amounts of flavonoids, ellagitannins, ellagic acid, as well as triterpenes, acylated triterpenes and unidentified nonpolar compounds (Tables 1, 4). The nonpolar fraction (GL) consisted mainly of triterpenes and acylated triterpenes, but numerous other nonpolar constituents were not identified (Tables 1, 5). The fraction also contained remnants of proanthocyanidins, catechin and unidentified polar compounds.

Effects on oxidative stress biomarkers in human plasma *in vitro*

As demonstrated in Figure 1A, only the phenolic fraction from twig reduced plasma lipid peroxidation stimulated

Table 2. Secondary metabolites in the phenolic fraction of sea buckthorn leaves.

t_R [min]	[M-H]- (m/z)	$[M-H]^-$ formula	Tentative identification	Relative peak area (%)	Ref.
1.0	331.0665	$C_{13}H_{15}O_{10}$	GalA-Hex	2.1	–
1.3	305.0666	$C_{15}H_{13}O_7$	(Epi) gallocatechin	1.6	–
2.3	633.0734	$C_{27}H_{21}O_{18}$	Strictinin or isomer	5.2	[21,22]
2.5	935.0785	$C_{41}H_{27}O_{26}$	Stachyurin, casuarinin	10.3	[21,22]
	1103.0858	$C_{48}H_{31}O_{31}$	Hippophaenin B or isomers		–
3.2	785.0843	$C_{34}H_{25}O_{22}$	Ellagitanin	1.5	–
3.8	935.0793	$C_{41}H_{27}O_{26}$	Casuarictin or isomer	3.4	[21,22]
4.1	1117.0998	$C_{49}H_{35}O_{31}$	Ellagitannin	1.6	–
4.6	1085.0731	$C_{46}H_{29}O_{30}$	Ellagitannin	1.4	–
4.8	300.9982	$C_{14}H_5O_8$	Ellagic acid	1.2	–
5.4	609.1451	$C_{27}H_{29}O_{16}$	Q-3-*O*-rutinoside	1.3	[11]
5.5	463.0868	$C_{21}H_{19}O_{12}$	Q-Hex	1.3	–
5.7	623.1604	$C_{28}H_{31}O_{16}$	I-dHex-Hex	1.8	–
5.9	623.1606	$C_{28}H_{31}O_{16}$	I-3-*O*-Glc-7-*O*-Rha	2.0	[11]
6.4	961.2606	$C_{44}H_{49}O_{24}$	I-dHex-Hex-Hex-FerA	1.1	–
6.8	623.1607	$C_{28}H_{31}O_{16}$	I-3-*O*-rutinoside	1.1	[11]
9.8	593.1298	$C_{30}H_{25}O_{13}$	K-Hex-pCouA	2.3	[11]
10.3	593.1297	$C_{30}H_{25}O_{13}$	K-Hex-pCouA	1.2	–
12.6	789.2606	$C_{38}H_{45}O_{18}$	I-dHex-Hex-166	1.0	[11]
12.7	789.2602	$C_{38}H_{45}O_{18}$	I-dHex-Hex-166	1.9	[11]
13.3	1235.6061	$C_{59}H_{95}O_{27}$	Triterpene saponin	1.2	–
14.6	1381.6639	$C_{65}H_{105}O_{31}$	Triterpene saponin	1.1	–
16.2	1219.6106	$C_{59}H_{95}O_{26}$	Triterpene saponin	1.2	–
16.5	1459.7109	$C_{71}H_{111}O_{31}$	Triterpene saponin	2.1	–
16.9	1313.6519	$C_{65}H_{101}O_{27}$	Triterpene saponin	1.6	–
17.7	1401.7059	$C_{69}H_{109}O_{29}$	Triterpene saponin	2.1	–
17.8	1297.6559	$C_{65}H_{101}O_{26}$	Triterpene saponin	1.6	–
18.5	487.3418	$C_{30}H_{47}O_5$	Triterpene	3.5	–
19.5	1239.6523	$C_{63}H_{99}O_{24}$	Triterpene saponin	1.0	–
23.0	471.3474	$C_{30}H_{47}O_4$	Triterpene	1.3	[11]

The listed compounds correspond to UHPLC-charged aerosol detector peaks with area ≥ 1% of the total peak area.
166: Linalool-1-oic acid; dHex: Deoxyhexose; FerA: Ferulic acid; GalA: Gallic acid; Glc: Glucose; Hex: Hexose; I: Isorhamnetin; K: Kaempferol; pCouA: p-Coumaric acid; Q: Quercetin; Rha: Rhamnose.

by H_2O_2/Fe. However, the fraction's activity has not been concentration-dependent (% of inhibition of this process was about 15% for all tested concentrations). Moreover, the nonpolar fraction from twig (only at the highest used concentration - 50 μg/ml) inhibited plasma lipid peroxidation induced by H_2O_2/Fe (Figure 1B). On the other hand, in this model, the nonpolar fraction from leaf inhibited this process at all tested concentrations (Figure 1B). The phenolic fraction and nonpolar fraction from twig were found to protect human plasma proteins against H_2O_2/Fe-induced carbonylation (Figure 2A & B), for example, in the presence of the phenolic fraction at the highest used concentration - 50 μg/ml, this process was reduced about 35% (Figure 2A). The nonpolar fraction from leaf reduced this process only at the highest concentration - 50 μg/ml (Figure 2B). In addition, the phenolic fraction from leaf had no effect on plasma protein carbonylation induced by H_2O_2/Fe (Figure 2A).

Another set of experiments, focused on the determination of protein thiols (Figure 2C & D), showed that only the phenolic fraction from twig (at high tested concentrations: 5, 10 and 50 μg/ml) and the phenolic fraction from leaf (at the highest tested concentration - 50 μg/ml) reduced the thiol oxidation induced by H_2O_2/Fe (Figure 2C).

Effects on hemostatic parameters of plasma

Analysis of the effect of the phenolic fractions and the nonpolar fractions from *E. rhamnoides* (L.) A. Nelson leaf and twig (at a dose range 0.5–50 μg/ml; incubation time 30 min) on the coagulation properties of plasma showed that the phenolic fraction from leaf significantly prolonged the PT (Figure 3A). Moreover, the nonpolar fraction

Table 3. Secondary metabolites in the nonpolar fraction of sea buckthorn leaves.

t_R [min]	[M-H]. (m/z)	$[M-H]^-$ formula	Tentative identification	Relative peak area (%)	Ref.
2.5	935.0785, 1103.0858	$C_{41}H_{27}O_{26}$, $C_{48}H_{31}O_{31}$	Stachyurin, casuarinin hippophaenin B or isomers	1.2	[21,22]
16.5	1459.7109	$C_{71}H_{111}O_{31}$	Triterpene saponin	1.6	–
16.7	1313.6537	$C_{65}H_{101}O_{27}$	Triterpene saponin	1.8	–
16.9	1313.6541	$C_{65}H_{101}O_{27}$	Triterpene saponin	2.0	–
17.7	1297.6602	$C_{65}H_{101}O_{26}$	Triterpene saponin	1.300	–
17.7	1401.7086	$C_{69}H_{109}O_{29}$	Triterpene saponin	4.2	–
17.8	1297.6603	$C_{65}H_{101}O_{26}$	Triterpene saponin	2.0	–
18.0	1151.6021	$C_{59}H_{91}O_{22}$	Triterpene saponin	1.1	–
18.4	1255.6474	$C_{63}H_{99}O_{25}$	Triterpene saponin	4.7	–
18.5	487.3440	$C_{30}H_{47}O_5$	Triterpene	18.3	–
19.5	1239.6549	$C_{63}H_{99}O_{24}$	Triterpene saponin	4.3	–
20.1	1093.5979	$C_{57}H_{89}O_{20}$	Triterpene saponin	3.6	–
22.4	471.3479	$C_{30}H_{47}O_4$	Triterpene	2.1	[11]
22.4	471.3479	$C_{30}H_{47}O_4$	Triterpene	3.0	[11]
22.6	633.3793	$C_{39}H_{53}O_7$	Acylated triterpene†	2.2	[23]
23.0	471.3480	$C_{30}H_{47}O_4$	Triterpene	13.1	[11]
23.2	471.3481	$C_{30}H_{47}O_4$	Triterpene	7.1	[11]

The listed compounds correspond to UHPLC-charged aerosol detector peaks with area ≥ 1% of the total peak area.
†Acylated with phenolic acids.

Table 4. Secondary metabolites in the phenolic fraction of sea buckthorn twigs.

t_R [min]	[M-H]. (m/z)	$[M-H]^-$ formula	Tentative identification	Relative peak area (%)	Ref.
1.4	305.0659	$C_{15}H_{13}O_7$	(Epi)gallocatechin	6.8	[28]
1.5	593.1289	$C_{30}H_{25}O_{13}$	(Epi)C-(epi)GC	3.6	[29]
1.6	881.1921	$C_{45}H_{37}O_{19}$	(Epi)C-(epi)C-(epi)GC	1.8	[29]
1.8	881.1922	$C_{45}H_{37}O_{20}$	(Epi)C-(epi)C-(epi)GC	1.1	[29]
2.2	577.1340	$C_{30}H_{25}O_{12}$	Dimeric proanthocyanidin	8.8	[27,29]
2.3	289.0709	$C_{15}H_{13}O_6$	Catechin	10.0	[28]
2.7	1153.2607	$C_{60}H_{49}O_{24}$	Tetrameric proanthocyanidin	2.9	[29]
3.1	865.1973	$C_{45}H_{37}O_{18}$	Trimeric proanthocyanidin	3.6	[27,29]
3.3	577.1344	$C_{30}H_{25}O_{12}$	Dimeric proanthocyanidin	2.5	[27,29]
3.6	1153.2605	$C_{60}H_{49}O_{24}$	Tetrameric proanthocyanidin	1.2	[29]
4.6	865.1973	$C_{45}H_{37}O_{18}$	Trimeric proanthocyanidin	1.4	[27,29]
4.8	300.9983	$C_{14}H_5O_8$	Ellagic acid	1.2	–
11.3	582.2600	–	Nitrogen-containing compound	1.6	–
11.8	612.2710	–	Nitrogen-containing compound	2.0	–
12.1	642.2822	–	Nitrogen-containing compound	3.2	–
12.5	672.2918	–	Nitrogen-containing compound	3.9	–
18.5	487.3430	$C_{30}H_{47}O_5$	Triterpene	3.0	–

The listed compounds correspond to UHPLC-charged aerosol detector peaks with area ≥ 1% of the total peak area.
(Epi)C: (Epi)catechin; (Epi)GC: (Epi)gallocatechin.

from twig also prolonged the APTT (Figure 3D). However, none of the tested fractions changed the TT (Figure 3E & F).

Table 6 shows comparative action of the phenolic fractions, the nonpolar fractions and the butanolic extracts from *E. rhamnoides* (L.) A. Nelson leaf and twig (at the concentration of 50 µg/ml) on biomarkers of oxidative stress and parameters of coagulation. For example, the butanolic extract from twig had stronger antioxidant properties (measured by the level of TBARS) than other tested fractions (from twig and leaf) (Table 6). On the other hand, in the presence of the butanolic extract from the leaves, inhibition of protein carbonylation was stronger than other

Table 5. Secondary metabolites in the nonpolar fraction of sea buckthorn twigs.

t_R [min]	[M-H]. (m/z)	$[M-H]^-$ formula	Tentative identification	Relative peak area (%)	Ref.
18.5	487.3437	$C_{30}H_{47}O_5$	Triterpene	5.87	–
19.5	487.3428	$C_{30}H_{47}O_5$	Triterpene	1.53	–
22.5	471.3469	$C_{30}H_{47}O_4$	Triterpene	3.38	[11]
23.0	471.3464	$C_{30}H_{47}O_4$	Triterpene	6.45	[11]
23.2	471.3470	$C_{30}H_{47}O_4$	Triterpene	8.46	[11]
24.9	617.3839	$C_{39}H_{53}O_6$	Acylated triterpene†	1.86	[23]
25.1	617.3836	$C_{39}H_{53}O_6$	Acylated triterpene†	3.14	[23]
25.3	617.3842	$C_{39}H_{53}O_6$	Acylated triterpene†	2.82	[23]
25.4	617.3846	$C_{39}H_{53}O_6$	Acylated triterpene†	2.04	[23]
25.8	455.3527	$C_{30}H_{47}O_3$	Triterpene	2.33	–
26.0	617.3839	$C_{39}H_{53}O_6$	Acylated triterpene†	11.47	[23]

The listed compounds correspond to UHPLC-charged aerosol detector peaks with area ≥ 1% of the total peak area.
†Acylated with phenolic acids.

Table 6. Comparative effects of phenolic fractions, nonpolar fractions, butanolic extracts from sea buckthorn twig and leaf at the highest tested dose (50 μg/ml) on selected parameters of oxidative stress and hemostasis.

	Parameters of oxidative stress			Parameters of hemostasis		
	Inhibition of lipid peroxidation induced by H_2O_2/Fe (%)	Increase of protein thiol groups in plasma treated with H_2O_2/Fe (%)	Inhibition of protein carbonylation induced by H_2O_2/Fe (%)	Prolongation of APTT (%)	Prolongation of PT (%)	Prolongation of TT (%)
Phenolic fraction from twig (a)	19.9 ± 4.5 ($p > 0.05$; a vs b, e, f)	75.0 ± 18.9 ($p < 0.05$; a vs d)	33.4 ± 6.7 ($p > 0.05$; a vs b, c, e)	–	–	–
Non-polar fraction from twig (b)	20.9 ± 2.5 ($p > 0.05$; b vs e, f)	–	28.1 ± 4.9 ($p > 0.05$; b vs c)	5.3 ± 2.4 ($p > 0.05$; b vs f)	–	–
Butanolic extract from twig (c)	38.9 ± 8.5 ($p < 0.05$; c vs a, b, e, f)	–	30.4 ± 8.2 ($p > 0.05$; c vs d)	21.3 ± 5.7 ($p < 0.05$; c vs b, f)	–	–
Phenolic fraction from leaf (d)	–	55.7 ± 17.9	–	–	8.9 ± 4.4	–
Nonpolar fraction from leaf (e)	25.5 ± 6.3 ($p > 0.05$; e vs f)	–	26.4 ± 5.4	–	–	–
Butanolic extract from leaf (f)	25.7 ± 10.7	–	41.2 ± 9.7 ($p < 0.05$; f vs a, b, c, e)	9.8 ± 3.4	–	–

Results are given as means ± standard error (n = 5–10).
APTT: Activated partial thromboplastin time; PT: Prothrombin time; TT: Thrombin time.

fractions (Table 6). Moreover, only phenolic fractions from twig and leaf reduced the oxidation of protein thiols in plasma treated with H_2O_2/Fe (Table 6), and the phenolic fractions from twig had stronger effect on this process than the phenolic fraction from leaf (Table 6).

We observed that butanolic extract from twig prolonged APTT stronger than butanolic extract from leaf or the nonpolar fraction from twig (Table 6). In addition, only the phenolic fraction from left statistically significant prolonged PT (Table 6).

Discussion

Modern phytotherapy (uses in prophylaxis and treatment of different diseases, including cardiovascular diseases) often recommends the use of various parts of plants in the form of standardized preparations containing purified and concentrated bioactive components, for example, phenolic compounds. Our previous experiments have established that standardized phenolic fraction (rich in nonacylated and acylated flavonoids and nonpolar compounds) and standardized nonpolar fraction from sea buckthorn berries are able to reduce the oxidative stress in human plasma *in vitro*. Moreover, we have observed that especially triterpenes and their derivatives possess antioxidant activity [15]. Results of Michel *et al.* [20] indicate that other parts of sea buckthorn (leaf, stem, root and seed) have different biological properties. The main objective of our present studies was to examine chemical composition, antioxidant and anticoagulant properties of phenolic fraction and nonpolar fraction from sea buckthorn leaf and twig. LC–

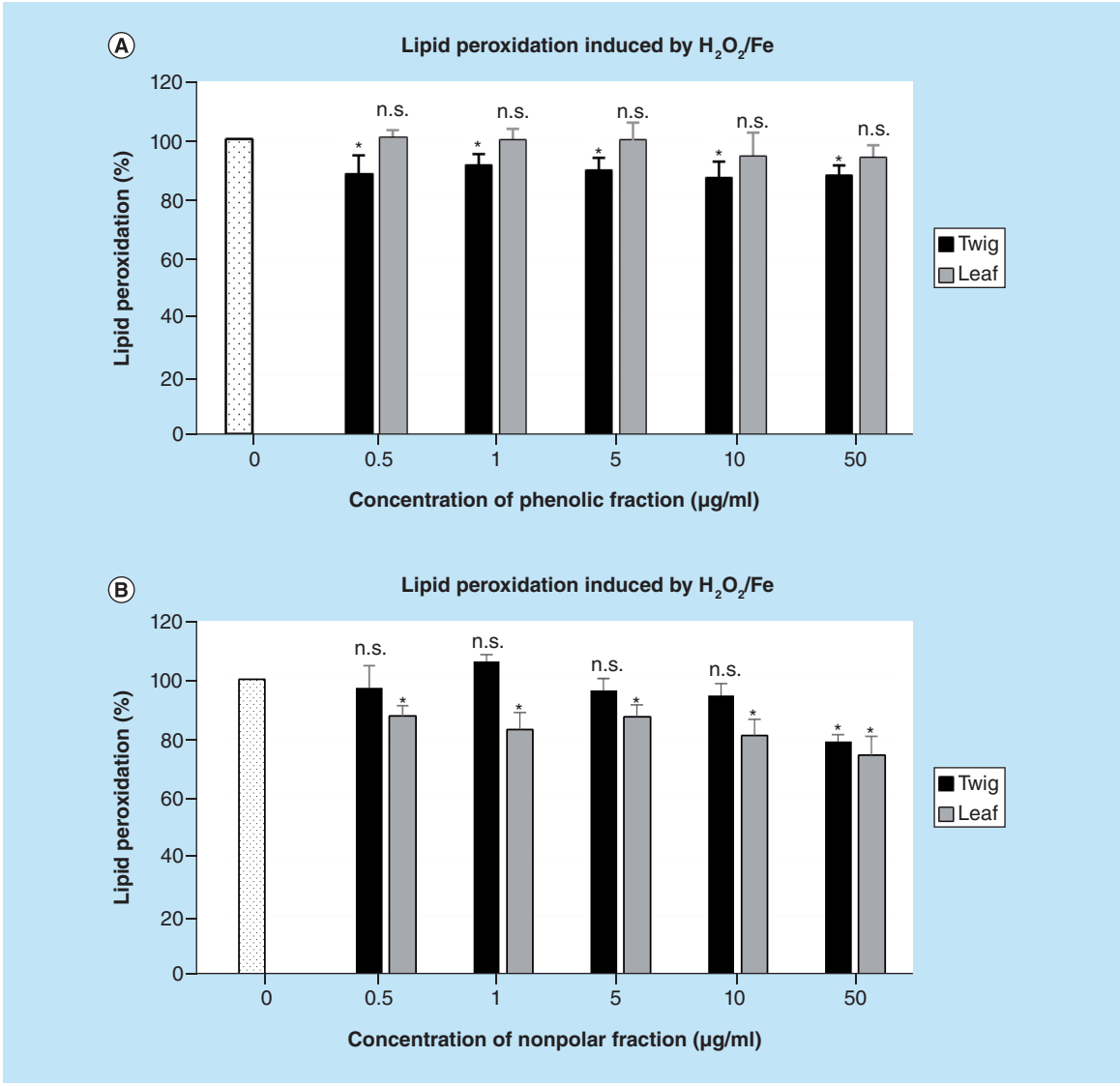

Figure 1. Effects of the phenolic fractions (A) and the nonpolar fractions (B) of *E. rhamnoides* (L.) A. Nelson leaf and twig (0.5–50 µg/ml) on plasma lipid peroxidation induced by H_2O_2/Fe. In these experiments, the TBARS level (a marker of lipid peroxidation) in control samples (plasma treated with H_2O_2/Fe) was 1.621 ± 0.344 nmol TBARS/ml of plasma, and was expressed as 100% of TBARS level. Data represent means ± standard error of 5–10 independent experiments. * $p < 0.05$ (vs control), n.s. (not statistical) - $p > 0.05$ (vs control).
n.s.: Not statistical; TBARS: Thiobarbituric acid reactive substances.

MS analysis demonstrated very significant differences in chemical composition of the tested fractions from sea buckthorn leaves and twigs. Hydrolysable tannins, flavonoids, triterpene saponins and some unidentified polar compounds were the dominant compounds in the phenolic fraction of sea buckthorn leaves. Triterpenes, their acylated derivatives and triterpene saponins were main constituents of the nonpolar fraction of sea buckthorn leaves. While phenolic compounds of sea buckthorn leaves, including ellagitannins and acylated flavonoids, have been well characterized in the literature [10,11,21,22], data about their less polar constituents are scarce. Triterpenes and acylated triterpenes are similar to those detected in the sea buckthorn fruit, or isolated from the sea buckthorn bark leaves [11,19,23]. In contrast, though the presence of triterpene saponins in sea buckthorn leaves was discovered before, on the basis of simple laboratory tests [24], it seems these compound were not described in more detail in the available literature. However, triterpene saponins of similar kind were earlier isolated from sea buckthorn seeds [25,26].

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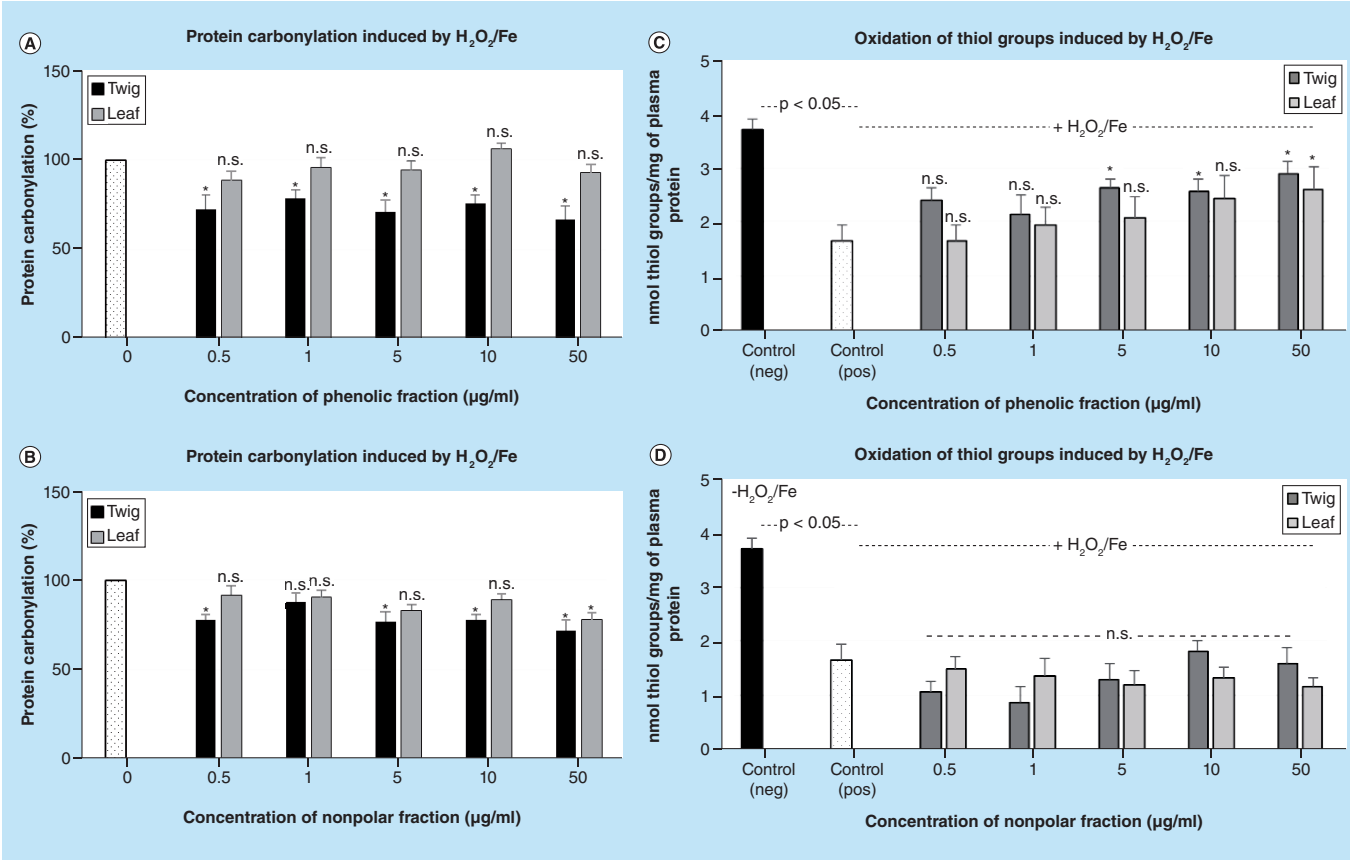

Figure 2. Effects of the phenolic fractions (A & C) and the nonpolar fractions (B & D) of *E. rhamnoides* (L.) A. Nelson leaf and twig (0.5–50 µg/ml) on plasma protein carbonylation and oxidation of thiol groups induced by H_2O_2/Fe. For protein carbonylation, the level of carbonyl groups (a marker of protein oxidation) in control samples (plasma treated with H_2O_2/Fe) was 38.4 ± 4.1 nmol carbonyl group/mg of plasma protein, and was expressed as 100% of level of carbonyl groups. For thiol groups, control negative (neg) refers to plasma not treated with H_2O_2/Fe, whereas control positive (pos) to plasma treated with H_2O_2/Fe. Data represent means ± standard error of 5–10 independent experiments. * $p < 0.05$ (vs control), n.s. - $p > 0.05$ (vs control).
n.s.: Not statistical

Proanthocyanidins and catechin were the dominant compounds in the phenolic fraction of sea buckthorn twigs. Unlike the phenolic fraction of sea buckthorn leaves, it contained only trace amounts of flavonoids and practically devoid of saponins. Similar flavan-3-ols and proanthocyanidins were earlier detected in sea buckthorn bark, twigs and fruit [27–29]. As regards the nonpolar fraction, consisting mainly of triterpenes and acylated triterpenes, the twig triterpenes and acylated triterpenes were generally similar to those from sea buckthorn leaves (as described above), bark and fruit [19,23].

The composition of the leaf and the twig fractions appears to offer promise with regard to their potential application as antioxidant and anticoagulant agents, which may be used in prophylaxis and treatment of cardiovascular diseases. It is important that in our *in vitro* model, the range of tested concentrations of tested plant fractions (0.5–50 µg/ml) in human plasma may be achieved by way of their oral supplementation [30,31]. The antioxidant properties of the four tested fractions were verified in biological model – in human plasma exposed to oxidative stress induced by physiological oxidant, H_2O_2/Fe *in vitro*. The present experiment employs the three parameters frequently used in research on oxidative stress – the level of TBARS, and levels of carbonyl and thiol groups to study some of these effects on lipid peroxidation and protein damages. Our results revealed differences in antioxidant activity between the phenolic fraction and the nonpolar fraction from sea buckthorn leaf in plasma treated with H_2O_2/Fe. For example, it is demonstrated by our tests that the nonpolar fraction from leaf exerted stronger inhibitory action on H_2O_2/Fe – induced plasma lipid peroxidation than the phenolic fraction from leaf, especially at the dose 50 µg/ml (e.g., the inhibition of lipid peroxidation was about 25% for the nonpolar fraction). In addition, the nonpolar fraction (rich in triterpenes and their derivatives) from sea buckthorn twig, like

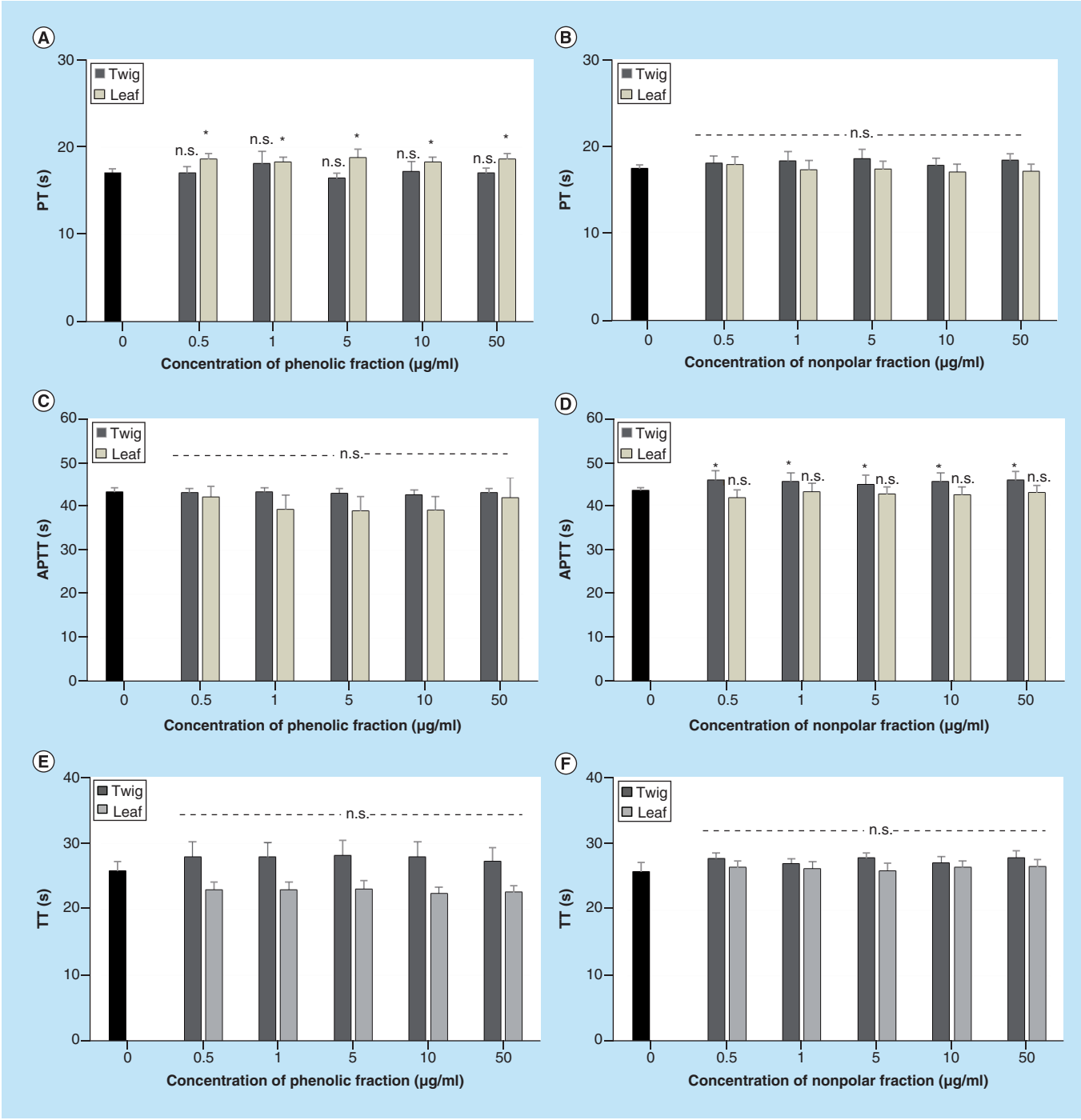

Figure 3. Effects of the phenolic fractions (A, C & E) and the nonpolar fractions (B, D & F) of *E. rhamnoides* (L.) A. Nelson leaf and twig (0.5–50 µg/ml) on selected hemostatic parameters of plasma: APTT, PT and TT. Data represent means ± standard error of 7–10 independent experiments. * $p < 0.05$ (vs control), n.s. - $p > 0.05$ (vs control).
APTT: Activated partial thromboplastin time; n.s. Not statistical; PT: Prothrombin time; TT: Thrombin time.

leaf had antioxidant properties (measured by the level of TBARS and carbonyl groups) in human plasma treated with H_2O_2/Fe. These results are generally in line with literature reports. For example, results of Yang *et al.* [23] demonstrated the antioxidant activity of triterpenoids isolated from branch bark of extract of sea buckthorn. On the other hand, we observed that the phenolic fractions from leaf and twig had stronger protector actions on thiol group oxidation in plasma treated with H_2O_2/Fe than the nonpolar fractions. However, only the phenolic fraction

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from sea buckthorn twig reduced plasma lipid peroxidation and protein carbonylation. We suppose that strong antioxidant potential of the phenolic fractions from twig (observed in three used tests) may depend on the presence of proanthocyanidins and catechin. It is important that proanthocyanins are known to be one of the most powerful natural antioxidants.

