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Polifenolowe ekstrakty z różnych części mniszka pospolitego *(Taraxacum officinale)* jako modulatory hemostazy

Polyphenolic extracts from different parts of dandelion *(Taraxacum officinale)* as haemostasis modulators

Praca doktorska

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pod kierunkiem
dr hab. Beaty Olas, prof. UŁ

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Dziękuję moim ***Rodzicom***
za pomoc w realizowaniu moich celów
i nieustanne wsparcie, za co jestem
Im ogromnie wdzięczna.
Braciom *za słowa otuchy i cenne rozmowy.*
Przyjaciołom i znajomym
za ich obecność w ciężkich chwilach i kibicowanie.

W sposób szczególny dziękuję
Pani dr hab. Beacie Olas, prof. UŁ
za opiekę promotorską, cierpliwość
oraz nieocenioną pomoc w trakcie
przygotowywania niniejszej rozprawy doktorskiej.

Dziękuję również ***pracownikom IUNG*** *w Puławach*
za życzliwość, miłą atmosferę pracy
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Dorobek naukowy wchodzący w skład rozprawy doktorskiej

- ✓ **Prace doświadczalne**

[1]Lis B., Jędrejek D., Stochmal A., Olas B. 2018. „Assesment of effects of phenolic fractions from leaves and petals of dandelion in selected components of hemostasis" *Food Research International* 107, 605-612 **(IF$_{2018}$= 3,579; MNiSW= 40** – według punktacji MNiSW z dnia 9.12.2016r.**)**

[2]Jędrejek D., **Lis B.**, Rolnik A., Stochmal A., Olas B. 2019. „Comparative phytochemical, cytotoxicity, antioxidant and haemostatic studies of *Taraxacum officinale* root preparations" *Food and Chemical Toxicology* 126, 233-247 **(IF$_{2019}$= 4,679; MNiSW= 40** – według punktacji MNiSW z dnia 9.12.2016r.**)**

[3]Lis B., Rolnik A., Jędrejek D., Soluch A., Stochmal A., Olas B. 2019. „Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study" *Journal of Functional Foods* 59, 16-24 **(IF$_{2019}$= 3,701; MNiSW= 100** – według punktacji MNiSW z dnia 31.07.2019r**.)**

[4]Lis B., Jędrejek D., Mołdoch J., Stochmal A., Olas B. 2019. „The anti-oxidative and hemostasis-related multifunctionality of L-chicoric acid, the main component of dandelion: an *in vitro* study of its cellular safety, antioxidant and anti-platelet properties, and effect on coagulation" *Journal of Functional Foods* 59, 16-24 **(IF$_{2019}$= 3,701; MNiSW= 100** – według punktacji MNiSW z dnia 31.07.2019r**.)**

[5]Lis B., Jędrejek D., Rywaniak J., Soluch A., Stochmal A., Olas B. 2020. „Flavonoid preparations from *Taraxacum offcinale* L. fruits – a phytochemical, antioxidant and hemostasis studies" *Molecules* 25, 1-33 **(IF$_{2019}$= 3,267; MNiSW= 100** – według punktacji MNiSW z dnia 31.07.2019r.**)**

- ✓ **Praca przeglądowa**

[6]Lis B., Olas B. 2019. „Pro-health activity of dandelion (*Taraxacum officinale* L.*)* and its food products - history and present" *Journal of Functional Foods* 59, 40-48 **(IF$_{2019}$= 3,701; MNiSW= 100** – według punktacji MNiSW z dnia 31.07.2019r.**)**

Sumaryczny współczynnik oddziaływania (*ang.* Impact Factor, IF) publikacji (w roku opublikowania) wchodzących w skład rozprawy doktorskiej wynosi **22,628**. Całkowita liczba punktów za publikacje stanowiące rozprawę doktorską, według listy czasopism punktowanych MNiSW (dla roku wydania) wynosi **480**.

Współczynnik IF podano zgodnie z rokiem wydania publikacji. W przypadku punktów MNiSW wykorzystano listę z dnia 9.12.2016r. – dla prac opublikowanych przed 31.07.2019r., natomiast do pozostałych prac opublikowanych po 31.07.2019r. użyto listę z dnia 31.07.2019r.

Pozostały dorobek naukowy

A) PUBLIKACJE

1. **Lis B**., Olas B. 2016. „Działanie mniszka pospolitego (*Taraxacum officinale*) na układ sercowo - naczyniowy" Spojrzenie młodych naukowców na rozwój nauk biologicznych i chemicznych. Materiały pokonferencyjne. Wyd. CreativeTime. Kraków 20-25 **(IF_{2016}= 0; MNiSW= 0)**

2. **Lis B.**, Grabek-Lejko D. 2016. „Mniszek lekarski *(Taraxacum officinale)* – potencjalne właściwości prozdrowotne" *Nauka Przyroda Technologie* 10, 1-15 **(IF_{2016}= 0; MNiSW= 9)**

3. Jędrejek D., Kontek B., **Lis B**., Stochmal A., Olas B. 2017. „Evaluation of antioxidant activity of phenolic fractions from the leaves and petals of dandelion in human plasma treated with H_2O_2 and H_2O_2/Fe" *Chemico-Biological Interactions* 262, 29-37 **(IF_{2017}= 3,497; MNiSW= 30)**

4. Olas B., Żuchowski J., **Lis B**., Skalski B., Kontek B., Grabarczyk Ł., Stochmal A. 2018. „Comparative chemical composition, antioxidant and anticoagulant properties of phenolic fraction (a rich in non-acylated and acylated flavonoids and non-polar compounds) and non-polar fraction from *Elaeagnus rhamnoides* (L.) A. Nelson fruits" *Food Chemistry* 247, 39-45 **(IF_{2018}= 5,399; MNiSW= 40)**

5. **Lis B.** 2018. „Mniszek pospolity- roślina bogata w przeciwutleniacze". Rozdział w monografii: Wybrane substancje o znaczeniu biologicznym - spojrzenie młodych naukowców. Wyd. CreativeTime. Kraków 87-93 **(IF_{2018}= 0; MNiSW= 0)**

6. Skalski B., **Lis B.,** Pecio Ł., Kontek B., Olas B., Żuchowski J., Stochmal A. 2019. „Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – Induced oxidative stress and changes in hemostasis" *Food and Chemical Toxicology* 125, 614-620 **(IF_{2019}= 4,679; MNiSW= 40)**

7. Skalski B., Kontek B., **Lis B.,** Olas B., Żuchowski J., Stochmal A. 2019. „Biological properties of *Elaeagnus rhamnoides* (L.) A. Nelson twig and leaf extracts" *BMC Complementary and Alternative Medicine* 19, 1-12 **(IF_{2019}= 3,020; MNiSW= 40)**

8. Majewski M., **Lis B.**, Juśkiewicz J., Ognik K., Borkowska-Sztachańska M., Jedrejek D., Stochmal A., Olas B. 2020. „Phenolic fractions from dandelion leaves and petals as modulators of the antioxidant status and lipid profile in an *in vivo* study" *Antioxidants 9*, 1-13 **(IF_{2020}= 4,520 ; MNiSW= 100)**

9. Majewski M., **Lis B.**, Olas B., Ognik K., Juśkiewicz J. 2020. „Dietary supplementation with copper nanoparticles influences the markers of oxidative stress and modulates vasodilation of thoracic arteries in young Wistar rats" *PLoS ONE* 15, 1-16 **(IF_{2020}= 2,740; MNiSW= 100)**

10. Majewski M., **Lis B.**, Juśkiewicz J., Ognik K., Jędrejek D., Stochmal A., Olas B. 2021. „The composition and vascular/antioxidant properties of *Taraxacum officinale* flower

water syrup in a high-fat diet using an obese rat model" *Journal of Ethnopharmacology* 265, 1-10 **(IF$_{2020}$= 3,690; MNiSW= 140)**

11. Lis B., Rywaniak J., Jędrejek D., Szustka A., Stochmal A., Olas B. 2021. „Anti-platelet potential of fractions from various organs of dandelion (*Taraxacum officinale* L.) in whole blood" *Journal of Functional Foods* 59, 16-24 **(IF$_{2020}$= 3,701; MNiSW= 100)**

Sumaryczny współczynnik oddziaływania (*ang.* Impact Factor, IF) publikacji (w roku opublikowania) wchodzących w skład całego dorobku wynosi **53,874**. Całkowita liczba punktów za publikacje stanowiące dorobek naukowy, według listy czasopism punktowanych MNiSW (dla roku wydania) wynosi **1079**.

B) KOMUNIKATY ZJAZDOWE

✓ WYSTĄPIENIA USTNE

1. **Lis B.**, Jędrejek D., Stochmal A., Kontek B., Olas B. „Wpływ ekstraktów wyizolowanych z mniszka lekarskiego (*Taraxacum officinale)* na peroksydację lipidów osocza w warunkach *in vitro".* V Konferencja Biologii Molekularnej, Łódź 7-9.04.2016 r.

2. **Lis B**., Jędrejek D., Stochmal A., Olas B. „Badanie antypłytkowych właściwości fenolowych frakcji z liści i płatków mniszka pospolitego". Naturalne substancje roślinne – aspekty strukturalne i aplikacyjne. Puławy, 6-8.09.2017 r.

3. **Lis B.** „Polifenolowe ekstrakty z różnych organów mniszka pospolitego *(Taraxacum officinale)* jako modulatory hemostazy". IX Sesja Magistrantów i Doktorantów. Łódź, 21.06.2018 r.

4. **Lis B.**, Jędrejek D., Stochmal A., Olas B. „Wpływ frakcji wyizolowanych z owoców mniszka pospolitego na czasy krzepnięcia osocza oraz adhezję płytek krwi w warunkach *in vitro".* V Krajowa Konferencja „Naturalne substancje roślinne – aspekty strukturalne i aplikacyjne". Puławy, 18.09.-20.09.2019 r.

✓ POSTERY

1. **Lis B.**, Olas B. „Działanie mniszka pospolitego (*Taraxacum officinale)* na układ sercowo-naczyniowy". Konferencja Młodych Naukowców. Kraków, 22.10.2016 r.

2. **Lis B.,** Jędrejek D., Stochmal A., Olas B. „Wpływ fenolowych frakcji z płatków i liści mniszka pospolitego (*Taraxacum officinale)* na właściwości hemostatyczne osocza". Bioopen. Łódź, 11-12.05.2017 r.

3. Olas B., **Lis B.,** Skalski B., Stochmal A., Żuchowski J. „*Hippophae rhamoides L.* fruit as a modulator of hemostatic parameters of human plasma *in vitro".* New and Old Phytochemicals: Their role In Ecology, Veterinary and Welfare. Chieti, Włochy 17-19.09.2017 r.

4. Skalski B., Kontek B., **Lis B.**, Olas B., Stochmal A., Żuchowski J. „Changes in human plasma protein carbonylation induced by fractions from the leaves and twigs of *Hippophae rhamnoides* L". Chieti, Włochy 17-19.09.2017 r.

5. Rolnik A., **Lis B.,** Jędrejek D., Stochmal A., Olas B. „Wpływ fenolowych frakcji z korzeni mniszka pospolitego (*Taraxacum officinale*) na aktywność hemostatyczną osocza". Młodzi Naukowcy w Polsce- Badania i Rozwój. Warszawa, 17.11.2017 r.

6. Rolnik A., **Lis B.,** Jędrejek D. „Fitochemiczna analiza i wpływ różnych preparatów z korzeni mniszka pospolitego (*T. officinale)* na czas trombinowy osocza ludzkiego". Bioopen. Łódź, 24-25.05.18 r.

7. **Lis B.,** Jędrejek D., Olas B. „Protective activity of various *Taraxacum officinale* root preparations against human plasma protein damages *in vitro"*. IV International Conference of Cell Biology. Kraków, 11-12.05.2018 r.

8. **Lis B.,** Majewski M., Jędrejek D., Stochmal A., Olas B. „Określenie właściwości biologicznych surowicy szczurów karmionych frakcją z liści i płatków mniszka pospolitego (*Taraxacum officinale)"*. IV Konferencja dla Młodych Naukowców nt. Nowe Wyzwania dla Polskiej Nauki. Wrocław, 8.12.2018 r.

9. **Lis B.,** Jędrejek D., Stochmal A., Olas B. „Właściwości biologiczne kwasu cykoriowego wyizolowanego z liści mniszka pospolitego *(Taraxacum officinale)*". IV Konferencja dla Młodych Naukowców nt. Nowe Wyzwania dla Polskiej Nauki. Wrocław, 8.12.2018 r.

10. **Lis B.,** Jędrejek D., Stochmal A., Olas B. „Wpływ kwasu cykoriowego na adhezję płytek krwi". Bioopen. Łódź, 30-31.05.2019 r.

11. **Lis B.,** Jędrejek D., Stochmal A., Olas B. „Protective activity of various *Taraxacum officinale* fruit fractions against lipid damages in blood platelets". V International Conference of Cell Biology. Kraków, 10-12.05.2019 r.

12. **Lis B.,** Jędrejek D., Stochmal A., Olas B. „Effect of dandelion fruit fractions on the biological activity of plasma treated with H_2O_2/Fe". Natural Products in Drug Discovery and Human Health. Lizbona, Portugalia, 27.07.-1.08.2019 r.

Wstęp

Hemostaza pełni ważną rolę w utrzymaniu równowagi w całym organizmie. Stanowi zespół mechanizmów obronnych warunkujących płynność krążącej krwi w ustroju, natomiast w sytuacji uszkodzenia ściany naczynia krwionośnego, zabezpiecza jego strukturę tworząc skrzep (Hrachovinova, 2018). Do głównych składowych tego układu należą m.in. płytki krwi, czynniki układu krzepnięcia i fibrynolizy, jak i ściana naczyń krwionośnych. Komponenty te oddziałują na siebie wzajemnie, przez co możliwa jest równowaga hemostatyczna w ustroju (Hvas, 2016; van der Meijden i Heemskerk, 2019).

Najmniejsze fragmenty komórek w organizmie człowieka, jakimi są płytki krwi, powstają z megakariocytów w szpiku kostnym i zawierają trzy rodzaje ziarnistości tj. ziarnistości gęste, ziarnistości α oraz lizosomy. Z ziarnistości gęstych uwalniane są takie substancje jak serotonina, adenozyno-5'-diforforan (ADP) i histamina. Natomiast, w ziarnistościach α obecne są czynniki krzepnięcia, cząsteczki adhezyjne i modulujące układ odpornościowy (np. P-selektyna (CD62P), fibrynogen, β-tromboglobulina czy trombospondyna). Co więcej, nieliczne ziarnistości lizosomalne są miejscem występowania glikohydrolaz, jak i zdegradowanych enzymów (Xu i in., 2016; Kerris i in., 2020). Podstawowa rola płytek krwi w hemostazie, a przede wszystkim w hemostazie pierwotnej (przed uruchomieniem kaskady krzepnięcia) opiera się na trzech etapach, tj. adhezji, sekrecji i agregacji. W pierwszym etapie hemostazy pierwotnej następuje przyleganie płytek krwi do miejsca uszkodzonej warstwy śródbłonkowej, poprzez wytworzenie kompleksu receptorów płytek krwi z odsłoniętymi włóknami kolagenu. W rezultacie powstałego wiązania płytek krwi z uszkodzoną tkanką dochodzi do ich aktywacji pod wpływem różnych agonistów, tj. kolagenu, trombiny czy ADP (Hrachovinova, 2018; Neubauer i Zieger, 2021). W dalszej kolejności dochodzi do zmiany kształtu płytek krwi oraz intensywnego uwalnianie związków obecnych w ziarnistościach. Degranulacja jest możliwa w obecności jonów Ca^{2+} i prowadzi do reorganizacji płytek krwi oraz ma wpływ na amplifikację procesu aktywacji. W wyniku licznych interakcji między płytkami następuje nieodwracalna agregacja, czego rezultatem jest powstanie hemostatycznego czopu płytkowego. Do istotnych elementów tego etapu należy receptor płytkowy GP IIb/IIIa (m.in. dla fibrynogenu), którego zmiana konformacji warunkuje wiązanie z płytkami krwi. Końcowy etap polegający na wzmocnieniu czopu płytkowego nierozpuszczalnymi włóknami fibryny generowanymi przez kaskadę krzepnięcia, czego efektem jest powstanie skrzepu ostatecznego określany jest hemostazą wtórną (Korzonek-Szlacheta i in., 2018; van der Meijden i Heemskerk, 2019).

Jedną z cech układów biologicznych jest ich niestabilność, co oznacza, że są podatne na działanie różnych czynników, w tym reaktywnych form tlenu (RFT), które są zdolne do modyfikacji struktur i funkcji wszystkich makrocząsteczek komórkowych, takich jak: węglowodany, białka, lipidy czy DNA (Yang i Lian, 2020). Co więcej, stres oksydacyjny może również wpływać na hemostazę poprzez indukowanie zmian w procesie krzepnięcia czy aktywacji płytek krwi. Zarówno fizyczne, jak i psychiczne czynniki stresogenne powodują aktywację płytek krwi, co przejawia się zmianami w ich bioaktywności i agregacji. Brak zachowanej równowagi między produkcją RFT, a obecnością antyoksydantów (substancji przeciwutleniających) przyczynia się do występowania stresu oksydacyjnego, co dodatkowo może prowadzić do rozwoju procesów patologicznych warunkujących powstawanie miażdżycy w przebiegu ostrych zespołów wieńcowych oraz mózgowych incydentów niedokrwiennych (Korzonek-Szlacheta, 2018; Yang i Lian, 2020).

Według Światowej Organizacji Zdrowia to choroby sercowo-naczyniowe są główną przyczyną zgonów na świecie niezmiennie od dwudziestu lat. Mimo, iż obecnie obserwuje się ogromny rozwój medycyny, innowacyjnych metod leczenia, jak i większą skuteczność wykrywania schorzeń to jednak wciąż umiera na świecie prawie 18 mln ludzi z powodu chorób układu krążenia (WHO, 2020).

