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Analiza składu chemicznego preparatów z warzyw z rodziny dyniowatych (*Cucurbitaceae*) i astrowatych (*Asteraceae*) i ich wpływ na wybrane elementy hemostazy

Analysis of the chemical composition of preparations of vegetables from cucurbits (*Cucurbitaceae*) and asteraceae (*Asteraceae*) family and their influence on selected elements of hemostasis

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- "Comparative phytochemical, antioxidant and hemostatic studies of preparations from selected vegetables from *Cucurbitaceae* family" **A. Rolnik**, I. Kowalska, A. Soluch, A. Stochmal, B. Olas; Molecules 2020, 25, 1-20 (IF= 4,412 (2020); liczba punktów MEiN=140 (2021)) **(praca 3)**
- "Antioxidant and hemostatic properties of preparations from *Asteraceae* family and their chemical composition – Comparative studies" **A. Rolnik**, A. Soluch, I. Kowalska, B. Olas; Biomedicine & Pharmacotherapy 2021, 142, 1-10 (IF=6,529 (2020); liczba punktów MEiN=100 (2021)) **(praca 4)**
- "Preparations from selected cucurbit vegetables as antiplatelet agents" **A. Rolnik**, B. Skalski, A. Stochmal, B Olas; Scientific Reports 2022, 1, 1-15 (IF=4,996 (2021); liczba punktów MEiN=140 (2021)) **(praca 5)**
- "The *in vitro* anti-platelet activities of plant extracts from the *Asteraceae* family", **A. Rolnik**, A. Stochmal, B. Olas; Biomedicine & Pharmacotherapy 2022, 149, 1-8 (IF=6,529 (2020); liczba punktów MEiN=100 (2021)) **(praca 6)**

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Pozostały dorobek naukowy

- "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, 19, 1-17 (IF=4,412 (2021); liczba punktów MEiN=140 (2021))
- "Comparative phytochemical, cytotoxicity, antioxidant and hemostatic studies of *Taraxacum officinale* root preparations" D. Jedrejek, B. Lis, **A. Rolnik**, A. Stochmal, B. Olas; Food and Chemical Toxicology 2019; 126, 233-247 (IF=4,400 (2021); liczba punktów MEiN=100 (2022))
- "Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study" B. Lis, **A. Rolnik**, D. Jedrejek, A. Soluch, A. Stochmal, B. Olas; Journal of Functional Foods 2019; 59, 16-24 (IF=3,701 (2021); liczba punktów MEiN=100 (2022))
- "Quercetin and kaempferol derivatives isolated from aerial parts of *Lens culinaris* Medik as modulators of blood platelet functions" **A. Rolnik**, J. Żuchowski, A. Stochmal, B. Olas; Industrial Crops and Products 2020; 152, 1-8 (IF=5,645 (2021); liczba punktów MEiN=200 (2022))
- "Modulation of oxidative stress and hemostasis by flavonoids from lentil aerial parts" J. Żuchowski, **A. Rolnik**, W. Adach, A. Stochmal, B. Olas; Molecules 2021; 26, 2, 497 (IF=4,412 (2021); liczba punktów MEiN=140 (2021))
- "Antioxidant and anticoagulant effects of phenylpropanoid glycosides isolated from broomrapes (*Orobanche caryophyllacea*, *Phelipanche arenaria*, and *P. ramosa*)" B. Skalski, S. Pawelec, D. Jedrejek, **A. Rolnik**, R. Pietukhov, R. Piwowarczyk, A. Stochmal, B. Olas; Biomedicine & Pharmacotherapy 2021, 139, 111618 (IF=6,529 (2020); liczba punktów MEiN=100 (2021))
- "Multifunctional compounds in the extract from mature seeds of *Vicia faba var. minor*: Phytochemical profiling, antioxidant activity and cellular safety in human selected blood cells in *in vitro* trials" M. Kowalczyk, **A. Rolnik**, W. Adach, M. Kluska, M. Juszczak, Ł. Grabarczyk, K. Woźniak, B. Olas, A. Stochmal, Biomedicine & Pharmacotherapy 2021, 139, 111718 (IF=6,529 (2020); liczba punktów MEiN=100 (2021))

- “Beneficial *in vitro* effects of a low myo-inositol dose in the regulation of vascular resistance and protein peroxidation under inflammatory conditions” **A. Rolnik**, B. Olas, J. Szablińska-Piernik, LB. Lahuta, A. Rynkiewicz, P. Cygański, K. Socha, L. Gromadziński, M. Thoene, M. Majewski, Nutrients 2022; 14, 1118, 1-13 (IF=5,719 (2020); liczba punktów MEiN=140 (2022))

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WSTĘP

Choroby układu sercowo-naczyniowego, niezmiennie od wielu lat, są główną przyczyną śmierci na świecie. Tylko w Europie są powiązane z 46% wszystkich zgonów. Podstawą rozwoju chorób układu sercowo-naczyniowego są zaburzenia hemostazy, czyli procesu odpowiadającego za utrzymanie płynności krwi w organizmie i tamowanie krwawienia w przypadku przerwania ściany naczynia. W prawidłowych warunkach, integralność uszkodzonej ściany jest przywracana poprzez tworzenie skrzepu w miejscu urazu, a następnie rozpuszczanie skrzepu, zapewniając przepływ krwi w zdrowym naczyniu. Zaburzenia w tych procesach mogą skutkować nadmiernym krwawieniem lub zakrzepicą. Zakrzepica prowadzi do niedrożności naczyń i do blokowania normalnego przepływu krwi, co może powodować niedotlenienie tkanki (Versteeg i wsp. 2013; Hou i wsp. 2015; Manon-Jensen i wsp. 2016). Zaburzenia hemostazy, takie jak zakrzepica tętnic, czy niedotlenienie mięśnia sercowego, często mogą być uzależnione od przyczyn środowiskowych. Do najpoważniejszych zalicza się niezdrowy tryb życia, połączony z nieprawidłową dietą (McFadyen i wsp. 2018).

Płytki krwi i ich aktywacja odgrywają kluczową rolę w hemostazie pierwotnej, w trakcie której płytki łączą się z odsłoniętą macierzą komórkową, poprzez adherencję do białek macierzy, tworząc tym samym pierwotny skrzep, zapobiegający utracie krwi w miejscu uszkodzenia naczynia. W kolejnym etapie hemostazy, płytki pełnią dodatkową funkcję, jako mediatorzy dla czynników kaskady krzepnięcia (Clemetson 2012). W warunkach prawidłowego przepływu, nieaktywne płytki krwi poruszają się swobodnie w naczyniach krwionośnych i nie wchodzą w kontakt ze śródbłonkiem naczyń krwionośnych. Do ich aktywacji, w tym adhezji, dochodzi po interakcji z receptorami w macierzy zewnątrzkomórkowej. W przepływie żylnym są to receptory kolagenu, fibronektyny oraz lamininy, natomiast w przepływie tętniczym adhezja płytek jest uzależniona od wiązania czynnika von Willebranda do glikoproteinowego receptora GPIb-IX-V. W przypadku przerwania ściany naczynia, kolagen wiąże się z krążącym w krwi czynnikiem von Willebranda, co umożliwia przyłączenie z kompleksem GPIb-IX-V (Sang i wsp. 2021). Interakcja płytek krwi z kolagenem powoduje aktywację fosfolipazy Cγ, która hydrolizuje 4,5-bisfosforan fosfatydyloinozytolu do trójfosforanu inozytolu (IP3) i 1,2-diacyloglicerolu (DAG). IP3 wiąże się z retikulum endoplazmatycznym w błonie komórkowej, co skutkuje wyrzutem jonów wapnia do cytoplazmy. Związany z błoną DAG razem z jonami wapnia aktywuje kinazę białkową C, prowadząc do zmiany kształtu płytek i uwolnienia zawartości ich

ziarnistości, w tym agonistów, takich jak adenozynodifosforan (ADP). Dodatkowo, adhezja płytek krwi do kolagenu jest wzmocniona poprzez interakcję z integryną $\alpha_2\beta_1$ (Sang i wsp. 2021).

Najważniejszym receptorem decydującym o agregacji płytek krwi jest glikoproteina GPIIb/IIIa (integryna $\alpha_{IIb}\beta_3$), która może wiązać się z fibrynogenem. Ponadto, ekspozycja GPIIb/IIIa jest istotnym markerem aktywacji płytek krwi. Funkcja GPIIb/IIIa opiera się na dwukierunkowym przewodzeniu sygnałów przez błonę komórkową, ułatwiając interakcję z potencjalnymi agonistami. Nieaktywna glikoproteina ma ograniczoną zdolność powinowactwa do ligandów, ponieważ domena wiążąca RGD (sekwencja zawierająca argininę, glicynę i kwas asparaginowy) w integrynie jest ukryta (zwinięta). Sygnały wewnętrzne w komórce prowadzą do zmian konformacyjnych integryny, powodując rozwinięcie domeny RGD, co nasila powinowactwo GPIIb/IIIa do wiązania ligandów (Armstrong i Peter 2012; Yun i wsp. 2016) . Innym markerem aktywacji płytek krwi może być selektyna P. W trakcie aktywacji płytek krwi dochodzi do zwiększenia jej ekspozycji, poprzez przetransportowanie jej z α-ziarnistości na powierzchnię płytki (Yun i wsp. 2016).

Zaburzenia procesu hemostazy i ryzyko rozwoju chorób sercowo-naczyniowych mogą być między innymi wywołane przez stres oksydacyjny, czyli brak równowagi pomiędzy procesami antyoksydacyjnymi a prooksydacyjnymi w organizmie. Jedną z przyczyn powstawania stresu oksydacyjnego jest formowanie reaktywnych from tlenu (RFT) w komórkach. Płytki krwi w trakcie aktywacji uwalniają RFT do środowiska, np. w czasie wiązania GPVI do kolagenu (Lambrechts i wsp. 2015; Koenig-Oberhuber i Filipovic 2016). Po ekspozycji płytek na działanie agonistów, oksydaza NAD(P)H jest głównym enzymem odpowiedzialnym za produkcję RTF, które z kolei wpływają na aktywację integryny $\alpha_{IIb}\beta_3$. Anionorodnik ponadtlenkowy powstający w płytkach w warunkach hipoksji-reoksygenacji może ulec konwersji do nadtlenku, co może powodować zwiększoną podatność płytek krwi na agregację indukowaną kolagenem i kwasem arachidonowym. Ponadto, nadtlenek wodoru powoduje utlenianie grup tiolowych w białkowej domenie fosfatazy tyrozynowej. Proces ten z kolei nasila wrażliwość płytek krwi na aktywację kolagenem. Utlenianie grup tiolowych w białkach osocza dodatkowo wpływa na osłabienie odpowiedzi płytek krwi na działanie agonistów (Han i wsp. 2020).

Choroby układu sercowo-naczyniowego są poważnym zagrożeniem dla życia człowieka, a skutki udarów i zawałów są często odczuwane przez długie lata. Pacjenci zmuszeni są stosować terapie farmakologiczne przez całe życie. Część pacjentów cierpiących

na choroby układu sercowo-naczyniowego można poddać terapii prewencyjnej, opierającej się na zmianach behawioralnych, w tym żywieniowych, czy rewaskularyzacji chirurgicznej. Wszyscy jednak zmuszeni są stosować leki (Pepió Vilaubí i wsp. 2018). Wiele terapii stosowanych w chorobach układu krążenia opiera się na ograniczaniu aktywności płytek krwi. Aspiryna jest jednym z najstarszych i najlepiej poznanych leków o działaniu przeciwpłytkowym. Jej mechanizm działania opiera się na nieodwracalnej inhibicji cyklooksygenazy (COX), w szczególności COX1, a tym samym aspiryna blokuje syntezę tromboksanu A_2. Aspiryna jest stosowana w terapii chorób sercowo-naczyniowych ograniczając patologiczną agregację płytek krwi. Dodatkowo, jest szeroko stosowanym środkiem przeciwbólowym i przeciwzapalnym. Aspiryna jest jedną z podstawowych form terapii farmakologicznej w większości krajów, ze względu na niskie koszty wytwarzania i prosty sposób stosowania, ale u około jednej trzeciej pacjentów powoduje skutki uboczne, w postaci nadmiernych krwawień. Dodatkowo, pewien odsetek pacjentów jest odporny na działanie aspiryny. Może być to powiązane z niektórymi chorobami, jak cukrzyca czy ostre zespoły wieńcowe. Efekty uboczne, wywołane zażyciem aspiryny, występują również u osób, u których wykryto polimorfizmy dla COX1 i receptorów glikoproteinowych (Lambrechts i wsp. 2015; Koenig-Oberhuber i Filipovic 2016).

Kolejna grupa leków o działaniu antypłytkowym, to leki blokujące receptor P_2Y_{12}, będący receptorem dla ADP. Leki blokujące receptor P_2Y_{12} dzieli się na dwie klasy: tienopirydyny i cyklopentylotriazolopirymidyny. Wszystkie leki należące do klas tienopirydyn dostarczane są do organizmu jako nieaktywne metabolity, które są aktywowane przez esterazy osoczowe lub wątrobowy cytochrom P3A4/3A5. Następie, aktywne metabolity nieodwracalnie wiążą się do receptora P_2Y_{12}. Copidogrel należy do najpopularniejszych przedstawicieli tienopirydyny. Cangrelor, będący pochodną cyklopentylotraizolopirymidyny, również blokuje receptor P_2Y_{12}, ale w porównaniu do clopidegrolu jego działanie jest w pełni odwracalne. Obydwa rodzaje leków często stosowane są razem z aspiryną, jako podwójna terapia przeciwpłytkowa u osób po zawale mięśnia sercowego (McFadyen i wsp. 2018). Kolejnym przykładem terapii, bazującej na mechanizmie antypłytkowym, jest wiązanie leku do GPIIb/IIIa, uniemożliwiając agregację płytek krwi do fibrynogenu. Do tej grupy należą takie leki, jak: abciximab czy tirofiban. Abciximab jest monoklonalnym przeciwciałem mysim i wykazuje najwyższe powinowactwo do receptora GPIIb/IIIa (Koenig-Oberhuber i Filipovic 2016). Nie tylko terapia z zastosowanie aspiryny, ale również inne stosowane terapie antypłytkowe dają efekty niepożądane w postaci zwiększonego ryzyka krwawień w trakcie

operacji, zdarzeń niedokrwiennych i hamowania prawidłowej agregacji płytek krwi. Dodatkowo, przerwanie terapii antypłytkowej może wiązać się z ponownym wystąpieniem zawału mięśnia sercowego lub zakrzepicy (Rengasamy i wsp. 2019). W chorobach układu sercowo-naczyniowego stosuje się również leki bazujące na właściwościach antykoagulacyjnych, których działanie opiera się na inhibicji elementów kaskady krzepnięcia, uniemożliwiając powstawanie skrzepu. Terapie antykoagulacyjne wykazują działanie natychmiastowe, w szczególności w przypadku ostrej choroby zakrzepowo-zatorowej, natomiast pacjenci prewencyjnie przyjmują leki przez wiele lat. Obecnie najczęściej stosowanymi lekami jest nisko-cząsteczkowa heparyna i fondaparynuks. Antykoagulacyjne działanie heparyny opiera się na wiązaniu i aktywowaniu czynnika antytrombinowego, co z kolei przyspiesza inaktywację osoczowych czynników kaskady krzepnięcia, w tym II, IX, X, XIIa. Fondaparynuks wiąże się selektywnie do antytrombiny III, powodując neutralizację czynnika Xa (Koenig-Oberhuber i Filipovic 2016).

Świadomość społeczna na temat roli zdrowej diety w codziennym życiu wzrasta z roku na rok, stąd coraz większe zainteresowanie produktami naturalnymi dobrej jakości i dietą, opierającą się w przeważającym stopniu na owocach i warzywach. Zalecana dawka warzyw w codziennej diecie to minimum 200 g według Światowej Organizacji Zdrowia (Olas 2018; Salas-Salvadó i wsp. 2018). Warzywa i owoce mogą być elementami składowymi nutraceutyków i żywności funkcjonalnej. Ich aktywność biologiczna jest skorelowana z zawartością metabolitów wtórnych, takich jak: flawonoidy, terpenoidy, seskwiterpeny, saponiny, itp. Ponadto, skład chemiczny jest często uzależniony od rodziny, a nawet gatunku. Właściwości prozdrowotne owoców i warzyw są w większości dobrze poznane, ale nadal brakuje danych literaturowych na temat ich wpływu na pewne aspekty zdrowotne, np. choroby układu sercowo-naczyniowego (Mokganya i Tshisikhawe 2019, Olas 2019).

Do najliczniejszych rodzin warzyw na świecie należą rodzina dyniowatych i astrowatych. Warzywa należące do obydwu rodzin od setek lat są stałymi elementami różnorodnych diet na całym świecie. Ich uprawa w danym kraju, uzależniona jest w dużym stopniu od warunków klimatycznych i zapotrzebowania danego regionu (**praca 1 i 2**).

Rodzina dyniowatych (*Cucurbitaceae*) składa się z ponad 800 gatunków, a jej przedstawiciele występują na całym świecie, z czego około 300 jest wykorzystywanych przez człowieka, 150 jest uprawianych na szeroką skalę i zaledwie 30 ma znacznie w światowej produkcji żywności. Do najpopularniejszych jadalnych przedstawili należy: dynia, arbuz, melon, ogórek czy cukinia. Większość warzyw należących do tej rodziny, poza szerokim

zastosowaniem przemysłowym, charakteryzuje się również właściwościami prozdrowotnymi, powiązanymi z obecnością metabolitów wtórnych w ich składzie chemicznym (Saboo i wsp. 2013). Warzywa te zawierają również błonnik i minerały, takie jak: miedź, cynk czy magnez, a ich nasiona, w szczególności nasiona dyni, są bardzo dobrym źródłem białka. W swoim składzie warzywa zawierają także tokoferole i karetonoidy, które są dobrymi antyoksydantami (Gohari Ardabili i wsp. 2011; Patel i Rauf 2017). Wiele gatunków warzyw dyniowatych było już wykorzystywanych w medycynie tradycyjnej ze względu na swoje właściwości prozdrowotne. Na przykład, w Chinach, Indiach, Meksyku i Argentynie dynie wykorzystywano do leczenia zakażeń pasożytniczych, a jej nasiona, jako dodatek do diety dla osób z zaburzeniami pracy pęcherza moczowego i nerek. *Cirtullus colocynthis,* zwany arbuzem kolokwintem, uprawianym w krajach arabskich, wykorzystywano, jako środek przeciwbólowy, poronny i przeczyszczający. W Indiach, *Trichosanthes cucumerina,* potocznie nazywana gurdlina ogórkowata, stosowana była, jako lekarstwo na bóle głowy, gorączkę i biegunkę (Salehi i wsp. 2019). Powszechne zastosowanie warzyw dyniowatych w medycynie tradycyjnej wzbudziło zainteresowanie naukowców nad ich aktywnością biologiczną. Do najlepiej opisanych należy aktywność przeciwcukrzycowa. Na przykład, nasiona dyni zawierają polisacharydy związane z białkami, powodujące wzrost insuliny w osoczu, a także kwas p-aminobenzoesowy, sterole i pektyny, które regulują poziom glikemii u pacjentów spożywających pokarmy bogate w błonnik. Cukinia również zawiera związki, które powodują wzrost poziomu insuliny w osoczu (Rajasree i wsp. 2016). Wiele gatunków warzyw z rodziny dyniowatych ma również właściwości przeciwnowotworowe. Dynia ogromna jest źródłem związków hamujących rozwój komórek nowotworowych. Cucurbitacyny, czyli związki obecne tylko w warzywach dyniowatych, charakteryzują się aktywnością antynowotworową poprzez hamowanie cyklu komórkowego w fazie G2/M, prowadząc do apoptozy komórek nowotworowych. Cucurbitacyny wykazują również właściwości przeciwzapalne, po przez hamowanie aktywności mediatorów zapalnych, takich jak syntaza tlenku azotu, czynnika martwicy nowotworów α (ang. tumor necrosis factor-α, TNF-α) czy cyklooksygenaza-2. Warzywa dyniowate, ze względu na obecność terpenów w składzie, naruszają błonę komórkową bakterii i grzybów, powodując zahamowanie wzrostu grzybów i bakterii (**praca 1**).

Rośliny należące do rodzinny astrowatych, podobnie jak dyniowate były wykorzystywane w medycynie tradycyjnej od ponad 300 lat, ze względu na swoją aktywność przeciwzapalną, przeciwdrobnoustrojową, antyoksydacyjną i hepatoprotekcyjną (García-

Herrera i wsp. 2014). To jedna z największych kwiatowych rodzin roślin, z ponad 25000 gatunkami na świecie, z czego przeważająca część rośnie w klimacie tropikalnym lub subtropikalnym. Do najpopularniejszych gatunków należy cykoria, sałata, rumianek, mniszek pospolity czy stokrotka (Achika i wsp. 2014; Nikolić i Stevović 2015). Wielu przedstawicieli tej rodziny powinno być elementem codziennej diety ze względu na szereg makro- i mikroelementów w ich składzie. W 100 g suchej masy jadalnych części roślin zawarte jest od 0,40 do 6,13 g białka i od 2,55 do 13,44 g błonnika. Dodatkowo, są one dobrym źródłem witamin A, B i C, oraz sodu, potasu, wapnia czy magnezu. Na przykład, cykoria zawiera 22,15 mg witaminy C w 100 g suchej masy, a jej korzenie są doskonałym źródłem inuliny, której zawartość w 100 g świeżych korzeni waha się pomiędzy 11-20 g zależnie od pory roku. Fakt ten czyni korzenie cykorii dobrym substytutem kawy bezkofeinowej dla cukrzyków. Jadalną częścią topinamburu są korzenie, które również są dobrym źródłem inuliny, a także potasu i magnezu. Korzeń topinamburu często wykorzystywany jest w wyrobach cukierniczych, ze względu na niską kaloryczność i teksturę naśladującą teksturę tradycyjnego tłuszczu (García-Herrera i wsp. 2014; Perović i wsp. 2021). Wiele gatunków warzyw z rodziny astrowatych charakteryzuje się aktywnością farmakologiczną, której podstawą są metabolity wtórne, takie jak saponiny, sterole, kwasy fenolowe czy olejki eteryczne. Do metabolitów wtórnych odpowiedzialnych w znaczącym stopniu za właściwości prozdrowotne roślin z rodziny astrowatych należą laktony seskwiterpenowe, odpowiedzialne za gorzki posmak w roślinach i wspomagające procesy trawienne i apetyt (Koc i wsp. 2015). Aktywność antyoksydacyjna tych warzyw, objawiająca się zdolnością do wychwytywania wolnych rodników lub blokowania formowania rodników tlenowych, jest powiązana z zawartością związków fenolowych. Im wyższe stężenie tych związków tym lepsza aktywność. Korelację taką można zaobserwować w przypadku aktywności antyoksydacyjnej preparatów z mniszka pospolitego. Ekstrakty z kwiatów mniszka, z pośród ekstraktów innych części morfologicznych tej rośliny, zawierają najwyższe stężenie związków fenolowych. Ich aktywność antyoksydacyjna polega m.in. na ochronie superkręconego DNA przed pęknięciami spowodowanymi rodnikami hydroksylowymi. Dodatkowo, ograniczają utlenianie lipidów i karbonylację białek w osoczu (Hu 2018; Lis i wsp. 2018). Na aktywność przeciwzapalną roślin, należących do astrowatych, wpływ ma obecna w ich składzie arcetina, związek charakteryzujący się zdolnością do ograniczania produkcji mediatorów zapalnych, w tym interleukiny IL-6 i IL1β, prostaglandyny E_2 (PGE_2), czy czynnika martwicy nowotworów (TNF-α). Arcetina działa hamująco na przesyłanie sygnału w szlaku NFkβ (ang. czynnik transkrypcyjny kβ), powodując tłumienie działania cyklooksygenazy 2 (Tourchi i wsp. 2016) **(praca 2)**.

Więcej informacji na temat właściwości prozdrowotnych warzyw z rodziny dyniowatych i rodziny astrowatych jest opisana w pracach 1 i 2.

CEL PRACY

I. Analiza składu chemicznego preparatów z wybranych warzyw z rodziny *Cucurbitaceae* i *Asteraceae*.

II. Ocena oddziaływania związków zawartych w preparatach z wybranych warzyw z rodzin dyniowatych i astrowatych z wybranymi elementami hemostazy, w tym płytkami krwi w badaniach *in vitro*.

III. Wybór preparatu roślinnego charakteryzującego się najwyższą aktywnością biologiczną pod kątem chorób układu sercowo-naczyniowego.

Założenia podstawowe:

- Analiza fitochemiczna preparatów z wybranych warzyw z rodzin dyniowatych i astrowatych.
- Ocena poziomu biomarkerów stresu oksydacyjnego w osoczu w obecności preparatów z wybranych warzyw z rodzin dyniowatych i astrowatych, w warunkach *in vitro.*
- Ocena wpływu preparatów z wybranych warzyw z rodzin dyniowatych i astrowatych na proces krzepnięcia w warunkach *in vitro.*
- Analiza wpływu preparatów z wybranych warzywna z rodzin dyniowatych i astrowatych na różne etapy aktywacji płytek krwi i przemiany biochemiczne zachodzące w tych komórkach w warunkach *in vitro.*
- Oznaczenie aktywności zewnątrzkomórkowej dehydrogenazy mleczanowej w płytkach krwi, w celu sprawdzenia toksyczności preparatów z wybranych warzyw z rodzin dyniowatych i astrowatych, w warunkach *in vitro.*

Hipoteza badawcza: Preparaty z wybranych warzyw z rodziny dyniowatych i rodziny astrowatych wykazują aktywność antyoksydacyjną, antypłytkową i przeciwzakrzepową.

MATERIAŁY I METODY:

Materiał roślinny

Z rodziny dyniowatych wybrano:

- Dynię (bez pestek) (*Cucurbita pepo*)
- Cukinię (z pestkami) (*Cucurbita pepo* L. *convar. Giromontina)*
- Ogórka (z pestkami) (*Cucumis* sativus L.)
- Patisona białego (bez pestek) (*Cucurbita pepo* L. var. *patisoniana*)
- Patisona żółtego (bez pestek) (*Cucurbita pepo* L. var. *patisoniana*)

Z rodziny astrowatych wybrano:

- Liście cykorii (*Cichorium intybus L.)*
- Liście sałaty zielonej (*Lactuca sativa* L.)
- Liście sałaty czerwonej (*Lactuca sativa* L. *var. crispa*)
- Bulwy topinamburu (*Helianthus tuberosus* L.)
- Owoce i korzenie mniszka pospolitego *(Taraxacum officinale)*

Materiał roślinny tworzyły jadalne organy warzyw dwóch rodzin dyniowatych i astrowatych. Warzywa pozyskano z marketów oraz od lokalnych rolników w trakcie ich optymalnego rozwoju. Korzenie i owoce (czyli niełupki) mniszka pospolitego zebrano w okolicach Rzeszowa.

Materiał biologiczny

Materiałem biologicznym była krew pełna, pobrana w Regionalnym Centrum Krwiodawstwa i Krwiolecznictwa i Szpitalu im. Dr Rydygiera w Łodzi. Krew pobierano na dwa rodzaje antykoagulantów: roztwór CPDA (cytrynian, fosforan, dekstroza; 9:1, v/v, krew/CPD) oraz antykoagulant BAPA (benzylsulfonyl-D-argininyl-prolyl-4 amidinobenzylamide). Krew pobierano od zdrowych wolontariuszy, którzy przez dwa tygodnie przed pobraniem nie przyjmowali leków/suplementów o działaniu antypłytkowym ani substancji uzależniających, takich jak: alkohol, tytoń, nikotyna. Z krwi wyizolowano osocze ubogopłytkowe, bogatopłytkowe oraz płytki krwi metodą wirowania różnicowego (Kłyszejko-Stefanowicz, 2013).

Badania zostały przeprowadzone za zgodą Komisji Bioetycznej Uniwersytetu Łódzkiego na podstawie protokołu nr 8/KBBN-UŁ/III/2018.

Metody:

Metodyka przeprowadzona w IUNG-PIB w Puławach:

- ✓ Przygotowanie materiału roślinnego do badań (ekstrakcja)
- ✓ Analiza fitochemiczna (jakościowa i ilościowa) materiału roślinnego wykonana techniką wysokosprawnej chromatografii cieczowej ze spektrometrem masowym (ang. high-performance liquid chromatography-mass spectrometry) HPLC-MS
- ✓ Analiza całkowitego potencjału antyoksydacyjnego z wykorzystaniem techniki cienkowarstwowej chromatografii z 2,2-difenylo-1-pikrylohydrazylem DPPH (TLC-DPPH•)

Metodyka przeprowadzona w Katedrze Biochemii Ogólnej, Wydział BIOŚ UŁ:

W celu oceny wpływu preparatów roślinnych na biomarkery stresu oksydacyjnego w osoczu zmierzono poziom:

- ✓ peroksydacji lipidów metodą z kwasem tiobarbiturowym (TBA),
- ✓ grup karbonylowych w białkach metodą kolorymetryczną z 2,4-dinitrofenylohydrazyną (DNPH),
- ✓ grup tiolowych w białkach metodą kolorymetryczną z kwasem 5,5'-ditiobis(2-nitrobenzoesowym) (DTNB, odczynnik Ellmana),
- ✓ zdolność absorbcji rodników tlenowych (ang. oxygen radical absorbance capacity ORAC)" "OxiSelect™ ORAC Activity Assay Kit"
- ✓ całkowity potencjał antyoksydacyjny (ang. total antioxidant capacity) TAC "Antioxidant Assay Kit".

Dodatkowo, analizie poddano wpływ preparatów na elementy hemostazy, takie jak:

- ✓ czasy krzepnięcia tj. czas trombinowy (*ang.* thrombin time, TT), czas protrombinowy (*ang.* prothrombin time, PT) i czas częściowej tromboplastyny po aktywacji (*ang.* activated partial tromboplastin time, APTT) metodą koagulometryczną w osoczu,
- ✓ tworzenie skrzeplin w warunkach przepływu krwi z wykorzystaniem techniki microchipów we krwi pełnej (*ang.* total thrombus formation analysis system, T-TAS),

- ✓ adhezję płytek krwi do kolagenu i fibrynogenu metodą statyczną na podstawie aktywności kwaśnej fosfatazy w przemytych płytkach krwi,
- ✓ ekspozycję selektyny P i zmiany konformacji receptora GPIIb/IIIa na powierzchni płytek krwi metodą cytometrii przepływowej we krwi pełnej,
- ✓ przemianę arachidonaniu w płytkach krwi metodą z kwasem tiobarbiturowym (TBARS).

Analiza toksyczności preparatów z wybranych warzyw na płytki krwi:

- ✓ aktywność zewnątrzkomórkowej dehydrogenazy mleczanowej (ang. lactate dehydrogenase, LDH).

Przygotowanie materiału roślinnego:

W pracy (3 i 4) opisano ekstrakcję materiału roślinnego. 1 mg liofilizatu poddano ekstrakcji z wykorzystaniem ekstraktora Dionex ASE 200 Accelerated Solvent Extraction System. Warunki ekstrakcji: rozpuszczalnikiem był 80% metanol, ciśnienie 1500 psi, temperatura 40ºC, 3 cykle ekstrakcji. Uzyskane preparaty oczyszczono z cukrów z wykorzystanie ekstrakcji SPE, następnie rozpuszczono w 70% metanolu za pomocą ultradźwięków. Z uzyskanego preparatu pobrano 150 µl do analizy HPLC-MS.

Przygotowanie materiału roślinnego do analiz aktywności biologicznej

Uzyskane preparaty z testowanych warzyw rozpuszczono w 50% DMSO. Stężenie końcowe preparatu w próbce wynosiło od 1-50 µg/ml. Końcowe stężenie DMSO w próbie nie przekraczało 0,05%, a jego ewentualny efekt był analizowany we wszystkich badaniach dotyczących aktywności biologicznej **(praca 3 i 4)**.

WYNIKI

Analiza fitochemiczna preparatów z wybranych warzyw

Analizy jakościowej i ilościowej preparatów z warzyw dyniowatych i astrowatych dokonano w Zakładzie Biochemii i Jakości Plonów w Instytucie Uprawy Nawożenia i Gleboznawstwa – Państwowym Instytucie Badawczym w Puławach. Analizę przeprowadzono z wykorzystaniem wysokosprawnej chromatografii cieczowej sprzężonej ze spektrometrią masową na chromatgrafie Dionex UltiMate 3000RS z detektorem CAD, na kolumnie Acquity UPLC BEH C18, w temperaturze 60ºC, dla preparatów z warzyw dyniowatych **(praca 3)** oraz kolumnie Acquity UPLC HSS T3, w temperaturze 50ºC dla preparatów z warzyw astrowatych **(praca 4)**. Faza ruchoma zawierała wodę i acetonitryl. Związki zostały zidentyfikowane na podstawie spektrometrii mas, techniką jonizacji ESI (elektrorozpylanie). W preparatach z warzyw z rodziny dyniowatych scharakteryzowano 36 metabolitów wtórnych. W przeważającym stopniu zaobserwowano różnice pomiędzy zawartością związków w składzie poszczególnych preparatów warzyw przynależnych do jednej rodziny. Większość scharakteryzowanych związków należała do: glikozydów fenyloetanoidowych, flawonoidów, kwasów tłuszczowych, lipidów oraz glicerofosfolipidów. Najmniejszą różnorodność fitochemiczną wykazują preparaty z dyni i cukinii. W ich składzie pośród związków fenolowych zidentyfikowano identyczne pochodne kempferolu i kwasu synapinowego. Pozostałe preparaty zawierają wiele związków należących do grupy fenyloetanoidów, występujących w formie glikozydów oraz flawonoidy, między innymi: rutynę, ksantoraminę, primulawerynę, 3-O-rutyno-izoramnetynę, czy kwercytynę. Spośród aminokwasów zidentyfikowano głównie fenyloalaninę i tryptofan, obecne we wszystkich preparatach. Najwyższą zawartością i różnorodnością kwasów tłuszczowych charakteryzuje się preparat z cukinii. Zidentyfikowano również kilka związków, na przykład w preparacie z dyni obecny jest ester 3,4-dihydroksyfenylo-1-metylowy kwasu karbaminowego, w obydwu patisonach monoglukozyd sekoizolarycyrezinolu, a we wszystkich preparatach kwas azelainowy **(praca 3).** W preparatach z warzyw z rodziny astrowatych scharakteryzowano 44 metabolity wtórne, które dzieliły się na trzy główne grupy: kwasy fenolowe, flawonoidy, laktony seskwiterpenów. Dodatkowo zidentyfikowano obecność aminokwasów, kwasów tłuszczowych i lipidów. Największą różnorodnością charakteryzuje się preparat z sałaty czerwonej, a najmniejszą topinamburu. Najliczniejszą grupę stanowiły kwasy fenolowe, spośród których zidentyfikowano pochodne kwasu hydroksycynamonowego, takie jak: izomery kwasu chlorogenowego i kwas 1,5-dikawoilochinowy, a także pochodnych kwasu

ferulowego, kwasu p-kumarowego i kwasu cykoriowego. 12 spośród wszystkich związków należących do kwasów fenolowych zidentyfikowano w sałacie czerwonej, w tym kwas kaftarowy, który obecny był tylko w sałacie czerwonej. Wszystkie scharakteryzowane flawonoidy obecne były tylko w preparatach z dwóch gatunków sałat. Przeważającą część stanowiły O-glikozydy kwercetyny lub kempferolu, dodatkowo często połączone były z kwasem glukuronowym. Większość z dziewięciu zidentyfikowanych laktonów seskwiterpenowych wykryto w preparacie z liści cykorii. Głównie są to pochodne laktucyny i laktukopikryny. W preparacie z topinamburu nie wykryto żadnego związku należącego do laktonów seskwiterpenowych. Preparaty z liści cykorii i bulw topinamburu zawierały dwa zidentyfikowane aminokwasy, tryptofan i metylotryptofan. W lipidach przeważały glicerofosfolipidy, obecne we wszystkich przebadanych preparatach z warzyw z rodziny astrowatych **(praca 4).**

Analiza właściwości antyoksydacyjnych preparatów z warzyw z rodziny dyniowatych i warzyw z rodziny astrowatych

W celu oceny właściwości antyoksydacyjnych preparatów z wybranych warzyw wykonano analizę potencjału wychwytywania wolnych rodników z wykorzystaniem chromatografii cienkowarstwowej z 2,2-difenylo-1-pikrylohydrazolem (TLC-DPPH•). Chromatografia cienkowarstwowa to prosta i szybka metoda rozdziału związków. Zastosowanymi wzorcami był kwas chlorogenowy oraz rutyna. Spośród warzyw dyniowatych najwyższą aktywność do wychwytywania wolnych rodników miał preparat z cukinii, nieco niższą preparat z patisona żółtego, a najniższą ogórek. Potencjał antyoksydacyjny preparatu z cukinii jest powiązany z wysoką zawartością flawonoidów, obecnych tylko w tym preparacie, takich jak: rutyna czy ksantoramina. Równie wysoka aktywność preparatu z patisona żółtego wynika z wysokiej zawartości pochodnych kwasu benzoesowego, głównie kwasu 3-(β-D-glukopiranozylo)-2-hydroksybenzoesowygo (**praca 3**). W przypadku preparatów z warzyw astrowatych najwyższą aktywnością antyoksydacyjną charakteryzuje się preparat z sałaty czerwonej, a najniższą preparat z topinamburu, co jest skorelowane z ich składem chemicznym. Preparat z sałaty czerwonej charakteryzował się największą różnorodnością w składzie chemicznym, ponadto najwyższym stężeniem związków fenolowych (**praca 4**).

Ocenę potencjału antyoksydacyjnego wykonanego także w osoczu z wykorzystaniem analizy ORAC (OxiSelect™ ORAC Activity Assay) oraz TAC (Total Antioxidant Assay KIT). Niestety, zarówno w analizie ORAC, jak i TAC nie zaobserwowano

istotnych statystycznie zmian w stosunku do kontroli, czyli osocza nietraktowanego preparatami z wybranych warzyw z rodziny astrowatych i dyniowatych **(praca 3 i 4)**. Dokonano również analizy wpływu preparatów roślinnych na poziom innych biomarkerów stresu oksydacyjnego w osoczu, w warunkach *in vitro*. Induktorami stresu oksydacyjnego była mieszanina 4,7 mM H_2O_2, 3,8 mM $FeSO_4$, 2.5 mM EDTA. Poziom peroksydacji lipidów określono na podstawie reakcji z kwasem triobarbiturowym. Zaobserwowano, że preparaty z warzyw dyniowatych w zakresie stężeń 1-50 µg/ml istotnie zahamowały peroksydację lipidów osocza traktowanego H_2O_2/Fe^{2+}. W przypadku preparatów z warzyw z rodziny astrowatych istotne zahamowanie peroksydacji lipidów zaobserwowano tylko dla cykorii, sałaty zielonej i topinamburu w najwyższym stężeniu (50 µg/ml). Spośród preparatów z warzyw z rodziny astrowatych istotne właściwości protekcyjne względem grup tiolowych w białkach osocza zaobserwowano dla wszystkich badanych preparatów (w stężeniach 5, 10 i 50 µg/ml dla preparatu z cykorii, w stężeniach 1, 10 i 50 µg/ml dla preparatu z sałaty czerwonej, w stężeniach 10 i 50 µg/ml dla preparatu z topinamburu i najniższym stężeniu dla preparatu z sałaty zielonej). W przypadku preparatów z warzyw z rodziny dyniowatych istotne właściwości protekcyjne względem grup tiolowych w białkach osocza zaobserwowano tylko dla preparatu z dyni, ogórka i patisona żółtego. Istotne statystycznie zahamowanie karbonylacji białek w osoczu w stosunku do próby kontrolnej (osocza z induktorem stresu oksydacyjnego H_2O_2/Fe^{2+}) zaobserwowano dla preparatów z warzyw z rodziny dyniowatych: z dyni (w stężeniach 5 i 50 µg/ml) oraz ogórka, patisona białek i żółtego w najwyższym stężeniu (50 µg/ml). Istotne statystycznie zahamowanie karbonylacji białek w osoczu w stosunku do próby kontrolnej (osocza z induktorem stresu oksydacyjnego H_2O_2/Fe^{2+}) zaobserwowano dla wszystkich preparatów astrowatych, w tym cykorii i sałaty zielonej w stężeniach 10 i 50 µg/ml, oraz sałaty czerwonej i topinamburu w najwyższym stężeniu (50 µg/ml) **(praca 3 i 4)**. Zaobserwowane istotne zamiany już w niskich stężeniach 1 i 5 µg/ml tylko dla preparatu z patisona żółtego sugerują, że charakteryzuje go najsilniejsza aktywność antyoksydacyjna w badaniach *in vitro* spośród preparatów z warzyw z rodziny dyniowatych. Jako jedyny z przebadanych preparatów z warzyw dyniowatych w swoim składzie zawiera glukozyd fenylopropanoidowy. Związek ten charakteryzuje się zdolnością do hamowanie peroksydacji lipoprotein o niskiej gęstości i chelatowania jonów metali, co powiązane jest z obecnością grup fenyloetanoidowych w jego strukturze (López-Munguía i wsp. 2011).

Najlepszymi właściwościami antyoksydacyjnymi spośród preparatów z warzyw astrowatych charakteryzował się preparat z cykorii, w którym zidentyfikowano między innymi

antocyjany, jako główną grupę związków. Silne właściwości antyoksydacyjne cykorii są związane z obecnością antocyjanów. Mulabagal i wsp. (2009) również wykazał hamujące działanie preparatu z cykorii na peroksydację lipidów w osoczu (Mulabagal i wsp. 2009) **(praca 4).**

Analiza właściwości antykoagulacyjnych preparatów z warzyw z rodziny dyniowatych i warzyw z rodziny astrowatych

Analizę wpływu preparatów z wybranych warzyw w zakresie stężeń 1-50 µg/ml na właściwości koagulacyjne osocza wykonano poprzez pomiar czasu protrombinowego, czasu trombinowego oraz czasu częściowej tromboplastyny po aktywacji z wykorzystaniem koagulometru Optic Coagulation Analyser (Kselmed, Grudziądz, Polska). Zarówno, w przypadku preparatów z warzyw z rodziny dyniowatych, jak i astrowatych nie zaobserwowano zmian istotnych statystycznie w stosunku do osocza nietraktowanego preparatami roślinnymi **(praca 3 i 4)**.

Do oceny procesu tworzenia skrzepu w pełnej krwi traktowanej preparatami z wybranych warzyw wykorzystano system T-TAS, który umożliwia analizę przepływu krwi w czasie rzeczywistym w warunkach półfizjologicznych. W badaniu zastosowano chipy pokryte kolagenem, które służą do wizualizacji skrzepliny płytek krwi. Uzyskane wyniki przedstawiono, jako wartość wyliczoną na podstawie parametru AUC_{10} (Area Under the Curve). W przypadku preparatów z warzyw z rodziny dyniowatych zaobserwowano istotne zahamowanie powstawania skrzepu dla 4 preparatów (dyni, ogórka, patisona żółtego i patisona białego) w najwyższym stężeniu (50 µg/ml) **(praca 5)**. Preparaty z warzyw z rodziny astrowatych nie wykazały istotnych zmian w stosunku do próby kontrolnej, natomiast istotne zahamowanie procesu zaobserwowano dla preparatu z korzeni mniszka pospolitego w stężeniu 50 µg/ml **(praca 6)**.