A novel finding of this study is that only the phenolic fraction from sea buckthorn left prolonged the clotting time – the PT (which is a measure of the efficiency of extrinsic coagulation system). We suppose that anticoagulant properties of this fraction may be associated with the modulation prothrombin activity or clotting factors V, VII and X by bioactive compounds presented in this fraction. For the first time, we demonstrated that the nonpolar fraction from sea buckthorn twig has also anticoagulant properties, because it prolongs the APTT, which is a measure of the efficiency of the intrinsic mechanism of activation of prothrombin, excluding blood platelets. We may suppose that triterpenes, acylated triterpenes or some unidentified nonpolar compounds had the greatest impact on APTT, being the major constituents of the fraction.

Our present experiment shows that sea buckthorn leaf and twig are a rich source of secondary metabolites possessing antioxidant and anticoagulant activities, but the effect of pure compound (which are presented at the highest concentrations) still remain to be examined. However, greater beneficial actions have been associated with the antioxidants obtained from whole plant extracts, fractions or other products than those obtained singly [32,33]. We suggest that especially the phenolic fraction from sea buckthorn twig can be a new source of natural components, demonstrating antioxidant properties. In addition, the nonpolar fraction from sea buckthorn twig (rich in triterpenes and acylated triterpenes) is also promising plant material exerting not only antioxidant, but also anticoagulant properties, beneficial in the prophylaxis and treatment of cardiovascular disorders. However, the identification of individual secondary metabolites, which are responsible for different biological properties (including their effects on blood platelet functions, which are engaged in hemostasis and pathological conditions (e.g., atherosclerosis)) of sea buckthorn leaf and twig should be done in further. In addition, our experiments would further determine the possible effects of used fractions on the level of glutathione and activity of antioxidant enzymes (in plasma and blood cells) and mechanism behind their antioxidant properties.

Future perspective

The obtained results may help to better understand mechanism of bioactivity of preparations obtained from sea buckthorn. It may also contribute to the broader use of different parts of this useful plant as a source of nutraceuticals or medicinal compounds. Sea buckthorn fruits are often harvested by cutting whole twigs, so the use of twigs and leaves of this plant as a source of bioactive secondary metabolites could be also a way to manage harvest waste.

Summary points

Sea buckthorn & health

- Sea buckthorn berries are recognized as a rich source of health – promising substances including phenolic compounds.
- Other parts of sea buckthorn – leaves and twigs also contain phenolic compounds.

Chemical profile & biological activity (*in vitro*) of sea buckthorn

- The phenolic and nonpolar fractions of leaves and twigs were investigated.
- LC–MS analysis showed significant differences in chemical composition of tested fractions.
- Tested fractions had different biological properties, including antioxidant and anticoagulant activities *in vitro*.
- Both fractions of twigs reduced H_2O_2/Fe-induced human plasma protein carbonylation.

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Financial & competing interests disclosure

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Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – Induced oxidative stress and changes in hemostasis

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ABSTRACT

The objective of this study is to investigate the biological effects of phenolic compounds extracted from the sea buckthorn berries on oxidative stress and hemostasis. The sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) berries are rich in flavonoids and non-polar compounds. In this study, the activity of the phenolic fraction from the sea buckthorn berries was evaluated *in vitro* in comparison with three phenolic compounds: isorhamnetin (compound 1) and its two new derivatives: compound 2 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-rhamnoside) and compound 3 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-(3'''-isovaleryl)-rhamnoside). The impact of these phenolic compounds and the phenolic fraction against the effect of the donor of hydroxyl radicals - H_2O_2/Fe on proteins and lipids in human plasma was measured. Additionally, the aim of the study was to determine the effect of these phenolic compounds and the phenolic fraction on various typical hemostasis parameters. Our results show that the used derivatives of isorhamnetin possess different biological properties (e.g. antioxidant, anti-platelet and anticoagulant). The tested compounds can be seen as new natural beneficial compounds to be used in prevention and treatment of cardiovascular diseases.

1. Introduction

For a long time, herbal remedies have been used as therapeutics due to their relatively fewer side effects, however, the knowledge on their biological effects is yet limited. Various organs of *Elaeagnus rhamnoides* (L.) A. Nelson (sea buckthorn), including berries, are a rich source of bioactive compounds, including phenolic compounds, which are beneficial for human health (Malinowska and Olas, 2016; Olas, 2016 and 2018a and b). Nevertheless, the effect of different organs of sea buckthorn on components of hemostasis (e.g. plasma, blood platelets or other blood cells), is not yet clear. Moreover, most of the studies have been carried out using different kinds of extracts or fractions from the sea buckthorn. Their composition has not always been determined either, and their bioactive constituents have not been identified, including in the manuscripts by Michel et al. (2012) and Chen et al. (2013). Our earlier results demonstrated that the phenolic fraction (rich in non-acylated and acylated flavonols and non-polar compounds) from the sea buckthorn berries acts as an antioxidant and anticoagulant (Olas et al., 2018). In addition, using LC-MS analysis we were able to observe that various glycosides of isorhamnetin and quercetin, including compounds acylated with an unidentified aliphatic acid, were the dominant constituents of this fraction (Olas et al., 2018).

Various berries, including the sea buckthorn berries, are potent candidates for functional food products especially bearing in mind the fact that epidemiological studies indicate that diets rich in berry phenolic antioxidants may reduce the oxidative stress (Olas, 2018b). In addition, berries (in different forms: fresh, juice or medicinal product) may play an important role in the modulation of hemostasis, in particular the various steps of blood platelet activation, including blood platelet aggregation (Olas, 2017). In this study, we focused on biological actions of two isorhamnetin derivatives: compound 2 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-rhamnoside) and compound 3 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-(3'''-isovaleryl)-rhamnoside), which were isolated from the phenolic fraction from the sea buckthorn berries (Zuchowski et al. (sub.)) (Fig. 1). The action of isorhamnetin derivatives was compared to biological effects of isorhamnetin - compound 1, which, among others, protects human retinal pigment epithelium cells from oxidative stress induced by H_2O_2 (Wang et al., 2018), and the phenolic fraction, which was used to isolate the tested phenolic compounds. Biological properties were studied *in vitro* using various tests (based on human plasma and human blood platelets) to observe its protective properties against oxidative damage to protein

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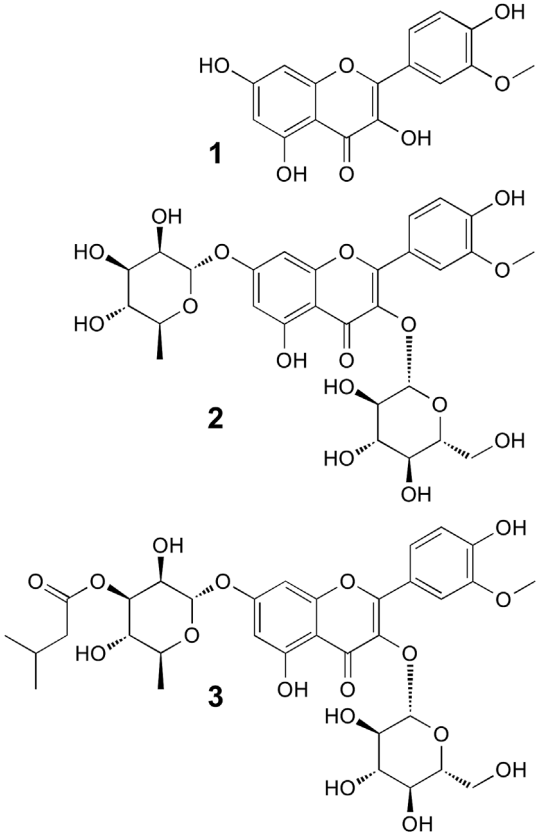

Fig. 1. Chemical structure of isorhamnetin (compound 1) and its derivatives: compound 2 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-rhamnoside) and compound 3 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-(3‴-isovaleryl)-rhamnoside) isolated from the phenolic fraction of *E. rhamnoides* (L.) A. Nelson berries.

and lipid components of plasma, as well as their influence on the hemostasis: selected parameters of coagulation - the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT); and one of the steps of blood platelet activation – platelet aggregation stimulated by various physiological agonists (ADP, collagen and thrombin), because oxidative stress is involved in pathogenesis of numerous diseases, including cardiovascular disorders, which, in addition, are very often associated with changes in hemostasis. Moreover, the objective was also to investigate the value of isorhamnetin, its derivatives and the examined phenolic fraction as potential functional food ingredients, since no undesired or toxic effects (including hematological or chemical effects) have been associated with the consumption of various berries (e.g. sea buckthorn berries) or their products (Olas, 2017 and 2018b).

2. Material and methods

2.1. Chemicals

Dimethylsulfoxide (DMSO), thiobarbituric acid (TBA), thrombin, formic acid (LC-MS grade), isorhamnetin, and H_2O_2 have been purchased from Sigma (St. Louis, MO., USA). Methanol (isocratic grade) and acetonitrile (LC-MS grade) have been acquired from Merck (Darmstadt, Germany). Other reagents represented analytical grade and were provided by commercial suppliers, including POCh, (Poland), Acros (Poland), and Chempur (Poland). Adenosine-5′-diphosphate (ADP) and collagen have been purchased from Chrono-Log (USA).

Derivatives of isorhamnetin: compound 2 and 3 were isolated from the phenolic fraction from sea buckthorn berries as described earlier (Zuchowski et al. (sub.)).

2.2. Plant material

The sea buckthorn (*E. rhamnoides* (L.) A. Nelson) berries have been obtained from a horticultural farm in Sokółka, Podlaskie Voivodeship, Poland (53°24′N, 23°30′E). The fruits were ground frozen in a meat grinder, lyophilized (Gamma 2–16 LSC, Christ, Osterode am Harz, Germany), and stored in a refrigerator.

2.3. Preparation and quantification of the phenolic fraction

The phenolic fraction of the sea buckthorn berries was prepared as described above (Olas et al., 2018). Various glycosides of isorhamnetin and quercetin, including compounds acylated with an unidentified aliphatic acid, were the dominant constituents of this fraction. Moreover, the preparation also contained small amounts of putative triterpenes and acylated triterpenes (Olas et al., 2018).

2.4. Stock solutions of tested compounds and plant fraction

Stock solutions of the *E. rhamnoides* (L.) A. Nelson phenolic fractions, isorhamnetin and its derivatives were made in 50% DMSO. The final concentration of DMSO in samples was lower than 0.05% and its effects were determined in all experiments.

2.5. Blood samples

Fresh human blood or plasma have been obtained from regular, medication-free donors from the Regional Center for Transfusion Medicine in Lodz (Poland) as well as peripheral blood from non-smoking men and women (collected into CPD solution (citrate/phosphate/dextrose; 9:1; v/v blood/CPD) or CPDA solution (citrate/phosphate/dextrose/adenine; 8:5:1; v/v; blood/CPDA)). All samples were drawn in the morning from fasting donors. Donors had not taken any medications or addictive substances (including alcohol, tobacco, antioxidant supplementation, aspirin or any other anti-platelet drugs) prior to the blood collection. Our analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research. Moreover, the protocol was approved by the Committee for Research on Human Subjects of the University of Lodz number 3/KBBN-UŁ/II/2016.

2.6. Isolation of plasma and blood platelets

Human platelet-rich plasma (PRP) and blood platelets were isolated by differential centrifugation of blood as described by Wachowicz and Kustron (1992). The platelet pellet was washed with modified Tyrode's buffer (pH 7.4) twice; afterwards, the platelets were suspended in the same buffer. The concentration of platelets in suspensions (used in the experiments), estimated spectrophotometrically (Walkowiak et al., 1989), amounted to 2.5–3 × 10^8/mL. Suspensions of blood platelets or plasma were incubated (30 or 60 min, at 37 °C) with:

- *E. rhamnoides* (L.) A. Nelson fraction, isorhamnetin and its derivatives at the final concentrations of 5 and 10 μg/mL
- *E. rhamnoides* (L.) A. Nelson fraction, isorhamnetin and its derivatives at the final concentrations of 5 and 10 μg/mL plus 4.7 mM H_2O_2/3.8 mM Fe_2SO_4/2.5 mM EDTA.

The protein concentration was calculated according to the procedure devised by Whitaker and Granum (1980), on the basis of absorbance measurements at 280 nm (in tested samples).

2.7. Markers of oxidative stress

2.7.1. Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring the concentration

of TBARS. Incubation of plasma (control, plant fraction/tested compound and H_2O_2/Fe-treated plasma) was stopped by cooling the samples in an ice bath. Samples of plasma were transferred to an equal volume of cold 15% (v/v) trichloroacetic acid in 0.25 M HCl and 0.37% thiobarbituric acid in 0.25 M HCl, immersed in a boiling water bath for 10 min, and then centrifugated at 10,000 × *g* for 15 min, 18 °C. Absorbance was measured at 535 nm (the SPECTROstar Nano Microplate Reader - BMG LABTECH Germany) (Wachowicz, 1984; Bartosz, 2008). The TBARS concentration was calculated using the molar extinction coefficient (ε = 156,000 $M^{-1}cm^{-1}$).

2.7.2. The carbonyl group measurement

The detection of carbonyl groups in proteins was carried out according to Levine et al. (1990) and Bartosz (2008). The carbonyl group concentration was calculated using a molar extinction coefficient (ε = 22,000 $M^{-1}cm^{-1}$), and the level of carbonyl groups was expressed as nmol carbonyl groups/mg of protein. Carbonyl content was determined using the SPECTROstar Nano Microplate Reader- BMG LABTECH Germany.

2.7.3. The thiol group determination

The thiol group content was measured spectrophotometrically (the SPECTROstar Nano Microplate Reader- BMG LABTECH Germany) by absorbance at 412 nm with Ellman's reagent: 5,5′-dithio-bis-(2-nitrobenzoic acid). The thiol group concentration was calculated using a molar extinction coefficient (ε = 13,600 $M^{-1}cm^{-1}$) (Ando and Steiner, 1973a and b; Bartosz, 2008). The level of thiol groups was expressed as nmol thiol groups/ml of plasma.

2.8. Parameters of hemostasis

2.8.1. The measurement of prothrombin time

Human plasma (50 μL) was added to measuring cuvette and incubated for 2 min at 37 °C on a block heater. The measuring cuvette was transferred to the measuring holes and 100 μL of Dia-PT liquid (commercial thromboplastin) was added. The PT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) (Malinowska et al., 2012).

2.8.2. The measurement of thrombin time

Human plasma (50 μL) was added to a measuring cuvette and incubated for 1 min at 37 °C on a block heater. The measuring cuvette was transferred to the measuring holes and 100 μL of thrombin was added (final concentration - 5 U/mL). The TT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) (Malinowska et al., 2012).

2.8.3. The measurement of APTT

Human plasma (50 μL) was added to a measuring cuvette and incubated with 50 μL of Dia-PTT liquid (commercial preparation) for 3 min at 37 °C on block heater. The measuring cuvette was transferred to the measuring holes and 50 μL of 25 mM $CaCl_2$ was added. The APTT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) (Malinowska et al., 2012).

2.8.4. The measurement of blood platelet aggregation

Platelet aggregation was measured turbidimetrically in PRP or blood platelets in Tyrode's buffer using the optical Chrono-Log aggregometer (Chrono-Log, Havertown, PA, USA).

After the pre-incubation procedure for the PRP samples, ADP (10 μM) or collagen (2 μg/mL) were added and blood platelet aggregation measured for 10 min. The aggregometer was calibrated each time (100% aggregation) on blood platelet poor plasma (PPP).

After the pre-incubation procedure for the blood platelet samples, thrombin (1 U/ml) was added and blood platelet aggregation measured for 10 min. The aggregometer was calibrated each time (100% aggregation) on Tyrode's buffer.

2.9. Data analysis

Statistical analysis was carried out using several tests. In order to eliminate uncertain data, the Q-Dixon test was performed. All the values in this study were expressed as a mean value ± SEM (n = 3–12 independent experiments). The statistically significant differences were assessed by applying the paired Student's *t*-test or by applying one-way ANOVA test followed by a multicomparison Tukey's test; and the significance level was $p < 0.05$.

3. Results

3.1. Effects of phenolic compounds/phenolic fraction from E. rhamnoides (L.) A. Nelson berries on oxidative stress markers in human plasma

In comparison to the control (control (neg) – untreated) samples, H_2O_2/Fe stimulated plasma exhibited a markedly raised concentration of TBARS (the marker of lipid peroxidation) and carbonyl groups in plasma proteins (the marker of protein oxidation), as well as a decrease in thiol groups in plasma proteins (the marker of protein oxidation) (Fig. 2). In the presence of isorhamnetin and its derivatives: compound 2 and 3 (at concentrations: 5 and 10 μg/mL), plasma lipid peroxidation induced by H_2O_2/Fe was significantly reduced (Fig. 2A). All used phenolic compounds effectively reduced plasma lipid peroxidation by about 30% at 10 μg/mL (for isorhamnetin and compound 3) and about 40% at 10 μg/mL (for compound 2) (Fig. 2A, Table 1). In this model, compound 2 (at the highest used concentration – 10 μg/mL) had stronger antioxidant properties than isorhamnetin and compound 3 (Fig. 2A, Table 1). However, the phenolic fraction from *E. rhamnoides* (L.) A. Nelson berries (at both tested concentrations: 5 and 10 μg/mL) did not change the level of plasma lipid peroxidation induced by H_2O_2/Fe (Fig. 2A).

Compound 2 and 3, and the phenolic fraction from *E. rhamnoides* (L.) A. Nelson berries (at both tested concentrations: 5 and 10 μg/mL) were found to protect the plasma against H_2O_2/Fe – induced thiol group oxidation in proteins, however, isorhamnetin (5 and 10 μg/mL) had no effect on this process (Fig. 2B, Table 1). In the other experiment, all tested compounds (isorhamnetin and its derivatives) and the phenolic fraction from *E. rhamnoides* (L.) A. Nelson berries reduced the protein carbonylation induced by H_2O_2/Fe by about 25% at 5 μg/mL and about 30% at 10 μg/mL (Fig. 2C, Table 1).

3.2. Effects of phenolic compounds/phenolic fraction from E. rhamnoides (L.) A. Nelson berries on hemostasis

Analysis of the effect of the tested phenolic compounds from *E. rhamnoides* (L.) A. Nelson berries (at concentrations: 5 and 10 μg/mL) on the coagulation activity of human plasma showed that only compound 3 significantly prolonged the thrombin time. Isorhamnetin and compound 2 did not change the thrombin time (Fig. 3A, Table 1). Moreover, in the presence of compound 3, the action was stronger than with isorhamnetin and compound 2 (Fig. 3A, Table 1). On the other hand, all tested phenolic compounds did not change the prothrombin time and the activated partial thromboplastin time (Fig. 3B and C). In addition, tested compound 3 (at concentrations – 5 and 10 μg/mL) did not prolong the thrombin time of human plasma, when the mixture of tested compound and thrombin (earlier pre-incubated thrombin with compound 3, and then added to the plasma) was used (Fig. 4).

In our measurements, we found that all the tested compounds (at the concentration – 10 μg/mL) did not change blood platelet aggregation stimulated by ADP or collagen (Fig. 5). On the other hand, isorhamnetin and compound 3 inhibit this process induced by thrombin (inhibition of blood platelet aggregation stimulated by thrombin was about 25%) (Fig. 5, Table 1).

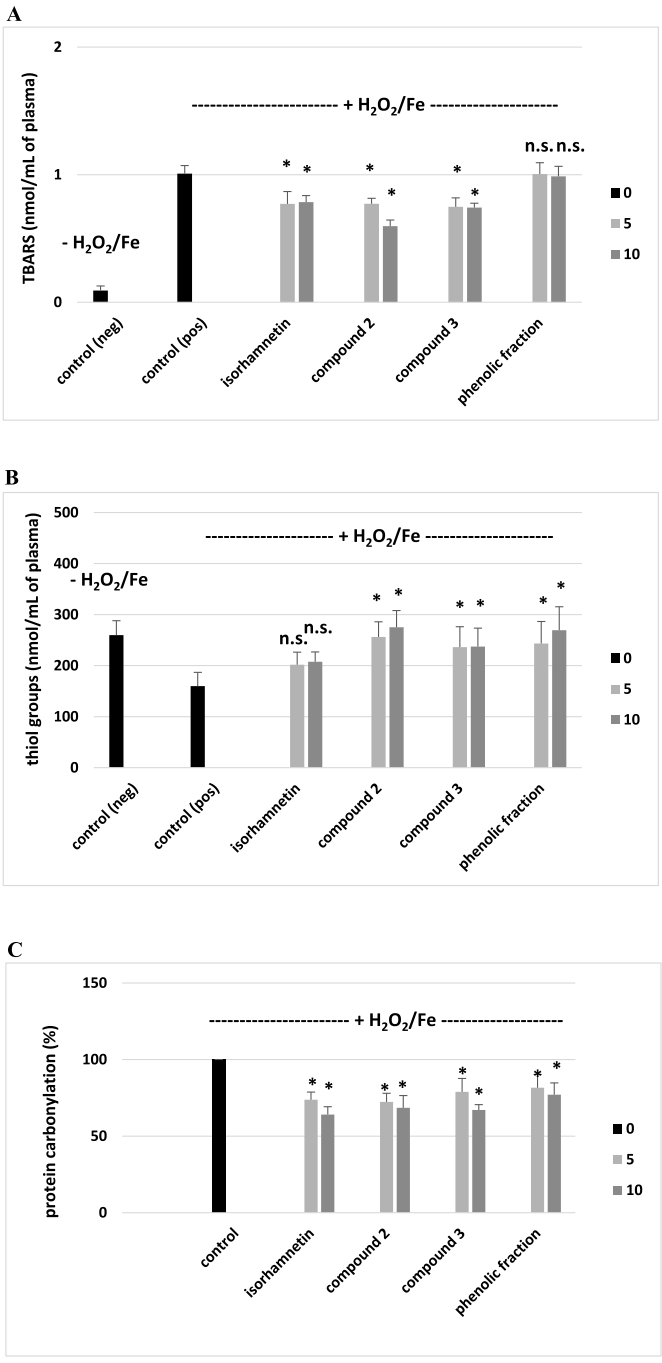

Fig. 2. Effects of isorhamnetin, its derivatives and the phenolic fraction of *E. rhamnoides* (L.) A. Nelson berries (5 and 10 μg/mL; 60 min) on plasma lipid peroxidation induced by H_2O_2/Fe (A), on the level of protein thiol groups in plasma treated with H_2O_2/Fe (B) and on plasma protein carbonylation induced by H_2O_2/Fe (C; in these experiments, the level of carbonyl groups in control samples: for plasma not treated with H_2O_2/Fe was 25.9 ± 4.9 nmol/mg of plasma proteins, and for plasma treated with H_2O_2/Fe was 61.4 ± 12.4 nmol/mg of plasma proteins and was expressed as 100% of protein carbonylation). Data represent means ± SEM of 5–12 independent experiments. Control negative (neg) refers to plasma not treated with H_2O_2/Fe, whereas control positive (pos) to plasma treated with H_2O_2/Fe ($p < 0.01$). *$p < 0.05$ (*vs.* control (pos)), n.s. – $p > 0.05$ (*vs.* control (pos)).

Table 1 demonstrated comparative effects of isorhamnetin and its derivatives (at the highest used concentration – 10 μg/mL) on selected parameters of oxidative stress and hemostasis. For example, compound 3 had stronger anti-coagulant and anti-aggregatory properties than

Table 1
Comparative effect of isorhamnetin and its derivatives (at the highest concentration - 10 μg/mL) on selected parameters of oxidative stress and hemostasis. Data represent means ± SE of 3–12 independent experiments.

Tested phenolic compound	Parameters of oxidative stress	Parameters of hemostasis			
	Inhibition of lipid peroxidation induced by H_2O_2/Fe (%)	Inhibition of protein carbonylation induced by H_2O_2/Fe (%)	The level of protein thiol groups (nmol/ml of plasma) in plasma treated with H_2O_2/Fe	Prolongation of TT (%) (Compound was incubated with plasma for 30 min and then thrombin was added)	Prolongation of TT (%) (The solution of thrombin was incubated for 30 min with tested fraction and then the mixture was added to the human plasma)	Inhibition of blood platelet aggregation induced by thrombin (%)
isorhamnetin (a)	29.7 ± 5.3 ($p > 0.05$; a *vs.* c; $p < 0.05$; a *vs.* b)	36.1 ± 5.1 ($p > 0.05$; a *vs.* b, c)	201.8 ± 24.6 ($p > 0.05$; a *vs.* c; $p < 0.05$; a *vs.* b)	3.5 ± 2.8 ($p < 0.05$; a *vs.* b; $p < 0.01$; a *vs.* c)	-	27.7 ± 4.9 ($p > 0.05$; a *vs.* b, c)
	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	No effect *vs.* control	No effect *vs.* control		Positive effect (anti-aggregatory action) *vs.* control
compound 2 (b)	40.6 ± 4.0 ($p < 0.05$; b *vs.* a, c)	37.6 ± 8.0 ($p > 0.05$; b *vs.* a. c)	266.1 ± 29.8 ($p < 0.05$; b *vs.* a, c)	10.3 ± 5.9 ($p < 0.05$; b *vs.* a, c)	-	17.1 ± 5.8 ($p > 0.05$; a *vs.* b, c)
	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	No effect *vs.* control		No effect *vs.* control
compound 3 (c)	29.6 ± 6.4 ($p > 0.05$; c *vs.* a; $p < 0.05$; c *vs.* b)	31.0 ± 3.5 ($p > 0.05$; c *vs.* a, b)	236.2 ± 40.0 ($p > 0.05$; c *vs.* a; $p < 0.05$; c *vs.* b)	24.2 ± 4.4 ($p < 0.01$; c *vs.* a; $p < 0.05$; c *vs.* b)	No effect *vs.* control	26.5 ± 5.6 ($p > 0.05$; a *vs.* b, c)
	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (anticoagulant action) *vs.* control		Positive effect (anti-aggregatory action) *vs.* control

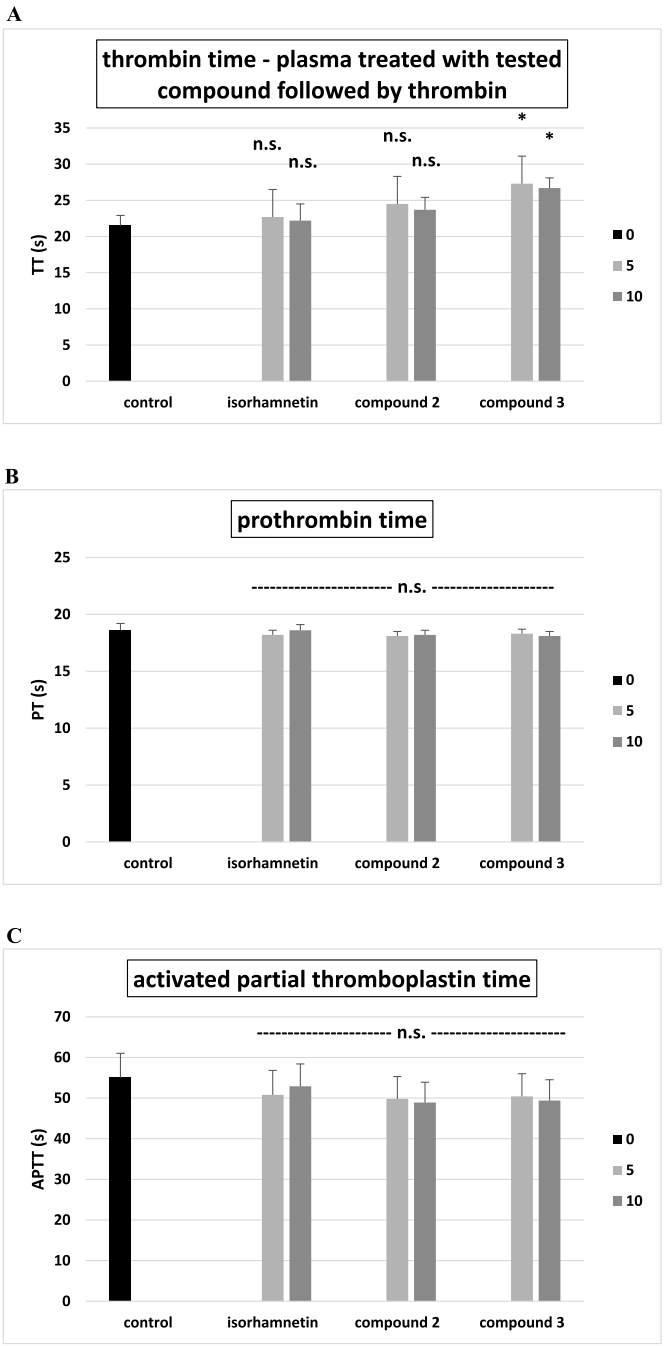

Fig. 3. Effects of isorhamnetin and its derivatives (5 and 10 μg/mL; 30 min) on the hemostatic parameters of human plasma (TT (A), PT (B), APPT (C)). Tested phenolic compounds were incubated with plasma for 30 min and then thrombin was added. Data represents means ± SEM of 4–6 independent experiments. *p < 0.05 *vs.* control, n.s. p > 0.05 *vs.* control.

compound 2. On the other hand, compound 2 had stronger antioxidant activity than compound 3 and isorhamnetin.