Jedną z najczęściej stosowanych metod leczenia w chorobach o podłożu sercowo-naczyniowym jest terapia lekami przeciwzakrzepowymi i przeciwpłytkowymi, stanowiąca tzw. „złoty standard" (Korzonek i Zembik, 2015; Neubauer i Zieger, 2021). Przykładami takich leków są kwas acetylosalicylowy (aspiryna), klopidogrel (tienopirydyna), warfaryna, dabigatran, riwaroksban i apiksaban, których działanie opiera się na różnych mechanizmach. Aspiryna działa hamująco na aktywność cyklooksygenazy obecnej w płytkach krwi, pośrednio hamując również wytwarzanie tromboksanu, natomiast klopidogrel blokuje receptory płytkowe P_2Y_{12}. Warfaryna zaliczana jest do antagonistów witaminy K i ma wpływ na hamowanie syntezy czynników krzepnięcia (II, VII, IX i X) w wątrobie. Natomiast, takie preparaty jak dabigatran, riwaroksban i apiksaban należą do antykoagulantów nowej generacji (*ang.* novel orac anticoagulants, NOAC) i działają selektywnie względem jednego czynnika krzepnięcia. Na przykład dabigatran jest inhibitorem trombiny, a riwaroksban i apiksaban są inhibitorami czynnika Xa (Byon i in., 2019; Abadie i in., 2020). Na rynku farmaceutycznym dostępnych jest wiele leków przeciwpłytkowych jednak ich stosowanie wywołuje u wielu pacjentów niepożądane efekty uboczne (głównie są to powikłania krwotoczne i niedokrwienne), przez co wciąż trwają próby wynalezienia skutecznego antidotum (Milling i Ziebell, 2020; Fredenburgh i Weitz, 2021).

Jednym z profilaktycznych zaleceń ekspertów Europejskiego Towarzystwa Kardiologicznego jest urozmaicona dieta bogata w szeroką gamę owoców, warzyw czy roślin leczniczych, które są źródłem naturalnych przeciwutleniaczy czy związków o aktywności antypłytkowej, takich jak polifenole. Związki te stanowią największą i najbardziej rozpowszechnioną grupę, która zaliczana jest do substancji biologicznie czynnych (Jankowski, 2017). Są to organiczne związki chemiczne z grupy fenoli posiadające co najmniej dwie grupy hydroksylowe, które łączą się z pierścieniem benzenowym. W zależności od obecnej liczby grup hydroksylowych, jak i rodzaju połączenia pierścieni aromatycznych wyodrębnia się dwie klasy związków tj. flawonoidy i kwasy fenolowe (Mężyńska i Brzóska, 2016). Wśród flawonoidów wyróżnia się flawony, flawonony, flawonole, flawanole i izoflawony. Natomiast, kwasy fenolowe dzieli się na kwasy hydroksybenzoesowe i hydroksycynamonowe. Związki te hamują zmiany oksydacyjne w lipidach czy białkach, a to jest podstawowy mechanizm w zmianach śródbłonkowych zachodzących w miażdżycy (Abbas i in., 2017; Neubauer i Zieger, 2021). Liczne badania kliniczne, jak i epidemiologiczne dostarczają dowodów, iż można zapobiec rozwojowi chorób cywilizacyjnych poprzez wdrożenie do swojego stylu życia dwóch podstawowych elementów tj. systematycznego wysiłku fizycznego oraz wzbogacenie diety w produkty zasobne w związki polifenolowe (Mężyńska i Brzóska, 2016; Cao i in., 2019; Fraga i in., 2019; Yamagata, 2019).

Przykładem rośliny o wielokierunkowym działaniu prozdrowotnym jest mniszek pospolity *(Taraxacum officinale)*. To szeroko rozpowszechniona na całym świecie bylina należąca do rodziny astrowatych *(Asteraceae),* która jest bogatym źródłem zarówno kwasów fenolowych (kwas cykoriowy, kwas chlorogenowy), flawonoidów (pochodne luteoliny, kwercetyny), jak i terpenów (laktony seskwiterpenowe). Dodatkowo, mniszek pospolity to skarbnica witamin (A, C, E, K i B) i składników mineralnych (wapń, sód, magnez, żelazo, miedź, krzem, cynk, mangan) (Schutz i in., 2006; Gonzales-Castejon i in., 2012). Ze względu na wysoką zawartość składników odżywczych liście mniszka pospolitego często są składnikiem sałatek, a korzenie zasobne w inulinę stosowane są jako substytut kawy lub herbaty. Ponadto, z kwiatów mniszka przyrządza się syrop, herbatę czy wino. Oprócz walorów smakowych mniszek pospolity od wielu wieków był wykorzystywany jako remedium na dolegliwości takie jak np. zaburzenia wątroby, pęcherzyka żółciowego czy nerek. Zarówno badania *in vitro,* jak i *in vivo* potwierdzają aktywność biologiczną rośliny w różnych jednostkach chorobowych (Wirngo i in., 2016; Xie i in., 2018; Abdel-Magied i in., 2019; Gao i in., 2019; Miłek i in., 2019; Sekhon-Loodu i Rupasinghe, 2019; Yoon i Park, 2019; Ignat i in., 2021). Za dobroczynny wpływ mniszka odpowiadają związki chemiczne zawarte w roślinie. Są

to m.in. laktony seskwiterpenowe, które wykazują działanie przeciwzapalne i antybakteryjne, jak i triterpeny czy fitosterole o działaniu przeciwmiażdżycowym. Innym przykładem są kwasy fenolowe o właściwościach antyoksydacyjnych i immunostymulujących oraz kumaryny o działaniu przeciwnowotworowym, przeciwzapalnym, przeciwbakteryjnym i przeciw-zakrzepowym. W mniszku obecne są również flawonoidy wykazujące aktywność antyoksydacyjną. Ponadto, korzenie mniszka są zasobne w inulinę, która ma działanie probiotyczne, hipoglikemiczne i wzmacniające układ odpornościowy (Schutz i in., 2006; Gonzales-Castejon i in., 2012).

W pracy numer [6] wchodzącej w skład rozprawy doktorskiej skoncentrowano się na przedstawieniu historii, jak i aktualnej wiedzy dotyczącej aktywności biologicznej mniszka pospolitego, która została potwierdzona w wielu badaniach zarówno w modelu *in vitro,* jak i *in vivo.*

Mimo wielu doniesień i prowadzonych badań nad aktywnością biologiczną mniszka pospolitego, nadal występują luki w wiedzy na temat wpływu preparatów otrzymanych z różnych organów tej rośliny na układ hemostazy. Dlatego też, istotą niniejszej pracy było pogłębienie wiedzy dotyczącej działania różnych pod względem składu chemicznego preparatów z mniszka pospolitego wyizolowanych z liści, płatków, korzeni i owoców na wybrane parametry stresu oksydacyjnego oraz hemostazy. Wszystkie użyte do badań preparaty zostały poddane analizie fitochemicznej i udostępnione przez Instytut Uprawy Nawożenia i Gleboznawstwa w Puławach, a ich charakterystyka chemiczna została przedstawiona w Tabeli 1.

Tabela 1. Porównanie profilu fitochemicznego badanych preparatów z różnych organów mniszka pospolitego *(T. officinale)* tj. liści, płatków, korzeni i owoców (opracowano na podstawie publikacji nr 1, 2 i 5 wchodzących w skład pracy doktorskiej).

Organy *T. officinale* / Nazwa klasy/związków chemicznych	LIŚCIE		PŁATKI		KORZENIE					OWOCE						
	nazwa frakcji		nazwa frakcji		nazwa frakcji					nazwa ekstraktu			nazwa frakcji			
	A (50%)	B (85%)	A (50%)	B (85%)	A	B	C	D	E	E1	E2	E3	A	B	C	D
KWASY FENOLOWE	++++	+++	+++	-	-	+	++	++	++	++	++++	+++	++++	++++	+++	++++
hydroksyfenylooctowe pochodne inozytolu	-	-	-	-	-	+	++	+	-	-	-	-	-	-	-	-
pochodne kwasów hydroksycynamonowych	+	+	+	-	-	-	+	++	++	+	+	+	-	-	-	-
kwas cykoriowy	+++	++	++	-	-	-	-	-	++	++	++++	+	-	-	-	-
FLAWONOIDY	+	++	+	++++	-	-	-	-	-	+	+	+++	++++	++++	++++	++++
luteolina	-	-	-	++++	-	-	-	-	-	+	-	+	++++	-	-	-
TERPENY	-	-	-	-	++	++	-	-	-	+	-	+	-	-	-	-
laktony seskwiterpenowe	-	-	-	-	++	++	-	-	-	+	-	+	-	-	-	-

- brak grupy/związku chemicznego w badanym preparacie;
+ obecność związków chemicznych w badanym preparacie (poziom metabolitów zaznaczono w tabeli w zależności od stężenia i oznaczono jako + < ++ < +++ < ++++).

Cel pracy

Idea podjęcia prac badawczych powstała w oparciu o wstępnie uzyskane wyniki badań (Jędrejek i in., 2017), analizę profilu fitochemicznego poszczególnych organów mniszka pospolitego wykonanej przez IUNG-PIB w Puławach, jak i dostępne dane literaturowe wskazujące na potencjał mniszka pospolitego w różnych schorzeniach u podstawy, których leży stres oksydacyjny (Schutz i in, 2006; Gonzalez-Castejon i in., 2012).

Uważa się, że stres oksydacyjny przyczynia się do rozwoju różnych schorzeń, w tym chorób układu sercowo-naczyniowego. Dlatego też zbadano wpływ preparatów (ekstraktów i frakcji) z mniszka na wybrane parametry stresu oksydacyjnego w osoczu, jak i płytkach krwi. Doświadczenia wykonano w warunkach *in vitro* oznaczając poziom anionorodnika ponadtlenkowego w płytkach krwi, peroksydacji lipidów, grup tiolowych i karbonylowych w białkach osocza i płytkach krwi. W przeprowadzonych badaniach induktorem stresu oksydacyjnego była mieszanina 4,7 mM H_2O_2, 3,8 mM $FeSO_4$ i 2,5 mM EDTA stanowiąca donor jednej z najbardziej reaktywnych cząstek w układach biologicznych czyli rodnika hydroksylowego (•OH), który powstaje w organizmie w tzw. reakcji Fentona ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+ OH^- + •OH$).

Innym kluczowym aspektem badań była ocena skuteczności preparatów z mniszka pospolitego względem parametrów hemostazy. W tym celu przeprowadzono pomiary agregacji i adhezji płytek krwi, czasów krzepnięcia, ekspresji selektyny P (CD62P) i receptora GPIIb/IIIa (wiązanie PAC-1) na powierzchni płytek krwi z użyciem metody cytometrii przepływowej oraz tworzenia skrzepliny w warunkach pół-fizjologicznych z wykorzystanie techniki microchipów. Dodatkowo, wykonano oznaczenie aktywności zewnątrzkomórkowej dehydrogenazy mleczanowej w celu sprawdzenia cytotoksyczności badanych preparatów względem płytek krwi. Co więcej, analizie właściwości biologicznych poddano kwas cykoriowy, który został wyizolowany z 50% frakcji liści mniszka pospolitego.

Materiały i metody badawcze

Materiałem do badań była krew pełna (pobrana na roztwór CPD (cytrynian, fosforan, dekstroza; 9:1, v/v; krew/CPD) z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi oraz ze Szpitala im. Dr Rydygiera w Łodzi (pobrana na roztwór CPDA-1 (cytrynian,

fosforan, dekstroza, adenina; 8,5:1; v/v; krew/CPDA-1)). Krew pobierano od zdrowych ochotników, którzy nie przyjmowali żadnych leków ani substancji uzależniających (w tym tytoniu, alkoholu, suplementacji antyoksydacyjnej oraz aspiryny czy innych leków przeciwpłytkowych). Z krwi izolowano osocze ubogopłytkowe, osocze bogatopłytkowe oraz płytki krwi metodą wirowania różnicowego (Kłyszejko-Stefanowicz, 2013). Przeprowadzone badania zostały wykonane za zgodą Komisji Bioetycznej Uniwersytetu Łódzkiego na podstawie protokołu nr 2/KBBN-UŁ/II/2016.

Materiał roślinny tj. korzenie, liście, płatki i owoce mniszka pospolitego został zebrany w okolicach Rzeszowa. Analiza składu chemicznego została wykonana w Zakładzie Biochemii i Jakości Plonów, w Instytucie Uprawy Nawożenia i Gleboznawstwa w Puławach-Państwowym Instytucie Badawczym. Otrzymano preparaty z ww. organów mniszka pospolitego różne pod względem składu chemicznego. Dodatkowo, z frakcji 50% liści mniszka pospolitego wyizolowano kwas cykoriowy (2,3–O–dikawoilo–L–winowy). W badaniach stosowano preparaty roślinne o stężeniach końcowych: 0,5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml i 50 µg/ml (co odpowiada stężeniom uzyskiwanym podczas suplementacji związkami fenolowymi w osoczu). Inkubacja osocza/krwi pełnej/płytek krwi z badanymi preparatami roślinnymi/kwasem cykoriowym wynosiła 30 min. w temperaturze 37^{o}C.

W pracy wykonano doświadczenia określające wpływ preparatów z liści, płatków, korzeni i owoców mniszka pospolitego *(T. officinale)* oraz kwasu cykoriowego na **parametry stresu oksydacyjnego:**

- ✓ Oznaczenie poziomu peroksydacji lipidów osocza i płytek krwi metodą z kwasem tiobarbiturowym (TBA),
- ✓ Oznaczenie poziomu grup karbonylowych w białkach osocza i płytek krwi metodą kolorymetryczną z 2,4-dinitrofenylohydrazyną (DNPH),
- ✓ Oznaczenie poziomu grup tiolowych w białkach osocza i płytek krwi metodą kolorymetryczną z kwasem 5,5'-ditiobis(2-nitrobenzoesowym) (DTNB, odczynnik Ellmana),
- ✓ Oznaczenie zawartości anionorodnika ponadtlenkowego w płytkach krwi metodą redukcji cytochromu c.

Ponadto, określono wpływ preparatów z liści, płatków, korzeni i owoców mniszka pospolitego *(T. officinale)* oraz kwasu cykoriowego na **parametry hemostazy:**

- ✓ Pomiar adhezji płytek krwi do kolagenu i fibrynogenu metodą statyczną z oznaczeniem aktywności kwaśnej fosfatazy,
- ✓ Pomiar agregacji płytek krwi metodą turbidymetryczną,
- ✓ Oznaczenie ekspresji selektyny P i receptora GPIIb/IIIa na powierzchni płytek krwi metodą cytometrii przepływowej,
- ✓ Oznaczenie czasów krzepnięcia tj. czasu trombinowego (*ang.* thrombin time, TT), czasu protrombinowego (*ang.* prothrombin time, PT) i czasu częściowej tromboplastyny po aktywacji (*ang.* activated partial tromboplastin time, APTT) metodą koagulometryczną,
- ✓ Oznaczenie i analiza powstających skrzeplin w warunkach przepływu krwi z wykorzystanie techniki microchipów (*ang.* total thrombus formation analysis system, T-TAS).

Dodatkowo, określono wpływ preparatów z liści, płatków, korzeni i owoców mniszka pospolitego *(T. officinale)* oraz kwasu cykoriowego na **parametry cytotoksyczności:**

- ✓ Oznaczenie aktywności zewnątrzkomórkowej dehydrogenazy mleczanowej (*ang.* lactate dehydrogenase, LDH) metodą Wróblewskiego i La Due.

Omówienie prac wchodzących w skład rozprawy doktorskiej

Analiza fitochemiczna badanych preparatów z mniszka pospolitego

Wstępne badania nad składem chemicznym badanych organów z mniszka pospolitego przeprowadzone we współpracy z IUNG-PIB w Puławach wykazały, iż są one zasobne w różnorodne substancje, w tym związki polifenolowe. Z korzeni mniszka pospolitego wyodrębniono pięć frakcji A – E. Potwierdzono w nich obecność 100 różnych metabolitów wtórnych, w tym pochodnych kwasów hydroksycynamonowych, laktonów seskwiterpenowych i hydroksyfenylooctowych pochodnych inozytolu **(praca numer [2]).**

W liściach i płatkach mniszka pospolitego zidentyfikowano 52 związki fenolowe wśród, których dominowały kwasy fenolowe i ich pochodne (25 związków) oraz flawonoidy (27 związków). Analiza chromatograficzna wykazała, że w szczególności 85% frakcja z płatków mniszka stanowiła bogate źródło flawonoidów, gdzie głównym komponentem była luteolina. Natomiast, w tej frakcji nie stwierdzono kwasów fenolowych, które były obecne w pozostałych preparatach. Ponadto, w 50% frakcjach z liści i płatków oraz w 85% frakcji

z liści mniszka dominującym związkiem był kwas cykoriowy (Jędrejek i in., 2017). Dlatego też, związek ten postanowiono wyizolować z 50% frakcji z liści mniszka i porównać jego aktywność biologiczną względem zarówno frakcji z liści, jak i płatków **(praca numer [4])**.

Ostatnim elementem charakterystyki fitochemicznej organów *T. officinale* była analiza składu owoców. Otrzymano metanolowy ekstrakt z owoców mniszka (E1), który był bogaty w związki polifenolowe. Następnie E1 rozdzielono na ekstrakt kwasów hydroksy-cynamonowych (E2) i ekstrakt flawonoidów (E3). W kolejnym kroku, z ekstraktu flawonoidowego (E3) wyodrębniono cztery frakcje, w których dominowały poszczególne związki tj. A – frakcja luteoliny, B – frakcja filonotisflawonu, C – frakcja flawonolignanów i D – frakcja aglikonów flawonowych **(praca numer [5]).**

Ocena właściwości antyoksydacyjnych badanych preparatów z mniszka pospolitego w osoczu i płytkach krwi

Analizę efektywności działania antyoksydacyjnego badanych preparatów z mniszka pospolitego przeprowadzono na podstawie oznaczeń różnych markerów stresu oksydacyjnego. Zbadano między innymi peroksydację lipidów określoną jako poziom substancji reagujących z kwasem tiobarbiturowym (TBARS), utlenienie białek w oparciu o oznaczenia poziomu grup tiolowych i grup karbonylowych oraz poziom generowania anionorodnika ponadtlenkowego w płytkach krwi. W prowadzonych przeze mnie doświadczeniach w warunkach *in vitro* modelem badawczym było osocze, jak i płytki krwi, gdzie warunki stresu oksydacyjnego pełnił dodatek mieszaniny H_2O_2/Fe^{2+} generującej rodnik hydroksylowy. Jest to jedna z najbardziej reaktywnych cząsteczek, która posiada niesparowany elektron na zewnętrznej orbicie. Dzięki tej właściwości może tworzyć wiązania chemiczne z biomolekułami i prowadzić do ich nieodwracalnych uszkodzeń. Źródłem nadtlenków lipidowych mogą być błony komórkowe w skład, których wchodzą wielonienasycone kwasy tłuszczowe. Stymulacja specyficznych receptorów na powierzchni komórki aktywuje fosfolipazę A_2, co w efekcie prowadzi do uwolnienia kwasu arachidonowego z błony komórkowej. Dalsze etapy metabolizmu kwasu arachidonowego prowadzą do produkcji m.in. tromboksanu A_2 (Trostchansky i in., 2019).