Analiza zmian aktywacji płytek krwi w obecności preparatów z warzyw z rodziny dyniowatych i astrowatych

Analizie poddano między innymi zdolność płytek krwi do adhezji do dwóch białek adhezyjnych: kolagenu i fibrynogenu, w obecności preparatów z wybranych warzyw. Płytki krwi uzyskano metodą wirowania różnicowego ze świeżo pobranej krwi od zdrowych dawców. Poziom adhezji mierzono przez pomiar aktywności kwaśnej fosfatazy metodą kolorymetryczną, a wyniki przedstawiono jako procent od uzyskanego poziomu adhezji płytek krwi nietraktowanych preparatami roślinnymi. Adhezję do kolagenu prowadzono w dwóch

układach: (1) płytki niestymulowane i (2) stymulowane trombiną, w przypadku fibrynogenu również zastosowano dwa układy: (1) płytki krwi stymulowane ADP oraz (2) płytki krwi stymulowane trombiną **(praca 5 i 6).** W przypadku preparatów z warzyw z rodziny dyniowatych istotne zahamowanie adhezji niestymulowanych płytek krwi do kolagenu zaobserwowano dla preparatów z dyni, cukinii i patisona białego w stężeniu 50 µg/ml, oraz dla ogórka w stężeniach 5 i 50 µg/ml, a dla płytek krwi stymulowanych trombiną istotne zahamowanie zaobserwowano dla preparatów z dyni i ogórka w obydwu stężeniach (5 i 50 µg/ml) oraz dla preparatu z patisona żółtego w najwyższym stężeniu (50 µg/ml). Istotne zahamowanie poziomu adhezji do fibrynogenu w układzie z płytkami krwi stymulowanymi trombiną stwierdzono w obydwu stężeniach (5 i 50 µg/ml) dla preparatów z cukinii i patisona białego, oraz w najwyższym stężeniu (50 µg/ml) dla dyni i patisona żółtego. Nie zaobserwowano natomiast istotnych zmian w drugim układzie dla fibrynogenu, gdzie płytki krwi były stymulowane ADP **(praca 5).** Wszystkie testowane preparaty z wybranych warzyw z rodziny astrowatych oraz preparat z korzeni mniszka pospolitego istotnie zahamowały poziom adhezji płytek krwi do kolagenu. W płytkach krwi niestymulowanych istotne zahamowanie zaobserwowano dla preparatów z cykorii i zielonej sałaty w obydwu stężeniach (5 i 50 µg/ml), a dla preparatów z sałaty czerwonej, topinamburu i korzeni mniszka pospolitego w najwyższym stężeniu (50 µg/ml). W przypadku adhezji płytek krwi do fibrynogenu, w układzie z płytkami stymulowanych trombiną istotne zahamowanie stwierdzono w obydwu stężeniach dla preparatów z cykorii i topinamburu, natomiast w najwyższym stężeniu dla preparatów z obydwu gatunków sałat i korzeni mniszka pospolitego. Również w układzie z płytkami krwi stymulowanymi trombiną zaobserwowano istotne zahamowanie adhezji dla wszystkich przebadanych preparatów, w tym w obydwu stężeniach dla preparatu z cykorii, sałaty zielonej, sałaty czerwonej, a w najwyższym stężeniu dla topinamburu, korzeni i owoców mniszka pospolitego. Natomiast w układzie z płytkami krwi stymulowanymi ADP tylko preparat z owoców mniszka pospolitego nie wykazał istotnych zmian w stosunku do kontroli **(praca 6).**

Aktywację płytek w krwi pełnej pod wpływem preparatów z testowanych warzyw oceniono również na podstawie pomiarów poziomu ekspozycji P selektyny oraz zmian w konformacji receptora GPIIb/IIIa. Pomiary wykonywano techniką cytometrii przepływowej we krwi pełnej. Krew inkubowano z preparatami w stężeniach 5 i 50 µg/ml, przez 30 min, w temperaturze 37ºC. Zarówno ekspozycja P selektyny, jak i zmiany konformacyjne receptora GPIIb/IIIa badano w 4 układach: (1) z płytkami niestymulowanymi, (2) z płytkami

stymulowanymi 10 μM ADP, (3) z płytkami stymulowanymi 20 μM ADP oraz (4) z płytkami stymulowanymi 10 μg/ml kolagenem. Do analizy zmian w ekspozycji P selektyny zastosowano przeciwciało CD62-P PE, a zmian w konformacji receptora GPIIb/IIIa zastosowano przeciwciało PAC-1 FITC (**praca 5 i 6**). Trzy preparaty z wybranych warzyw z rodziny dyniowatych (ogórek, patison żółty i patison biały) w stężeniu 50 μg/ml powodowały istotne obniżenie zdolności receptora GPIIb/IIIa do wiązania PAC-1 w płytkach krwi aktywowanych 10 μM ADP. W pozostałych układach nie zaobserwowano żadnych istotnych zmian. Nie zaobserwowano też istotnego wpływu preparatów na ekspozycję P selektyny **(praca 5).** W przypadku warzyw z rodziny astrowatych również nie stwierdzono istotnych zmian w ekspozycji P selektyny oraz zmian w konformacji receptora GPIIb/IIIa, natomiast dla preparatów z owoców i korzeni mniszka pospolitego zaobserwowano istotne obniżenie ekspozycji P selektyny oraz zmian w konformacji GPIIb/IIIA w układzie z płytkami krwi stymulowanymi kolagenem. Dodatkowo, preparat z owoców mniszka pospolitego ograniczył zmiany konformacyjne receptora GPIIb/IIIa w układzie z płytkami krwi stymulowanymi 20 μM ADP **(praca 6).**

Analiza przemiany arachidonaniu (enzymatycznej peroksydacji lipidów) w płytkach krwi w obecności preparatów z wybranych warzyw z rodziny dyniowatych i astrowatych

Aktywacja płytek krwi jest powiązana z przemianami biochemicznymi, prowadzącymi m. in. do metabolizm kwasu arachidonowego, w trakcie którego powstaje tromboksan A_2. Do oceny metabolizmu kwasu arachidonowego w płytkach krwi inkubowanych z preparatami z wybranych warzyw z rodziny dyniowatych i rodziny astrowatych wykorzystano metodę z kwasem triobarbiturowym. Płytki krwi aktywowano trombiną. Wszystkie badane preparaty z warzyw z rodziny dyniowatych w stężeniach 5 i 50 μg/ml istotnie obniżyły poziom peroksydacji kwasu arachidonowego w płytkach krwi. Najwyższą aktywność miał preparat z patisona żółtego, dla którego stwierdzono aż w 85% zahamowanie peroksydacji w stosunku do próby kontrolnej (płytek krwi aktywowanych trombiną) (**praca 5).** Dwa preparaty z warzyw z rodziny astrowatych także istotnie zahamowały peroksydację kwasu arachidonowego, w tym preparat z sałaty czerwonej w stężeniu 50 μg/ml, a preparat z topinamburu w stężeniach 5 and 50 μg/ml. W przypadku preparatu z topinamburu zaobserwowano zahamowanie peroksydacji na poziomie 60%. Efekt ten powiązany jest z obecnością kwasów fenolowych, w tym kwasu 5-kawoilochinowego, kwasu kawowo-winowego, kwasu 3-kawoilochinowego, flawonoidów, w tym pochodnych kwercetyny i laktonów seskwiterpenowych. Związki te prawdopodobnie modulują aktywność płytek krwi

poprzez interakcję z metabolizmem kwasu arachidonowego na etapie modulacji aktywności cyklooksygenazy **(praca 6).**

Ocena cytotoksyczności preparatów z wybranych warzyw z rodzin dyniowatych i astrowatych

Ocenę toksyczności preparatów z wybranych warzyw względem płytek krwi przeprowadzono poprzez oznaczenie aktywności zewnątrzkomórkowej dehydrogenazy mleczanowej (ang. *lactate dehydrogenase*, LDH). Jest to marker uszkodzenia komórek, pozwalający na określenie toksyczności preparatów względem płytek krwi. Płytki krwi wyizolowano ze świeżej krwi pobranej od zdrowych dawców, a następnie inkubowano z preparatami z wybranych warzyw przez 30 min, w temp. 37ºC. Zarówno, preparaty z warzyw z rodziny dyniowatych, jak i preparaty z warzyw z rodziny astrowatych nie spowodowały istotnych zmian w poziomie dehydrogenazy mleczanowej, co wskazuje na brak toksyczności względem płytek krwi **(praca 5 i 6).**

WNIOSKI:

- Zarówno warzywa z rodziny dyniowatych, jak i astrowatych mają bogaty i różnorodny skład chemiczny. W obydwu rodzinach warzyw zidentyfikowano aminokwasy, kwasy tłuszczowe, liczne związki fenolowe, takie jak flawonoidy i kwasy fenolowe.

- Preparaty z wybranych warzyw z rodziny dyniowatych i warzyw z rodziny astrowatych wykazują aktywność antyoksydacyjną i antypłytkową, w tym antyadhezyjną. Potencjalny antypłytkowy mechanizm ich działania może obejmować: hamowanie przemiany kwasu arachidonowego w płytkach krwi, blokując powstawanie tromboksanu A_2. W przypadku warzyw z rodziny dyniowatych potencjalny mechanizm może obejmować inhibicję zmian konformacyjnych receptora GPIIb/IIa.

- Najwyższym potencjałem antypłytkowym i antyoksydacyjnym spośród preparatów z warzyw z rodziny dyniowatych charakteryzuje się patison żółty, co jest skorelowane z szeroką gamą związków fenolowych, obecnych w jego składzie chemicznym.

- Spośród preparatów z warzyw z rodziny astrowatych najwyższą aktywność antyoksydacyjną w osoczu wykazała cykoria, co skorelowane jest z wysoką zawartością kwasów fenolowych i antocyjanidynów. Natomiast najlepszą aktywnością antypłytkową (w tym antyadhezyjną) charakteryzuje się preparat z sałaty czerwonej, co skorelowane jest wysoką z zawartością kwasów fenolowych.

STRESZCZENIE

Choroby układu sercowo-naczyniowego od wielu lat są w czołówce przyczyn zgonów na całym świecie. Ich podłożem są zaburzenia procesu hemostazy, które mogą być z kolei spowodowane nieprawidłową aktywacją płytek krwi, prowadzącą do powstawania zakrzepów i zatorów w naczyniach krwionośnych. Dodatkowym podłożem dla chorób układu sercowo-naczyniowego jest stres oksydacyjny. Ponadto, jednym z najważniejszych czynników ryzyka wystąpienia i rozwoju tych chorób jest nieprawidłowa dieta, bogata w wysoko przetworzone produkty, nasycone kwasy tłuszczowe i węglowodany proste. Badania naukowe ostatnich lat sugerują, że dieta bogata w owoce i warzywa może mieć nie tylko prewencyjne, ale również terapeutyczne oddziaływania w chorobach układu krążenia.

Warzywa z rodzin dyniowatych i astrowatych, od wielu lat, są stałym elementem diety w różnych częściach świata. Dodatkowo, ze względu na swoją aktywność biologiczną, stosowane były już w medycynie tradycyjnej. Współczesne dane literaturowe potwierdzają ich szeroką aktywność prozdrowotną, w tym antyoksydacyjną, przeciwzapalną i przeciwnowotworową. Nadal jednak jest bardzo mało doniesień na temat ich wpływu na parametry hemostazy.

Głównymi celami niniejszej pracy była analiza składu chemicznego preparatów z wybranych warzyw dyniowatych i astrowatych oraz ocena ich wpływu na wybrane parametry hemostazy w układzie *in vitro.* Materiał roślinny stanowiły jadalne elementy wybranych warzyw z rodziny dyniowatych (dynia, cukinia, ogórek patison biały oraz patison żółty) oraz z rodziny astrowatych (cykoria, sałata zielona, sałata czerwona, topinambur). Dodatkowym, porównawczym materiałem roślinnym były korzenie i owoce mniszka pospolitego. Materiał badawczy stanowiła też krew pełna, osocze ubogopłytkowe oraz płytki krwi wyizolowane ze świeżo pobranej krwi od zdrowych dawców.

Analizę składu chemicznego wykonano techniką HPLC-MS. Dokonano też oceny aktywności antyoksydacyjnej badanych preparatów, poprzez analizę całkowitego potencjału antyoksydacyjnego z wykorzystaniem metody TLC-DDPH•, jak również poprzez pomiary różnych biomarkerów stresu oksydacyjnego w osoczu. Ocenę właściwości antypłytkowych wykonano stosując analizę wpływu preparatów roślinnych na adhezję płytek krwi do kolagenu i fibrynogenu, ekspozycję selektyny P i zmiany konformacji receptora GPIIb/IIIa na powierzchni płytek krwi metodą cytometrii przepływowej oraz przemiany arachidonaniu w płytkach krwi. Analizę właściwości antykoagulacyjnych preparatów wykonano poprzez

pomiary czasów krzepnięcia w osoczu oraz ocenę tworzenia skrzepliny w warunkach przepływu krwi z wykorzystaniem systemu T-TAS w krwi pełnej.

Zastosowane badania obrazują bogaty skład chemiczny preparatów. Zidentyfikowano obecność aminokwasów, kwasów tłuszczowych oraz związków fenolowych, w tym kwasów fenolowych i flawonoidów. W przeprowadzonych badaniach *in vitro* uzyskano istotny wpływ preparatów z warzyw dyniowatych i astrowatych na wybrane elementy hemostazy. Ponadto, wszystkie testowane preparaty roślinne wykazują aktywność antyoksydacyjną w osoczu. Charakteryzują się również aktywnością antypłytkową. Najwyższą aktywność antyoksydacyjną spośród warzyw dyniowatych posiada patison żółty, natomiast spośród warzyw astrowatych najwyższą aktywnością antyoksydacyjną charakteryzowała się cykorią, a najwyższą aktywnością antypłytkową sałata czerwona. Aktywności biologiczne tych roślin są silnie skorelowane z ich składem chemicznym.

SUMMARY

Cardiovascular diseases have been in the top ten reason of death around the world for many years. Their background is based on hemostasis disorders, which can be causes by dysfunction of blood platelets, leading to blood clots and blockages in blood vessels. Additional reason of cardiovascular diseases is oxidative stress. One of the main risk factors of development of cardiovascular diseases is unhealthy diet, rich in ultra-process food, saturated fatty acids and simple carbohydrates. Studies suggests that diet rich in fruits and vegetables can have not only preventive action, but also therapeutic effect on the circulatory system.

Vegetables from *Cucurbitaceae* and *Asteraceae* families are part of everyday diets around the world for many years. Additionally, due to its biologically activity, they were often used in the traditional medicine. Nowdays literature data confirm their broad pro-health activity, including antioxidant, anti-inflammatory and antitumor activity. However, there are still very few reports on their influence on the parameters of hemostasis.

The main aims of this dissertation were the analysis of chemical composition of preparation from selected vegetables from *Cucurbitaceae* and *Asteraceae* family and their effect on selected hemostasis parameters. Plant material was the edible elements of selected vegetables from the *Cucurbitaceae* family (pumpkin, zucchini, cucumber, white pattypan squash and yellow pattypan squash) and from the *Asteraceae* family (chicory, green lettuce, red lettuce, Jerusalem artichoke), additional plant material was dandelion roots and fruits. The biological material consisted of whole blood, plasma and blood platelets isolated from healthy donors.

For analysis of chemical composition HPLC-MS methods was used. Evaluation of antioxidant activity was measure with technique TLC-DDPH•, and the measurement of various biomarkers of oxidative stress in human plasma in *in vitro* models. Analysis of antiplatelet activity was measure by analysis of effect of tested preparations on adhesion of blood platelets to collagen and fibrinogen, P-selectin exposure, and changes in GPIIb/IIIa receptor conformation on the platelet surface by flow cytometry and arachidonic acid transformation in platelets. The anticoagulant properties of the preparations were analyzed by measuring plasma coagulation times and assessing thrombus formation under blood flow conditions using the T-TAS system in whole blood.

The conducted research shows the presence of several compounds belonging to the derivatives of phenolic acids and flavonoids, as well as amino acids and fatty acids in the

composition of preparations belonging to the cucurbit's family and the *Asteraceae* family. Additionally, they confirm the significant influence of preparations made of cucurbits and *Asteraceae* on selected elements of hemostasis. All tested preparations show antioxidant activity in plasma. Additionally, they are also characterized by anti-platelet activity. Yellow pattypan squash was characterized by the best activity among cucurbits, while among *Asteraceae* vegetables, chicory had the highest antioxidant activity, and red lettuce had the highest antiplatelet activity. Their biological activities are strongly correlated with their chemical composition.

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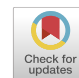
Review Article

Vegetables from the *Cucurbitaceae* family and their products: Positive effect on human health



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ABSTRACT

The *Cucurbitaceae* family is a large group of crops with more than 800 species known worldwide. Vegetables from this family have been used for centuries, not only for consumption, but also for their medicinal value. The most characteristic cucurbits are pumpkin and cucumber, which are cultivated and consumed in many parts of the world. Seeds from cucurbits have many health benefits and are a popular snack. Cucurbit plants are rich in carotenoids, terpenoids, saponins, and phytochemicals. Vegetables from the *Cucurbitaceae* family have a positive influence on human health, and various studies have clearly indicated that cucurbit vegetables have antioxidant, antidiabetic, antiinflammatory, and purgative properties. This mini review evaluates the current literature about vegetables from the *Cucurbitaceae* family and their products, in addition to their positive effect on human health.

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Introduction

Diet has a key role in human health. A healthy diet depends on its quality, which means a healthy energy balance, and a variety of nutrients. An easy way to achieve diversity in a diet is to look at groups of food that can be translated without a problem into a dietary recommendation. A significant example of this kind of food is vegetables. The recommendation is to eat at least 200 g of different vegetables per day [1]. In many parts of the world, wild edible vegetables are not the only source of food, but are also used as nutraceutical and functional foods [2]. Recent clinical and epidemiologic studies indicate that different vegetables and their products contain phyto-protective compounds that possess various biological activities, which may have a beneficial effect on human health. However, the mechanism of their action have not been clearly demonstrated [3].

In many countries, especially developing ones, almost 80% of the population choose traditional medicine over health care in their country. Bioactive natural compounds are the basic source for the development of modern pharmaceutical agents. All around the world, natural and conventional drug are used in 50% of total drug use [4]. Medical plants are cheaper than most chemical-based medicine. Observational studies have shown that the protective effect of a daily intake of vegetables for the prevention of store. The reason for this effect is based on vegetables as the main source of potassium, which positively influences blood pressure. Some studies have indicated that the regular consumption of vegetable juice may reduce blood pressure [1,5].

The present mini review examines the current literature concerning the positive role of vegetables from the *Cucurbitaceae* family for human health.

Characteristic of *Cucurbitaceae* family

Cucurbitaceae is a large family of plants, also known as cucurbits, with 130 genera and 800 species. The name of the *Cucurbitaceae* family came from Latin, where the word *corbis* means bottle or basket, cucurbits were used in various ways in the past (e.g., mature vegetables served as containers or even musical instruments) [6]. *Cucurbitaceae* are the most diverse plant family and are cultivated around the world in a variety of environmental conditions. More than 300 plant species are used by humans, but only 150 species are cultivated expansively, and 30 of these are crucial for the global food production. The leading producers of *Cucurbitaceae* are Turkey, China, India, and the United States. The cultivation of cucurbits for food goals started more than 3000 years ago in Western Asia [6].

Cucurbits come from a big and meaningful group of vegetables, and the most common are pumpkin, melon, watermelon, and cucumber. Many different cucurbits are consumed around the world, and the majority of the plants are medicinally valuable. These plants were used in traditional medicine as a remedy for various disease for ages, particularly within Chinese and Ayurvedic

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systems. Some reports show the many important physiological properties, such as cardiovascular, hepatoprotective, immunoregulatory, and anti-inflammatory activities. *Cucurbita pepo* was used in traditional medicine in countries such as China, India, Argentina, Brazil, and Mexico to, for example, treat patients internally and externally for worms and parasites. In Africa, pumpkin seeds are used to treat tapeworm, whereas in other folkloric medicine, seeds are part of their treatment for bladder and kidney disorders [7–9].

Bitter apple (*Cirtullus colocynthis*) is a plant that belongs to the *Cucurbitaceae* family and grows plentifully in Arabian countries. In traditional medicine, this plant was used as a hair growth promoter, purgative, analgesic, and abortifacient agent. In Native America, cucurbits were used in traditional medicine to treat urinary ailments and intestinal worms. The seeds from cucurbits were used to cure high blood pressure and prevent kidney stones. In southeastern Europe, *Cucurbita pepo* has helped heal prostate enlargements and irritable bladders [10]. Snake gourd (*Trichosanthes cucumerina*) is prescribed by local healers in India to cure headaches, abdominal tumors, fever, diarrhea, and skin allergies [11].

Melothria heterophylla is a scandent herb that belongs to the *Cucurbitaceae* family and grows in many different part of India. The herb was used in traditional medicine for purgative and invigorating effects. The leaves from this plant were used to prepare juices known for their antiinflammatory properties [12]. *Citrullus colocynthis* is another plant that belongs to the *Cucurbitaceae* family, and although native to Africa and tropical Asia, the plant is now also popular in the Mediterranean basin. Its leaves were used as a cholagogue and treatment for cough, and its roots were a remedy for breast inflammation as well as uterine and arthritic pain [13]. *Momordia charanti*a belong to cucurbits and is known as bitter ground or African cucumber. All fruits from *Momordia* are rich in many health-promoting, nutritional compounds and phytochemical agents. Bitter ground was used in folk medicine for numerous diseases, such as hypertension, cancer, obesity, as well as viral and bacterial infections. In Africa, the plant was used for syphilis, worm infections, inflammation, and skin disease [14]. Additionally, cucurbits are a good source of nutrients and nonnutritive compounds, such as proteins, carbohydrates, vitamins, and minerals [9,15].

The *Cucurbitaceae* family has an important economic role because vegetables and fruits from this family are used in various food products. Cucurbits can be used not only as a food source, but also in the cosmetic industry. Cucumber is often added to skin products because of its healing, cooling, and soothing properties, and is also an ingredient in many natural soaps. Mature fruits of *Luffa aegyptiaca* were used as natural sponges throughout history owing to their structure [16,17].

During the food production of cucurbits, a lot of fruits and vegetables are intended for disposal. Most of this waste are peels, seeds, and fruits, which do not satisfy esthetic demands but are still a source of compounds, such as polyphenols, and show antioxidant and antiinflammatory properties, which also makes cucurbits attractive for the cosmetic industry [18].

Botanic characteristics

Cucurbitaceae are mostly annual and rare perennial plants. Their stems are herbaceous and angular, usually trailing or climbing through tendrils; the leaves are generally lobed or divided, reticulated, and palmately veined with a long and hollow petiole. Both stems and leaves are full of juicy sap. Owing to the storage of water and food, the roots and branch thicken. *Cucurbitaceae* flowers are mostly white or yellow and generally unisexual. Both female and male flowers occur on the same plant. The inflorescence is large and showy. Its fruits are soft and fleshy, particularly indehiscent and often enormous, round size, with variations in shape, size, and color patterns in different species within this family [19].

Cucurbita pepo, also known as pumpkin, is one of the most well-known vegetables worldwide from the *Cucurbitaceae* family owing to its typical, big, ovoid-elliptical shape. *Cucurbita pepo* has three different color patterns, with the most recognized one being orange and the others light or dark green with longitudinal white lines or stripes and minute white and green spots. Pumpkin is an annual creeping plant that is immune to low and high temperature. Most of its parts, from a fleshy shell to its seeds, are edible. In many countries, oil from the seeds is often consumed, as well as the dried seeds as a healthy snack [20,21].

One of the lesser popular vegetables in the *Cucurbitaceae* family is zucchini, also known as courgette. Its shape resembles a ridged cucumber, but zucchini is available in yellow and green colors. Botanically, this vegetable is considered a fruit, but in gastronomic terms, this is a vegetable. Zucchini has a firm texture with ripened fruit and a characteristic flower [18,22,23].

Cucurbits in diets

Edible plants, derived from the *Cucurbitaceae* family, are a group of plants in five genera: *Cucurbita, Cucumis, Citrullus, Legenaria, and Sechium* [24]. The majority of the *Cucurbitaceae* family is a good source of proteins in a diet. Cucurbits, much like most vegetables, are a good source of dietary fiber, which provides a lot of healthy properties and helps reduce cholesterol, insulinemic response, and changes in the intestinal function. Cucurbits can prevent constipation and reduce the blood glucose level.

Seeds from cucurbits have one of the highest food values because they are rich in protein and minerals, such as copper, phosphorus, zinc, iron, and magnesium. The seeds are also a good source of carotenoids and tocopherols, particularly α- and γ-tocopherol. *Cucurbita moschota* seeds contain approximately 15.9 mg/100 g of total tocopherols. Cucurbit seeds have high essential fatty and linoleic acid, often taste sweet with a subtle nutty flavor, and are easily chewable. Pumpkin seeds are rich in dietary fiber, and flour from pumpkin seeds is often added to bakery products to enhance texture and flavor. Dietary fiber improves gel formation, as well as water and oil holding in baked goods. The fiber also improves nutritional quality owing to its health benefits [8,9,25]. Pumpkin seeds are used in the treatment of depression because they contain L-tryptophan, which raises serotonin levels in the brain (so-called happy hormone).

In Africa and the Middle West, seeds are used to produce cooking oil. In some countries, oil is even added to salads [15,20,24]. In many African regions, seeds from plants belonging to the *Cucurbitaceae* family are prescribed diets as a treatment for diabetes [26]. During the fall, pumpkin flavor is used as an addition to coffee. *Cucurbita pepo* is popular in the western world, but in Asia, *Cucurbita moschota* and *Cucurbita maxima* are consumed more often [21].

Cucumis sativus (i.e., cucumber) is one of the most popular vegetables around the world. More than 84 million tons of cucumbers are cultivated yearly and the biggest producer is China. Cucumbers are often eaten during the summer as a cooling food, and serve as popular additions to salads. Cucumber helps with indigestion and constipation [7,19,27]. Cucumber is a good source of potassium and vitamins C, K, and A. Additionally, cucumber is recommended as a dietary food owing to its 96% water content and only a few calories [19], and also has a positive effect on good looks. The regular

consumption of cucumber helps with skin problems, reduces swelling under the eyes, and even promotes hair growth [28].

Zucchini is popular among people on a diet due to its low amount of calories. For example, a medium-sized zucchini has only 25 calories. This is due to its high water content (approximately 96 %), but zucchinis still have high nutritious value because of their significant amount of potassium, folate, and vitamin A. Zucchinis also have a high content of magnesium and phosphorus, which are necessary to build and maintain healthy bones. Zucchini is also a very good source of vitamin C. The regular consumption of zucchinis helps treat asthma and can be used in the prevention of scurvy and bruising caused by a deficiency in vitamin C [23]. Zucchini can be eaten year round, but the most popular time is during the summer and early fall. The fleshy fruit is used for stir fry and pasta. The seeds are consumed along with the vegetable [9].

Fresh juice from *Momordica charantia* controls blood sugar and insulin levels, as well as improves the human digestive system. The daily dose of a *Momordica charantia* juice drink helps increase body stamina and prevents fatigue [4]. Bottle gourd is commonly cultivated in tropical areas of India, not only as an edible vegetable, but also a cardiotonic and aphrodisiac agent [11].

Cantaloupe is one of the most popular types of *Cucumis melon* around the world owing to its sweet, juicy taste and nutritional value. In 2016 alone, more than 1.9 million tons of melon were harvested in Europe. Cantaloupe is typically consumed fresh but can also be used to produce juice, salads, and desserts. Cantaloupe is a good source of vitamins A and E, magnesium, and potassium. Cantaloupe shows various health properties, such anticancer, antioxidant, diuretic, and antidiabetic activity. Studies have demonstrated that its peels and seeds, which were considered waste in the past, can be used in the food industry as nutraceutical products due to their composition. They are a source of polyphenols, including flavonoids (responsible for antioxidant activity) and tannis (responsible for antimutagenic and anticarcinogenic potentials) [29].

Chemical characteristics and health benefits of vegetables from the *Cucurbitaceae* family

The chemical composition of the *Cucurbitaceae* family consists of phytochemicals. They are nonnutritive compounds and occur naturally in plants. Some examples in plants are tannins, carbohydrates, saponins, and cardiac glycosides [8,25]. They contain a high level of bioactive compounds, such as triterpenes, sterol, and alkaloids. Terpenoids are biogenetically derived from active isoprene, and their basic structure is based on six isoprene units [30].

As a result of secondary metabolite cucurbitacin content, cucurbitacin terpenoids are highly oxygenated, manly, tetracyclic plant substances, but some have an extra ring because of formal cyclizations between C-16 and C-24. Their skeleton is based on 19-(10→9β)-abeo-10α-lanost-5-ene (Fig. 1). Cucurbitacin terpenoids are different from other terpenoids owing to the high degree of unsaturation and the presence of many acetoxy and keto groups. The level of cucurbitacin terpenoids depends on the plant tissue. The highest concentration is in mature fruit and the lowest in the seeds [19,31]. Cucurbitacin terpenoids are characterized by a bitter taste. They typically show toxicity, but in the right concentration, they display a potential to treat various pathologies, such as inflammation or autoimmune disease. In vivo toxicity reports show a range of toxicity dose between 2 and 12.5 mg/kg. Cucurbitacins are divided into 12 different categories from A to T, but cucurbitacins B and E possess the most promising properties and are used in clinical studies [8,19,30,31].

Most vegetables from the *Cucurbitaceae* family are rich in bioactive compounds that are responsible for the yellow-red pigmentation called carotenoids. More than 700 carotenoids occur naturally, but only 50 are known to be absorbed, metabolized, and used by the human body for health benefits. In Cucurbits, they appear as α-carotene, β-carotene, and lutein zeaxanthin [30,32]. Cucurbits can be a source of polysaccharides in a diet [33]. The total content of carotenoids in Cucurbita pepo range from 171.9 to 461.9 μg/g of fruits dry extract, and in *Cucurbita moschata* from 234.21 to 404.98 μg/g of fruits dry extract. The volume of carotenoids is often higher in the peel than the flesh. For example, in Cucurbita moschata this value is even 10 fold [17].

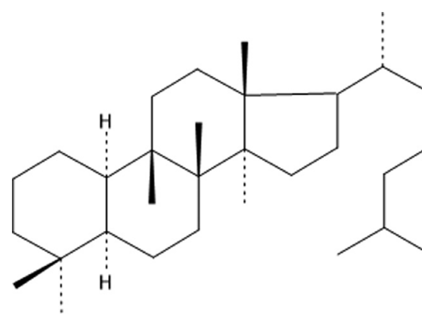

Fig. 1. Structure of cucurbita.

The entire *Cucurbitaceae* family shows numerous health benefits, but each vegetable or fruit has influence on human health in their own way (Table 1). Pumpkin shows a stimulant effect on the central nervous system and can be used for alternative treatment in conditions related to dizziness [8,33]. The fruits of leaf ground, also known as *Cucurbita ficifiola*, are used in some countries in medicine to heal wounds and hemorrhoids. Cucumber is rich in tannins and phytosterols. Tannins have astringent properties that hasten the healing process of wounds and are a potential metal ion chelator. Phytosterol shows significant hypocholesterolemic effects [8,16]. *Bryonia dioica*, also known as red bryony, is a source of remedy for anthropic arthritics and bronchial asthma and is often used as a powerful cure for snake bites [4]. *Mukai maderaspatana* has been used as cough medicine, and the roots can be chewed on to relieve toothaches [11]. Cucurbitacins are characterized by a bitter taste, and are even considered the most bitter-tasting compound in plants, which is the reason for their purgative properties. Cucurbitacins also stimulate gastric secretion [8,19,30,31].

Ischemic heart disease has been the main cause of death around the world for 15 years, and stroke was in second place in the top 10 world causes of deaths. In 2016 alone, 15.2 million people died worldwide from these two diseases [34]. The *Cucurbitaceae* family consists of many phytochemicals, which influence the cardiovascular system, such as saponins and cardiac glycosides (often used in the treatment of heart diseases). Saponins have the ability to coagulate red blood and help stop bleeding. Cucurbitacin also has an anti-atherosclerotic effect owing to its inhibiting lipid oxidation product (mostly malonaldehyde and 4-hydroxynonenal) [8,19,25,30]. *Momoridace balsamina* contains glycosides in its leaves and seeds. Glycosides are often used to treat cardiac diseases. These compounds increase the force of heart contractions owing to its increasing calcium-induced calcium release. Courgettes also help prevent heart disease and related symptoms, such as high cholesterol [8,23,33].

Table 1
Various biologic activates of the *Cucurbitaceae* family (in vitro and in vivo experiments)

Plant	Length of study	Experimental model	Effect	Reference
Cucumis melo Cantaloupe (seeds and peels)	–	2,2-diplenyl-1-picrylhydrazyl radical scavenging activity and ferric reducing antioxidant power (in vitro)	Antioxidant (Antioxidant power peels:12.27 ± 1.22 mg ascorbic acid equivalent/g of extract seeds: 0.31 ± 0.02 mg ascorbic acid equivalent/g of extract)	[29]
Cucurbita moschata (extracts)	48 h	Human cancer cell line: Hela, HCT-8, HepG-2 (in vitro)	Anticancer (cytotoxic activity to cancer cell)	[37]
Cucurbita moschata (Cucurmosin)	–	Cancer cell line: K562, B16, A 549 (in vitro)	Anticancer (works like ribosome-inactivating protein, inhibited cell tumor cell growth)	[36]
Cucurbita pepo	24, 48, 72 h	HaCat human keratinoytes cell line (in vitro)	Anticancer activity	[18]
Momordia charantia	–	Macrophageslike cells RAW 264,7 (in vitro)	Antiinflammatory activity suppressed tumor necrosis factor-α production and nuclear factor kappa-light-chain enhancer of activated B-cell DNA binding activity	[38]
Cucurbita ficifolia (fruit)	30 d	Male rats (in vivo)	Antidiabetic and antioxidant (reduce lipid peroxidation in pancreatic tissue and plasma glucose)	[35]
Cucurbitaceae family (seeds)	7 d	Male mice (in vivo)	Antidiabetic (goblins in seeds show antihyperglycemic activities)	[26]
Cucurbita moschata	4 mo	12 y old Asian patient with diabetes (in vivo)	Antidiabetic (decreased of glycosylated hemoglobin)	[10]
Melothria heterophylla	3 h	Rats model (in vivo)	Antiinflammatory (hinders release or action of prostaglandin)	[12]
Momordia charantia	3 mo	Patients with knee osteoarthritis (in vivo)	Antiinflammatory (improvement in knee osteoarthritis, reduction in analgesic score)	[14]
Cucurbita moschata	–	*Fusarium oxysporum Candida albicans* (in vivo)	Antimicrobial (synergism with chitin syntheses inhibitor)	[39]

Antioxidant activity

Oxidative stress is a lack of balance between pro- and antioxidant levels in favor of pro-oxidants. Oxidative stress is a harmful condition for the entire body and indications of disease, such as cancer or obesity. Cucurbits, owing to their various bioactive components, display antioxidant effects (e.g., compounds such as cucurbitacins B and E or ellagitannins, which belong to tannins and have a free radical scavenging ability) [8,20,31]. Most vegetables from the *Cucurbitaceae* family are also rich in carotenoids, which improve food health and stability because of their antioxidant power. Pumpkin oil is a good source of squalene and is a linear triterpenoid that plays an important role in maintaining oxidation stability of oil owing to its antioxidant properties [30,32].

Courgette shows antioxidant activities owing to its β-carotene content [19,23]. Compounds contained in the seeds power and pulp of bitter gourd display antioxidant activity by inhibiting lipid peroxidation [14]. Vella at el. (2019) demonstrated the antioxidant activity of extracts from the peels and seeds of cantaloupe melon in in vitro studies using 2,2-diplenyl-1-picrylhydrazyl radical scavenging activity and ferric reducing antioxidant power. Both extracts from peels and seeds displayed antioxidant properties owing to their high flavonoids content. Antioxidant power (measured by ferric reducing antioxidant power) in peels amounts to 12.27 ± 1.22 mg of ascorbic acid equivalent/g of extract, and in seeds to 0.31 ± 0.02 mg ascorbic acid equivalent/g of extract (Table 1).

Xia and Wang (2007) [35] have shown the antioxidant activates of *Cucurbita ficifolia* due to a reduction of lipid peroxidation in in vivo studies. The researchers used 24 male Sprangue-Dawley rats, and divided the animals in four groups: Normal rats as a control group, normal rats treated with *Cucurbita ficifolia* extract, diabetic rats treated with *Cucurbita ficifolia* extract, and untreated diabetic rats. All animals were fed daily *Cucurbita ficifolia* extracts for 30 d. The lipid peroxidation was measured with the thiobarbituric acid method. The results showed a significant decrease in lipid peroxidation compared with untreated diabetic rats (Table 1).

Antidiabetic activity

More than 380 million people suffer from diabetes, which is a huge financial and health care burden on the health care system. More and more researchers look for plants as a solution for diabetes therapy, among others in the cucurbit family [33]. Pumpkin has been shown to have significant antidiabetic effects. Protein-bound polysaccharides isolated from pumpkin seeds possess hypoglycemic activity, such as increasing insulin in plasma. Pumpkin also contains other biologically active components, such as para-aminobenzoic acid and sterols [15], and is rich in pectin, which controls glycemic levels and decreases the need for insulin in patients who consume fiber-rich foods [8,33].

Momordica charanita produce a variety of bioactive phytochemicals, such as triterpenoids, steroids, saponins, and alkaloids. The plant is often used in the treatment of diabetes owing to the properties of polysaccharides in unripe fruit. Even some molecules from this fruit have shown antidiabetic activity, including polypeptide-p, vicine, and charatin [4,33]. Courgette has also demonstrated antidiabetic properties [23]. Teugwa et al. (2019) proved that globulin isolated from seeds from the *Cucurbitaceae* family has antidiabetic activity. Using an oral glucose tolerance test, hypoglycemic properties were performed on 24 male Wistar rats in an in vivo model (Table 1). In vivo studies reported on a clinical case of a young Asian patient with diabetes who ate 200 g of pumpkin for 4 mo. Researchers observed a decrease in glycosylated hemoglobin from 10.8% to 8.5% after 2 mo (Table 1) [10]. Moreover, there are no recent in vitro studies on this subject.

Anticancer property

Cancer has many different variations and is one of the most common diseases around the world. Saponin lowers the risk of

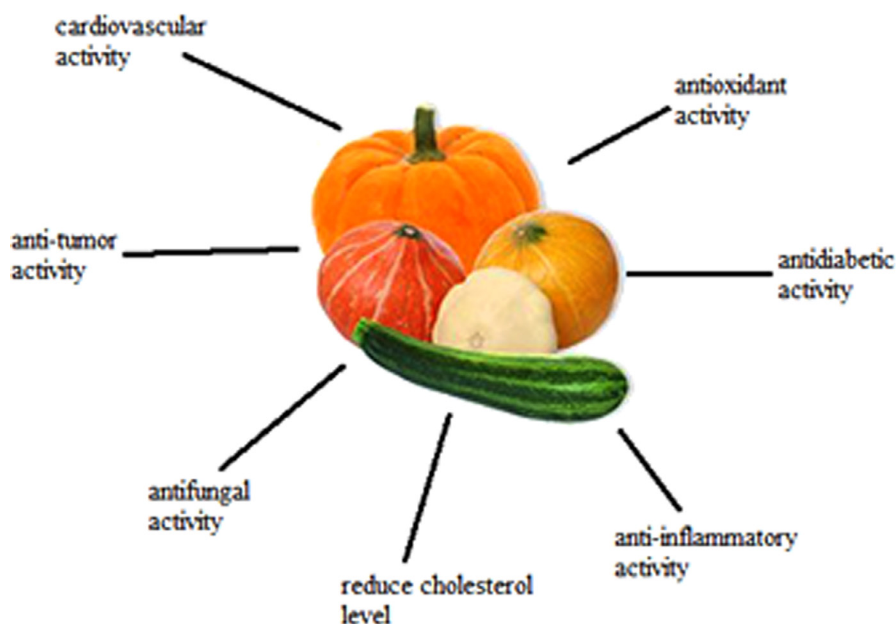

Fig. 2. The positive effect on the human body.

cancer as a result of antimutagenic and antitumor activities. Cucurbitacins have shown an antitumor effect because they induce apoptosis in cancer cells by stopping cell cycles at the G2/M phase [8,30,31]. Polysaccharides act as hypoglycemic agents and have an antitumor effect. Mukai maderaspatana is a source of ergosterol that shows anticancer activities. Cucurbita andrena display anticancer activities by their ability to inhibit cyclo-oxygenase-2 [10,13]. Hou et al. (2008) demonstrated the anticancer activity of *Cucurbita moschata* against human leukemia cells K562, murine melanoma cells B16, and lung adenocarcionoma cells A549 in an in vitro model using a standard MTT assay. Compounds isolated in *Cucurbita moschata* can inhibit cell tumor growth by working like ribosome-inactivating protein (Table 1).

Hemsleya amabilis belongs to the *Cucurbitaceae* family, grows in the tropical and subtropical regions of China, and is commonly known as *xue don*. Feng et al. (2019) showed that components isolated from Hemsleya amabilis display anticancer activity using an cytotoxic assay-MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) procedure with human cancer cell lines HCT-8, Hela and HepG-2 in in vitro studies. The researchers proved that isolated compounds belong to Cucurbitane triterpenes and exhibits significant cytotoxic activity against human tumor cell lines. The half maximal inhibitory concentration value ranged from 5.9 to 33.9 μM compared with the positive control (cisplatin; Table 1). Piccollela et al. (2019) demonstrated a cytotoxicity of zucchini extracts on HaCat human kerationcytes cell line in in vitro studies. A maximum of 70% of cytotoxicity showed a texted dose of 500 μg/mL after 48 and 72 h (Table 1). However, there are no recent in vivo studies on this subject.

Antiinflammatory activities

Cucurbits are a source of cucurbitacins, which show toxicity, but in the right concentration they display a potential to treat various pathologies, such as inflammation or autoimmune diseases. The most promising cucurbitacins, B and E, demonstrate antiinflammatory activity owing to the inhibition of mediators of inflammation, such as tumor necrosis factor-α, cyclo-oxygenase-2, and nitric-oxe synthase-2 [30,31]. Cucurbits can be a source of polysaccharides in a diet. This compound can generate a change in biologic functions in an organism, modulate macrophages, and thereby modulate the immune system and reduce inflammation [33].

Fig. 2.

Cardiac glycosides, contained in *Momoridace balsamina* leaves and seeds, protect against lethal endotoxemia and have antiinflammatory activity [8]. *Mukai maderaspatana* is a popular plant in India, especially in the Erode District, and a source of ergosterol (compound that shows antiinflammatory and anticancer activities) [10]. Kobori et al. (2008) demonstrated the antiinflammatory properties of *Momordia charantia* extracts in RAW 264.7 macrophages-like cells in an in vitro model. Using gene expression analysis, the researchers proved that a butanol fraction of *Momordia charantia* extract suppressed lipopolysaccharide-induced tumor necrosis factor α production in RAW 264.7 and suppressed the nuclear factor kappa-light-chain enhancer of activated B-cell DNA binding activity (Table 1).