4. Discussion

It was demonstrated that the dietary intake of flavonoids from vegetables and fruits may reduce cardiovascular diseases (Eccleston et al., 2002; Giampieri et al., 2012). The sea buckthorn berries are a good source of various flavonoids, which show beneficial effects on health, including cardiovascular system (Cheng et al., 2003). Various flavonol glycosides from the sea buckthorn berries were identified using

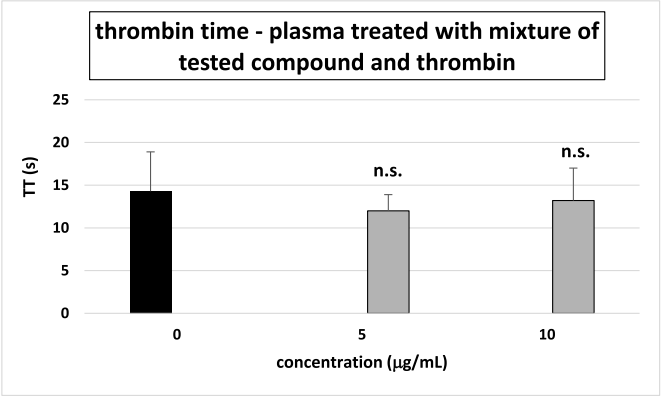

Fig. 4. Effects of compound 2 (5 and 10 μg/mL; 30 min) on the thrombin time of human plasma. The solution of thrombin was incubated for 30 min with tested compound 2 and then the mixture was added to the human plasma. Data represents means ± SEM of 4–6 independent experiments. n.s. p > 0.05 *vs.* control.

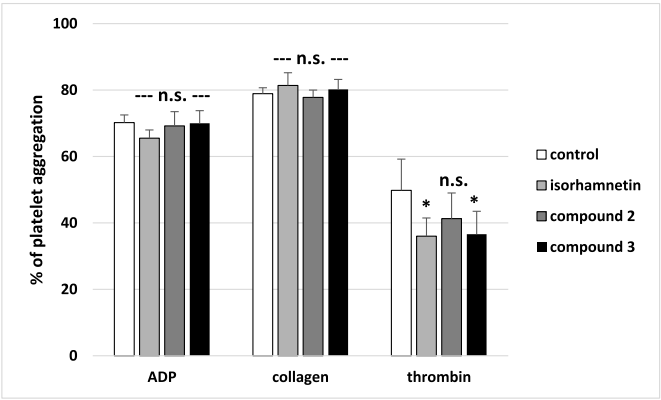

Fig. 5. Effects of isorhamnetin and its derivatives (10 μg/mL; 30 min) on blood platelet aggregation induced by ADP or collagen (in platelet-rich plasma) or by thrombin (in blood platelets in Tyrode's buffer). Data represents means ± SEM of 3–5 independent experiments. *p < 0.05 *vs.* control, n.s. p > 0.05 *vs.* control.

chromatographic methods by various authors (Gulieyev et al., 2004; Zheng et al., 2009; Korekar et al., 2011; Fang et al., 2013; Teleszko et al., 2015; Olas et al., 2016, 2017 and 2018; Guo et al., 2017). It is very important that the total flavonoids from the aqueous ethanol extract of sea buckthorn berries have been clinically used for the treatment of cardiovascular diseases in China since 1980 (Wang et al., 1993, 2000). Luo et al. (2015) observed that isorhamnetin inhibited atherosclerotic plaque development in apolipoprotein E knockout (ApoE−/−) mice by phosphatidylinositol 3-kinase/protein kinase B signal transduction and heme oxygenase-1 induction. Results of Guo et al. (2017) indicate that isorhamnetin protects against cardiac hypertrophy through blocking PI3K-AKT pathway. Various other experiments showed that isorhamnetin has anticancer activity (Teng et al., 2006; Li et al., 2014, 2015). In addition, isorhamnetin ameliorates lipopolysaccharide (the main component in Gram-negative bacteria) – induced inflammatory response through down-regulation of nuclear factor-kappa B signaling (Li et al., 2016a). Shi et al. (2018) have also observed that isorhamnetin is a potent immunesuppressive agent by inhibiting dendritic cells activation and trafficking. Another experiment of Li et al. (2016b) showed that isorhamnetin (especially at concentration 100 μM) has the protective role on human brain microvascular endothelial cells from cytotoxicity induced by methylglyoxal and oxygen-glucose deprivation.

Sanchez et al. (2007) observed that isorhamnetin inhibits the

production of reactive oxygen species (ROS). Sun et al. (2012) also demonstrated that this compound inhibits the H_2O_2 action by scavenging free ROS. Moreover, results of other authors (Sea et al., 2016; Wang et al., 2018; Zhao et al., 2018) indicate that isorhamnetin has antioxidant properties *in vitro*. Similar effects have been observed in our present experiments *in vitro*. In the present study, three various assays (lipid peroxidation, carbonyl and thiol groups determined) have been used to study the antioxidant properties of isorhamnetin and its two derivatives in human plasma treated with H_2O_2/Fe under *in vitro* conditions. Our results revealed differences in antioxidant properties between tested phenolic compounds in human plasma treated with H_2O_2/Fe. It seems possible that the difference in chemical structure of the tested compounds may justify this observation. For the first time, it is demonstrated by our three tests that compound 2 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-rhamnoside) had stronger antioxidant properties than compound 3 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-(3'''-isovaleryl)-rhamnoside) and isorhamnetin.

Berries have been reported to exert the highest antioxidant activity among all kinds of fruits (Olas, 2018b). However, phenolic compounds isolated from berries are very often known to be less effective antioxidant or anti-platelet factors than berries and their food products (Chong et al., 2010; Olas, 2017 and 2018a and b). On the other hand, we report for the first time that isorhamnetin and its two derivatives have often stronger or similar antioxidant properties than used phenolic fraction. We suppose that the antioxidant potential of tested phenolic fraction from the sea buckthorn berries fraction may depend on the presence of flavonoids (especially isorhamnetin and its two used derivatives), which may act as hydroxyl radical scavengers.

Berry phenolic compounds not only possess antioxidant activities, but often demonstrate anti-platelet or anti-coagulant properties. A key novel finding of our experiment is a demonstration of the anti-platelet (anti-aggregatory) and the anti-coagulant properties, observed in the form of inhibited blood platelet aggregation stimulated by thrombin, and prolonged clotting time – the thrombin time, recorded for tested compound 3. However, we did not observe that compound 2 prolonged the TT of human plasma when this compound was preincubated with thrombin. We suppose that anti-coagulant activity of compound 3 is not associated with a modulation of thrombin activity. On the other hand, results of Choi et al. (2016) indicate that flavonoids may inhibit the enzymatic activity of thrombin. Thrombin as serine protease plays not only important functions in coagulation process, but it is also a blood platelet activator.

Blood platelets are the smallest un-nucleated blood cells, which play a significant function in hemostasis. Moreover, these cells have a fundamental role in acute coronary syndromes pathogenesis. Numerous endogenous agonists (named also stimuli, activator) induce blood platelet signal transduction *via* their receptors, including cascade of platelet activation, among them not only thrombin, but also ADP and collagen. Present *in vitro* study was designed to estimate the anti-platelet actions of isorhamnetin and its derivatives isolated from the phenolic fraction of *E. rhamnoides* (L.) A. Nelson berries. For the first time, it has been demonstrated through our test that isorhamnetin and its derivative – compound 3 exerted inhibitory action on thrombin – stimulated blood platelet aggregation. Moreover, we observed that none of the tested phenolic compounds blocked the inhibition of human blood platelet aggregation stimulated by other physiological agonists: ADP and collagen. It may suggest that isorhamnetin and compound 3 could modulate blood platelet activation by interfering with thrombin receptors on blood platelets. In addition, different action of tested compounds on platelet aggregation (measured in PRP and in blood platelets in Tyrode's buffer) may also depend on their binding ability to various components of plasma (Dangles et al., 2001).

The bioavailability and toxicity of phenolic compounds are important elements in the evaluation of their biological activities under different conditions, including *in vitro* and *in vivo* models (Manach et al., 2004, 2005). Flavonoids exhibit a low bioavailability, e.g. the oral bioavailability of quercetin aglycon is only about 1% in humans (Khaled et al., 2003). However, bioavailability of phenolic compounds differs from one berry to another. Recently, the results of Guo et al. (2017) have demonstrated that after enzymatic digestion, the phenolic compounds were quite different from the chemical extracts from the sea buckthorn berries, and more flavonoid aglycones were released, whereas less total phenolics, flavonoid glycosides and phenolic acids were detected. However, the cellular antioxidant property of berries was significantly enhanced by digestion. It is also important that there is information about the toxicity of berry phenolic compounds (Olas, 2017 and 2018b).

In our experiments, human plasma and blood platelets were preincubated with phenolic compounds or the phenolic fraction from the sea buckthorn berries in two concentrations (5 and 10 μg/mL). The concentration of phenolic substances (5 μg/mL) is likely to occur in plasma *in vivo* after oral supplementation can reach up to 5–7 μM (10 μM of isorhamnetin is around 3 μg/mL). However, they depend on the food, e.g. total plasma concentration of caffeoylquinic acids can reach about 5 μg/mL (Farah et al., 2008). Therefore, lower concentration of tested compounds or the phenolic fraction (5 μg/mL), used in our study model, appears to correspond to the physiological concentration of plant-derived phenolic compounds available after oral administration.

In conclusion, this is the first paper presenting a multi-method research study evaluating the antioxidant, anti-platelet and anti-coagulant properties of isorhamnetin and its two derivatives isolated from the phenolic fraction of *E. rhamnoides* (L.) A. Nelson berries. Our results demonstrate that there is a novel potential of these compounds in prevention and treatment of cardiovascular diseases. However, compound 3 is a better anti-coagulant and shows stronger anti-platelet activity than compound 2, yet compound 2 is a more efficient antioxidant than compound 3 and isorhamnetin.

Declaration of interest statement

None to declare.

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Transparency document

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Anti-Platelet Properties of Phenolic Extracts from the Leaves and Twigs of *Elaeagnus rhamnoides* (L.) A. Nelson

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Abstract: Sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) is a small tree or bush. It belongs to the Elaeagnaceae family, and has been used for many years in traditional medicine in both Europe and Asia. However, there is no data on the effect of sea buckthorn leaves and twigs on the properties of blood platelets. The aim of the study was to analyze the biological activity of phenolic extracts from leaves and twigs of sea buckthorn in blood platelets in vitro. Two sets of extracts were used: (1) phenolic compounds from twigs and (2) phenolic compounds from leaves. Their biological effects on human blood platelets were studied by blood platelet adhesion, platelet aggregation, arachidonic acid metabolism and the generation of superoxide anion. Cytotoxicity was also evaluated against platelets. The action of extracts from sea buckthorn twigs and leaves was compared to activities of the phenolic extract (a commercial product from the berries of *Aronia melanocarpa* (Aronox®) with antioxidative and antiplatelet properties. This study is the first to demonstrate that extracts from sea buckthorn leaves and twigs are a source of bioactive compounds which may be used for the prophylaxis and treatment of cardiovascular pathologies associated with blood platelet hyperactivity. Both leaf and twig extracts were found to display anti-platelet activity in vitro. Moreover, the twig extract (rich in proanthocyanidins) displayed better anti-platelet potential than the leaf extract or aronia extract.

Keywords: antiplatelet activity; adhesion; aggregation; *E. rhamnoides*; blood platelets

1. Introduction

Platelets are highly reactive cells activated through various specific membrane receptors by physiological agonists, such as adenosine diphosphate (ADP), thrombin and collagen, as well as non-physiological agonists. They also play an important role in hemostasis, this being the regulation of the flowing properties of blood. In the presence of agonists, blood platelets respond by adhering to various adhesive proteins, including collagen, forming platelet aggregates and secreting various compounds from granules. Moreover, various biochemical processes such as phosphoinositide hydrolysis, arachidonic metabolism and eicosanoid biosynthesis, and reactive oxygen species (ROS) generation, are involved in platelet activation [1,2]. However, uncontrolled platelet activation is also an important risk factor of cardiovascular diseases. For example, blood platelets may form pathogenic thrombi, which are responsible for acute ischemic events [2]. In developed countries, the greatest single cause of mortality is due to cardiovascular conditions, such as atherosclerosis and thrombosis; these are responsible for about 50% of all deaths each year in Europe [3–5].

The most widely-known and popular anti-platelet drug is acetylsalicylic acid (aspirin), which acts as a cyclooxygenase inhibitor, an enzyme involved in eicosanoid synthesis. The most common side effects of aspirin are indigestion, stomach aches and bleeding [1,2]. Hence, there is great interest in identifying new anti-platelet agents without side effects. Many experiments, both in vitro and in vivo, suggest that berries may contain substances that affect the functioning of blood platelets, including their high phenolic content [6]. Various berries, including aronia berries (*Aronia melanocarpa*), blueberries (*Vacconium myrtillus*) and grapes (*Vitis*) have been found to possess antioxidant and antiplatelet activities [6–8]. Studies indicate that the effects of these fruits on blood platelet activation are dependent on not only the concentration of berry phenolics or the class of phenolic compounds, but also the type of berry and the form of food products or medical preparations [6]. In addition, the consumption phenolic compounds present in fresh berries or berry products, such as berry extracts, have not been associated with any unwanted or toxic activity, including hematological or urinary effects [7–17].

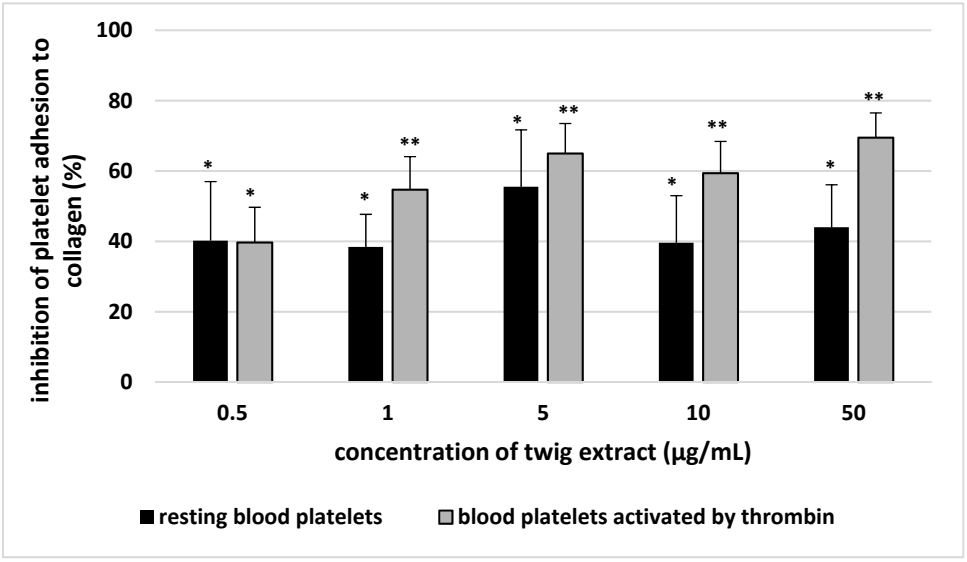
Dietary supplements, including commercial products made from aronia berries (Aronox®) may inhibit platelet activation, by reducing platelet aggregation or eicosanoid synthesis [10]. Various studies have shown that sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) berries and their products may have therapeutic and protective properties against cardiovascular diseases [11–15]. Sea buckthorn is a small tree or bush. It belongs to the Elaeagnaceae family, and it has been used for many years in traditional medicine in both Europe and Asia. Sea buckthorn fruits have been used for treating various diseases, including cardiovascular diseases, for many years and are described in Chinese medical literature [6,8]. The therapeutic potential of sea buckthorn oils against cardiovascular diseases has been associated with its high unsaturated fatty acid content [8]. In addition, the phenolic-rich fraction of sea buckthorn berries has also been found to demonstrate anti-platelet activity [16], and sea buckthorn leaves and twigs contain various bioactive compounds, including phenolic compounds, with antioxidant and anticoagulant properties [17]. However, the mechanism behind their influence on blood platelet activation remains unknown. Therefore, the aim of the present study was to determine the biological activity of extracts from the leaves and twigs of sea buckthorn against blood platelets in vitro. The following battery of standard tests was used to obtain a broad overview of the key mechanisms behind the beneficial action of phenolic compounds on cardiovascular diseases: blood platelet adhesion to collagen type I and fibrinogen, blood platelet aggregation induced by various physiological agonists, metabolism of thiol groups and glutathione (GSH) in tested blood cells, nonenzymatic lipid peroxidation in resting platelets, arachidonic acid metabolism (enzymatic lipid peroxidation) in platelets activated by thrombin, and platelet superoxide anion (O_2^-) production. In addition, the cellular safety of tested extracts was evaluated in vitro using a cytotoxicity test against human blood platelets, measuring extracellular lactate dehydrogenase (LDH) activity. The action of extracts from sea buckthorn twigs and leaves was compared to activities of the phenolic extract, a commercial extract from the berries of *Aronia melanocarpa* (Aronox®) with antioxidative and antiplatelet properties [18–20].

2. Results

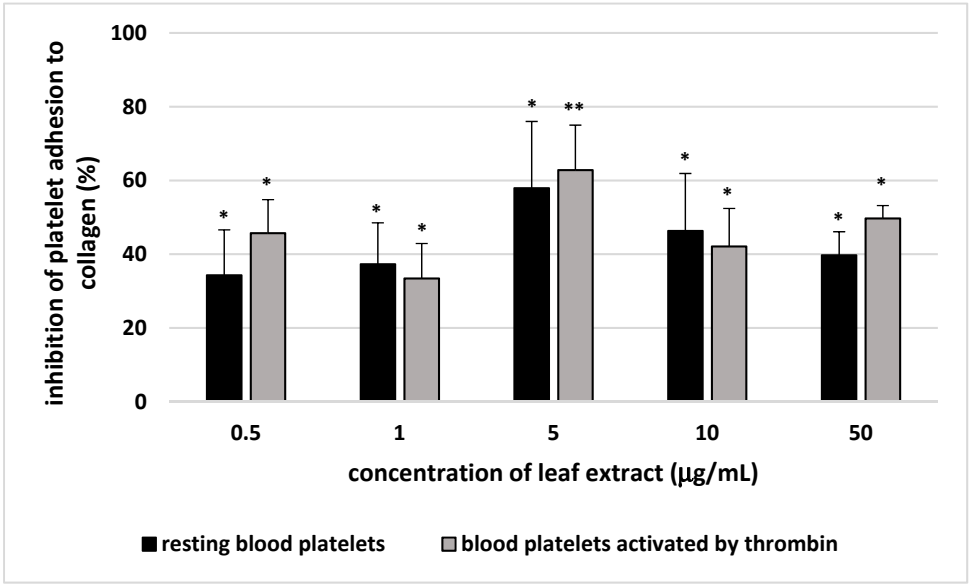
Our results show significantly lower adhesion to collagen of resting blood platelets and thrombin-activated platelets following preincubation with 0.5–50 μg/mL twig and leaf extract (Figure 1; Table 1). The percentage inhibition of adhesion of thrombin- or ADP-activated platelets to fibrinogen is given in Figure 2. At the highest tested concentration (50 μg/mL), the sea buckthorn twig extract demonstrated greater inhibition of thrombin-activated platelets to collagen or fibrinogen than the leaf extract. The twig extract demonstrated 69.5 ± 7.0% ($p < 0.02$) inhibition of adhesion to collagen and 62.6 ± 9.0% ($p < 0.02$) inhibition of adhesion to fibrinogen.

The next part of the study examined the potential of the twig and leaf extracts (at 10 and 50 μg/mL) to reduce platelet aggregation stimulated by different agonists, i.e., ADP, collagen and thrombin. The tested extracts were not found to display any anti-aggregatory properties when ADP and collagen were used as agonists ($p > 0.05$). However, both 10 and 50 μg/mL leaf extract inhibited

thrombin-stimulated platelet aggregation, as did the twig extract at the higher concentration of 50 μg/mL ($p < 0.05$) (Figure 3). For example, the percentage inhibition of thrombin-stimulated platelet aggregation was 35.5 ± 9.1% ($p < 0.05$) for twig extract and 29.9 ± 8.9% ($p < 0.05$) for leaf extract at a concentration of 50 μg/mL (Figure 3).



(**A**)

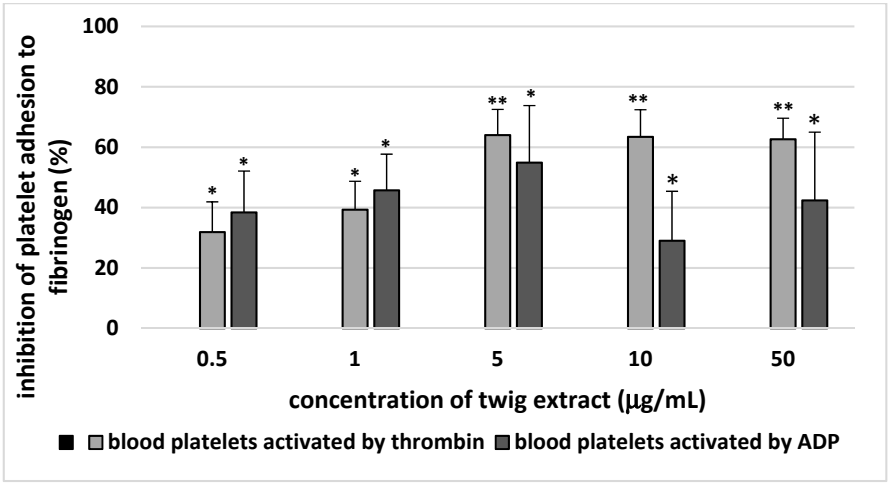


(**B**)

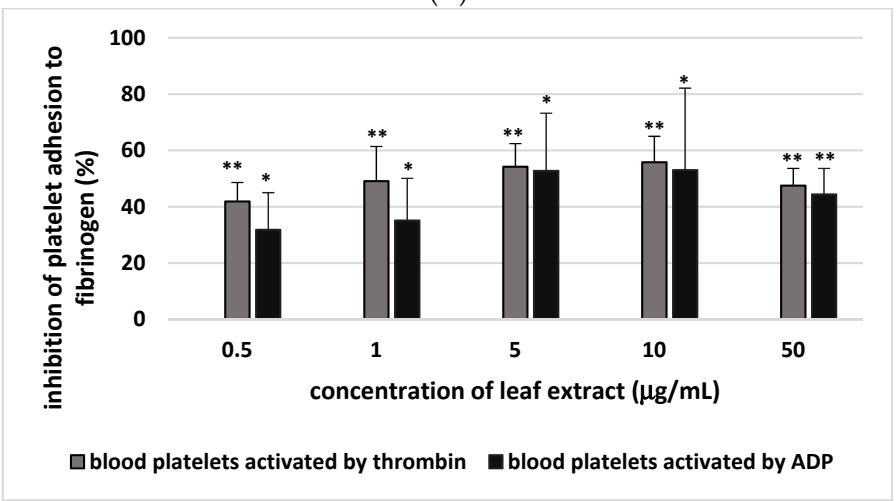
Figure 1. Twig (**A**) and leaf extract (**B**) (0.5–50 μg/mL; 30 min) on adhesion of resting blood platelets and thrombin-activated platelets to collagen. The inhibition of platelet adhesion by the plant extracts is expressed as the percentage of that recorded for control blood platelets (without the plant extract)–positive control. Data represent mean ± standard error (SE) of 5 (for resting platelets) and 9 (for thrombin-activated platelets) healthy volunteers (each experiment performed in triplicate). * $p < 0.05$, ** $p < 0.02$ (vs. control platelets).

Table 1. Comparison of the effects of sea buckthorn twig and leaf extracts and aronia berry extract at the concentration (10 μg/mL) on blood platelet adhesion. Results are given as means ± SE of 5–9 healthy volunteers (experiments done in triplicate).and expressed in percentage (%) difference between the control and tested extracts.

	Inhibition of Resting Blood Platelet Adhesion to Collagen (%)	Inhibition of Thrombin-Activated Platelets to Collagen (%)	Inhibition of Thrombin-Activated Platelets to Fibrinogen (%)	Inhibition of ADP-Activated Platelets to Fibrinogen (%)
Sea buckthorn twig extract (a)	39.6 ± 13.4 ($p > 0.05$, a vs. b, c)	59.4 ± 9.0 ($p < 0.05$, a vs. b, c)	63.4 ± 5.3 ($p < 0.05$, a vs. b, c)	29.0 ± 16.4 ($p > 0.05$, a vs. b, c)
Sea buckthorn leaf extract (b)	46.3 ± 15.6 ($p > 0.05$, b vs. c)	42.1 ± 10.3 ($p > 0.05$, b vs. c)	55.8 ± 9.2 ($p < 0.05$, b vs. c)	53.0 ± 29.1 ($p > 0.05$, b vs. c)
Aronia berry extract (c)	24.5 ± 11.4	34.9 ± 12.9	32.1 ± 17.4	30.7 ± 15.9



(**A**)



(**B**)

Figure 2. Twig (**A**) and leaf extract (**B**) (0.5–50 μg/mL; 30 min) on adhesion of thrombin/adenosine diphosphate (ADP)-activated platelets to fibrinogen. Inhibition of platelet adhesion by the plant extract is expressed as the percentage of that recorded for control blood platelets (without the plant extract)–positive control. Data represent mean ± SE of 5 (for ADP-activated platelets) and (for thrombin-activated platelets) healthy volunteers (each experiment performed in triplicate). * $p < 0.05$, ** $p < 0.02$ (vs. control platelets).

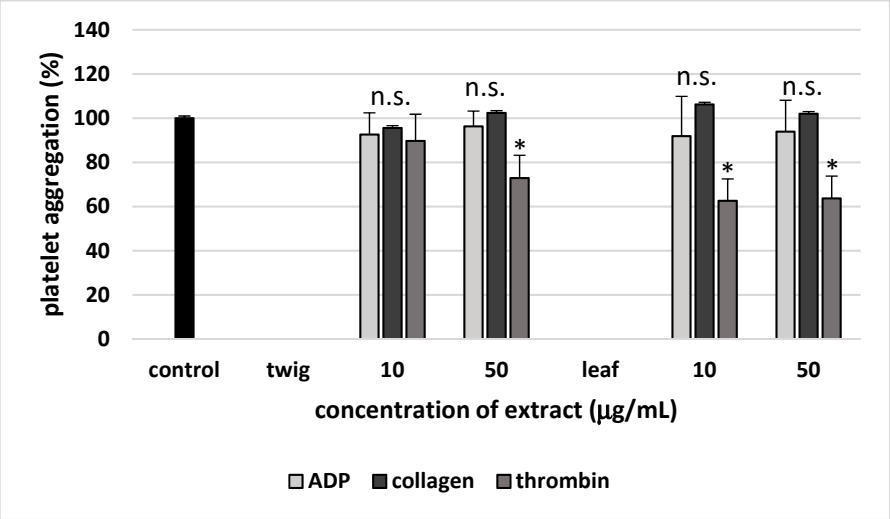
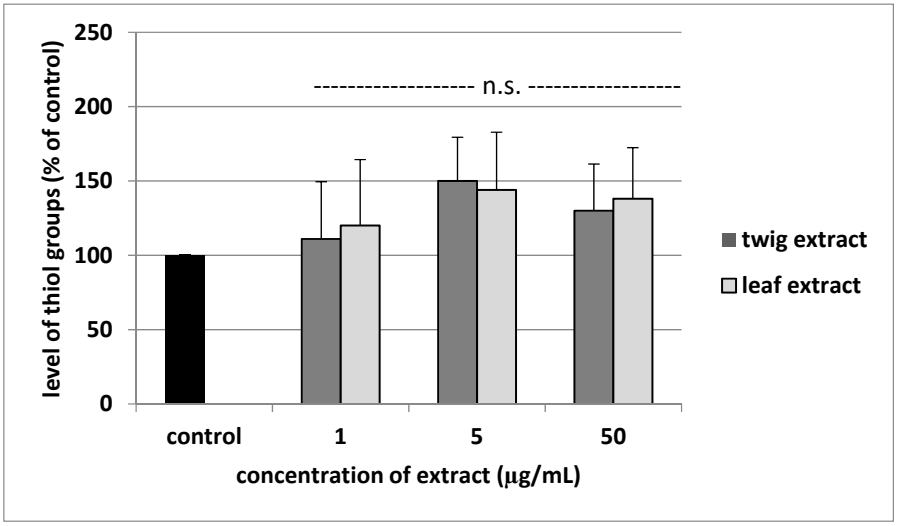


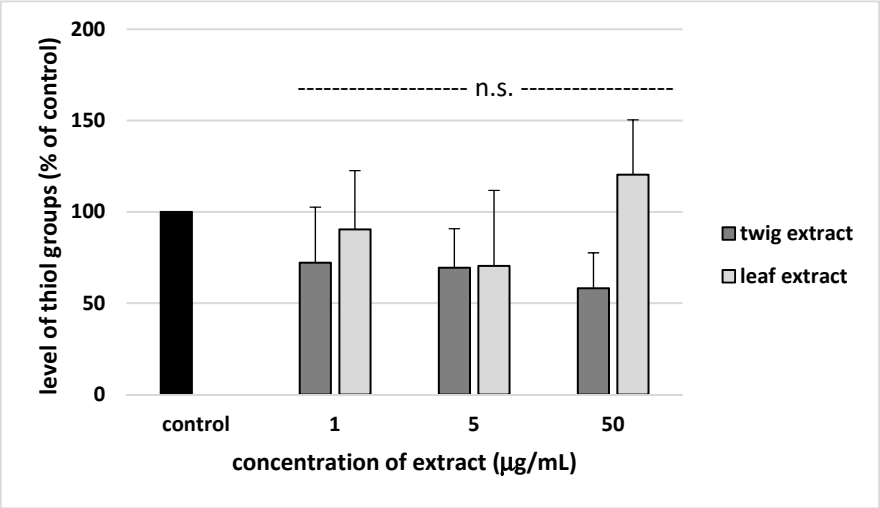
Figure 3. Effects of twig and leaf extract (10 and 50 µg/mL; 30 min) on blood platelet aggregation stimulated by different agonists: 10 µM ADP, 2 µg/mL collagen and 1 Unit/mL thrombin. Data represent mean ± SE of 5 (for thrombin-activated platelets) and 9 (for ADP or collagen-activated platelets) healthy volunteers (each experiment performed in triplicate). Neither concentration of the tested extract (10 and 50 µg/mL) had a statistically significant effect on aggregation stimulated by ADP and collagen compared to control platelets ($p > 0.05$ (n.s.)). However both concentrations of the tested extract (10 and 50 µg/mL) had a statistically significant effect on aggregation stimulated by thrombin compared to controls (* $p < 0.05$).

No change was observed in platelet GSH concentration or thiol group number in platelet proteins following exposure to the two tested sea buckthorn extracts at concentrations between 1–50 µg/mL ($p > 0.05$) (Figure 4).