Analiza aktywności badanych preparatów z korzeni mniszka pospolitego (frakcje A – E) wykazała, że wpływają one na obniżenie poziomu peroksydacji lipidów w osoczu w obecności H_2O_2/Fe^{2+}. Działanie antyoksydacyjne przy zastosowaniu niższego stężenia tj. 1 μg/ml ($p<0,01$) wykazała zarówno frakcja C (bogata w hydroksyfenylooctowe pochodne

inozytolu oraz kwasy chlorogenowe), jak i frakcja E (zasobna w hydroksycynamonowe pochodne kwasu winowego). Podobny efekt odnotowano w przypadku wszystkich frakcji (A – E) w wyższym stężeniu tj. 5 μg/ml. Natomiast, najsilniejszy efekt wszystkich testowanych frakcji odnotowano w stężeniu 50 μg/ml ($p<0,001$). Niemniej jednak, najlepsze wyniki uzyskano dla frakcji C oraz dla frakcji A i E (redukcja o ponad 30% w porównaniu z kontrolą pozytywną, którą stanowiło osocze traktowane H_2O_2/Fe^{2+}) **(praca numer [2]).**

Co więcej, wolne rodniki mogą wpływać destrukcyjnie na białka poprzez uszkodzenia oksydacyjne grup tiolowych czy powstawanie grup karbonylowych. W efekcie dochodzi do zmian w strukturze i funkcjonowaniu tych molekuł. Zaobserwowano, że wszystkie badane frakcje z korzeni mniszka miały ochronny wpływ na grupy tiolowe w białkach osocza w warunkach stresu oksydacyjnego. Jednak, najlepszą aktywność w stężeniach 0,5 – 50 μg/ml wykazała frakcja A (bogata w aminokwasowe pochodne laktonów seskwiterpenowych). Natomiast, frakcja B (zawierająca laktony seskwiterpenowe i hydroksyfenylooctowe pochodne inozytolu) wykazała efekt ochronny w stężeniach 10 i 50 μg/ml, a frakcja D (zawierającą kwas chlorogenowy) w dawkach 0,5, 1, 10 i 50 μg/ml. Dodatkowo, frakcja C zadziałała najlepiej w niższych dawkach tj. 0,5 – 5 μg/ml, a frakcja E wykazywała znaczącą aktywność zarówno w najniższym stężeniu (0,5 μg/ml), jak i najwyższych dawkach (10 i 50 μg/ml). Ponadto, wszystkie badane frakcje z korzeni mniszka hamowały wzrost karbonylacji białek osocza indukowanej przez H_2O_2/Fe^{2+}, jednak proces ten nie zawsze był istotny statystycznie **(praca numer [2]).**

Stres oksydacyjny również wpływa destrukcyjnie na płytki krwi wpływając na wzmożoną aktywację enzymów, wzrost stężenia jonów wapniowych, uaktywnienie metabolizmu kwasu arachidonowego czy wytwarzanie izoprostanów. Wyniki przeprowadzonych przeze mnie badań wskazują, że użyte preparaty z korzeni mniszka istotnie hamowały peroksydację lipidów płytek krwi w warunkach stresu oksydacyjnego. Odnotowano, że w stężeniu 50 μg/ml inhibicja ta wynosiła około 70% w porównaniu z kontrolą traktowaną H_2O_2/Fe^{2+} dla wszystkich pięciu frakcji (A – E). Ponadto, ochronny wpływ na grupy tiolowe białek w płytkach krwi poddanych działaniu H_2O_2/Fe^{2+} wykazały dwie badane frakcje: A i E, w najniższym testowanym stężeniu tj. 10 μg/ml. Co więcej, frakcja C hamowała karbonylację białek w płytkach krwi poddanych działaniu H_2O_2/Fe^{2+} w dwóch badanych stężeniach (10 i 50 μg/ml), podczas gdy frakcja A była aktywna tylko w najwyższej dawce (50 μg/ml), a frakcja D w niższym stężeniu (10 μg/ml). Natomiast, w przypadku pozostałych dwóch frakcji (B i E) nie zaobserwowano istotnych zmian **(praca numer [3])**.

W płytkach krwi, podobnie jak i w innych komórkach mogą być wytwarzane reaktywne formy tlenu. W wyniku stymulowania płytek krwi przez trombinę dochodzi do generowania m.in. anionorodnika ponadtlenkowego ($O_2^{\bullet-}$). Proces ten wiąże się m.in. z metabolizmem kwasu arachidonowego, który może zachodzić w obecności cyklooksygenazy lub 12-lipoksygenazy. Ponadto, powstawanie $O_2^{\bullet-}$ w płytkach krwi może odbywać się poprzez aktywację oksydazy NADPH (Olas i Wachowicz, 2003; Masselli i in., 2020). Dlatego też w dalszej kolejności postanowiono zmierzyć poziom $O_2^{\bullet-}$ zarówno w spoczynkowych płytkach krwi, jak i aktywowanych trombiną w obecności badanych frakcji z korzeni mniszka. Wyniki badań wskazują, że tylko frakcja A w stężeniu 10 μg/ml miała istotny wpływ na zmniejszenie poziomu $O_2^{\bullet-}$ w spoczynkowych płytkach krwi ($p<0,05$). Natomiast, w płytkach krwi stymulowanych trombiną zaobserwowano, że wszystkie frakcje w obu badanych stężeniach tj. 10 i 50 μg/ml zwiększają produkcję anionorodnika ponadtlenkowego. Zaobserwowany efekt może być związany z silną stymulacją trombiny, która łącząc się z receptorem PAR (*ang.* protease activated receptor) prowadzi do translokacji podjednostki NADPH ($p67^{phox}$) przy jednoczesnym uwolnieniu anionorodnika ponadtlenkowego. Badane frakcje z korzeni mniszka pospolitego wykazują różnice w składzie chemicznym, co może też tłumaczyć ich różną aktywność biologiczną, w tym przeciwutleniającą. Podsumowując, frakcje A i C wykazywały najlepsze działanie antyoksydacyjne w osoczu i płytkach krwi poddanych działaniu H_2O_2/Fe^{2+} w porównaniu do pozostałych badanych frakcji (B, D i E) **(praca numer [3])**.

W kolejnym etapie zbadano wpływ preparatów z liści i płatków mniszka pospolitego na stres oksydacyjny w płytkach krwi. Najsilniejszy efekt hamujący peroksydację lipidów w płytkach krwi poddanych działaniu H_2O_2/Fe^{2+} wykazała 50% frakcja z liści i 50% frakcja z płatków (obie w dawce 50 μg/ml), w porównaniu z innymi frakcjami. Preparaty te spowodowały spadek peroksydacji lipidów o około 40% w porównaniu do kontroli. Dodatkowe badania wykazały, że po upływie 30 minut inkubacji płytek krwi z 50% frakcją z liści (w stężeniach 1 i 50 μg/ml), ilość grup tiolowych w białkach płytek krwi wyraźnie wzrosła w porównaniu do kontroli traktowanej H_2O_2/Fe^{2+}. Natomiast, zastosowanie preparatów z liści i płatków mniszka nie wpłynęło istotnie ($p>0,05$) na zmiany w poziomie karbonylacji białek w płytkach krwi. Co więcej, odnotowano również istotne hamowanie tworzenia anionorodnika ponadtlenkowego dla czterech testowanych frakcji roślinnych w badanym stężeniu 10 μg/ml w nieaktywowanych płytkach krwi, jak i aktywowanych trombiną ($p<0,05$). Frakcje bogate w pochodne kwasów hydroksycynamonowych (tj. 50% frakcja z liści i 50% frakcja z płatków) okazały się być bardziej efektywne pod względem aktywności

przeciwutleniającej, niż frakcje bogate we flawonoidy. Ponadto, w 50% frakcjach z liści i płatków odnotowano największą zawartość kwasu cykoriowego względem całkowitej zawartości związków polifenolowych obecnych w preparatach. Na podstawie otrzymanych wyników stwierdzono, że to obecność a co za tym idzie aktywność kwasu cykoriowego może odgrywać główną rolę jako czynnik antyoksydacyjny **(praca numer [1])**.

Kolejny aspekt oceny aktywności antyoksydacyjnej dotyczył działania kwasu cykoriowego, który stanowił główny komponent badanych ekstraktów z owoców oraz frakcji z liści i płatków mniszka. Kwas cykoriowy jest naturalnie występującym kwasem fenolowym, który obecny jest również w sałacie, trawie morskiej, skrzypie, paproci czy bazylii (Lee i Scagel, 2013). Na podstawie przeprowadzonych badań, zaobserwowano istotne działania antyoksydacyjne kwasu cykoriowego. Kwas cykoriowy w dawce 50 μg/ml, wpłynął na zmniejszenie karbonylacji indukowanej przez H_2O_2/Fe^{2+} w białkach osocza o około 25% w porównaniu z kontrolą ($p<0,01$). Ponadto, potwierdzono ochronny efekt względem grup tiolowych w białkach osocza poddanych działaniu H_2O_2/Fe^{2+}. Proces utleniania był znacząco hamowany w zakresie stężeń od 1 do 50 μg/ml. Co więcej, kwas cykoriowy w dawce 50 μg/ml wpłynął na obniżenie peroksydacji lipidów o około 70% w płytkach krwi traktowanych H_2O_2/Fe^{2+} ($p<0,001$). Dodatkowo, związek ten w stężeniu 10 μg/ml chronił grupy tiolowe w płytkach krwi poddanych działaniu H_2O_2/Fe^{2+} ($p<0,05$). Natomiast, nie wykazano istotnego wpływu na poziom grup karbonylowych **(praca numer [4]).**

Ostatni etap oceny aktywności antyoksydacyjnej dotyczył działania preparatów pozyskanych z owoców mniszka. W porównaniu do korzeni, liści i płatków mniszka są one najmniej poznanym organem. Dlatego też, w ramach niniejszej pracy doktorskiej poszerzono wiedzę na temat działania tych preparatów, które mogą również stanowić dobre źródło związków o wielorakiej aktywności biologicznej. W przypadku trzech testowanych ekstraktów (E1 – E3) oraz dwóch frakcji z owoców mniszka bogatych w luteolinę (frakcja A) oraz aglikony flawonowe (frakcja D) odnotowano hamowanie peroksydacji lipidów osocza w obecności H_2O_2/Fe^{2+} w najwyższym badanym stężeniu tj. 50 μg/ml. Natomiast, preparaty zawierające filonotisflawony (frakcja B) oraz flawonolignany (frakcja C) z owoców mniszka nie wykazały działania przeciwutleniającego. Ponadto, ekstrakty E2 i E3 oraz frakcje B, C i D redukowały utlenianie grup tiolowych białek w osoczu poddanym działaniu H_2O_2/Fe^{2+}. Co więcej, ekstrakt bogaty we flawonoidy (E3) redukował ten proces w stężeniach 10 i 50 μg/ml. Odnotowano również, że wszystkie badane preparaty z owoców mniszka w stężeniu 50 g/ml hamowały karbonylację białek w osoczu poddanym działaniu H_2O_2/Fe^{2+}.

Dodatkowo, dwa ekstrakty (E1 i E3) były również aktywne w niższym badanym stężeniu (10 g/ml). Stwierdzono, że najlepsze właściwości antyoksydacyjne wykazuje ekstrakt E2 oraz frakcja A – bogata w luteolinę. Ponadto, w przypadku ekstraktu bogatego w pochodne kwasów hydroksycynamonowych (E2), gdzie głównym komponentem jest kwas cykoriowy potwierdzono silny efekt antyoksydacyjny w osoczu, co jest zgodne z wcześniejszymi obserwacjami **(praca numer [5]).**

Ocena właściwości antypłytkowych badanych preparatów z mniszka pospolitego

W dalszej części badań skupiono się na zbadaniu wpływu różnych preparatów z mniszka pospolitego na hemostatyczną aktywność płytek krwi. W tym etapie badań prowadzonych w warunkach *in vitro* modelem badawczym były płytki krwi, osocze bogatopłytkowe, jak i krew pełna. Ocenę przeprowadzono na podstawie następujących etapów aktywacji płytek krwi tj. adhezji, agregacji i sekrecji związków obecnych w ich ziarnistościach pod wpływem różnych agonistów takich jak trombina, ADP i kolagen. Użycie w przeprowadzonych badaniach poszczególnych agonistów pomogło w uzyskaniu wstępnej informacji dotyczącej szlaków aktywacji płytek krwi, które mogły być modulowane przez testowane preparaty z mniszka. Uważa się, że najbardziej efektywnym aktywatorem płytek krwi jest trombina, która działa na receptory aktywowane przez proteazy tj. PAR w szczególności u ludzi są to PAR-1 i PAR-4. Z kolei, dla ADP są to receptory $P2Y_1$ i $P2Y_{12}$, a dla kolagenu - glikoproteina GP VI oraz kompleks GP Ia/IIa i kompleks GP Ib/IX/V (Sambrano i in., 2001; Rivera i in., 2009). Ponadto, aktywacja płytek krwi w obecności wybranych preparatów z owoców mniszka była badana na podstawie ekspresji selektyny P i receptora GPIIb/IIIa na powierzchni płytek krwi z zastosowaniem metody cytometrii przepływowej.

W pierwszej kolejności, ocenie właściwości przeciwpłytkowych poddano preparaty z korzeni mniszka (frakcje A – E, w stężeniach 10 i 50 μg/ml). Na podstawie przeprowadzonych doświadczeń stwierdzono, że adhezja spoczynkowych płytek krwi do kolagenu w obecności wszystkich preparatów z korzeni mniszka była istotnie hamowana. Z kolei, tylko frakcje fenolowe tj. frakcja C (w stężeniu 50 μg/ml), oraz frakcje D i E (w stężeniach 10 i 50 μg/ml) były najsilniejszymi inhibitorami adhezji płytek krwi do kolagenu, indukowanej trombiną. Natomiast, adhezja płytek krwi do fibrynogenu indukowana trombiną była istotnie hamowana przez frakcję D (w stężeniach 10 i 50 μg/ml) oraz przez pozostałe trzy frakcje (B, C i E) w najwyższym stężeniu tj. 50 μg/ml. Co więcej, najlepsze działanie hamujące adhezję płytek

krwi do fibrynogenu indukowanej ADP wykazywała frakcja E w obu badanych dawkach (10 i 50 μg/ml) **(praca numer [3])**.

Jednym z czynników ryzyka rozwoju chorób sercowo-naczyniowych są też zaburzenia związane z nadmierną agregacją płytek krwi. Przeprowadzone przeze mnie badania wskazują, że trzy frakcje z korzeni mniszka tj. frakcja A, B i C w stężeniu 50 μg/ml hamują agregację płytek krwi stymulowanych 10 μM ADP o około 20% w porównaniu z kontrolą. Natomiast, żaden z badanych preparatów nie wpływał istotnie na agregację płytek krwi stymulowaną 2 μg/ml kolagenem **(praca numer [3])**, ani 1 U/ml trombiny **(praca numer [2]).** Uważa się, że jednym z możliwych mechanizmów działania antyagregacyjnego testowanych preparatów z korzeni mniszka może być interakcja związków wchodzących w skład roślinnych preparatów z receptorami ADP na błonie płytek krwi **(praca numer [3])**.

Aktywacja płytek krwi wiąże się z metabolizmem kwasu arachidonowego, podczas którego powstaje tromboksan A_2. Wyniki badań wskazują, że testowane preparaty z korzeni mniszka (A – E; w stężeniach 10 i 50 μg/ml) nie mają istotnego wpływu na ten proces. Świadczy to o braku zdolności związków zawartych w badanych preparatach do modulowania aktywności płytek krwi poprzez ingerencję w metabolizm kwasu arachidonowego. Podsumowując, najlepsze działanie przeciwpłytkowe wykazała frakcja C, w której największą zawartość stanowią hydroksyfenylooctowe pochodne inozytolu i kwasy chlorogenowe. Przypuszcza się, że to alkohol cukrowy (inozytol) odpowiada za efekt przeciwpłytkowy, gdyż inne fenolowe składniki, tj. pochodne kwasów hydroksycynamonowych we frakcjach D i E nie wykazały tak wysokiej aktywności **(praca numer [3])**.