The antiinflammatory effect of bitter gourd was shown in in vivo studies of patients with knee osteoarthritis. After 3 mo of daily intake of *Momordia charantia* supplements, significant improvements in knee osteoarthritis were observed (Table 1) [14]. Mondal et al. (2019) displayed the antiinflammatory effect of *Melothria heterophylla* in an in vivo model using carrageenan-induced rats paw Edema. A total of 36 rats were divided in six groups in this study. The decreased effect of edema obtained from the compound isolated from *Melothria heterophylla* was similar to that from a standard drug (Table 1).

Antimicrobial activity

Cucurbits are rich in terpenoids, which have a negative effect on fungi and bacteria owing to the membrane disruption and inhibitory effects [8,20]. Cucurbitacins stimulate gastric secretion. In addition, these compounds exert antifeedant for insects. Bitter apple is rich in cucurbitacin E 2-O-β-D-glucopyranoside and ursolic acid. Both compounds have strong antimicrobial properties [11]. Pumpkin extracts effectively inhibited a growth of *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus,* and *Bacillus*

subtilis. The mechanisms of antimicrobial properties are not fully known to date, but studies have shown that whole extract works better than isolated compounds [10].

Cheong et al. (1997) showed the antifungal effect of 28-kDa antifungal PR-5 protein isolated from *Cucurbita moschata* leaves in an in vivo model. The Inn agar-disc plate method PR-5 protein significantly inhibited the growth of *Fusarium oxysporum*. The isolated protein also inhibited the growth of *Candida albicans* owing to its synergism with a chitin syntheses inhibitor called nikkomycin (Table 1).

Conclusions

Today, more and more people base their diet on fruit and vegetables. The *Cucurbitaceae* family is known and cultivated around the world. The most popular vegetables from this family are cucumber and pumpkin, but squash and zucchini are increasingly used in the kitchen. Seeds have also become popular and healthy snack [7]. In traditional medicine, cucurbits were used to treat bladder and kidney stones and for their purgative properties [9,15]. Cucurbits are a good source of nutrients, such as proteins, fiber, vitamins, and polysaccharides, but also show medical value, such as hepatoprotective and antiinflammatory activities.

Owing to the high content of carotenoids, phytochemicals and terpenoids cucurbits have demonstrated antioxidant and anticancer properties. The *Cucurbitaceae* family, especially pumpkin, is known for its antidiabetic benefits [9,20]. Cucurbits are more and more used for their pharmacological properties (e.g., addition for treatment of diabetes). Some isolated compounds can have pharmacotherapeutic effects in low urinary tract disease and inflammation, such as *Momordica charantia* supplements in knee osteoarthritis [14,17]. Antimicrobial properties are the reason for the extensive use of pumpkin in patients with stomach and intestinal disorders [10].

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Review

The Plants of the *Asteraceae* Family as Agents in the Protection of Human Health

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Abstract: The *Asteraceae* family is one of the largest flowering plant families, with over 1600 genera and 2500 species worldwide. Some of its most well-known taxa are lettuce, chicory, artichoke, daisy and dandelion. The members of the *Asteraceae* have been used in the diet and for medicine for centuries. Despite their wide diversity, most family members share a similar chemical composition: for example, all species are good sources of inulin, a natural polysaccharide with strong prebiotic properties. They also demonstrate strong antioxidant, anti-inflammatory and antimicrobial activity, as well as diuretic and wound healing properties. Their pharmacological effects can be attributed to their range of phytochemical compounds, including polyphenols, phenolic acids, flavonoids, acetylenes and triterpenes. One such example is arctiin: a ligand with numerous antioxidant, antiproliferative and desmutagenic activities. The family is also a source of sesquiterpene lactones: the secondary metabolites responsible for the bitter taste of many plants. This mini review examines the current state of literature regarding the positive effect of the *Asteraceae* family on human health.

Keywords: *Asteraceae* family; human health; antioxidant activity

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1. Introduction

Plants have long played a crucial role in the development of medicine, primarily due to their ability to synthesize secondary metabolites with potentially significant biological activity. In traditional medicine, plants were used in various ways to treat many different ailments. According to the World Health Organization, over 80% of the global population still depends on traditional and folk medicine, most of which is based on plant remedies. Drugs based on plants used in traditional medicine are often cheaper than normal drugs, are easily accessible and have fewer side effects than their synthetic alternatives. Many traditional medicinal plants have recently been analyzed using more modern methods, leading to the discovery of many promising compounds. These plant-derived compounds can be used in the modification of existing drugs or the design of completely new ones [1,2].

The majority of *Asteraceae* family members have therapeutic applications, and have a long history in traditional medicine: some members have been cultivated for more than 3000 years for edible and medical purposes. They are most common in arid and semi-arid regions of subtropical areas, but are known and distributed throughout the word. The *Asteraceae* family members show a wide range of anti-inflammatory, antimicrobial, antioxidant and hepatoprotective activities (Figure 1) [3]. This paper reviews the current state of up-to-date literature concerning the positive effect of plants, particularly the vegetables, from the *Asteraceae* family on human health.

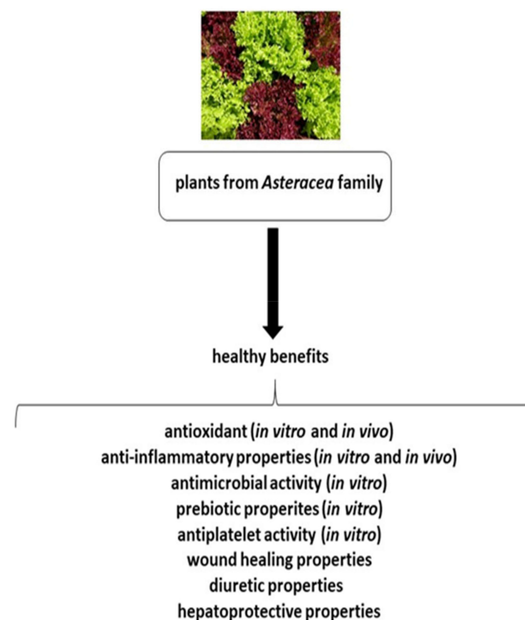

Figure 1. The positive effects of plants from the *Asteraceae* family on human health.

2. Characteristics of the *Asteraceae* Family

The *Asteraceae* family, often known as the sunflower family, is one of the largest flowering plant families, including over 1600 genera and 25,000 species worldwide. It includes a number of well-known species, such as chicory, sunflower, lettuce, coreopsis, dahlias and daisy, as well as a number of plants of medicinal significance, such as wormwood, chamomile and dandelion [4]. For example, *Carduus* species have often been used as antihemorroidal and cardiotonic remedies in traditional medicine, and *Onopordum tauricum* as a remedy for liver disease. The flowers and roots from *Onopordum acanthium* were used as antipyretic and diuretic agents, and *Centaurea solstitalis* is used in folk medicine in Turkey to treat stomach problems, abdominal pain, herpes infections and the common cold [5]. *Tanacetum parthenium,* also known as feverfew in folk medicine and medieval aspirin, has been used as a remedy for headaches, migraine, nausea, vomiting, stomach-aches, rheumatism and other inflammations [6].

Another plant with practical uses is *Bidens pilosa*, also known as Spanish needles, which grows mostly in subtropical and tropical regions. It has been used as a remedy for liver problems and to lower blood pressure, and is a major ingredient in herbal infusions in Taiwanese folk medicine. In addition, *Carthamus tinctorius* (safflower) is a treatment for rheumatism and osteoporosis in Korean herbal medicine [7], and the juice from *Emilia sonchifolia* roots is used to treat dysentery in Chinese medicine and as a remedy for diarrhea in Nepalese medicine [3].

Cichorium intybus (chicory) is used in traditional medicine as a remedy for inflammatory inflammation and liver disorders, and is also used to treat gallstones, gout, rheumatism and appetite loss. Tonics from *C. intybus* have also been used to treat enlarged spleen and fever in Indian Ayurveda medicine, and a decoction from leaves was used as a cure for rheumatism and gout [8,9].

Many plants from the Asteraceae family have been used in traditional medicine in Turkey. Tea prepared from *Achillea aleppica* and *Achillea biebersteinii* was recommended for abdominal pain. The aerial parts from *Chrysophthalmum montanum* were boiled and applied to wounds and other injuries. The roots were often eaten to reduce high blood pressure. *Matricaria aurea* was recommended in the diet twice a day for bronchitis, sore throat and cough. The seeds of *Notobasis syriaca* were used as remedies for liver disease [10].

3. Botanically Characteristics of the *Asteraceae* Family

The *Asteraceae* family is widely distributed throughout the world in a variety of ecological habitats, except Antarctica. They are found in forest habitats, high altitude grasslands and even urban green spaces, but they are much less common in tropical areas [11]. The morphology of the *Asteraceae* plants is also diverse. Some species are trees reaching more than 30 m, such as *Dasyphyllum excelsum* in Chile or *Vernonia arborea* in Malaysia; however, many others are shrubs, like rabbit brush or rosette-trees, and most are perennial or less annual herbs, ranging from 1–3 m tall sunflowers and to almost sessile forms. The smallest examples are those of the genus *Mnioides* found in the Peruvian Andes [11].

The form of the leaves varies widely: while most are large, others are small and spiny, and some are nonexistent, with their function being taken over by a green stem. Most leaves are covered with an indumentum and hairs of all lengths and colors [11]. Most have a flat cluster of small flowers of various colors. A good example is the Jerusalem artichoke, with thin, yellow flowers on a tall stalk [3,12].

4. Nutritional Value of *Asteraceae* Family

Many species of the *Asteraceae* can be included in a regular, healthy diet. A study of the *Asteraceae* by García-Herrera et al. [13] found the protein content to range from 0.4 to 6.13 g per 100 g of edible parts and fiber from 2.55 to 13.44 g. The roots, leaves and flowers are also good sources of Na, K, Ca and Mg, and of vitamins A, B, C and D. Most plants have a low fat content [13].

Crepis vesicaria and *Sonchus oleraceus* both grow in the Mediterranean area. Both are considered wild edible plants and are often used as additions to salads in Italian cuisine, and both are good sources of vitamin A: 100 g of *C. vesicaria* leaves provide 50% of the recommended daily allowance (RDA) of vitamin A and *S. oleraceus* provides over 80%. Additionally, both species contain high levels of thiamine: 200 g of material supplies 15% of the RDA of thiamine. In addition, 200 g of *S. oleraceus* supplies almost 14 mg of lutein per day, which has been associated with a reduction of age-related macular degeneration [14].

Artemisia absinthium is used as a flavoring agent in various wines and spirits, and is an important addition to absinthe. *Carthamus tinctorius* (safflower) is especially popular in Portugal, where the seeds are used for cheese manufacture and the leaves are used as food colorants. The young leaves of *Inula crithmoides* are eaten raw, and the fresh shoots can be added to salad or pickled [7].

Cichorium intybus, or chicory, contains 22.15 mg of vitamin C per 100 g of dry matter and more than 60% of its total organic acid content is malic acid. Chicory has various uses in the kitchen: the green leaf is a basic ingredient in salads and a popular addition in sandwiches. The roots are used as caffeine-free coffee substitutes. Chicory extracts can be add to nonalcoholic and alcoholic beverages to improve their taste [8,15,16]. Chicory root is a one of the biggest natural sources of inulin. The content of inulin varies from 11–20 g on 100 g of fresh roots and around 44% on dry root weight. The amount of inulin can change depending on season and is the lowest during autumn [17].

Cynara cardunculus (artichoke), has been consumed for centuries. In ancient times, rich Greeks and Romans consumed immature flowers as high-quality vegetables on special occasions and the mature flowers were used as milk coagulants in cheese production. Nowadays, the flowers are often eaten as frozen and canned delicacies, and are often used for plant-based milk and cheese. The flowers of *Tagetes erecta*, commonly known as the Mexican marigold, are often used as food colorants; they are also added to poultry feed to decrease egg cholesterol level and improve egg yolk pigmentation [18].

Helianthus tuberosus, Jerusalem artichoke, is also a versatile choice in cuisine. Its edible parts are the tubers, which contain vitamins and minerals such as potassium and phosphorous. It is also a source of inulin, a complex carbohydrate which can promote good health in humans; it is believed that 100 g of Jerusalem artichoke tuber provides almost 10 g of inulin. Inulin increases the absorption of calcium, magnesium and various other

minerals. Due to its low calorific value and ability to emulate the texture of traditional fat, it is used as an effective substitute for regular sugar and fat in cookies, cakes and breads. Jerusalem artichoke tubers can be used to enhance the characteristics of fermented milk products: in Canada, their juice is fermented and consumed as a prebiotic drink with blueberry juice [12].

5. Chemical Characteristics and Health Benefits of Vegetables from the *Asteraceae* Family

Many species of *Asteraceae* demonstrate various pharmacological activities, which have been attributed to their phytochemical components, including essential oils, lignans, saponins, polyphenolic compounds, phenolic acids, sterols and polysaccharides (Figures 2 and 3) [5]. A study of various members of the *Asteraceae* family, viz. *Cirsium arvense*, *Onoporidium acanthium*, *Centaurea solstitailis* and *Carduus acanthoides*, found the total phenolic content extract to range from 8.035 to 90.305 mg GAE/L (milligrams of gallic acid equivalent of plant extract), and total flavonoid content from 18.031 to 185.437 mg QE/L (milligram of quercetin equivalent of plant extract) [5].

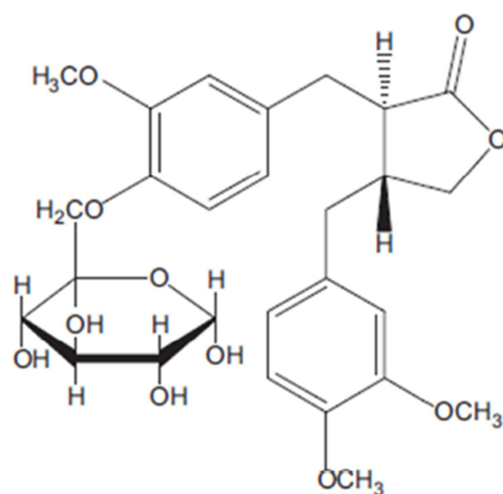

Figure 2. Chemical structure of Arctiin according to Tourchi, Arslan and Iranshahi [2].

A wide range of phenolic compounds are found, including chicoric acid, kaempferol and its derivatives, luteolin and its derivatives, quercetin, and apigenin and its derivatives. They are also found in the underground parts of plants; for example, chicory root is a source of many acids such as caffeic acid, chlorogenic acid and isovanillic acid [16,19]. In addition, a number of triterpenes, such as taraxacin, taraxacin acid, fardiol, arnidiol, taraxasterol, α-amiryn and β-amiryn, have been identified in *Taraxacum* spp.: an important member of the family. Many plants are also sources of malic acid, fumaric acid, citric acid and ascorbic acid [20].

Arctiin is a lignan, a glucoside of artigenin, found in many species of *Asteraceae*, particularly *Centaurea imperialis*, *Forsythia viridissima* and *Saussuerea heteromallav* and was first isolated from *Arctium lappa*. Arctiin possesses a number of pharmacological effects including cytotoxicity, antiproliferative and desmutagenic activity; it also acts as a platelet activating factor antagonist and calcium antagonist (Figure 2) [2].

Of the 1100 known acetylenes, i.e., molecules with biological activity, around 200 have been found in the tribes of the *Asteraceae*, including the *Astereae*, *Cynereae*, *Anthemideae* and *Heliantheae*. Each tribe has its own original set of acetylene metabolites, and hence can be used for chemotaxonomy. Although they share the same basic general chemical structure, based on two or more triple bonds, the compounds are diverse and included a range of aliphatic and cyclic structures containing sulfur, nitrogen and oxygen. Acetylenes demonstrate various cytotoxic, anti-inflammatory and antibiotic effects, among others [21].

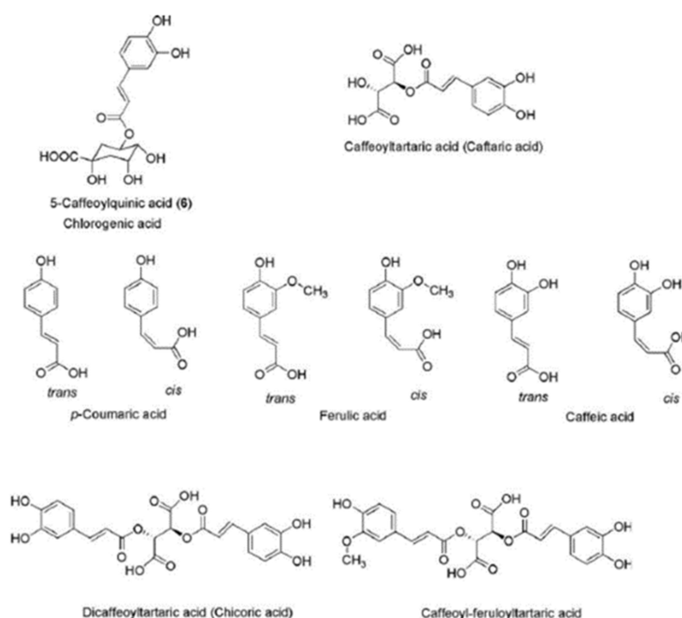

Figure 3. Chemical structure of hydroxycinnamic acid derivatives according to Jaiswal, Kiprotich and Kuhnert [22].

The plants of the *Asteraceae* family are rich sources of chloregenic acid: a hydroxycinnamic acid derivative formed by the reaction between quinic acid and a specific trans-cinnamic acid, such as ferulic, caffeic or *p*-coumaric acid. Chloregenic acids have been found to possess antiviral, antioxidant, antimutagenic, anti-inflammatory and radical-scavenging activities [22]. Various hydroxycinnamic acid derivatives, especially caffeic acid esters, have been found in the leaf (214 mg/g of dry weight) and petal fractions (420 mg/g of dry weight) of dandelion [23].

One of the most important groups of compounds in the *Asteraceae* family is that of the sesquiterpene lactones. They are terpenoids, which represent half of all the sesquiterpenes in the terpenoids group. Their chemical structure is based on a 15-carbon atom skeleton formed around three isoprene units [24]. Sesquiterpene lactones are colorless, have a bitter taste, and are present at particularly high levels in the genera *Vernonia*, *Ambrosia*, *Parthenium* and *Artemisia* [6]. Sesquiterpene lactones promote appetite and digestion due to their bitter taste. Chicory roots are a rich source of sesquiterpene lactones like lactucin, 8-deoxylactucin,13-dihydro-8-deoxylactucin, lactucopicrin,13- dihydrolactucopicrin, jacquinelin, crepidiaside B, lactuside A [17].

Artemisia absinthium is a perennial herb commonly known as wormwood. It is often added to biological sprays against pests due to its odor; however, it also demonstrates a range of health properties, including diuretic, digestive, balsamic and depurative effects. It is also recommended as a supplement in leukemia treatment. The aerial part of the plant exhibits snake antivenom activity.

Erigeron canadensis shows antiplatelets and anticoagulant activity, especially induced by the cyclooxygenases pathway induced by arachidonic acid. The preparation from this plant can inhibit plasma clot formation in prothrombin time and partial thromboplastin time in human plasma. It has also demonstrated significant anti-IIa activity mediated by cofactor II of heparin [25].

Acmella oleracea is a source of spilanthol, a compound belonging to N-alkyladmides. It shows diuretic activity and is used in oral health care, often as an addition to toothpaste [7]. Topical application of *Achillea kellalensis* flowers on a wound can hasten healing, due to their flavonoid content. *Achillea millefolium* extract has an estrogenic effect, thanks to its content of phytoestrogens such as apigenin and luteolin; these have a stronger binding affinity to β estrogen receptors than estradiol [3]. *Calendula officinalisis* demonstrates wound healing properties and antibacterial and antiviral activity [8].

Many *Asteraceae*, especially Taraxacum spp., Reicardia picroides, Sonchus oleraceus and Picris echioides show bacteriostatic and bacterial potency against Salmonella tymphimurium, Bacillus aureus, Escherichia coli and Staphylococcus aureus. They have also demonstrated antifungal activity against Penicillium ochrochloron (Table 1) [19].

Various plants from the *Asteraceae* family demonstrate antimicrobial activity in vitro. An antimicrobial screening assay found ethanol extract from *Ageratum conyzoides* and *Tagetes erecta* to demonstrate antimicrobial properties against a broad spectrum of Gram-positive and Gram-negative bacteria. *T. erecta* was also found to inhibit the growth of *P. aeruginosa* [26]. Chicory also demonstrated antimicrobial effect, due to inhibitory effect on various Gram-positive and Gram-negative bacteria, *Aspergillus niger* and *Sachharomyces cerevisiae* [17].

Taraxacum officinale, dandelion, shows strong diuretic activity, probably due to its high potassium content. It can also improve the regenerative capacity of the liver: it was found to suppress monophosphate-activated protein kinase in the livers of mice fed a high-fat diet [20,27]. Lis and Olas [28] reported that dandelion roots demonstrated antiplatelet activity in vitro, based on measurements of acid phosphatase activity during blood platelet adhesion to collagen and fibrinogen; the strongest antiplatelet activity was demonstrated by a fraction with high hydroxyphenylacaetate inositol ester content (Table 1).

Cynara cardunculus, artichoke, has demonstrated hepatoprotective, hypocholesterolemia, hypolipidemic and hypoglycemic properties, which have been attributed to its high phenolic compound content. Artichoke can also serve as a source of dietary prebiotic [18].

Achillea cucullata is a Turkish and Iranian species with antimicrobial activity. *A. cucullate* extract has been found to inhibit the growth of Gram-positive bacteria like *Staphylococcus aureus* and *Enterococcus faecalis*, and Gram-negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* in vitro. It can also inhibit the growth of *Candida albicans* (Table 1) [1]. *Helianthus tuberosus*, Jerusalem artichoke, has demonstrated prebiotic properties, which have been attributed to its inulin content: inulin improves the survival of *Lactobacillus paracasei* BGP1 and *Lactobacillus plantarum* CIDCA8327 strains, and enhances their resistance to gastrointestinal conditions (Table 1) [29].

Sylibum marianum is also known as milk thistle. Its major source of silymarin, a mixture of silibinin A and B, silydianin and silychristin. Milk thistle demonstrated various biological activity, including hepatoprotective, cardioprotective and cytoprotective effects. Milk thistle has antidotal and protective effects against numerous biological toxins, like mycotoxin, bacterial toxin and even snake venoms. Silymarin present in milk thistle has shown antioxidant activity against lipid peroxidation induced by aflatoxins. Silymarin also suppressed lipopolysaccharide-induced neuroinflammatory impairment. Beside natural toxins, milk thistle also has a protective effect against various chemical toxic agents, like aluminum, copper, cadmium and lead [30].

Table 1. Various health properties of the *Asteraceae* revealed by in vitro and in vivo experiments.

Plants (Preparation/Extract)	Chemical Characteristic of Preparation/Extract	Type of Research	Biological Activity	References
Extract from seeds of *H. cretica*, *H. graecum*, *P. echioides*, *R. picroides*, *S. hispanicus*, *S. oleraceus*, *U. picroides* and *T. officinale*	α- and β-tocopherols (18.32 and 16.31 μg/100 g fresh weight) oxalic acid (972 mg/100 g fresh weight)	In vitro	Antimicrobial activity (bacteriostatic and bactericidal potency against *Bacillus areus*, *Salmonella tymphimurium*, *Escherichia coli*, *Penicilium funiculosum*)	[19]
Extract from Jerusalem artichoke	Inulin (isolated from roots)	In vitro (*L. paracasei* BGP1 and *L. plantarum* CIDCA8327 strain)	Prebiotic properties (inulin improved bacterial growth)	[29]
Aqueous extract from *Achillea cucullata*	The total phenol content (53.807 ± 0.059 mg GAE/g dry weight) the total flavonoid content (21.372 ± 0.026 mg QE/g)	In vitro	Antimicrobial activity (inhibitory effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa*)	[1]

Table 1. *Cont.*

Plants (Preparation/Extract)	Chemical Characteristic of Preparation/Extract	Type of Research	Biological Activity	References
Extract from the roots from *Taraxacum officinale* (5 fractions)	Hydroxycinnamic acids, hydroxyphenylacetic acid derivatives, sesquiterpene lactones	In vitro	Antiplatelet activity (inhibitory effect on blood platelet adhesion to endothelial cells)	[28]
Aqueous extract from *Achillea cucullata*	The total phenol content (53.807 ± 0.059 mg GAE/g dry weight) the total flavonoid content (21.372 ± 0.026 mg QE/g)	In vitro	Antioxidant activity (DDPH free radical scavenging activity)	[1]
Extract from leaf from *Cichorium intybus*	Anthocyanins, (the major–Cyanidin-3-O-(6″-malonyl-β-glucopyranoside))	In vitro	Antioxidant activity (anthocyanins in leaf have free radical scavenging ability)	[9]
Extract from leaf and petals from *Taraxacum officinale*	Hydroxycitric acids: in the leaf fraction 420 mg/g dry weight (the main component-l-chicoric acid 350 mg/g dry weight); in the petal fraction 214 mg/g dry weight	In vivo (18 male albino Wistar rats)	Antioxidant activity (the level of biomarkers of oxidative stress in blood plasma)	[23]
Extract from *Cynara scolymus*	Phenolic acids (mainly chlorogenic acid, cynarin and caffeic acid), sesquiterpene lactones,	In vivo (60 male and 60 female Winstar rats)	Anti-inflammatory activity (increase in total leukocyte and lymphocyte counts)	[31]
Extract from *Cichorium intybus*	Phenolic acids, sesquiterpene lactones, β-sitosterol	In vivo (6-week-old male mice)	Anti-inflammatory activity (increased level of IL-12)	[32]

5.1. Antioxidant Activity

Extracts from the plants of the *Asteraceae* family demonstrate free radical scavenging ability, which has been attributed to their phenolic compound content. The phenolic compounds act by improving the endogenous antioxidant system, chelating the metal ions and avoiding the formation of free radicals. For example, arctiin has been found to significantly slow increases in intracellular reactive oxygen species (ROS) generation induced by H_2O_2: a process which often mediates sudden cell cycle arrest or cell death [2,25,33]. In addition, the lipophilic compounds isolated from feverfew can decrease human neutrophil oxidative burst activity [8].

Extracts from *T. officinale* flowers can inhibit supercoiled DNA breakage in vitro induced by hydroxyl and peroxyl radicals, and reduce lipid and protein oxidation in plasma in vitro. The polysaccharide fraction from the roots also appears to improve antioxidant protection mechanisms in an acetaminophen-induced oxidative injury model in mice [20,27].

The effect of dandelion on the antioxidant profile of blood plasma and urine samples, and blood plasma lipid level was investigated in vivo. Three groups of six male albino Wistar rats were included in the study. One group was supplemented with dandelion leaf extract, another with dandelion petal extract and a control group which did not receive either. The results indicated a decrease in blood plasma lipid levels and lower oxidative stress in blood plasma, as indicated by thiol group levels and protein carbonylation inhibition (Table 1) [23].

Antioxidant activity in fresh chicory leaves was determined by evaluating lipid peroxidation inhibitory activity in vitro using fluorescence spectroscopy and liposome oxidation. The 250 µg/mL leaf extract preparation inhibited 88% of lipid peroxidation. The chromatographic profiles of the plants indicated high levels of anthocyanins, which are known to demonstrate strong antioxidant activity (Table 1) [9].

Antioxidant activity is strongly and positively correlated with phenolic content. An in vitro study found high levels of both in the ethyl acetate fraction from Jerusalem artichoke leaves [34].

Silybum marianum owes its strong antioxidant ability to silibinin (SBN), a flavonolignan isolated from its fruits and seeds. Silibinin demonstrates strong scavenging potential for most free radicals, such as peroxyl radical and hydroxyl radicals. It also inhibits the NF-kB pathway by treating and attenuating the inflammatory reaction that stimulated

atherosclerosis. In in vivo experiments, SBN has been shown to protect mouse and rat liver against the toxic effects of carbon tetrachloride and alcohol [35].

Aqueous ethanolic extract of *Achillea cucullata* has been found to demonstrate antioxidant potential in vitro based on DDPH free radical scavenging assay. The findings indicate an IC_{50} value of 132.55 ± 0.026 μg/mL for the extract, compared to 7.548 ± 0.047 μg/mL for the strong antioxidant gallic acid (Table 1) [1].

Artemisia absinthium methanolic extract after oral administration at doses of 100 and 200 mg/kg showed scavenging activity on superoxide anion radicals, by restoring superoxide dismutase and glutathione levels and decreasing the level of thiobarbituric acid reactive substances. This leads to the inhibition of oxidative stress caused by cerebral ischemia and reperfusion [25].

5.2. Anti-Inflammatory Activity

Cynara scolymus, artichoke, has been found to demonstrate anti-inflammatory activity in vivo in a study of 60 male and 60 female Wistar rats. The animals were treated with 1, 2 or 4 g/kg body weight of *Cynara scolymus* extract for 28 days. Regular treatment with the extract increased total lymphocyte and leukocyte count, interleukin-12 (IL-12) and phagocyte activity, had an immunostimulant effect, as indicated by hemogram, serum biochemistry, lymphoid organ weight, macrophage and neutrophil oxidative burst, and specific humoral immune response (Table 1) [31].

The anti-inflammatory activity of 0.1–100 μg/mL *Cichorium intybus*, i.e., chicory, extract was demonstrated in in vivo studies based on six-week-old male C57BL/6 and BALB/c mice. The chicory extract increased production of IL-12 by dendritic cells, i.e., antigen-presenting cells in the immune system (Table 1). Additionally, higher concentrations of extract inhibited allogenic T cell proliferation, but increased the level of IFN-γ at lower concentrations [32]. Chicory extract has also been found to lower the concentration of certain cytokines, such as the anti-inflammatory interleukin-4 [8,34,35].

Arctiin plays a crucial role in the anti-inflammatory activities of the *Asteraceae*, due to its ability to inhibit production of inflammatory mediators, including the interleukins IL-6 and IL-1β, prostaglandin E_2 (PGE_2), tumor necrosis factors (TNF-α) and nitric oxide. Arctiin also inhibits the translocation pathway of nuclear factor (NF)-kβ, leading to suppression of cyclooxygenase-2 (COX-2) [2].

The methanol extract from *Emilia sonchifolia* demonstrates anti-inflammatory effects by inhibition of edema induced by carrageenan [3]. Oleamide isolated from burdock can reduce the production of TNF-α and IL-4 [8].

Taraxacum species also demonstrate anti-inflammatory activity: extracts from dandelion flowers prevent the production of proinflammatory cytokines like PGE_2 and suppresses COX-2 and iNOS; in addition, taraxasterol isolated from dandelion inhibits the production of TNF-α, IL-1β, PGE_2, nitric oxide and IL-6 by preventing NF-kβ translocation in LPS-induced RAW264.7 macrophage models [20].

5.3. The Application of Asteraceae in Human Health

Nowadays, there is an increasing interest in the role of diet in human health and therapy based on natural remedies in the treatments for many ailments. It is proven that a diet rich in plants, the best source of antioxidants, plays a dominant role in preventing these diseases. For example, inulin isolated from dandelion roots is used for microbiological production of a high fructose syrup, as a replacement for the traditional one, and plays a role in the prevention of diabetes and obesity. Coffee from dandelion roots is a great alternative for normal coffee, due to the lack of narcotic effect. In the USA, preparations from dandelion leaves are an addition to health food products and supplements for diuretic problems [28]. Chicory also is a valuable source for new health food products and functional food. The roots from chicory are a healthy replacement for white flour and fat in cracker production, due to a high level of dietary fiber and inulin. They are in addition to various low-calorie sweeteners to increase dietary fiber content [17]. Jerusalem artichoke also is

a source of remedies for various diseases. In Russia, the flowers are used for tea, which, used daily, helps to improve the immune system in the body, provides an energy boost and prevents kidney disorders. Tubers of Jerusalem artichoke are recommended in the diet for obesity, as they cause a feeling of satiation [36].

However, further studies the *Asteraceae* family should be conducted to fully understand the potential uses as a prevention for many diseases or in the development of new drugs.

6. Conclusions

The *Asteraceae* family is the most varied and cosmopolitan family of flowering plants. Many of its species have been used in traditional medicine since ancient times. Nowadays, the growing need for more natural sources of medicine has driven scientific interest towards the *Asteraceae* family. Studies have demonstrated that their extracts have a positive impact on human health, thanks to their antioxidant, anti-inflammatory and antimicrobial activities [8].

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Abbreviations

MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
ROS	reactive oxygen species
SBN	silibinin
IL	interleukin
IFN-γ	interferon γ
PGE_2	prostaglandin E_2
COX	cyclooxygenase
iNOS	nitric oxide synthase
NF-kβ	nuclear factor kappa-light-chain-enhancer of activated B cells
LPS	lipopolysaccharide

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Comparative Phytochemical, Antioxidant and Haemostatic Studies of Preparations from Selected Vegetables from *Cucurbitaceae* Family

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Abstract: The aim of this study was to provide detailed insight into the chemical composition and activity of five cucurbit vegetable preparations (pumpkin, zucchini, cucumber, white and yellow pattypan squash), each containing various phytochemical compounds with potential use against oxidative stress induced by the hydroxyl radical donors in human plasma in vitro. We studied the antiradical capacity of vegetable preparations using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. As oxidative stress may induce changes in hemostasis, our aim included the determination of their effect on three selected hemostatic parameters of plasma, which are three coagulation times: PT (prothrombin time), APTT (activated partial thromboplastin time) and TT (thrombin time). However, none of used vegetable preparations changed APTT, PT or TT compared to the control. The phytochemical composition of the tested preparations was determined by UPLC-ESI-QTOF-MS. In our in vitro experiments, while all five tested preparations had antioxidant potential, the preparation from yellow pattypan squash showed the strongest potential. All cucurbit vegetable preparations inhibited lipid peroxidation. Only zucchini did not have an effect on protein carbonylation and only yellow pattypan squash inhibited thiol oxidation. The antioxidant activity of cucurbits appears to have triggered significant interest in multiple applications, including CVDs (cardiovascular diseases) associated with oxidative stress, which can be treated by supplementation based on these vegetables.

Keywords: oxidative stress; plasma; coagulation; UPLC-ESI-QTOF-MS analysis; *Cucurbitaceae* family

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1. Introduction

Lifestyle factors, including nutrition, play an important role in the etiology and treatment of cardiovascular diseases (CVDs) [1]. Recent results have demonstrated that certain vegetables (for example onion, garlic, tomato and beetroot) and their products may act as mediators in the prevention and treatment of cardiovascular diseases by various mechanisms [1–4]. The cardioprotective actions of vegetables may include lowering of blood pressure, improving endothelial function, modifying lipid metabolism and reducing oxidative stress [2,3]. The regular intake of vegetable products rich in phenolic compounds is associated with a reduced risk of cardiovascular diseases [1,4]. However, the effect of vegetables from the *Cucurbitaceae* family on parameters of oxidative stress and hemostasis is not always well documented. In addition, the chemical components of preparations (for example extracts and fractions) isolated from these vegetables have not been adequately described.

The *Cucurbitaceae* family is a large group of crops, with 800 species. The most popular cucurbits are pumpkin, cucumber, melon and watermelon. They are cultivated and consumed in various parts

of the world. Moreover, they are used in traditional medicine, for example in China, India, Mexico and Brazil [5]. Some results demonstrate that these vegetables have hepatoprotective, cardiovascular and anti-inflammatory properties [6,7]. These actions are commonly linked with chemical components. They are also rich in different phytochemicals [8].

It is known that polyphenol-rich extracts may alleviate the negative impact of oxidative stress and hemostasis [9]. Cucurbit preparations were investigated in the present work (in vitro model). This study aimed to investigate the in vitro protective effects of five cucurbit vegetable preparations: pumpkin (*Cucurbita pepo*; fruit without seeds), zucchini (*Cucurbita pepo convar. giromontina*; fruit with seeds), cucumber (*Cucumis sativus*; fruit with seeds), white pattypan squash (*Cucurbita pepo var. patisoniana*; fruit without seeds) and yellow pattypan squash (*Cucurbita pepo var. patisoniana*; fruit without seeds). We measured different parameters of oxidative stress: lipid peroxidation determined by thiobarbituric acid reactive substances (TBARS); thiol group level; protein carbonylation; oxygen radical antioxidant capacity (ORAC); and total antioxidant capacity of plasma. In addition, we also studied the antiradical capacity of preparations from cucurbit vegetables using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test. As oxidative stress may induce changes in hemostasis [10], another aim of our experiments was to determine the effect of the five vegetable preparations on three selected hemostatic parameters of human plasma: activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) in an in vitro model.

2. Results

2.1. Chemical Characteristic of Vegetable Preparations

The information on the occurrence of phytochemicals in the five cucurbit vegetable preparations with their UPLC-ESI-QTOF-MS data is shown in Table 1. A total of 36 phytochemicals were characterized, and most could be identified by comparing their retention times, MS spectra and MS/MS fragmentation and literature data [10–15]. As shown by the UPLC-ESI-QTOF-MS analysis, the five cucurbit vegetable preparations differed in their final qualitative chemical composition (Figure 1A–E), except for the two pattypan squash varieties (Figure 1B,C), whose composition was somewhat similar, with corresponding base-peak chromatograms (BPC) in the negative ionization mode of the five tested preparations. Among the identified metabolites were analytes belonging to different compound classes. The vast majority were representatives of groups such as phenylethanoid glycosides, flavonoids, fatty acids and lipids. The last class of compounds occurred in a significant amount in all the analyzed profiles and was identified as glycerophospholipids. The pumpkin (Figure 1D) and cucumber (Figure 1E) preparations showed the smallest phytochemical diversity. In these extracts, among the phenols, derivatives of kaempferol and synapic acid were identified. The extracts of zucchini (Figure 1A), white pattypan squash (Figure 1C) and yellow pattypan squash (Figure 1B) also contained phenolic compounds known as phenylethanoids, all of them occurring in the form of glycosides. Among the more known flavonoids, quercetin-3-O-rutinoside (rutin), 7-methylquercetin-3-galactoside-6″-rhamnoside-3‴-rhamnoside (xanthorhamnin), methyl 5-methoxy-2-[(6-*O*-pentopyranosylhexopyranosyl)oxy]benzoate derivative (primulaverin), isorhamnetin 3-*O*-rutinoside (narcissin), hesperetin 7-*O*-(2″,6″-di-*O*-α-rhamnopyranosyl)-β-glucopyranoside and quercetin 3,3′-dimethyl ether 7-rutinoside were identified, with all these metabolites only being found together in the extract from zucchini. Amino acids were interpreted as two compounds, L-phenylalanine glycoside located in the extracts from pumpkin, cucumber and white pattypan squash, and L-tryptophan glycoside identified in the fractions from pumpkin, zucchini and white pattypan squash. The largest amount of compounds from the group of fatty acids was detected in the most diverse phytochemical profile of zucchini i.e., all-*cis*-6,9,12-octadecatrienoic acid (γ-linolenic acid) derivative, (9Z, 12Z)-octadeca-9,12-dienoic acid (linoleic acid) derivative and other not identified octadecadienoic acid derivatives. Two of the last compounds described were also present in the other four preparations. Many individual compounds were also identified, namely

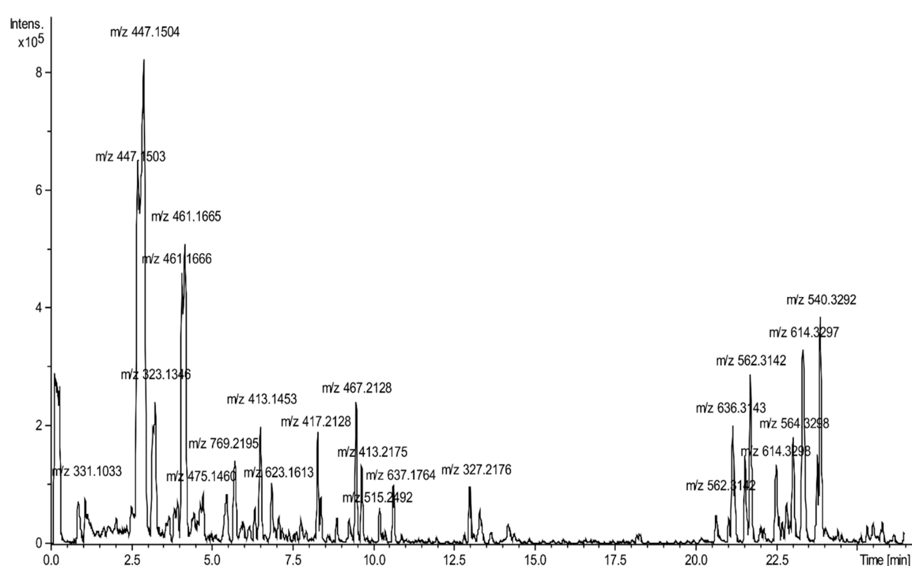
3-(β-D-glucopyranosyloxy)-2-hydroxybenzoic acid in the preparation from yellow pattypan squash; 3,4-dihydroxyphenyl-1-methyl ester-carbamic acid in the preparation from pumpkin; cinncassiol A in the preparation from white pattypan squash; secoisolariciresinol monoglucoside in the preparations from both pattypan squashes; and, as the last identified active compound, nonanedioic acid (azelaic acid) was found in the all tested preparations.

Table 1. UPLC-ESI-QTOF-MS data of identified compounds and their presence (+) in five cucurbit vegetable preparations.