(**A**)

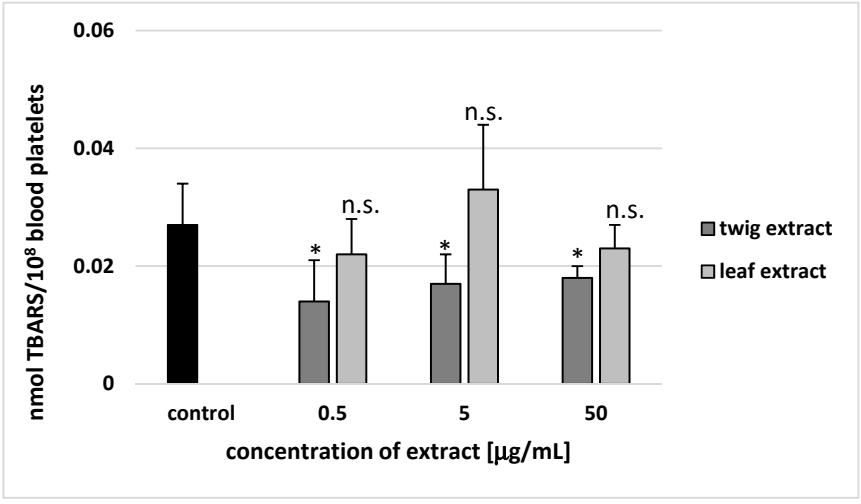
Figure 4. *Cont.*



(**B**)

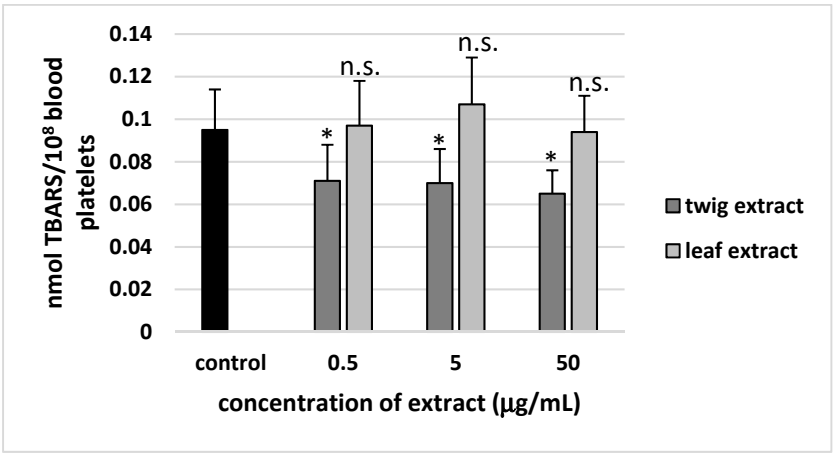
Figure 4. Twig and leaf extract (1, 5 and 50 µg/mL; 30 min) on the level of thiol groups in glutathione (GSH) fraction (**A**) and protein fraction (**B**) isolated from blood platelets. Data represent mean ± SE of 3 (for GSH) and 4 (for protein fraction) healthy volunteers (each experiment done in triplicate). In these experiments, the level of GSH fraction in control sample (positive control–blood platelets not treated with plant extract) was 5.7 ± 0.8 nmol GSH/mL of platelets, and was expressed as 100% (**A**); the level of thiol groups in protein fraction in control sample (positive control–blood platelets not treated with plant extract) was 112.4 ± 17.4 nmol GSH/mL of platelets, and was expressed as 100% (**B**). None of three different concentrations of the tested extract (1, 5 and 50 µg/mL) had a statistically significant effect compared to controls ($p > 0.05$ (n.s.)).

As demonstrated in Figure 5A,B, no change in the thiobarbituric acid reactive substances (TBARS) level was observed in the resting blood platelets or the thrombin-activated blood platelets following incubation with the leaf extract at concentrations of 0.5, 5 or 50 µg/mL ($p > 0.05$). On the other hand, all used concentrations of twig extract (0.5, 5 and 50 µg/mL) significantly reduced lipid peroxidation in both the resting and the thrombin-activated platelets ($p < 0.05$) (Figure 5A,B). At the highest-used concentration of twig extract (50 µg/mL), inhibition of lipid peroxidation was found to be about 40% for both resting platelets and those activated by thrombin (Figure 5A,B). In addition, 50 µg/mL twig extract demonstrated stronger inhibition than leaf extract at the same concentration.



(**A**)

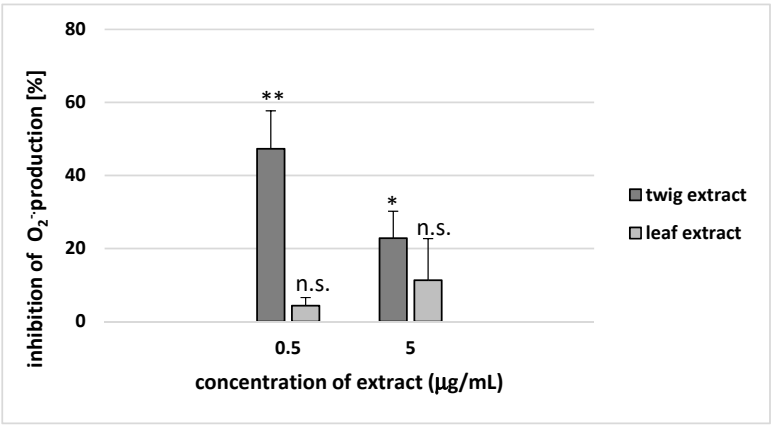
Figure 5. *Cont.*



(**B**)

Figure 5. Twig and leaf extract (0.5, 5 and 50 µg/mL; 30 min) on lipid peroxidation in resting platelets (**A**) and in blood platelets activated by thrombin (**B**). In these experiments, blood platelets not treated with plant extract were used as control samples (positive control). Data represent mean ± SE of 6 healthy volunteers (each experiment done in triplicate). The three different concentrations of the twig extract (0.5, 5 and 50 µg/mL) had a statistically significant compared to controls (* $p < 0.05$). However, none of the three different concentrations of the leaf extract (0.5, 5 and 50 µg/mL) had any statistically significant effect compared to controls ($p > 0.05$ (n.s.)).

Only the twig extract was found to significantly reduce the process of O_2^- production in resting platelets and activated platelets (Figure 6A,B).



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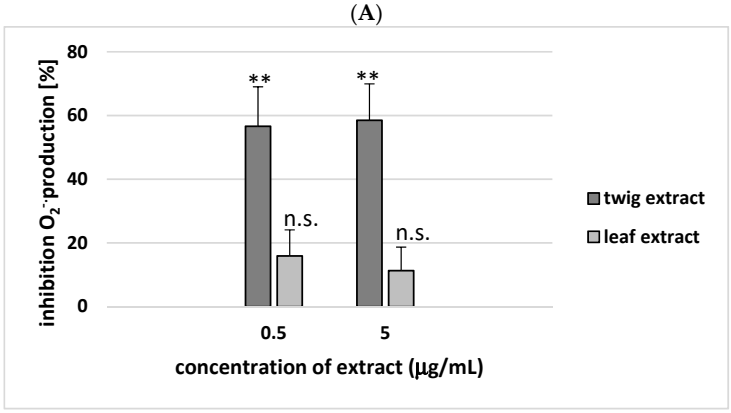

(**B**)

Figure 6. Effects of twig and leaf extract (0.5 and 5 µg/mL; 30 min) on O_2^- production in resting platelets

(**A**) and in blood platelets activated by thrombin (**B**). Data represent mean ± SE of 5 healthy volunteers (each experiment done in triplicate). In these experiments, the O_2^- level in control samples (positive control – blood platelets not treated with plant extract) was 0.592 ± 0.321 nmol/10^8 platelets (for resting platelets) and 1.222 ± 0.434 nmol/10^8 platelets (for thrombin-activated platelets). Inhibition of O_2^- production was expressed as a percentage of that recorded for positive control (platelets without tested extracts). The effects of the two different concentrations of twig extract (0.5 and 5 μg/mL) were significantly different to controls (* $p < 0.05$; ** $p < 0.02$). The two different concentrations of leaf extract (0.5 and 5 μg/mL) demonstrated no statistically significant effect compared to control platelets ($p > 0.05$ (n.s.)).

Regarding the cytotoxicity of the extracts, none were found to cause lysis of blood platelets ($p > 0.05$) (Figure 7).

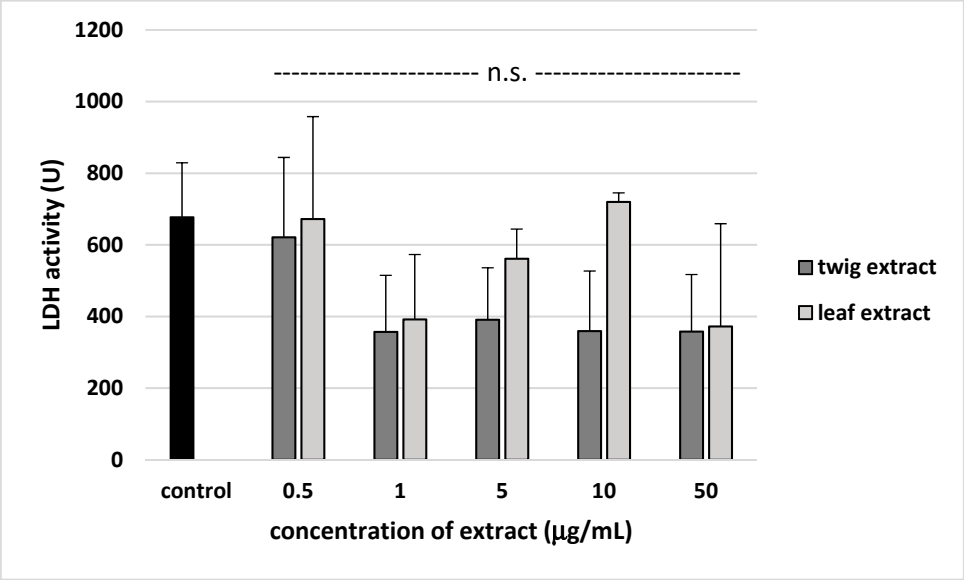

Figure 7. The toxic effects of twig and leaf extract (0.5–50 μg/mL; 30 min) against human blood platelets. In these experiments, blood platelets not treated with plant extract were used as control samples (positive control). Data represent mean ± SE of 6 healthy volunteers (each experiment performed in triplicate). None of the five different concentrations of the tested extract (0.5, 1, 5, 10 and 50 μg/mL) had any statistically significant effect compared to controls ($p > 0.05$ (n.s.)).

In comparative experiments (for blood platelet adhesion), the extract of sea buckthorn twigs (at the tested concentration, 10 μg/mL) turned out to be more effective than 10 μg/mL aronia extract ($p < 0.05$) (Table 1).

3. Discussion

In addition to multivitamin and multimineral compounds, demand is growing for supplements based on plant sources. One such plant is sea buckthorn, which offers great promise as a supplement, mainly due to its high concentrations of vitamin C, tocopherols and carotenoids, as well as its unique profile of lipids (especially unsaturated fatty acids) and various bioactive compounds, including phenolics, believed to be good for human health [8,21,22], in both berries and berry products [8,21].

Sea buckthorn berries are therefore good candidates for functional food production. Recently, other studies have indicated that sea buckthorn leaves and twigs are also good sources of phenolic compounds with various biological activities, including antioxidant and anticoagulant properties [17,23–28]. However, the effect of extracts from sea buckthorn leaves and twigs on blood platelet activation, which play an important role in various cardiovascular diseases, has not yet been studied. Therefore, the

main aim of our in vitro study was to examine the anti-platelet properties of sea buckthorn leaf and twig extracts.

A significant new finding is that these extracts demonstrate antiadhesive activity in the tested system of isolated washed human blood platelets, with the tested extracts reducing blood platelet adhesion to collagen and fibrinogen. In addition, both tested extracts inhibited the aggregation of platelets following thrombin stimulation; interestingly, this inhibition was not observed in the platelets activated by collagen or ADP. It is possible that that tested extracts might interact with the plasma proteins present in platelet-rich plasma (PRP), thus preventing anti-aggregatory activity.

Although blood platelet function is known to involve thiol groups [29,30], our findings do not suggest that the sea buckthorn twig and leaf extracts influenced the levels of thiol groups, and that the extracts probably do not modulate platelet activation by thiol groups. In addition, the tested extracts did not change the platelet concentration of GSH: an important physiological antioxidant.

Blood platelet activation is associated with arachidonic acid metabolism, in which different intermediate products, including pro-thrombotic thromboxane A_2 (TXA_2) are produced. Thromboxane A_2 is an unstable compound, which is metabolized to inactive thromboxane B_2 after about 30 s. In the present experiments, TBARS concentration was used as an indicator of enzymatic peroxidation of arachidonic acid in the thrombin-stimulated platelets. The twig extract was found to reduce the thrombin-induced enzymatic cascade of arachidonic acid metabolism in blood platelets. It is possible that this extract may restore the level of platelet response by helping maintain the redox balance in thrombin-activated blood platelets.

It is known that ROS, which may behave as secondary signaling molecules, are generated both in resting platelets and those activated by various agonists, including thrombin. For example, O_2^- generation is associated with the enzymatic pathway of arachidonic acid metabolism. A reduction in O_2^- production was observed in thrombin-activated blood platelets treated with the twig extract, which was accompanied by a decrease in TBARS production. This inhibition of TBARS production and O_2^- generation by the twig extract suggests that it may also inhibit the thrombin-activated arachidonic acid pathway. It is also possible that the tested leaves and twig extracts may influence platelet reactivity by modifying other signal pathways, not only ROS level, e.g., through the inhibition of enzymatic peroxidation of lipids (TXA_2 biosynthesis), or modifying the expression of platelet receptors.

The differences in blood platelet activation displayed by the extracts may be accounted for by differences in their phenolic profiles. For example, the greater potency of the twig extract may be associated with its higher proanthocyanidin concentration compared to the leaf extract; for example, it demonstrated greater ability to encourage the inhibiting of platelet adhesion (stimulated by thrombin) to type I collagen. Type I is the most prevalent form of collagen in the arterial vessels changed by atherosclerosis.

Two important aspects of the use of natural compounds as drugs or supplements are their toxicity and bioavailability; these parameters are often determined for phenolics intended for use as ingredients of supplements or drugs in in vitro and in vivo models. Our present results demonstrate that none of the tested sea buckthorn extracts induced damage to human blood platelets within the whole tested concentration range. As the concentrations of sea buckthorn extracts used in the study may be achievable in blood during their oral supplementation [31–33], we can confirm that sea buckthorn leaves and twigs are safe for use in supplements. Gupta et al. [34] also demonstrated no cytotoxicity and side effects for sea buckthorn leaves following oral administration.

A novel finding of our present study is that sea buckthorn twig extract (at the used concentration, 10 µg/mL), similar to well-known aronia berry extract, has anti-platelet potential. It is also an interesting that sea buckthorn twig extract had stronger anti-adhesive activity than aronia berry extract.

The bioavailability of phenolic compounds varies according to food source. Moreover, it may also depend on the presence of various other compounds in the food matrix, including those with anti-platelet properties [35]. Tormanovic et al. [36] indicate that hippuric acid, a phenolic compound,

acts as a key metabolite following consumption of fruits, including berries; this exerts anti-platelet activity by blocking the ADP receptors present on blood platelets [37].

Chong et al. [38] indicate that anthocyanidins and procyanidins have a beneficial impact on pathologies of the cardiovascular system, including platelet hyperactivity. Reis et al. [39] also report that anthocyanins have a beneficial cardiovascular effect in animal and human studies. In addition, phenolic extracts from berries and commercial products made from berries (for example from aronia berries, Aronox®, which has a high concentration of anthocyanidins) have been found to be more effective anti-platelet factors than pure phenolic compounds in both in vitro and in vivo models [6,38,40,41]. These findings may suggest that phenolic compounds have synergistic inhibitory actions. Our present findings regarding the extract of sea buckthorn twigs, which is rich in proanthocyanidins, are consistent with those of these studies. Therefore, we suppose that this extract may also demonstrate anti-platelet potential in an in vivo model. The action of the tested ellagitannin-rich extract from sea buckthorn leaves on blood platelet activation may depend on the interaction of the ellagitannins with thrombin and other proteins. Dong et al. [42] report that these compounds may inhibit the catalytic activity of thrombin. However, no information exists on the effect of urolithins, metabolites produced in the gut following consumption of ellagitannins, upon blood platelet function.

Our present findings shed new light on the anti-platelet potential of sea buckthorn twig and leaf extracts, particularly those of the twig extract. In future, both may be recommended in the prevention and treatment of cardiovascular diseases associated with hyperactivation of platelets. In addition, our findings may assist the development of further potential anti-platelet supplements or potent drugs against cardiovascular diseases as alternatives to classical drugs such as aspirin, which often induce side-effects. It is important that fruits are harvested together with leaves and twigs; these by products represent a rich source of additional safe phenolic compounds with anti-platelet potential, which would otherwise be regarded as production waste.

4. Materials and Methods

4.1. Chemicals

ADP was obtained from Chrono-Log Corporation (Havertown, USA). Thrombin was purchased from BioMed Lublin, Poland. Collagen type I, bovine serum albumin (BSA), cytochrome C, 5,5′-dithio-bis(2-nitro-benzoic acid), and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Fibrinogen was isolated from pooled citrated human plasma by cold ethanol precipitation followed by ammonium sulphate fractionation at 26% saturation at 4 °C, according to Doolittle [43]; its concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 1.55 for 1 mg/mL solution. The concentration of purified human fibrinogen in the reaction system was 2 mg/mL. All other reagents represented analytical grade and were provided by commercial suppliers.

The content of phenolics in the phenolic-rich powder aronia berr extract (commercial product: Aronox® by Agropharm Ltd., Poland; batch No. 020/2007k) amounted to 309.6 mg/g of extract, including phenolic acids (isomers of chlorogenic acid), 149.2 mg/g of extract, anthocyanins (anthocyanin glycosides: cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, cyanidin 3-xyloside), 110.7 mg/g, and flavonoids (quercetin glycosides), 49.7 mg/g of extract. The high-performance liquid chromatography (HPLC) determination of this extract was described previously [18–20].

4.2. Plant Material

Sea buckthorn twigs and leaves were obtained from a horticultural farm in Sokółka, Podlaskie Voivodeship, Poland (53°24′N, 23°30′E), the largest Polish producer of sea buckthorn fruits. The plant material was identified by Mr. Stanislaw Trzonkowski, the owner of the farm. Voucher specimens have been deposited at the Institute of Soil Science and Plant Cultivation, Sate Research Institute, Pulawy, Poland (IUNG/HRH/2015/2).

4.3. Chemical Characteristics of Extracts from Sea Buckthorn Twigs and Leaves

The extracts from leaves and twigs of sea buckthorn were prepared as previously described (Sadowska et al., 2017). Briefly, freeze-dried leaves and air-dried twigs were milled in a laboratory mill (Retsch ZM200, Germany). The powdered plant material (140 g of the leaves or 200 g of the twigs) was extracted with 3 L (in three batches) of 80% methanol (*v/v*), for 48 h, at room temperature; the extraction was assisted by ultrasonic treatment (6 × 10 min.). After filtration, the methanol extracts were defatted with n-hexane. The defatted extract was concentrated under reduced pressure, the residue was resuspended in Milli-Q water, acidified with formic acid and subjected to n-butanol extraction. The butanol extracts obtained were rotary evaporated, and the residue was suspended in Milli-Q water and freeze-dried. Their composition was determined by reverse-phase ultra high-performance liquid chromatography–mass spectrometry (UHPLC–MS), using the ACQUITY UPLC^{TM} system (Waters, Milford, MA, USA), coupled with and ACQUITY TQD (Waters) triple quadrupole mass detector. Samples were chromatographed using an ACQUITY BEH C18 (100 mm × 2.1 mm, 1.7 µm; Waters) column. MS analyses were performed using negative and positive ion mode. More details of the applied analytical method are presented elsewhere [26]. Constituents of the extracts were classified and identified on the basis of their ultraviolet (UV) and MS spectra (including in-source fragmentation), authentic standards, as well as literature data [44–47]. UV-DAD detection (range: from 190 to 480 nm) was used for semi-quantitation of phenolic compounds. The content of individual hydrolysable tannins, flavonoids and proanthocyanidins was expressed as gallic acid, rutin and epicatechin equivalents, respectively, and was determined on the basis of calibration curves. The peak integration of hydrolysable tannins was performed at 270 nm, flavonoids were determined at 350 nm, and proanthocyanidins at 280 nm. Shown results are means ± SD of three replications.

The principal constituents of sea buckthorn leaf extract were ellagitannins (259.6 ± 3.1 mg/g). The total flavonoid content was 74.7 ± 0.7 mg/g. Catechin and proanthocyanidins were also detected, their total content was 7.2 ± 0.2 mg/g The sea buckthorn twig extract consisted mainly of B–type proanthocyanidins and catechin (total content 597.1 ± 10.2 mg/g). Ellagic acid and its glycosides were also present (the total content 22.4 ± 0.11 mg/g). Flavonoids were present in trace amounts (the total content was 1.7 ± 0.4 mg/g). Major phenolic constituents of the sea buckthorn leaf extract and the twig extract are shown in Tables 2 and 3, respectively. More details on the composition can be found elsewhere [26].

Table 2. Major phenolic compounds (above 5 µg/mg) of the sea buckthorn leaf extract.

No.	Compounds (Tentative Identification)	t_R (min)	λ max (nm)	$[M - H]^-$ $[M + H]^+$	Fragment Ions (+) (*m/z*)	Concentration (µg/mg)
1	strictinin/isomer	13.92	220, 270	633 635	153, 277, 303, 447	6.6 ± 0.3 [a]
2	strictinin/isomer	14.12	220, 270	633 635	153, 277, 303, 447	7.4 ± 0.3 [a]
3	stachyurin/isomer	14.61	222, 270	935 937	153, 277, 345, 617	14.2 ± 0.5 [a]
4	casuarinin/isomer	15.13	227, 270	935 937	153, 255, 345, 617	24.5 ± 0.5 [a]
5	casuarinin/isomer	15.34	230, 270	935 937	153, 255, 345, 617	39.4 ± 0.5 [a]
6	hippophaenin B/isomer	17.17	224, 270	1103 1105	153, 345, 471, 617	8.8 ± 0.2 [a]

Table 2. *Cont.*

No.	Compounds (Tentative Identification)	t_R (min)	λ max (nm)	$[M-H]^-$ $[M+H]^+$	Fragment Ions (+) (*m*/*z*)	Concentration (μg/mg)
7	hippophaenin B/isomer	17.30	224, 270	1103 1105	153, 345, 471, 617	19.6 ± 1.0 [a]
8	ellagic acid-Hex	19.30	253, 261	463 465	153, 303	7.4 ± 0.2 [a]
9	casuarictin/isomer	21.56	224, 270sh	935 937	153, 277, 303, 447, 785	21.7 ± 0.2 [a]
10	Q-Hex-dHex	23.31	255, 352	609 611	303, 449	5.9 ± 0.0 [b]
11	ellagic acid-Pen	24.02	253, 361	433 435	303	8.0 ± 0.2 [a]
12	ellagic acid	24.37	253, 366	301 303		14.0 ± 0.3 [a]
13	I-3-*O*-Hex-7-*O*-dHex	26.31	255, 350	623 625	317, 463	8.4 ± 0.0 [b]
14	I-3-*O*-Glc-7-*O*-Rha	27.33	255, 352	623 625	317, 463	15.9 ± 0.1 [b]
15	K-Hex-pCouA	43.85	266, 314	593 595	147, 287	5.4 ± 0.1 [b]

Hex—hexose; dHex—deoxyhexose; Glc—glucose; I—isorhamnetin; K—kaempferol; pCouA—*p*-coumaric acid; Q—quercetin; Pen—pentose; Rha—rhamnose; [a] gallic acid equivalent; [b] rutin equivalent. The presented data is an updated version of a table published in the supplementary materials of the article by Sadowska et al. (2017).

Table 3. Major phenolic compounds (above 5 μg/mg) of the butanol extract of sea buckthorn twigs.

No.	Compounds (Tentative Identification)	t_R (min)	λ max (nm)	$[M-H]^-$ $[M+H]^+$	Fragment Ions (+) (*m*/*z*)	Concentration (μg/mg)
1	gallocatechin-catechin	5.38	270, 300sh	593 595	259, 305, 465	9.2 ± 0.4 [b]
2	gallocatechin-catechin	5.85	270, 300sh	593 595	259, 305, 465	8.0 ± 0.4 [b]
3	dimeric proanthocyanidin	9.71	200, 279	577 579	289	58.4 ± 0.7 [b]
4	catechin	10.86	200, 278	289 291	139	76.1 ± 0.8 [b]
5	trimeric proanthocyanidin	11.37	200, 279	865 867	139, 289, 579	23.3 ± 0.6 [b]
6	tetrameric proanthocyanidin	14.90	200, 278	1153 1155	289, 577, 865	12.3 ± 0.2 [b]
7	trimeric proanthocyanidin	16.14	200, 278	865 867	289, 577	13.8 ± 0.3 [b]
8	dimeric proanthocyanidin	16.77	200, 278	577 579	291	7.4 ± 0.2 [b]
9	tetrameric proanthocyanidin	19.23	200, 279	1153 1155	289, 577, 865	11.8 ± 0.2 [b]
10	ellagic acid	24.46	253, 366	301 303		7.5 ± 0.1 [a]

[a] gallic acid equivalent; [b] epicatechin equivalent; The presented data is an updated version of a table published in the supplementary materials of the article by Sadowska et al. (2017).

Stock solutions of the twig extract and leaf extract were made in 50% DMSO. The final concentration of DMSO in samples was lower than 0.05% and its effect was determined in all experiments. A stock solution of aronia berry extract was made in H_2O.

4.4. Blood Platelet Isolation

Fresh human blood was obtained from 9 healthy volunteers (non-smokers and non-drugs, including supplements with anti-platelet and antioxidative properties; median age = 27) in the Lodz Medical Center (Lodz, Poland); the samples were collected in CPD solution (citrate/phosphate/dextrose; 9:1; *v*/*v* blood/CPD). The samples were not pooled. To obtain platelet-rich plasma, whole blood was centrifuged (1200 rpm, 15 min, 25 °C). The platelet titer was determined spectrophotometrically using a Helios α spectrophotometer at a wavelength λ = 800 nm [48]. The suspension obtained was diluted with Barber buffer (0.14 M NaCl, 0.014 M Tris, 5 mM glucose, pH 7.4) to final concentration of 2×10^8 cells/mL. Analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research, and approved by the Committee on the Ethics of Research in Human Experimentation of the University of Lodz (resolution No. 3/KBBN-UŁ/II/2016). The first, participants provided verbal consent to the researchers, and later participants provided written the documents.

The platelet suspension was incubated for 30 min at 37 °C with extracts from individual parts of sea buckthorn at final concentrations of 0.5, 1.0, 5.0, 10 and 50 μg/mL. Moreover, blood platelets were incubated for 30 min at 37 °C with aronia extract at the final concentration of 10 μg/mL.

4.5. Platelet Adhesion

Platelet adhesion was evaluated by measuring the activity of the platelet exoenzyme (acid phosphatase). Platelets were dissolved with Triton X-100. The formation of *p*-nitrophenol was measured at λ = 405 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany) following the addition of *p*-nitrophenylphosphate, the phosphatase substrate. The color reaction is created by the addition of 2 M NaOH. The absorbance of control blood platelets (without tested extracts) was expressed as 100% [16,49].

4.6. Platelet Aggregation

Platelet aggregation was measured by turbidimetry in platelet-rich plasma or in a platelet suspended in Barber's buffer using the optical Chrono-Log aggregometer (Chrono-Log, Havertown, PA, USA) [50].

Samples were prepared with 594 μL platelet-rich plasma (PRP) plus 6 μL extracts, or platelets suspended in Barber buffer and 6 μL tested extracts. In addition, a control sample without extract was prepared. Such prepared samples (with extract and without extract) were incubated at 37 °C for 30 min.

After incubation, 5 μL ADP (final concentrations 10 μM) or 5 μL collagen (final concentration–2 μg/mL) was added to the platelet rich plasma (PRP) for 10 min. The aggregometer was calibrated against the poor platelet plasma (100% aggregation).

After incubation, 5 μL thrombin (final concentration 1 Unit/mL) was added for 10 min to the platelets suspended in Barber's buffer. The aggregometer was calibrated against Barber's buffer (100% aggregation).

In these experiments, the blood platelets or PRP not treated with plant extract were used as control samples (positive control), and the rate of agonist-induced aggregation for the control sample (in the absence of plant extract) was 100%.

4.7. Glutathione and Thiol Group Measurement

The concentrations of thiol groups in platelet proteins and the glutathione concentration was measured spectrophotometrically using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany) at λ = 412 nm with Ellman's reagent: 5,5′-dithio-bis-(2-nitrobenzoic acid. The thionitrobenzoate protein derivative and the yellow thionitrobenzoic anion were formed as a result of the reaction of Ellaman's digestion with ionised thiol groups. The concentration of thiol groups was calculated using a molar absorption coefficient (ε = 13,600 $M^{-1}cm^{-1}$) [51,52].

4.8. Lipid Peroxidation Measurement

Lipid peroxidation was determined by measuring TBARS concentration. First, 15% trichloroacetic acid and 0.37% thiobarbituric acid were added to the test samples. The samples were heated in a heating block at 100 °C for 10 min. After cooling, the test samples were centrifuged (10,000 rpm, 15 min, 18 °C). The absorbance of the supernatant was measured at λ = 535 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany) [17,18,53,54].

4.9. Superoxide Anion Measurement

The superoxide anion level in blood platelets was determined by spectrophotometric measurement of the reduction of ferricytochrome c to ferrocytochrome. Cytochrome c (160 μM) was added to two platelet suspensions: one stimulated by thrombin and another that was unstimulated (resting). The samples were centrifuged (2000× *g*). The absorbance of the supernatant was measured at λ = 550 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). The O_2^- determination uses the molar absorption coefficient for cytochrome C, which is 18,700 $M^{-1}cm^{-1}$ [22,55].

4.10. Lactate Dehydrogenase (LDH) Activity Measurement

Determination of the activity of lactate dehydrogenase (LDH) released from platelets is a measure of the toxicity of the tested extracts against platelets. The test samples were centrifuged (15 min, 25 °C, 2500 rpm). The microtiter plate was loaded with 270 μL of 0.1 M phosphate buffer, 10 μL of supernatant and 10 μL of NADH. After a 20-min incubation at room temperature, 10 μL of pyruvate (5 mg) was added and the absorbance was measured. The reading was repeated for 10 min in every minute using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany) at λ = 340 nm [56].

4.11. Data Analysis

Several tests were used to perform the statistical analysis. In order to eliminate uncertain data, the Q-Dixon test was performed. All the values in this study were expressed as mean ± SE; n–number of blood donors. Statistical analysis was performed with one-way analysis of variance (ANOVA) for repeated measurements.

Author Contributions: B.S. designed the study and did experimental work. B.K. collaborated in analysis of results. A.R. did experimental work. B.O. collaborated analysis as well as performed a critical version of the manuscript. A.S. collaborated of chemical characteristics of plant extracts and performed a critical version of the manuscript. J.Ż. did experimental work and analyses of chemical characteristics of plant extracts and draft the manuscript. All authors read and approved the final manuscript.

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Abbreviations

ADP—adenosine diphosphate; BSA—bovine serum albumin; CPD—citrate/phosphate/dextrose; DMSO—dimethylsulfoxide; GSH—glutathione; LDH—lactate dehydrogenase; O_2^-—superoxide anion; PRP—platelet-rich plasma; ROS—reactive oxygen species; TBARS—thiobarbituric acid reactive substances; TXA_2—thromboxane A_2.

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Sample Availability: Samples of the *Elaeagnus rhamnoides* (L.) A. Nelson extracts are available from the authors.