Kolejny aspekt badań dotyczył aktywności przeciwpłytkowej kwasu cykoriowego, którą porównano względem frakcji z liści i płatków mniszka. W przeprowadzonym badaniu dotyczącym adhezji spoczynkowych płytek krwi do kolagenu nie zaobserwowano istotnego wpływu kwasu cykoriowego w żadnym z użytych dawek (0,5, 10 i 50 μg/ml). Natomiast, w wyższych stężeniach (10 i 50 μg/ml) kwas cykoriowy hamował adhezję płytek krwi do kolagenu indukowaną trombiną o około 20%. Ponadto, w przeprowadzonych doświadczeniach również stwierdzono, że testowane frakcje z liści i płatków mniszka posiadają aktywność przeciwpłytkową. Obserwacje te dotyczyły zarówno adhezji spoczynkowych płytek krwi, jak i stymulowanych trombiną do kolagenu, jednak inhibicja ta nie zawsze była istotna statystycznie. Dodatkowo, kwas cykoriowy w najwyższym stężeniu 50 μg/ml hamował adhezję płytek krwi aktywowanych trombiną czy ADP do fibrynogenu (o 20 % ($p<0,05$) dla trombiny oraz 41% ($p<0,01$) dla ADP). Podobny efekt przeciwpłytkowy odnotowano w obecności frakcji

z liści i płatków mniszka. Co więcej, proces agregacji płytek krwi traktowanych 10 μM ADP był najsilniej hamowany w najwyższym badanym stężeniu (50 μg/ml) przez 50% frakcję z liści i płatków mniszka oraz 85% frakcję z liści. Natomiast, najlepsze działanie antyagregacyjne wykazała frakcja 50% z płatków mniszka, która powodowała 30% inhibicję tego procesu **(praca numer [4]).**

Dalsze badania pokazały, że zastosowanie kwasu cykoriowego w stężeniu 50 μg/ml także istotnie hamuje enzymatyczną peroksydację lipidów. Odnotowano spadek poziomu TBARS o 20% w porównaniu z kontrolą (płytek krwi stymulowanych trombiną). Porównując działanie badanych preparatów okazało się, że aktywność przeciwpłytkowa kwasu cykoriowego była słabsza w porównaniu do 50% frakcji z liści. Taka sytuacja może być spowodowana tym, że związki fenolowe wchodzące w skład frakcji mogą działać ze sobą na zasadzie synergizmu, czego rezultatem są lepsze właściwości biologiczne. Ponadto, 50% frakcja z liści zawiera m.in. pochodne kwasów fenolowych i flawonoidów (w tym luteolinę), które mogą mieć silniejsze działanie niż pojedynczy związek **(praca numer [4])**.

W kolejnym etapie badań zaobserwowano, że testowane preparaty z owoców mniszka (tj. E2 i E3 oraz frakcje A, B i C) znacząco wpływają na hamowanie adhezji płytek krwi do fibrynogenu aktywowanych ADP. Co więcej, frakcje A i B wykazywały wysoką aktywność w obu badanych stężeniach - 10 i 50 μg/ml, natomiast pozostałe preparaty (E2, E3 oraz frakcja C) hamowały adhezję tylko w stężeniu 50 μg/ml. Ponadto, trzy frakcje flawonoidowe (A – C) w obu badanych dawkach tj. 10 i 50 μg/ml wykazywały działanie hamujące w przypadku adhezji płytek krwi aktywowanych trombiną. Natomiast, frakcja D wykazywała aktywność antyadhezyjną tylko w stężeniu 10 μg/ml. Dodatkowo, zastosowanie preparatów bogatych we flawonoidy tj. ekstraktu E3 i frakcji A – D (w dawce 50 μg/ml) istotnie wpłynęło na obniżenie peroksydacji lipidów w płytkach krwi traktowanych trombiną. Na przykład, frakcja A bogata w luteolinę (10 i 50 μg/ml) hamowała ten proces o około 60% w porównaniu do kontroli (płytek krwi stymulowanych trombiną). Na podstawie uzyskanych wyników stwierdzono, że to flawonoidy obecne w owocach mniszka mają wpływ na aktywację płytek krwi poprzez modulowanie metabolizmu kwasu arachidonowego **(praca numer [5])**.

Następnie, do dalszych badań wybrano trzy preparaty z owoców mniszka o najwyższym potencjale antyoksydacyjnym i przeciwpłytkowym. Były to ekstrakty E2 i E3 oraz frakcja A bogata w luteolinę. Wpływ wybranych preparatów z owoców mniszka (w stężeniu 10 i 50 μg/ml) na płytki krwi oceniano za pomocą cytometrii przepływowej, analizując aktywację kompleksu GPIIb/IIIa (wiązanie PAC-1) i ekspresję selektyny P (CD62P)

w próbkach krwi pełnej zawierających spoczynkowe płytki krwi, jak i płytki krwi stymulowane przez ADP lub kolagen. Technika cytometrii przepływowej może być wykorzystywana zarówno w diagnostyce, jak i w badaniach podstawowych, a w tym przypadku umożliwiła pomiar aktywności biologicznej płytek krwi w środowisku naturalnym, tj. bezpośrednio po pobraniu krwi. Uzyskane wyniki badań pokazały, że ekspresja GPIIb/IIIa na płytkach krwi aktywowanych 20 μM ADP zmniejsza się o 49% tylko w obecności ekstraktu E2 w stężeniu 50 μg/ml. Ponadto, odnotowano hamowanie wiązania PAC-1 w płytkach krwi aktywowanych 10 μg/ml kolagenem w obecności ekstraktu E2 w stężeniu 50 μg/ml (spadek o 35%) i frakcji A w stężeniu 50 μg/ml (spadek o 40%). Na podstawie uzyskanych wyników stwierdzono, że hamowanie adhezji płytek krwi do fibrynogenu przez testowane preparaty może mieć związek z niską ekspresją receptora GPIIb/IIIa. Innym badanym markerem aktywacji płytek krwi była P-selektyna, której ekspresja w płytkach krwi aktywowanych 10 μM ADP była hamowana pod wpływem frakcji A w dawce 50 μg/ml. Co więcej, frakcja A również wpłynęła na inhibicję ekspresji CD62P o 53% w płytkach krwi aktywowanych 10 μg/ml kolagenem. Podobne działanie zaobserwowano w obecności ekstraktu E2, który spowodował spadek ekspresji selektyny P o 48% ($p<0,05$) **(praca numer [5]).**

Ocena wpływu testowanych preparatów z mniszka pospolitego na właściwości hemostatyczne osocza krwi- analiza właściwości antykoagulacyjnych

Kardioprotekcyjne działanie substancji pochodzenia roślinnego może dotyczyć nie tylko hamowania aktywacji płytek krwi, ale również hamowania aktywacji białek kaskady krzepnięcia krwi. Dlatego też, w końcowym etapie badań określono wpływ preparatów z mniszka w warunkach *in vitro* na aktywność hemostatyczną osocza, w odniesieniu do trzech wybranych parametrów hemostatycznych, tj. czasu częściowej tromboplastyny po aktywacji, czasu protrombinowego i czasu trombinowego.

Analiza wpływu na aktywność koagulacyjną ludzkiego osocza wykazała, że osiem badanych preparatów, tj. 85% frakcja z liści, 50% i 85% frakcje z płatków oraz wszystkie frakcje z korzeni mniszka (A – E) istotnie wydłużały czas trombinowy w zakresie stężeń 0,5-50 μg/ml. Dodatkowo zaobserwowano, że wszystkie badane frakcje z liści i płatków (w najwyższym stężeniu tj. 50 μg/ml), jak i dwie frakcje z korzeni mniszka bogate w pochodne kwasów hydroksycynamonowych tj. frakcja D (w stężeniu 10 i 50 μg/ml) i frakcja E (w stężeniu 50 μg/ml) istotnie wydłużały czas TT osocza, gdy były preinkubowane z trombiną. Przypuszcza się, że właściwości antykoagulacyjne badanych preparatów, a także mechanizm

odpowiedzialny za ten proces, są związane z modulacją aktywności trombiny i najprawdopodobniej ma to związek z hamowaniem jej aktywności, co w rezultacie przekłada się na spowolnienie powstawania skrzepu w osoczu. Natomiast, zastosowanie ww. preparatów nie miało istotnego wpływu na zmiany zarówno czasu PT, jak i APTT **(praca numer [1] i [2])**.

Natomiast, w przypadku badanych ekstraktów i frakcji z owoców (E1 – E3; frakcje A - D) oraz dla kwasu cykoriowego nie odnotowano działania antykoagulacyjnego w żadnym z badanych parametrów **(praca numer [4] i [5])**.

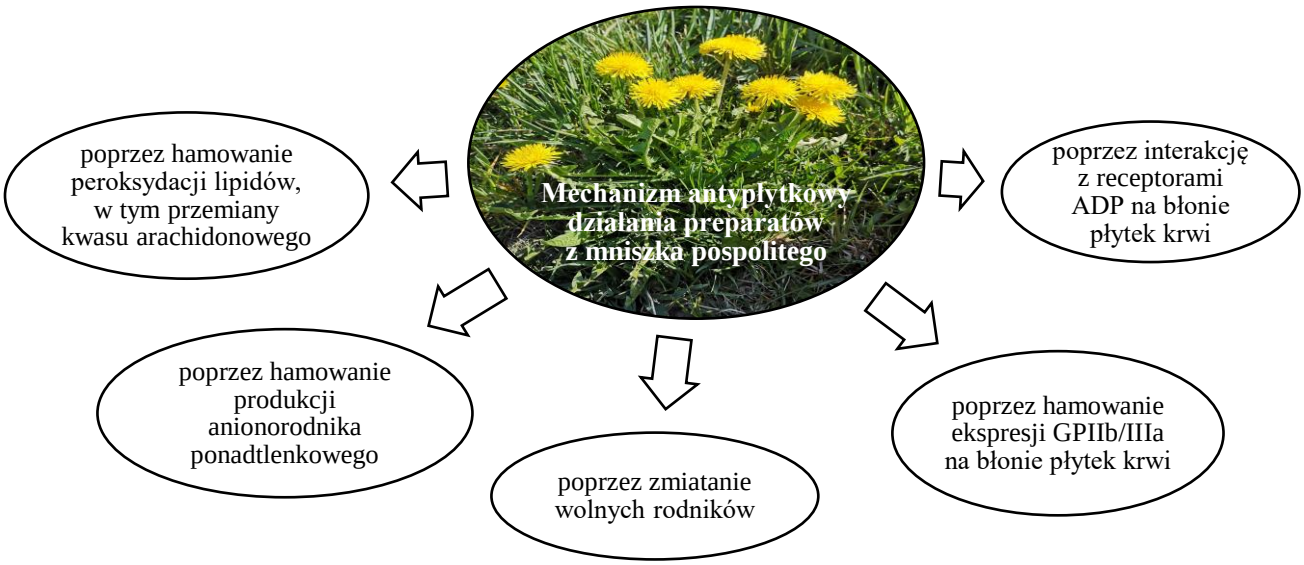
Dodatkowym elementem było sprawdzenie wpływu wybranych preparatów z owoców mniszka (E2, E3 i frakcja A) na układ hemostazy pierwotnej za pomocą nowatorskiej metody z wykorzystaniem microchipu. Metoda ta była zastosowana w warunkach półfizjologicznych w momencie przepływu krwi przez sztuczne naczynia krwionośne z użyciem microchipów pokrytych kolagenem. Stwierdzono, że wszystkie preparaty z owoców mniszka pospolitego (E2, E3 i frakcja A; w stężeniu 50 μg/ml) wykazują potencjał przeciwzakrzepowy. Prawdopodobnie aktywność ta jest uwarunkowana obecnością pochodnych kwasu kawowego (głównie kwasu cykoriowego), pochodnych flawonowych, jak i luteoliny. Jednak aktywność ta nie była istotna statystycznie względem kontroli ($p>0,05$) **(praca numer [5])**.

Ocena cytotoksyczności badanych preparatów z mniszka pospolitego

Jednym z etapów niniejszej pracy doktorskiej było zbadanie wpływu preparatów z mniszka pospolitego, jak i kwasu cykoriowego na zewnątrzkomórkową aktywność dehydrogenazy mleczanowej (LDH) – marker uszkodzenia komórek. Na podstawie przeprowadzonych badań, stwierdzono, że żaden z testowanych preparatów mniszka pospolitego, jak i kwas cykoriowy (w zakresie stężeń 0,5 – 50 μg/ml) nie powodowały lizy płytek krwi, określanej jako wyciek dehydrogenazy mleczanowej do środowiska pozakomórkowego **(praca numer [1], [2], [4] i [5]).**

Wnioski

1. Wszystkie preparaty z mniszka użyte do badań są bezpieczne i nie wykazują cytotoksyczności względem płytek krwi.
2. Wykazują one aktywność antyoksydacyjną, antypłytkową oraz przeciwzakrzepową. Właściwości te zależą od składu chemicznego badanych preparatów z mniszka pospolitego. Na przykład, preparaty z liści, płatków oraz owoców mniszka, które są bogate w pochodne kwasów hydroksycynamonowych mają lepszą aktywność przeciwutleniającą w porównaniu do preparatów zasobnych we flawonoidy. Najlepsze działanie kardioprotekcyjne, tj. aktywność przeciwutleniającą, przeciwpłytkową i antykoagulacyjną wykazały liście mniszka, w których zidentyfikowano największą zawartość kwasu cykoriowego.
3. Mechanizmy działania preparatów z mniszka pospolitego na płytki krwi są wielokierunkowe, na przykład preparaty z korzeni, liści i płatków wykazują efekt antyagregacyjny w skutek interakcji związków zawartych w preparatach z receptorami ADP na błonie płytek krwi. Działanie przeciwpłytkowe badanych preparatów z liści, płatków i owoców mniszka pospolitego zostało też skorelowane z hamowaniem kaskady przemian kwasu arachidonowego w płytkach krwi (Ryc. 1).



Ryc. 1 Wielokierunkowe działanie antypłytkowe preparatów z mniszka pospolitego.

Streszczenie

Według Światowej Organizacji Zdrowia to choroby sercowo-naczyniowe są główną przyczyną zgonów na którą umiera średnio 18 mln ludzi. Uważa się, że to stres oksydacyjny stoi u podłoża wielu chorób cywilizacyjnych. Jednym z istotnych zaleceń ekspertów Europejskiego Towarzystwa Kardiologicznego jest profilaktyka żywieniowa oparta przede wszystkim na urozmaiconej diecie, jak i suplementacji preparatami roślinnymi, które bogate są w szeroką gamę naturalnych przeciwutleniaczy czy związków o aktywności antypłytkowej, takich jak substancje polifenolowe.

Przykładem rośliny o wielokierunkowym działaniu zdrowotnym jest mniszek pospolity *(T. officinale)*. To szeroko rozpowszechniona na całym świecie bylina należąca do rodziny astrowatych *(Asteraceae)*, która jest bogatym źródłem zarówno kwasów fenolowych, flawonoidów, terpenów, jak i witamin czy składników mineralnych. Oprócz walorów smakowych mniszek pospolity od wielu wieków był wykorzystywany jako remedium na wiele różnych dolegliwości. Jednak, mimo wielu doniesień i prowadzonych badań nad aktywnością mniszka pospolitego, nadal występują luki w wiedzy na temat wpływu preparatów z tej rośliny na układ hemostazy. Dlatego też, głównym celem niniejszej pracy doktorskiej było zbadanie aktywności preparatów (ekstraktów i frakcji) wyizolowanych z korzeni, liści, płatków i owoców mniszka pospolitego *(T. officinale)* oraz kwasu cykoriowego pod kątem wybranych parametrów stresu oksydacyjnego i hemostazy w warunkach *in vitro*.

Analiza składu chemicznego poszczególnych preparatów była prowadzona we współpracy z Instytutem Uprawy Nawożenia i Gleboznawstwa w Puławach. Otrzymane ekstrakty, jak i frakcje były zasobne w pochodne kwasów hydroksycynamonowych, jak i flawonoidów. W przeprowadzonych doświadczeniach użyto preparaty w zakresie stężeń 0,5 – 50 μg/ml, co odpowiada stężeniom uzyskiwanym podczas suplementacji związkami fenolowymi w osoczu. Wpływ preparatów z mniszka na stres oksydacyjny zbadano oznaczając poziom anionorodnika ponadtlenkowego w płytkach krwi, peroksydacji lipidów, grup tiolowych i karbonylowych w białkach osocza i płytkach krwi. Induktorem stresu oksydacyjnego była mieszanina H_2O_2/Fe^{2+} stanowiąca donor rodnika hydroksylowego. Natomiast kluczowym aspektem badań była ocena skuteczności preparatów z mniszka pospolitego względem parametrów hemostazy. W tym, celu przeprowadzono pomiary agregacji i adhezji płytek krwi, czasów krzepnięcia, ekspresji selektyny P i receptora GPIIb/IIIa na powierzchni płytek krwi z użyciem metody cytometrii przepływowej oraz tworzenia

skrzepliny w warunkach pół-fizjologicznych z wykorzystanie techniki microchipów. Dodatkowo, wykonano oznaczenie aktywności zewnątrzkomórkowej dehydrogenazy mleczanowej w celu sprawdzenia cytotoksyczności badanych preparatów względem płytek krwi.

Uzyskane wyniki potwierdzają, iż wszystkie preparaty z mniszka pospolitego użyte do badań są bezpieczne i nie powodują cytotoksyczności względem płytek krwi. Ponadto, posiadają one aktywność antyoksydacyjną, ponieważ wpływają istotnie na hamowanie peroksydacji lipidów i karbonylacji białek, a także chronią białka osocza i płytek krwi w warunkach stresu oksydacyjnego. Właściwości te skorelowano z obecnością aktywnych składników chemicznych w badanych frakcjach i ekstraktach z mniszka. Oprócz działania przeciwutleniającego, testowane preparaty wykazały inne właściwości kardioprotekcyjne, tj. aktywność przeciwpłytkową i antykoagulacyjną. Ich wpływ na układ hemostazy był oparty na różnych mechanizmach m.in. na hamowaniu kaskady przemian kwasu arachidonowego czy interakcji z receptorami ADP na błonie komórkowej płytek krwi. Wśród wszystkich testowanych preparatów z mniszka pospolitego, najlepsze działanie antyoksydacyjne, jak i przeciwzakrzepowe zaobserwowano w przypadku frakcji z liści mniszka, w których też zidentyfikowano największą zawartość kwasu cykoriowego.

Podsumowując, preparaty z mniszka pospolitego mogą stanowić wartościowe źródło naturalnych substancji, których działanie może zostać wykorzystane w terapii chorób u podłoża, których stoi stres oksydacyjny. Co więcej, naturalne substancje czynne biologicznie mogą stanowić suplement diety wspomagający leczenie dolegliwości ze strony układu sercowo- naczyniowego, które związane są m.in. z nadreaktywnością płytek krwi.