Nr	Rt (min)	Identified Compound	Compound Class	[M-H], m/z, ESI Neg.	Major MS-MS Fragments	Pumpkin	Cucumber	Zucchini	White Pattypan Squash	Yellow Pattypan Squash
1	1.14	3-(*β*-D-glucopyranosyloxy)-2-hydroxybenzoic acid	benzoic acid derivative	315.071649	152, 315	-	-	-	-	+
2	1.47	fructosyl L-phenylalanine	amino acid	326.124313	164, 236, 326	+	+	-	+	+
3	1.87	L-tryptophan glycoside	amino acid	365.134743	116, 203, 275, 365	+	-	-	-	-
4	2.45	salicylic acid *O*-glycoside	phenolic acid	299.077241	137, 299	-	+	-	-	-
5	3.87	zizybeoside I	phenylethanoid glycoside	431.155861	147, 431	-	-	+	-	-
6	4.23	forsythoside E (isomer I)	phenylethanoid glycoside	461.166185	147, 309, 461	-	-	+	+	+
7	4.35	cinncassiol A	diterpenoid	381.191905	289, 381	-	-	-	+	-
8	4.39	sinapic acid hexoside	phenolic acid	431.192317	223, 385, 431	+	-	-	-	-
9	4.43	hydrangeifolin I	phenylpropanoid glycoside	415.160971	269, 415, 461	-	-	-	+	+
10	4.55	shimaurinoside B	megastigmane glycosides	381.176621	249, 381, 427	+	-	-	-	-
11	4.65	kaempferol derivative	flavonoid	450.117527	145, 285, 450	-	+	-	-	-
12	4.68	primulaverin derivative	flavonoid	475.146049	133, 295, 323, 475	-	-	+	-	-
13	4.75	adenostemmoic acid C	diterpenoid	367.212612	287, 303, 367	-	-	-	-	+
14	5.50	rutin	flavonoid	609.146766	301, 609	-	-	+	-	-
15	5.69	secoisolariciresinol monoglucoside	lignan	523.217892	165, 361, 523	-	-	-	+	+
16	5.75	xanthorhamnin	flavonoid	769.219503	299, 314, 769	-	-	+	-	-
17	6.54	unidentified	iridoid glycoside	413.145347	269, 311, 351, 413	-	-	+	+	+
18	6.88	isorhamnetin 3-*O*-rutinoside	flavonoid	623.161334	299, 315, 623	-	-	+	-	-
19	7.21	azelaic acid	dicarboxylic acid	187.097798	125, 187	+	+	-	-	+
20	8.41	hesperetin 7-*O*-(2′′,6′′-di-*O*-*α*-rhamnopyranosyl)-*β*-glucopyranoside	flavonoid	739.244676	295, 471, 559, 739	-	-	+	-	-

Table 1. *Cont.*

Nr	Rt (min)	Identified Compound	Compound Class	[M-H], m/z, ESI Neg.	Major MS-MS Fragments	Pumpkin	Cucumber	Zucchini	White Pattypan Squash	Yellow Pattypan Squash
21	9.68	octadecadienoic acid derivative	fatty acid	413.217479	209, 371, 413	-	-	+	-	-
22	10.66	quercetin 3,3′-dimethyl ether 7-rutinoside	flavonoid	637.176449	299, 313, 329, 637	-	-	+	-	-
23	13.04	octadecadienoic acid derivative	fatty acid	327.218013	211, 327	+	-	+	+	+
24	14.17	octadecadienoic acid derivative	fatty acid	329.233674	211, 329	-	+	+	+	+
25	20.68	glycerophospholipid	lipid	636.316333	277, 474, 636	-	+	+	+	+
26	21.19	glycerophospholipid	lipid	636.315949	277, 474, 636	+	+	+	+	+
27	21.26	glycerophospholipid	lipid	562.315676	277, 505, 562	+	+	+	+	+
28	21.57	γ-linolenic acid derivative	fatty acid	721.364358	277, 397, 721	-	-	+	-	+
29	22.54	glycerophospholipid	lipid	638.331427	152, 279, 476, 638	+	+	+	+	+
30	22.63	glycerophospholipid	lipid	564.331348	279, 504, 564	+	+	+	+	+
31	23.03	γ-linolenic acid derivative	fatty acid	559.311446	277, 559	-	-	+	-	-
32	23.06	linoleic acid derivative	fatty acid	564.329757	279, 504, 564	+	+	+	+	+
33	23.27	glycerophospholipid	lipid	614.330267	255, 452, 614	+	+	+	+	+
34	23.82	glycerophospholipid	lipid	452.277974	255, 452	-	-	+	+	+
35	23.89	glycerophospholipid	lipid	540.330582	255, 480, 540	+	+	+	+	+
36	24.46	glycerophospholipid	lipid	566.346313	281, 506, 566	+	+	-	+	+



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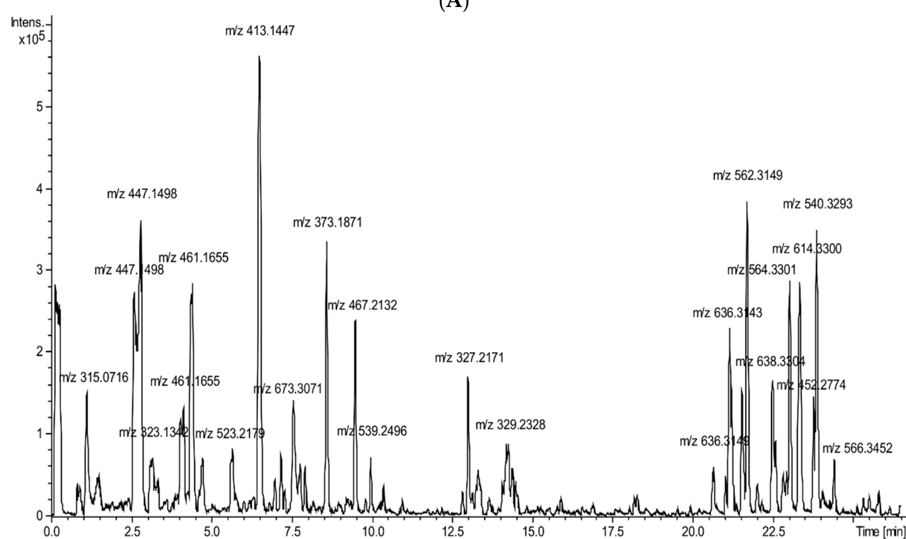

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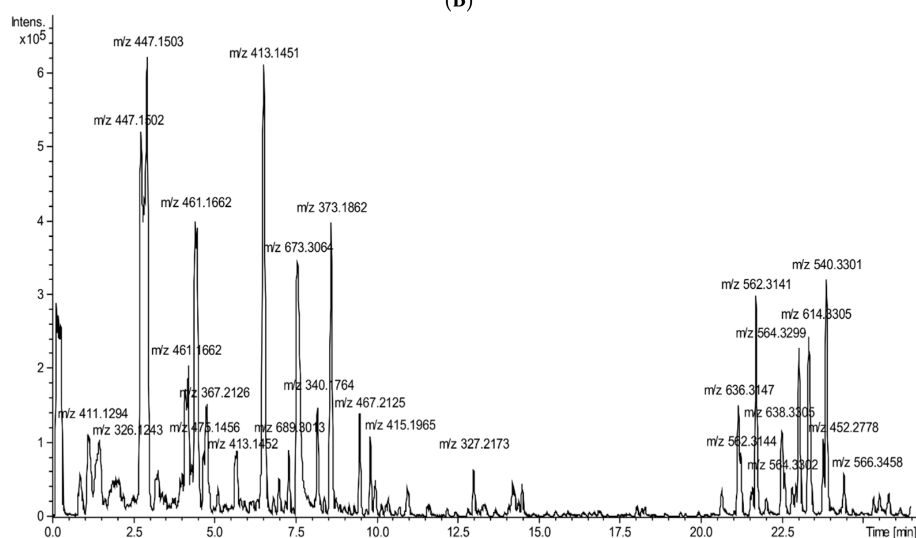


(**C**)

Figure 1. *Cont*.

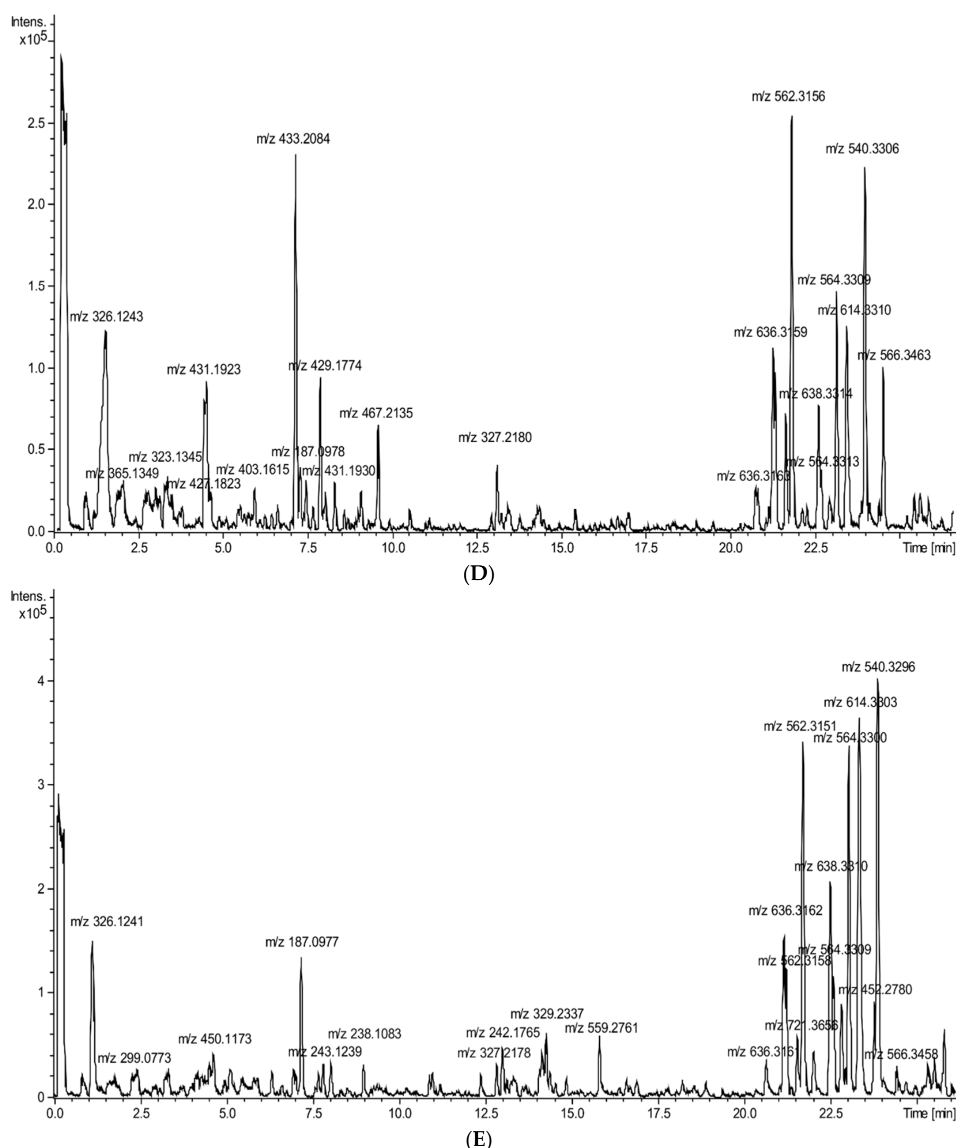


Figure 1. The base-peak chromatogram (BPC) of the five cucurbit vegetable preparations, obtained using high resolution UHPLC-ESI-QTOF-MS in negative ionization mode: preparation from zucchini (**A**), preparation from yellow pattypan squash (**B**), preparation from white pattypan squash (**C**), preparation from pumpkin (**D**), and preparation from cucumber (**E**).

2.2. *Effects of Vegetable Preparations on Hemostatic Parameters of Human Plasma*

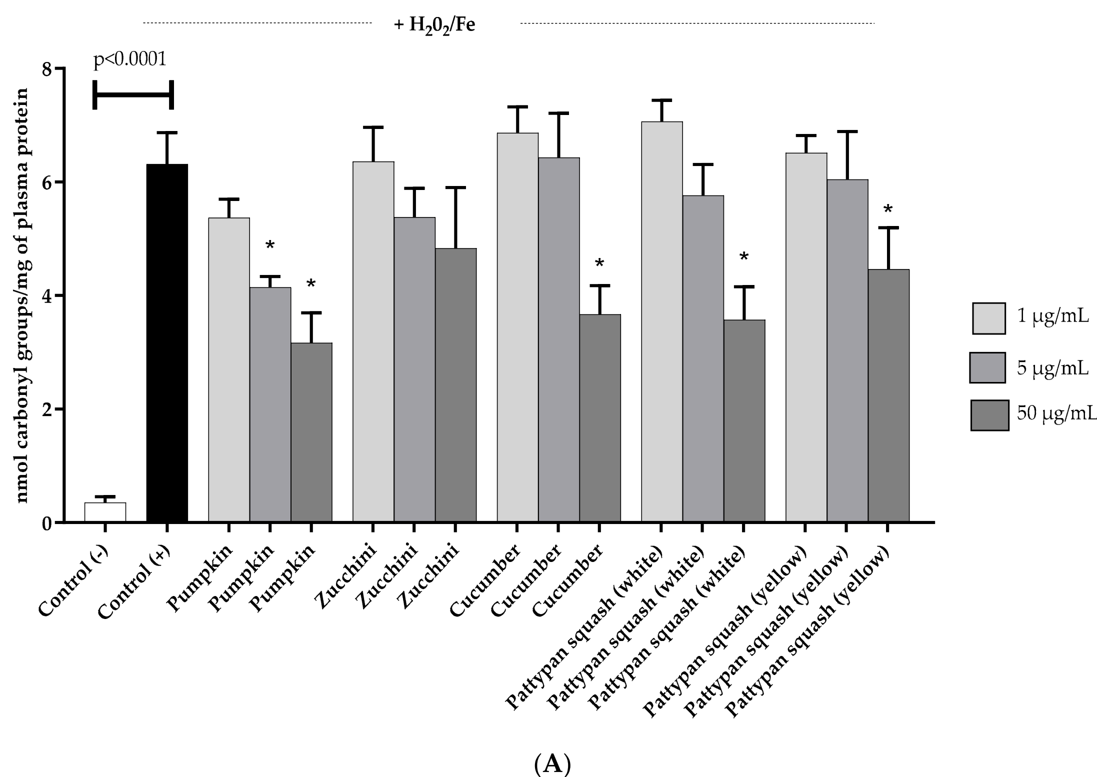
The analysis of the effect on the coagulation properties of human plasma demonstrated that none of the tested cucurbit vegetable preparations (concentration range 1–50 µg/mL; incubation time 30 min) changed APTT, PT or TT compared with control (plasma without cucurbits vegetables preparations ($p > 0.05$).

2.3. *Effects of Vegetable Preparations on Oxidative Stress Parameters*

The effect of the five cucurbit vegetable preparations (concentration range 1–50 µg/mL; incubation time 30 min) on the level of the three biomarkers of oxidative stress in human plasma was studied in vitro. We observed that the tested vegetable preparations did not exert any significant effect on oxidative stress in human plasma not treated with H_2O_2/Fe (data not demonstrated). On the other hand, exposure of plasma to H_2O_2/Fe (a strong oxidant) resulted in significant protein carbonylation, oxidation of thiol groups, and enhanced levels of lipid peroxidation (Figure 2A–C). As demonstrated in Figure 2A,

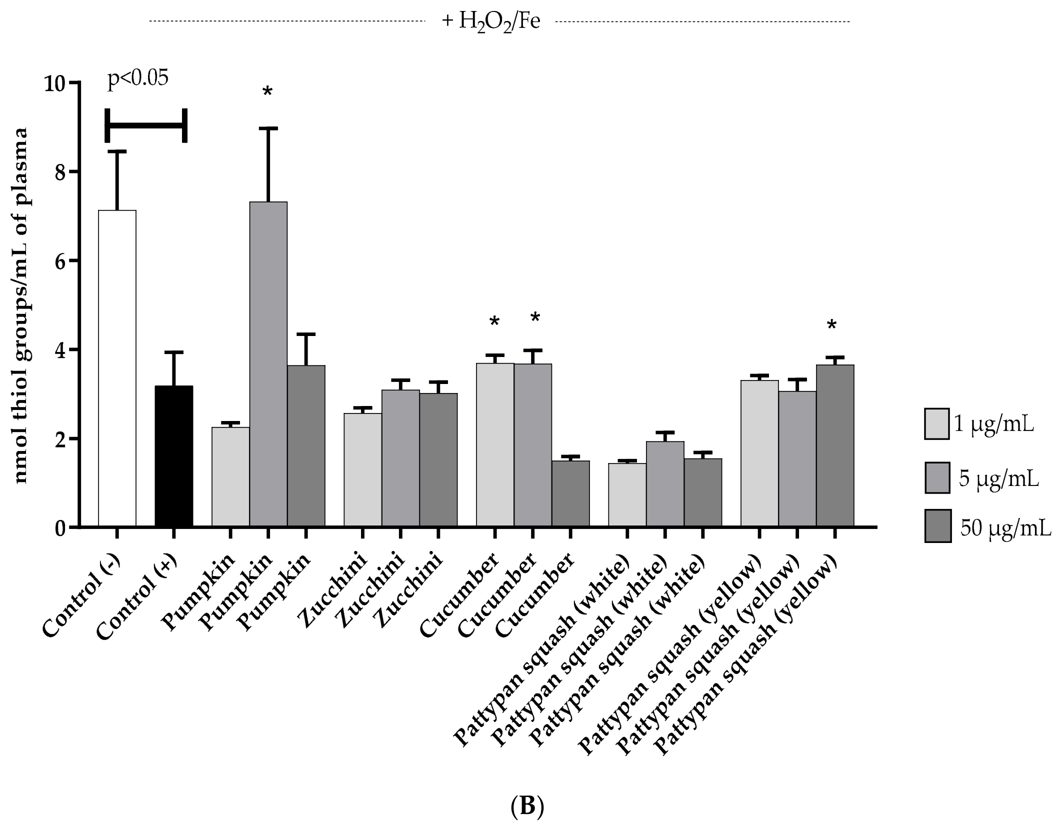
only four vegetable preparations (pumpkin, cucumber, white pattypan squash and yellow pattypan squash at the highest concentration of 50 µg/mL) reduced plasma protein carbonylation induced by H_2O_2/Fe. The best result was obtained for the pumpkin preparation (reduction of this process by more than 60% in comparison to the control positive) (Figure 2A). As shown in Figure 2B, the tested vegetable preparations had different effects on the oxidation of the protein thiols in plasma treated with H_2O_2/Fe. No positive effect was observed for two of the tested preparations (zucchini and white pattypan squash). On the other hand, for the yellow pattypan squash preparation, only at the highest dose (50 µg/mL) was it able to protect plasma against H_2O_2/Fe-induced oxidation of protein thiols. The preparation from cucumber exerted the strongest effect at lower doses (1 and 5 µg/mL) (Figure 2B). The best result was obtained for the pumpkin preparation at the concentration of 5 µg/mL (Figure 2B). Additionally, the activity of all tested vegetable preparations was not concentration-dependent for the 30 min incubation time (Figure 2B). Moreover, four of the tested preparations (zucchini, cucumber, white pattypan squash, and yellow pattypan squash) significantly ($p < 0.05$) inhibited plasma lipid peroxidation induced by H_2O_2/Fe starting from a dose of 1 µg/mL (Figure 2C). The pumpkin preparation showed the highest activity at higher tested concentrations (5 and 50 µg/mL) ($p < 0.05$) (Figure 2C). However, the cucurbits preparations at both concentrations (5 and 50 µg/mL) had non-significant influence on the antioxidant capacity of the ORAC plasma measures or, in concentration 50 µg/mL, the total antioxidant capacity in vitro (Figure 3A,B).

The antiradical capacities of the five vegetable preparations are also shown in Figure 3C. The order of the DPPH scavenging activity for the preparations was as follows: cucumber (0.007 ± 0.00) < pumpkin (0.052 ± 0.00) < white pattypan squash (0.064 ± 0.01) < yellow pattypan squash (0.086 ± 0.01) < zucchini (0.093 ± 0.02) (Figure 3C).



(**A**)

Figure 2. *Cont.*



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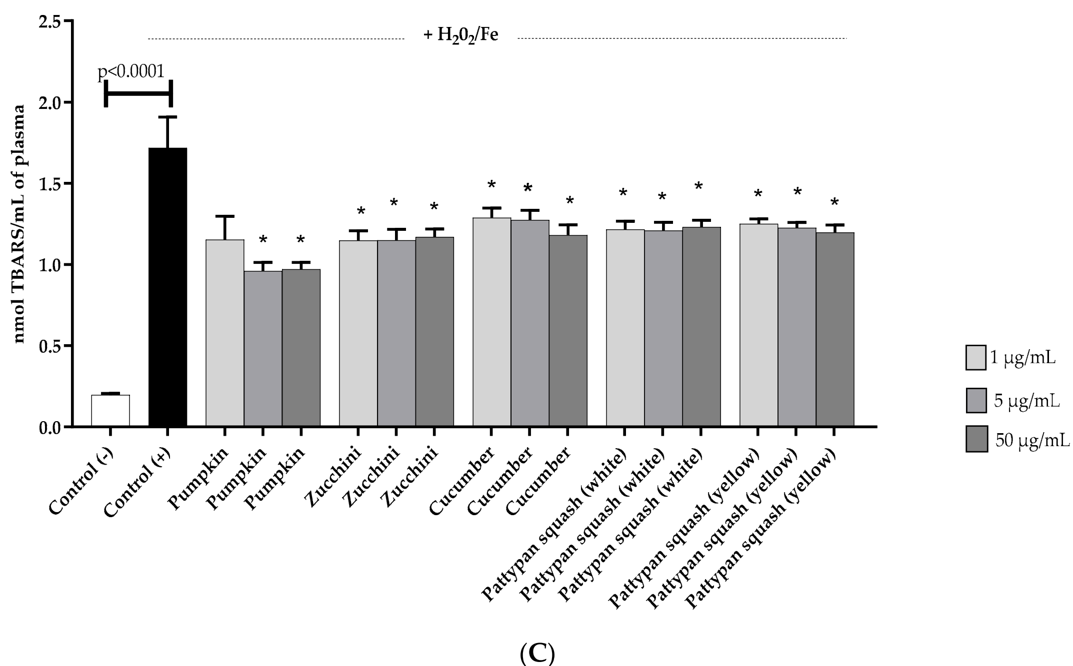
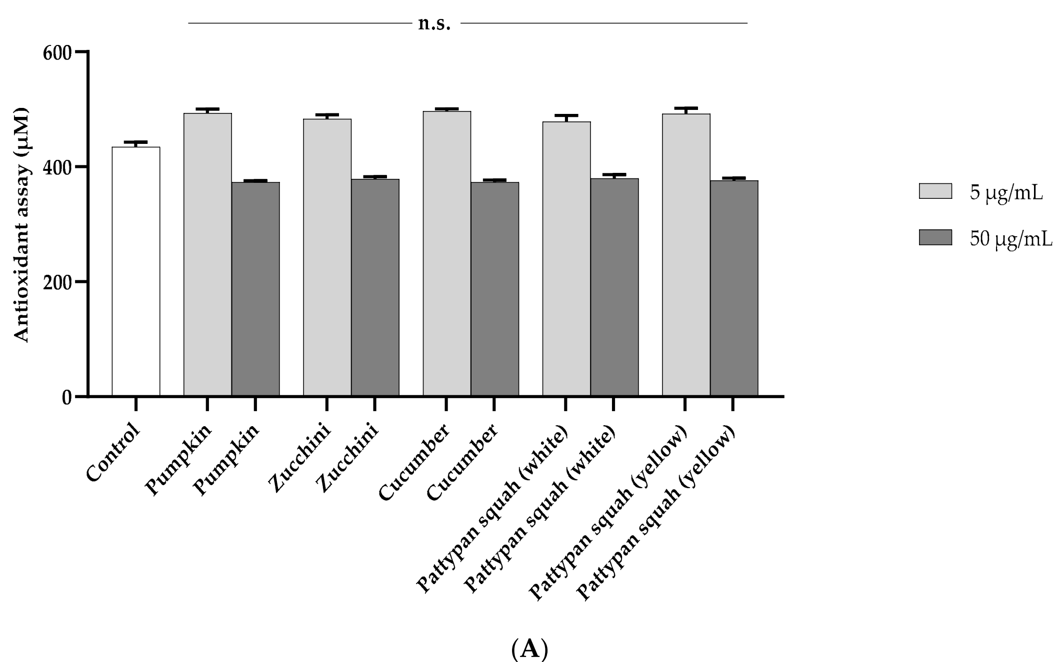


(**C**)

Figure 2. Effects of the five cucurbit vegetable preparations (concentration range 1–50 µg/mL, pre-incubation time 5 min) on the oxidative damages of plasma protein–protein carbonylation, in plasma treated with H_2O_2/Fe (incubation time-25 min) (**A**); on the oxidative damages of plasma proteins, the level of thiol groups in plasma treated with H_2O_2/Fe (incubation time 25 min) (**B**); and on lipid peroxidation in plasma treated with H_2O_2/Fe (incubation time 25 min) (**C**). Results are given as mean ± SE ($n = 6$). Control negative refers to plasma not treated with H_2O_2/Fe, whereas control positive to plasma treated with H_2O_2/Fe. One-way ANOVA followed by a multicomparison Tukey test and Kruskal–Wallis test: * $p < 0.05$, compared with positive control (treated with H_2O_2/Fe).

Table 2 demonstrates the comparative effects of the five tested vegetable preparations (at the highest used concentration 50 µg/mL) on selected biomarkers of oxidative stress in human plasma treated with H_2O_2/Fe. Table 2 gives a full picture of comparison between the effect of all selected vegetables on different biomarkers of oxidative stress in human plasma. We observed that the yellow pattypan squash preparation had a stronger antioxidant potential than the other four preparations. The yellow pattypan squash preparation inhibited oxidative stress induced by H_2O_2/Fe in all the in vitro tests (Table 2).



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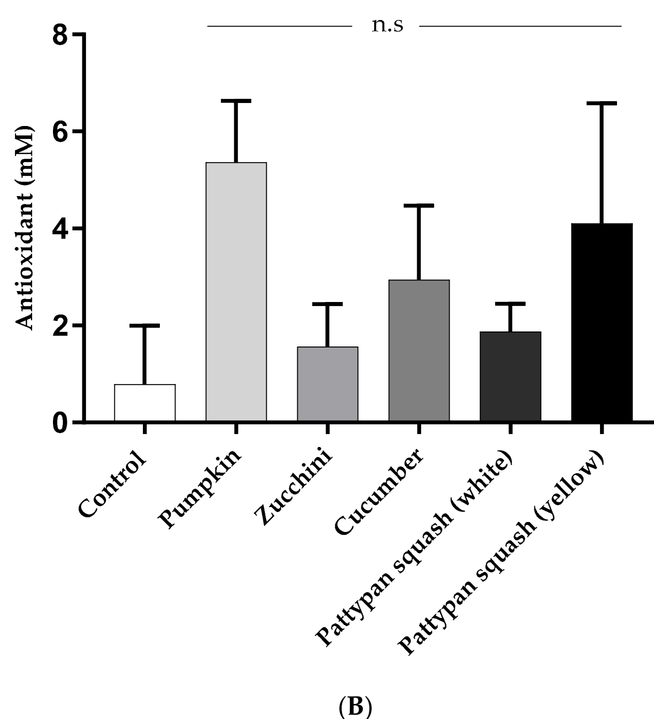

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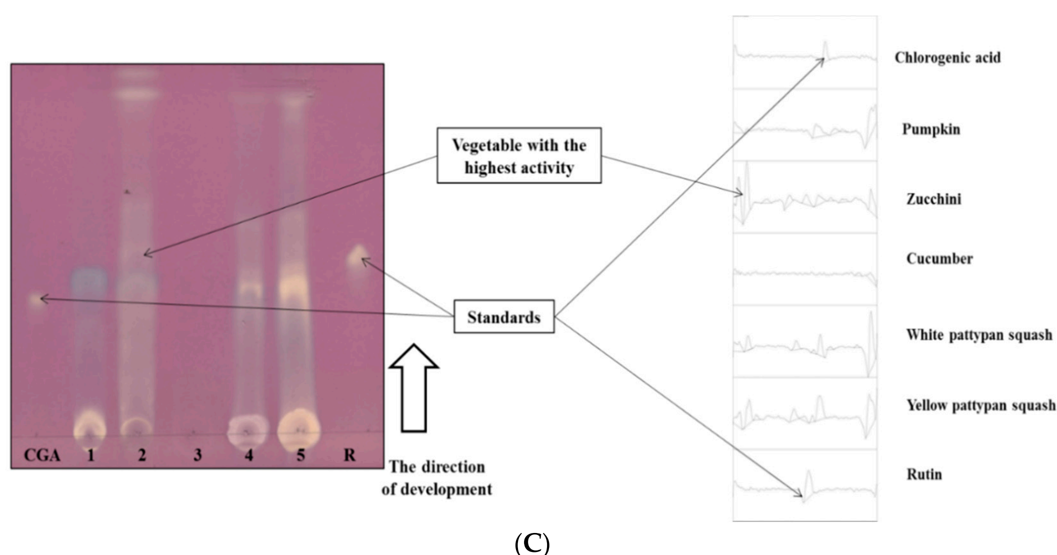
Figure 3. *Cont.*



(**C**)

Figure 3. Effects of the five cucurbit vegetable preparations (incubation time 30 min) on the plasma antioxidant capacity, measured as ORAC (oxygen radical antioxidant capacity) (**A**); on total antioxidant capacity of plasma (**B**). Results are given as mean ± SE (n = 5). One-way ANOVA followed by a multicomparison Tukey test and Kruskal–Wallis test, compared with control. The use of TLC-DPPH• assay for the detection of antioxidant activity of preparations from: 1-pumpkin, 2-zucchini, 3-cucumber, 4-white pattypan squash, 5-yellow pattypan squash. Standards: chlorogenic acid (CGA) and rutin (R) (**C**).

Table 2. Comparative effects of the five cucurbit vegetable preparations on oxidative stress in plasma treated with H_2O_2/Fe (tested concentration 50 µg/mL).

Preparation	Protein Carbonylation	Thiol Oxidation	Lipid Peroxidation
from fruit with seeds			
Zucchini	No effect	No effect	Positive action-inhibition of this process (anti-oxidative potential)
Cucumber	Positive action-inhibition of this process (anti-oxidative potential)	No effect	Positive action-inhibition of this process (anti-oxidative potential)
from fruit without seeds			
Pumpkin	Positive action-inhibition of this process (anti-oxidative potential)	No effect	Positive action-inhibition of this process (anti-oxidative potential)
White pattypan squash	Positive action-inhibition of this process (anti-oxidative potential)	No effect	Positive action-inhibition of this process (anti-oxidative potential)
Yellow pattypan squash	Positive action-inhibition of this process (anti-oxidative potential)	Positive action-inhibition of this process (anti-oxidative potential)	Positive action-inhibition of this process (anti-oxidative potential)

3. Discussion

Preparations from fruits and vegetables constitute a basis for modern phototherapy, as they contain concentrated active components, including phenolic compounds. The antioxidant or prooxidant properties of selected vegetables from the *Cucurbitaceae* family were studied in an in vitro model of human plasma exposed to oxidative stress. The stress conditions were induced by H_2O_2/Fe, the donor of hydroxyl radicals (one of the strongest oxidative agents) involved in the pathophysiology of various processes. Hydroxyl radicals may interact with plasma lipids and proteins. The destructive effect of OH is reflected in the increased plasma levels of oxidative stress parameters such as TBARS, oxidation of thiol groups and protein carbonylation. The levels of these biomarkers have been connected with the progress or poorer prognosis of various diseases, for example cardiovascular diseases [16]. In our in vitro experiments, all tested preparations exerted a protective action in plasma proteins and lipids against hydroxyl radicals. Therefore, our present studies indicate that the tested vegetables from the

Cucurbitaceae family may be promising candidates for the prevention and treatment of CVDs associated with oxidative stress.

The complex chemical composition of the preparations are likely related to their ability to modulate oxidative stress, and hence reduce the risk of cardiovascular diseases. On the other hand, the preparations neither failed to induce the total antioxidant capacity of plasma nor led to higher values of ORAC. However, in the DPPH free radical scavenging activity test, all five of the preparations differed in chemical composition and demonstrated antiradical properties. The zucchini preparation showed the highest radical scavenging effect of the preparations, because its phytochemical profile was the richest in terms of the presence of flavonoid compounds (Table 1). In general, the antioxidant activity of flavonoids depends on the structure and substitution pattern of the hydroxyl groups. The essential requirement for effective radical scavenging is the 4-carbonyl group in ring C and 3′,4′-orthodihydroxy configuration in ring B. The presence of the 3-OH group or 3- and 5-OH groups, giving a catechol-like structure in ring C, is also beneficial for the antioxidant activity of flavonoids. The presence of the C2–C3 double bond configured with a 4-keto arrangement is known to be responsible for electron delocalization from ring B and increases the radical-scavenging activity. In the absence of the o-dihydroxy structure in ring B, a catechol structure in ring A can compensate for flavonoid antioxidant activity. Quercetin has a catechol structure in ring B, as well as a 2,3-double bond in conjunction with a 4-carbonyl group in ring C, allowing the delocalization of the phenoxyl radical electron to the flavonoid nucleus. The combined presence of a 3-hydroxy group with a 2,3-double bond additionally increases the resonance stabilization for electron delocalization; hence, it has a higher antioxidant value. Flavonols (e.g., quercetin, isorhamnetin) have a hydroxyl group at position 3. Kim and Lee [17] suggest a structurally important role of the 3-OH group in the chroman ring responsible for the enhancement of antioxidant activity. The antioxidant activity of the zucchini preparation is a result of rutin, isorhamnetin 3-*O*-rutinoside, hesperetin 7-*O*-(2′′,6′′-di-*O*-α-rhamnopyranosyl)-β-glucopyranoside, xanthorhamnin and quercetin 3,3′-dimethyl ether 7-rutinoside contents. The results of Iswaldi et al. [14] showed that sixteen flavonoids and other polar compounds with their derivatives were identified in the whole zucchini vegetables. The yellow pattypan squash preparation also showed a high antioxidant activity, which was significantly influenced by the very high content of benzoic acid derivatives, mainly 3-(β-D-glucopyranosyloxy)-2-hydroxybenzoic acid [14].

The results of the inorganic experimental system (DPPH test) did not quite coincide with those obtained in the biological experimental system (human plasma treated with a strong physiological inducer of oxidative stress, H_2O_2/Fe). However, the tested preparations showed antioxidant activities of different levels in vitro. In this biological system, in vitro, the yellow pattypan squash preparation had the strongest antioxidant properties, and inhibited oxidative stress induced by H_2O_2/Fe in all the tests: plasma lipid peroxidation, oxidation of thiol groups in plasma proteins and plasma protein carbonylation. In addition, it seems to be important that the preparations often demonstrated antioxidant activity even at low concentrations: 1 and 5 μg/mL. The best antioxidant activity of yellow pattypan squash may be due to the presence of a benzoic acid derivative level. The antioxidant activity of benzoic acid derivatives correlates with the presence of the phenolic group and the position of the hydroxyl group. Velika and Kron [18] discovered that derivatives with a blocked hydroxyl group showed lower antioxidant properties than derivatives with a blocked carbonyl group. The pattypan squash yellow preparation, in comparison to the other preparations, contain phenylpropanoids glycoside, which could be another reason for its antioxidant activity in plasma. Phenylpropanoid glycoside showed a strong antioxidant activity, such as inhibition of oxidation of low-density lipoprotein through free radical scavenging and metal ion chelation, which correlates with the presence of the phenylpropanoid and phenylethanoid groups in the structure. Thuan et al. [19] demonstrated that phenylpropanoid glycoside isolated from *Picria tel-ferae* can inhibit lipid peroxidation in an in vitro model using TBARS assay [19,20].

The pumpkin preparation inhibited protein carbonylation and lipid peroxidation in the plasma in the in vitro model. The pumpkin preparation was the only one to have a high content of phenolic

acid, which lead to the display of antioxidant properties. Xanthopoulou et al. [21] proved a correlation between phenolic compound content and the antioxidant activity of pumpkin. The antioxidant activities of pumpkin seed extracts were determined using a DPPH free radical assay. The results showed that pumpkin seed extract demonstrated a phenol concentration-dependent antiradical activity [21]. The cucumber and white pattypan squash preparations showed similar effects to the pumpkin. Both preparations inhibited protein carbonylation and lipid peroxidation in plasma. The zucchini preparation only inhibited lipid peroxidation in plasma. Zucchini contain the highest level of flavonoids of all the tested preparations. Khennouf et al. [22] reported that flavonoids have a strong free radical scavenging ability and can inhibit xanthine oxidase activity, a source of oxygen free radicals. Flavonoids also inhibit lipids, but at a lower level than other secondary plant metabolites, such as tannins and phenolic compounds [22]. Other researchers also observed that preparations from vegetables of the *Cucurbitaceae* family may reduce oxidative stress in vitro, and in the in vivo models. For example, *Cucurbita maxima* pumpkin pectin inhibited oxidative stress induced by 2,2′-azobis(2-methylpropionamide) dihydrochloride in cell cultures (cell lines HT-29, human colon adenocarcinoma), and MDCK1 (canine kidney epithelium). Oxidative stress was measured by the production of intracellular reactive species [23]. In another in vitro model, Shayesteh et al. [24] also demonstrated the protective effects of pumpkin fruit extract against oxidative stress. Cumene hydroperoxide and glyoxal were used as inductors of oxidative stress in freshly isolated rat hepatocytes. The tested extract (50 μg/mL) reduced oxidative stress as measured by various parameters, including lipid peroxidation, reactive oxygen species production and glutathione depletion. The same activity was observed by Bahramsoltani et al. [25]. Moreover, the results of Abarikwu et al. [26] demonstrated that fluted pumpkin seeds (200 mg/kg body wt.) protect against busulfon-induced oxidative stress in adult mice. The effect of ethanolic extract of fluted pumpkin seeds was investigated after 40 days of oral administration. Ghahremanloo et al. [27] have also observed that pumpkin extract ameliorates oxidative stress in obese rats, leading to decreased cardiovascular disease risk in obesity. Three groups of obese rats received hydroalcoholic extract of pumpkin as one daily dose of 100, 200 and 400 mg/kg, respectively. At the end of six weeks, the parameters of oxidative stress were measured [27].

Oxidative stress may alter the coagulation process, and this may lead to the development of CVDs [16]. However, it is not known whether the tested preparations are associated with the modulation coagulation process, or whether they have an antithrombotic activity. For the first time, in our present study on these five preparations, we found that they did not change the coagulation system, and did not show anticoagulant or procoagulant potential in the in vitro model. Therefore, we suggest that the preparations may be a source of antioxidants that do not incur the risk of bleeding or thrombosis [16].

4. Materials and Methods

4.1. Chemicals

Methanol and acetonitrile, HPLC grade, were purchased from Merck (Darmstadt, Germany). Formic acid, LC-MS grade, was purchased from Sigma-Aldrich, (St. Louis, MO, USA). Ultrapure water was obtained in-house with a purification system (Milli-Q-Simplicity-185, Millipore Corp.). Dimethylsulfoxide (DMSO), thiobarbituric acid (TBA) and H_2O_2 were purchased from Sigma (St. Louis, MO, USA). Other reagents were of analytical grade and were provided by commercial suppliers, including POCh, (Poland), Acros (Poland) and Chempur (Poland).

4.2. Plant Material

Five cucurbit vegetable types were selected, and subjected to freeze-drying (CHRIST Gamma 2-16 LSC Freeze Dryers, Osterode am Harz, Germany): pumpkin (*Cucurbita pepo* L., fruit without seeds); zucchini (*Cucurbita pepo* L. *convar. Giromontina*, fruit with seeds); cucumber (*Cucumis sativus* L., fruit with seeds); white pattypan squash (*Cucurbita pepo* L. var. *patisoniana*, fruit without seeds) and

yellow pattypan squash (*Cucurbita pepo* L. var. *patisoniana*, fruit without seeds). The plant material was stored at the Department of Biochemistry and Crop Quality of the Institute in Puławy, Poland.

4.3. Extraction and Preparation of Vegetable Preparations

Plant materials (1 mg of each of the freeze-dried vegetables) were extracted using an automatic extractor, Dionex ASE 200 Accelerated Solvent Extraction System. The extraction process conditions were as follows: extraction solvent: 80% methanol, solvent pressure: 1500 psi, extraction cell temperature: 40 °C, extraction cycles: 3. The extracts were evaporation dried under reduced pressure, at 40 °C (Heidolph Hei-Vap Advantage, rotary evaporator). The five extracts were purified, mainly from sugars, by solid phase extraction (SPE). Specialized metabolite fractions were eluted from Oasis Extraction Cartridges (Waters, MA, USA) with 6 mL of 85% methanol. The evaporated eluate was dissolved in 70% MeOH. Next, the samples were cleaned in an ultrasonic bath for 5 min at 30 °C (SONOREX DIGITEC DT 510 H, Bandelin, Germany) and centrifuged for 5 min at 10,000 rpm at 20 °C (laboratory centrifuge: Polygen Sigma 3-16 KL, Sigma, Germany). Finally, 150 µL of the supernatant from each sample was subjected to HPLC-MS analysis.

4.4. Phytochemical Profiling

The quantitative analysis of each preparation was carried out by ultra-high resolution mass spectrometry (UHRMS) using a Dionex UltiMate 3000RS (Thermo Scientific, Darmstadt, Germany) system with a charged aerosol detector (CAD) interfaced with a high-resolution quadrupole time-of-flight mass spectrometer (HR/Q-TOF/MS, Impact II, Bruker Daltonik GmbH, Bremen, Germany). The chromatographic separation was performed on an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 µm, Waters, Manchester, UK), with the column temperature maintained at 60 °C. The mobile phases were acidified (0.1% formic acid) water (solvent A) and acidified (0.1% formic acid) acetonitrile (solvent B). The chromatographic method consisted of the following linear gradient: 7% B from 0 to 0.5 min and then the concentration of B was increased to 80% from 0.5 to 26 min. The sample injection volume was 1.0 µL and the flow rate was set at 500 µL/min. Compounds were analyzed based on data from the mass spectra. Electrospray ionization (ESI) was performed in negative ion mode. The mass scan range was set at 50–2000 *m*/*z*. Ion source parameters were as follows: capillary voltage 3.0 kV, collision energy20.0 eV, dry gas 6.0 L/min and dry temperature 200 °C. Data acquisition and processing were performed using DataAnalysis 4.3 (Bruker Daltonik GmbH, Bremen, Germany). Determination of molecular formula was carried out by mass accuracy, adduct, and fragment information using SmartFormula.

4.5. Stock Solutions of Vegetable Preparation

The stock solutions of vegetable preparations, used in the tests for biological activity, were made in 50% DMSO. The final concentration of DMSO in the samples (human plasma) was lower than 0.05% and its effects were determined in all experiments.

4.6. Human Plasma Isolation

Human blood or plasma were obtained from six regular donors (non-smoking men and women) to a blood bank (Lodz, Poland) and a medical center (Lodz, Poland). Blood was collected as a 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). Donors had not taken any medication or addictive substances (including tobacco, alcohol and antioxidant supplementation) for at least two week before a donation. 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 ref. 8/KBBN-UŁ/III/2018). The plasma was isolated by differential centrifuging as described earlier [28]. The plasma was incubated (30 min, at 37 °C; for hemostatic parameters) with vegetable preparations at the final concentrations of 1–50 µg/mL. Human plasma was also pre-incubated (5 min, at 37 °C; for parameters of oxidative stress) with vegetable preparations

at the final concentrations of 1–50 µg/mL, and then treated with 4.7 mM H_2O_2/3.8 mM Fe_2SO_4/2.5 mM EDTA (25 min, at 37 °C).

The protein concentration, determined by measuring absorbance at 280 nm according to the procedure of Whitaker and Granum [29], was measured using Bradford protein assay [30].

4.7. Markers of Oxidative Stress

4.7.1. Lipid Peroxidation Measurement

Lipid peroxidation was quantified by measuring the concentration of TBARS, according to the method described by Wachowicz [31] and Bartosz [32]. After 30 min of incubation with a preparation from Cucurbitaceae vegetables at the final concentrations of 1–50 µg/mL, the samples were mixed with an equal volume of cold 15% (*v*/*v*) trichloroacetic acid ($C_2HCl_3O_2$) in 0.25M HCl and 0.37% (*v*/*v*) TBA in 0.25 M HCl, and then immersed in a boiling water bath for 15 min. After cooling, the absorbance was measured at 535 nm using the SPECTROstar Nano Microplate Reader (BMG LABTECH, Ortenberg, Germany). The TBARS concentration was calculated using the molar extinction coefficient (ε = 156,000 M^{-1} cm^{-1}) and was expressed as nmol/mL of plasma.