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Response of blood platelets to phenolic fraction and non-polar fraction from the leaves and twigs of *Elaeagnus rhamnoides* (L.) A. Nelson *in vitro*

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ABSTRACT

Sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) is a shrub with lanceolate leaves and orange fruits. In traditional Chinese medicine, sea buckthorn organs, especially fruits have been used to treat different diseases, for example cardiovascular disorders. *In vitro* studies indicate that the phenolic-rich fraction of sea buckthorn fruits demonstrates anti-platelet activity. The aim of the present study was therefore to determine the influence of phenolic and non-polar fractions isolated from the leaves and twigs of sea buckthorn on various parameters of human blood platelets *in vitro*. Plant material consisted of four different fractions: (1) the phenolic fraction isolated from the leaves, (2) the phenolic fraction isolated from the twigs, (3) the non-polar fraction isolated from leaves and (4) the non-polar fraction from twigs. The chemical composition of the tested fractions was determined using reversed phase UHPLC-HRMS/MS. The fractions from twigs were found to have stronger anti-platelet properties than those from leaves, and all tested fractions were found to be safe for the blood platelets. The tested fractions from the sea buckthorn, especially the non-polar fraction from the twigs, may potentially be a source of compounds with antiplatelet activity.

1. Introduction

Blood platelets are the smallest, non-seminal morphotic elements of bone marrow and peripheral blood. The cells are known to play an important role in hemostasis, particularly so upon activation. They may be activated by the interaction of an agonist, such as adenosine diphosphate (ADP), collagen or thrombin, with a receptor on the surface of the platelet. Following activation, taking place through various pathways, such as the arachidonic acid pathway, nitric oxide pathway or the serotonic pathway, or by the action of reactive oxygen species (ROS) [1], the platelet plays a key role in plate plug formation and the repair of damaged blood vessel walls [2,3].

However, the presence of abnormalities in platelet morphology, platelet dysfunction, excessive or insufficient levels of platelets, a lack or dysfunction of receptors, or disturbances in the arachidonic acid metabolism can lead to numerous cardiovascular diseases [4]. Dysregulation of blood platelet activity, particularly blood platelet aggregation, is linked to the progression of atherosclerosis [1]. Although anti-platelet agents, such as aspirin, are able to inhibit various steps of platelet activation, including platelet aggregation, their action typically induces a number of side effects, including bleeding. Therefore, there is great interest in identifying plant supplements or food products with anti-platelet activity [5,6], with recent studies focusing on the chemical composition and biological properties of sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) organs, especially its fruits [7–14].

Sea buckthorn is a shrub with lanceolate leaves and orange fruits. This plant belongs to the *Elaeagnaceae* family. It is distributed throughout Asia and Europe. Sea buckthorn has been used in traditional medicine for centuries. Its therapeutic potential has been already observed in traditional Chinese medicine. For example, people used various its organs, especially berries for treating different diseases, including hypertension, skin diseases and problems with the digestive

Abbreviations: ADP, adenosine diphosphate; BSA, bovine serum albumin; CPDA1, citrate/phosphate/dextrose/adenine; DMSO, dimethylsulfoxide; DTNB, 5,5'-dithio-bis(2-nitro-benzoic acid); LDH, lactate dehydrogenase; $O_2^{-\cdot}$, superoxide anion; MDA, malonic dialdehyde; NADH, reduced form of nicotinamide adenine dinucleotide; NAD^+, oxidized form of nicotinamide adenine dinucleotide; PBS, phosphate buffered saline; PRP, platelet-rich plasma; ROS, reactive oxygen species; TCA, trichloroacetic acid; TBA, thiobartbituric acid; TBARS, thiobarbituric acid reactive substances; TBS, Tris-buffered saline; TXA_2, thromboxane A_2

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Table 1
Secondary metabolites in the phenolic fraction of sea buckthorn leaf extract; the listed compounds correspond to Charged Aerosol Detector peaks with area ≥1 % of the total peak area.

Peak	RT [min]	Area Frac. %	λ_{max} nm	$[M-H]^-$ (*m/z*)	MS/MS fragments	Formula	Error (ppm)	Tentative identification
1	0.8	8.1		377.0854*	341.1092	$C_{12}H_{22}O_{11}$	0.6	dihexose
				191.0562		$C_7H_{12}O_6$	−0.3	quinic acid / isomer
2	1.0	2.1	270	331.0664	271.0481, 169.0152, 125.0243	$C_{13}H_{16}O_{10}$	2.1	gallic acid hexoside / isomer
				783.0684	481.0603, 300.9988	$C_{34}H_{24}O_{22}$	0.3	pedunculagin / isomer
3	1.3	1.6	270sh	305.0666		$C_{34}H_{24}O_{22}$	0.1	(epi)gallocatechin
				633.0740	463.0556, 300.9986	$C_{27}H_{22}O_{18}$	−1.8	strictinin / isomer
7	2.3	5.2	270	633.0733	463.0520, 300.9994	$C_{27}H_{22}O_{18}$	0.0	strictinin / isomer
8	2.5	10.2	270	935.0789	783.0677, 633.0745, 571.0728, 482.0629, 300.9993, 275.0197	$C_{41}H_{28}O_{26}$	0.8	casuarinin / isomer
				1103.0851	1059.0978, 935.0841, 783.0733, 633.0551, 571.0754, 300.9996, 275.0206	$C_{48}H_{32}O_{31}$	0.03	hippophaenin B or isomer
10	3.2	1.5	270sh	785.0846	633.0743, 483.0760, 300.9995	$C_{34}H_{26}O_{22}$	−0.4	tellimagrandin I / isomer
14	3.8	3.4	270	935.0795	633.0738, 463.0529, 300.9991	$C_{41}H_{28}O_{26}$	0.1	casuarictin / isomer
15	4.1	1.6	270sh	1117.0998	935.0789, 633.0739, 300.9988, 275.0188	$C_{49}H_{34}O_{31}$	1.2	ellagitannin
19	4.6	1.4	270	1085.0731	450.9935, 299.0188	$C_{48}H_{30}O_{30}$	1.6	ellagotannin
21	4.8	1.2	254, 367	300.9983		$C_{14}H_6O_8$	2.4	ellagic acid
24	5.4	1.3	254, 350	609.1451	300.0267#, 271.0240	$C_{27}H_{30}O_{16}$	1.7	Q-3-O-rutinoside
25	5.5	1.3	255, 351	463.0869	300.0263#, 271.0234	$C_{21}H_{20}O_{12}$	2.8	Q-Hex
26	5.7	1.8	254, 354	623.1604	477.1026, 461.1067, 313.0343#	$C_{28}H_{32}O_{16}$	2.1	I-dHex-Hex
28	5.9	2.0	254, 355	623.1606	477.1030, 461.1073, 313.0343#	$C_{28}H_{32}O_{16}$	1.8	I-3-O-Glc-7-O-Rha
32	6.4	1.1	255, 230	961.2606	815.2020, 639.1512, 460.0980, 313.0340#	$C_{44}H_{50}O_{24}$	1.4	I-dHex-Hex-Hex-FerA
35	6.8	1.1	254, 355	623.1607	315.0500	$C_{28}H_{32}O_{16}$	1.7	I-3-O-rutinoside
50	9.8	2.3	266, 315	593.1298	447.0923, 285.0394	$C_{30}H_{26}O_{13}$	0.5	K-Hex-pCouA
53	10.3	1.2	266, 315	593.1297	447.0931, 285.0391	$C_{30}H_{26}O_{13}$	0.6	K-Hex-pCouA
67	12.6	1.0	253,355	789.2606	626.2002, 477.1036, 313.0353#	$C_{38}H_{46}O_{18}$	0.7	I-dHex-166-Hex
68	12.7	1.8	253,355	789.2613	627.2084, 477.1026, 313.0352#	$C_{38}H_{46}O_{18}$	−0.2	I-dHex-166-Hex
71	13.3	1.2		1235.6061	1073.5568, 911.5061, 749.4491, 603.3899, 471.3478	$C_{59}H_{96}O_{27}$	0.4	triterpenoid saponin
78	14.6	1.1		1381.6639	1057.5599, 733.4533, 587.3952, 455.3528	$C_{65}H_{106}O_{31}$	0.4	triterpenoid saponin
81	16.2	1.2		1219.6106	1057.5599, 895.5096, 733.4530, 587.3947, 455.3524	$C_{59}H_{96}O_{26}$	0.9	triterpenoid saponin
82	16.5	2.1		1459.7109	807.4532, 661.3947, 529.3527, 469.3330, 183.1015	$C_{71}H_{112}O_{31}$	0.4	triterpenoid saponin
84	16.9	1.6		1313.6519	985.5027, 823.4472, 661.3946, 601.3758, 529.3550, 469.3308, 183.1026	$C_{65}H_{102}O_{27}$	1.3	triterpenoid saponin
87	17.7	2.1		1401.7059	915.5453, 749.4464, 603.3893, 471.3472, 183.1020	$C_{69}H_{110}O_{29}$	0.1	triterpenoid saponin
88	17.8	1.6		1297.6563	1135.6125, 973.5466, 807.4536, 661.3963, 529.3516, 469.3322, 183.1013	$C_{65}H_{102}O_{26}$	1.8	triterpenoid saponin
92	18.5	3.5		487.3418	409.3105	$C_{30}H_{48}O_5$	2.3	triterpenoid
95	19.5	1.0		1239.6512	1077.6005, 915.5478, 749.4475, 603.3886, 471.3468, 183.1018	$C_{63}H_{100}O_{24}$	0.7	triterpenoid saponin
106	23.0	1.3		471.3478		$C_{30}H_{48}O_4$	0.4	triterpenoid

* - a Cl adduct; # - a radical aglycone ion; dHex – deoxyhexose; Hex – hexose; pCouA – coumaric acid; FerA – ferulic acid; 166 – (-)-linalool-1-oic acid / isomer ($C_{10}H_{16}O_3$).

system [15,16]. China was the first country to recognize this plant as a drug, being formally included in the Chinese pharmacopoeia in 1977 [17]. Research from recent years also demonstrate that various sea buckthorn preparations may be used in the treatment of abnormal cholesterol concentrations, skin burns and atopic dermatitis [7–14]. Chinese medicinal literature has demonstrated the use of sea buckthorn fruits for treating various illnesses, including cardiovascular disease [18,19]. Moreover, different forms of sea buckthorn products are available in China, for example Flavonoid Tablets used to treat ischemic cardiopathy [20]. Our earlier studies indicate that the phenolic-rich fraction of sea buckthorn fruits demonstrates anti-platelet activity *in vitro* [21]. In addition, and sea buckthorn leaves and twigs contain phenolic compounds with antioxidant and anticoagulant properties [22], and the non-polar fraction from sea buckthorn twigs has been found to prolong prothrombin time and activated partial thromboplastin time in *in vitro* model [22]; however, the mechanism behind the activity of these fractions on blood platelet activation remains unknown. Therefore, the aim of the present study was to determine the effect of the phenolic and non-polar fractions from sea buckthorn leaves and twigs on blood platelet activation *in vitro*. More specifically, it examines the influence of the fractions on blood platelet adhesion to two adhesive proteins, fibrinogen and collagen type I, this being the most prominent collagen type in the arterial wall in vessels changed by atherosclerosis, blood platelet aggregation induced by various physiological agonists (thrombin, ADP and collagen), non-enzymatic lipid peroxidation in resting platelets, arachidonic acid metabolism (enzymatic lipid peroxidation) in platelets activated by thrombin, and platelet superoxide anion ($O_2^{-\cdot}$) generation. In addition, the study also investigates the *in vitro* cytotoxicity of the tested plant fractions against human blood platelets by measuring extracellular lactate dehydrogenase (LDH) activity.

2. Materials and methods

2.1. Chemicals

Thrombin was obtained from BioMed Lublin, Poland. ADP and collagen were purchased from Chrono-Log Corporation (Havertown, USA). Dimethylsulfoxide (DMSO), 5,5'-dithio-bis(2-nitro-benzoic acid) (DTNB), bovine serum albumin (BSA), cytochrome C and collagen type I were obtained from Sigma-Aldrich (St. Louis, MO., USA). Fibrinogen was isolated from freshly-drawn human blood at low temperatures (4 °C) using reagents with an appropriate concentration of ethanol, according to Doolittle et al. [23]. The obtained fibrinogen solution was diluted to a concentration of 2 mg/mL.

Table 2
Secondary metabolites in the non-polar fraction of sea buckthorn leaf extract; the listed compounds correspond to Charged Aerosol Detector peaks with area ≥1 % of the total peak area.

Peak	RT [min]	Area Frac. %	λ_{max} nm	$[M-H]^-$ (*m/z*)	MS/MS fragments	Formula	Error (ppm)	Tentative identification
4	2.5	1.2	~270	935.0801	783.0699, 633.0738, 481.0621, 300.9996, 275.0204	$C_{41}H_{28}O_{26}$	−0.5	stachyurin / isomer
				1103.0858	1059.0974, 935.0786, 783.0715, 633.0732, 300.9995, 275.0197	$C_{48}H_{32}O_{31}$	−0.3	hippophaenin / isomer
46	16.5	1.6		1459.7150	1297.6596, 973.5559, 807.4544, 661.3974, 601.3746, 529.3546, 469.3330, 369.2816, 183.1026	$C_{71}H_{112}O_{31}$	−2.4	triterpenoid saponin
47	16.7	1.8		1313.6537	1151.6000, 985.5061, 763.4265, 661.3956, 601.3749, 529.3555, 469.3332, 221.0675, 183.0140	$C_{65}H_{102}O_{27}$	−0.1	triterpenoid saponin
48	16.9	2.0		1313.6548	1151.600, 1147.5542, 985.5055, 827.4989, 661.3976, 601.3753, 469.3331, 369.2804, 183.1036	$C_{65}H_{102}O_{27}$	−0.9	triterpenoid saponin
50	17.7	1.3		1297.6601	1135.6041, 973.5575, 969.5075, 747.4322, 601.3765, 529.3584, 469.3326, 183.1039	$C_{65}H_{102}O_{26}$	−1.1	triterpenoid saponin
51	17.7	4.2		1401.7051	1239.6541, 915.5450, 749.4476, 603.3911, 471.3482, 183.1037	$C_{69}H_{110}O_{29}$	0.7	triterpenoid saponin
52	17.8	2.0		1297.6603	1135.6055, 973.5521, 969.5064, 807.4540, 661.3964, 601.3763, 529.3523, 469.3324, 183.1039	$C_{65}H_{102}O_{26}$	−1.2	triterpenoid saponin
53	18.0	1.1		1151.6038	985.5035, 823.4423, 763.4300, 661.3976, 601.3767, 529.3557, 469.3330, 183.1034	$C_{59}H_{92}O_{22}$	−2.6	triterpenoid saponin
55	18.4	4.7		1255.6475	1093.5914, 769.4896, 765.449, 603.3885, 471.3462, 183.1023	$C_{63}H_{100}O_{25}$	0.5	triterpenoid saponin
56	18.5	18.3		487.3440		$C_{30}H_{48}O_5$	−2.3	triterpenoid
60	19.5	4.3		**1239.6549**	1077.5983, 915.5494, 749.4449, 603.3920, 471.3496, 183.1031	$C_{63}H_{100}O_{24}$	−1.4	triterpenoid saponin
				487.3438		$C_{30}H_{48}O_5$	−1.8	triterpenoid
63	20.1	3.6		1093.5972	931.5451, 769.4896, 603.3932, 471.3500, 183.1042	$C_{57}H_{90}O_{20}$	−1.8	triterpenoid saponin
81	22.4	2.1		471.3480		$C_{30}H_{48}O_4$	−0.1	triterpenoid
77	22.4	3.0		471.3479		$C_{30}H_{48}O_4$	0.3	triterpenoid
79	22.6	2.2	311	633.3793	469.3332, 163.0398, 145.0290	$C_{39}H_{54}O_7$	0.6	$C_{30}H_{48}O_5$-pCouA
81	23.0	13.1	323	**471.3478**	423.3275	$C_{30}H_{48}O_4$	0.3	triterpenoid
				663.3906	193.0506, 175.0403	$C_{40}H_{56}O_8$	−0.5	$C_{30}H_{48}O_5$-FerA
82	23.2	7.1	307	**471.3487**		$C_{30}H_{48}O_4$	−1.5	triterpenoid
				633.3802	469.3323, 163.0396, 145.0288	$C_{39}H_{54}O_7$	−0.9	$C_{30}H_{48}O_5$-pCouA
99	26.12	6.5	n.r.	**455.3533**		$C_{30}H_{48}O_3$	−0.6	oleanolic acid, ursolic acid
				617.3853	453.3370 (4), 163.0391 (1), 145.0297 (24)	$C_{39}H_{54}O_6$	−0.9	$C_{30}H_{48}O_4$-pCouA

pCouA – coumaric acid; FerA – ferulic acid; **bold** – main constituent; n.r. – not registered.

Table 3
Secondary metabolites in the phenolic fraction of sea buckthorn twig extract; the listed compounds correspond to Charged Aerosol Detector peaks with area ≥1 % of the total peak area.

Peak	RT (min)	Area Frac. (%)	λ_{max} nm	$[M-H]^-$ (*m/z*)	MS/MS fragments	Formula	Error (ppm)	Tentative identification
1	0.8	14.7	274	191.0561		$C_7H_{12}O_6$	0.3	Quinic acid / isomer
				377.0855*	341.1077, 215.0323	$C_{12}H_{22}O_{11}$	0.4	dihexose
				609.1245	423.0720, 305.0663, 177.0185	$C_{30}H_{26}O_{14}$	0.9	(epi)GC-(epi)GC
2	1.1	6.9	274	337.1398	241.0982, 199.0872			unidentified
				609.1248	423.0721, 305.0668, 177.0200, 125.0238	$C_{30}H_{26}O_{14}$	0.2	(epi)GC-(epi)GC
3	1.4	6.8	274	305.0659	219.0654, 125.0249	$C_{15}H_{14}O_7$	2.5	(epi)gallocatechin
4	1.5	3.6	274	593.1289	423.0711, 305.06559, 177.0191, 125.0242	$C_{30}H_{26}O_{13}$	1.9	(epi)C-(epi)GC
5	1.6	1.8	274	881.1921	711.1345, 593.1292, 423.0712, 305.0656, 287.0556, 177.0195, 125.0237	$C_{45}H_{38}O_{19}$	1.6	(epi)C-(epi)C-(epi)GC
6	1.8	1.1	278	881.1925	711.1342, 593.1299, 467.0985; 423.0723, 305.0666, 287.0558, 177.0188, 125.0244	$C_{45}H_{38}O_{19}$	1.0	(epi)C-(epi)C-(epi)GC
7	2.2	8.8	278	577.1343	451.1027, 425.0864, 407.0758, 289.0705, 125.0243	$C_{30}H_{26}O_{12}$	1.4	(epi)C-(epi)C
8	2.3	10.0	278	289.0709	245.0815, 123.0448	$C_{15}H_{14}O_6$	2.9	catechin
10	2.7	2.9	278	1153.2607	525.0781, 407.0770, 289.0701, 243.0288, 125.0256	$C_{60}H_{50}O_{24}$	1.1	(epi)C-(epi)C-(epi)C-(epi)C
11	3.1	3.6	278	865.1973	695.1395, 575.1201, 407.0775, 287.0557, 243.0295, 175.0398, 125.0250	$C_{45}H_{38}O_{18}$	1.4	(epi)C-(epi)C-(epi)C
12	3.3	2.5	278	577.1342	451.1028, 425.0874, 407.0770, 289.0712, 125.0249	$C_{30}H_{26}O_{12}$	1.6	(epi)C-(epi)C
14	3.6	1.2	278	1153.2607	407.0777, 289.0694, 243.0290, 175.0396, 125.0258	$C_{60}H_{50}O_{24}$	1.1	(epi)C-(epi)C-(epi)C-(epi)C
20	4.6	1.4	278	865.1973	695.1402, 587.1187, 407.0764, 299.9902, 289.0705, 125.0243	$C_{45}H_{38}O_{18}$	1.4	(epi)C-(epi)C-(epi)C
21	4.8	1.2	254, 367	300.9987		$C_{14}H_6O_8$	0.9	ellagic acid
36	11.3	1.6	295	582.2600	462.2028, 342.1440, 316.1669, 145.0286, 119.0502	$C_{34}H_{37}N_3O_6$	1.6	tricoumaroyl spermidine
37	11.8	2.0	295, 310	612.2710	492.2134, 462.2031, 342.1452, 316.1661, 145.0281, 134.0366, 119.0502	$C_{35}H_{39}N_3O_7$	0.8	feruloyl dicoumaroyl spermidine
38	12.1	3.2	295, 314	642.2822	522.2240, 492.2140, 372.1559, 330.1453, 175.0388, 134.0364, 119.0503	$C_{36}H_{41}N_3O_8$	−0.2	diferuloyl coumaroyl spermidine
39	12.5	3.9	295, 319	672.2918	522.2240, 496.2465, 372.1562, 330.1457, 304.1664, 175.0392, 134.0365	$C_{37}H_{43}N_3O_9$	1.2	triferuloyl spermidine
45	18.5	3.0		487.3430		$C_{30}H_{48}O_5$		triterpenoid

* - a Cl^- adduct; (epi)C – (epi)catechin; (epi)GC – (epi)gallocatechin.

Table 4
Secondary metabolites in the non-polar fraction of sea buckthorn twig extract; the listed compounds correspond to Charged Aerosol Detector peaks with area ≥1 % of the total peak area.

Peak	RT (min)	Area Frac. %	λ_{max} nm	$[M-H]^-$ (m/z)	MS/MS fragments (%)	Formula	Error (ppm)	Tentative identifification
16	18.5	5.9		487.3432		$C_{30}H_{48}O_5$	−0.6	triterpenoid
21	19.5	1.5		487.3428		$C_{30}H_{48}O_5$	0.3	triterpenoid
31	22.5	3.4		471.3471		$C_{30}H_{48}O_4$	1.8	triterpenoid
34	23.0	6.5		471.3465		$C_{30}H_{48}O_4$	3.1	triterpenoid
35	23.2	8.6		471.3470		$C_{30}H_{48}O_4$	2.2	triterpenoid
41	24.9	1.9	307	617.3838	453.3346, 163.0399, 145.0284	$C_{39}H_{54}O_6$	1.5	$C_{30}H_{48}O_4$-pCouA
42	25.1	3.1	323	**633.3794**	453.3369, 179.0349, 161.0235	$C_{39}H_{54}O_7$	0.5	$C_{30}H_{48}O_4$-CafA
				521.3106	447.2746, 345.2060, 271.2275, 175 0390	$C_{29}H_{46}O_8$	2.7	unidentified
43	25.3	2.8	311	617.3843	453.3374, 163.0396, 145.0284	$C_{39}H_{54}O_6$	0.8	$C_{30}H_{48}O_4$-pCouA
44	25.4	2.0	311	**617.3845**	453.3372, 163.0396, 145.0284	$C_{39}H_{54}O_6$	0.5	$C_{30}H_{48}O_4$-pCouA
				471.2750	295.2255, 247.0981, 175.0394	$C_{28}H_{40}O_6$	0.5	unidentified
47	25.8	2.3		**455.3526**		$C_{30}H_{48}O_3$	1.0	triterpenoid
				447.2746	345.2071, 271.2276, 193.0597, 175.0402	$C_{26}H_{40}O_6$	1.4	unidentified
48	26.0	11.5	311	617.3845	453.3366, 163.0400, 145.0287	$C_{39}H_{54}O_6$	0.5	$C_{30}H_{48}O_4$-pCouA
49	26.1	30.6	n.r.	**455.3534**		$C_{30}H_{48}O_3$	−0.8	ursolic acid, oleanolic acid
				617.3852		$C_{39}H_{54}O_6$	−0.6	$C_{30}H_{48}O_4$-pCouA

pCouA – coumaric acid; CafA – caffeic acid; **bold** – main constituent; n.r. – not registered.

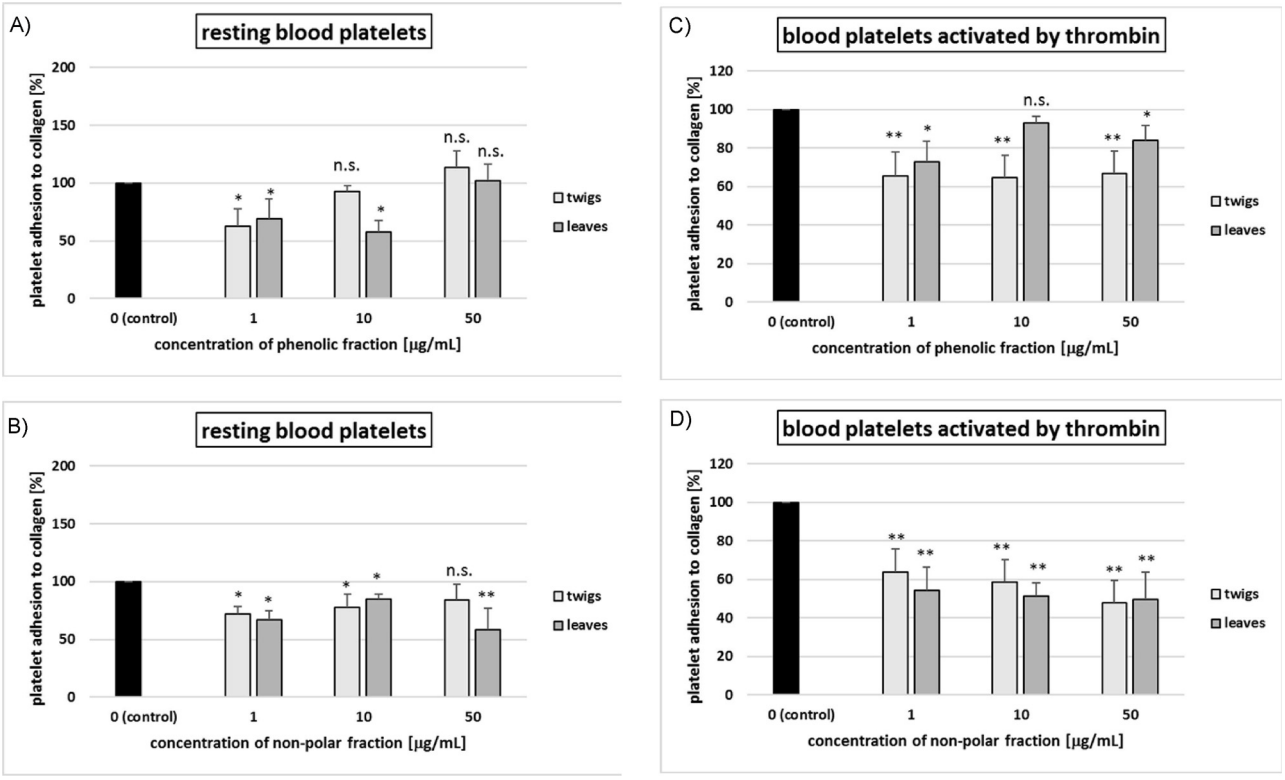

Fig. 1. Effects of phenolic fractions from sea buckthorn twigs and leaves (A and C) (1–50 µg/mL; 30 min) and non-polar fractions from sea buckthorn twigs and leaves (B and D) (1–50 µg/mL; 30 min) on collagen adhesion of resting and thrombin-activated platelets. Blood platelets not treated with plant fractions were used as control samples (positive control). Adhesion is expressed as a percentage of the control samples (100 %). Data represent mean ± SE of 9 healthy volunteers (experiments performed in triplicate). Action of the twig or leaf fractions was compared to control: *$p < 0.05$, **$p < 0.02$.

2.2. Plant material

Whole branches of sea buckthorn were obtained during the harvest time (August 2015) from a horticultural farm in Sokółka, Poland (53°24'N, 23°30'E), the biggest producer of sea buckthorn in Poland. The studied fractions from various sea buckthorn organs have been prepared at the Institute of Soil Science and Plant Cultivation – Sate Research Institute, Puławy, Poland (IUNG/HRH/2015/2). The leaves and fruit were hand-picked at the laboratory. The leaves were subsequently freeze-dried and milled in a laboratory mill (Retsch ZM200, Haan, Germany); twigs were air-dried at 40 °C and subsequently powdered, using a pair of laboratory mills (Retsch SM300, ZM200). The milled plant material was stored in a freezer.

2.3. Chemical characteristics of the phenolic fraction and non-polar fraction from sea buckthorn twigs and leaves

The tested fractions from the leaves and twigs of sea buckthorn were prepared and analyzed as described previously [22]. Briefly, the leaves (284 g) were extracted with 5 L (in three portions) of 80 % methanol (v/v), at room temperature (48 h), with three cycles (10 min) of ultrasonication (3 × 10 min). The ground twigs (680 g) were extracted with

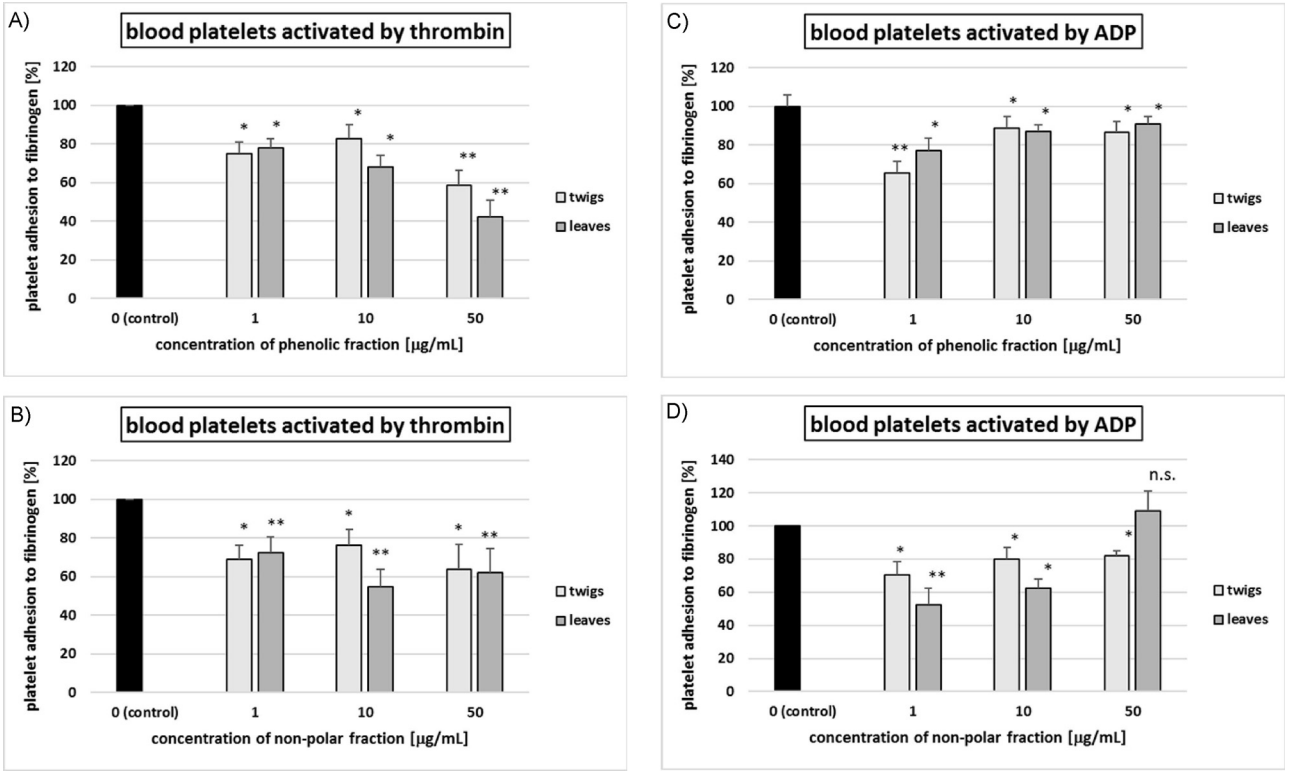

Fig. 2. Effects of phenolic fractions from sea buckthorn twigs and leaves (A and C) (1–50 µg/mL; 30 min) and non-polar fractions from sea buckthorn twigs and leaves (B and D) (1–50 µg/mL; 30 min) on fibrinogen adhesion of thrombin/ADP-activated platelets. Blood platelets not treated with plant fraction were used as control samples (positive control). Adhesion is expressed as a percentage of the control samples (100 %). Data represent mean ± SE of 9 healthy volunteers (experiments performed in triplicate). Action of the twig or leaf fractions was compared to control: *$p < 0.05$, **$p < 0.02$.