Summary

According to the World Health Organization, cardiovascular diseases are the leading cause of death for an average of 18 million people. Oxidative stress is believed to be at the core of many civilization diseases. One of the important recommendations of the European Society of Cardiology experts is the nutritional prevention based primarily on a varied diet, as well as supplementation with plant preparations that are rich in a wide range of natural antioxidants or compounds with antiplatelet activity, such as polyphenolic substances.

An example of a plant with multidirectional health benefits is the dandelion (*T. officinale*). This perennial plant, which belongs to the *Asteraceae* family, is widespread throughout the world and is a rich source of phenolic acids, flavonoids, terpenes, as well as vitamins and minerals. In addition to its taste, the dandelion has been used for many centuries as a remedy for many different ailments. However, despite many reports and ongoing studies on dandelion activity, there are still gaps in the knowledge of the plant's effects on the hemostatic system. Therefore, the main objective of this dissertation was to investigate the activity of preparations (extracts and fractions) isolated from roots, leaves, petals and fruits of dandelion *(T. officinale)* and chicoric acid for selected oxidative stress and hemostasis parameters *in vitro*.

Analysis of chemical composition of different preparations was performed in cooperation with the Institute of Soil Science and Plant Cultivation in Puławy. The obtained extracts and fractions were rich in derivatives of hydroxycinnamic acids and flavonoids. In the experiments, the preparations were used in the concentration range of 0.5 - 50 μg/ml, which corresponds to the concentrations obtained during supplementation with phenolic compounds in plasma. The effect of dandelion preparations on oxidative stress was studied by determining the levels of superoxide anion radical in platelets, lipid peroxidation, thiol and carbonyl groups in plasma proteins and platelets. The inducer of oxidative stress was the H_2O_2/Fe^{2+} mixture as a hydroxyl radical donor. On the other hand, the key aspect of the study was to evaluate the efficacy of dandelion preparations against haemostasis parameters. Therefore, the platelet aggregation and adhesion, clotting time, P-selectin and GPIIb/IIIa receptor expression on the platelet surface were measured using flow cytometry and thrombus formation under semi-physiological conditions with the use of microchip technique. In addition, determination of extracellular lactate dehydrogenase activity was performed to check the cytotoxicity of the tested preparations against platelets.

The obtained results confirm that all dandelion preparations used in this study are safe and do not cause cytotoxicity against platelets. In addition, they have antioxidant activity because they have significant effect on inhibition of lipid peroxidation and protein carbonylation; and they protect plasma and platelet proteins under oxidative stress conditions. These properties were correlated with the presence of active chemical constituents in the tested dandelion fractions and extracts. Besides of antioxidant activity, the tested preparations showed other cardioprotective properties, i.e. antiplatelet and anticoagulant properties. Their influence on haemostasis was based on different mechanisms including inhibition of arachidonic acid metabolism cascade or interaction with ADP receptors on platelet cell membrane. Among all the dandelion preparations tested, the best antioxidant as well as anticoagulant activity was observed in the case of fractions from dandelion leaves, in which also the highest content of chicoric acid was identified.

To sum up, dandelion preparations may be a valuable source of natural substances whose effects may be used in the therapy of diseases underlying oxidative stress. Moreover, natural biologically active substances may be a dietary supplement supporting the treatment of cardiovascular ailments, which are related to, among others, platelet hyperreactivity.

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Assessment of effects of phenolic fractions from leaves and petals of dandelion in selected components of hemostasis

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ABSTRACT

Aerial parts and roots of *Taraxacum officinale* (dandelion) have been found to be rich sources of polyphenols, including cinnamic acid derivatives, flavonoids and triterpenoids, which exert different biological activities, such as anti-inflammatory, anticancer and antimicrobial. Additionally, the whole plant is recognized as safe and well tolerated by humans, with no reported adverse effects. Nowadays, dandelion is a commonly available dietary supplement and a component of pharmaceutical preparations used for the treatment of bladder, liver, and spleen. Nevertheless, the effect of dandelion on blood platelets and plasma – components of hemostasis involved in the functioning of a cardiovascular system and linked with various cardiovascular diseases, has not been studied yet. Thus, the main objective of our *in vitro* experiments was to examine the anti-platelet and antioxidant properties of four standardized dandelion phenolic fractions, *i.e.* leaves 50% and 85% methanol fractions, and petals 50% and 85% methanol fractions, in blood platelets. Additionally, aforementioned plant preparations were investigated for hemostatic activity in plasma, using three selected hemostatic parameters: the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT). None of the studied dandelion fractions, caused the damage of human blood platelets, at the whole tested range. The inhibition of lipid peroxidation in platelets treated with H_2O_2/Fe (the donor of OH·) was observed for two fractions: leaves and petals 50% fractions, both at the dose 50 μg/mL. Analysis of the effect on the coagulation activity of human plasma demonstrated that three fractions: petals 50% fraction, and leaves and petals 85% fractions, significantly prolonged the thrombin time, at the whole tested range. On the contrary, none of the fractions changed the APTT and the PT. The obtained results demonstrate that dandelion preparations, based on aerial parts, especially rich in hydroxycinnamic acid derivatives (leaves and petals 50% fractions) are promising plant materials exerting both antioxidant and anticoagulant activities of the hemostatic system that is beneficial in the prevention and treatment of cardiovascular diseases.

1. Introduction

A number of completed and ongoing studies, based on both *in vitro* and *in vivo* experiments, provide evidence that plant extracts and plant based food products, such as jams and juices; and phytopharmacological preparations, rich in phenolic compounds can reduce risk of cardiovascular disease (Chong, Macdonald, & Lovegrove, 2010; McEwen, 2014). In the last two decades, several investigations aimed at cognition of chemical composition and bioactivity of dandelion (*Taraxacum officinale*) have been conducted (Hu & Kitts, 2005; Yarnell & Abascal, 2009; Gargouri et al., 2012; Tettey, Ocloo, Nagajyothi, & Lee, 2014; Hassan, El-Kholy, & Galal, 2015; Martinez et al., 2015). As a result, the aerial parts of dandelion have been characterized as rich in phenolic compounds, especially hydroxycinnamic acid derivatives and flavonoid glycosides (Chen, Inbaraj, & Chen, 2012; Hudec et al., 2007; Jedrejek, Kontek, Lis, Stochmal, & Olas, 2017; Kim et al., 2008; Schütz, Kammerer, Carle, & Schieber, 2005; Williams, Goldstone, & Greenham, 1996). Due to exerted bioactivities, and no reported adverse effects in humans (Martinez et al., 2015; Sweeney, Vora, Ulbricht, & Basch, 2005; Yarnell & Abascal, 2009), the plant is commonly available food ingredient and component of medical preparations. Moreover, extracts from dandelion organs and derived products, such as teas, honey and tinctures, are listed on the US Food and Drug Administration list of foods and supplements generally recognized as safe (E-CFR, 2015). For

Abbreviations: APTT, activated partial thromboplastin time; DMSO, dimethylsulfoxide; H_2O_2, hydrogen peroxide; GSH, glutathione; GRAS, generally recognized as safe; HDL, high density lipoprotein; LDH, lactate dehydrogenase; LDL, low density lipoprotein; MDA, malonyldialdehyde; O_2^{-}·, superoxide anion; PT, prothrombin time; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TT, thrombin time; VLDL, very low density lipoprotein

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example, roasted and ground dandelion root is a popular in the USA substitute of tea and coffee, although devoid of stimulant properties, being a source of nutrients and minerals (García-Carrasco, Fernandez-Dacosta, Dávalos, Ordovás, & Rodriguez-Casado, 2015). Moreover, dried leaves of dandelion can be added to drinks and alcoholic beverages. In turn, dandelion flowers are used for the production of wine and syrup, and to improve the smell and taste of foods, such as sweets, cakes, jellies, frozen dairy desserts and cheese (Schütz, Carle, & Schieber, 2006).

Our previous *in vitro* experiments demonstrated that standardized dandelion phenolic fractions from leaves and petals are able to reduce the oxidative stress (*i.e.* lipid peroxidation and protein carbonylation) in human plasma (Jedrejek et al., 2017). However, their effect on blood platelets, another important element of hemostasis, was not investigated. Blood platelets are the smallest morphotic elements of the blood and they are mainly involved in the coagulation process but are also engaged in various pathological conditions, such as thrombosis and atherosclerosis (Blockmans, Deckmyn, & Vermylen, 1995).

The aim of presented study was to examine the *in vitro* activity of four standardized phenolic fractions from dandelion (leaves and petals 50% methanol fractions and leaves and petals 85% methanol fractions) on a nonenzymatic lipid peroxidation in resting blood platelets, and enzymatic lipid peroxidation – arachidonic acid metabolism in platelets activated by a thrombin (physiological agonist), in response to the level of thiobarbituric acid reactive substances (TBARS). Additionally, their antioxidant effect in blood platelets, associated with a generation of superoxide anion ($O_2^{-}\cdot$), activity against the effect of the donor of hydroxyl radicals – H_2O_2/Fe on blood platelet lipids and platelet proteins (protein carbonylation and the number of protein thiol groups), was also measured. Another aim was to investigate the effect of dandelion fractions on the extracellular lactate dehydrogenase (LDH) activity – a marker of cell damage; and also the effect on thiol group metabolism in platelets, because changes of thiol redox potential in blood platelets can play an important role in the mechanism of their activation. Finally, *in vitro* effect of plant preparations on the hemostatic activity of plasma, in relation to three selected hemostatic parameters, *i.e.* the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT), was determined.

2. Materials and methods

2.1. Chemicals

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

2.2. Plant material

The aerial parts of dandelion were harvested on a farm located in south-eastern Poland (50.114175°N, 21.911738°E). Petals were collected on April 29, 2015 (at the time of dandelion flowering), and leaves on May 28, 2015 (after plant flowering). Subsequently, plant material was freeze-dried, powdered, and finally used for extraction and fractionation.

2.3. Extraction and preparation of dandelion phenolic fractions

Leaves and petals of dandelion were first extracted with 80% methanol under reflux, subsequently obtained raw extracts were evaporated and loaded onto a short glass column filled with Cosmosil C18-PREP 140 (140 μm, 6 × 10 cm, Nacalai Tesque Inc., Kyoto, Japan). The column was first washed with water, and then with 50% MeOH, followed by 85% MeOH to elute phenolic compounds. Thus, four different dandelion polyphenolic fractions were obtained, *i.e.* leaves 50% and 85% methanol fractions (A and C, respectively), and petals 50% and 85% methanol fractions (B and D, respectively). The detailed information on extraction and fractionation of plant material can be found in our previous publication (Jedrejek et al., 2017). Fractions A–D were characterized using LC-PDA-MS/MS technique. The chromatographic analyses demonstrated that phenolic acids, especially hydroxycinnamic acid derivatives, were dominant compounds in three fractions: 50% fraction from leaves, 50% fraction from petals and 85% fraction from leaves, where total amounts equaled 418.64 mg/g of dry weight (main component L-chicoric acid, about 350 mg/g d.w.) and 214.33 mg/g d.w. (main component L-chicoric acid, about 115 mg/g d.w.) and 305.17 mg/g d.w. (main component L-chicoric acid, about 195 mg/g d.w.), respectively. Additionally, 85% fraction from leaves contained also significant amounts of flavonoid glycosides, total amount equaled 160.72 mg/g d.w. (main component luteolin-*O*-hexoside, about 80 mg/g d.w.). In turn, 85% fraction from petals contained exclusively flavonoids, total amount equaled 633.71 mg/g d.w. (main component luteolin aglycone, about 470 mg/g d.w.). The concentrations of phenolic acids, flavonoids and total phenolic compounds in 50% and 85% leaf and petal fractions of dandelion is presented in Table 1. The more detailed characterization of the above fractions has been described earlier (Jedrejek et al., 2017).

A stock solution of phenolic fractions was made in 50% DMSO. The final concentration of DMSO in samples was lower than 0.05% and its effects were determined in all experiments.

2.4. Blood platelets and plasma isolation

Fresh human plasma and blood were obtained from regular, medication-free donors at a blood bank and a Medical Center (Lodz, Poland). Peripheral blood was also obtained from non-smoking men and women (collected into CPD solution (citrate/phosphate/dextrose; 9:1; v/v blood/CPD) or CPDA solution (citrate/phosphate/dextrose/adenine; 8.5:1; v/v; blood/CPDA)). They had not taken any medications or addictive substances (including tobacco, alcohol, antioxidant supplementation and aspirin or any other anti-platelet drugs). The protocol was approved by the Committee for Research on Human Subjects of the University of Lodz number 2/KBBN-UŁ/II/2016. Platelet-rich plasma (PRP) was prepared by centrifugation of fresh human blood at 1200 × *g* for 12 min at room temperature. Platelets were then sedimented by centrifugation at 2300 × *g* for 15 min at room temperature. The platelet pellet was washed with modified Tyrode's buffer (pH 7.4) twice; afterward, the platelets were suspended in the same buffer. The concentration of platelets in suspensions (used in the experiments), estimated spectrophotometrically (Walkowiak, Michalak, Koziołkiewicz, & Cierniewski, 1989), amounted to 2.5–3 × 10^8/mL.

Suspensions of blood platelets or plasma were incubated (30 min, at 37 °C) with:

- 50% fraction from leaves of dandelion at the final concentrations of 0.5–50 μg/mL
- 85% fraction from leaves of dandelion at the final concentrations of 0.5–50 μg/mL
- 50% fraction from petals of dandelion at the final concentrations of 0.5–50 μg/mL
- 85% fraction from petals of dandelion at the final concentrations of 0.5–50 μg/mL.

Suspensions of blood platelets were pre-incubated (30 min, at 37 °C) with:

- 50% fraction from leaves of dandelion at the final concentrations of 1–50 μg/mL
- 85% fraction from leaves of dandelion at the final concentrations of

Table 1
The concentration of phenolic compounds in 50% and 85% leaf and petal fractions from dandelion (Jedrejek et al., 2017; modified).

Type of phenolic fraction from dandelion	50% fraction from leaves (A) (mg/g d.w. ± SD)	85% fraction from leaves (C) (mg/g d.w. ± SD)	50% fraction from petals (B) (mg/g d.w. ± SD)	85% fraction from petals (D) (mg/g d.w. ± SD)
Total phenolic acids	418.64 ± 5.67	305.17 ± 4.41	214.33 ± 5.18	–
Therein L-chicoric acid	351.58 ± 4.72	196.07 ± 1.11	117.17 ± 3.18	–
Total flavonoids	19.17 ± 0.64	160.72 ± 2.54	38.73 ± 2.55	633.71 ± 4.02
Therein luteolin	–	–	–	472.46 ± 2.26
Total phenolic compounds	437.81 ± 6.31	465.89 ± 6.95	253.06 ± 7.73	633.71 ± 4.02
Description	Phenolic acid rich fraction	Phenolic acid and flavonoid rich (mixed) fraction	Phenolic acid rich fraction	Flavonoid rich fraction

1–50 μg/mL
- 50% fraction from petals of dandelion at the final concentrations of 1–50 μg/mL
- 85% fraction from petals of dandelion at the final concentrations of 1–50 μg/mL.

and then treated with thrombin (5 U/mL, 5 min, at 37 °C).

Suspensions of blood platelets were pre-incubated (5 min, at 37 °C) with:

- 50% fraction from leaves of dandelion at the final concentrations of 1–50 μg/mL
- 85% fraction from leaves of dandelion at the final concentrations of 1–50 μg/mL
- 50% fraction from petals of dandelion at the final concentrations of 1–50 μg/mL
- 85% fraction from petals of dandelion 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 was calculated by measuring the absorbance of tested samples at 280 nm, according to the procedure of Whitaker and Granum (1980).

2.5. Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring the concentration of TBARS. Incubation of blood platelets was stopped by cooling the samples in an ice-bath. Samples of platelets were transferred to an equal volume of cold 15% (v/v) trichloroacetic acid in 0.25 M HCl and centrifuged at 8000 × *g* for 10 min. One volume of clear supernatant was mixed with 0.2 volume of 0.37% thiobarbituric acid in 0.25 M HCl immersed in a boiling water bath for 15 min. Then the cooled samples were centrifuged at 8000 × *g* for 5 min and the absorbance was measured at 535 nm (the SPECTROstar Nano Microplate Reader - BMG LABTECH Germany) (Bartosz, 2008; Wachowicz, 1984). The TBARS concentration was calculated using the molar extinction coefficient ($\varepsilon = 156{,}000\ M^{-1}\ cm^{-1}$). The level of TBARS (as nmol TBARS/mg of platelet protein) was expressed as a percentage (%) of control sample.

2.6. Superoxide anion measurement

Cytochrome c reduction was used to measure $O_2^{-}\cdot$ generation in control and in platelets incubated with the tested fractions, as described earlier (Olas et al., 1999; Wachowicz, Olas, Zbikowska, & Buczynski, 2002). Briefly, an equal volume of modified Tyrode's buffer, containing cytochrome *c* (160 μM), was added to a platelet suspension. After incubation, the platelets were sedimented by centrifugation at 2000 × *g* for 5 min and the supernatants were transferred to cuvettes. Cytochrome c reduction was measured spectrophotometrically at 595 nm. To calculate the molar concentration of $O_2^{-}\cdot$ the molar extinction coefficient for cytochrome *c* of $18{,}700\ M^{-1}\ cm^{-1}$ was used.

2.7. Carbonyl groups measurement

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

2.8. Thiol groups determination

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

2.9. The measurement of prothrombin time

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

2.10. The measurement of thrombin time

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

2.11. The measurement of APTT

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

2.12. LDH activity measurement

Extracellular LDH activity was measured by following a decrease in absorbance at the wavelength of λ-340 nm (the SPECTROstar Nano Microplate Reader - BMG LABTECH Germany) resulting from the NADH oxidation (Wroblewski & Ladue, 1955).

2.13. Data analysis

Statistical analysis was done using several tests. In order to eliminate uncertain data, the Q-Dixon test was performed. All the values in this study were expressed as a mean ± standard error (S.E.). Differences within and between groups were assessed by applying one-way ANOVA test followed by a multicomparison Tukey's test using STATISTICA 12. Differences for $O_2^{-}\cdot$ generation were assessed by Mann-Whitney *U* test. Values marked with the same letter in a given figure panel do not differ significantly from each other.