4.7.2. Carbonyl Group Measurement

The carbonyl groups were determined in plasma proteins according to Levine et al. [33] and Bartosz [32]. The absorbance measurement (at λ = 375 nm) was performed using the SPECTROstar Nano Microplate Reader (BMG LABTECH, Ortenberg, Germany). The carbonyl group concentration was calculated using a molar extinction coefficient (ε = 22,000 M^{-1} cm^{-1}) and was expressed as nmol/mg of plasma protein.

4.7.3. Thiol Group Measurement

The thiol group content was measured spectrophotometrically (absorbance at λ = 412 nm), using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Ortenberg, Germany), with 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) according to the method described by Ando and Steiner [34,35] and Bartosz [32]. The thiol group concentration was calculated using a molar extinction coefficient (ε = 13,600 M^{-1} cm^{-1}) and was expressed as nmol/mL of plasma protein.

4.7.4. TLC-DPPH• Test

The free radical scavenging potential of the pumpkin, zucchini, cucumber, white and yellow pattypan squash preparations was assessed by means of a simple benchtop TLC-DPPH• bioassay with ImageJ program. This method, with small modifications, has been found suitable for the analysis of complex samples, as proved in our previous publications [36–38].

Preparations (5 mg/mL) and standard compounds, chlorogenic acid ((1*S*,3*R*,4*R*,5*R*)-3-{[(2*E*) -3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid) and rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]-4*H* -chromen-4-one), 1 mg/mL, were prepared and applied to aluminum-backed silica gel (60 F254, Merck) chromatographic plates, with an 8 mm gap between them, and at 10 mm from both the left and low edges, using a micropipette with a scale. The plates were developed in vertical chambers pre-saturated for 15 min with the optimized mobile phase: acetonitrile:chloroform:water:formic acid (80:10:10:5, *v*/*v*/*v*/*v*). The plates were developed at a distance of 90 mm and dried in a hood for 30 min before derivatization. Next, the TLC plates were immersed for 5 s in a freshly prepared 0.2% (*w*/*v*) methanolic DPPH• solution. After removing the DPPH• excess, the plates were stored in the dark for 30 min and then scanned by means of a flatbed scanner. Compounds with the ability to scavenge free radicals emerged as a yellow band against a purple background. The test was performed in triplicate.

4.7.5. Image Processing Procedure

The results of the TLC–DPPH• test were documented by flat-bed scanning, saved in the form of jpg image files and further processed by means of an open source and free program, ImageJ, developed at the National Institute of Health in the USA.

For the DPPH• staining, the results change over time, and therefore it is crucial to precisely define the time that elapses between immersion and documentation. The results were documented every 10 min for an hour, and after the comparison it was decided to process the images taken 30 min after staining. Subsequently the images chosen were processed by means of the ImageJ program, with the use of a modified procedure following Olech et al. [39]. In summary, the color images were converted to 8-bit type images (Image/Type/8-bit). These images were denoised by applying the following steps: Process/Filters/Median/Radius-20 pixels. The baseline drift was removed (Process/FFT/Bandpass Filter/Filter large structures down to-120 pixels; filter small structures up to-0 pixels). The images processed in this way were then inverted (Edit/Invert). In order to change the videoscan images into chromatograms, resembling those obtained in high-performance liquid chromatography (HPLC), a rectangular selection tool was used to outline the tracks. The line profile plots were obtained in the same way as described in the original procedure [39]. The areas under the common peaks were measured and compared with the area obtained for chlorogenic acid and rutin, a compound with a recognized free radical scavenging potential.

4.7.6. ORAC Assay

ORAC is based on a hydrogen atom transfer (HAT) process, with oxidation of a fluorescent probe by peroxyl radicals. The role of the antioxidant in the assay is to block peroxyl radical oxidation of the fluorescent probe until the whole antioxidant activity in the sample is complete. The sample antioxidant activity is associated with the fluorescence decay curve, which is represented as the area under the curve (AUC), used to measure total peroxyl radical antioxidant activity and compare to the antioxidant standard curve of Trolox, a water-soluble tocopherol analogue. The Trolox curve was prepared on the same plate as all samples and according to the instructions supplied in the kit. The procedure for the ORAC assay was performed on plasma according to the instructions supplied with the Oxygen Radical Antioxidant Capacity (ORAC) Assay kit from CELL BIOLABS, INC (San Diego, USA). The kit included a 96-well microtiter plate with clear bottom black plate, fluorescein probe 100×, free radical initiator, antioxidant standard (Trolox™), and assay diluent (4×). The samples at concentration 5 and 50 µg/mL were dissolved in the ratio 1:100 [40].

4.7.7. Total Antioxidant Capacity of Plasma

Total antioxidant capacity of plasma (with a preparation from *Cucurbitaceae*) can be measured by the antioxidant ability of metmyoglobin to inhibit the oxidation of 2,2′-azino-di-[3-etyhylbenthiazoline sulphonated] (ATBS) in the sample. The amount of ABTS can be monitored by measuring the absorbance at 750 nm. The antioxidant in the sample causes suppression of the absorbance to a degree proportional to its concentration. The antioxidant capacity of the sample is compared with that of Trolox, a water-soluble tocopherol analogue. The Trolox curve was prepared on the same plate as all samples and according to instructions supplied in kit. The procedure for total antioxidant capacity was performed on plasma according to the instructions supplied with the Antioxidant Assay KIT from Cayman Chemical (Ann Arbor, MI, USA). The kit included Antioxidant Assay Buffer (10×), Antioxidant Assay Chromogen, Antioxidant Assay Metmyoglobin, Antioxidant Assay Trolox, Antioxidant Assay Hydrogen Peroxide, and a 96-well solid plate. The samples at concentration 50 µg/mL were dissolved 1:20 [41].

4.8. Parameters of Coagulation

4.8.1. Measurement of Prothrombin Time (PT)

The PT was determined coagulometrically using an Optic Coagulation Analyser, model K-3002 (Kselmed, Grudziadz, Poland), according to the method described by Malinowska et al. [42]. Briefly, after 30 min of treatment with a *Cucurbitaceae* preparation, the human plasma (50 µL) was incubated for 2 min at 37 °C and then, directly before measurement, 100 µL of Dia-PT liquid (commercial preparation: Kselmed, Grudziadz, Poland) was added.

4.8.2. Measurement of Thrombin Time (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. [42]. Briefly, after treatment with the *Cucurbitaceae* preparation, the human plasma (50 µL) was incubated for 1 min at 37 °C and then, directly before measurement, 100 µL of thrombin was added (final concentration was 5 U/mL).

4.8.3. Measurement of Activated Partial Thromboplastin Time (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. [42]. Briefly, after treatment with the *Cucurbitaceae* preparation, the human plasma (50 µL) was incubated with 50 µL of Dia-PTT liquid (commercial thromboplastin: Kselmed, Grudziadz, Poland) for 3 min at 37 °C and then, directly before measurement, 50 µL of 25 mM CaCl2 was added.

4.9. Data Analysis

Several tests were used to carry out the statistical analysis. All the values in this study were expressed as mean ± SE. The results were first evaluated for normality with the Kołmogorowa–Smirnowa test and equality of variance with the Levine test. Statistically significant differences were assessed by applying the ANOVA test (significance level was $p < 0.05$), followed by a Tukey multiple comparisons test or Kruskal–Wallis test.

5. Conclusions

The present paper is the first detailed study of these five preparations from selected vegetables from the *Cucurbitaceae* family and provides new insights into their phytochemical composition and biological activity (using human plasma). In this work, the UHPLC-ESI-QTOF-MS system was used to identify different compounds in the tested preparations. The results revealed that these preparations have various bioactive compounds with antioxidant activities for use in the prophylaxis and treatment of diseases involving oxidative stress, including CVDs. Although antioxidant properties were demonstrated in an in vitro model, the real effect of these extracts should be verified in an in vivo model.

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Sample Availability: Samples of pumpkin (*Cucurbita pepo*; fruit), zucchini (*Cucurbita pepo convar. giromontina*), cucumber (*Cucumis sativus*), white pattypan squash (*Cucurbita pepo var. patisoniana*) and yellow pattypan squash (*Cucurbita pepo var. patisoniana*) are available from the authors.

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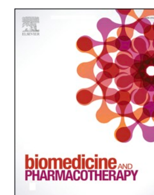

Antioxidant and hemostatic properties of preparations from *Asteraceae* family and their chemical composition – Comparative studies

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A R T I C L E I N F O

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A B S T R A C T

Asteraceae, known as sunflower family, is one of the largest flowering plants family around the world. Sunflower family contains numerous phytochemical compounds. The aim of this study was to describe phytochemical characteristics and investigate the effect of four sunflower vegetable preparations (extracts): chicory leaves (*Cichorium intybus*), green lettuce leaves (*Lactuca sativa*), red lettuce leaves (*Lactuca sativa* var. *crispa*) and sunchoke roots (*Helianthus tuberosus*) on different biomarkers of oxidative stress in human plasma in *in vitro* model. The antioxidant potential was also tested using the DPPH method. The phytochemical composition of the tested preparations was determined by UPLC-ESI-QTOF-MS. All the tested extracts demonstrated antioxidant activity in human plasma. We have observed chicory's and sunchoke's extracts had strongest antioxidant properties in the used models with human plasma. None of the tested vegetables changed ORAC and TAC *in vitro*. The obtained results suggest that sunflower vegetables might help to prevent oxidative stress related with cardiovascular diseases.

1. Introduction

Asteraceae family, also known as sunflower family, is one of the largest flowering plants family around the world. It includes over 1600 genera and 2500 species, with chicory, lettuce, daisies, dandelion and artichoke as most known representatives. Plants from this family are distributed on every continent, except for Antarctica, and grow in every type of habitat, due to their easy ecological adaptability and cosmopolitan distribution. Their morphology is impressively diverse and covers 1 cm high herbs and over 30 m high trees [1,2]. *Asteraceae* family due to its high biodiversity, contains numerous phytochemical compounds, among others polyphenols, phenolic acids, flavonoids, sesquiterpene lactones, essential oils, saponins and lignans. The chemical composition of plants from sunflower family is the reason for their biological activity. Many species were used in traditional medicine. *Centaurea solstitalis* were used as a remedy for stomach problems, infections, cold and to treating abdominal pain in Turkey. *Cichorium intybus* were used for liver disorder, gallstones, rheumatism and appetite loss. Nepalese drink juice form roots of *Emilia sonchifolia* for fever [3,4]. The chamomile (*Matricaria chamomille*) is another medicinal herbal native in southern and western Europe, North Africa, both Americas and Asia, well known for thousands of years. It has mainly the anti-inflammatory and antiseptic activity. A tea made from chamomile flowers was used for nausea and diarrhea, the sluggish digestion. In many countries, it was suggested as treatment for urinary tract inflammation and painful menstruation. The drug powered was often applied to treat skin eruptions, inflammation in eyes and for faster wound healing [5]. Over the years researchers have analyzed the traditional use of plants, which led to discovery of many promising compounds and growing interest in therapeutic used of members of *Asteraceae* family [6]. Many members of sunflower family demonstrated pharmacological activity. *Cichorium intybus,* also known as chicory, shows

Abbreviations: DMSO, dimethylsulfoxide; TBA, thiobarbituric acid; UHRMS, ultra-high-resolution mass spectrometry; RLL, red lettuce leaves; GLL, green lettuce leaves; CL, chicory leaves; SR, sunchoke roots; CPD, citrate/phosphate/dextrose; 9:1, v/v blood/CPD; HAT, hydrogen atom transfer; AUC, area under the curve; ORAC, Oxygen Radical Antioxidant Capacity; ATBS, 2,2′-azino-di-[3-etyhylbenthiazoline sulphonated]; PHA, phenolic acids; FL, flavonoids; SL, sesquiterpene lactones; ROS, reactive oxygen species; NOX, NADPH oxidase; MPO, myeloperoxidase; LOX, lipoxygenases; eNOS, nitric oxide synthase; 8-iso-PGF2α, urinary 8-iso-prostaglandin F2α.

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anti-inflammatory activity, by lowering the level of cytokines, including interleukin 4, inhibiting proliferation of allogeneic T cells, and increasing interferon γ. In *in vivo* studies, an extract from chicory increased the production of interleukin 12 by dendritic cells in male mice. In *in vitro* studies extract from fresh leaves form chicory inhibited lipid peroxidation, one of biomarkers of oxidative stress, thanks to high anthocyanin levels in plants [7–10]. The Jerusalem artichoke also showed strong antioxidant activity, which is correlated with phenolic compounds in chemical composition. Additionally, Jerusalem artichoke demonstrated strong prebiotic properties, especially for *Lactobacillus* sp., because it contains inulin (about 18%), a polysaccharide that improved a survival of *Lactobacillus* sp. and enhanced resistance to gastrointestinal conditions [11]. Dandelion shows antiplatelet activity, due to its ability to inhibit blood platelet adhesion to collagen and fibrinogen. It also can improve hepatic regenerative capacity [12,13].

The aim of our present experiments was to describe phytochemical characteristics and investigate the effects of four plant preparations (extracts) from *Asteraceae* vegetables: chicory leaves (*Cichorium intybus*), green lettuce leaves (*Lactuca sativa*), red lettuce leaves (*Lactuca sativa* var. *crispa*) and sunchoke roots (*Helianthus tuberosus*) on different biomarkers of oxidative stress, including the level of thiobarbituric acid reactive substances (TBARS) – biomarker of lipid peroxidation, the level of thiol groups, protein carbonylation, total antioxidant capacity (TAC) and oxygen radical antioxidant capacity in human plasma *in vitro*. Since oxidative stress can modulate hemostasis, our work is also devoted to the effect of these preparations on hemostatic properties of plasma. For our research, we selected the plants not only popular in everyday diet in Poland, but also cheap and easy to cultivate, which can improved further research on bigger scale. All the tested vegetables showed health biological activity but their influences on biomarkers of oxidative stress in hemostasis process have not been studied yet.

2. Materials and methods

2.1. Chemicals

Methanol, acetonitrile and HPLC grade, were purchased from Merck (Darmstadt, Germany). Formic acid, LC-MS grade, was purchased from Sigma-Aldrich, (St. Louis, MO, USA). Ultrapure water was obtained inhouse with a purification system (Milli-Q-Simplicity-185, Millipore Corp.). Reference standard chlorogenic acid (5-caffeoylquinic acid) was purchased from Fluka AG (Switzerland). Dimethylsulfoxide (DMSO), thiobarbituric acid (TBA) and H_2O_2 were purchased from Sigma (St. Louis, MO., USA). Other reagents were of analytical grade and were provided by commercial suppliers, including Chempur (Poland), POCh, (Poland) and Acros (Poland).

2.2. Plant material

Four vegetable types: leaves of chicory (*Cichorium intybus* L.), leaves of green lettuce (*Lactuca sativa* L.), leaves of red lettuce (*Lactuca sativa* L. var. *crispa*) and roots of sunchoke (*Helianthus tuberosus* L.) were bought in a supermarket, and then were frozen and lyophilized (CHRIST Gamma 2-293 16 LSC Freeze Dryers, Osterode am Harz, Germany). The plant material has been deposited at the Department of Biochemistry and Crop Quality of the Institute in Puławy, Poland.

2.3. Extraction and preparation of vegetable preparations (extracts)

2.3.1. Preparation of samples for phytochemical profiling and quantitative determination of major phenolic compounds

Plant material was extracted using automatic extractor, Dionex ASE 200 (Accelerated Solvent Extraction System). The extraction process conditions were described below: extraction solvent 80% methanol, solvent pressure 1500 psi, extraction cell temperature 40 °C, extraction cycles 3. The extracts were evaporated and dried under reduced pressure, at 40 °C (Heidolph Hei-Vap Advantage, rotary evaporator). All the obtained crude extracts were kept in a refrigerator.

Before analysis, 1 mL of 70% methanol was added to 10 mg of each dry extract, and samples were sonicated for 15 min at 25 °C for better dissolution (sonicator SONOREX DIGITEC DT 510H, Bandelin, Germany). Then, those extracts were purified by solid phase extraction (SPE). The samples were loaded onto the Oasis HLB column (Extraction Cartridges, Waters, Massachusetts, USA), which were washed with water sugars removal, and secondary metabolites fractions were eluted with acidified (0.1% formic acid) 85% methanol for separation from the chlorophyll. The eluates were evaporated and dissolved in 1 mL of 70% methanol, and next centrifuged for 10 min at 11,000 rpm (laboratory centrifuge Polygen Sigma 3-16 KL, Sigma, Germany). Finally, 5 μL of the supernatant from each sample was subjected to UHPLC-ESI-QTOF-MS for the qualitative analysis, and 2.5 μL to UHPLC-MS analysis for the quantitative determination of major phenolic acids.

2.4. Phytochemical profiling and quantification of major phenolic compounds

2.4.1. Ultra-high-resolution mass spectrometry UHPLC-ESI-QTOF-MS for the qualitative analysis

The qualitative analysis of the four aqueous-methanol extracts were carried out on UHRMS (Ultra High Resolution Mass Spectrometry) on a Dionex UltiMate 3000RS (Thermo Scientific, Darmstadt, Germany) system with a CAD (Charged Aerosol Detector) interfaced with a High-Resolution Quadrupole Time-Of-Flight Mass Spectrometer (HR/Q-TOF/MS, Impact II, Bruker Daltonik GmbH, Bremen, Germany). The chromatographic separation was performed on an Acquity UPLC HSS T3 column (150 × 2.1 mm, 1.8 μm, Waters, Manchester, UK), the column temperature was maintained at 50 °C. The mobile phase consisted of: acidified (0.1% formic acid) water (solvent A) and acidified (0.1% formic acid) acetonitrile (solvent B) at a flow rate of 500 μL/min. The chromatographic method consisted of in the following linear gradient: 2% B from 0 to 0.30 min, and the concentration of B was then increased to 99% from 0.30 to 22.01 min. The sample injection volume was 5.0 μL.

The compounds were analyzed based on data from mass spectra. Electrospray ionization (ESI) was performed in negative ion mode. The mass scan range was set at 80–2000 *m/z*. Ions source parameters; capillary voltage 3.0 kV, dry gas 6.0 L/min and dry temperature 200 °C. In addition, the analytes were identified based on data from UV spectrum. The PDA was operated in the range of 190–750 nm. Data processing was performed using DataAnalysis 4.3 (Bruker Daltonik GmbH, Bremen, Germany).

2.4.2. Ultra high pressure liquid chromatography (UHPLC-MS) conditions

The quantitation of the 6 identified phenolic acids were performed by ACQUITY UPLC system, equipped with a PDA and a triple quadrupole mass detector (TQD, Waters, Milford, MA, USA). Separation of compounds was applied using an Acquity UPLC HSS C18 column (100 × 2.1 mm, 1.8 μm particle size; Waters, Manchester, UK) with a gradient mobile phase. Solvent A – water with 0.1% formic acid and solvent B – acetonitrile with 0.1% formic acid were used as follows: 10–22% in 7,5 min at a flow rate of 0.5 mL/min. The column temperature was maintained at 30 °C. The injection volume was set to 2.5 μL. Compounds were interpreted based on the data from mass spectra. ESI ionization was performed in negative ion mode. PDA was operated in the range of 191–480 nm, with a resolution of 3.6 nm. Data processing was performed using MassLynx V4.1 software, Waters.

The quantitative analysis of the phenolic acids were performed based on data from UV spectra about the wavelength of 320 nm. The quantitative determinations of compounds were carried out by an external standard method and results expressed as mg per gram of extracts. The linearity of the method was shown using a calibration curve with seven known concentrations of standard in the range of 10–200 μg/mL, the linear correlation coefficient for the curve was ($R^2 = 0.999$). According

to the calibration equation (y = 260.3x − 129.31), the phenolic acids content of extracts from red lettuce leaves (RLL), green lettuce leaves (GLL), chicory leaves (CL) and sunchoke roots (SR) were calculated as shown in Table 2.

2.5. Stock solutions of vegetable preparations (extracts)

All of the vegetables preparation were dissolved in 50% DMSO to prepared stock solution used for tests biological activity. The final concentration of DMSO in the samples was lower than 0.05% and its effects were determined in all experiments.

2.6. Human plasma isolation

Human blood or plasma were obtained from eight regular donors (non-smoking men and women) a blood bank (Lodz, Poland) and a medical center (Lodz, Poland). Blood was collected as a 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). Donors had not taken any medication or addictive substances (including tobacco, alcohol and antioxidant supplementation) for a week before donation. 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 ref. 8/KBBN-UŁ/III/2018). The plasma was isolated by differential centrifuging as described earlier [14]. For hemostatic parameters, the plasma was incubated for 30 min at 37 °C with vegetable preparations at the final concentrations of 1–50 μg/mL. Human plasma was also pre-incubated (5 min, at 37 °C; for parameters of oxidative stress) with vegetable preparations at the final concentrations of 1–50 μg/mL, and then treated with 4.7 mM H_2O_2/3.8 mM Fe_2SO_4/2.5 mM EDTA (25 min, at 37 °C). The protein concentration, determined by measuring absorbance at 280 nm (in the tested samples), was calculated according to the procedure of Whitaker and Granum [15].

2.7. Markers of oxidative stress

2.7.1. Lipid peroxidation measurement

The level of lipid peroxidation in human plasma was quantified by measuring the concentration of thiobarbituric acid reactive substances (TBARS), according to the method described by Wachowicz [16] and Bartosz [17]. After 30 min of incubation with a preparation from *Asteraceae* vegetables, the samples were mixed with an equal volume of cold 15% (v/v) trichloroacetic acid in 0.25 M HCl and 0.37% (v/v) TBA in 0.25 M HCl, and then immersed in a boiling water bath for 15 min. After cooling, the absorbance was measured at 535 nm using the SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). The TBARS concentration was calculated using the molar extinction coefficient (ε = 156,000 M^{-1} cm^{-1}), and was expressed as nmol/mL of plasma.

2.7.2. Carbonyl group measurement

The carbonyl groups were determined in plasma proteins according to Levine et al. [18] and Bartosz [16]. The absorbance measurement (at λ = 375 nm) was performed using the SPECTROstar Nano Microplate Reader (BMG LABTECH). The carbonyl group concentration was calculated using a molar extinction coefficient (ε = 22,000 M^{-1} cm^{-1}), and was expressed as nmol/mg of plasma protein.

2.7.3. Thiol group measurement

The thiol group content was measured spectrophotometrically (absorbance at λ = 412 nm), using a SPECTROstar Nano Microplate Reader (BMG LABTECH), with 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) according to the method described by Ando and Steiner [19,20] and Bartosz [17]. The thiol group concentration was calculated using a molar extinction coefficient (ε = 13,600 M^{-1} cm^{-1}), and was expressed as nmol/mL of plasma protein.

2.7.4. TLC-DPPH• test and image processing by means of the ImageJ program

Thin-layer chromatography coupled with image processing can be applied for screening the antioxidant activity of compounds present in chicory, green lettuce, red lettuce and sunchoke preparations. Using this method it is possible to determine the activity of the compound mixture without the need to isolate single standards.

Standard compounds (chlorogenic acid and rutin – 1 mg/mL, 4 μL on a plate) and the analyzed *Asteraceae* vegetable phenolic fraction: chicory, green lettuce, red lettuce and sunchoke in concentration: 0.85, 0.95, 0.93, 0.82 mg/mL, respectively; were applied to aluminum-backed silica gel (60 F254, Merck) chromatographic plates in amounts: 25, 15, 15 and 25 μL, respectively. The plates were developed in vertical chambers by means of optimized mobile phase: acetonitrile: chloroform: water: formic acid (60:10:15:5, v/v/v/v) to a distance of 90 mm and dried under a fume cupboard. Next, the TLC plates were immersed for 5 s in a freshly prepared 0.2% (w/v) methanolic DPPH• solution. After removing the DPPH• excess, the plates were stored in the dark for 30 min and then scanned by means of a flatbed scanner. Compounds with the ability to scavenge free radicals emerged as a yellow band against a purple background. The test was performed in triplicate. Image processing by means of the ImageJ program is described in detail in an earlier publication of Rolnik et al. [21].

2.7.5. ORAC assay

ORAC is based on a hydrogen atom transfer (HAT) process, with oxidation of a fluorescent probe by peroxyl radicals. The role of the antioxidant in the assay is to block peroxyl radical oxidation of the fluorescent probe until the whole antioxidant activity in the sample is complete. The sample antioxidant activity is associated with the fluorescence decay curve, which is represented as the area under the curve (AUC), used to measure total peroxyl radical antioxidant activity and compare to the antioxidant standard curve of Trolox. The procedure for the ORAC assay was performed on plasma according to the instructions supplied with the Oxygen Radical Antioxidant Capacity (ORAC) Assay kit from CELL BIOLABS, INC (San Diego, USA). The kit included a 96-well microtiter plate with clear bottom black plate, fluorescein probe 100X, free radical initiator, antioxidant standard (Trolox™), and assay diluent (4X). The samples were dissolved in the ratio 1:100 [22].

2.7.6. Total antioxidant capacity of plasma

Total antioxidant capacity of plasma (with a preparation from *Asteraceae*) can be measured by the antioxidant ability of metmyoglobin to inhibit the oxidation of 2,2′-azino-di-[3-etyhylbenthiazoline sulphonated] (ATBS) in the sample. The amount of ABTS can be monitored by measuring the absorbance at 750 nm or 405 nm. The antioxidant in the sample causes suppression of the absorbance to a degree proportional to its concentration. The antioxidant capacity of the sample is compared with that of Trolox. The procedure for total antioxidant capacity was performed on plasma according to the instruction supplied with the Antioxidant Assay KIT from Cayman Chemical (Ann Arbor, MI, USA). The kit included Antioxidant Assay Buffer (10X), Antioxidant Assay Chromogen, Antioxidant Assay Metmyoglobin, Antioxidant Assay Trolox, Antioxidant Assay Hydrogen Peroxide, and 96-well solid plate. The samples were dissolved 1:20 [23].

2.8. Parameters of coagulation

2.8.1. Measurement of prothrombin time (PT)

The PT was determined coagulometrically using an Optic Coagulation Analyser, model K-3002 (Kselmed, Grudziadz, Poland), according to the method described by Malinowska et al. [24]. Briefly, after 30 min of treatment with a *Asteraceae* preparation, the human plasma (50 μL)

Table 1
Phytochemical characteristics of the crude extracts (preparations) from four plants of the *Asteraceae* family identified by UPLC-QTOF MS/MS.

RT (min)	Proposed compound	Compound class	[M-H]⁻ *m/z*	MS2 [M-H]⁻ (*m/z*)	Ion formula [-H]	Ref.	RLL	GLL	CL	SR
6.36	L-tryptophan	amino acids	203	203, 116	C11H11N2O2	not found	–	–	+	+
6.48	3- caffeoylquinic acid	phenolic acids	353	353, 191, 179, 135	C16H17O9	Papetti et al., 2017; Corazzone et al., 2013; Jaiswal et al., 2011	–	–	+	–
7.6	methylentryptophan	amino acids	215	215, 171, 142, 116	C12H11N2O2	not found	–	–	+	+
7.77	5-caffeoylquinic acid	phenolic acids	353	353, 191	C16H17O9	Xue et al., 2019	+	+	+	+
8.02	4-caffeoylquinic acid	phenolic acids	353	353, 191, 179, 173, 135	C16H17O9	Zhang et al., 2013	–	–	–	+
8.14	zeylanidine A	sesquiterpene lactones	349	349, 277, 215, 200	C17H17O8	Chen et al., 1998	–	–	+	–
8.3	cichorioside B	sesquiterpene lactones	439	439, 277, 215, 197	C22H29O12	Seto et al., 1988	–	+	+	–
8.65	lactucin derivative	sesquiterpene lactones	347	347, 275, 213	C17H15O8	Ferioli et al., 2015	–	–	+	–
8.72	1-caffeoylquinic acid	phenolic acids	353	353, 191	C16H17O9	Chen et al., 2014	+	+	+	+
8.9	cis-5-p-coumaroylquinic acid (1)	phenolic acids	337	337, 191	C16H17O8	Jaiswal et al., 2011	+	+	–	–
9.02	11,13-dihydrolactucin	sesquiterpene lactones	277	277, 215	C15H17O5	Ferioli et al., 2015	–	–	+	–
9.48	lactucin	sesquiterpene lactones	275	275, 257, 213	C16H17O7	Ferioli et al., 2015	–	–	+	–
9.59	5-O-feruloylquinic acid	phenolic acids	367	367, 191, 173	C17H19O9	Papetti et al., 2017	–	–	+	–
9.75	cis-5-p-coumaroylquinic acid (2)	phenolic acids	337	337, 191	C16H17O8	Jaiswal et al., 2011	+	+	–	–
10.1	dicaffeoyltartaric acid (chicoric acid) (1)	phenolic acids	473	473, 311, 293, 179, 149	C22H17O12	Papetti et al., 2017; Corazzone et al., 2013	+	+	+	–
10.12	caffeoyltartaric acid (caftaric acid)	phenolic acids	311	311, 179	C13H11O9	Moreno-Escamilla et al., 2020	+	–	–	–
10.13	dicaffeoyltartaric acid derivative	phenolic acids	710	710, 473, 311, 293, 179, 149	C32H38O18	Papetti et al., 2017; Corazzone et al., 2013	+	–	–	–
10.14	dicaffeoyltartaric acid (chicoric acid) (2)	phenolic acids	473	473, 311, 293, 179, 149	C22H17O12	Papetti et al., 2017; Corazzone et al., 2013	+	–	–	–
10.3	quercetin 3-O-glucoside (1)	flavonols	463	463, 301	C21H19O12	Moreno-Escamilla et al., 2020	–	+	–	–
10.54	dicaffeoyltartaric acid (chicoric acid) (3)	phenolic acids	473	473, 311, 293, 179, 149	C22H17O12	Papetti et al., 2017; Corazzone et al., 2013	+	+	–	–
10.76	quercetin 3-O-glucuronide	flavonols	477	477, 301	C21H17O13	Moreno-Escamilla et al., 2020	+	+	–	–
10.88	quercetin 3-O-glucoside (2)	flavonols	463	463, 300	C21H19O12	Moreno-Escamilla et al., 2020	+	+	–	–
10.93	kaempherol-3-O-glucuronide	flavonols	461	461, 285	C21H17O12	Moreno-Escamilla et al., 2020	+	+	–	–
11.16	caffeoyltartaric-p-coumaroyl acid (1)	phenolic acids	457	457, 295, 293, 179, 163	C22H17O11	Moreno-Escamilla et al., 2020	+	+	–	–
11.25	quercetin malonylglucoside	flavonols	549	549, 505, 300	C24H21O15	Moreno-Escamilla et al., 2020	+	+	–	–
11.28	quercetin acetylgalactoside (1)	flavonols	505	505, 300	C23H21O13	not found	+	–	–	–
11.33	3,4-dicaffeoylquinic acid	phenolic acids	515	515, 353, 335, 191, 179, 173, 135	C25H23O12	Chen et al., 2014; Zhang et al., 2013; Jaiswal et al., 2011	–	–	+	+
11.54	1,3-dicaffeoylquinic acid	phenolic acids	515	515, 353, 191, 179, 135	C25H23O12	Chen et al., 2014; Zhang et al., 2013; Jaiswal et al., 2011	+	+	+	+
11.57	quercetin acetylgalactoside (2)	flavonols	505	505, 300	C23H21O13	not found	+	+	–	–
11.69	caffeoyltartaric-p-coumaroyl acid (2)	phenolic acids	457	457, 295, 293, 179, 163	C22H17O11	Moreno-Escamilla et al., 2020	–	+	–	–
11.71	8-deoxylactucin	sesquiterpene lactones	259	259, 215	C15H15O4	Ferioli et al., 2015	–	+	–	–
11.75	3,5-dicaffeoylquinic acid	phenolic acids	515	515, 353, 191, 179, 135	C25H23O12	Chen et al., 2014; Zhang et al., 2013; Jaiswal et al., 2011	+	–	+	+
11.93	jacquinelin derivative	sesquiterpene lactones	469	469, 423, 349, 261, 199, 113	C22H29O11	Kisiel et al., 1997; Graziani et al., 2015	+	+	+	–
12.03	4,5-dicaffeoylquinic acid	phenolic acids	515	515, 353, 335, 191, 179, 173, 135	C25H23O12	Chen et al., 2014; Zhang et al., 2013	–	–	–	+
13.87	11,13-dihydrolactucopicrin 15-oxalate	sesquiterpene lactones	483	483, 277, 215	C25H23O10	Chadwick et al., 2013	–	+	+	–
15.17	11,13-dihydrolactucopicrin	sesquiterpene lactones	411	457, 411, 277, 215	C23H23O7	Ferioli et al., 2015	–	–	+	–
15.97	octadecadienoic acid derivative	fatty acids	327	327, 211, 171	C18H31O5	Levandi et al., 2009; Hamdy et al., 2018	+	+	+	–
16.81	pinellic acid	fatty acids	329	329, 229, 211	C18H33O5	Levandi et al., 2009; Hamdy et al., 2018	+	+	+	–
20.95	linolenic acid derivative	fatty acids	721	721, 397, 277	C34H57O16	Herrero et al., 2007	+	–	–	–
21.08	hydroxyoctadecenoic acid	fatty acids	297	297, 265, 116	C18H33O3	Levandi et al., 2009	–	–	+	+
21.41	glycerophospholipid	lipids	476	476, 279, 196	C23H43NO7P	Herrero et al., 2007	–	–	–	+
21.43	glycerophospholipid	lipids	564	564, 504, 279	C27H51NO9P	Herrero et al., 2007	+	–	–	+

(*continued on next page*)

Table 1 (*continued*)

RT (min)	Proposed compound	Compound class	$[M-H]^-$ *m/z*	MS2 $[M-H]^-$ (*m/z*)	Ion formula [-H]	Ref.	RLL	GLL	CL	SR
21.69	glycerophospholipid	lipids	540	540, 480, 255	C25H51NO9P	Herrero et al., 2007	+	–	–	+
21.84	octadecadienoic acid derivative	fatty acids	311	311	C18H31O4	Levandi et al., 2009	+	+	+	+

RLL – red lettuce leaves.
GLL – green lettuce leaves.
CL – chicory leaves.
SR – sunchoke roots.
(+) – present.
(–) – not present.

was incubated for 2 min at 37 °C and then, directly before measurement, 100 µL of Dia-PT liquid (commercial preparation: Kselmed, Grudziadz, Poland) was added.

2.8.2. Measurement of thrombin time (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. [24]. Briefly, after treatment with the *Asteraceae* preparation, the human plasma (50 µL) was incubated for 1 min at 37 °C and then, directly before measurement, 100 µL of thrombin was added (final concentration was 5 U/mL).

2.8.3. Measurement of activated partial thromboplastin time (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. [24]. Briefly, after treatment with the *Asteraceae* preparation, the human plasma (50 µL) was incubated with 50 µL of Dia-PTT liquid (commercial thromboplastin: Kselmed, Grudziadz, Poland) for 3 min at 37 °C and then, directly before measurement, 50 µL of 25 mM $CaCl_2$ was added.

2.9. Data analysis

Several tests were used to carry out the statistical analysis. All the values in this study were expressed as mean ± SE. The results were first evaluated for normality with the Kołmogorowa–Smirnowa test and equality of variance with the Levine test. Statistically significant differences were assessed by applying the ANOVA test (significance level was $p < 0.05$), followed by a Tukey multiple comparisons test or Kruskal–Wallis test.

3. Results

3.1. Chemical characteristic of vegetable preparations (extracts)

An overview of all characterized compounds in *Asteraceae* extracts by HPLC-ESI-Q-TOF-MS using the negative mode is given in Table 1 and Fig. 1. These compounds are shown with their retention time, *m/z* experimental, molecular formula generated by the software for the detected deprotonated molecule, MS/MS fragments and the proposed assignment. In the present work, 44 phytochemicals have been tentatively identified in extracts from red lettuce leaves, green lettuce leaves, chicory leaves and sunchoke roots by using the combination of MS and MS/MS data and the information previously reported in the literature. Due to their structural characteristics, the identified analytes were divided into three main classes of metabolites: phenolic acids (PHA), flavonoids (FL) and sesquiterpene lactones (SL). In addition to the dominant groups described above, the presence of amino acids, fatty acids and lipids was also demonstrated. The greatest number of the identified compounds was found in the red lettuce extract (25 analytes), while the sunchoke preparation showed the lowest phytochemical differentiation (14 analytes). The most numerous group is PHA, including eighteen interpreted compounds. Most of them are hydroxycinnamic acids, such as isomers of caffeoylquinic acid (chlorogenic acid) and dicaffeoylquinic acid, derivatives of ferulic acid, coumaric acid and chicoric acid. The red lettuce extract showed the greatest phytochemical differentiation (12 identified compounds), incl. caffeoylquinic and dicaffeoylquinic acid isomers, *cis*-5-*p*-coumaroylquinic acid, chicoric acid, caftaric acid (red lettuce only) and caffeoyltartaric-*p*-coumaroyl acid. All the interpreted FL are flavonols, and their presence was only demonstrated in lettuce extracts. The dominant compounds are *O*-glycosides with quercetin and kaempferol aglycones. Moreover, in addition to the flavonol glycosides, there are also combinations with glucuronic acid, such as; quercetin-3-*O*-glucuronide and kaempferol-3-*O*-glucuronide. Among the nine identified SL (mainly derivatives of lactucin and lactucopicrin), the most were present in the chicory leaves extract (8 compounds). However, in the extracts of green and red lettuce, there were much less of them. No compounds of this class were detected in the sunchoke extract. Two compounds from the group of amino acids have been identified in the chicory leaves and sunchoke roots extracts; L-tryptophan and methylentryptophan. Fatty acids and lipids were also detected among the interpreted analytes.

3.2. Effects of vegetable preparations (extracts) on hemostatic parameters of human plasma

The analysis of the effect on the coagulation properties of human plasma demonstrated that none of the tested vegetable preparations (concentration range 1–50 µg/mL; incubation time 30 min) changed APTT, PT or TT ($p > 0.05$) (Fig. 2).

3.3. Effects of vegetable preparations (extracts)on oxidative stress parameters

As demonstrated in Fig. 3A, four used vegetable preparations (especially at the highest concentrations) reduced plasma protein carbonylation induced by H_2O_2/Fe. For example, three tested preparations (from green lettuce, red lettuce and sunchoke) at the highest used concentration (50 µg/mL) inhibited this process by about 50% compared to plasma treated with only H_2O_2/Fe (Fig. 3A). All the tested reparations also inhibited the oxidation of plasma protein thiols induced by H_2O_2/Fe, but this effect was not always statistically significant (Fig. 3B). Moreover, three tested preparations (from chicory, green lettuce and sunchoke) altered the level of TBARS in plasma treated with H_2O_2/Fe (Fig. 4). However, neither of the tested preparations (50 µg/mL) changed ORAC and TAC *in vitro* (Fig. 5A and B). TLC-DPPH• method, coupled with image processing, has been applied to quantitatively measure direct antioxidant properties of the four *Asteraceae* vegetable preparations (Fig. 5C). The activity against chlorogenic acid was as follows: sunchoke (0.140 ± 0.01) < green lettuce (0.683 ± 0.02) < chicory (0.738 ± 0.03) < red lettuce (0.807 ± 0.03). The highest antioxidant activity of the red lettuce preparation was influenced by the highest content of phenolic acids (Table 2).

Table 3 compares the effect of four tested vegetable preparations at a

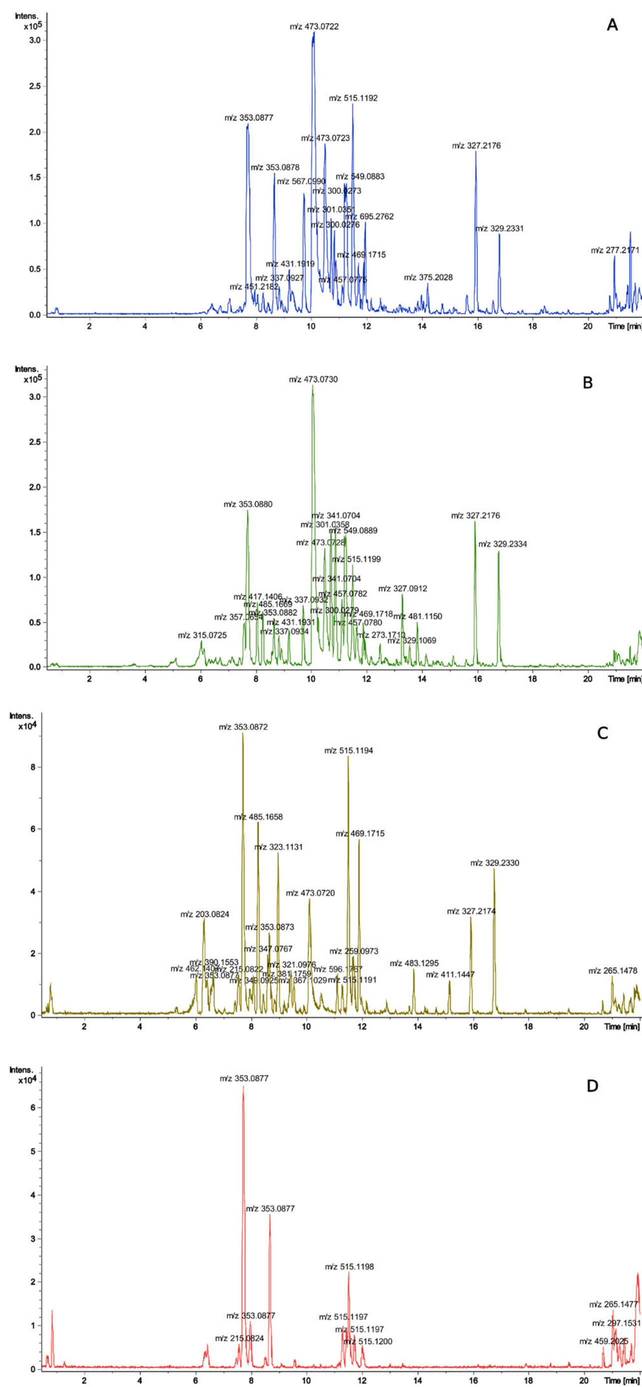

Fig. 1. The base-peak chromatograms (BPC) of aqueous methanol extracts (preparations) from leaves of red lettuce (A), leaves of green lettuce (B), leaves of chicory (C) and roots of sunchoke (D), obtained using high resolution UHPLC-ESI-QTOF-MS in negative ionization mode.

concentration 50 μg/mL on the selected biomarkers of oxidative stress in human plasma treated with H_2O_2/Fe. We have observed that two preparations (form chicory and sunchoke) had stronger anti-oxidant properties than preparations from green lettuce and red lettuce. Preparations from chicory and sunchoke had antioxidant activity in three used models: lipid peroxidation; protein carbonylation and oxidation of protein thiols.