14 L of 80 % methanol (in three portions), as described above. Filtered extracts were concentrated by rotary evaporation (40 °C) and defatted with hexane. Organic solvents were removed in a rotary evaporator, the residue was subsequently resuspended in Milli-Q water and extracted with butanol. The butanol extracts were rotary evaporated to remove the solvent, the residue was suspended in water and 20 % *tert*-butanol, and lyophilized. The procedures yielded 12.42 *g* of the dry leaf extract and 24.64 *g* of the twig extract. A 12 g portion of the leaf extract was suspended in 600 ml of 50 % methanol, shaken, ultrasonicated for 2 min and centrifuged. The supernatant, containing mainly phenolic compounds, was dried in a rotary evaporator, dissolved in 20 % *tert*-butanol and lyophilized, to yield 11.37 g of the phenolic fraction. The dried pellet was dissolved in a mixture of *tert*-butanol and water and lyophilized (0.63 g). The twig extract was fractionated in the same way (14 g was mixed with 700 ml of 50 % methanol), and it yielded 13.07 *g* of the phenolic fraction and 0.83 g the nonpolar fraction. The chemical composition of the tested fractions was determined by reverse phase UHPLC-MS/MS, using a Thermo Ultimate 3000RS (Thermo Fischer Scientific, MS, USA) UHPLC system, equipped with a charged aerosol detector, and a diode array detector. The system was hyphenated with a Bruker Impact II Q-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Chromatographic separations were carried out on an ACQUITY BEH C18 column (2.1 × 150 mm, 1.7 µm; Waters, MA, USA). The chemical composition of the fractions was identified based on their MS and UV spectra, as well as literature data. The relative content of individual compounds was measured on the basis of CAD chromatograms and expressed as a percentage of the total peak area.

Plant fractions used in *in vitro* experiments were dissolved in 50 % DMSO. The final concentration of DMSO in the test samples was 0.05 % and its effects were determined in all experiments.

2.4. Blood platelet isolation

Fresh human blood was collected from healthy volunteers (median age = 28, non-smokers and non-drugs or supplements with anti-platelet and antioxidant activities). Blood was collected on the CPDA1 anticoagulant (citrate/phosphate/dextrose/adenine; 8.5:1; v/v; blood/CPDA). The blood was centrifuged (1200 rpm, 15 min, 25 °C), and the platelet rich plasma (PRP) was centrifuged again (1200 rpm, 15 min, 25 °C) to obtain platelets, which were then resuspended in Barber's buffer. Platelet titer was determined spectrophotometrically at wavelength (λ) of 800 nm, and then adjusted to a final concentration of 2×10^8 cells/mL [24]. Platelet-rich plasma or blood platelets concealed in Barber's buffer were incubated (30 min, 37 °C) with the tested fractions from the sea buckthorn organs in final concentrations in the samples: 1.0; 10; 50 µg/mL.

The study was performed with the consent of the bioethical commission of the University of Lodz (3/KBBN-UŁ/II/2016).

2.5. Platelet aggregation

Platelet aggregation was measured by turbidimetry in platelet-rich plasma, or in a platelet suspension in Barber's buffer using a Chrono-Log (Whole Blood Lumi-Aggregometer (Chrono-Log, Havertown, Pa)) [25].

Test samples were prepared: 594 µl of PRP and 6 µl of fraction or 594 µl of platelets suspended in Barber's buffer and 6 µl of fraction. At the same time, a control sample was prepared without the tested fraction.

After incubation, 5 µl ADP (final concentration 10 µM) or 5 µl collagen (final concentration 2 µg/mL) was added to the test samples (PRP and fraction). The level of aggregation was measured for 10 min. The aggregometer was calibrated against the platelet-poor plasma (100 %

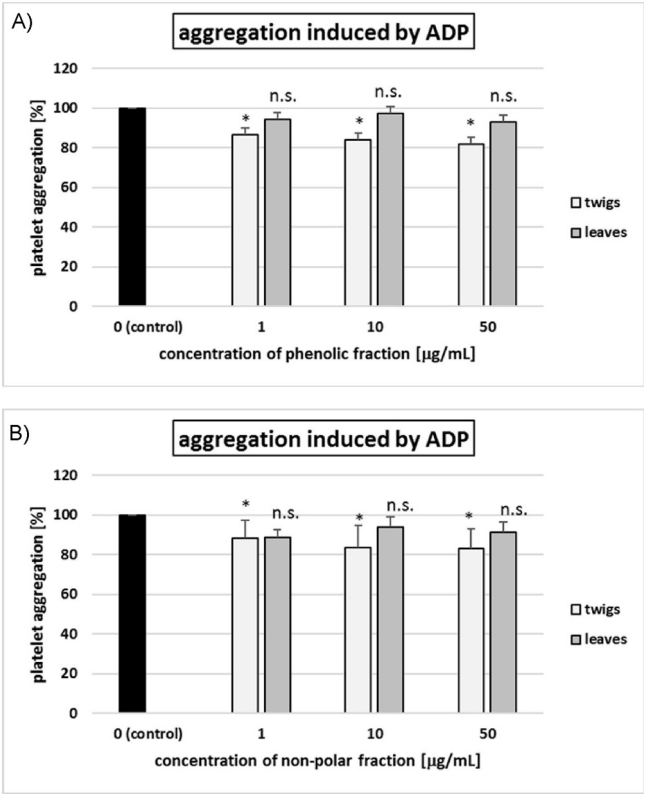

Fig. 3. Effects of phenolic fractions from sea buckthorn twigs and leaves (A) (1–50 µg/mL; 30 min) and non-polar fractions from sea buckthorn twigs and leaves (B) (1–50 µg/mL; 30 min) on blood platelet aggregation stimulated by 10 µM ADP. Data represent mean ± SE of 8 healthy volunteers. In this experiment, PRP not treated with plant fraction was used as control samples (positive control); the rate of agonist-induced aggregation for the control sample was regarded as 100 %. Action of the twig or leaf fractions was compared to control: *$p < 0.05$.

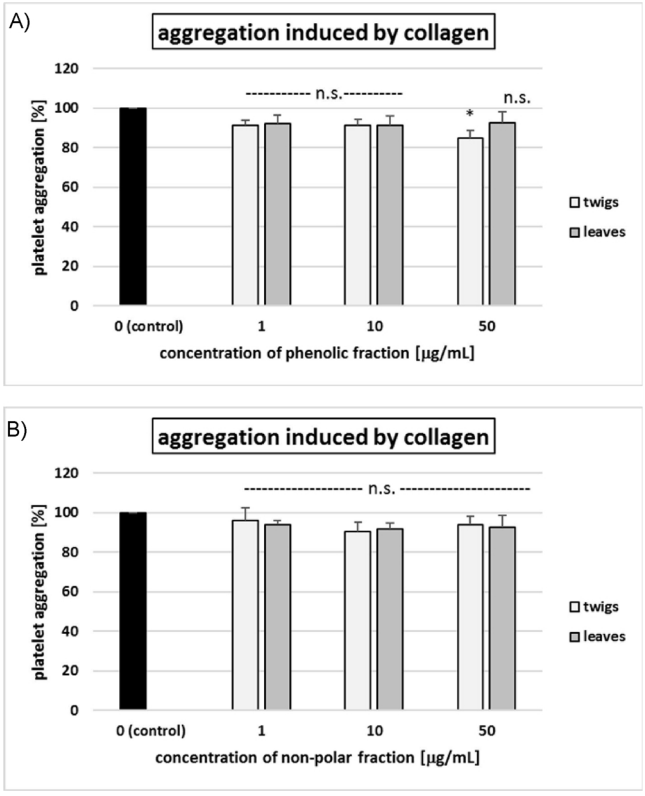

Fig. 4. Effects of phenolic fractions from sea buckthorn twigs and leaves (A) (1–50 µg/mL; 30 min) and non-polar fractions from sea buckthorn twigs and leaves (B) (1–50 µg/mL; 30 min) on blood platelet aggregation stimulated by 2 µg/mL collagen. Data represent mean ± SE of 6 healthy volunteers. PRP not treated with plant fraction was used as control samples (positive control), and the rate of agonist-induced aggregation for the control sample was assumed to be 100 %. Action of the twig or leaf fractions was compared to control: *$p < 0.05$.

aggregation).

After incubation, 5 µl thrombin (final concentration 1 Unit/mL) was added to the test samples (platelets suspended in Barber's buffer and fraction). The level of aggregation was measured for 10 min using an aggregometer calibrated against Barber's buffer (100 % aggregation). The method is described in more detail by Skalski et al. [26].

2.6. Platelet adhesion

The test determined the activity of platelet acid phosphatase by measuring the formation of p-nitrophenols at wavelength $\lambda = 405$ using SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). 96-well plates were coated with adhesive proteins (100 µl fibrinogen at final concentration 2 mg/mLand collagen at final concentration 0.04 mg/mL). The plate was incubated at 4 °C for 24 h. After incubation, non-absorbed proteins were removed from the plate. Plate wells were washed 250 µl of Tris-buffered saline (TBS) three times. 200 µl of 1 % albumin (BSA solution) was applied to the plate and the plate was incubated at 37 °C for 2 h. After incubation, BSA was removed and then the plate was washed with TBS buffer with ions (0.1 mM $CaCl_2$; 0.1 mM $MgCl_2$). Prepared test samples (720 µl of platelets suspended in Barber's buffer; 8 µl of tested extracts) and 50 µl of thrombin (final concentration of 0.2 U/mL) or ADP (final concentration 30 µM) were added to the plate. The plate was incubated at 37 °C for 1 h. After incubation, unadherved platelets were removed from the wells and the wells were washed three times with phosphate buffered saline (PBS) (250 µl per well). The citrate buffer (0,1 % Triton and 5 mM p-nitrophenyl phosphate) was added to the wells, and the plate was incubated at room temperature for 1 h. 100 µl of 2 M NaOH was added to each well. The results are expressed as a percentage (control without tested fraction - 100 %) [27].

2.7. LDH activity measurement

The prepared samples (blood platelets concealed in Barber's buffer with tested fractions) were centrifuged (2500 rpm, 15 min, 25C). A 96-well plate was loaded with 270 µl of 0.1 M phosphate buffer pH 7,4 (0,69 g NaH_2PO_4 x H_2O + 25 ml H_2O (1); 7,16 g NaH_2PO_4 x 12 H_2O + 100 ml H_2O (2); 19 ml (1) +81 ml (II) filled with water to 200 ml), followed by 10 µl of the supernatant and 10 µl of NADH solution (5 mg NADH +2 ml 0,1 M phosphate buffer pH 7,4) respectively. The plate was incubated at room temperature for 20 min and 10 µl of pyruvate solution (5 mg puryvate +2 ml 0,1 M phosphate buffer pH 7,4) was added. The reaction rate of the formation of NAD^+ and lactate was measured at a wavelength (λ) of 340 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). The absorbance readings were repeated every minute for 10 min [28].

2.8. Lipid peroxidation measurement

Lipid peroxidation was determined by measuring the colored product resulting from the condensation of one molecule of malondialdehyde (MDA) with two molecules of thiobarbituric acid (TBA). Two batches of samples were prepared (resting platelets and platelets stimulated with thrombin at a final concentration of 10 Unit/mL), and 15 % trichloroacetic acid (TCA) and 0.37 % TBA were added. The

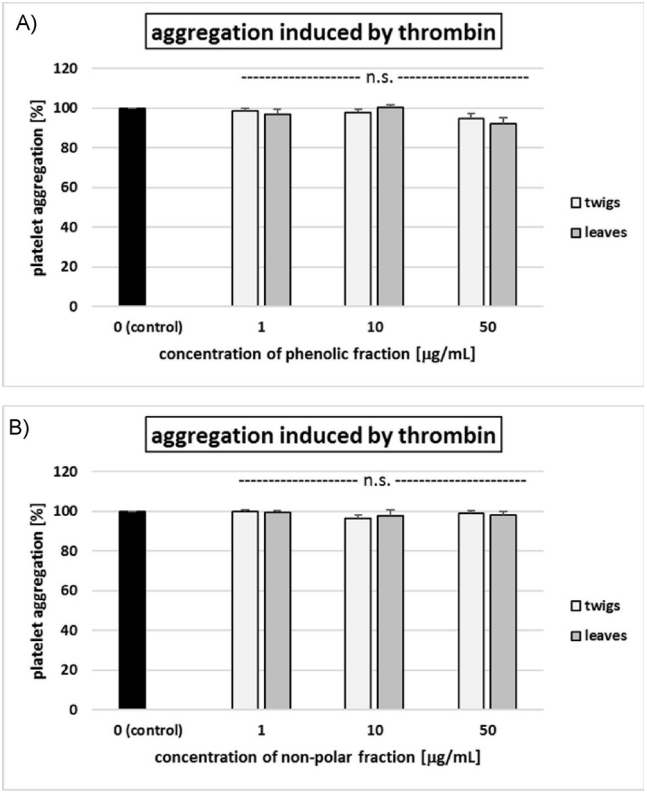

Fig. 5. Effects of phenolic fractions from sea buckthorn twigs and leaves (A) (1–50 µg/mL; 30 min) and non-polar fractions from sea buckthorn twigs and leaves (B) (1–50 µg/mL; 30 min) on blood platelet aggregation stimulated by 1 Unit/mL thrombin. Data represent mean ± SE of 6 healthy volunteers. Washed blood platelets not treated with plant fraction were used as control samples (positive control), and the rate of agonist-induced aggregation for the control sample was assumed to be 100 %. No concentration of any tested fraction (1, 10 and 50 µg/mL) had a statistically significant effect on thrombin-stimulated aggregation (action of the twig or leaf fractions was compared to control: $p > 0.05$ (n.s.)).

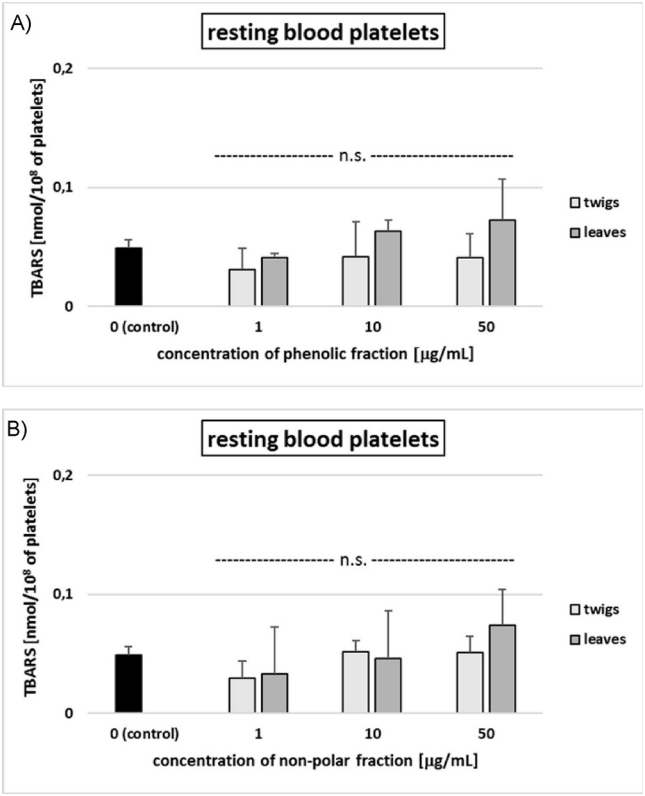

Fig. 6. Effects of phenolic (A) and non-polar fractions (B) from twigs and leaves (1–50 µg/mL; 30 min) on lipid peroxidation in resting platelets. In these experiments, blood platelets not treated with plant fraction were used as control sample. Data represent mean ± SE of 6 healthy volunteers (experiments done in triplicate). No concentration of any of the tested fractions (1, 10 and 50 µg/mL) had a statistically significant effect on this process (compared to control -$p > 0.05$ (n.s.)).

samples were vented and boiled for 10 min; they were then cooled at 4 °C and centrifuged (1000 rpm, 15 min, 18 °C). The absorbance of the supernatant was measured at a wavelength (λ) of 535 nm. The TBARS concentration was calculated using the molar extinction coefficient ($\varepsilon = 156{,}000\ M^{-1}\,cm^{-1}$). [29,30].

2.9. Superoxide anion measurement

The superoxide anion created in blood platelets stimulated by thrombin (10 Unit/mL) reacts with ferricytochrome c introduced into the mixture to produce ferrocytochrome c. The level of ferrocytochrome c can be determined by the difference in the absorbance spectrum compared to ferricytochrome c. Briefly, the samples were treated with 300 µl of cytochrome c and centrifuged (1500 rpm/5 min/25 °C). The absorbance of the obtained supernatant was then measured at a wavelength $\lambda = 550$ nm. The amount of superoxide anion was calculated using using the molar extinction coefficient ($\varepsilon = 18{,}700\ M^{-1}\,cm^{-1}$) [31].

2.10. Data analysis

To eliminate uncertain data, the Q-Dixon test was performed. All the values in this study were expressed as mean ± SE; n – number of blood donors. Statistical analysis was performed with one-way ANOVA for repeated measurements.

3. Results

The main constituents of the phenolic fraction of sea buckthorn leaves were hydrolysable tannins (31.3 % of the total peak area), non-acylated and acylated flavonol glycosides (24.5 % of the total peak area), and triterpenoid saponins (15 % of the total peak area), while the non-polar fraction contained mainly triterpenoid saponins (30.5 % of the total peak area), as well as triterpenoids and acylated triterpenoids (50.7 % of the total peak area). B-type proanthocyanidins and catechin (54.3 % of the total peak area) were dominant components of the phenolic fraction of sea buckthorn twigs. The non-polar fraction of sea buckthorn twigs consisted mainly of triterpenoids and acylated triterpenoids (89 % of the total peak area). Tables 1–4 present the composition of the phenolic and non-polar fraction of sea buckthorn leaves and sea buckthorn twigs, with the latest update. A more detailed description and discussion of results can be found in the work of Skalski et al. [22].

The study examined the effect of the two types of fraction from the twigs and leaves (concentration range 1 − 50 µg/mL) on the selected steps of blood platelet activation, *viz.* platelet adhesion to collagen and fibrinogen and platelet aggregation, as well as selected biochemical processes, *viz.* non-enzymatic lipid peroxidation, enzymatic lipid peroxidation and $O_2^{-\cdot}$ production, *in vitro*.

The adhesion of resting blood platelets to collagen was significantly inhibited by incubation with the phenolic fractions from the sea buckthorn twigs (1 µg/mL), and the leaves (1 and 10 µg/mL) (Fig. 1A); it was also inhibited by the non-polar leaf fractions at all tested concentrations (1, 10 and 50 µg/mL) and the twig fractions at 1 and 10 µg/mL (Fig. 1B). In contrast, for the thrombin-activated platelets, the

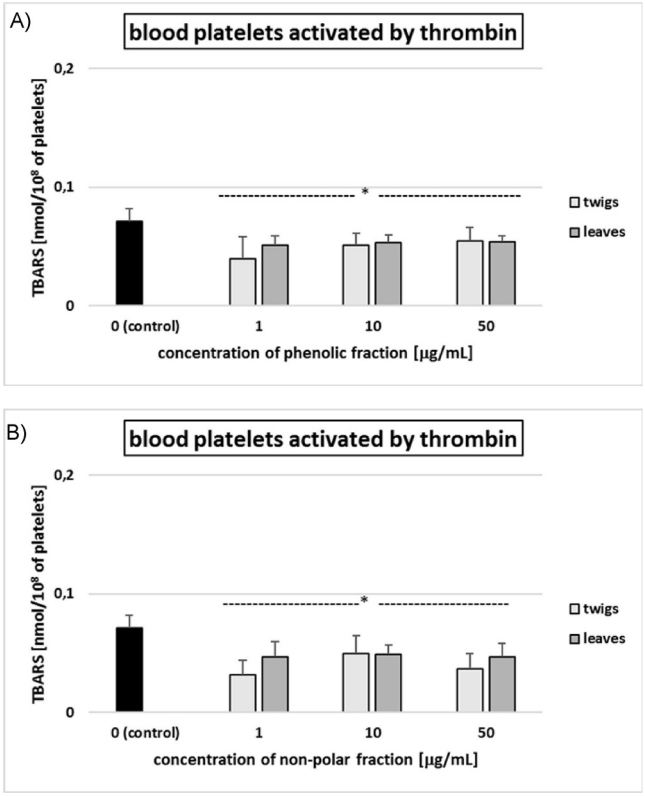

Fig. 7. Effects of phenolic (A) and non-polar fractions (B) from twigs and leaves (1–50 µg/mL; 30 min) on lipid peroxidation in platelets activated by thrombin. In these experiments, blood platelets not treated with plant fraction were used as control sample. Data represent mean ± SE of 6 healthy volunteers (experiments done in triplicate). All three concentrations of the twig and leaf fractions (1, 10 and 50 µg/mL) had a statistically significant effect compared to control (*$p < 0.05$).

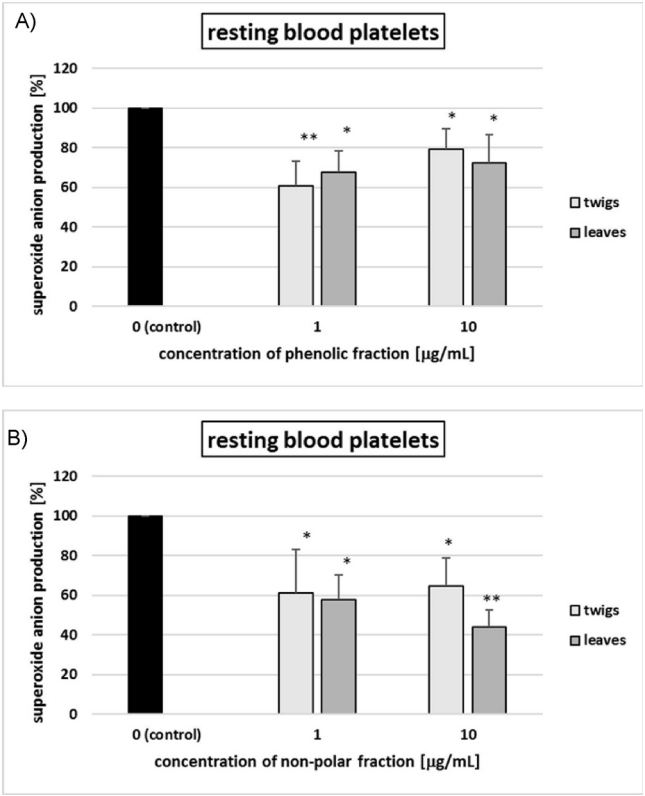

Fig. 8. Effects of phenolic (A) and non-polar fractions (B) from twigs and leaves (1 and 10 µg/mL; 30 min) on $O_2^{-\cdot}$ production in resting platelets. Data represent mean ± SE of 8 healthy volunteers (experiments performed in triplicate). In these experiments, the $O_2^{-\cdot}$ level in control (blood platelets not treated with plant fraction) was 0.457 ± 0.256 nmol/10^8 resting platelets. In the graphs, $O_2^{-\cdot}$ production is expressed as a percentage of control (100 %). The two concentrations of twig or leaf fractions (1 and 10 µg/mL) gave significantly different results to control (*$p < 0.05$; ** $p < 0.02$).

phenolic twig fraction inhibited binding to collagen at all tested concentrations (1, 10 and 50 µg/mL) (Fig. 1C) while the phenolic leaf fraction did so at only 1 and 50 µg/mL (Fig. 1C). For example, at the highest tested concentration (50 µg/mL), the sea buckthorn twig phenolic fraction demonstrated greater inhibition of thrombin-activated blood platelets to collagen than leaf phenolic fraction (Fig. 1C). Interestingly, all non-polar fractions from both twigs and leaves inhibited binding at all tested concentrations (Fig. 1D). The twig and leaf non-polar fractions (50 µg/mL) demonstrated about 50 % inhibition of adhesion of thrombin-activated platelets to collagen (Fig. 1D).

In addition, the adhesion of thrombin or ADP-activated platelets to collagen was significantly inhibited by all used fractions (Fig. 2A, B, C and D). Only the non-polar leaf fraction, administered at the highest concentration (50 µg/mL) had no statistically significant effect (Fig. 2D).

While both the phenolic and non-polar fractions of the sea buckthorn twigs inhibited ADP-stimulated aggregation (Fig. 3A and B). For example, the percentage inhibition of ADP-stimulated blood platelet aggregation was about 20 % for the phenolic fraction from twigs at a concentration of 50 µg/mL (Fig. 3A). In addition, they generally had no effect on aggregation induced by collagen or thrombin (Figs. 4 AB and 5 AB). Only the phenolic fraction from leaves at the highest test concentration (50 µg/mL) significantly inhibited collagen-stimulated aggregation (by about 20 %) (Fig. 4A).

Although none of the fractions were found to change the level of TBARS in resting blood platelets (Fig. 6A and B), all were observed to significantly inhibit enzymatic lipid peroxidation in thrombin-activated platelets at all tested concentrations (1–50 µg/mL) (Fig. 7A and B). At the highest-used concentration of the phenolic fractions form leaves and twigs (50 µg/mL) inhibition of lipid peroxidation stimulated by thrombin was found to be about 20 % (Fig. 7A).

All used plant fractions (1 and 10 µg/mL) significantly reduced the amount of $O_2^{-\cdot}$ in resting platelets and thrombin-activated platelets (Fig. 8 and 9); however, none induced lysis of blood platelets (Fig. 10AB).

Table 5 compares the effects of phenolic and non-polar fractions (at a tested concentration of 10 µg/mL) from the leaves and twigs on platelet adhesion, platelet aggregation, enzymatic lipid peroxidation and O_2^{-} production *in vitro*. Of the four tested fractions, the non-polar twig fraction displayed the strongest anti-platelet properties; in addition, it was only found to be ineffective on platelet aggregation stimulated by collagen and thrombin.

4. Discussion

Due to its medicinal activity and the high nutritional value of its fruits, the last few decades have seen a growth of interest in the use of sea buckthorn. However, as well as its fruits, its other organs are commonly used in traditional medicine, especially in China [15,16]. Various preparations from sea buckthorn, including phenolic extracts and fractions, have been demonstrated to have antimicrobial, antiviral, antioxidant and anticoagulant activities [13,14,22].

Various cardiovascular diseases are characterized by the presence of activated blood platelets in the circulatory system, and platelet hyperactivation and their hyperaggregation are known to be significant risk factors [32]. Although various cardiovascular disorders caused by platelet hyperactivation can be prevented and treated with the use of

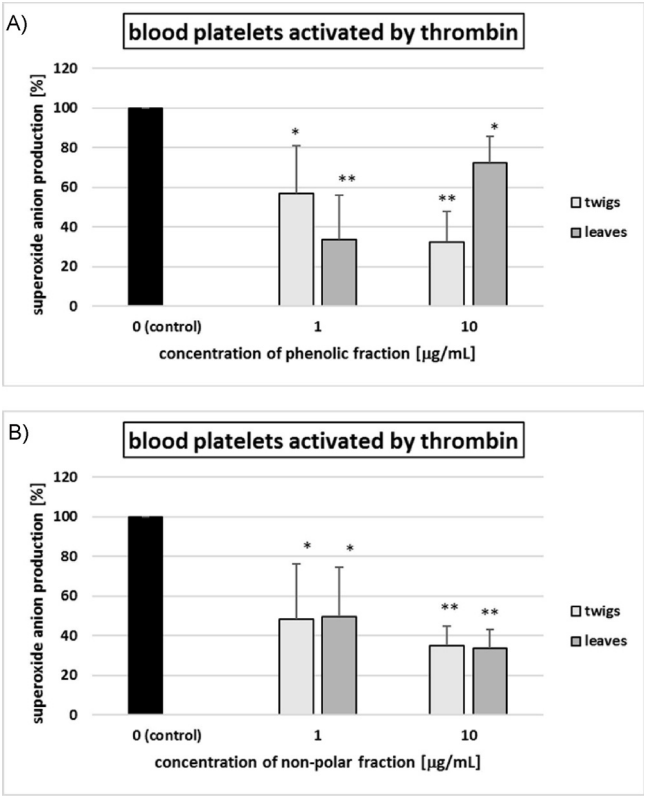

Fig. 9. Effects of phenolic (A) and non-polar fractions (B) from twigs and leaves (1 and 10 µg/mL; 30 min) on $O_2^{-\cdot}$ production in platelets activated by thrombin. Data represent mean ± SE of 8 healthy volunteers (experiments performed in triplicate). In these experiments, the $O_2^{-\cdot}$ level in positive control samples (blood platelets not treated with plant fraction) was 0.898 ± 0.311nmol/10^8 platelets activated by thrombin. In the graphs, the $O_2^{-\cdot}$ production is expressed as a percentage of control values (100 %). The twig and leaf fractions gave significantly different results to control at concentrations of 1 and 10 µg/mL; * $p < 0.05$; ** $p < 0.02$).

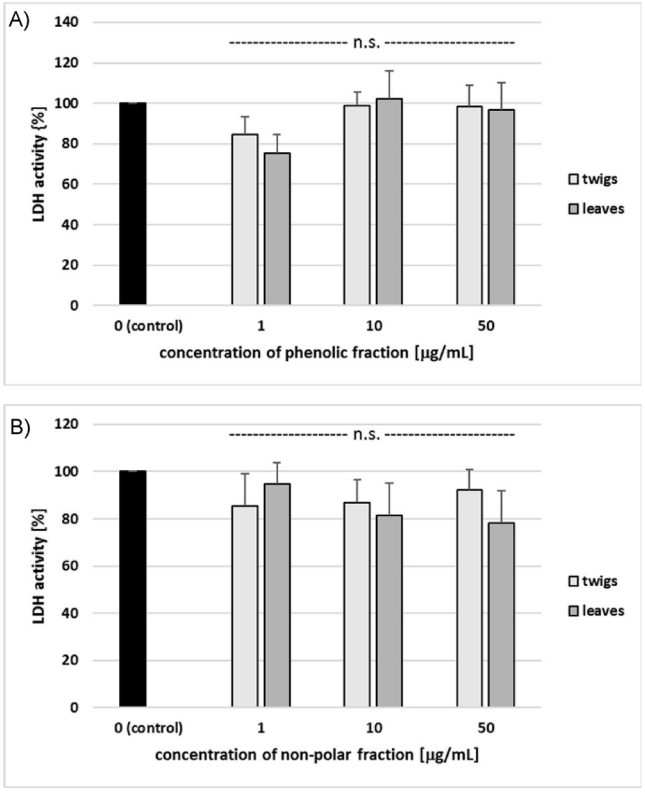

Fig. 10. The toxic effects of twig and leaf extract (0.5–50 µg/mL; 30 min) against human blood platelets. In these experiments, blood platelets not treated with plant extract were used as control sample. Data represent mean ± SE of 5–6 healthy volunteers (experiments performed in triplicate). In the graphs, the LDH activity is expressed as a percentage of the control sample (100 %). None of the five different concentrations of the tested fraction (0.5, 1, 5, 10 and 50 µg/mL) had any statistically significant effect compared to controls ($p > 0.05$ (n.s.)).

medications inhibiting blood platelet activation, little is known of the effects of sea buckthorn leaves and twigs, and their constituent compounds, on the activation and biochemical processes of blood platelets. Nevertheless, previous LC–MS analyses have revealed significant differences in chemical composition between phenolic and non-polar fractions obtained from sea buckthorn leaves and twigs [22]. These fractions have also been found to possess different biological properties, such as antioxidant and anticoagulant activities, *in vitro*; for example, both the phenolic and non-polar fractions from twigs reduced human plasma protein carbonylation induced by H_2O_2/Fe: a hydroxyl radical donor [22].