3. Results

The anti-platelet and antioxidant activities of phenolic fractions from leaves and petals of dandelion (at the dose range of 1–50 µg/mL) in blood platelets were studied *in vitro*. The cytotoxicity of plant fractions on human blood platelets was also evaluated. We observed that none of the dandelion preparations caused the lysis of platelets, determined as a leakage of lactate dehydrogenase into the extracellular medium, at all tested range (data not shown).

As shown in Fig. 1, only fraction C (leaves 85% fraction) significantly modulated non-enzymatic lipid peroxidation in resting blood platelets (at the dose 50 µg/mL), while other fractions relatively did not cause any effect. In turn, only fraction B (petals 50% fraction) reduced enzymatic lipid peroxidation (peroxidation of arachidonic acid) in blood platelets activated by thrombin, in a noticeable way (at the dose 50 µg/mL), compared to other fractions (Fig. 2). As demonstrated in Fig. 3, fraction A (leaves 50% fraction) and B (petals 50% fraction) had the strongest inhibition effect on lipid peroxidation in platelets treated with H_2O_2/Fe, both at the dose 50 µg/mL, compared to other fractions. Both fractions, A and B, resulted in about 40% decrease in lipid peroxidation compared to control (Fig. 3).

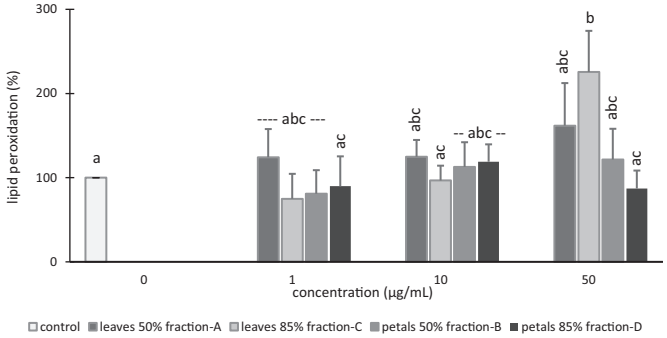


Fig. 1. Effects of phenolic fractions from leaves (A and C fraction) and petals (B and D fraction) from dandelion (concentration range 1–50 µg/mL) on lipid peroxidation in resting blood platelets. The value of the control sample was 0.742 ± 0.396 nmol/mg of platelet protein, and was expressed as 100% of TBARS level. Results are expressed as a percentage of TBARS level, and given as means ± S.E. (n = 4–14). Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).

Exposure of blood platelets to strong oxidant - H_2O_2/Fe, resulted in an enhanced level of protein carbonyl groups and the oxidation of protein thiol groups in blood platelets (Figs. 4 and 5A). As demonstrated in Figs. 4 and 5A, none of the four tested fractions did inhibit protein carbonylation and the oxidation of thiol groups induced by H_2O_2/Fe ($p > 0.05$). However, after 30 min incubation of resting platelets with fraction A (leaves 50% fraction), at two tested concentrations: 1 and 50 µg/mL, the amount of thiol groups in blood platelet proteins increased noticeably, compared to other fractions (Fig. 5B).

Another set of experiments was focused on the determination of $O_2^{-}\cdot$ level in resting blood platelets and blood platelets activated by thrombin, treated with tested fractions at the concentration 10 µg/mL, however, it was observed for all fractions that after a 30-min incubation time the amount of $O_2^{-}\cdot$ in both models did not diminish statistically significant ($p > 0.05$) (Figs. 6 and 7).

Analysis of the effect on the coagulation activity of human plasma demonstrated that three fractions, *i.e.* fraction C (leaves 85% fraction),

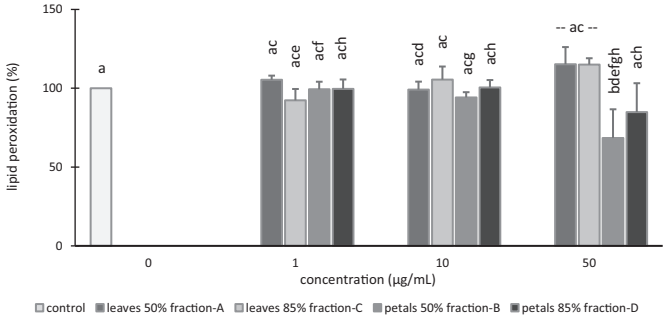


Fig. 2. Effects of phenolic fractions from leaves and petals from dandelion (concentration range 1–50 µg/mL) on lipid peroxidation in blood platelets activated by thrombin. In these experiments, the TBARS level (a marker of lipid peroxidation) in control samples (blood platelets activated by thrombin) was 1.429 ± 0.321 nmol/mg of platelet protein and was expressed as 100% of TBARS level. Results are given as means ± S.E. (n = 6–13). Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).

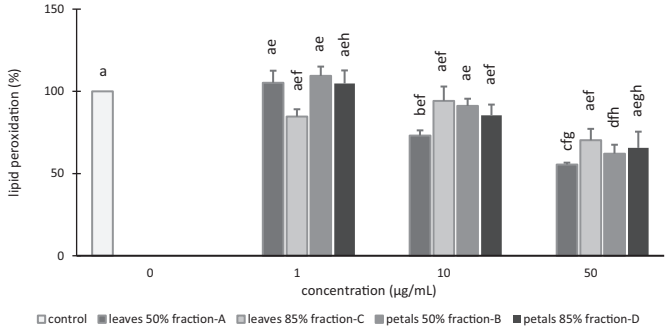


Fig. 3. Effects of phenolic fractions from leaves and petals from dandelion (concentration range 1–50 μg/mL) on lipid peroxidation in blood platelets treated with H_2O_2/Fe. In these experiments, the TBARS level (a marker of lipid peroxidation) in control samples (blood platelets treated with H_2O_2/Fe) was 2.223 ± 0.439 nmol/mg of platelet protein and was expressed as 100% of TBARS level. Results are given as means ± S.E. (n = 4). Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).

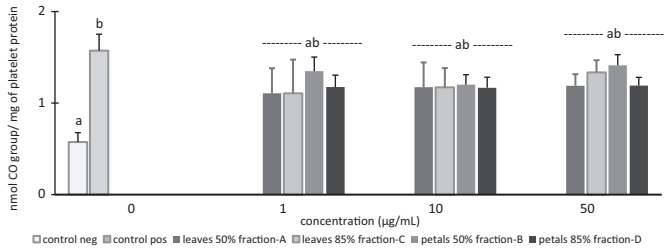
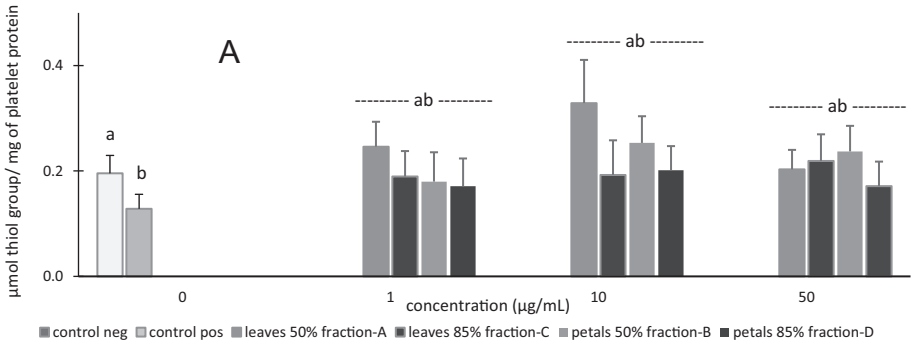


Fig. 4. Effects of phenolic fractions from leaves and petals from dandelion (concentration range 1–50 μg/mL) on protein carbonylation in blood platelets treated with H_2O_2/Fe. Results are given as means ± S.E. (n = 3–4). Control negative (neg) refers to platelets not treated with H_2O_2/Fe, whereas control positive (pos) to platelets treated with H_2O_2/Fe. Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).



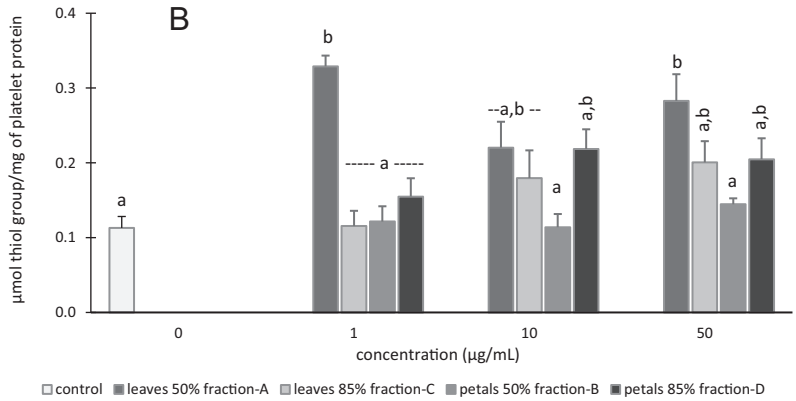


Fig. 5. Effects of phenolic fractions from leaves and petals from dandelion (concentration range 1–50 μg/mL) on the level of thiol groups in platelets' protein treated with H_2O_2/Fe (A) and in resting blood platelets (B). Results are given as means ± S.E. (n = 3–6). Control negative (neg) refers to platelets not treated with H_2O_2/Fe, whereas control positive (pos) to platelets treated with H_2O_2/Fe. Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).

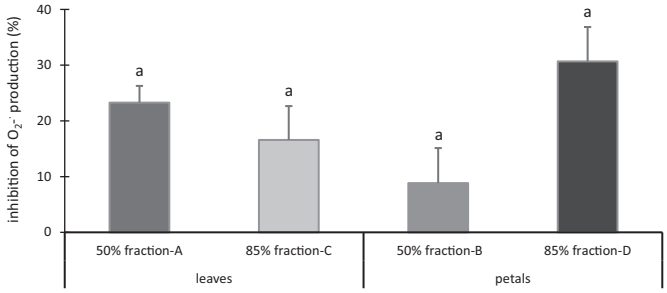


Fig. 6. Effects of phenolic fractions from leaves and petals from dandelion (concentration 10 μg/mL) on $O_2^{-}\cdot$ production in resting blood platelets. In these experiments, the $O_2^{-}\cdot$ level in control samples was 0.946 ± 0.421 nmol/mg of platelet protein. Inhibition of $O_2^{-}\cdot$ production was expressed as a percentage of that recorded for platelets (without tested fraction). Data represent means ± S.E. (n = 3–4). Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).

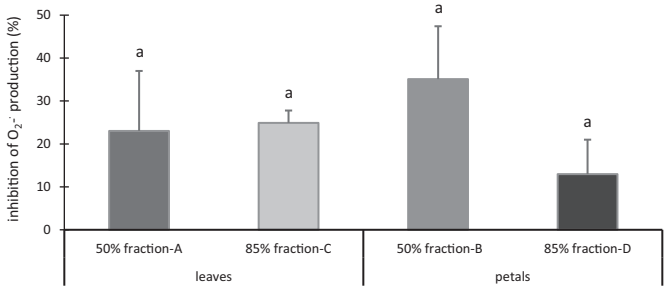


Fig. 7. Effects of phenolic fractions from leaves and petals from dandelion (concentration 10 μg/mL) on $O_2^{-}\cdot$ production in blood platelets activated by thrombin. In these experiments, the $O_2^{-}\cdot$ level in control samples was 1.752 ± 0.641 nmol/mg of platelet protein. Inhibition of $O_2^{-}\cdot$ production was expressed as a percentage of that recorded for platelets activated by thrombin (without tested fraction). Data represent means ± S.E. (n = 4). Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).

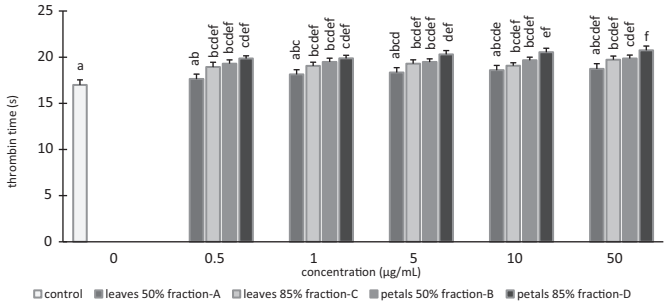


Fig. 8. Effects of phenolic fractions from leaves and petals from dandelion (concentration range 0.5–50 μg/mL) on the thrombin time of human plasma. Tested fractions were incubated with plasma for 30 min and then thrombin was added. Data represent means ± S.E. ($n = 8$). Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).

B (petals 50% fraction) and D (petals 85% fraction) significantly prolonged the thrombin time, at the whole tested range 0.5–50 μg/mL (Fig. 8, Table 2). However, none of the investigated dandelion preparations changed the activated partial thromboplastin time and the prothrombin time (data not presented). In addition, four tested fractions significantly prolonged the thrombin time of plasma, when the mixture of tested fraction and thrombin (earlier pre-incubation of thrombin with plant fraction, and then adding to the plasma) was used, for all fractions at the dose 50 μg/mL (Fig. 9). Moreover, Table 2 demonstrates comparative effects of phenolic fractions from leaves and petals of dandelion at the concentration of 50 μg/mL in selected components of hemostasis (blood platelets and plasma). For example, two tested fractions (A and B) had stronger anti-platelet and antioxidant properties, than fraction C and D.

4. Discussion

An example of plants multi-target tendency is a dandelion. The first mention of the medicinal use of dandelion dates back to the turn of 10th and 11th centuries when Arabic physicians used this plant in the treatment of liver and spleen diseases (Chatterjee, Ovadje, Mousa, Hamm, & Pandey, 2011). An interesting aspect of the beneficial effect of dandelion extracts on human health is their protective effect on the cardiovascular system. It has been proven that the enrichment of the diet of rats with leaves and roots of dandelion affects the lipid profile of the body. In the *in vivo* experiment performed by Arafa, Massoudel-Said, Abdel-Rahman, and Abdel-Megeid (2010) the rats were administrated with carbon tetrachloride (CCl_4) what caused an increase in the level of cholesterol, low density lipoprotein (LDL), very low density lipoprotein (VLDL) fractions and triglycerides, whereas a high density lipoprotein (HDL) fraction decreased in their blood. It is well known that changes of these parameters significantly contribute to the development of heart diseases, including atherosclerosis. Subsequently, the diet of rats was supplemented with dandelion roots (10 g of roots/200 g of bread) which resulted in significant decrease of cholesterol (by 19.9%), LDL (by 38.7%) and triglycerides and VLDL (both by 16.3%), and significant increase of HDL (by 13.5%) in their plasma, compared to control group (Arafa et al., 2010). Similar effects have been observed by Korean scientists (Choi et al., 2010). Moreover, they observed the inhibition of the formation of atherosclerotic plaques by 58%, in relation to the control group (Choi et al., 2010). Additionally, it has been demonstrated that phenolic fractions from dandelion petals and leaves reduce the level of oxidative stress markers in human plasma treated with H_2O_2 or H_2O_2/Fe^{2+} under *in vitro* conditions (Jedrejek et al., 2017). Despite these findings, the mechanisms of antioxidant protection of dandelion phenolic fractions for blood platelet components, especially lipids and proteins, and for the hemostatic function of blood platelets and plasma, remained unidentified.

Observed changes in hemostasis are very often correlated with

Table 2
Comparative effects of phenolic fractions from leaves (A and C) and petals (B and D) from dandelion at the highest tested dose (50 μg/mL) in selected components of hemostasis.

Type of phenolic fraction from dandelion	50% fraction from leaves (A)	85% fraction from leaves (C)	50% fraction from petals (B)	85% fraction from petals (D)
Blood platelets				
Toxicity to the blood platelets	Non-toxic	Non-toxic	Non-toxic	Non-toxic
Lipid peroxidation in resting blood platelets	No effect	Negative action – stimulation of lipid peroxidation (prooxidative properties)	No effect	No effect
Lipid peroxidation in blood platelets activated by thrombin	No effect	No effect	Positive action – inhibition of lipid peroxidation (anti-platelet properties)	No effect
Lipid peroxidation in blood platelets treated with H_2O_2/Fe	Positive action – inhibition of lipid peroxidation (antioxidative properties)	No effect	Positive action – inhibition of lipid peroxidation (antioxidative properties)	No effect
Protein carbonylation in blood platelets treated with H_2O_2/Fe	No effect	No effect	No effect	No effect
Oxidation of protein thiols in blood platelets treated with H_2O_2/Fe	No effect	No effect	No effect	No effect
Protein thiol groups in resting blood platelets	Increase in thiol groups	No effect	No effect	No effect
Plasma				
Thrombin time (plasma treated with tested fraction and then thrombin)	No effect	Positive action – prolongation of TT (anticoagulant properties)	Positive action – prolongation of TT (anticoagulant properties)	Positive action – prolongation of TT (anticoagulant properties)
Thrombin time (plasma treated with mixture (tested fraction plus thrombin))	Positive action – prolongation of TT (anticoagulant properties)	Positive action – prolongation of TT (anticoagulant properties)	Positive action – prolongation of TT (anticoagulant properties)	Positive action – prolongation of TT (anticoagulant properties)

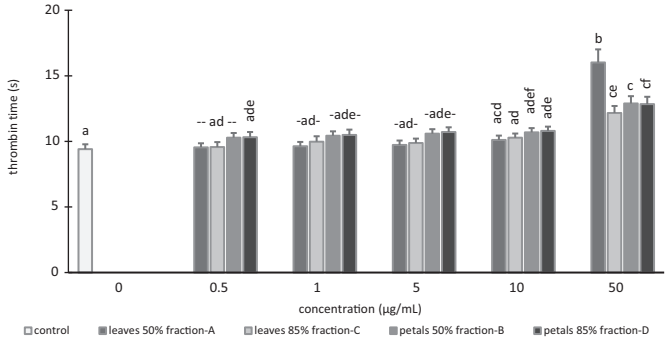


Fig. 9. Effects of phenolic fractions from leaves and petals from dandelion (concentration range 1–50 µg/mL) on the thrombin time of human plasma. The solution of thrombin was incubated for 30 min with tested fractions and then the mixture was added to the human plasma. Data represent means ± S.E. (n = 8). Values marked with the same letter in a given figure panel do not differ significantly from each other ($p < 0.05$).

oxidative stress, and may lead to the development of cardiovascular diseases. In our *in vitro* experiments, a human blood platelets models were used because this is a vital component of hemostasis. In general, it is not recommended to evaluate the antioxidant and anti-platelet properties of plant extracts by only a single method due to the complex nature of phytochemicals. Therefore, in the present study a four different assays, *i.e.* generation of superoxide anion, lipid peroxidation, carbonyl and thiol groups' determination, have been engaged. At the beginning of our experiment, we confirmed that an addition of H_2O_2/Fe to human blood platelets results in a significant increase in the level of different biomarkers of oxidative stress, similarly to the previous test with human plasma (Jedrejek et al., 2017). Present *in vitro* study was also designed to estimate the antioxidant effects of phenolic fractions from dandelion leaves and petals on the changes of platelet lipids and protein of human blood platelets stimulated by physiological oxidant - H_2O_2/Fe. For example, it is shown by our test that two fractions characterized as phenolic acid rich fractions, *i.e.* fraction A (leaves 50% fraction) and B (petals 50% fraction), exerted stronger inhibitory action on H_2O_2/Fe-induced lipid peroxidation in human blood platelets than flavonoid containing fractions, at the dose 50 µg/mL. These results are consistent with other studies on the role of dandelion polyphenols as protective agents against oxidative stress. Cho et al. (2002) have observed that dandelion water extract lowered the hepatic lipid peroxidation in diabetic rats. In turn, the results of Kim et al. (2008) have indicated that dandelion phenolic fractions, rich in cinnamic acid derivatives, inhibited phosphatidylcholine peroxidation.