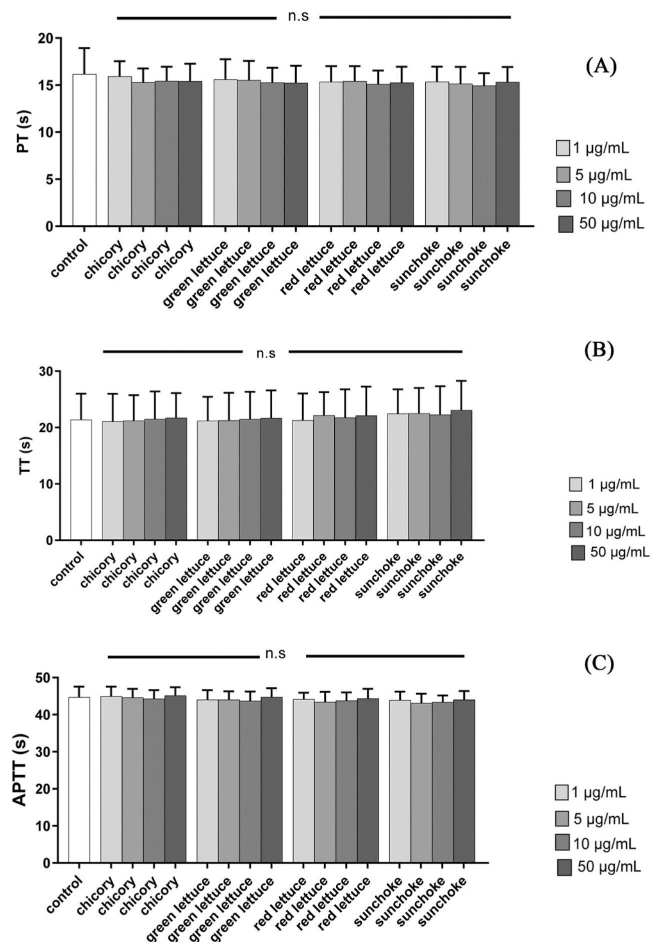

Fig. 2. Effects of the four vegetable preparations (concentration range 1–50 μg/mL, incubation time – 30 min) on the hemostatic parameters of human plasma: PT (A), TT (B), and APTT (C). Data are expressed as mean ± SE (n = 8). One-way ANOVA followed by a multicomparison Tuckey test and Kruskal–Wallis test p > 0.05 (n.s.), compared with control.

4. Discussion

Numerous studies have shown a strong connection between oxidative stress and cardiovascular disease. Oxidative stress is created by imbalance between the production of reactive oxygen species (ROS) and antioxidant activity. In a normal psychological condition ROS, the concentration is modulated by the enzymatic and non-enzymatic antioxidant system and oscillates at a controlled level. The antioxidant status can be influenced by triggers of xenobiotics, such as smoking, drugs, excessive drinking, radiation and other environmental agents. The enzyme system consists mainly of four enzymes: NADPH oxidase (NOX), myeloperoxidase (MPO), lipoxygenases (LOX) and uncoupled endothelial nitric oxide synthase (eNOS). MPO is involved in atherosclerotic disease due to its involvement in ROS formation as isoprostanes and superoxide anion. NADPH has severe isoforms responsible for ROS formation, which are crucial in creating active eicosanoids, such as isoprostanes and thromboxane [25]. ROS play an important role in the progressive pathology of atherosclerosis, due to their involvement in endothelial dysfunction, inflammatory process, blood platelet aggregation and vascular smooth muscle cell proliferation. The development of atherosclerosis is often caused by the accumulation of low-density lipoproteins in the sub endothelium, due to their oxidation by ROS, which leads to generation of foam cells and creation atherosclerotic plaques [26]. ROS level may increase due to the failure of a homeostatic condition, such as dyslipidaemia, obesity, diabetes, hypertension and acute

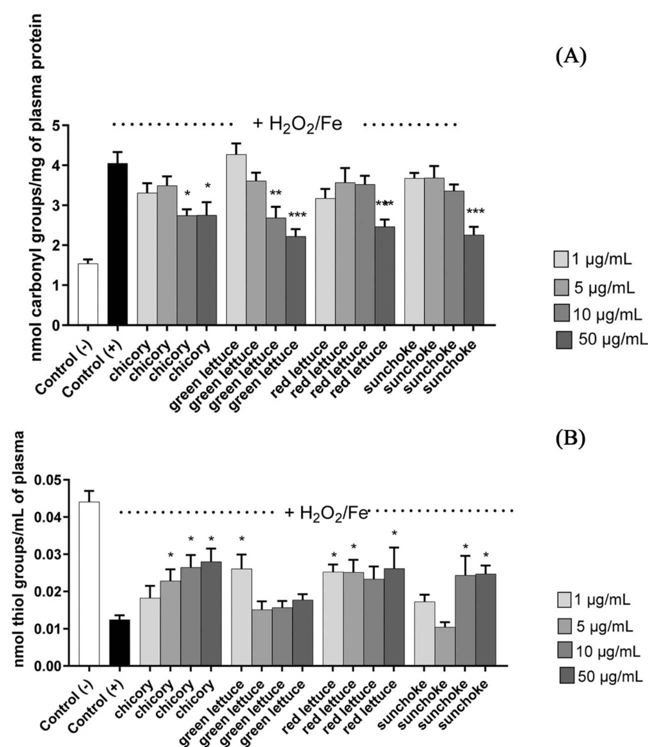

Fig. 3. Effects of the four vegetable preparations (concentration range 1–50 μg/mL, pre-incubation time – 5 min) on the oxidative damages of plasma proteins – protein carbonylation, in plasma treated with H_2O_2/Fe (incubation time – 25 min) (A), and on the oxidative damages of plasma proteins – the level of thiol groups in plasma treated with H_2O_2/Fe (incubation time – 25 min) (B). Results are given as mean ± SE (n = 8). Control negative refers to plasma not treated with H_2O_2/Fe, whereas control positive to plasma treated with H_2O_2/Fe. One-way ANOVA followed by a multicomparison Tuckey test and Kruskal–Wallis test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with positive control (treated with H_2O_2/Fe).

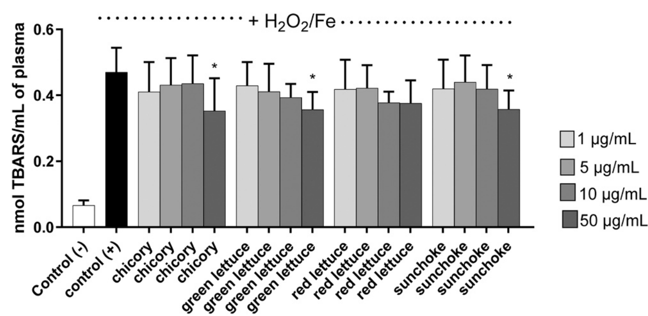

Fig. 4. Effects of the four vegetable preparations (concentration range 1–50 μg/mL, pre-incubation time – 5 min) on lipid peroxidation in plasma treated with H_2O_2/Fe (incubation time – 25 min). Results are given as mean ± SE (n = 8). Control negative refers to plasma not treated with H_2O_2/Fe, whereas control positive to plasma treated with H_2O_2/Fe. One-way ANOVA followed by a multicomparison Tuckey test and Kruskal–Wallis test: *$p < 0.05$, compared with positive control (treated with H_2O_2/Fe).

conditions. It is also associated with risk factors, such as metabolic syndrome, atrial fibrillation, and peripheral artery disease. Risk factors can be detected due to their association with biomarkers of cardiovascular disease, such as oxidation of low-density lipoproteins, higher levels of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in urine, and serum levels of soluble NOX2-derived peptide. 8-iso-$PGF_{2\alpha}$ is a derivative of arachidonic acid oxidation [25].

The antioxidant and anticoagulant activity of preparation from selected vegetables from *Asteraceae* family, including chicory, green and red lettuce and sunchule, were studied in *in vitro* model of human plasma exposed to oxidative stress induced by H_2O_2/Fe-a hydroxyl radical donor. Hydroxyl radicals induce oxidative stress in the plasma by increasing the level of biomarkers, such as lipid peroxidation, protein carbonylation, and oxidation of thiol groups. In our *in vitro* studies, all the tested extracts demonstrated protective activity in plasma lipids and proteins against hydroxyl radicals. All the tested vegetables have a diverse chemical composition, which is connected with their biological activity, including antioxidant action. In our studies, we identified three main classes of metabolites: phenolic acid, flavones and sesquiterpene lactones. Red lettuce showed the greatest phytochemical diversity, and the smallest sunchoke (Table 1). The obtained results were confirmed by TLC-DPPH• method, were the lowest activity against chlorogenic acid was shown by sunchoke, and the highest for red lettuce. However, neither preparation resulted in higher plasma values or induced the total antioxidant activity of the plasma.

Flavonoids are plant secondary metabolites found in fruits and vegetables. Their amount in plants may vary depending on the species, plant parts, degree of maturity, type of cultivation and edaphoclimatic conditions. Most are derived from malonyl-coenzyme-A and p-coumaroyl-CoA. Their structure includes three phenolic rings A, B and C in which ring A is condensed with ring C and ring B has 2-position as a substitute. They play a crucial role in the human diet due to their participation in the antioxidant network along with other biological antioxidants in the human body, like vitamins E and C. They act as free radical scavengers by donating an electron or hydrogen atom [27,28]. Tamayose et al. [29] isolated twelve chlorogenic acid derivates and two flavones from *Moquiniastrum floribundum*, a plant belonging to *Asteraceae* family and study their cytotoxic activity against DPPH radical. The results showed, that all compounds are able to trap free radical, but the chlorogenic acids have the strongest antiradical activity, which is connected with antioxidant activity [29]. Dietary flavonoids act as dietary antiatherosclerotic agents by inhibiting of oxidation of low-density lipoproteins in the vascular system. This ability is based on their physical nature. Lipid peroxidation can be initiated by the oxidation of a radical chain mediated by the radical of peroxide lipids in phospholipid bilayers or aqueous radicals from extracellular fluids. It is assumed that the structure of the flavonoid diphenylpropane interacts with phospholipid bilayers or is inserted inside the bilayers [27].

Our biological studies were not consistent with the results TLC-DPPH• method, because red lettuce has the lowest antioxidant activity in human plasma. Red lettuce extract was only one with no significant effect on lipid peroxidation. The rest inhibit lipid peroxidation at the highest concentration. All vegetable preparations inhibit protein carbonylation at the highest concentration, additionally chicory, while green lettuce demonstrated that effect in 10 μg/mL concentration. Chicory and red lettuce in almost every concentration had a strong significant effect on the recently tested biomarker of oxidative stress, the level of thiol groups. Sunchoke showed this effect only in the highest concentration, while green lettuce in the lowest. Mulabagal et al. [7] also demonstrated antioxidant effect of chicory in *in vitro* studies by evaluation of lipid peroxidation inhibitory activity. The chicory extract and fractions were tested at a concentration of 250 μg/mL. All preparations showed an inhibitory effect on lipid peroxidation. These studies found that anthocyanins were the main phenolic compounds in chicory leaves, which may be the cause of their antioxidant activity [7].

All the obtained results suggest that although the extracts of selected vegetables demonstrated antioxidant activity in human plasma, in the *in vitro* model they did not influence the coagulation system, nor did they show any anticoagulant or procoagulant activity. In addition, the results of the preliminary assessment of the antioxidative action of the tested extracts by the TLC-DPPH• method did not coincide with the results obtained in the biological experimental system with the use of plasma. Hence, it can be suggested that other groups of compounds (not only phenolic compounds) may be responsible for their antioxidant

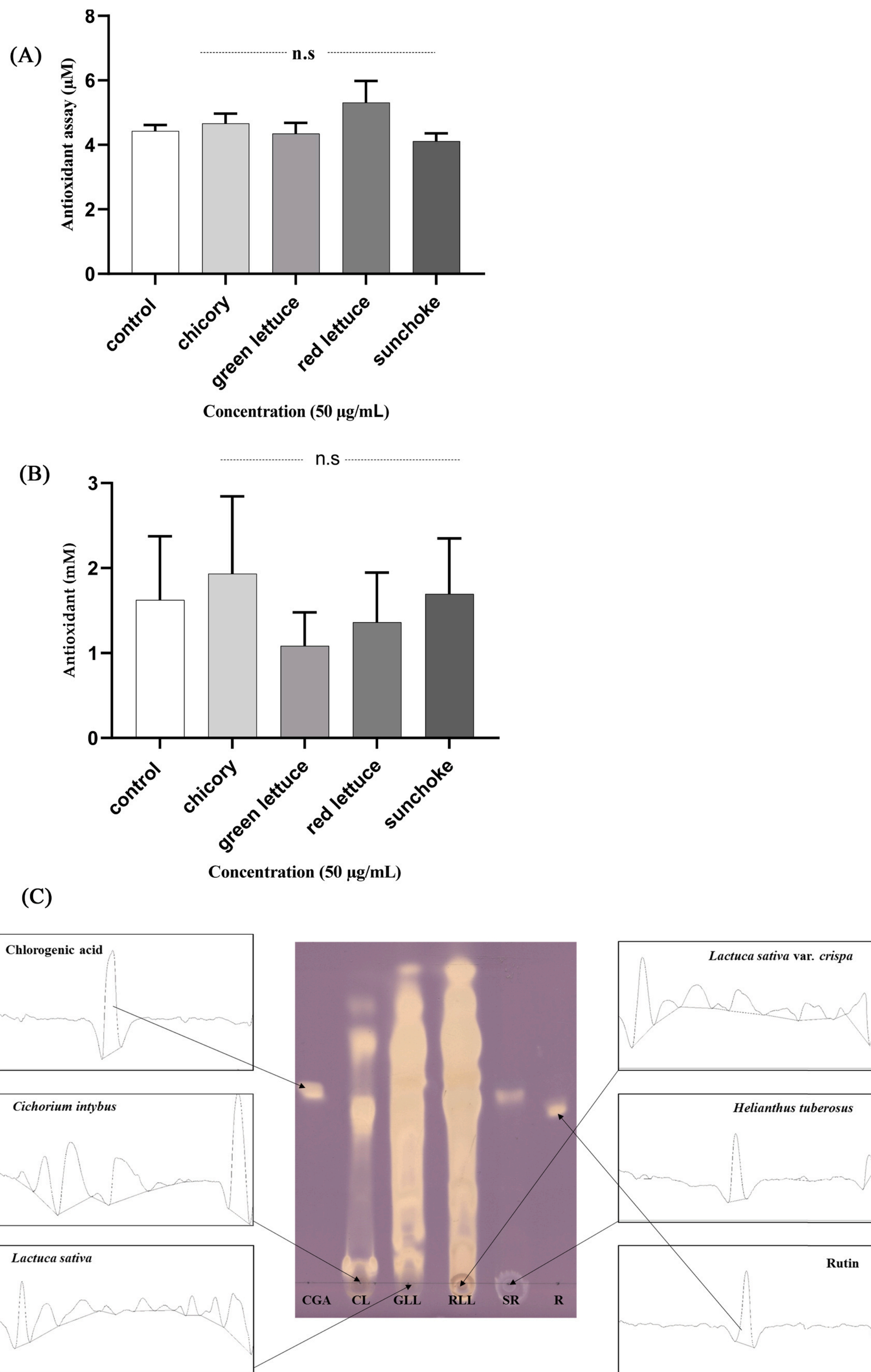

Fig. 5. Effects of the four vegetable preparations (incubation time – 30 min) on the plasma antioxidant capacity, measured as ORAC (A); on total antioxidant capacity of plasma (B). Results are given as mean ± SE (n = 6). One-way ANOVA followed by a multicomparison Tuckey test and Kruskal–Wallis test, compared with control. The use of TLC-DPPH$^{\bullet}$ test (C) for the study of antioxidant activity of chicory (CL), green lettuce (GLL), red lettuce (RLL) and sunchoke (SR). Standards: chlorogenic acid (CGA) and rutin (R).

Table 2
Average contents of 6 phenolic acids in extracts from four plants of the *Asteraceae* family.

Sample	Content [mg/100 g of extract]						Total [mg/100 g of extract]
	5-CQA (*m/z* 354)	CQA (*m/z* 354)	CQA (*m/z* 354)	Chicoric acid (*m/z* 474)	DiCQA (*m/z* 516)	DiCQA (*m/z* 516)	
RLL	2.27 ± 0.3	(–)	(< LOQ)	4.56 ± 0.63	0.74 ± 0.01	(< LOQ)	7.57
GLL	0.46 ± 0.01	(–)	(< LOQ)	2.26 ± 0.1	0.18 ± 0.004	(< LOQ)	2.90
CL	0.12 ± 0.001	(< LOQ)	(< LOQ)	0.21 ± 0.003	0.13 ± 0.003	(< LOQ)	0.46
SR	0.07 ± 0.0006	(< LOQ)	(–)	(–)	(< LOQ)	(–)	0.07

RLL – red lettuce leaves.
GLL – green lettuce leaves.
CL – chicory leaves.
SR – sunchoke roots.
CQA – caffeoylquinic acid.
DiCQA – dicaffeoylquinic acid.
(< LOQ) – below limit of quantification.
(–) – not present.

Table 3
Comparative effects of the four vegetable preparations on oxidative stress in plasma treated with H_2O_2/Fe (tested concentration 50 μg/mL).

Preparation from	Protein carbonylation	Thiol oxidation	Lipid peroxidation
Chicory	Antioxidant properties – inhibition of this process	Antioxidant properties – inhibition of this process	Antioxidant properties – inhibition of this process
Green lettuce	Antioxidant properties – inhibition of this process	No effect	Antioxidant properties – inhibition of this process
Red lettuce	Antioxidant properties – inhibition of this process	Antioxidant properties – inhibition of this process	No effect
Sunchoke	Antioxidant properties – inhibition of this process	Antioxidant properties – inhibition of this process	Antioxidant properties – inhibition of this process

properties.

CRediT authorship contribution statement

Agata Rolnik: Methodology, Formal analysis, Investigation, Writing – original draft. **Agata Soluch:** Methodology, Formal analysis. **Iwona Kowalska:** Methodology, Formal analysis, Writing – original draft. **Beata Olas:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of conflicting interests

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

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Preparations from selected cucurbit vegetables as antiplatelet agents

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Increased blood platelet activation plays an important role in cardiovascular diseases (CVDs). Recent experiments indicate that certain fruits and vegetables, including onion, garlic, and beetroot, have anti-platelet potential and therefore may reduce the likelihood of CVDs. While vegetables from the *Cucuritaceae* family are known to exerting beneficial antioxidant and anti-inflammatory effects, their effects on blood platelet activation are poorly understood. Therefore, the aim of the present study was to determine the effect on platelet adhesion of preparations from selected cucurbits: pumpkin (*Cucurbita pepo*; fruit without seeds), zucchini (*Cucurbita pepo convar. giromontina*; fruit with seeds), cucumber (*Cucumis sativus*; fruit with seeds), white pattypan squash (*Cucurbita pepo var. patisoniana*; fruit without seeds) and yellow pattypan squash (*Cucurbita pepo var. patisoniana*, fruit without seeds). It also evaluates the activity of these preparations on enzymatic lipid peroxidation in thrombin-activated washed blood platelets by TBARS assay. The study also determines the anti-platelet properties of these five cucurbit preparations in whole blood by flow cytometry and with the total thrombus-formation analysis system (T-TAS) and evaluates the cytotoxicity of the tested preparations against platelets based on LDH activity. The results indicate that the yellow *Cucurbita pepo var. patisoniana* preparation demonstrated stronger anti-platelet properties than the other tested preparations, reducing the adhesion of thrombin-activated platelets to collagen/fibrinogen, and inhibiting arachidonic acid metabolism and GPIIb/IIIa expression on 10 µM ADP-activated platelets. None of the preparations was found to cause platelet lysis. Our findings provide new information on the anti-platelet activity of the tested cucurbit preparations and their potential for treating CVDs associated with platelet hyperactivity.

It is important to stop bleeding promptly after vessel injury, maintain blood in a fluid state, and eliminate blood clots when integrity is restored to the vascular system. This operation, known as *hemostasis*, is underpinned by a range of highly-conserved mechanisms in which blood plays a key role[1,2]. Under physiological conditions, vascular integrity is restored by clot formation at the injured site, and to prevent thrombosis, this process is balanced by various anticoagulant and antiplatelet mechanisms. An imbalance between the anticoagulant and procoagulant mechanisms may result in hemorrhage or excessive thrombosis. Thrombosis is a life-threatening occlusion of vessels, in which the normal feedback controls used to regulate thrombus size and stability no longer appear to function appropriately[3,4]. Blood platelets play a primary role in the first wave of hemostasis, also known as *primary hemostasis*; they also play an indirect one in the second wave, as mediators of the blood coagulation pathway. *Primary hemostasis* refers to the early stages of hemostasis when coagulation has to develop sufficiently to prevent blood loss during an injury. During this process, blood platelets interact with the exposed matrix, where they adhere to various adhesive proteins, including collagen[1,2].

Blood platelets are the smallest cells in the circulation system, with a diameter of approximately 1–2 µm, and play a crucial role in hemostasis by repairing injuries to blood vessels. During their 8- to 12-day lifespan, they are mostly inactive[1]; however, they usually become activated after interacting with the protein receptors on the surface of the endothelium. This activation initiates a coagulation cascade: after adhesion to the extracellular matrix, von Willebrand factor from the exposed collagen forms a bridge with the platelet receptor complex glycoprotein (GP) Ib-IX-V. The collagen also binds to other platelet receptors, such as GPIa/IIa and GPVI leading to platelet activation and the release of P-selectin from α-granules in the platelets[5].

The principal ligament for fibrinogen is the GPIIb/IIIa receptor (integrin $\alpha_{IIb}\beta_3$), also known as CD41/CD61. It is a heterodimeric complex formed by the synthesis of a single IIb and single IIIs subunit. Beside fibrinogen, it can bind to fibronectin, von Willebrand factor, and vitronectin. The key function of integrin is to promote platelet aggregation by conducting bidirectional signals across the plasma membrane, made possible by its role

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in facilitating interactions with potential ligands. During its resting state, GPIIb/IIIa is bent and its "headpiece" is closed, thus reducing its affinity for physiological ligands; however, stimulation by certain stimulatory signals, such as from an inside-out signal, causes conformation changes that expose the extracellular binging domain[6,7].

Platelet activation can be stimulated by multiple pathways; however, two key routes are connected with signal transduction pathways and have their foundation in membrane glycoproteins that are solely expressed in platelets. One pathway is based on the activation of platelets through G protein-coupled receptors, leading to the release of ADP and thromboxane A_2, resulting in an increase of cytosolic calcium concentration. This initiates specific signaling pathways, and causes further platelet activation. Alternatively, the coagulation pathway generates thrombin, a highly potent platelet activator which is needed to convert fibrinogen into fibrin to stabilize the platelet "plug". Thrombin activates blood platelets through protease activated receptors (PAR) on the platelet surface[1]. After activation, the platelets release P-selectin from α-granules to the surface. P-selectin is a ligand responsible for the interaction between platelets, leukocytes and endothelial cells, and plays a key role in linking hemostasis and inflammation[7].

In addition, platelet activation can result in the activation of cytosolic phospholipase A_2 (cPLA$_2$), which generates arachidonic acid from phospholipid membranes. After formation, arachidonic acid is available for oxidization by cyclooxygenase (COX-1) or lipoxygenase (12-LOX). The results of oxidation are dependent on the type of blood cell. While oxidized arachidonic acid generates prostaglandin E_2 and leukotriene B_4 in leukocytes, it results in the production of thromboxane A_2 and 12-hydroxy-5,8,10,14-eicosatetraenoic (12-HETE) in platelets[8]. Arachidonic acid can be also oxidized by cytochrome P 450, a heme-containing enzyme found in different tissues[9]. Hemostasis is not the only function of platelets, due to their high sensitivity to different diseases states, which make them one of the most accessible markers. Platelets interact with leukocytes and endothelium cells and their reactivity for various pathogenesis states are widely dependent upon active markers, including CD36, CD41, CD42a, CD42b, CD61. Platelets are also able to release and transfer many substances, which interact with endothelium cells. They can store high amount of amyloid precursor protein and its metabolism may accumulated Aβ in the brain, leading to its vasculature through blood brain barrier. In renal diseases platelets are in the presence of toxic products in the circulation leading to the form of bleeding diatheses, which are hemorrhage and other pathological feature like thrombocytopenia and glomerular thrombosis. Moreover, blood platelets play also an important role in metastasis[10].

The members of the *Cucurbitaceae* family are rich in phenolic acids, flavonoids and terpenoids, all of which show strong antioxidant activity and may influence various parts of hemostasis. Oxidative stress is a risk factor in cardiovascular disease and hemostasis disorders. The components of cucurbits have been found to have a positive effect on biomarkers of oxidative stress in plasma[11]. Indeed, our previous studies have found selected vegetables from the *Cucurbitaceae* family, including pumpkin, zucchini, cucumber, yellow pattypan squash and white pattypan squash, to contain various secondary metabolites with antioxidant activity[11]; however, the influences of these vegetable preparations on the biological properties of blood platelets remain unknown. Therefore, to continue our previous research, the present study examined the anti-platelet potential of the same five cucurbit preparations, viz. pumpkin (*Cucirbita pepo*; fruit without seeds), zucchini (*Cucurbita pepo convar. giromontina*; fruit with seeds), cucumber (*Cucumis sativus*; fruit with seeds), white pattypan squash (*Cucurbita pepo var. patisoniana*; fruit without seeds) and yellow pattypan squash (*Cucurbita pepo var. patisoniana, fruit without seeds*) in tested washed human blood platelets and human whole blood in vitro. In addition to their anti-adhesive action, the present study also examines the activity of these preparations on enzymatic lipid peroxidation—arachidonic acid metabolism in thrombin-activated washed blood platelets based on thiobarbituric acid reactive substances (TBARS) assay. In addition, the present study also determines the anti-platelet properties of these five cucurbit preparations in whole blood using flow cytometry and a total thrombus-formation analysis system (T-TAS) and evaluates their cytotoxicity against platelets based on extracellular lactate dehydrogenase (LDH) activity. The action of cucurbit preparations was compared to commercial product—Aronox (*Aronia melanocarpa* berry extract with anti-platelet and antioxidant activities[12,13].

Materials and methods

Chemical reagents. Dimethylsulfoxide (DMSO), bovine serum albumin (BSA), thiobarbituric acid (TBA), fibrinogen were acquired from Sigma-Aldrich (St. Louis, MO., USA). All other reagents were purchased from commercial suppliers, including POCH (Poland), Chempur (Poland), Chrono-log (Poland), and Kselmed (Poland). Ultrapure water was prepared in-house using a Milli-Q water purification system (Millipore, Milford, MA, USA).

A stock solution of commercial product—Aronox (*Aronia melanocarpa* berry extract, Agropharm Ltd., Poland) was prepared in H_2O.

Plant material. *Obtained vegetable preparations.* Five of the most well-known and easily-available types of cucurbit vegetables were selected for the study, these being pumpkin (*Cucurbita pepo* L., fruit without seeds); zucchini (*Cucurbita pepo* L. *convar. Giromontina*, fruit with seeds); cucumber (*Cucumis sativus* L., fruit with seeds); white pattypan squash (*Cucurbita pepo* L. *var. patisoniana*, fruit without seeds) and yellow pattypan squash (*Cucurbita pepo* L. *var. patisoniana*, fruit without seeds). All obtained materials were bought from organic farming in Poland 51°09′15.0″N 21°59′47.1″E, in 2019. The samples were shredded, frozen and freeze dried (CHRIST Gamma 2–16 LSC Freeze Dryer, Osterode am Harz, Germany), and stored in the Department of Biochemistry and Crop Quality of the Institute in Puławy, Poland. The plants were identified by Katarzyna Adamczyk: the owner of a private farm. The sample voucher for this material has been deposited in the Institute's collection under the deposit number 42/2019/IUNG, 43/2019/IUNG, 44/2019/IUNG, 45/2019/IUNG, and 46/2019/IUNG, respectively. All plant studies involved in the research were carried out in accordance with

Identified compound	Compound class	Cucurbit preparation				
		Pumpkin	Cucumber	Zucchini	White pattypan squash	Yellow pattypansqaush
3-(β-D-glucopyranosyloxy)-2-hydroxybenzoic acid	Benzoicacidderivative	–	–	–	–	+
Fructosyl L-phenylalanine	Amino acid	+	+	–	+	+
L-tryptophan glycoside	Amino acid	+	–	+	–	–
Zizybeoside I	Phenylethanoid glycoside	–	–	+	–	–
Forsythoside E (isomer I)	Phenylethanoid glycoside	–	–	+	+	+
Hydrangeifolin I	Phenylpropanoid glycoside	–	–	–	+	+
Sinapic acid hexoside	Phenolic acid	+	–	+	–	–
Salicylic acid O-glycoside	Phenolic acid	–	+	–	–	–
Quercetin 3,3′-dimethyl ether 7-rutinoside	Flavonoids	–	–	+	–	–
Kaempferol derivative	Flavonoid	–	+	–	–	–
Primulaverin derivative	Flavonoid	–	–	+	–	–
Rutin	Flavonoid	–	–	+	–	–
Adenostemmoic acid C	Diterpenoids	–	–	–	–	+
Octadecadienoic acid derivative	Fatty acid	+	+	+	+	+
γ-Linolenic acid derivative	Fatty acid	–	–	+	–	+
Linoleic acid derivative	Fatty acid	+	+	+	+	+
Glycerophospholipid	Lipid	+	+	+	+	+

Table 1. Identified compounds and their presence (+) in five cucurbit vegetable preparations based on Rolnik et al.[10].

relevant institutional, national or international guideline. The entire section was previously described in Rolnik et al.[11].

Extraction and chemical analysis of vegetable preparations. The extraction process was performed based on the following conditions: extraction solvent: 80% methanol, solvent pressure: 1500 psi, extraction cell temperature: 40 °C, extraction cycles: three, using an automatic extractor (Dionex ASE 200 Accelerated Solvent Extraction System). The extracts were dried by evaporation under reduced pressure, at 40 °C (HeidolphHei-Vap Advantage, rotary evaporator). The five preparations were purified from mostly sugars using solid phase extraction (SPE), as described previously[11].

The most diverse phytochemical profile was demonstrated by the zucchini preparation, and the least by the cucumber. Almost all identified compounds could be classified as phenylethanoids, flavonoids, glycoside lipids or fatty acids. The pumpkin and cucumber contained, *inter alia*, kaempferol and synaptic acid; while the other three preparations only contained phenylethanoids as glycosides.

Both identified phenylethanoid glycosides, zizybeoside I and forsythoside E (isomer I), were present in zucchini. Of the identified phenolic acids, the sinapic acid hexoside was found in pumpkin and the salicylic acid O-glycoside in cucumber. Both pattypan squashes contain diterpenoids: cinncassiol A in the white pattypan and adenostemmoic acid C in the yellow pattypan. Among the flavonoids,7-methylquercetin-3-galactoside-6″-rhamnoside-3‴-rhamnoside, quercetin-3-O-rutinoside(rutin),isorhamnetin 3-O-rutinoside (narcissin) and-hesperetin 7-O-(2″,6″-di-O-α-rhamnopyranosyl)-β-glucopyranoside, were identified.

Glycerophospholipids were identified in all cucurbit preparations. Fatty acids such as linoleic acid and octadecadienoic acid derivatives were also found in all the tested vegetables; however, the γ-linolenic acid derivative was present only in zucchini and yellow pattypan squash (Table 1)[11].

Stock solutions of vegetable preparation. To analyse the biological activity, stock solutions of the vegetable preparations were dissolved in 50% DMSO. The final concentration of DMSO in the samples (human plasma) was lower than 0.05% and its effects were determined in each experiment.

Blood and blood platelets.

Isolation of blood platelets. Human blood was collected from healthy, medication-free volunteers in the Medical Center in Lodz; all of whom reported not smoking or consuming alcohol. The blood was collected into tubes with citrate/phosphate/dextrose/adenine (CPDA) anticoagulant. Blood platelets were separated from fresh blood through differential centrifugation, as described previously[14,15]. Following this, the platelets were suspended in Barber's buffer, in a modified Tyrode's buffer (0.14 M NaCl, 0.014 M Tris,

10 mM glucose; pH 7.4). The amount of platelets used for the test reached $1.5–2.0 \times 10^8$/mL and were measured using a UV–Visible Helios α spectrophotometer at 800 nm. For each experiment, blood or blood platelets were incubated for 30 min, at 37 °C with vegetable preparations at final concentrations of 5 and 50 µg/mL or aronia berry extract at final concentration of 50 µg/mL.

Confirmation by human participants. All experiments were approved by the University of Lodz Committee for Research on Human Subjects and carried out under permission number 8/KBBN-UŁ/III/2018.

We confirm that all experiments were performed in accordance with relevant guidelines and regulations. All donors were informed about the purpose of the study and gave their informed consent to participate.

Effect of vegetable preparations on hemostasis parameters. *Flow cytometry.* To study the effects of the cucurbit preparations on the reactivity and activation of resting and stimulated blood platelets, whole blood models were used. Firstly, whole blood was incubated with preparations from selected cucurbit vegetables for 15 min at 37 °C, and then for another 15 min at room temperature (RT) with the addition of 10 and 20 µM ADP or collagen as platelet agonists. After incubation, the tested samples were diluted tenfold in sterile PBS with Mg^{2+}, and then stained with 3 µL of anti-CD61/PerCP, anti-CD62/PE, or PAC-1/FITC antibodies for 30 min at RT in the dark. Isotype controls were also prepared; these contained resting blood samples stained with 3 µL of anti-CD61/PE and isotype control antibodies marked with FITC/PEisotype. Finally, all samples were fixed with 1% CellFix for 60 min at 37 °C.

The platelets were counted using an LSR II Flow Cytometer (Becton Dickinson, San Diego, CA, USA), based on the fluorescence of 10,000 platelets (CD61/PerCP positive objects). The platelets were distinguished from other blood cells by a forward light scatter (FCS) vs. side light scatter (SSC) plot on a log/log scale (first gate) and by positive staining with monoclonal anti-CD61/PerCP antibodies (second gate). The percentages of CD62P-positive and PAC-1-positive platelets were calculated in each sample. All results were analyzed using FlowJo _v.10.7.2 (Becton Dickinson, San Diego, CA, USA)[12,15,16,17].

Total Thrombus Formation Analysis System (T-TAS). T-TAS was used to determine the thrombus formation process under flow conditions using the PL-chip microchip coated with collagen. Fresh whole blood collected on BAPA (benzylsulfonyl-D-arginyl-prolyl-4-amidinobenzylamide) was incubated with preparations from the five cucurbit samples for 30 min at 37 °C. Then the samples were transferred to the PL-chip. The results were recorded as AUC_{10} i.e., Area Under the Curve[18].

Platelet adhesion. Platelet adhesion was measured based on the activity of exoenzyme acid phosphatase in platelets. The plates were coated with 0.04 mg/mL collagen or 2 mg/mL fibrinogen. After isolation from fresh blood, the blood platelets were incubated with selected cucurbit preparations for 30 min at 37 °C. Next samples, which contain platelets and cucurbits preparations were added on plates and left to adhere for hour at 37 °C. The platelets were then dissolved with Triton X-100 and treated with the phosphatase substrate (p-nitrophenylphosphate), resulting in the formation of p-nitrophenol. The level of p-nitrophenol were measured at $\lambda = 405$ nm using a SPECTROstarNanoMicroplate Reader (96-well microtiter plates, BMG LABTECH, Germany). To achieve a color reaction in the samples, 2 M NaOH was added. All readings were taken in reference to the control sample containing only blood platelets with Barber's buffer, in a modified Tyrode's buffer, whose expression was assumed to be 100%[13,19].

Effect of vegetable preparations on parameters of damages. *Activity of LDH.* The cytotoxic effects of the selected *Cucurbitaceae* preparations on blood platelets were evaluated based on the release of lactate dehydrogenase (LDH) from the platelets. After incubation, the test samples were centrifuged for 15 min at 25 °C at 2500 rpm, and 10 µL of supernatant was transferred to a microtiter plate. The plate was then loaded with 270 µL of 0.1 M phosphate buffer and 10 µL of NADH. After a 20-min incubation at room temperature, 10 µL of pyruvate (5 mg) was added and the absorbance measured immediately afterwards. The further readings were taken at one-minute intervals over a 10-min period. Absorbance was measured at $\lambda = 340$ nm using a SPECTROstarNanoMicroplate Reader (BMG LABTECH, Germany)[20,21].

Effect of vegetable preparations on lipid peroxidation. The level of lipid peroxidation on the blood platelets was determined based on thiobarbituric acid reactive substances (TBARS) content. The samples were mixed with 0.37% thiobarbituric and 15% trichloroacetic acid and heated for 10 min at 100 °C in a heating block. Following this, the samples were allowed to cool and centrifuged at 10,000 rpm for 15 min at 18 °C. The absorbance of the supernatant was measured at $\lambda = 535$ nm using a SPECTROstarNanoMicroplate Reader (BMG LABTECH, Germany)[22,23].

Data analysis. Several tests were used to carry out the statistical analysis. All the values were expressed as mean ± SD. First the results were checked for normality with the Kolmogorow-Smirnow test, and the equality of variance was determined with Levine's test. Statistically significant differences were identified using an ANOVA test (assuming a significance level of $p < 0.05$), followed by either Tukey's multiple comparisons test or the Kruskal–Wallis test as appropriate.

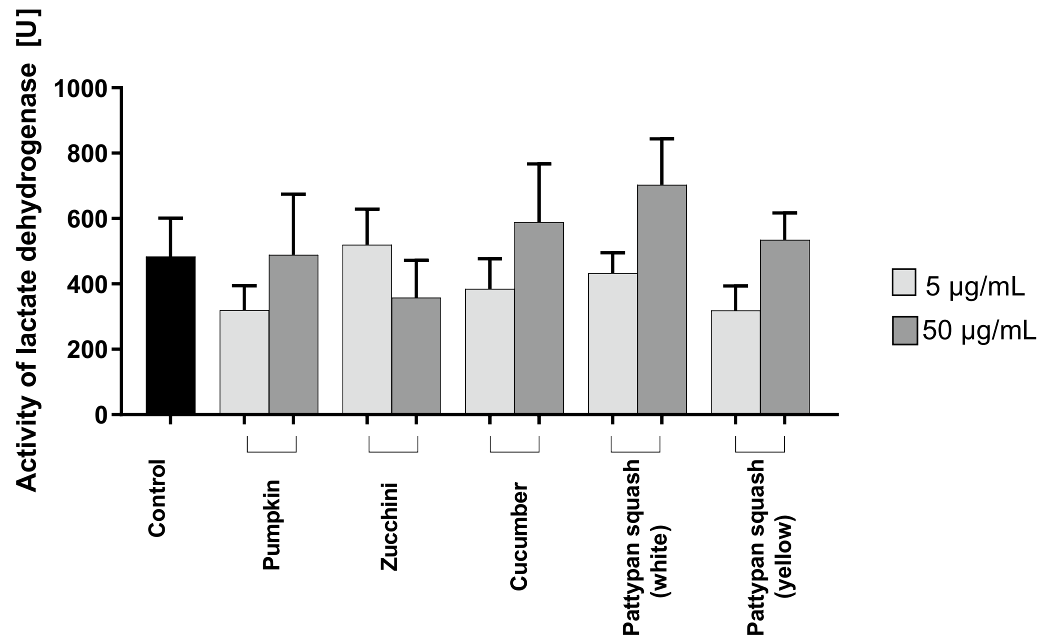

Figure 1. Effects of the five cucurbit vegetable preparations (concentrations 5 and 50 μg/mL, incubation time—30 min) on damage to human blood platelets. Results are given as mean ± SD (n = 4); the control sample (platelets without plant preparation). There wasn't any statistically significant between effect of 5 and 50 μg/mL ($p > 0.05$). The baseline spectral reading (absorbance) for plant preparations range between 0.009 and 0.0245.

Results

Effect of vegetable preparations on parameters of damages.

To determine the toxic effect of all the tested cucurbit preparations on human blood platelets, the level of extracellular LDH activity was measured. The results indicate no significant difference in blood platelet viability after exposure to the used plant preparations at 5 and 50 μg/mL compared to control, however, these changes were not statistically significant (Fig. 1).

Effect of vegetable preparations on platelet adhesion.

The anti-adhesive properties of five plant preparations were studied in vitro using washed blood platelets. The results were presented as percent of level of adhesion for control samples. The obtained results showed the level of adhesion of resting platelets to collagen was significantly inhibited after pre-incubation with four tested preparations: pumpkin (50 μg/mL), zucchini (50 μg/mL), cucumber (5 and 50 μg/mL), and pattypan squash (white) (50 μg/mL) (Fig. 2A).

In the case of the thrombin-activated blood platelets, both the pumpkin and cucumber preparations (5 and 50 μg/mL) also demonstrated inhibitory properties (Fig. 2B). Reduced adhesion was also observed for the pumpkin, cucumber, zucchini, pattypan squash (white), and pattypan squash (yellow) in other model (Fig. 3A); however, these changes were not always statistically significant, including cucumber (5 and 50 μg/mL), pumpkin (5 μg/mL), and pattypan squash (white) (5 μg/mL) (Fig. 3A).

For the ADP-stimulated platelets, all of the tested plant preparations (5 and 50 μg/mL) have not anti-adhesive properties (Fig. 3B).

Effect of vegetable preparations on lipid peroxidation.

As shown in Fig. 4, all the tested plant preparations (5 and 50 μg/mL) altered the level of lipid peroxidation in platelets stimulated by thrombin. Most significantly, the pattypan squash (yellow) preparation (50 μg/mL) demonstrated 85% inhibition relative to the positive control.

Effect of vegetable preparations on the hemostasis parameters in whole blood.

The samples treated with five plant preparations demonstrated different levels of blood platelet activation (measured by flow cytometry) compared with untreated whole blood control samples (Figs. 5 and 6). Only three preparations demonstrated clear changes at the highest tested concentration (50 μg/mL): the cucumber, the pattypan squash (white), and pattypan squash (yellow) preparations significantly reduced PAC-1 binding in platelets activated by 10 μM ADP (Fig. 5B).

Four cucurbit preparations (pumpkin, cucumber, pattypan squash (white), and pattypan squash (yellow)) significantly changed AUC_{10} values measured by T-TAS in whole blood (Fig. 7).