None of the four types of sea buckthorn fractions tested in the present study were found to cause damage to the blood platelets, determined as leakage of lactate dehydrogenase into the extracellular medium, throughout the tested concentration range (1 − 50 µg/mL), and we can confirm that sea buckthorn leaves and twigs are safe for use in supplements. Of these four fractions, the phenolic leaf fraction was rich in hydrolysable tannins while the phenolic twig fraction was rich in proanthocyanidins and catechin; both the polar and non-polar leaf fractions were rich in triterpenoids and their derivatives. In addition, the concentrations of the phenolic fractions from sea buckthorn leaves and twigs used in the study may be achievable in blood during their oral supplementation [33–35].

Following induction by thrombin and other agonists, platelet activation is associated with arachidonic acid metabolism and the synthesis of eicosanoids such as thromboxane A_2; it also plays a role in enzymatic lipid peroxidation and the generation of ROS, which may act as secondary messengers; however, non-enzymatic lipid peroxidation and ROS production is also observed in resting blood platelets. Our present findings confirm that sea buckthorn leaves and twigs and their secondary metabolites demonstrate anti-oxidative potential in an *in vitro* human blood platelet model: each of the four tested fractions inhibited the production of superoxide anions, as measured by cytochrome c reduction (Figs. 8 and 9, Table 5). However, none of the tested fractions (from leaves and twigs) inhibited lipid non-enzymatic peroxidation in resting blood platelets. In addition, all tested plant fractions reduced enzymatic lipid peroxidation in blood platelets stimulated by thrombin (Figs. 6 and 7, Table 5). This may suggest that the constituents of the phenolic and non-polar fractions are able to modulate platelet activity by interfering with the metabolism of arachidonic acid, and that they may influence platelet reactivity by modifying ROS level and modulating the expression of platelet receptors.

The phenolic fractions and the non-polar fractions in the present study displayed different antioxidant properties towards resting platelets and thrombin-activated platelets. For example, at a dose of 10 g/mL, the non-polar leaf fraction, rich in triterpenes and their derivatives, exerted stronger inhibitory action on O_2^- production (about 60 % inhibition of O_2^- production) in resting platelets than the phenolic leaf fraction, which is rich in hydrolysable tannins (about 15 % inhibition). Similar results were observed in blood platelets activated by thrombin.

These findings are in agreement with those obtained previously that found the non-polar fraction from sea buckthorn leaves to inhibit oxidative stress in plasma treated with H_2O_2/Fe to a greater degree than the phenolic fraction [22]. Similar results concerning the role of triterpenoids isolated from the branch bark of sea buckthorn and their

Table 5
Comparison of the effects of phenolic and non-polar fractions from sea buckthorn twigs and leaves (at a concentration of 10 μg/mL) on various steps of blood platelet activation and biochemical processes in blood platelets. Data represent mean ± SE. This inhibition of studied processes by the plant fractions is expressed as the percentage of that recorded for control platelets or PRP (without the plant fraction).

Experiment	Tested plant fraction at the level of 10 μg/mL			
	Phenolic fraction from leaves	Phenolic fraction from twigs	Non-polar fraction from leaves	Non-polar fraction from twigs
Adhesion of resting platelets to collagen (inhibition of this process (%))	42.1 ± 9.7 ($p < 0.05$)	5.5 ± 2.8 ($p > 0.05$)	12.3 ± 3.1 ($p < 0.05$)	21.9 ± 3.4 ($p < 0.05$)
Adhesion of thrombin-activated platelets to collagen (inhibition of this process (%))	6.2 ± 2.9 ($p > 0.05$)	38.9 ± 5.1 ($p < 0.05$)	46.2 ± 4.8 ($p < 0.05$)	40.4 ± 6.1 ($p < 0.05$)
Adhesion of thrombin-activated platelets to fibrinogen (inhibition of this process (%))	35.2 ± 7.7 ($p < 0.05$)	20.1 ± 3.5 ($p < 0.05$)	52.2 ± 7.1 ($p < 0.05$)	25.5 ± 4.9 ($p < 0.05$)
Adhesion of ADP-activated platelets to fibrinogen (inhibition of this process (%))	15.6 ± 3.2 ($p < 0.05$)	14.4 ± 3.5 ($p < 0.05$)	40.4 ± 8.1 ($p < 0.05$)	19.5 ± 2.2 ($p < 0.05$)
Aggregation of ADP-stimulated platelets (inhibition of this process (%))	3.3 ± 1.7 ($p > 0.05$)	19.2 ± 3.3 ($p < 0.05$)	4.9 ± 2.0 ($p > 0.05$)	17.4 ± 3.0 ($p < 0.05$)
Aggregation of collagen-stimulated platelets (inhibition of this process (%))	6.2 ± 2.5 ($p > 0.05$)	7.2 ± 3.2 ($p > 0.05$)	8.0 ± 3.7 ($p > 0.05$)	10.2 ± 4.2 ($p > 0.05$)
Aggregation of thrombin-stimulated platelets (inhibition of this process (%))	5.3 ± 3.5 ($p > 0.05$)	6.3 ± 3.1 ($p > 0.05$)	5.9 ± 4.1 ($p > 0.05$)	2.8 ± 2.0 ($p > 0.05$)
Lipid peroxidation in thrombin-activated platelets (inhibition of this process (%))	23.2 ± 5.1 ($p < 0.05$)	27.4 ± 5.5 ($p < 0.05$)	30.1 ± 4.9 ($p < 0.05$)	29.8 ± 3.8 ($p < 0.05$)
$O_2^{\cdot -}$ production in resting platelets (inhibition of this process (%))	35.2 ± 6.6 ($p < 0.05$)	42.7 ± 7.1 ($p < 0.05$)	44.8 ± 8.1 ($p < 0.05$)	46.7 ± 9.1 ($p < 0.05$)
$O_2^{\cdot -}$ production in thrombin-activated platelets (inhibition of this process (%))	70.2 ± 12.1 ($p < 0.05$)	50.4 ± 10.7 ($p < 0.05$)	74.9 ± 15.8 ($p < 0.05$)	75.5 ± 16.7 ($p < 0.05$)

antioxidant properties were obtained by Yang et al. [36], who report that the antioxidant potential of tritrepenoids may be determined by the presence of a hydroxylated aromatic ring, the number of hydroxyl groups bound to it, and position of these groups.

Our present results are also the first to indicate that sea buckthorn leaf and twig fractions are rich in various secondary metabolites with anti-platelet properties. An interesting finding is that both the phenolic and non-polar leaf and twig extracts display anti-adhesive activity in an experimental system of isolated washed human blood platelets in the presence of two adhesive proteins: collagen type I and fibrinogen. However, the tested fractions differed in their anti-adhesive potential; for example, while the non-polar leaf and twig fractions inhibited blood platelet adhesion in all used models (Table 5), the phenolic leaf and twig fractions reduced platelet adhesion in all but one, with this model depending on the type of fraction. In addition, the non-polar fractions also displayed stronger anti-adhesive properties than the phenolic fractions (Figs. 1 and 2).

This is also the first study to examine the anti-aggregative action of tested plant fractions in two sets of platelet sample treated with their agonists: ADP and collagen in platelet-rich plasma, and thrombin in washed blood platelets. In contrast to the anti-adhesive action, only the two twig fractions caused significant inhibition of platelet aggregation stimulated by ADP. This anti-aggregative action may be enabled by an interaction between their constituent compounds with ADP receptors on the platelet membrane.

A novel finding of our study is that all the tested fractions isolated from twigs and leaves have anti-platelet properties. Various studies have found that procyanidins and anthocyanidins have beneficial effects on the cardiovascular system, and that these benefits are derived through inhibition of platelet activation [4,5,37]. However, the mechanism behind the antiplatelet activity of triterpenoids and their derivatives is not fully understood and requires further research. A recent *in vitro* study based on turbidimetry found diterpene esters from green Arabica coffee beans to inhibit platelet aggregation at a concentration of 3×10^{-4} g/mL [38].

Our present findings confirm those of our previous studies, which indicate that compounds extracted from the various organs of sea buckthorn, *viz.* the fruits, leaves and twigs, exhibit anti-platelet properties. We suppose that anti-platelet potential of tested fractions may be associated with modulation of the metabolism of arachidonic acid, changes in ROS concentration and the expression of blood platelets receptors. In addition, these compounds are potentially valuable objects for future studies aimed at developing new drugs or food supplements intended for treating diseases related to cardiovascular disease and other disorders associated with platelet hyperactivation.

Declaration of Competing Interest

None to declare.

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Anti-Platelet Properties of Phenolic and Nonpolar Fractions Isolated from Various Organs of *Elaeagnus rhamnoides* (L.) A. Nelson in Whole Blood

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Abstract: Sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) is a shrub growing in coastal areas. Its organs contain a range of bioactive substances including vitamins, fatty acids, various micro and macro elements, as well as phenolic compounds. Numerous studies of sea buckthorn have found it to have anticancer, anti-ulcer, hepatoprotective, antibacterial, and antiviral properties. Some studies suggest that it also affects the hemostasis system. The aim of the study was to determine the effect of six polyphenols rich and triterpenic acids rich fractions (A–F), taken from various organs of sea buckthorn, on the activation of blood platelets using whole blood, and to assess the effect of the tested fractions on platelet proteins: fraction A (polyphenols rich fraction from fruits), fraction B (triterpenic acids rich fraction from fruits), fraction C (polyphenols rich fraction from leaves), fraction D (triterpenic acids rich fraction from leaves), fraction E (polyphenols rich fraction from twigs), and fraction F (triterpenic acids rich fraction from twigs). Hemostasis parameters were determined using flow cytometry and T-TAS (Total Thrombus-formation Analysis System). Additionally, electrophoresis was performed under reducing and non-reducing conditions. Although all tested fractions inhibit platelet activation, the greatest anti-platelet activity was demonstrated by fraction A, which was rich in flavonol glycosides. In addition, none of the tested fractions (A–F) caused any changes in the platelet proteome, and their anti-platelet potential is not dependent on the P2Y12 receptor.

Keywords: sea buckthorn; flow cytometry; T-TAS; haemostasis; electrophoresis

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1. Introduction

Anticoagulant and antiplatelet drugs play an important role in the prevention and treatment of cardiovascular thrombotic events caused by various mechanisms. However, these drugs may also induce various side effects, which are well described [1]. In addition, certain dietary components, including phenolic compounds and supplements with antiplatelet properties may also reduce platelet activation, and may have a significant influence on the prophylaxis and treatment of cardiovascular diseases (CVDs) [2–4]. Some of the most promising sources of active compounds for the prevention and treatment of CVDs may be the organs and fruits of sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) [5–7].

The medicinal values of sea buckthorn organs, especially the fruits, have been confirmed both by traditional medicine and by scientific reports [4,8]. Our previous in vitro

findings, based on the analysis of washed blood platelets and platelet-rich plasma, demonstrate that both polyphenols rich and triterpenic acids rich fractions from sea buckthorn leaves and twigs modulate the coagulation process in human plasma and modulate human platelet function [9,10]. In addition, our earlier results indicate that both types of fraction from sea buckthorn fruits possess anticoagulant and antioxidant properties [4]. However, their mechanisms of action in whole blood are not known. Therefore, the aim of the present in vitro study was to determine the anticoagulant and anti-platelet properties of selected polyphenols rich and triterpenic acids rich fractions isolated from different parts of *Elaeagnus rhamnoides* (L.) A. Nelson.

Six fractions were studied: fraction A—polyphenols rich fraction from fruits, rich in non-acylated and acylated flavonoids and nonpolar compounds; fraction B—triterpenic acids rich fraction from fruits; fraction C—polyphenols rich fraction from leaves; fraction D—triterpenic acids rich fraction from leaves; fraction E—polyphenols rich fraction from twigs; fraction F—triterpenic acids rich fraction from twigs. The analysis was performed in whole blood using flow cytometry and total thrombus-formation analysis system (T-TAS). The effect of the plant fractions on blood platelet function was determined by flow cytometry, i.e., by quantifying the cell-surface expression of the platelet activation markers P-selectin and GPIIb/IIIa, both in unstimulated platelets and in those treated with the agonists ADP (adenosine diphosphate) and collagen. Blood platelet function was also assessed based on vasodilator-stimulated phosphoprotein (VASP) phosphorylation in blood platelets. The aim of our study was also to identify changes in blood platelet proteomes following treatment with sea buckthorn fractions A–F.

2. Results

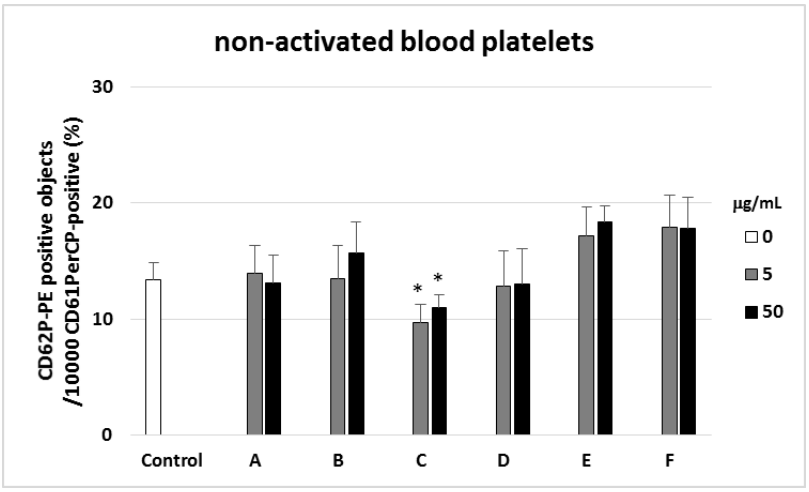
The results indicated altered blood platelet activation states in all samples treated with tested plant fractions (A–F) compared with platelets (without plant fractions); this was true for both samples treated with agonist (ADP or collagen) and resting platelets (Figures 1–3). However, these changes were not always statistically significant. Treatment with fraction C significantly reduced the expression of CD62P by about 30% for 5 μg/mL, and about 20% for 50 μg/mL in resting blood platelets (Figure 1A).

PAC-1 binding was found to be reduced by fractions A, B, and C, but increased by fraction F (Figure 2A). Moreover, fraction A was found to reduce PAC-1 binding in collagen-activated blood platelets when administered at both 5 μg/mL and 50 μg/mL, with a 30% reduction observed at 50 μg/mL (Figure 2D).

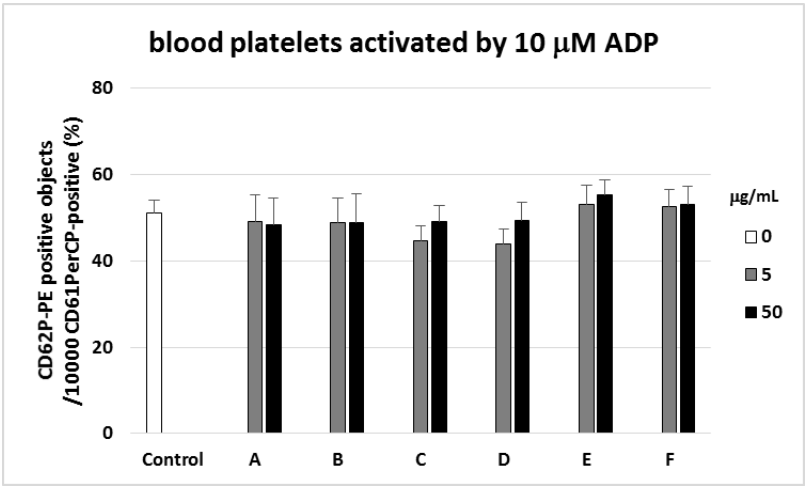
No differences were observed between the electropherograms of blood platelets treated with plant fractions (A–F) and the control samples (Figure 4). Nor were any differences observed between the PRI values of the samples treated with 50 μg/mL of the plant fractions (A–F) and the control samples (Figure 5).

Changes were also observed in the AUC_{10} measured by T-TAS (Figure 6). Four of the tested plant fractions (A, C, D, and E) markedly decreased AUC_{10} relative to control when administered at 50 μm/mL. However, no change was observed for the other two fractions (B and F) administered at the same concentration (Figure 6).

A



B



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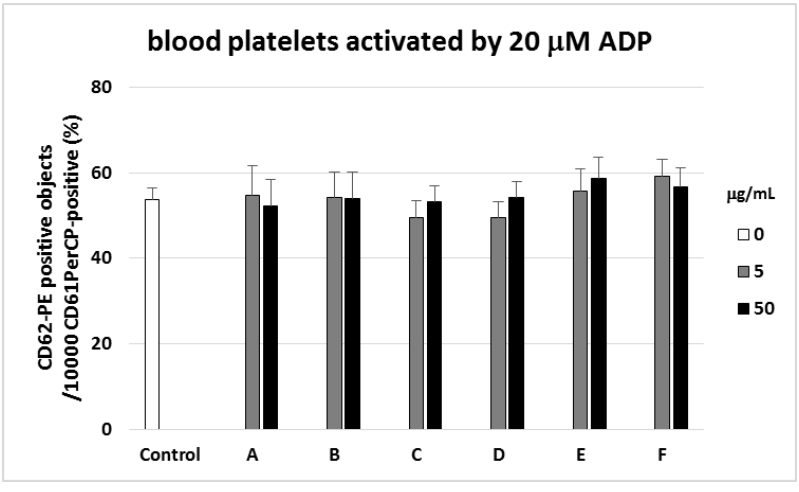


Figure 1. *Cont.*

D

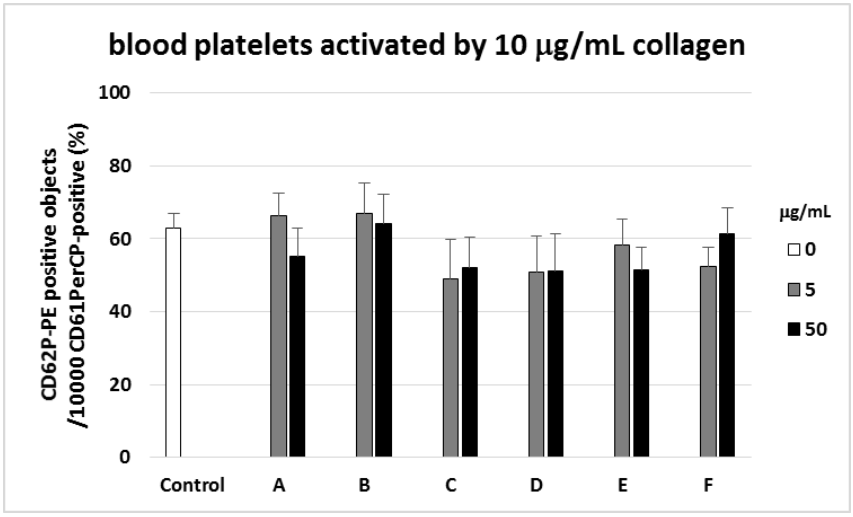
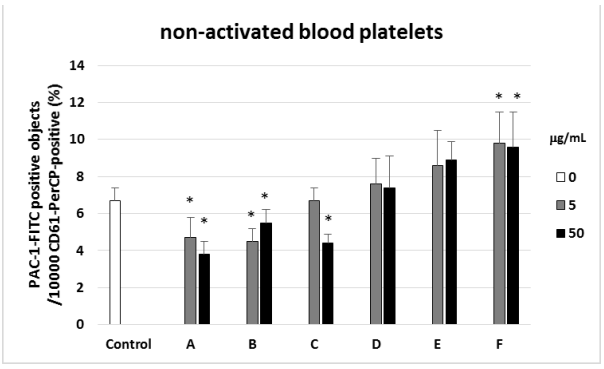


Figure 1. Effects of different plant fractions (5 and 50 μg/mL; 30 min) on expression of P-selectin on resting (**A**) or agonist-stimulated blood platelets: 10 μM ADP (adenosine diphosphate) (**B**), 20 μM ADP (**C**), and 10 μg/mL collagen (**D**) in whole blood samples. The blood platelets were distinguished based on the expression of CD61/PerCP. For each sample, 10,000 CD61-positive objects (blood platelets) were acquired. For the assessment of P-selectin expression, samples were labeled with fluorescently conjugated monoclonal antibody CD62P. Results are shown as the percentage of platelets expressing CD62P. Data represent mean ± SD of 6 healthy volunteers (each experiment performed in triplicate). * $p < 0.05$ (vs. control platelets–blood platelets without tested fraction).

A



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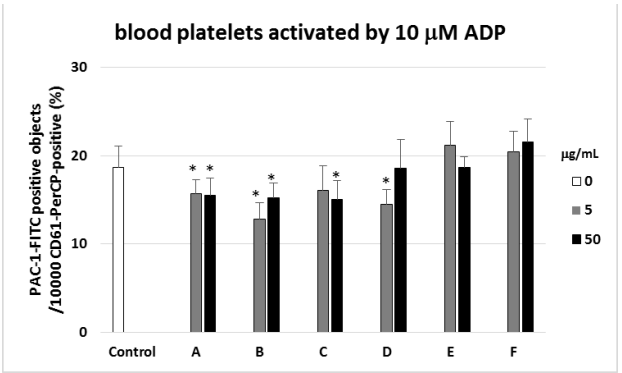
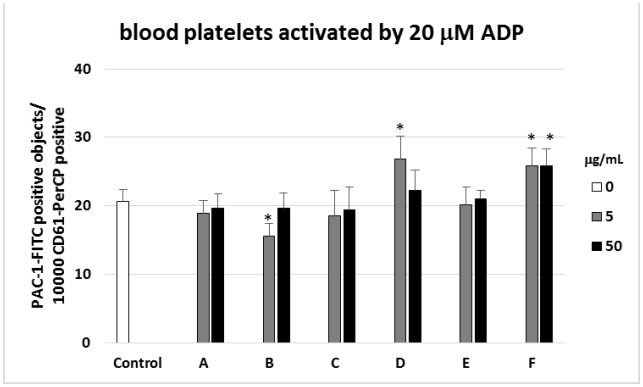


Figure 2. *Cont.*

C



D

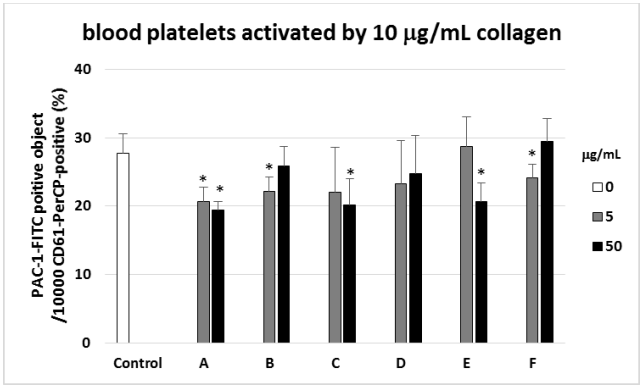


Figure 2. Effects of different plant fractions (5 and 50 μg/mL; 30 min) on expression of the active form of GPIIb/IIIa on resting (**A**) or agonist-stimulated blood platelets: 10 μM ADP (**B**), 20 μM ADP (**C**), and 10 μg/mL collagen (**D**) in whole blood samples. The blood platelets were distinguished based on the expression of CD61. For each sample, 10,000 CD61-positive objects (blood platelets) were acquired. For the assessment of GPIIb/IIIa expression, samples were labeled with fluorescently conjugated monoclonal antibody PAC-1/FITC. Results are shown as the percentage of platelets binding PAC-1/FITC. Data represent mean ± SD of 6 healthy volunteers (each experiment performed in triplicate). * $p < 0.05$ (vs. control platelets–blood platelets without tested fraction).

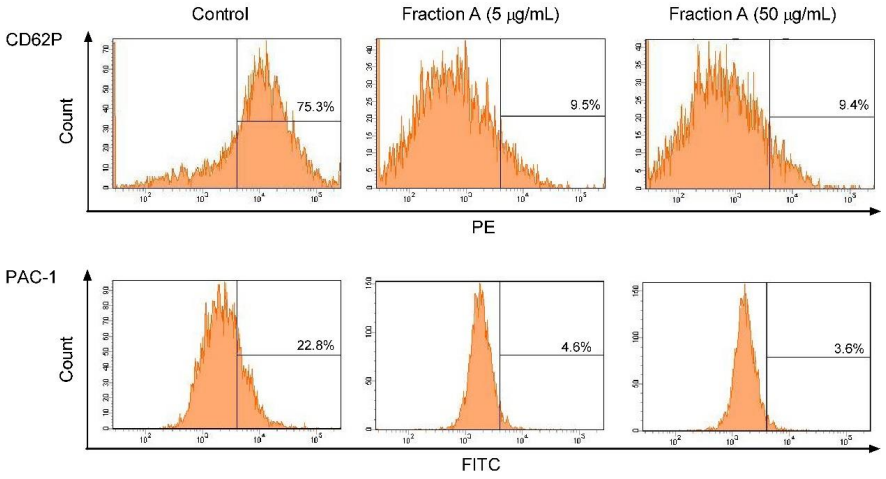


Figure 3. Effects of fraction A (concentration 5 and 50 μg/mL, incubation time—30 min) on the expression of P-selectin and the active form of GPIIb/IIIa on platelets stimulated by 10 μg/mL collagen in whole blood samples. Figure demonstrates selected diagrams.

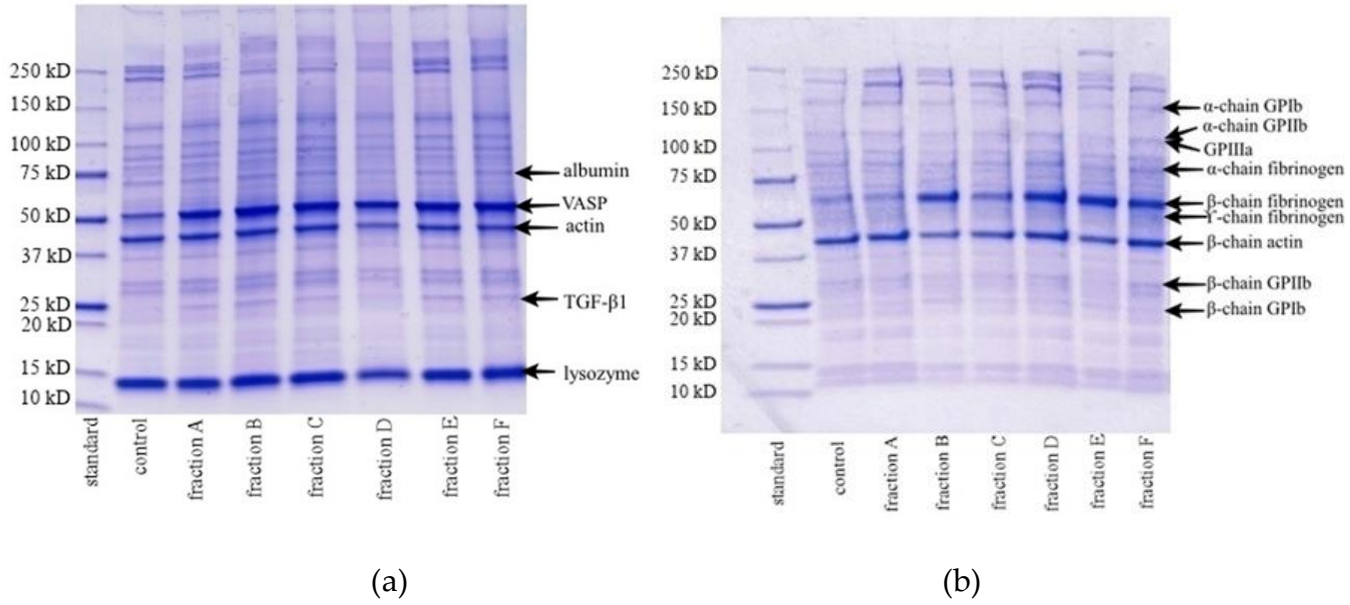


Figure 4. Electrophoretic patterns of blood platelet proteome in the presence of different plant fractions (50 μg/mL; 30 min): reducing conditions (**a**), non-reducing conditions (**b**).