The toxicity and bioavailability of phenolic compounds, and other natural products is a very important element in the evaluation of their biological properties under both *in vitro* and *in vivo* conditions. It should be emphasized that there is no reported information about the toxicity of dandelion tissues for animals and humans, especially in relation to their polyphenol content. The concentrations of tested fractions used in our experiments may be achievable in blood during their oral supplementation (Hirano, Mori, Kato, & Haga, 1978; Manach, Scalbert, Morand, Remsey, & Jimenez, 2004; Manach, Williamson, Morand, Scalbert, & Remsey, 2005). However, these compounds are also metabolized, and most of them appear in circulation as sulfate and glucuronidate derivatives, *i.e.* chicoric acid circulates as caffeic, dihydrocaffeic, ferulic, and dihydroferulic conjugates. On the other hand, a recent experiment (*in vitro*) by Baeza et al. (2017) demonstrates that dihydrocaffeic acid and dihydroferulic acid (at concentrations: 0.01–100 µg/mL) are more effective inhibitors of platelet activation than their phenolic precursors. Moreover, we observed that none of the tested dandelion phenolic fractions brought the damage of human blood platelets, at the whole tested range. Thus, we can confirm dandelion organs as a safe plant material. A key novel finding of this study is a demonstration of the anticoagulant activity, observed as prolonged clotting time – the thrombin time (TT), recorded for three tested fractions, *i.e.* fraction C (leaves 85% fraction), B (petals 50% fraction) and D (petals 85% fraction), at the whole tested range 0.5–50 µg/mL (Fig. 8). Additionally, we also observed that all dandelion fractions prolonged the TT of plasma when they were preincubated with thrombin – a multifunctional plasma serine protease with a central function in controlling hemostasis. We suppose that anticoagulant properties of tested phenolic fractions, and the mechanism responsible for this process, are associated with a modulation of thrombin activity (probably its inhibition). Yun, Cho, and Choi (2002) purified an anticoagulant protein (33 kDa) from *Taraxacum platycarpum*, which doubled APTT, PT, and TT of human plasma, probably also by inhibiting thrombin. In addition, TT changed more sensitively than APTT or PT, in response to the aforementioned protein of *T. platycarpum*.

Neef et al. (1996) demonstrated that ethanolic extracts of dandelion root have an inhibitory effect on human blood platelet aggregation (stimulated by ADP) – another important element of hemostasis. In this experiment, two different dandelion extracts were used, *i.e.* extract contained low-molecular polysaccharides (caused about 90% inhibition of platelet aggregation) and extract enriched with steroids and triterpenes (demonstrated about 80% inhibition of platelet aggregation). Both extracts were used at the dose of 0.04 g crude material/mL of platelet rich plasma. We report for the first time that phenolic fraction from dandelion petals (50% fraction – B, concentration 50 µg/mL) can effectively reduce enzymatic lipid peroxidation in blood platelets activated by thrombin. It may suggest that this fraction could modulate blood platelet activation by interfering with the metabolism of arachidonic acid.

We have observed that phenolic fractions from leaves and petals of dandelion differed in terms of investigated activities: anti-platelet, anticoagulant and antioxidant. Similarly, the differences in these activities were found between 50% and 85% fractions, for both leaves and petals (Table 2). The easiest way to explain these differences relates to different chemical profile of each tested fraction, which have been generally described as phenolic acid rich fractions (leaves and petals 50% fractions, A and B respectively), flavonoid rich fraction (petals 85% fraction, D), and mixed fraction (leaves 85% fraction, C). The total content of phenolic acids in fraction A and B and C was about 420 mg/g d.w., 215 mg/g d.w., and 305 mg/g d.w., respectively; which was about 95%, 85% and 65% of total polyphenols of fraction, respectively. Moreover, the main phenolic acid of fraction A and B and C was L-chicoric acid which represented about 80%, 45% and 40% of total polyphenols of the fraction, respectively. In turn, the total content of flavonoids in fractions C and D was about 160 mg/g d.w., and 630 mg/g d.w., respectively; which was about 35%, and 100% of total polyphenols of the fraction, respectively. Moreover, the main flavonoid of fraction C and D was luteolin (glycosylated form in fraction C, and aglycone in fraction D) which represented about 20%, and 75% of total polyphenols of the fraction, respectively. As shown in Table 2, fractions rich in cinnamic acid derivatives (fraction A and B) appeared to be more effective in terms of anti-platelet and antioxidant activity, than fractions rich in flavonoids (fraction C and D). Chicoric acid has been already recognized as strong antioxidant agent under *in vitro* conditions (Schlernitzauer et al., 2013; Thygesen, Thulin, Mortensen, Skibsted, & Molgaard, 2007), and our results confirm this. In turn, anticoagulant activity has been demonstrated by either fractions rich in phenolic acids (fraction B) or flavonoids (fraction D). Our results are also consistent with the literature. Results Hu and Kitts (2003) suggest that luteolin and luteolin-7-glucoside may decide about antioxidant activity of dandelion flowers. Afterwards, Benavente-Garcia and Castillo (2008) have described that flavonoids, including luteolin, have antioxidant and antithrombotic properties, even in low concentration. Guerrero et al. (2007) have also observed anti-platelet properties of luteolin, which was further confirmed by an experiment of Dell'Agli et al. (2008), in which the inhibition of blood platelet aggregation stimulated by thrombin have been demonstrated.

Previous studies (Lopez-Garcia, Kucekova, Humpolicek, Mlecek, & Saha, 2013; Schütz et al., 2005) together with present experiments indicate that dandelion is a rich source of phenolic compounds possessing a wide spectrum of biological activities, *i.e.* antioxidant, anti-platelet and anticoagulant; and the observed effects should be linked with the presence of various polyphenols. Moreover, we suggest that phenolic fractions from dandelion, especially leaves and petals 50% fractions, can be a new source of natural compounds showing co-operative activities: antioxidant, anti-platelet and anticoagulant, beneficial in the prevention and treatment of cardiovascular diseases, which are often associated with changes of hemostasis and oxidative stress.

Declaration of interest statement

None to declare.

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Comparative phytochemical, cytotoxicity, antioxidant and haemostatic studies of *Taraxacum officinale* root preparations

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ABSTRACT

Although the majority of potentially bioactive components of dandelion root have been described, revealing the presence of hydroxycinnamic acids (HCAs) and sesquiterpene lactones (SLs), new compounds are still being discovered, an example of which are the recently characterized 4-hydroxyphenylacetate inositol esters (PIEs). In this work, the dandelion root was separated into five preparations (A-E) differing in chemical content. A detailed LC-MS and chemical investigation of dandelion fractions allowed the identification of about 100 phytochemicals, including new compounds for the genus *Taraxacum*, and the plant kingdom, such as amino acid-SL adducts. In the DPPH● test, two preparations characterized by high content of HCAs (D and E) showed the highest free radical scavenging activity, while other demonstrated weaker action. In turn, in blood plasma, the best overall protective effect against oxidation by H_2O_2/Fe was obtained in the presence of preparations A (SL-amino acid adducts enriched fraction) and C (PIEs enriched fraction). A stronger anticoagulant effect was demonstrated for two preparations enriched with HCAs (D and E). None of dandelion root preparations caused the lysis of blood platelets, at all tested range (0.5–50 μg/mL). Our results demonstrate that dandelion roots are a safe and valuable source of different class natural compounds possessing antioxidant, anticoagulant and anti-platelet activities.

1. Introduction

The common dandelion (*Taraxacum officinale* L.) is considered a weed in many crops around the world, at the same time the plant has a long history as traditional herbal remedy to treat liver and gallbladder disorders, digestive ailments, diverse skin inflammations, and arthritic and rheumatic diseases (Martinez et al., 2015; Schütz et al., 2006). In the modern herbal medicine, all aforementioned functions of *T. officinale* have been upheld, and the dried leaves, flowers and roots, and extracts thereof are sold today as herbal teas, syrup and in the capsule form. Furthermore, dandelion is regarded as a fully nontoxic and entirely edible plant, and its aerial parts and root are the components of different food products, such as salads, wines, desserts, flavors, and coffee substitutes (Martinez et al., 2015).

The chemical composition of an entire plant (leaves, flowers, roots, and latex) has been studied extensively. Different tissues of dandelion reportedly contain an array of secondary metabolites, such as hydroxycinnamic acids (HCAs), flavonoids, sesquiterpene lactones (SLs), triterpenes, and coumarins (Kisiel and Barszcz, 2000; Schütz et al., 2005; Saeki et al., 2013; Huber et al., 2015; Jedrejek et al., 2017). Additionally, several studies demonstrated that the plant is also a rich source of vitamins, phytosterols, polysaccharides (inulin), various amino acids, and minerals (particularly potassium) (Williams et al., 1996; González-Castejón et al., 2012). Dandelion extracts and their components have been shown to exert a wide range of *in vitro* and *in vivo* biological actions, such as antioxidant, anti-inflammatory, anti-carcinogenic, anti-hyperglycemic, anti-thrombotic, antimicrobial and antiviral (Jeon et al., 2008; European Medicines Agency, 2009; Liu et al., 2010; You et al., 2010; He et al., 2011; Rehman et al., 2016; Jedrejek et al., 2017; Lis et al., 2018). Consequently, the use of dandelion preparations or the plant itself brings potentially a range of benefits for human health.

Although the major components of dandelion root have been described, new compounds are still being discovered, an example of

Abbreviations: APTT, activated partial thromboplastin time; HCA, hydroxycinnamic acid; H_2O_2, hydrogen peroxide; HPA, hydroxyphenylacetic acid; PIE, hydroxyphenylacetate inositol ester; PT, prothrombin time; SL, sesquiterpene lactone; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TT, thrombin time

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which are the recently characterized 4-hydroxyphenylacetate inositol esters (PIEs) (Kenny et al., 2014b; Huber et al., 2015). It can be assumed that most of the potentially bioactive constituents of dandelion root have been identified, however, the biological activity of many of these phytochemicals still remains unexplored. Additionally, recently, dandelion root has gained attention for its antioxidant, hepatoprotective, anticancer and antimicrobial activity (You et al., 2010; Ovadje et al., 2012; Kenny et al., 2014a; 2015; Esatbeyoglu et al., 2017; Xue et al., 2017).

Oxidative stress is considered as one of the key mechanisms correlated with human pathological processes, such as cancer, diabetes, and neurodegenerative disorders. Strong pro-oxidants have the ability to damage proteins and lipids, including those found in elements of the hemostatic system (plasma and platelets) which results in disturbance of hemostasis by changing their natural functioning and may contribute to the development of cardiovascular diseases (Xue et al., 2017). Oxidative stress is indicated by various biomarkers, which have not only diagnostic value but could conceivably be also useful indicators of the need for antioxidant supplementation. A number of natural antioxidants, especially polyphenols, have been shown to protect cell components (DNA, protein, and lipids) from oxidative stress by preventing the formation of free radicals or by detoxifying them, resulting in the prevention of a variety of pathophysiological processes (Lobo et al., 2010). In several *in vitro* and *in vivo* studies, different extracts from the root or whole dandelion plant have been determined to exert strong anti-oxidative action (You et al., 2010; Kenny et al., 2014a; Esatbeyoglu et al., 2017; Xue et al., 2017). This effect was attributed mainly to the hydroxycinnamic acids and flavonoid compounds present in them, while the contribution of other components, such as SLs and PIEs, has been much less recognized and studied.

This study aimed to investigate the *in vitro* protective effects of the five *T. officinale* root preparations, each containing a separate group of compounds, against oxidative stress stimulated by H_2O_2/Fe (donor of hydroxyl radicals) in human plasma. Three different markers of oxidation were measured, including lipid peroxidation (determined by thiobarbituric acid reactive substances - TBARS), protein carbonylation, and thiol group level. Additionally, another aim of our experiments was to determine the *in vitro* effect of dandelion root fractions on selected hemostatic parameters of plasma, including the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT); and platelets aggregation. Moreover, all tested preparations were evaluated for toxicity against blood platelets, by measuring the extracellular lactate dehydrogenase (LDH) activity – a marker of cell damage.

2. Materials and methods

2.1. Chemicals

Dimethylsulfoxide (DMSO), thiobarbituric acid (TBA), H_2O_2, formic acid (LC-MS grade), D-glucose, L-cysteine methyl ester hydrochloride, o-tolyl isothiocyanate, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Trolox were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (LC-MS grade), methanol (isocratic grade), tert-butanol and formic acid (98–100%) were acquired from Merck (Darmstadt, Germany). AccQ•Tag™ Ultra Eluent A and B, as well as pre-column AccQ•TagTM Ultra derivatization kit, were supplied by Waters (Milford, MA, USA). Ultrapure water was prepared in-house using a Milli-Q water purification system (Millipore Co.). All other reagents represented analytical grade and were provided by commercial suppliers.

2.2. Plant material

The dandelion root *(Taraxaci radix)* was collected in September 2016 from a natural source (south-eastern Poland) and identified by prof. Krzysztof Oklejewicz (Department of Botany, University of Rzeszów, Poland). A voucher specimen has been deposited at the Department of Biochemistry and Crop Quality of the Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy. Plant material was thoroughly washed with tap water, then cut into small pieces and freeze-dried (Gamma 2–16 LSC, Christ, Osterode am Harz, Germany). Dried roots were then pulverized (Grindomix GM200, Retsch, Haan, Germany) and stored in a refrigerator before extraction.

2.3. Preparation of the fractions A-E

The finely ground *Taraxacum officinale* L. roots (560 g) were thrice extracted with 80% methanol (v/v; 3 × 4 L), in total 36 h, at room temperature and using additionally sonication (18 × 10 min) to enhance the extraction efficiency. The methanol extracts were combined, filtered and concentrated with a vacuum evaporator (40 °C). Obtained residue (ca. 120 g) was suspended in 80% methanol (v/v; 1.5 L) and twice extracted with *n*-hexane (1.5 L) to remove fat-soluble compounds. The defatted extract was then concentrated in the rotary evaporator (40 °C), and the residue was resuspended in 5% methanol (v/v; 250 mL), and it was further purified by SPE on a short C18 column (6 × 4 cm, Cosmosil C18-PREP, 140 µm, Nacalai Tesque Inc., Kyoto, Japan) to remove carbohydrates (high content of sugars was shown by UPLC-PDA-MS analysis, data not shown). The column was earlier equilibrated with 4% methanol (v/v) acidified with 0.1% formic acid (v/v) and then 50 mL of sample was loaded; the sugars were washed with 4% methanol (v/v, 350 mL), and then column was washed with 80% methanol (v/v; 300 mL) to elute the compounds of interest. The eluates were combined, evaporated to dryness under the vacuum at 40 °C, and then the residue (about 12 g) was redissolved in 35 mL of methanol/tert-butanol (3:1, v/v) for further fractionation on Sephadex LH-20 (Sigma–Aldrich) column (80 × 2.8 cm i.d.). The injection volume was 6 mL and the column was eluted with isocratic methanol/tert-butanol (3:1, v/v) at a flow rate of 2.4 mL/min. The separation was monitored by UPLC-PDA-MS analysis on an ACQUITY BEH C18 column (50 × 2.1 mm, 1.7 µm, Waters). Thus, four pooled fractions (I-IV) were collected, concentrated in the rotary evaporator (40 °C), and then freeze-dried, which yielded 1.77 g, 2.36 g, 1.54 g, and 1.25 g, respectively. Due to the complex compositions (as shown by UPLC-PDA-MS analysis, data not shown), the four preparations (I-IV) were chromatographed again on a Sephadex LH-20 column. Fractions were dissolved in 6 mL of methanol/tert-butanol (3:1, v/v) and injected onto the column, which was eluted with isocratic methanol/tert-butanol (3:1, v/v) at a flow rate of 1.6 mL/min. The separation was monitored by UPLC-PDA-MS analysis on an ACQUITY BEH C18 column (50 × 2.1 mm, 1.7 µm, Waters). Altogether, five fractions (A-E) were collected, concentrated in the rotary evaporator (40 °C), and then freeze-dried, which yielded 0.80 g, 1.90 g, 1.07 g, 0.18 g, and 0.96 g, respectively.