The effects of all five tested plant preparations (at the highest tested concentration) on chosen blood platelet activation parameters are compared with aronia berry extract in Table 2. Of all preparations, the yellow pattypan squash preparation demonstrated the strongest anti-platelet properties, reducing the adhesion of thrombin-activated platelets to collagen/fibrinogen, and inhibiting arachidonic acid metabolism and GPIIb/IIIa expression on 10 μM ADP-activated platelets. In addition, aronia berry extract (50 μg/mL) decreased PAC-1 binding in

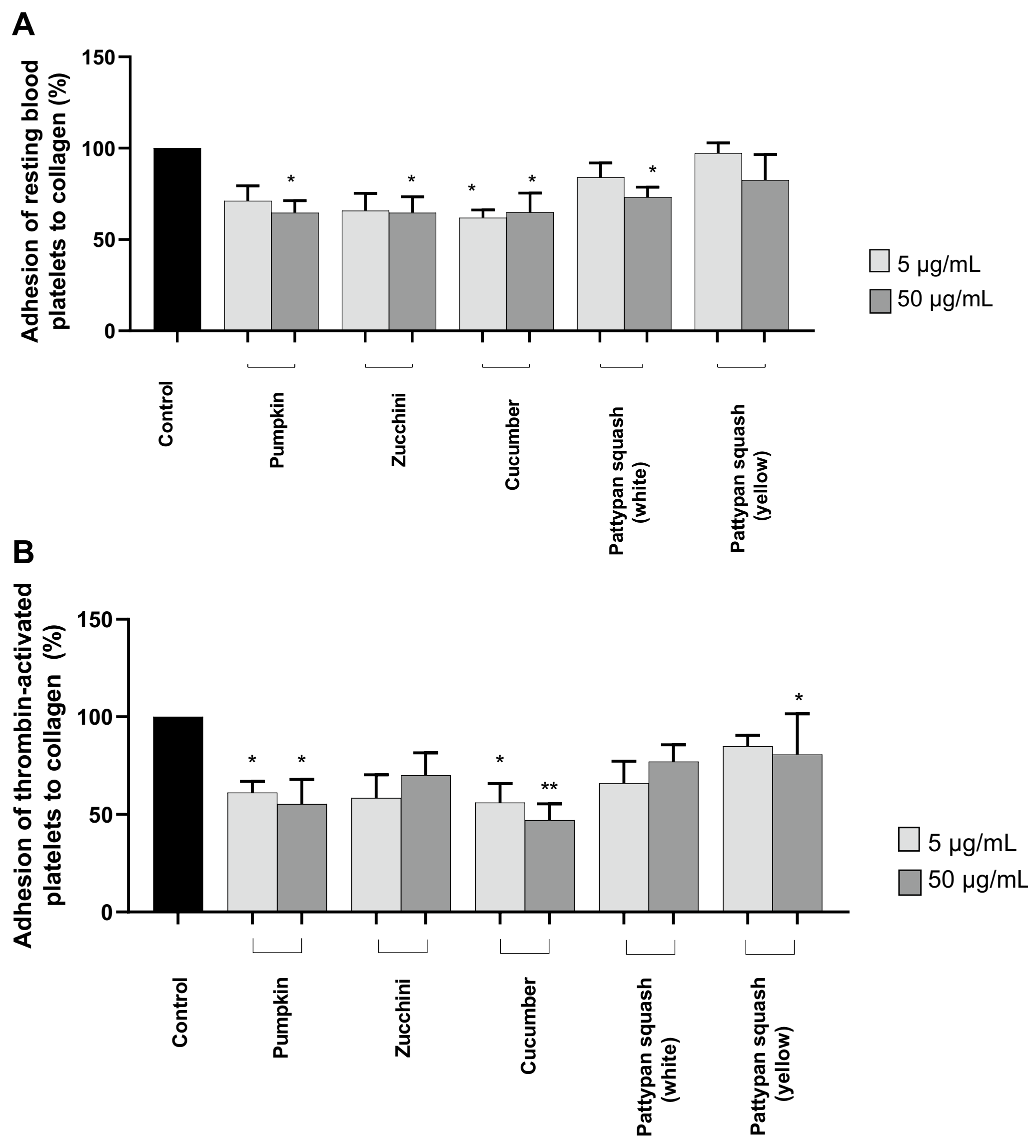

Figure 2. Effects of the five cucurbit vegetable preparations (concentrations 5 and 50 µg/mL, incubation time—30 min) on adhesion of resting platelets to collagen (**A**) or thrombin (final concentration 0.2 U/mL)—activated platelets (**B**). In the graphs, the adhesion is expressed as a percentage of the control sample (platelets without plant preparation). Results are given as mean ± SD (n = 5). Kruskal–Wallis test: $*p < 0.05$, $**p < 0.01$, compared with control (i.e. not treated with plant preparation). There wasn't any statistically significant between effect of 5 and 50 µg/mL ($p > 0.05$). The baseline spectral reading (absorbance) for plant preparations range between 0.00075 and 0.0095.

platelets activated by 20 µM ADP and collagen; it also reduced the expression of GPIIb/IIIa on platelets activated by 10 µM ADP, 20 µM ADP and collagen (data not presented).

Discussion

A fundamental part of the primary and secondary treatment of atherosclerotic thrombotic disease is the use of drugs affecting platelet function. Although several such drugs are available in the clinical armory, aspirin and clopidogrel have the most favorable profile of currently-used drugs and are the most widely-studied. Aspirin plays a crucial role in preventing thromboembolic complications from atherosclerotic disease. It is believed to prevent platelet activation by permanently inactivating key platelet enzymes. In contrast, clopidogrel offers slightly better effectiveness regarding the secondary prevention of vascular events. While it has no direct antiplatelet activity of its own, it stimulates metabolites, like free thiols group to bind to P_2Y_{12} platelet receptors to form disulfide bridges with extracellular cysteine residues, leading to irreversible inhibition of ADP-induced platelet aggregation, because the $P2Y_1$ receptor plays an significant role in ADP -induced activation of platelets[24].

In recent years the antiplatelet drugs are mostly related to role of mechanism of thrombus formation, which is exclusively expressed on blood platelet. Due to this important pharmacological research directions for treating

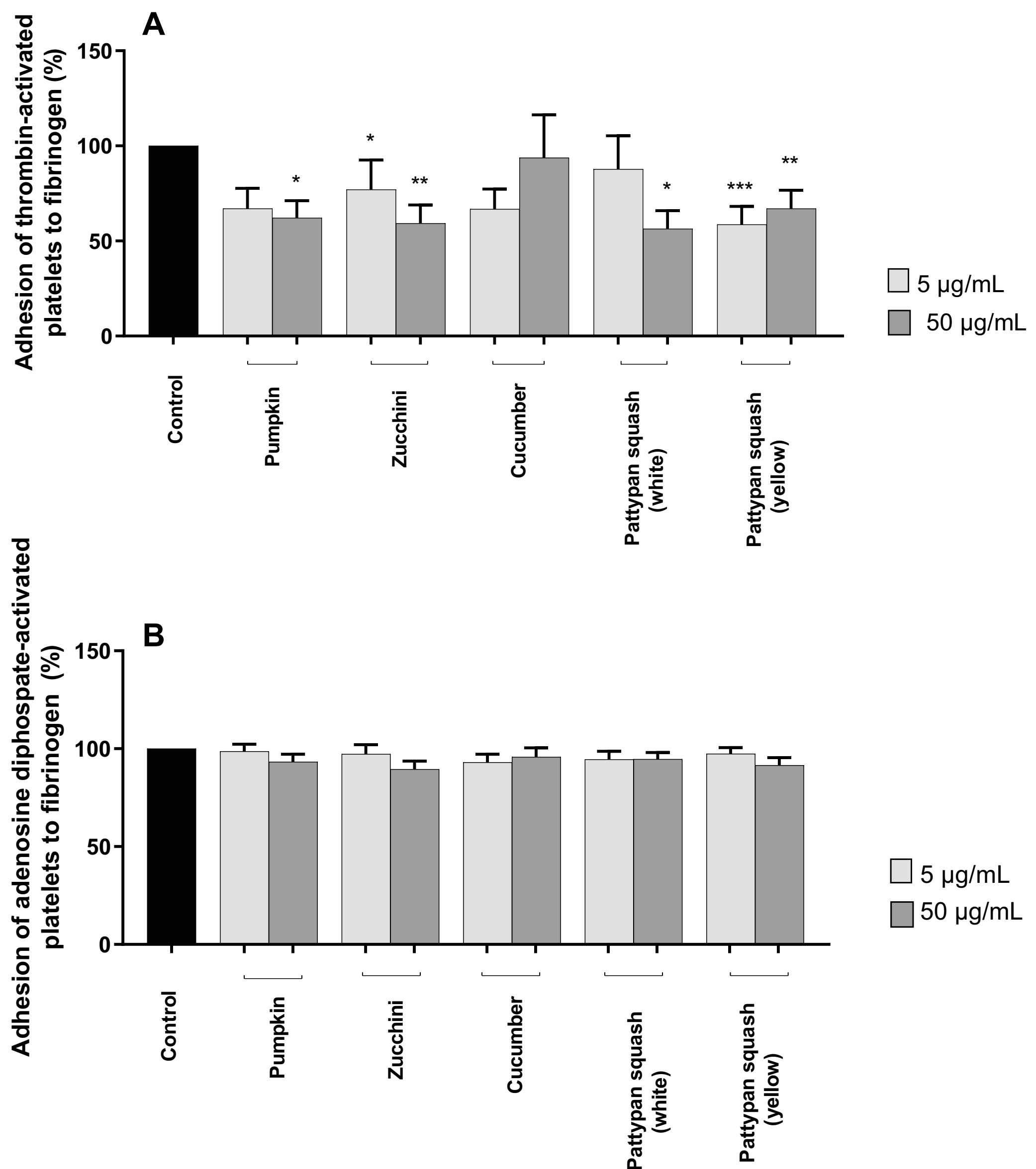

Figure 3. Effects of the five cucurbit vegetable preparations (concentrations 5 and 50 µg/mL, incubation time—30 min) on adhesion to fibrinogen and thrombin (final concentration 0.2 U/mL)—activated platelets (**A**) or ADP (final concentration 30 µM)—activated platelets (**B**). In the graphs, the adhesion is expressed as a percentage of the control sample (platelets without plant preparation). Results are given as mean ± SD (n = 5). Kruskal–Wallis test: *p < 0.05, **p < 0.01, ***p < 0.001, compared with control (i.e. not treated with plant preparation). There wasn't any statistically significant between effect of 5 and 50 µg/mL. There wasn't any statistically significant between effect of 5 and 50 µg/mL (p > 0.05). The baseline spectral reading (absorbance) for plant preparations range between 0.0025 and 0.0055.

hemostatic deficiencies are concerning the development of new drugs targeting, among others, the PAR1 thrombin receptor or GP VI platelet-specific collagen receptor[25]. The greatest adverse effect for antiplatelet drugs is the increased risk of bleeding, which is associated with an elevated risk of thrombosis. Antiplatelet therapy should inhibit platelet function during periods of high thrombotic risk. In addition, to avoid the risk of recurrence of ischemic events after premature cessation of medication or non-compliance, patients often require long-term antiplatelet therapy[24].

The Cucurbitaceae family includes a range of phytochemicals, such as saponins and cardiac glycosides, which are known to influence on the cardiovascular system and are often used in the therapy of heart disease. Saponins are able to coagulate blood and reduce bleeding, and the cucurbitacins can exert an anti-atherosclerotic effect due to their ability to inhibit lipid peroxidation products, like malonaldehyde[26,27]. Cucurbitacin B has a protective effect against cardiac hypertrophy by increase autophagy among cardiomyocytes; while hypertrophy

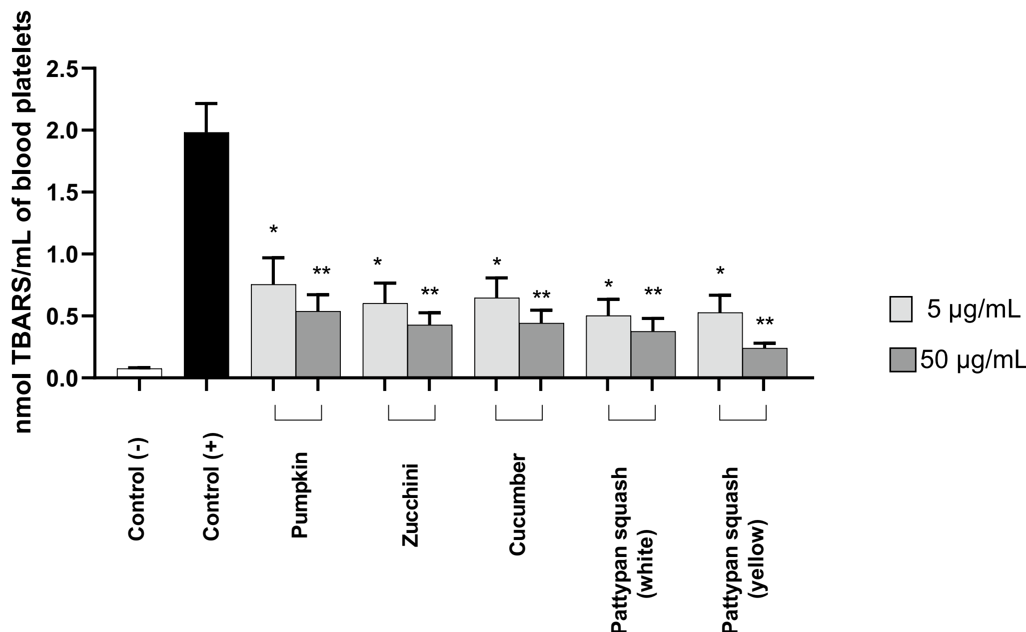

Figure 4. Effects of the five cucurbit vegetable preparations (concentrations 5 and 50 µg/mL) on lipid peroxidation in blood platelets activated by 5 U/mL thrombin (pre-incubation time with plant preparation—25 min; incubation time with thrombin—5 min). Results are given as mean ± SD (n = 5). *Control negative* refers to platelets not treated with thrombin, and *control positive* to platelets treated with thrombin. Kruskal–Wallis test: *p < 0.05, **p < 0.01. There wasn't any statistically significant between effect of 5 and 50 µg/mL (p > 0.05). The baseline spectral reading (absorbance) for plant preparations range between 0.00065 and 0.0035.

is a dynamic and adaptive process in physiological conditions, it can lead to pressure or volume overload, often resulting in heart failure if prolonged[28].

The leaves and seeds of *Momoridace balsamina* represent a ready source of glycosides, which can be used to treat cardiac diseases by intensifying the force of heart contraction, based on its influence on calcium release. It has also been found that zucchini can also help alleviate symptoms related with heart diseases, such as high cholesterol[27]. Other research indicates that ethanolic extract of *Lagenaria siceraria* (*Cucurbitaceae*) fruit inhibits ADP-induced platelet aggregation in vitro, and increases bleeding time and plasma re-calcification time in mice[29]. Other experiments have found cucumber sap extract to inhibit blood platelet aggregation induced by different agonists (ADP, collagen, and epinephrine) in platelet-rich plasma and to decrease plasma re-calcification time and prothrombin time[30]. In addition, a recent study by Sanzana et al. found that various pumpkin seed extracts (aqueous, ethanolic and methanolic extract) have anti-platelet properties in vitro[31]; the extracts inhibited platelet aggregation stimulated by ADP, collagen and thrombin receptor activator peptide 6 (TRAP-6) in vitro, and reduced P-selectin expression and GPIIb/IIIa activation on blood platelets stimulated by TRAP-6[31].

Our present findings, and those of our previous studies[11], suggest that the selected cucurbit preparations offer promise as candidates for reducing blood platelet activation. It is worth noting that the present study used a combination of flow cytometry and T-TAS to study platelet activation in its natural environment, i.e. after blood collection and incubation with plant preparations, and that is the first paper to examine the anti-platelet potential of the five tested cucurbit preparations in two in vitro models: one based on washed blood platelets and the other with whole blood. An important, and novel, finding is that three tested cucurbit preparations: the yellow pattypan squash, white pattypan squash, and cucumber, influenced blood platelet activation, as indicted by flow cytometry. In addition, four cucurbit preparations (pumpkin, cucumber, pattypan squash (white), and pattypan squash (yellow)) changed AUC_{10} values measured by T-TAS. The differences in anti-platelet potential observed between samples may be explained by their different chemical profiles. An important consideration is that the concentration of plant preparation (≤ 50 µg/mL) used in the study corresponds with physiological concentrations of phenolic compounds after oral administration[32].

Our results indicate that the yellow pattypan squash preparation appears to demonstrate the best anti-platelet properties of the tested cucurbits. The preparation was found to inhibit adhesion of thrombin-activated platelets to collagen and fibrinogen. It also significantly inhibited GPIIb/GPIIIa activation in ADP-activated platelets; it is likely that this inhibition may be responsible for the anti-adhesive potential of this preparation. This plant preparation was found to demonstrate anti-platelet potential measured by T-TAS. Again, its strong antiplatelet activity may well be correlated with its chemical composition. Preparations from pattypan squash are include a range of phenolic derivates, many of which show anti-platelet activity. One of the main compounds found in pattypan squash was phenylpropanoid glycoside, known to demonstrate anti-platelet activity[33]. Mesa et al. report that *Wendtia calycina* extract significantly reduced platelet aggregation induced by collagen in platelet-rich

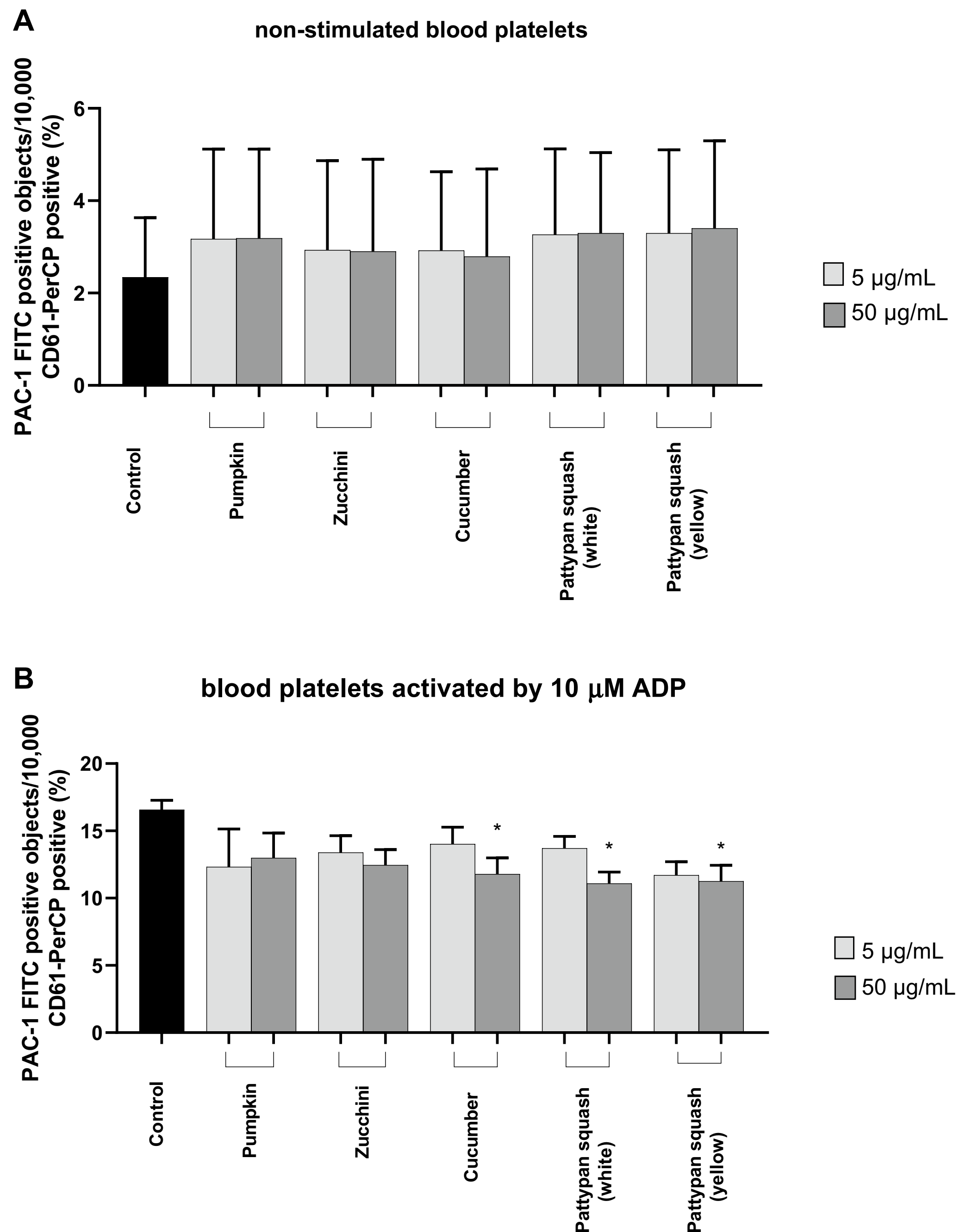

Figure 5. Effects of the five cucurbit vegetable preparations (concentrations 5 and 50 µg/mL, incubation time—30 min) on the expression of the active form of GPIIb/IIIa on resting (**A**) or agonist-stimulated blood platelets: 10 µM ADP (**B**), 20 µM ADP (**C**) and 10 µg/mL collagen (**D**) in whole blood samples. Additionally effects of three selected preparations (cucumber, pattypan squash white and yellow; 50 µg/mL; 30 min) on the expression of the active form of GPIIb/IIIa in platelets stimulated by 10 µM ADP in whole blood samples (**E**). This figure demonstrates selected diagrams (**E**). 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 the mean ± SD of six healthy volunteers (each experiment performed in triplicate). *$p < 0.05$ (vs. control platelets). There wasn't any statistically significant between effect of 5 and 50 µg/mL ($p > 0.05$).

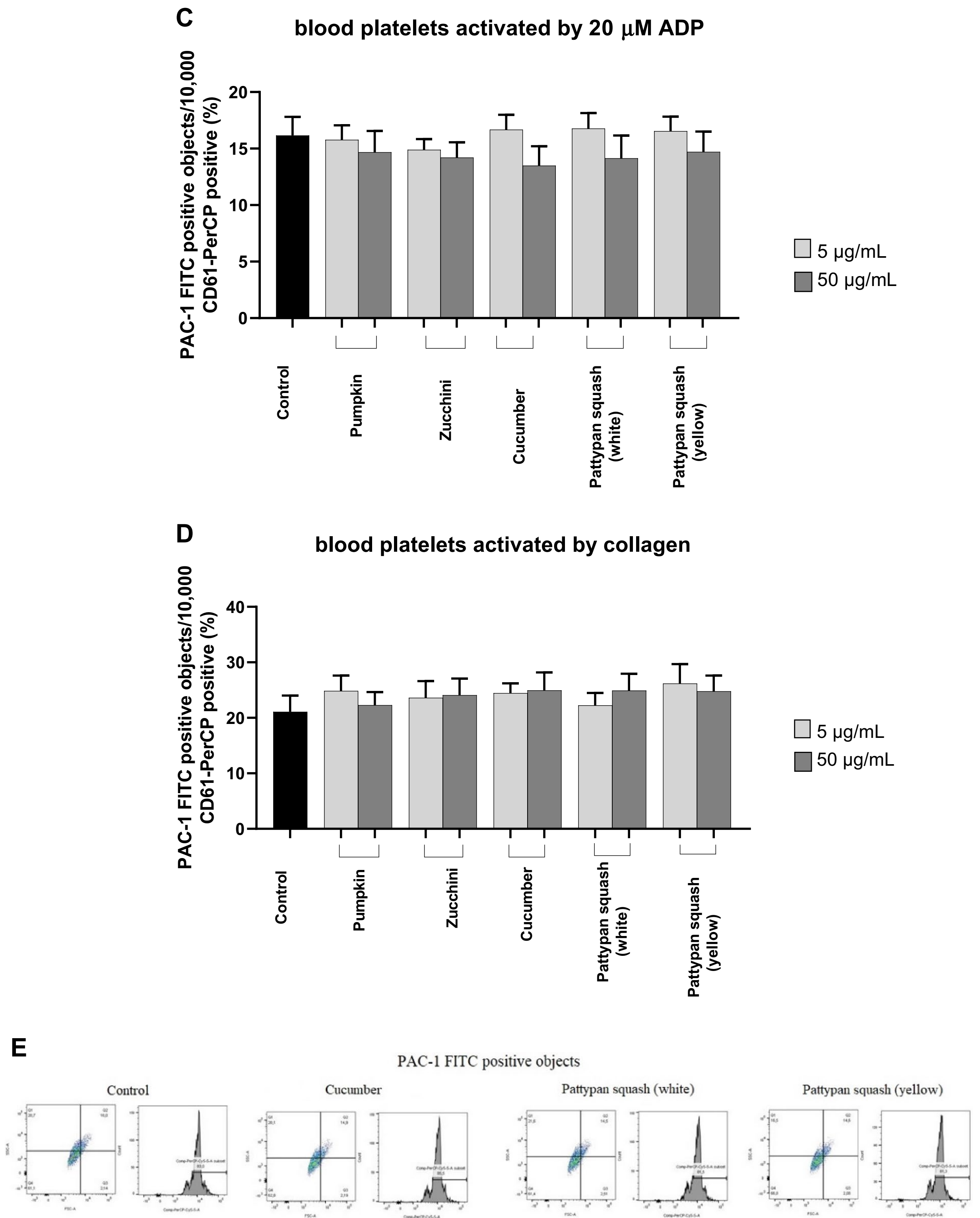

Figure 5. (continued)

plasma in vitro. This extract contains a high level of phenylpropanoid glycoside, and a certain amount of benzoic acid derivates[33], both of which were identified in pattypan squash (Table 1), with the benzoic acid derivative in the pattypan squash demonstrating a strong anti-aggregation effect[33]. The batch variability of plant matter is connected with the quantitative differences in chemical composition, due to the various plant cultivation methods and soil quality.

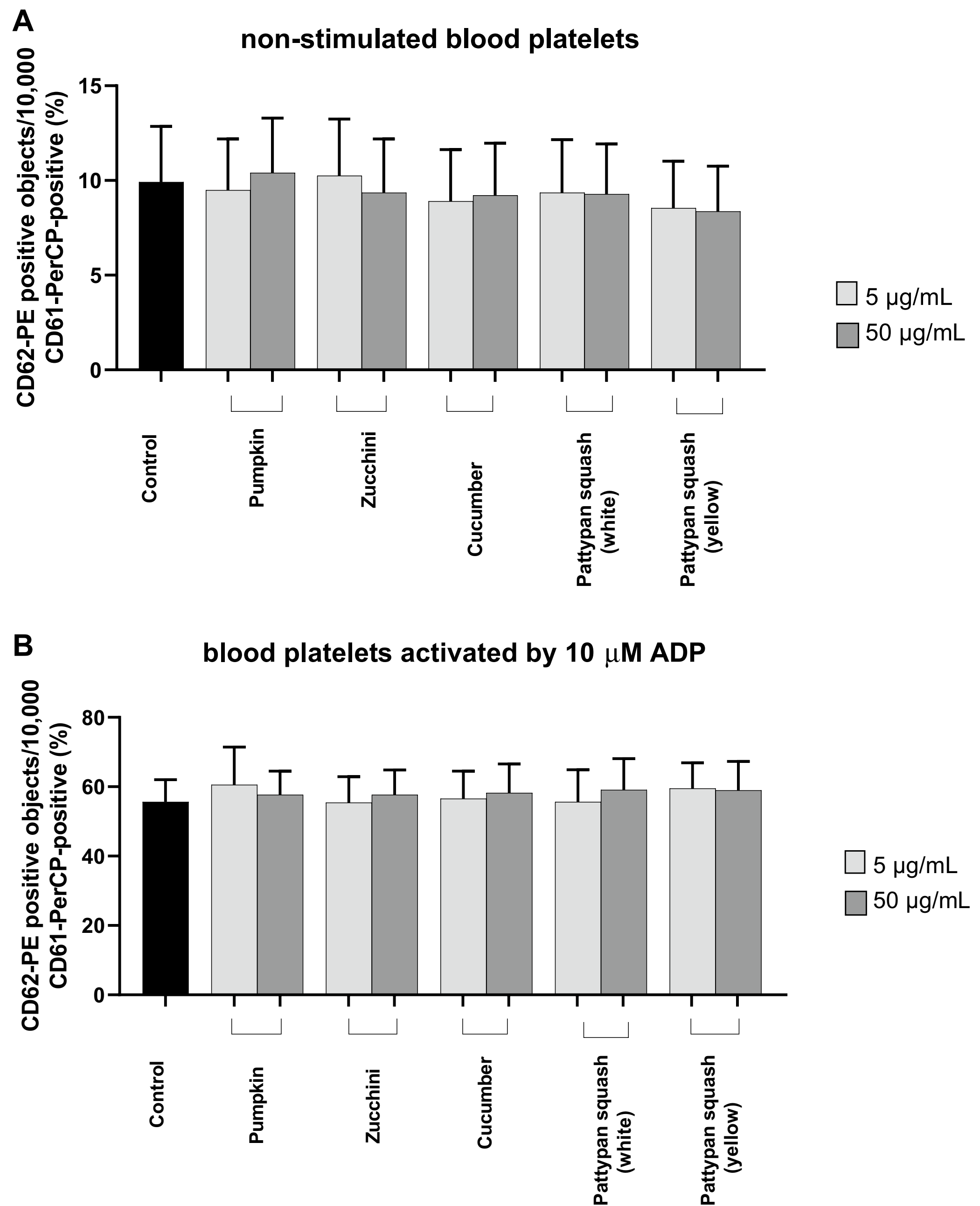

Figure 6. Effects of the five cucurbit vegetable preparations (concentrations 5 and 50 μg/mL, incubation time—30 min) on expression of P-selectin on resting (**A**) or agonist-stimulated blood platelets: 10 μM ADP (**B**), 20 μM ADP (**C**) and 10 μg/mL collagen (**D**) in whole blood samples. The blood platelets were distinguished based on the expression of CD61/PerCP. For each sample, 5000 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. Results are given as the mean ± SD of six healthy volunteers (each experiment performed in triplicate). There wasn't any statistically significant between effect of 5 and 50 μg/mL ($p > 0.05$).

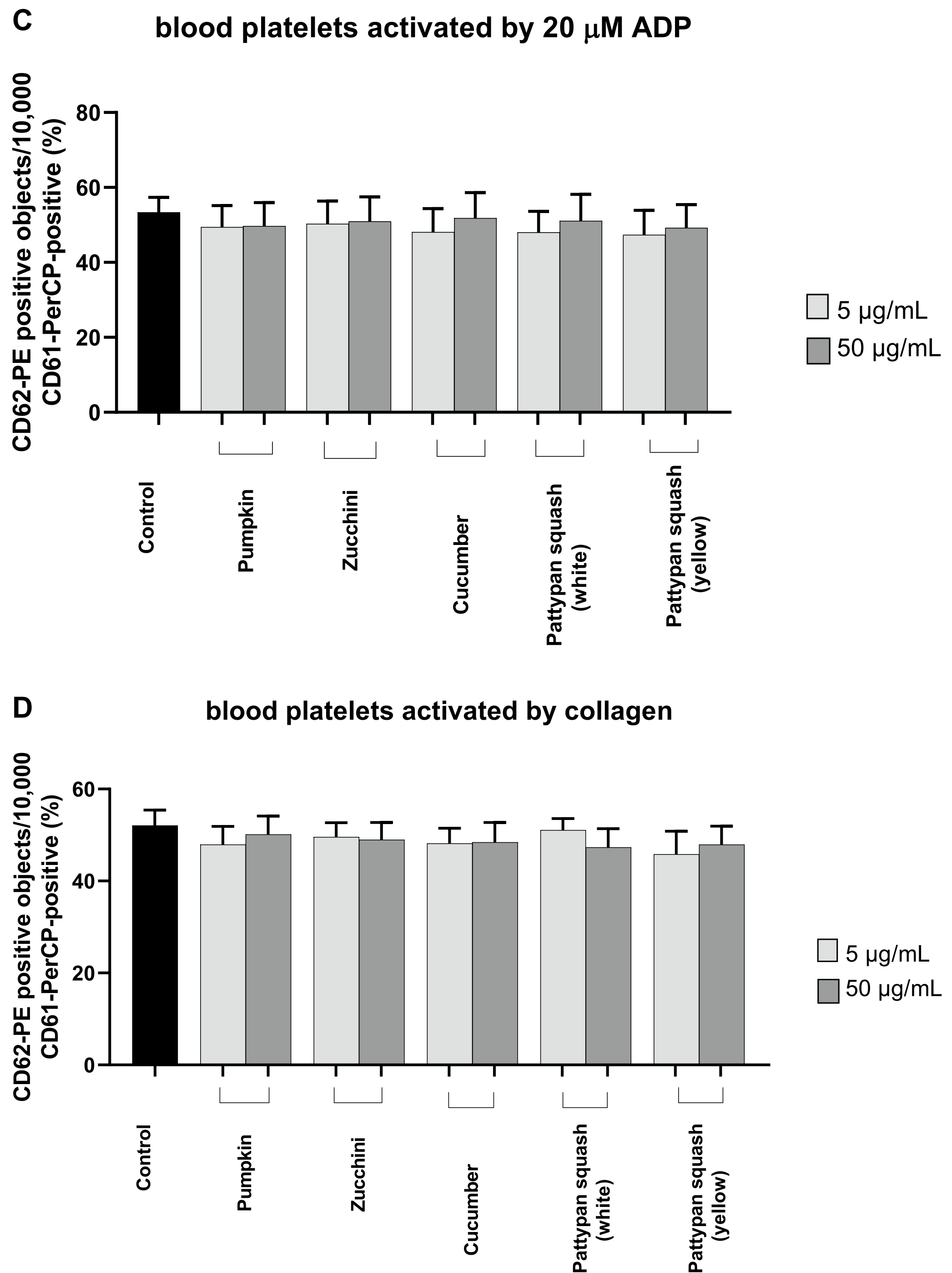

Figure 6. (continued)

Pattypan squash also contains various diterpenoids, which are also known to demonstrate anti-platelet activity (Table 1). For example, Thisoda et al.[34] found diterpenoids from *Andrographi spaniculate* extract to demonstrate anti-aggregatory effects of in vitro and to significantly inhibit thrombin-induced platelet aggegation[34]. However, further studies are needed to precisely identify the compound responsible for anti-platelet activity in pattypan squash. It may well be the case that this effect is derived from the synergistic action of multiple compounds together. Nevertheless, our results provide new information on the anti-platelet activity of the tested cucurbit preparations, especially the yellow pattypan squash preparation, and their possible use in CVDs associated with platelet hyperactivity.

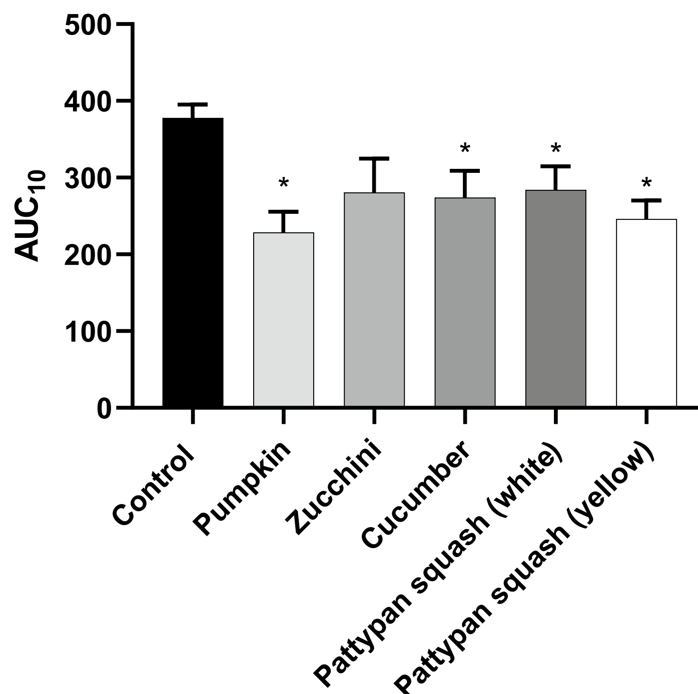

Figure 7. Effects of the five cucurbit vegetable preparations (concentration—50 µg/mL, incubation time—30 min) on the T-TAS using the PL-chip in whole blood samples. Whole blood samples were analyzed by the T-TAS at the shear rates of 1000 s^{-1} on the PL-chips. The area under the curve (AUC_{10}) in PL are shown as closed circles. Data represent the mean ± SD of eight healthy volunteers (each experiment performed in triplicate). *p < 0.05 (vs. control blood).

Parameter of platelet activation	Cucurbit preparation					
	Pumpkin	Zucchini	Cucumber	Pattypan squash (white)	Pattypan squash (yellow)	Aronia berry extract
Adhesion of thrombin-activated platelet to collagen	Inhibition of this process (anti-platelet potential)	No effect	Inhibition of this process (anti-platelet potential)	No effect	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)
Adhesion of thrombin-activated platelet to fibrinogen	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)	No effect	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)	No effect
Adhesion of ADP-activated platelet to fibrinogen	No effect	No effect	No effect	No effect	No effect	No effect
Arachidonic acid metabolism	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)	No effect
GPIIb/IIIa expression on 10 µM ADP-activated platelets	No effect	No effect	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)	Inhibition of this process (anti-platelet potential)	No effect

Table 2. Comparation of influence of the five cucurbit preparations (tested concentration—50 µg/mL) on selected properties of activated blood platelets[11,12].

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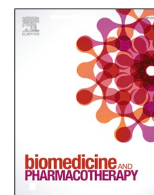

The in vitro anti-platelet activities of plant extracts from the *Asteraceae* family

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A B S T R A C T

Foods prepared from the *Asteraceae* family are known to exert in vitro antioxidant activity. For example, roots and fruit extracts from dandelion were found to possess antioxidant and anti-platelet potential in two in vitro models (washed blood platelets and whole blood). However, little is known of other extracts from the *Asteraceae*, such as chicory leaves, green lettuce leaves, red lettuce leaves, and Jerusalem artichoke roots, on the hemostatic system. Of all the tested extracts from the *Asteraceae*, dandelion root extract and dandelion fruit extracts appear to have the strongest anti-platelet potential in whole blood, while red lettuce leaves and Jerusalem artichoke roots demonstrated the strongest anti-platelet activity in washed blood platelets. Our results suggest that the members of the *Asteraceae* family, especially red lettuce leaves and Jerusalem artichoke roots, possess compounds that may exert beneficial anti-platelet effects. However, although *Asteraceae* plant organ extracts were found to demonstrate activity in vitro, further in vivo studies are needed to determine their true effects on cardiovascular disease.

1. Introduction

World Health Organization figures indicate that the leading causes of death globally are the various forms of cardiovascular disease (CVD), affecting about 20 million people each year. In response, the European Society of Cardiology recommends, among other things, the consumption of a varied diet and supplementation with fruit and plant extracts; these are known to be rich in various compounds with anti-platelet properties, such as phenolic compounds [1]. One such plant with potential multidirectional health benefits is dandelion (*Taracaxum officinale* L.), a member of the *Asteraceae* family [16].

The *Asteraceae* is one of the most diverse plant families, comprising nearly 1600 genera with 23 000 species; its members are broadly scattered around the word, being found in most climate conditions and habitats, including both forest and high altitude grasslands. Due to their cosmopolitan distribution and wide ecological adaptability, the members demonstrate considerable variety, from vines and trees, to annual and perennial herbs and shrubs. The *Asteraceae* comprises three large subfamilies: *Asteroideae, Barnadesioideae* and *Cichorioideae*. The most well-known species include lettuce and chamomile [21,22].

Asteraceae family members produce a wide range of compounds, whose activities are connected with the biological properties of their parent plants. Some species are an important part of the everyday diet of humans. The fiber content in the edible part has been found to range from 2.55 to 13.5 g per 100 g of dry mass and protein range from 0.4 to 6.13 g per 100 g (Garcia-Herrera et al., 2014). The edible parts of plants are source of numerous vitamins, including A, B and C, and various microelements, such as Na, Ca, Mg, and K [7].

All members are also ready sources of secondary compounds, such as phenolic compounds, with the highest concentrations being found in *Echinacea pallipad,* and the lowest in *Helianthus annuus* [8]. *E. pallipad* also demonstrated the highest levels of carotenoids. Most species have high levels of chlorogenic acid, chloric acid, caffeic acid and proantocyanidin [8]. Numerous *Asteraceae* species have medicinal applications and are have been implemented in the pharmaceutical, cosmetic and food industries.

Abbreviations: ADP, adenosine diphosphate; AUC, area under the curve; BSA, bovine serum albumin; CAD, corona-charged aerosol detector; CPD, citrate/phosphate/dextrose; CVDs, cardiovascular diseases; DAD, diode array detector; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DMSO, dimethylosulfoxide; FCS, forward light scatter; LDH, lactate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; SPE, solid phase extraction; SSC, side light scatter; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance.

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In addition, due to their high flavonoid and phenolic acid contents, these species demonstrate strong antioxidant activity; both groups of compounds are able to chelate metal ions, prevent the formation of free radicals and support the endogenous antioxidant system. The methanolic extract of *A. melanpodina* demonstrated more significant antioxidant activity than ascorbic acid in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, with the plant extract demonstrating a 100% chelating effect at a concentration of 400 μg/mL [21]. Many of the phenolic compounds in the plants also have anti-platelet potential. For example, some species contain arctiin, a glucoside of artigenin, which has been attributed with anti-inflammatory properties, among others. Arctiin also inhibits the production of inflammatory mediators, like prostaglandin E_2, interleukins IL-1β and IL-6 and tumor necrosis factor [29].

Our earlier results indicate that extracts obtained from various dandelion organs have antioxidant, anti-platelet and anticoagulant properties, and do not have cytotoxic effects against blood platelets ([12] and 2019; [15], 2019A and B, 2020, 2021). Furthermore, plant extracts from *Asteraceae* family, such as chicory leaves, green lettuce leaves, red lettuce leaves and Jerusalem artichoke roots, have been demonstrated to have antioxidant activity in human plasma in vitro [24]. However, there are still gaps in the knowledge of their effects on the hemostatic system, including blood platelet activation. Therefore, the main objective of this paper was to investigate the effect of four plant extracts from the *Asteraceae* family (chicory leaves, green lettuce leaves, red lettuce leaves, and Jerusalem artichoke roots) on hemostasis parameters against washed human blood platelets and whole human blood in vitro.

Various parameters were assessed to determine anti-platelet potential. To this end, two key techniques were used. (I) Flow cytometry was performed to determine the level of activation of resting or agonist (ADP or collagen)-stimulated platelets after incubation with tested plant extracts; briefly, this technique measured the cell-surface exposure of P-selectin (CD62P) and active GPIIb/IIIa (PAC-1 binding). (II) Thrombus formation was also measured to determine the influence of the tested plant extracts on whole blood. In addition, the influence of the plant extracts on platelet adhesion to fibrinogen and type I collagen (the predominant form of collagen in the arterial wall in vessels changed by atherosclerosis) and on the arachidonic acid metabolism (enzymatic lipid peroxidation) was determined in thrombin-activated blood platelets. Finally, the in vitro cytotoxicity of these plant extracts was also investigated.

A novel aspect of our study is that it compares the biological activity of four plant extracts from the *Asteraceae* (chicory leaves, green lettuce leaves, red lettuce leaves, and Jerusalem artichoke roots) with those of two dandelion extracts (i.e. from roots and fruits), which are known to display anti-platelet and anti-coagulant properties [13,17–20]. It is also important to note that all extracts were tested within the concentration range 5–50 μg/mL, which corresponds to the concentrations obtained in blood plasma during supplementation with phenolic compounds. In addition, the activity of the *Asteraceae* extracts was compared to that of a commercial product: Aronox (*Aronia melanocarpa* berry extract with anti-platelet properties) [23,28].

2. Materials and methods

2.1. Chemical reagents

Dimethylsulfoxide (DMSO, catalog number 67–68–5), fibrinogen (catalog number F8630), thiobarbituric acid (TBA, catalog number MFCD03939426), bovine serum albumin (BSA, catalog number A7030), 4-nitrophenyl phosphate (catalog number 333338–18–4), ADP (catalog number 119340–53–3), collagen (catalog number C8919) and thrombin (catalog number T9326) were acquired from Sigma- Aldrich (St. Louis, MO., USA). Antibodies (CD62-PE, PAC-1 FITC, CD61/PerCP) were acquired from Becton Dickinson (New Jersey, USA). PL-chip were purchased from Bionicum (Warsaw, Poland). All other reagents were purchased from commercial suppliers, including Chrono-log (Poland), POCH (Poland), Chempur (Poland) and Kselmed (Poland). Ultrapure water was prepared in-house using a Milli-Q water purification system (Millipore, Milford, MA, USA).