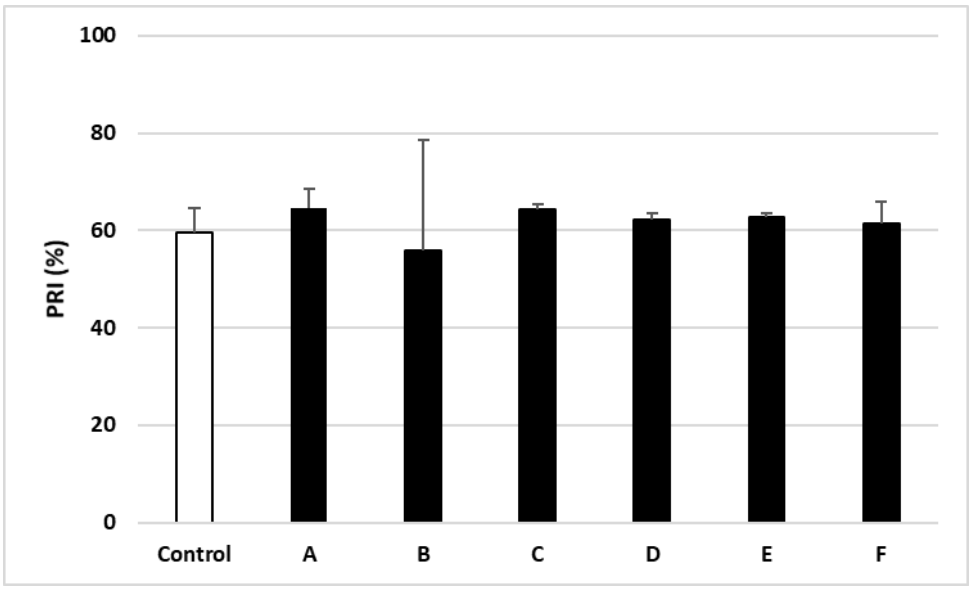
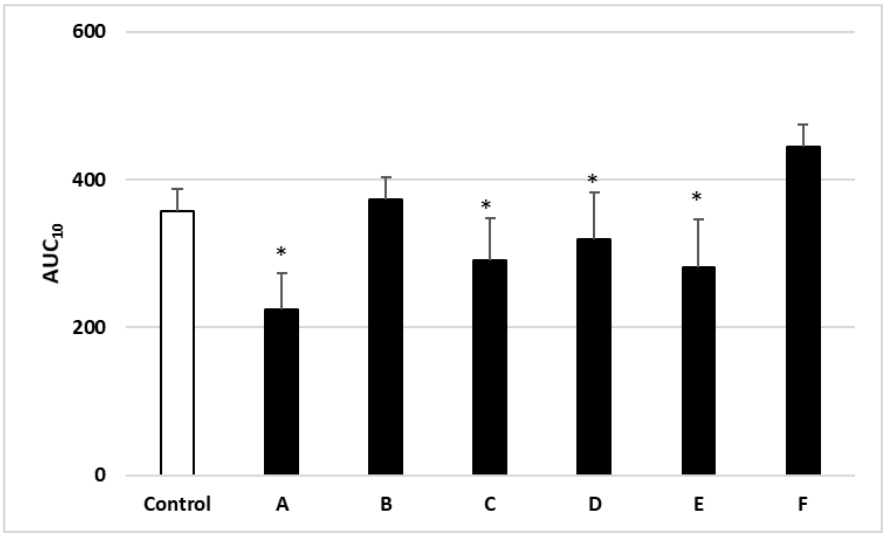
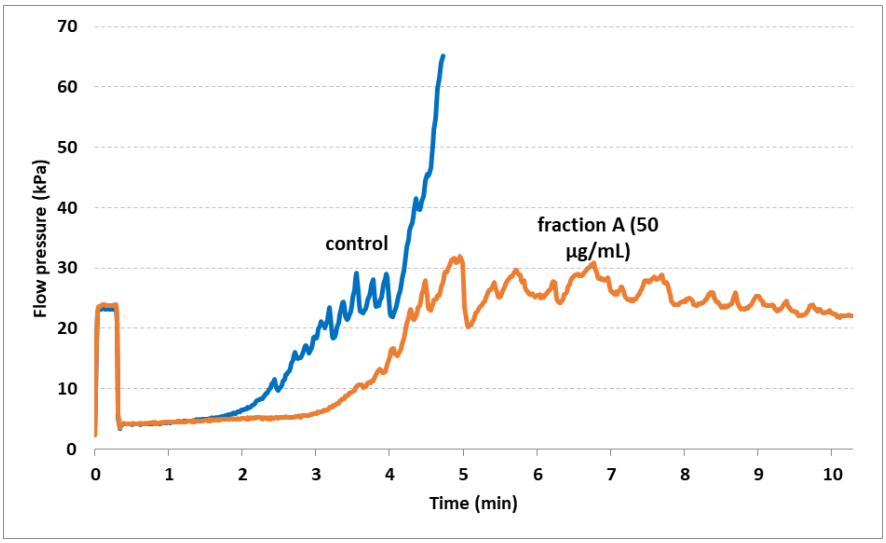


Figure 5. Effects of different plant fractions (50 μg/mL; 30 min) on vasodilator-stimulated phosphoprotein (VASP) phosphorylation in ADP—activated blood platelets. Data represent mean ± SD of 6 healthy volunteers (each experiment performed in triplicate).

A comparison of the effects of all six tested fractions (A–F) at the highest used concentration (50 μg/mL) on selected biomarkers of platelet activation, as measured by cytometric analysis and T-TAS, is given in Table 1. Of the six extracts, fraction A, i.e., the phenolic fraction from fruits, had the strongest anti-platelet potential. Fraction A inhibited PAC-1 expression in three used models: (1) non-activated platelets, (2) platelets activated by 10 μM ADP, (3) platelets activated by 10 μg/mL collagen. This fraction also demonstrated anti-coagulant potential, measured by T-TAS (Table 1).



(a)



(b)

Figure 6. Effects of different plant fractions (50 µg/mL; 30 min) on the T-TAS (Total Thrombus formation Analysis system) using the PL-chip (chip for analysis of platelet thrombus formation (primary hemostatic ability)) in whole blood samples (**a**). Whole blood samples were analyzed by the T-TAS at the shear rates of 1000 s^{-1} on the PL-chips. Area under the curve (AUC_{10}) in PL are shown as closed circles. Data represent mean ± SD of 6 healthy volunteers (each experiment performed in triplicate). * $p < 0.05$ (vs. control sample–whole blood without tested fraction). Figure 6 (**b**) demonstrates selected diagram for fraction A.

Table 1. A comparison of the effects of the phenolic fractions and the nonpolar fractions isolated from various organs of sea buckthorn (A–F, tested at 50 μg/mL) and aronia berry extract (50 μg/mL) on biomarkers of platelet activation measured by cytometric analysis and T-TAS.

Fraction	CD62P Expression				PAC-1 Expression				T-TAS	VASP Phosphorylation
	Non-Activated Platelets	Platelets Activated by 10 μM ADP	Platelets Activated by 20 μM ADP	Platelets Activated by 10 μg/mL Collagen	Non-Activated Platelets	Platelets Activated by 10 μM ADP	Platelets Activated by 20 μM ADP	Platelets Activated by 10 μg/mL Collagen		
A	No effect	No effect	No effect	No effect	Decrease (anti-platelet potential)	Decrease (anti-platelet potential)	No effect	Decrease (anti-platelet potential)	Anti-coagulant poteintial	No effect
B	No effect	No effect	No effect	No effect	Decrease (anti-platelet potential)	Decrease (anti-platelet potential)	No effect	No effect	No effect	No effect
C	Decrease (anti-platelet potential)	No effect	No effect	No effect	No effect	No effect	No effect	Decrease (anti-platelet potential)	Anti-coagulant poteintial	No effect
D	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	Anti-coagulant poteintial	No effect
E	No effect	No effect	No effect	No effect	No effect	No effect	No effect	Decrease (anti-platelet potential)	Anti-coagulant poteintial	No effect
F	No effect	No effect	No effect	No effect	Increase (pro-activation potential)	No effect	Increase (pro-activation potential)	No effect	No effect	No effect
Aronia berry extract	No effect	Decrease (anti-platelet potential)	Decrease (anti-platelet potential)	Decrease (anti-platelet potential)	No effect	No effect	Decrease (anti-platelet potential)	Decrease (anti-platelet potential)	No effect	No effect

Table 1 also shows comparative effect of six tested fractions (A–F; 50 µg/mL) and aronia berry extract (50 µg/mL) on the selected parameters of platelet activation.

3. Discussion

Shifts in hemostasis, including changes in blood platelet function, are well documented in cardiovascular diseases. A number of currently-used clinical antithrombotic agents, such as aspirin, are known for having side effects that may cause serious hematological risk, abnormality or gastrointestinal damage [11]. Therefore, plant preparations may represent an attractive alternative for antithrombotic agents as they demonstrate anti-platelet activity, and sometimes antioxidant potential, but without the side effects associated with artificial preparations.

To determine the influence of such natural preparations on selected aspects of hemostasis, such as platelet activation, the present study used a combination of flow cytometry and T-TAS to examine the effect of treating whole blood samples with six plant fractions isolated from different organs of *Elaeagnus rhamnoides* (L.) A. Nelson. T-TAS, a microchip-based flow chamber system that evaluates thrombogenicity in whole blood, may also be used to evaluate the influence of anti-thrombotic preparations on blood platelet activation and coagulation reactions over a collagen or collagen/tissue thromboplastin-coated surface [12]. The present experiment used surfaces coated with collagen for visualizing platelet thrombus formation in the presence of six tested plant fractions (A–F), four of which (A, C, D, and E) were found to demonstrate anti-coagulant potential.

Platelet activation was also assessed by flow cytometry analysis of P-selectin expression (CD62P) and activation of GPIIb/IIIa complex (PAC-1 binding) in whole blood samples, either those stimulated with ADP or collagen as agonists, and in unstimulated controls. Following platelet activation, the GPIIb/IIIa receptor undergoes conformational changes to reveal a ligand binding site specific for fibrinogen, among others. This site is vital for promoting blood platelet aggregation, which is recognized by PAC-1. In the present study, PAC-1 expression was found to be inhibited by exposure to the tested plant fractions, especially fraction A; this fraction has also previously been found to significantly lower blood platelet aggregation in washed blood platelets and platelet rich plasma [10]. Taken together, these findings suggest that inhibition of platelet aggregation may be associated with low expression of GPIIb/IIIa.

During platelet activation by agonists such ADP or collagen, blood platelets are known to release α-granules. The membranes of these α-granules include an adhesive protein called P-selectin, which is translocated to the surface during platelet activation. In the present studies, P-selectin expression was found to decrease in the presence of the tested fractions (A–F); however, this process was not always statistically significant.

Previous studies indicate that changes in blood platelet proteomes are often associated with the presence of cardiovascular disease [13]. However, no such change was observed in any of the samples treated with the tested plant fractions (A–F). In addition, blood platelet activation by ADP is known to be mediated by two receptors: P2Y1 and P2Y12. For example, P2Y12 receptor inhibition enhances VASP phosphorylation, a stage in platelet aggregation, whereas its activation is associated with VASP non-phosphorylation [14]. VASP phosphorylation assay is often used to study the interaction between anti-platelet drugs such as clopidogrel and the P2Y12 receptor [15]. Our present findings indicate that the anti-platelet potential of the tested plant fraction is not dependent on the P2Y12 receptor, i.e., no changes in VASP phosphorylation were observed.

In conclusion, both our present findings and those of previous studies [10,16] indicate that fractions isolated from various sea buckthorn organs, especially fraction A in the present study, demonstrate significant potency against platelet hyperactivation; however, their anti-platelet potential does not appear to act through the P2Y12 receptor. In addition, the high anti-platelet activity demonstrated by fraction A may be due to the presence of flavonol glycosides in the fraction, which may also be responsible for its antioxidant activity [17]. In addition, previous findings indicate that fraction A has anti-coagulant

and anti-platelet properties, which were observed in washed blood platelets [4,7]. For example, the phenolic fraction taken from fruits (fraction A) prolonged thrombin time and inhibited platelet adhesion in vitro, and changed the level of thiol groups in platelet proteins. Such action may be associated with the presence of flavonol glycosides [4]. A novel finding of this study is that fraction A, similarly to a commercial extract from the berries of *A. melanocarpa* (Aronox®); a known source of anthocyanins with different biological activities [17] has anti-platelet properties.

4. Materials and Methods

4.1. Chemicals

Flow cytometry reagents were purchased from Becton Dickinson (1329 W US-6, Holdrege, NE 68949, USA), PLT VASP/P2Y12 kit was acquired from BioCytex (140 Chemin de l'Armée d'Afrique, 13010 Marseille, France). The PL microchips and other equipment needed for the T-TAS equipment were purchased from Bionicum (Chełmska 21, 00-724 Warszawa, Poland). All reagents necessary for electrophoresis were provided by commercial suppliers, including BIO-RAD (Przyokopowa 33, 01-208 Warszawa, Poland), POCh (Gen. Sowińskiego 11, 44-121 Gliwice, Poland) and Sigma-Aldrich (2033 Westport Center Dr, St. Louis, MO 63146, USA). ADP was obtained from Chrono-Log Corporation (2 W Park Road, Havertown, PA 19083, USA). Collagen type I and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich.

A stock solution of commercial product–Aronox (by Agropharm Ltd., Poland; batch No 020/2007k, *Aronia melanocarpa* berry extract) was prepared in H_2O at a concentration of 5 mg/mL.

4.2. Plant Material

Sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson; syn. *Hippophae rhamnoides* L.) organs were obtained from a horticultural farm in Sokółka, Podlaskie Voivodeship, Poland (August, 2015) (53°24′ N, 23°30′ E), the biggest Polish plantation of sea buckthorn. Individual organs (fruits, leaves, and twigs) were transported to the Institute of Soil Science and Plant Cultivation–State Research Institute in Puławy, Poland.

4.3. Chemical Characteristics of Fractions from Sea Buckthorn Fruits, Twigs, and Leaves

Stock solutions of the tested fractions were made in 50% DMSO (*v/v*%). The final concentration of DMSO in samples was lower than 0.05% (*v/v*%) and its effect was determined in all experiments. Both phenolic and nonpolar fractions were obtained from sea buckthorn fruits, leaves, and twigs, and these were used for testing. The chemical composition of the tested fractions was determined using a Thermo Ultimate 3000RS UHPLC system (USA). Regarding the sea buckthorn fruits, the phenolic fractions are dominated by a range of flavonol glycosides, mainly isorhamnetin glycosides and acylated glycosides, while the nonpolar fractions demonstrate high levels of triterpenoids and acylated triterpenoids. For the leaves, the phenolic fraction contains a number of hydrolysable tannins and ellagic acid, together with both non-acylated and acylated flavonol glycosides, as well as triterpenoid saponins; in contrast, the nonpolar fraction is a source of triterpenoids and triterpenoid saponins. Finally, for the twigs, the phenolic fraction contains high levels of proanthocyanidins and catechin, as well as spermidine derivatives, which are acylated with coumarin and ferulic acid; the nonpolar extracts contain high levels of triterpenoids and acylated triterpenoids [4,10]. The content of dominant compounds in the tested fractions, expressed as a percentage of the total peak area, are given in Table 2.

Table 2. The content of dominant compounds in the tested fraction expressed as a percentage of the total peak area.

Fractions	Relative Peak Area %
Polyphenols rich fraction of sea buckthorn from fruits (fraction A)	
Flavonol glycosides, non-acylated and acylated	67.1
Triterpenoids and acylated triterpenoids	9.1
Triterpenic acids rich fraction of sea buckthorn fruits (fraction B)	
Triterpenoids and acylated triterpenoids	83.6
Flavonol glycosides, non-acylated and acylated	0.9
Polyphenols rich fraction of sea buckthorn from leaves (fraction C)	
Hydrolysable tannins and ellagic acid	31.3
Flavonol glycosides, non-acylated and acylated	24.5
Triterpenic acids rich fraction of sea buckthorn leaves (fraction D)	
Triterpenoids and acylated triterpenoids	50.7
Triterpenoid saponins	30.5
Polyphenols rich fraction of sea buckthorn from twigs (fraction E)	
Proanthocyanidins and catechin	54.3
Spermidine derivatives	10.7
Triterpenic acids rich fraction of sea buckthorn from twigs (fraction F)	
Triterpenoids and acylated triterpenoids	89.0
Catechin and proanthocyanidins	1.3

4.4. Blood and Blood Platelets Samples

Fresh human blood was collected from healthy volunteers (4 women, 4 men, the average age 28), who were not smoking or taking any drugs, including anti-platelet drugs and supplements at the time. The blood was collected in tubes with $CPDA_1$ anticoagulant (citrate/phosphate/dextrose/adenine; 8.5:1; *v/v*; blood/CPDA) (Sigma-Aldrich). The biological material was made available by the L. Rydygiera Medical Center in Łódź. The study was conducted with the consent of the local Bioethical Committee (UŁ3/KBBN-UŁ/II/2016, 12/10/2016).

The whole blood or washed blood platelets were incubated for 30 min at 37 °C with the tested fractions (final concentration: 5 and 50 µg/mL). Platelets were obtained by centrifugation (1200 rpm, 15 min, 25 °C) followed by suspension in Barber buffer (0.14 M NaCl, 0.014 M Tris, 5 mM glucose, pH 7.4).

4.5. Flow Cytometry Analysis

Changes in the platelet activation process were observed using an LSR II Flow Cytometer (Becton Dickinson, San Diego, CA, USA). Whole blood (150 µL) was incubated with extracts and fractions for 30 min at 25 °C. The samples were gently vortexed. After 15 min, the samples were treated with platelet agonists: ADP at final concentrations of 10 and 20 µM, or collagen at a final concentration of 10 µg/mL. The samples were then diluted 10-fold in sterile PBS with Mg^{2+}, and 3 µL of antibodies (CD61/PerCP; CD62/PE and PAC-1/FITC) were added to the cytometric tubes. The antibodies were then prepared for compensation settings and for isotype controls (CD61/PerCP, FITC isotype, PE isotype), following which, the samples were transferred to cytometric tubes and gently vortexed.

The samples were labelled in the dark for 30 min at 25 °C. The platelets were fixed in CellFix and incubated for one hour at 37 °C. All samples were vortexed before measurement. The obtained results were analysed using FACSDiva software [18,19].

4.6. Platelet VASP Phosphorylation

The specific ADP receptor for platelets (P2Y12) was monitored using a flow cytometry kit (PLT VASP/P2Y12). The test was carried out according to the manufacturer's instructions. The results are presented in the form of PRI (platelet reactivity index) [20].

4.7. Polyacrylamide gel Electrophoresis Analysis

In the first stage, cell lysates were prepared. Whole blood was centrifuged (1200 rpm, 15 min, 25 °C). The resulting platelet rich plasma was centrifuged as above. Low platelet plasma was collected and frozen. The platelets were suspended in Barber buffer. The plate suspension was incubated with test extracts and fractions for 30 min at 37 °C. After the incubation, the samples were centrifuged as above, and lysis buffer was added to the pellet. Samples were sonicated and centrifuged (5000 rpm, 5 min, 25 °C). The supernatant was transferred to new Eppendorf tubes. Protein separation was carried out under reducing and non-reducing conditions [21].

4.8. Total Thrombus-Formation Analysis System (T-TAS)

Platelet plug formation was measured in real time under blood flow conditions. Briefly, whole blood (400 µL) was incubated with test extracts and fractions, and 320 µL of blood was then transferred to the reservoir. Plug formation was determined using a PL chip based on the AUC_{10} (Area Under the Curve) parameter [22].

4.9. Data Analysis

The Q-Dixon test was performed to eliminate uncertain data. All the values in this study were expressed as mean ± SD; n—number of blood donors. Statistical analysis was performed with one-way ANOVA for repeated measurements.

Author Contributions: B.S. designed the study and did experimental work. J.R. did experimental work. A.S. (Aleksandra Szustka) did experimental work. J.Ż. prepared plant extracts and characterized their composition. A.S. (Anna Stochmal) collaborated in chemical characterization of plant extracts and performed a critical version of the manuscript. B.O. collaborated in analysis of results as well as performed a critical version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the local Bioethical Committee (UŁ3/KBBN-UŁ/II/2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data sets used and/or analysed in this study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ADP	adenosine diphosphate
AUC	Area Under the Curve
$CPDA_1$	citrate/phosphate/dextrose/adenine
CVDs	cardiovascular diseases
DMSO	dimethylsulfoxide
PRI	platelet reactivity indicator
T-TAS	Total Thrombus-formation Analysis System
VASP	vasodilator-stimulated phosphoprotein

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Oświadczam, że w pracy *„Biological properties of Elaeagnus rhamnoides (L.)A. Nelson twig and leaf extracts"*, B. Skalski, B. Kontek, B. Lis, B. Olas, Ł. Grabarczyk, A. Stochmal, J. Żuchowski. BMC Complementary and Alternative Medicine. 2019, 19(148), 1-12, mój udział wynosił 50% i obejmował planowanie i realizację pracy doświadczalnej (badanie stresu oksydacyjnego w osoczu w obecności ekstraktów z różnych organów rokitnika zwyczajnego), opracowanie wyników oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Phenolic fraction and nonpolar fraction from sea buckthorn leaves and twigs: chemical profile and biological activity"*, B. Skalski, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Future Medicinal Chemistry. 2018, 10(20), 2381-2394, mój udział wynosił 40% i obejmował planowanie i realizację pracy doświadczalnej (badanie stresu oksydacyjnego w osoczu oraz hemostazy osoczowej w obecności frakcji z różnych organów rokitnika zwyczajnego), opracowanie wyników oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – induced oxidative stress and changes in hemostasis"*, B. Skalski, B. Lis, Ł. Pecio, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Food and Chemical Toxicology. 2019, 125, 614-620, mój udział wynosił 35% i obejmował planowanie i realizację pracy doświadczalnej (badanie stresu oksydacyjnego w osoczu oraz badanie aktywacji płytek krwi w obecności izoramnetyny oraz jej dwóch pochodnych wyizolowanych z owoców rokitnika zwyczajnego) opracowanie wyników oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Anti-platelet properties of phenolic extracts from the leaves and twigs of Elaeagnus rhamnoides (L.) A. Nelson"*, B. Skalski, B. Kontek, A. Rolnik, B. Olas, A. Stochmal, J. Żuchowski. Molecules. 2019, 24(3620), 1-17, mój udział wynosił 40% i obejmował planowanie i realizację pracy doświadczalnej (badanie stresu oksydacyjnego w płytkach krwi, badanie aktywacji płytek krwi w obecności ekstraktów z różnych organów rokitnika zwyczajnego oraz ocena toksyczności testowanych ekstraktów), opracowanie wyników oraz przygotowanie manuskryptu.

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of Molecular Sciences. 2021, 22(3282) 1- 13, mój udział wynosił 40% i obejmował planowanie i realizację pracy doświadczalnej (badanie aktywacji płytek krwi w obecności frakcji z różnych organów rokitnika zwyczajnego oraz analiza elektroforetyczna białek płytkowych traktowanych testowanymi frakcjami), opracowanie wyników oraz przygotowanie manuskryptu.

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Oświadczam, że w pracy *„Phenolic fraction and nonpolar fraction from sea buckthorn leaves and twigs: chemical profile and biological activity"*, B. Skalski, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Future Medicinal Chemistry. 2018, 10(20), 2381-2394, mój udział wynosił 8% i obejmował opracowanie wyników dotyczących stresu oksydacyjnego.

Oświadczam, że w pracy *„Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – induced oxidative stress and changes in hemostasis"*, B. Skalski, B. Lis, Ł. Pecio, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Food and Chemical Toxicology. 2019, 125, 614-620, mój udział wynosił 7% i obejmował pomoc merytoryczną dotyczącą pomiaru agregacji płytek krwi.

Oświadczam, że w pracy *„Anti-platelet properties of phenolic extracts from the leaves and twigs of Elaeagnus rhamnoides (L.) A. Nelson"*, B. Skalski, B. Kontek, A. Rolnik, B. Olas, A. Stochmal, J. Żuchowski. Molecules. 2019, 24(3620), 1-17, mój udział wynosił 20% i obejmował realizację części doświadczalnej (badanie stresu oksydacyjnego w płytkach krwi w obecności frakcji z różnych organów rokitnika zwyczajnego) oraz pomoc merytoryczną.

Bogdan Kontek

B. Kontek

dr Joanna Rywaniak
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Łódź, 02.04.2021r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy *„Anti-platelet properties of phenolic and nonpolar fractions isolated from from various organs of Elaeagnus rhamnoides (L.) A . Nelson in whole blood”*, B. Skalski. J. Rywaniak, A. Szustka, J. Żuchowski, A. Stochmal, B. Olas. International Journal of Molecular Sciences. 2021, 22(3282) 1- 13, mój udział wynosił 20% i obejmował pomoc merytoryczną dotyczącą analizy aktywacji płytek krwi z wykorzystaniem cytometrii przepływowej, realizację części doświadczalnej (badanie aktywacji płytek krwi w obecności frakcji z różnych organów rokitnika zwyczajnego), opracowanie wyników oraz przygotowanie manuskryptu.

Joanna Rywaniak

Joanna Rywaniak

mgr Bernadetta Lis
Katedra Biochemii Ogólnej
Uniwersytet Łódzki

Łódź, 02.04.2021r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy *„Biological properties of Elaeagnus rhamnoides (L.)A. Nelson twig and leaf extracts"*, B. Skalski, B. Kontek, B. Lis, B. Olas, Ł. Grabarczyk, A. Stochmal, J. Żuchowski. BMC Complementary and Alternative Medicine. 2019, 19(148), 1-12, mój udział wynosił 10% i realizację części doświadczalnej (badanie hemostazy osoczowej) oraz opracowanie wyników.

Oświadczam, że w pracy *„Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – induced oxidative stress and changes in hemostasis"*, B. Skalski, B. Lis, Ł. Pecio, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Food and Chemical Toxicology. 2019, 125, 614-620, mój udział wynosił 27% i obejmował realizację części doświadczalnej (czasy krzepnięcia) oraz opracowanie wyników dotyczących hemostazy osoczowej.

Bernadetta Lis

Bernadetta Lis

mgr Agata Rolnik
Katedra Biochemii Ogólnej
Uniwersytet Łódzki

Łódź, 02.04.2021r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy *„Anti-platelet properties of phenolic extracts from the leaves and twigs of Elaeagnus rhamnoides (L.) A. Nelson”*, B. Skalski, B. Kontek, A. Rolnik, B. Olas, A. Stochmal, J. Zuchowski. Molecules. 2019, 24(3620), 1-17, mój udział wynosił 10% i obejmował realizację części doświadczalnej (oznaczenie adhezji płytek krwi w obecności ekstraktów z różnych organów rokitnika zwyczajnego) oraz opracowanie wyników.

Agata Rolnik

Rolnik Agata

mgr Aleksandra Szustka
Katedra Cytobiochemii
Uniwersytet Łódzki

Łódź, 02.04.2021r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy *„Anti-platelet properties of phenolic and nonpolar fractions isolated from from various organs of Elaeagnus rhamnoides (L.) A . Nelson in whole blood”*, B. Skalski. J. Rywaniak, A. Szustka, J. Zuchowski, A. Stochmal, B. Olas. International Journal of Molecular Sciences. 2021, 22(3282) 1- 13, mój udział wynosił 5% i obejmował pomoc merytoryczną podczas wykonywania analizy elektroforetycznej białek płytkowych traktowanych frakcjami z rokitnika zwyczajnego.

Aleksandra Szustka

Aleksandra Szustka

prof., dr hab. Anna Stochmal
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Państwowy Instytut Badawczy

Łódź, 02.04.2021r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy *„Biological properties of Elaeagnus rhamnoides (L.)A. Nelson twig and leaf extracts"*, B. Skalski, B. Kontek, B. Lis, B. Olas, Ł. Grabarczyk, A. Stochmal, J. Żuchowski. BMC Complementary and Alternative Medicine. 2019, 19(148), 1-12, mój udział wynosił 2% i obejmował pomoc merytoryczną oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Phenolic fraction and nonpolar fraction from sea buckthorn leaves and twigs: chemical profile and biological activity"*, B. Skalski, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Future Medicinal Chemistry. 2018, 10(20), 2381-2394, mój udział wynosił 2% i obejmował pomoc merytoryczną oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – induced oxidative stress and changes in hemostasis"*, B. Skalski, B. Lis, Ł. Pecio, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Food and Chemical Toxicology. 2019, 125, 614-620, mój udział wynosił 3% i obejmował pomoc merytoryczną oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Anti-platelet properties of phenolic extracts from the leaves and twigs of Elaeagnus rhamnoides (L.) A. Nelson"*, B. Skalski, B. Kontek, A. Rolnik, B. Olas, A. Stochmal, J. Żuchowski. Molecules. 2019, 24(3620), 1-17, mój udział wynosił 2% i obejmował pomoc merytoryczną oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Response of blood platelets to phenolic faction and non-polar fraction from the leaves and twigs of Elaeagnus rhamnoides (L.) A. Nelson in vitro"*. B. Skalski, A. Stochmal, J. Żuchowski, Ł. Grabarczyk, B. Olas. Biomedicine & Pharmacotherapy. 2019, 124(109897), 1-11, mój udział wynosił 2% i obejmował pomoc merytoryczną oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Anti-platelet properties of phenolic and nonpolar fractions isolated from from various organs of Elaeagnus rhamnoides (L.) A . Nelson in whole blood"*, B. Skalski. J. Rywaniak, A. Szustka, J. Żuchowski, A. Stochmal, B. Olas. International Journal of Molecular Sciences. 2021, 22(3282) 1- 13, mój udział wynosił 2% i obejmował pomoc merytoryczną oraz przygotowanie manuskryptu.

Anna Stochmal

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Łódź, 02.04.2021r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy *„Biological properties of Elaeagnus rhamnoides (L.)A. Nelson twig and leaf extracts"*, B. Skalski, B. Kontek, B. Lis, B. Olas, Ł. Grabarczyk, A. Stochmal, J. Żuchowski. BMC Complementary and Alternative Medicine. 2019, 19(148), 1-12, mój udział wynosił 15% i obejmował oznaczenie składu chemicznego testowanych preparatów oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Phenolic fraction and nonpolar fraction from sea buckthorn leaves and twigs: chemical profile and biological activity"*, B. Skalski, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Future Medicinal Chemistry. 2018, 10(20), 2381-2394, mój udział wynosił 30% i obejmował oznaczenie składu chemicznego testowanych preparatów oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – induced oxidative stress and changes in hemostasis"*, B. Skalski, B. Lis, Ł. Pecio, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Food and Chemical Toxicology. 2019, 125, 614-620, mój udział wynosił 8% i obejmował izolację testowanych związków oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Anti-platelet properties of phenolic extracts from the leaves and twigs of Elaeagnus rhamnoides (L.) A. Nelson"*, B. Skalski, B. Kontek, A. Rolnik, B. Olas, A. Stochmal, J. Żuchowski. Molecules. 2019, 24(3620), 1-17, mój udział wynosił 18% i obejmował oznaczenie składu chemicznego testowanych preparatów oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Response of blood platelets to phenolic faction and non-polar fraction from the leaves and twigs of Elaeagnus rhamnoides (L.) A. Nelson in vitro"*. B. Skalski, A. Stochmal, J. Żuchowski, Ł. Grabarczyk, B. Olas. Biomedicine & Pharmacotherapy. 2019, 124(109897), 1-11, mój udział wynosił 28% i obejmował oznaczenie składu chemicznego testowanych preparatów oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Anti-platelet properties of phenolic and nonpolar fractions isolated from from various organs of Elaeagnus rhamnoides (L.) A . Nelson in whole blood"*, B. Skalski. J. Rywaniak, A. Szustka, J. Żuchowski, A. Stochmal, B. Olas. International Journal of Molecular Sciences. 2021, 22(3282) 1- 13, mój udział wynosił 18% i obejmował oznaczenie składu chemicznego testowanych preparatów oraz przygotowanie manuskryptu.

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Łódź, 02.04.2021r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy *„Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – induced oxidative stress and changes in hemostasis”*, B. Skalski, B. Lis, Ł. Pecio, B. Kontek, B. Olas, J. Żuchowski, A. Stochmal. Food and Chemical Toxicology. 2019, 125, 614-620, mój udział wynosił 10% i obejmował izolację testowanych związków.

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Łódź, 02.04.2021r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy *„Biological properties of Elaeagnus rhamnoides (L.)A. Nelson twig and leaf extracts"*, B. Skalski, B. Kontek, B. Lis, B. Olas, Ł. Grabarczyk, A. Stochmal, J. Żuchowski. BMC Complementary and Alternative Medicine. 2019, 19(148), 1-12, mój udział wynosił 4% i obejmował pomoc merytoryczną oraz przygotowanie manuskryptu.

Oświadczam, że w pracy *„Response of blood platelets to phenolic faction and non-polar fraction from the leaves and twigs of Elaeagnus rhamnoides (L.) A. Nelson in vitro"*. B. Skalski, A. Stochmal, J. Żuchowski, Ł. Grabarczyk, B. Olas. Biomedicine & Pharmacotherapy. 2019, 124(109897), 1-11, mój udział wynosił 5% i obejmował pomoc merytoryczną oraz przygotowanie manuskryptu.

Łukasz Grabarczyk