A stock solution of Aronox (*Aronia melanocarpa* berry extract, Agropharm Ltd., Poland) was prepared in water.

2.2. Plant material

2.2.1. Acquisition of extracts from tested Asteraceae plants

Dandelion (*Taraxacum officinale* L.) was harvested on a farm located in south-eastern Poland (50°05′ N, 21°57′ E). The chicory leaves (*Cichorium intybus* L.), green lettuce leaves (*Lactuca sativa* L.), red lettuce leaves (*Lactuca sativa* L. *var. crispa*) and sunchoke roots (Jerusalem artichoke roots, *Helianthus tuberosus* L.) were purchased in a local farmers market during season. The plant material was freeze-dried (Gamma 2–16 LSC, Christ, Osterode am Harz, Germany), pulverized (Grindomix GM200, Retsch, Haan, Germany), and stored in a refrigerator before extraction. The resulting plant material has been deposited at the Department of Biochemistry and Crop Quality of the Institute in Puławy, Poland. The authentication of all plants was performed during chemical analysis, as described previously [24].

2.2.2. Extraction and chemical analysis of extracts from Asteraceae family

Extracts from chicory leaves, green lettuce leaves, red lettuce leaves, and Jerusalem artichoke roots, were extracted using a Dionex ASE 200 automatic extractor (Accelerated Solvent Extraction System). The following extraction process conditions were used: extraction solvent 80% methanol, solvent pressure 1500 psi, extraction cell temperature 40°C, three extraction cycles. Before analysis, 1 mL of 70% methanol was added to 10 mg of each dry preparation, and the samples were sonicated for 15 min at 25°C for better dissolution (SONOREX DIGITEC DT 510 H sonicator, Bandelin, Germany). The extracts were then purified by solid phase extraction (SPE).

The samples were loaded onto an Oasis HLB column (Extraction Cartridges, Waters, Massachusetts, USA). They were washed with water to remove any sugars, and the secondary metabolite extracts were eluted with acidified (0.1% formic acid) 85% methanol for separation from the chlorophyll. The eluates were evaporated and dissolved in 1 mL of 70% methanol, and next centrifuged for 10 min at 11,000 rpm (laboratory centrifuge Polygen Sigma 3–16 KL, Sigma, Germany).

Finally, 5 μL of the supernatant from each sample was subjected to UHPLC-ESI-QTOF-MS for the qualitative analysis, and 2.5 μL to UHPLC-MS analysis for the quantitative determination of major phytochemicals. The identified compounds were classified as phenolic acids, flavonoids and sesquiterpene lactones; however, fatty acids, and lipids were also present. The greatest diversity was found in the red lettuce preparation, and the least in the sunchoke extract. Among the phenolic acids, derivatives of hydroxycinnamic acid, caffeoylquinic acid, ferulic and coumaric acid predominated. Flavonoids were only observed in lettuce, among which flavonols predominated.

Part of this study is described in an earlier manuscript [24], which includes details about the chemical content of these extracts.

The finely ground *T. officinale* L. roots (560 g) were thrice extracted with 80% methanol (v/v; 3 × 4 L), for a total of 36 h, at room temperature; the samples were also subjected to sonication (18 ×10 min) to enhance the extraction efficiency. The UPLC-UV-MS analysis found the tested preparation to contain mostly hydroxyphenylacetic acid derivates, with hydroxyphenyl acetate inositol esters predominating. The entire process is described in more detail by Jedrejek et al. [13].

The fruits from dandelion were defatted by extraction with hexane under reflux; the material was then twice extracted with 80% methanol (v/v; 4 L × 2; 12 h × 2) at 30 °C and sonicated (12 × 10 min) to enhance extraction efficiency. The obtained material was washed (rinsed once) with 4% methanol and compounds of interest were eluted with 80% methanol, then filtered and concentrated with a vacuum evaporator.

After evaporation, the organic solvent, containing phenolic acid, was lyophilized (freeze-dried) to give 9.5 g of extracts. The dandelion fruit preparation was analyzed with a Thermo Ultimate 3000RS (Thermo Fischer Scientific, Waltham, MS, USA) chromatography system equipped with a diode array detector (DAD) and corona-charged aerosol detector (CAD), and coupled with a Bruker Impact II HD (Bruker, Billerica, MA, USA) quadrupole-time of flight (Q-TOF) mass spectrometer (MS). The dandelion fruit preparation contained the high level of phenolic acids. The whole process is described in more detail in Lis et al. [19]. Similar dandelion root and fruit extracts were obtained from other research groups [13,19].

2.2.3. Stock solutions of extracts from Asteraceae family

All of the *Asteraceae* family plant extracts were dissolved in 50% DMSO to prepare a stock solution to be used for testing biological activity. In all experiments, the extracts were used at final concentrations of 5 and 50 μg/mL. The final concentration of DMSO in the samples was lower than 0.05% and its effects were determined in all experiments.

3. Blood and blood platelets

3.1. Isolation of blood platelets

The samples of human blood were taken from regular, medication-free, non-drinking alcohol/non-smoking volunteers in the Medical Center in Lodz, Poland. The blood was collected into tubes with citrate/phosphate/dextrose/adenine (CPDA) anticoagulant. Blood platelets were detached from fresh blood by differential centrifugation as described previously [17,18]. The platelets were then suspended in Barber's buffer, a modified Tyrode's buffer (pH 7.4). The required amount of platelets, i.e. in the range $1.5–2.0 \times 10^8$/mL, was confirmed by spectrophotometric measurement in a UV-Visible Helios-α at 800 nm. In every experiment, blood or blood platelets were incubated for 30 min at 37 °C, either with *Asteraceae* extracts at final concentrations of 5 and 50 μg/mL, or aronia berry extract at a final concentration of 50 μg/mL.

All experiments were approved by the University of Lodz Committee for Research on Human Subjects and carried out under permission number 8/KBBN-UŁ/III/2018.

4. Effect of extracts from Asteraceae family on parameters of hemostasis

4.1. Flow cytometry

To determine the effect of *Asteraceae* extracts on the changes in activation and reactivity of resting and stimulated blood platelets, whole blood models were used. The whole blood samples were incubated with the *Asteraceae* extracts for 15 min at 37 °C; following this, platelet agonists (10 or 20 μM ADP or collagen) were added, and the mixture incubated for 15 min at room temperature (RT). The tested samples were then diluted 10-fold in sterile PBS (phosphate-buffered saline) with Mg^{2+}, and stained for 30 min at room temperature in the dark with 3 μL of anti-CD61/PerCP, anti-CD62/PE, or PAC- 1/FITC antibodies. Isotype controls were also prepared, containing resting blood samples, stained with 3 μL of anti-CD61/PE and isotype control antibodies marked with the FITC/PE isotype.

In the final step, all samples were fixed with 1% CellFix for 60 min at 37 °C. Platelets were counted based on the fluorescence of 5000 platelets (CD61/PerCP positive objects), using a LSR II Flow Cytometer (Becton Dickinson, San Diego, CA, USA) was used. The platelets were distinguished from other blood cells by a forward light scatter (FCS) vs. side light scatter (SSC) plot on a log/log scale (first gate) and by positive staining with monoclonal anti-CD61/PerCP antibodies (second gate). The percentages of CD62P-positive and PAC-1-positive platelets were calculated in each sample. The obtained data were analyzed using FlowJo software (Becton Dickinson, San Diego, CA, USA) [19,28].

4.2. Total thrombus formation analysis system (T-TAS®)

The thrombus formation process was measured under flow conditions using the T-TAS® system with a PL-chip microchip coated with collagen. Whole blood collected on BAPA (benzylsulfonyl-D-arginyl-prolyl-4-amidinobenzylamide) was incubated with extracts from tested *Asteraceae* plants for 30 min at 37 °C, and the samples were then transferred to the PL-chip. The results are given as AUC_{10} i.e., Area Under the Curve [10].

4.3. Platelet adhesion

Blood platelet adhesion was measured based on the activation of exoenzyme acid phosphatase in the blood platelets. After isolation from fresh blood and incubation with extracts from selected *Asteraceae* plants, the platelets were dissolved in Triton X-100 with phosphatase substrate (p-nitrophenylphosphate), resulting in the formation of p-nitrophenol. The level of p-nitrophenol was measured at λ = 405 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). The addition of 2 M NaOH facilitated a color reaction. A control sample was set up containing only blood platelets with Barber's buffer; this value assumed to be 100% [4].

5. Effect of extracts from the Asteraceae family on parameters of damages

5.1. Activity of LDH

The toxic effect of the selected *Asteraceae* vegetable extracts on blood platelets were analyzed by measurement of the activity of lactate dehydrogenase (LDH), the enzyme released from the platelets. After 30 min incubation at 37 °C, the test samples were centrifuged for 15 min at 25 °C in 2500 rpm. Following this, 10 μL of supernatant was transferred to a microtiter plate, which was then loaded with 270 μL of 0.1 M phosphate buffer and 10 μL of NADH. After a 20 min incubation at room temperature, 10 μL of pyruvate (5 mg) was added and the absorbance measured immediately afterwards. Readings were taken every minute for a 10-minute period. Absorbance was measured at λ = 340 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany) [31].

5.2. Effect of extracts from Asteraceae family on enzymatic lipid peroxidation

The level of lipid peroxidation in blood platelets incubated with selected *Asteraceae* extracts was determined based on the level of thiobarbituric acid reactive substances (TBARS). The samples were mixed with 15% trichloroacetic acid and 0.37% thiobarbituric, and then heated for 10 min at 100 °C in a heating block. The samples were then cooled and 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) [3,32].

5.3. Data analysis

Several tests were used to carry out the statistical analysis. All the values were expressed as mean±SD. First, the results were checked for normality with the Kołmogorow-Smirnow test, then equality of variance with the Levine test. Statistically significant differences were assessed by applying the ANOVA test (significance level was $p < 0.05$), followed by Tukey's multiple comparisons test, or accordingly, the Kruskal-Wallis test.

6. Results

The tested extracts were found to contain various biologically-active compounds including flavonoids and sesquiterpene lactones. The

chicory leaf preparation demonstrated the greatest chemical diversity and the Jerusalem artichoke preparation showed the least. The results are presented in Table 1.

The four plant extracts from the *Asteraceae* family (chicory leaves, green lettuce leaves, red lettuce leaves, and Jerusalem artichoke roots) did not influence AUC_{10} relative to control when administered at the highest concentration, 50 μg/mL, in whole blood in vitro (Fig. 1), i.e. they did not show any anticoagulant activity. These extracts did not appears to change the exposition of CD62P and PAC-1 in resting platelets or in ADP/collagen-activated platelets (Fig. 2).

The study also examined the effect of the four *Asteraceae* extracts on platelet adhesion to collagen and fibrinogen, in a washed blood platelet model. Although incubation with the four extracts at 5 and 50 μg/mL were found to inhibit the adhesion of both the resting and thrombin-activated platelets to collagen, variation was observed between the preparation, with the effect not always being statistically significant (Fig. 3 A and B). For example, the preparation from Jerusalem artichoke (5 and 50 μg/mL) demonstrated about 80% greater inhibition of thrombin-activated platelets to collagen than other used extracts (Fig. 3B). In addition, all four extracts significantly reduced adhesion of thrombin- and ADP-activated blood platelets to fibrinogen (Fig. 3 C and D).

All used plant extracts were found to inhibit enzymatic lipid peroxidation in thrombin-activated blood platelets, with red lettuce (50 μg/mL) and Jerusalem artichoke extracts (5 and 50 μg/mL) demonstrating a statistically significant effect. The Jerusalem artichoke preparation demonstrated about 60% inhibition of thrombin-activated lipid peroxidation at both 5 and 50 μg/mL (Fig. 4).

None of the plant extracts induced platelet lysis at either 5 or 50 μg/mL (Fig. 5).

The effects of the four plant extracts from *Asteraceae* family (chicory leaves, green lettuce leaves, red lettuce leaves, and Jerusalem artichoke roots) and two dandelion extracts (i.e. from roots and fruits) at the highest tested concentration (50 μg/mL) on blood platelet activation (using washed platelets and whole blood) are compared in Table 2. Of all tested extracts, the dandelion root and fruit extracts demonstrated the strongest anti-platelet potential in whole blood. However, the red lettuce leaf and Jerusalem artichoke root extracts demonstrated stronger anti-platelet activity than the other plant extracts and dandelion extracts against washed blood platelets (Table 2). The aronia berry extract (50 μg/mL, positive control) also demonstrated anti-platelet properties, decreasing PAC-1 binding in platelets activated by 20 μM ADP and collagen; it also reduced the expression of GPIIb/IIIa on platelets activated by 10 μM ADP, 20 μM ADP and collagen (data not presented).

7. Discussion

Blood is maintained in a fluid state inside its vessels and excessive bleeding after injury is prevented by the hemostatic system; a key component of this system is thrombogenicity. This process takes place in two waves, which occur simultaneously in a vessel. The first wave, also called primarily hemostasis, is based around platelet accumulation while the second is mediated by the blood coagulation pathway [9,27].

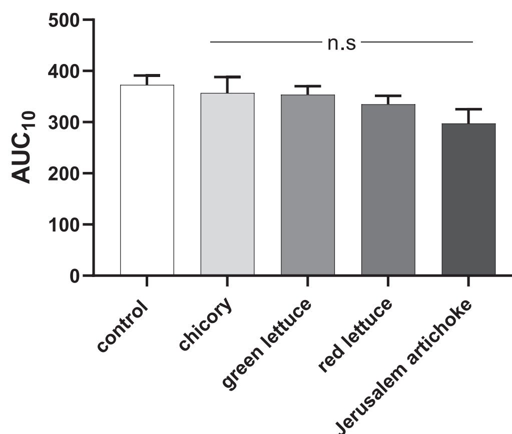

Fig. 1. Effects of the four plant extracts (50 μg/mL, incubation time – 30 min) on the T-TAS in whole blood samples. Data is given as the mean ± SD of four healthy volunteers (each experiment performed in triplicate). n.s – not significant ($p > 0.05$).

Blood platelets are small, irregular, anucleate fragments derived from megakaryocytes; their membrane contains glycoprotein, which is plays a role in platelet adhesion and cohesion through interaction with various ligands. Their main role in hemostasis is to ensure the integrity of the high-pressure circulatory system. In the event of the endothelial monolayer becoming disrupted, an interaction takes place between specific receptors on the platelet surface and sub-endothelial matrix protein, leading to platelet activation and aggregation, and thus thrombus formation [5].

Platelets also take part in the second wave of hemostasis, by modulating cell-based thrombin generation, which amplifies the blood coagulation cascade. The exposure of P-selectin, which is released from platelet granulates after activation, can potentiate thrombin generation by inducing a negative charge on the surface that harbors the coagulation factors [9]. Platelet activation takes place via a series of coagulation cascades. The first step is the adhesion of the platelets to the extracellular matrix; this is facilitated by a bridge being formed between exposed collagen and the glycoprotein (GP) Ib-IX-V receptor on the platelet membrane by von Willebrand factor. Collagen can also bind to two others receptors: GP Ia/IIa and GP IV. Activation leads to a change in platelet shape and the release of granule content. Another platelet receptor, GP IIb/IIIa, plays a role in thrombus stabilization by forming cross-links with fibrinogen or von Willebrand factor ([25,30]).

However, disturbances can occur in hemostasis system leading to atheromatous plaque thrombogenicity; such changes can result in the development of cardiovascular diseases (CVD) and ultimately, death. Although the mortality associated with CVD is decreasing in high-income countries, it continues to rise in most middle- and low- income countries. In Europe, despite the development of various pharmaceutical treatments and prophylaxes intended to improve the quality of life,

Table 1
Phytochemical characteristics of the extracts from four plants of the *Asteraceae* family identified by UPLC-QTOF 554 MS/MS (modified; [24]).

Proposed compound	Compound class	Red lettuce leaves	Green lettuce leaves	Chicory	Jerusalem artichoke
3- caffeoylquinic acid	phenolic acids	–	–	+	–
5-caffeoylquinic acid	phenolic acids	+	+	+	+
4-caffeoylquinic acid	phenolic acids	–	–	–	+
zeylanidine A	sesquiterpene lactones	–	–	+	–
lactucin derivative	sesquiterpene lactones	–	–	+	–
quercetin 3-O-glucoside	flavonol	–	+	–	–
kaempherol-3-O-glucuronide	flavonol	+	+	–	–
quercetin acetylgalactoside	flavonol	+	+	–	–
octadecadienoic acid derivative	fatty acids	+	+	+	–
pinellic acid	fatty acids	+	+	+	–

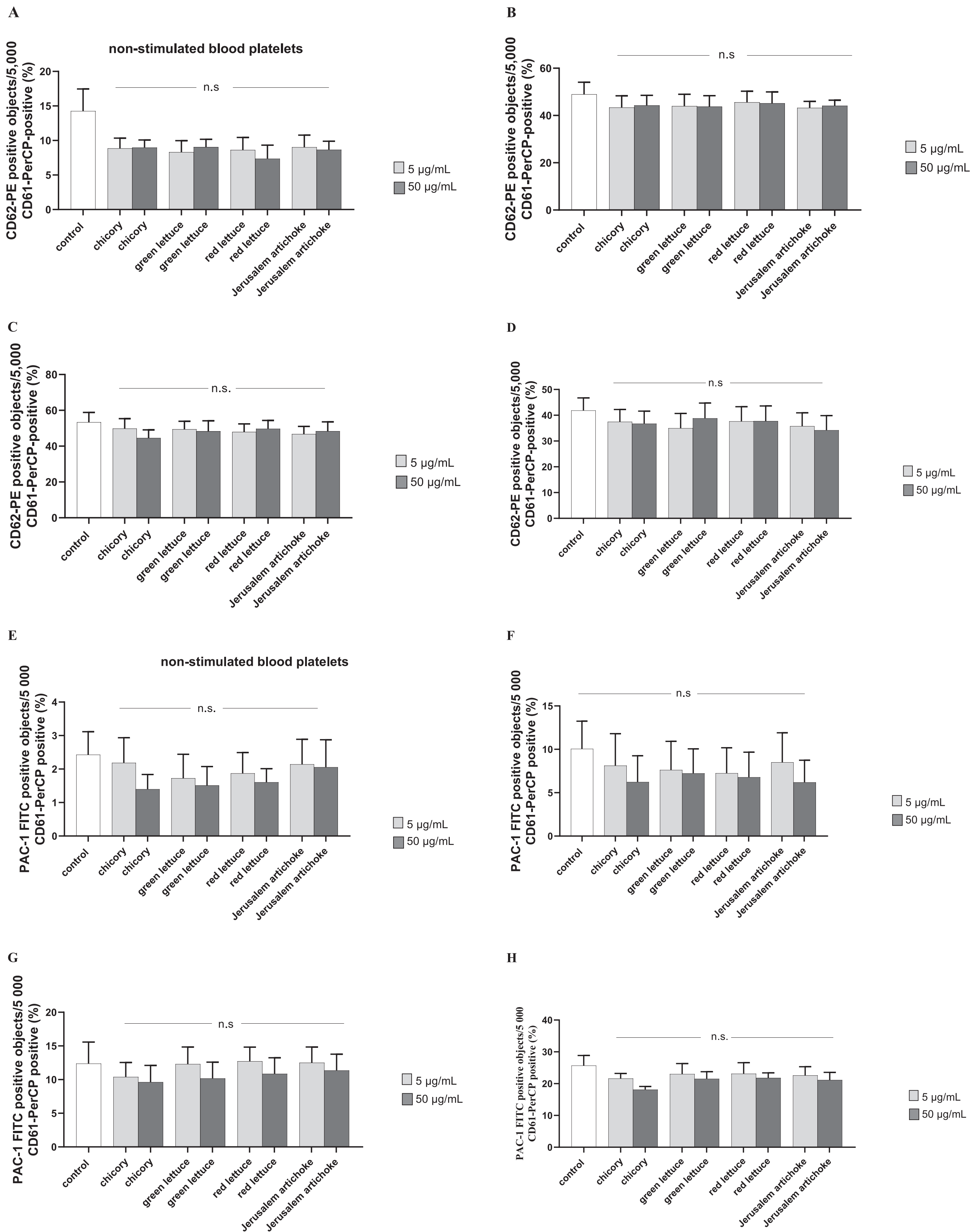

Fig. 2. Effects of the four plant extracts (5 and 50 μg/mL, incubation time – 30 min) on exposition of P-selectin on (A) resting or agonist-stimulated blood platelets: (B) 10 μM ADP, (C) 20 μM ADP and (D) 10 μg/mL collagen and on exposition of the active form of GPIIb/IIIa on (E) resting or agonist-stimulated blood platelets: (F) 10 μM ADP, (G) 20 μM ADP and (H) 10 μg/mL collagen in whole blood samples. Results are given as the mean ± SD of five healthy volunteers (each experiment performed in triplicate). n.s – not significant ($p > 0.05$).

Fig. 3. Effects of the four plant extracts (5 and 50 μg/mL, incubation time – 30 min) on adhesion of resting platelets to (A) collagen- or (B) thrombin (final concentration 0.2 U/mL) - activated platelets and on adhesion of blood platelets to fibrinogen, as well as (C) thrombin (final concentration 0.2 U/mL) - activated platelets or (D) ADP (final concentration 30 μM) - activated platelets. In the graphs, adhesion is expressed as a percentage of the control sample (platelets without plant preparation). Results are given as mean ± SD (n = 5). Kruskal-Wallis test: * $p < 0.05$, * * $p < 0.01$, compared with control (i.e. not treated with plant preparation); n.s – not significant ($p > 0.05$).

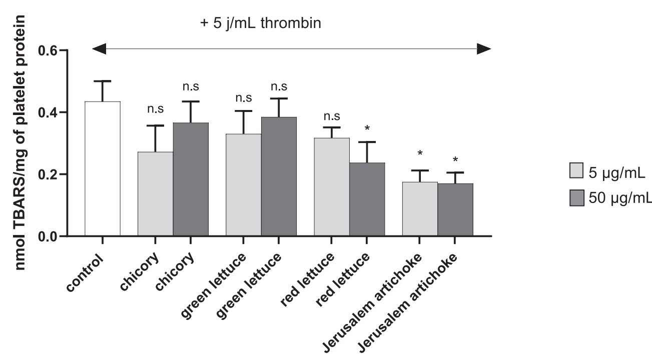

Fig. 4. Effects of the four plant extracts (5 and 50 μg/mL, incubation time – 30 min) on lipid peroxidation in platelets activated by thrombin. Results are given as mean ± SD (n = 5). Kruskal-Wallis test: * $p < 0.05$, compared with control (i.e. not treated with plant preparation); n.s – not significant ($p > 0.05$).

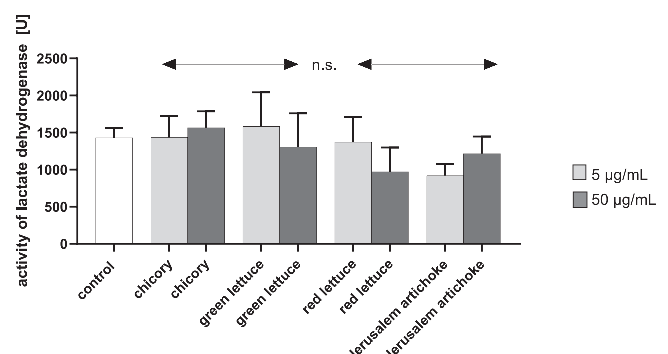

Fig. 5. The toxic effects of the four plant extracts (5 and 50 μg/mL, incubation time – 30 min) against human blood platelets. Results are given as mean ± SD (n = 5). None of the five extracts had any statistically significant effect compared to control n.s – not significant ($p > 0.05$).

almost 46% of deaths are connected with CVD. The most logical explanation is the prevalence of an unhealthy lifestyle connected with diet full of processed food with surplus of sugar, saturated fats and sodium [1,14].

Numerous risk factors are associated with the development of CVD, including obesity, diabetes a high pressure and sedentary lifestyle, smoking and excessive use of alcohol [25]. Even dietary habits are strongly correlated with incidence of cardiovascular disease [27]. A considerable body of evidence emphasizes the importance of everyday healthy nutrition in ensuring lifelong health and the prevention of various diseases, not only CVD. For example, people consuming the Mediterranean diet, characterized by a high nutrient content, including vitamins, polyphenols, unsaturated fats and dietary fiber, are less likely to develop CVD than those consuming a highly-processed diet [14]. Even Hippocrates recognized the potential of food products to prevent and sometimes even cure diseases as illustrated by his saying “Let food be thy medicine and medicine be thy food”.

Most unprocessed or low-processed food products, especially plant-

Table 2
A comparison of the effect of four plant extracts from *Asteraceae* family (chicory leaves, green lettuce leaves, red lettuce leaves, and Jerusalem artichoke roots) and two dandelion extracts (from roots and fruits) at the highest tested concentration - 50 µg/mL on parameters of blood platelet activation (using washed platelets and whole blood).

Tested extract	GPIIb/IIIa expression				P selectin expression				T-TAS	Adhesion to collagen		Adhesion to fibrinogen		Enzymatic lipid peroxidation
	Resting platelets	Platelets activated by 10 µM ADP	Platelets activated by 20 µM ADP	Platelets activated by collagen	Resting platelets	Platelets activated by 10 µM ADP	Platelets activated by 20 µM ADP	Platelets activated by collagen		Resting platelets	Platelets activated by thrombin	Platelets activated by thrombin	Platelets activated by ADP	Platelets activated by thrombin
Dandelion roots	No effect	No effect	No effect	Anti-platelet effect	No effect	No effect	No effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	No effect
Dandelion fruits	No effect	No effect	Anti-platelet effect	Anti-platelet effect	No effect	No effect	No effect	Anti-platelet effect	No effect	No study	No study	Anti-platelet effect	No effect	No effect
Chicory leaves	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	No effect
Green lettuce leaves	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	No effect
Red lettuce leaves	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect
Jerusalem artichoke roots	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect	Anti-platelet effect

based ones, contain biologically-active compounds known to bestow specific health-promoting benefits [1]. A strong body of evidence indicates that fruit and vegetable consumption lowers the risk of CVD. The American heart Association encourages the intake of fruits and vegetables as part of the everyday diet. Their protective effect may well be based on the antioxidant and anti-inflammatory potential of their biologically-active components. For example, potassium intake, present in many plants, reduces the risk of stroke or heart ischemia by reducing blood pressure; in addition, flavonoids and anthocyanins scavenge free radicals produced during atherogenesis, which plays a key role in CVD [1]. A number of dietary carbohydrates present in plants, including dietary fiber, are quite effective in managing hyperglycemia, while various oligosaccharides are known to play roles in managing the glycemic and insulin metabolism, both of which are risk factors for obesity and cardiovascular diseases [11].

One of the best-known species of *Asteraceae* is *T. officinale* (dandelion), which has been used in traditional medicine around the world for centuries. Recently, interest in its potential as a food product, pharmaceutical and cosmetic agent has grown due to its known biological activity and chemical composition, i.e. rich in phenolics and sesquiterpene lactones [16]. For example, dried leaves and petals are consumed as tea, which can be good source of potassium. The roasted roots can be drunk as a coffee replacement; this is especially valuable for diabetics due to its high inulin content: inulin has been found to improve the glycolipid metabolism in diabetic conditions, which is often related to CVD [16]. *T. officinale* extract is also known to possess significant antioxidant activity. The flower extract was found to reduce protein and lipid oxidation in plasma in vitro [12]. The dietary intake of *T. officinale* leaves and roots can reduce the risk of oxidative stress-related atherosclerosis thanks to its catechol, caffeic acid, m-coumaric acid, vanillic acid and syringic acid content; all these components favorably influence plasma antioxidant enzyme activities and lipid profiles [11].

Following on from our earlier results [24], the present study examined whether extracts from *Asteraceae* family members (chicory leaves, green lettuce leaves, red lettuce leaves, and Jerusalem artichoke roots) can effectively inhibit platelet activation using two in vitro models, viz. human washed blood platelets and human whole blood. Their effects on platelet activation in whole blood were determined based on exposure of P-selectin and the active form of GPIIb/IIIa on the platelet surface based on flow cytometry. The results indicate that not all tested plant extracts affected P-selectin and GPIIb/IIIa exposure. The strongest anti-platelet activity against whole blood was demonstrated by dandelion root and fruit extracts.

Our findings are the first to demonstrate that the tested plant extracts have significant anti-platelet activity in a washed platelet model. Of the tested from *Asteraceae* family members, the strongest anti-platelet potential was demonstrated by red lettuce leaves and Jerusalem artichoke roots, which effectively inhibited platelet adhesion to collagen and fibrinogen. They also inhibited the arachidonic acid metabolism in thrombin-activated platelets, as indicated by TBARS measurements. This finding suggests that the compounds present in these extracts may modulate platelet activation by interfering with arachidonic acid metabolism; these include various phenolic acids such as 5-caffeoylquinic acid, caffeoyltartaric acid and 1,3-dicaffeoylquinic acid, a number of flavonoids, such as quercetin derivates, and certain sesquiterpene lactones, including lactucin derivatives.

The differences in anti-platelet potential observed between the used plant extracts may be explained by their varied chemical profile. The high anti-platelet properties shown by red lettuce leaves and Jerusalem artichoke roots may be due to the high levels and considerable diversity of phenolic acids, such as caffeoylquinic acid. The same compound and its derivates were found in an coca extract. These compounds were able to suppress the exposure of P-selectin on platelets and potently inhibited COX-1 and COX-2 activity [6]. Red lettuce and Jerusalem artichoke are also rich in sesquiterpene lactones, which are known to have anti-platelets properties. A study of the aerial parts of *Capparis decidua*

found two sesquiterpene lactones to demonstrate anti-aggregatory activity during incubation with guinea pig platelet-rich plasma with the addition of an aggregating agent [26].

It is important to note that none of the tested plant extracts were found to cause damage to platelets, determined as leakage of LDH into the extracellular medium, and hence appear to be safe for use as supplements. However, it must be borne in mind that phenolic compounds often exhibit low bioavailability and hence must be administered at high doses; despite this, their metabolites sometimes demonstrate stronger anti-platelet properties than their precursors [2].

Our present results support previous observations that dandelion extracts, and those of other members of the *Asteraceae* family, possess a wide positive spectrum of activity. They also confirm the potential of these plants as promising components of functional foods intended to counter the onset of CVDs associated with blood platelet hyperactivation. However, although the tested extracts from the *Asteraceae* family were found to display various antioxidant and anti-platelet properties in vitro, their true effects on CVDs should be verified in in vivo studies.

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Conflict of interest statement

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

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CRediT authorship contribution statement

Agata Rolnik: Methodology, Formal analysis, Investigation, Writing – original draft. **Anna Stochmal:** Writing – original draft. **Beata Olas:** Conceptualization, Writing – original draft, Writing – review & editing.

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Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy "Comparative phytochemical, antioxidant and haemostatic studies of preparations from selected vegetables from *Cucurbitaceae* family" **A. Rolnik**, I. Kowalska, A. Soluch, A. Stochmal, B. Olas; Molecules 2020, 25, 1-20, mój udział 40% i obejmował opracowanie koncepcji badań, przygotowanie prób do analizy chemicznej, wykonanie analiz biologicznych (obejmujących parametrów stresy stresu oksydacyjnego oraz oznaczenie parametrów hemostazy, w tym czasów krzepnięcia), opracowanie wyników analiz biologicznych, przygotowanie analizy statystycznej, wykonanie rycin i przygotowanie części manuskryptu, odpowiedź na komentarze recenzentów.

Oświadczam, że w pracy "Antioxidant and hemostatic properties of preparations from *Asteraceae* family and their chemical composition – Comparative studies" **A. Rolnik**, A. Soluch, I. Kowalska, B. Olas; Biomedicine & Pharmacotherapy, 2021, 142, 111982, mój udział 45% i obejmował opracowanie koncepcji badań, przygotowanie prób do analizy chemicznej, wykonanie analiz biologicznych (obejmujących parametrów stresy stresu oksydacyjnego oraz oznaczenie parametrów hemostazy, w tym czasów krzepnięcia), opracowanie wyników analiz biologicznych, przygotowanie analizy statystycznej, wykonanie rycin i przygotowanie części manuskryptu, odpowiedź na komentarze recenzentów.

Oświadczam, że w pracy "Preparations from selected cucurbit vegetables as antiplatelet agents – the in vitro study" **A. Rolnik**, B. Skalski, A. Stochmal, B Olas; Scientific Reports 2021, 11 (1), 1-15, mój udział wynosił 50% i obejmował opracowanie koncepcji badań, przygotowanie prób do analizy, wykonanie analiz biologicznych (analizy aktywności płytek krwi po przez ocenę poziomu adhezji płytek krwi do kolagenu i fibrynogenu, ocenę poziomu ekspozycji glikoprotein GPIIb/IIIa i selektyny P, analizę przemiany arachidonaniu w płytkach krwi, analizę właściwości koagulacyjnych z wykorzystaniem systemu T-TAS oraz cytotoksyczności badanych preparatów), opracowanie wyników analiz biologicznych, przygotowanie analizy statystycznej wyników, wykonanie rycin i przygotowanie część manuskryptu, odpowiedź na komentarze recenzentów.

Oświadczam, że w pracy „The *in vitro* anti-platelet activities of plant extracts from the Asteraceae family" **A. Rolnik**, A. Stochmal, B. Olas; Biomedicine & Pharmacotherapy, 2022,149, 112809, mój udział wynosił 50% i obejmował opracowanie koncepcji badań, przygotowanie próbek do analizy, wykonanie analiz biologicznych (analizy aktywności płytek krwi po przez ocenę poziomu adhezji płytek krwi do kolagenu i fibrynogenu, ocenę poziomu ekspozycji glikoprotein GPIIb/IIIa i selektyny P, analizę przemiany arachidonaniu w płytkach krwi, analizę właściwości koagulacyjnych z wykorzystaniem systemu T-TAS oraz cytotoksyczności badanych preparatów), opracowanie wyników analiz biologicznych, przygotowanie analizy statystycznej wyników, wykonanie rycin i przygotowanie część manuskryptu, odpowiedź na komentarze recenzentów.

Oświadczam, że w pracy "The plants of the *Asteraceae* family as agents in the protection of human health" **A. Rolnik**, B. Olas; International Journal of Molecular Sciences 2021, 22, 1-10,

mój udział wynosił 60% i opracowanie koncepcji pracy przeglądowej, przygotowanie manuskryptu oraz rycin i tabelek, przygotowanie odpowiedzi na komentarze recenzentów.

Oświadczam, że w pracy "Vegetables from Cucurbitaceae family and their products; positive effect on human health" **A. Rolnik**, B. Olas Nutrition 2020, 78, 1-6, mój udział wynosił 60% i opracowanie koncepcji pracy przeglądowej, przygotowanie manuskryptu oraz rycin i tabelek, przygotowanie odpowiedzi na komentarze recenzentów.

Agata Rolnik

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Łódź, 12.09.2022

Oświadczenia o udziale w publikacjach

Oświadczam, że w pracy "Comparative phytochemical, antioxidant and haemostatic studies of preparations from selected vegetables from *Cucurbitaceae* family" A. Rolnik, I. Kowalska, A. Soluch, A. Stochmal, **B. Olas**; Molecules 2020, 25, 1-20, mój udział wynosił 20% i obejmował pomoc merytoryczną w opracowaniu koncepcji badań i pomoc w przygotowaniu części manuskryptu, w tym rozdziałów: wstęp, wyniki oraz dyskusja, pomoc w odpowiedzi na komentarze recenzentów.

Oświadczam, że w pracy "Antioxidant and hemostatic properties of preparations from *Asteraceae* family and their chemical composition – Comparative studies" A. Rolnik, A. Soluch, I. Kowalska, **B. Olas**; Biomedicine & Pharmacotherapy, 2021, 142, 1-10, mój udział wynosił 25% i obejmował pomoc merytoryczną w opracowaniu koncepcji badań, konsultacje merytoryczne, pomoc w przygotowaniu części manuskryptu, w tym rozdziałów wstęp, wyników oraz dyskusja, pomoc w odpowiedzi na komentarze recenzentów.

Oświadczam, że w pracy "Preparations from selected cucurbit vegetables as antiplatelet agents – the in vitro study" A. Rolnik, B. Skalski, A. Stochmal, **B Olas**; Scientific Reports 2021, 11 (1), 1-15, mój udział wynosił 25% i obejmował pomoc merytoryczną w opracowaniu koncepcji badań, konsultacje merytoryczne, pomoc w przygotowaniu części manuskryptu, w tym rozdziałów wstęp, wyników oraz dyskusja, pomoc w odpowiedzi na komentarze recenzentów.

Oświadczam, że w pracy „The in vitro anti-platelet activities of plant extracts from the *Asteraceae* family" A. Rolnik, A. Stochmal, **B. Olas**; Biomedicine & Pharmacotherapy, 2022,149, 1-15, mój udział wynosił 35% i obejmował pomoc merytoryczną w opracowaniu koncepcji badań, konsultacje merytoryczne, pomoc w przygotowaniu części manuskryptu, w tym wstęp, wyniki oraz dyskusja, pomoc w odpowiedzi na komentarze recenzentów.

Oświadczam, że w pracy "The plants of the *Asteraceae* family as agents in the protection of human health" A. Rolnik, **B. Olas**; International Journal of Molecular Sciences 2021, 22, 1-10, mój udział wynosił 40% i obejmował pomoc merytoryczną w opracowaniu koncepcji pracy przeglądowej, pomoc w przygotowaniu manuskryptu oraz pomoc w odpowiedzi na komentarze recenzentów.

Oświadczam, że w pracy "Vegetables from *Cucurbitaceae* family and their products; positive effect on human health" A. Rolnik, **B. Olas;** Nutrition 2020, 78, 1-6, mój udział wynosił 40% i obejmował pomoc merytoryczną w opracowaniu koncepcji pracy przeglądowej, pomoc w przygotowaniu manuskryptu i odpowiedzi na komentarze recenzentów.

Beata Olas

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Państwowy Instytut Badawczy

Puławy, 17.09.2022

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy "Comparative phytochemical, antioxidant and haemostatic studies of preparations from selected vegetables from *Cucurbitaceae* family" A. Rolnik, I. Kowalska, A. Soluch, **A. Stochmal**, B. Olas, Molecules 2020, 25, 1-20, mój udział wynosił 15% i obejmował pomoc merytoryczną w opracowaniu koncepcji badań, konsultacje merytoryczne, pomoc w przygotowaniu celu i hipotezy badawczej oraz przygotowanie części manuskryptu, w tym rozdziałów: wstęp, wyniki oraz dyskusja.

Oświadczam, że w pracy "Preparations from selected cucurbit vegetables as antiplatelet agents – the *in vitro* study" A. Rolnik, B. Skalski, **A. Stochmal**, B Olas; Scientific Reports 2021, 11 (1), 1-15, mój udział wynosił 15% i obejmował pomoc merytoryczna dotyczącą opracowania koncepcji badań, konsultacje merytoryczne, pomoc w przygotowaniu celu i hipotezy badawcze oraz przygotowanie części manuskryptu, w tym rozdziałów: wstęp, wyniki oraz dyskusja.

Oświadczam, że w pracy „The in vitro anti-platelet activities of plant extracts from the *Asteraceae* family" A. Rolnik, **A. Stochmal**, B. Olas; Biomedicine & Pharmacotherapy, 2022, 1-10, mój udział wynosił 15% i obejmował pomoc merytoryczną w opracowaniu koncepcji badań, konsultacje merytoryczne, pomoc w przygotowaniu celu i hipotezy badawcze, oraz przygotowanie części manuskryptu, w tym rozdziałów: wstęp, wyniki oraz dyskusja.

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Zakład Biochemii i Jakości Plonów,
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Państwowy Instytut Badawczy

Puławy, 30.08.2022

Oświadczenia o udziale w publikacjach

Oświadczam, że w pracy "Comparative phytochemical, antioxidant and haemostatic studies of preparations from selected vegetables from *Cucurbitaceae* family" A. Rolnik, **I. Kowalska**, A. Soluch, A. Stochmal, B. Olas; Molecules 2020, 25, 1-20, mój udział wynosił 15% i obejmował przygotowanie preparatów do analizy z materiału roślinnego, pomoc merytoryczną w opracowaniu koncepcji badań, wykonanie analizy TLC-DPPH, opracowanie części manuskryptu dotyczącego analizy chemicznej w rozdziałach: materiał i metody, wyniki oraz dyskusja.

Oświadczam, że w pracy " "Antioxidant and hemostatic properties of preparations from *Asteraceae* family and their chemical composition – Comparative studies" A. Rolnik, A. Soluch, **I. Kowalska**, B. Olas; Biomedicine & Pharmacotherapy, 2021, 142, 1-15, mój udział wynosił 20% i obejmował przygotowanie preparatów do analizy z materiału roślinnego, pomoc merytoryczną w opracowaniu koncepcji badań dotyczących analizy jakościowej prób, wykonanie analizy TLC-DPPH, opracowanie części manuskryptu dotyczącego analizy chemicznej w rozdziałach: materiał i metody, wyniki oraz dyskusja, pomoc w odpowiedzi na komentarze recenzentów.

Iwona Kowalska

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Instytut Uprawy Nawożenia i Gleboznawstwa,
Państwowy Instytut Badawczy

Łódź, 30.08.2022

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy „"Comparative phytochemical, antioxidant and haemostatic studies of preparations from selected vegetables from *Cucurbitaceae* family" A. Rolnik, I. Kowalska, **A. Soluch**, A. Stochmal, B. Olas; Molecules 2020, 25, 1-20, mój udział wynosił 10% i obejmował przygotowanie prób do analizy jakościowej, wykonanie analizy jakościowej, opracowanie wyników analizy jakościowej i przygotowanie części manuskryptu, w tym rozdziału materiały i metody oraz wyniki.

Oświadczam, że w pracy "Antioxidant and hemostatic properties of preparations from *Asteraceae* family and their chemical composition – Comparative studies" A. Rolnik, **A. Soluch**, I. Kowalska, B. Olas; Biomedicine & Pharmacotherapy, 2021, 142, 111982 mój udział wynosił 10% i obejmował przygotowanie prób do analizy jakościowej, wykonanie analizy jakościowej, opracowanie wyników analizy jakościowej i przygotowanie części manuskryptu, w tym rozdziału materiały i metody oraz wyniki.

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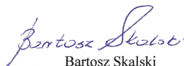
Łódź, 19.09.2022

Oświadczenie o udziale w publikacji

Oświadczam, że w pracy "Preparations from selected cucurbit vegetables as antiplatelet agents – the *in vitro* study" A. Rolnik, **B. Skalski**, A. Stochmal, B Olas; Scientific Reports 2021, 11 (1), 1-15, mój udział wynosił 10% i obejmował pomoc przy wykonywaniu analizy biologicznej (obejmującej techniki cytometrii przepływowej oraz systemu T-TAS), opracowania wyników analizy i pomoc w opracowaniu części manuskryptu obejmującego rozdziały: metody i materiały oraz wyniki.



Bartosz Skalski