2.4. Phytochemical profiling

2.4.1. UHPLC-MS/MS analysis

The chemical composition of the dandelion root fractions (A-E) was analyzed with an ACQUITY UPLC system (Waters), equipped with a photodiode array detector (PDA) and a tandem quadrupole mass spectrometer (TQD) using electrospray ionization (ESI) source. The samples were prepared in the concentration of 2 mg d.w./mL with 50% methanol, and then separated on an ACQUITY BEH C18 column (100 × 2.1 mm, 1.7 µm, Waters) at 40 °C. The injection volume was 2 µL. The mobile phase A consisted of 0.1% (v/v) formic acid in Milli-Q water, and the mobile phase B consisted of 0.1% (v/v) formic acid in acetonitrile. The separation was carried out at a flow rate of 0.4 mL/min and using a linear gradient from 3 to 35% of solvent B in solvent A for 23 min. The UV spectra were recorded within the range 190–490 nm (3.6 nm resolution). The MS analysis was performed in both negative and positive ion modes, using the following MS settings: scan range 100–1200 *m/z*; capillary voltage 2.8 kV (ESI neg) and 3.1 kV (ESI pos);

cone voltage 35 V (ESI neg) and 55 V (ESI pos); source temperature 140 °C; desolvation temperature 350 °C; desolvation gas flow 800 L/h, and cone gas flow 100 L/h. The MS/MS spectra were recorded in the negative ion mode, using argon as collision gas at a flow rate of 0.1 mL/min; collision energy was set at 28 V. Data acquisition and processing were performed using Waters MassLynx 4.1 software.

2.4.2. Acid hydrolysis of preparations A and B

After dissolving 3 mg of lyophilized fractions A and B in 1 mL of 2 M HCl, amino acid sesquiterpene lactone adducts and sesquiterpene lactone glycosides were hydrolyzed by heating to 100 °C for 2 h. After cooling, the samples were subjected to liquid-liquid extraction with ethyl acetate (3 × 1 mL), and the residue was dried under the stream of N_2 gas. Subsequently, the dry residue was dissolved in water (0.4 mL), neutralized with Amberlite IRA-400 (OH^- form) and dried under N_2 gas; for preparation A it was divided into two parts (separately for amino acid and sugar analysis) before drying. For amino acid analysis the dry residue was redissolved in water.

2.4.3. Amino acid analysis

The amino acid derivatization and LC analysis were performed according to the method described by the Waters application note (Aubin et al., 2007) and Liming et al. (2009), with slight modifications. Briefly, the aqueous layer containing amino acids (20 μL) was mixed with AccQ•Tag™ Ultra borate buffer (140 μL), and then 40 μL of derivatizing reagent (AccQ•Tag™ Ultra reagent in acetonitrile) was added. The mixture was then immediately vortexed, left to rest for 1 min and finally heated at 55 °C for 10 min. After cooling, the sample was analyzed by ACQUITY UPLC System (Waters). Chromatographic separation was performed on an ACQUITY AccQ•Tag™ Ultra column (100 × 2.1 mm, 1.7 μm; Waters). Ultraviolet detection was set at 260 nm, with a resolution of 3.6 nm. Details of the analysis can be found in the publications cited above (Aubin et al., 2007; Liming et al., 2009). Data acquisition and processing were performed using Waters MassLynx 4.1 software. As a result, on the basis of the retention time of authentic standards of amino acids (Amino acid hydrolysate standard, Waters) derivatized in the same way (glycine 3.30 min, glutamic acid 4.20 min, proline 5.60 min), proline (Pro), glutamic acid (Glu) and glycine (Gly) were identified in preparation A.

2.4.4. Sugar analysis

The sugar analysis was performed according to the method of Tanaka et al. (2007), with slight modifications. The dry aqueous layers containing monosaccharides, obtained after acid hydrolysis of preparations A and B, were dissolved in anhydrous pyridine (100 μL) containing L-cysteine methyl ester hydrochloride (0.5 mg) and heated at 60 °C for 1 h. Then the solution of o-tolyl isothiocyanate (0.5 mg) in pyridine (100 μL) was added, and the mixture was heated to 60 °C for another 1 h. After cooling, samples were analyzed by UPLC-PDA-ESI-MS/MS. Chromatographic separations were performed on an ACQUITY BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters). The PDA was operated in the range of 220–450 nm, with the resolution of 3.6 nm. Mass spectrometry analyses were performed applying ESI(+) ionization mode, using the SRM method. Details of the analysis found in the publication of Pérez et al. (2014). Data acquisition and processing were performed using Waters MassLynx 4.1 software. As a result, on the basis of the retention time of authentic standard of glucose (Glc) derivatized in the same way (D-Glc 10.40 min, L-Glc 10.18 min) D-glucose was identified in both A and B preparations.

2.5. Preparation of stock solutions for bioassay

Stock solutions of dandelion root fractions A-E were prepared with 50% (v/v) aq. DMSO, which is a universal solvent for many different plant substances. The final concentration of DMSO in the tested samples was below 0.05% (v/v). In addition, as repeatedly confirmed by our earlier studies, the above low concentration of DMSO added to plasma and blood platelets has no influence on the tested parameters of oxidative stress, coagulation and platelet activation (Lis et al., 2018; Olas et al., 2018).

2.6. DPPH free radical scavenging activity

The radical scavenging activity of dandelion root fractions against DPPH radical was determined using the method of Brand-Williams et al. (1995), with slight modifications as follows: 0.1 mL of appropriately diluted fraction (four different concentrations ranging from 0.03 to 2 mg/mL, dependently on the type of samples) or Trolox solution (32, 64, 128, 256, 511, and 767 μM) was added to 1.9 mL of DPPH solution (100 μM in methanol) in a cuvette. The reaction mixture was mixed gently, incubated at room temperature in the dark for 30 min, and then the absorbance was recorded at 517 nm using Thermo Scientific Evolution 260 Bio spectrophotometer. Control samples containing methanol instead of tested fractions were made.

The percentage of absorbance inhibition at 517 nm was calculated from the equation Inhibition (%) = $100 \times [(A_{control} - A_{sample})/A_{control}]$, where $A_{control}$ and A_{sample} are the absorbance values of the control and the test samples at $t = 30$ min, respectively. Standard curves were obtained for each fraction, and Trolox using the relationships of sample/standard concentration (μg/mL) to absorbance inhibition (%). To obtain Trolox Equivalent Antioxidant Capacities (TEAC) of the five tested fractions A-C a slopes of their curves were divided by slope of Trolox curve. The IC_{50} values of dandelion preparations on DPPH (defined as the concentration of sample necessary to cause 50% inhibition) were determined from the same curves (the percentage of scavenging activity plotted against the sample concentration), and Trolox was used as positive control.

2.7. Isolation of human plasma and blood platelets

Fresh human blood and plasma were obtained from a blood bank and Medical Center in Łódź (Poland), and they came from regular, medication-free donors. The protocol was approved by the Committee for Research on Human Subjects of the University of Lodz number 2/KBBN-UŁ/II/2016.

Blood platelets and plasma were isolated by differential centrifugation as described earlier (Lis et al., 2018). The platelets were suspended in modified Tyrode's buffer (pH 7.4). The concentration of blood platelets in suspensions (used in the experiments), estimated spectrophotometrically (Walkowiak et al., 1989), amounted to 2.5×10^8/mL.

Each sample (plasma and blood platelets) taken for analysis came from the different patient and was an independent trial. To measure parameters of hemostasis and cytotoxicity, the plasma or blood platelets were incubated at 37 °C for 30 min with the five tested preparations (concentration range 0.5–50 μg/mL). To measure the oxidative stress parameters, the plasma was pre-incubated at 37 °C for 5 min with the five tested preparations (concentration range 0.5–50 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), as previously described (Olas et al., 2018; Lis et al., 2018). Control negative refers to plasma not treated with H_2O_2/Fe, whereas control positive to plasma treated with H_2O_2/Fe.

The protein concentration was calculated by measuring the absorbance of tested samples at 280 nm, according to the procedure of Whitaker and Granum (1980) or using the Bradford assay (1976).

2.8. Markers of oxidative stress

2.8.1. Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring the concentration of TBARS, according to the method described by Wachowicz (1984) and Bartosz (2008). After incubation, 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.

2.8.2. Carbonyl group measurement

The carbonyl groups were determined in plasma protein according to Levine et al. (1990) and Bartosz (2008). The absorbance measurement (at 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.

2.8.3. Thiol group measurement

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

2.9. Parameters of hemostasis

2.9.1. The 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. (2012). Briefly, after 30 min of treatment with tested dandelion preparations the human plasma (50 µL) was incubated for 2 min at 37 °C, and then directly before measurement, a 100 µL of Dia-PT liquid (commercial preparation - Kselmed, Grudziadz, Poland) was added.

2.9.2. The 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. (2012). Briefly, after treatment with tested dandelion preparations the human plasma (50 µL) was incubated for 1 min at 37 °C, and then directly before measurement a 100 µL of thrombin was added (final concentration - 5 U/mL).

2.9.3. The 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. (2012). Briefly, after treatment with tested dandelion preparations 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, a 50 µL of 25 mM $CaCl_2$ was added.

2.9.4. The measurement of platelet aggregation stimulated by thrombin

Blood platelet aggregation was measured turbidimetrically in washed platelets using an optical Chrono-Log aggregometer (Chrono-Log, Havertown, PA, USA). The prepared washed platelet samples were prewarmed at 37 °C and stirred. After 5 min thrombin (1 U/ml) was added to washed platelet samples, and aggregation was measured for 10 min. The aggregometry was calibrated each time (100% aggregation) on Tyrode's buffer.

2.10. Toxicity against blood platelets – LDH measurement

Extracellular LDH activity was measured by following a decrease in absorbance at the wavelength of λ-340 nm (the SPECTROstar Nano Microplate Reader- BMG LABTECH Germany) resulting from the NADH oxidation (Wroblewski and Ladue, 1955).

2.11. Data analysis

Statistical analysis was performed using Statistica 10 (StatSoft). Normal distribution of data was checked by normal probability plots and the homogeneity of variance by Brown-Forsythe test. Differences within and between groups were assessed by applying one-way ANOVA followed by a multicomparison Duncan's test; for clarity reason, only the differences between the tested preparations and the control/control positive were marked. Results are presented as means ± SEM. Significance was considered at $p < 0.05$. Principal component analysis (PCA) was used as a descriptive tool to visualize the LC-UV-MS data in two dimensions, founding relationships between the variables (compounds) and the tested dandelion fractions. In order to eliminate uncertain data, the Q-Dixon test was performed.

3. Results

3.1. Chemical characteristics of the five dandelion root preparations (A-E)

Applied multiple fractionation procedures, engaging different chromatographic techniques, for the purification of dandelion methanolic root extract (E1), resulted in the five fractions A-E; corresponding scheme of the separation process is given in Fig. 1.

Major components of aforementioned preparations were tentatively identified and classified on the basis of their MS and UV spectra, chemical analysis (hydrolysis), as well as literature data (Kisiel and Barszcz, 2000; Schütz et al., 2005; Abu-Reidah et al., 2013; Kenny et al., 2014b). Additionally, the relative contribution of an individual compound in each preparation was evaluated on the basis of UV max plot chromatogram (full wavelength scan chromatogram from 190 to 490 nm) and expressed as a percentage of the total peak area. The

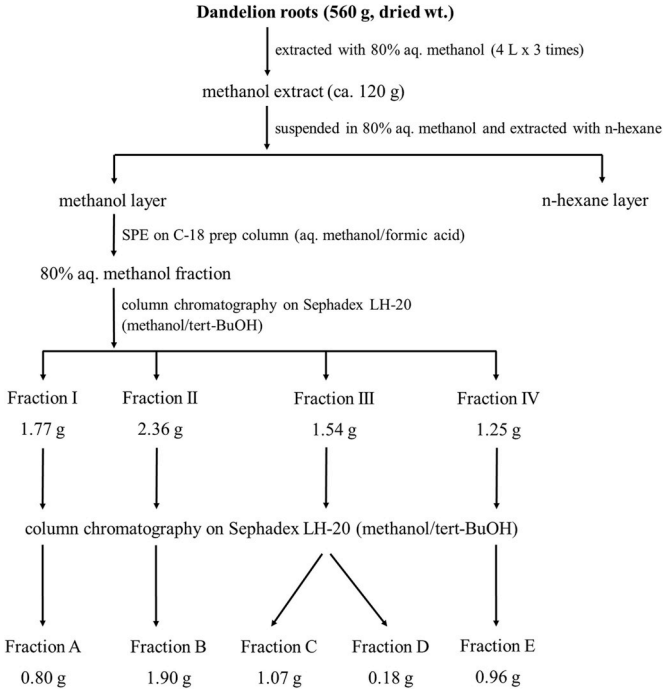

Fig. 1. Diagram of the receiving of the five dandelion root preparations (A–E).

Table 1
UPLC-PDA-MS/MS characteristic of major secondary metabolites and their relative content (expressed as a percentage of the total peak area on UV max plot chromatograms) in tested preparations from *T. officinale* roots.

Compd	Rt (min)	Identity	UVmax (nm)	$[M-H]^-$, *m/z*	$[M+H]^+$, *m/z*	MS/MS, *m/z*, ESI neg	Fr A (%Area UV)	Fr B (%Area UV)	Fr C (%Area UV)	Fr D (%Area UV)	Fr E (%Area UV)
1	1.86	hydroxyphenylacetate inositol ester	220, 275, 315sh	313, 627 $[2M-H]^-$	337 $[M+Na]^+$, 315	107[b], 151			0.75		
2	2.24	hydroxyphenylacetate inositol ester	220, 275, 315sh	313, 627 $[2M-H]^-$	337 $[M+Na]^+$, 315	107, 151			0.92		
3	2.68	caffeoyl-*O*-hexoside[a]	290, 310	341	343	135, 179			0.85		
4	3.23	hydroxyphenylacetate inositol ester	220, 275, 315sh	313, 627 $[2M-H]^-$	337 $[M+Na]^+$, 315	107, 151			1.84		
5	3.28	4-methoxycinnamoyltartaric acid	253, 320	309	311	133, 105				2.00	0.72
6	3.50	caffeoyl hexosyl-*O*-quinic acid	290sh, 325	515	539 $[M+Na]^+$, 517	191, 179, 353, 173, 161				0.43	
7	3.52	unidentified	215, 265, 320sh	359	383 $[M+Na]^+$	138, 182, 153, 197, 123		0.37	0.81		
8	3.72	caffeoyl-*O*-hexoside[a]	290, 310	341	343	135, 179, 161			2.61	1.13	
9	3.84	unidentified	280, 315	137	-	108, 92			0.84		
10	3.99	caffeoyl-*O*-hexoside[a]	290sh, 325	341	365 $[M+Na]^+$	161, 135, 179				0.30	
11	4.27	SL derivative (*m/z* 279-*O*-D-glucoside-proline conjugate)	215	556	558	114, 235, 279, 191, 394	0.74				
12	4.29	HPA derivative	220, 275	415	439 $[M+Na]^+$	101, 119, 113, 193, 165, 151		0.32			
13	4.58	caffeoyl-di-hexoside[a]	300br	549 $[M-H+HCO_2H]^-$, 503	527 $[M+Na]^+$	179, 161			0.28		
14	4.78	caffeoyl hexosyl-*O*-quinic acid	290sh, 320	515	-	191, 161			0.23		
15	4.92	feruloyl-*O*-hexoside	290, 325	355	-	134, 149, 193			0.72		
16	4.96	caffeoyl-*O*-hexoside[a]	290sh, 315	341	343	135, 179			3.29		
17	5.02	hydroxyphenylacetic acid[a]	220, 275, 315	151	-	not observed		3.12			
18	5.20	5-*O*-caffeoylquinic acid (chlorogenic acid)[a]	290sh, 325	353, 707 $[2M-H]^-$	355	191, 179			2.32	58.21	
19	5.46	caffeoyl-di-hexoside[a]	300br	503	-	161, 179, 323, 281, 341				3.84	
20	5.47	unidentified	220, 279	121	-	92		1.34			
21	5.48	caffeic acid[a]	290, 323	179	-	135, 134, 89			1.14		0.23
22	5.75	SL derivative	215	597	599	173, 435, 261, 131	1.08				
23	5.76	taraxacoside[a]	220, 275, 315sh	443 $[M-H+HCO_2H]^-$, 397	421 $[M+Na]^+$	175, 193, 165, 151, 113, 205		2.75	1.54		
24	6.20	unidentified	220, 275	419	397	not observed		0.50			
25	6.37	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447, 895 $[2M-H]^-$	449	151, 107, 133, 161			16.53		
26	6.86	coumaroylquinic acid	290sh, 310	337	-	191, 163, 119			0.35		
27	6.88	4-*O*-caffeoylquinic acid (cryptochlorogenic acid)[a]	290sh, 325	353	377 $[M+Na]^+$	173, 191				0.35	
28	7.00	SL derivative	220	512	514	217, 350, 392, 261	1.50				
29	7.09	SL derivative (*m/z* 261-*O*-D-glucoside-proline conjugate)	220	538	540	376, 114, 261, 217, 173	60.12				
30	7.34	coumaroyltartaric acid (coutaric acid)[a]	245, 290sh, 327	295, 591 $[2M-H]^-$	297, 319 $[M+Na]^+$	133, 179				12.54	
31	7.45	unidentified	220	431 $[M-H+HCO_2H]^-$, 385	409 $[M+Na]^+$	153, 205, 119	1.25				
32	7.53	unidentified	280, 315	423	-	225, 137, 165			0.22		
33	7.54	HCA	280, 325	463	487 $[M+Na]^+$	123, 149, 167, 311				0.72	
34	7.57	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447	471 $[M+Na]^+$	151, 107, 133, 161			0.46		
35	7.79	unidentified	265, 300	669	693 $[M+Na]^+$	489, 477, 373, 327			0.31		
36	7.99	feruloylquinic acid	290sh, 325	367	369, 391 $[M+Na]^+$	191, 93, 134			1.13		
37	8.09	4-methoxycinnamoyltartaric acid	290sh, 325	309	333 $[M+Na]^+$	161, 135, 133, 179				0.25	
38	8.43	HPA derivative	220, 275	475 $[M-H+HCO_2H]^-$, 429	453 $[M+Na]^+$	175, 151, 193, 165, 205		0.82			
39	8.44	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447, 895 $[2M-H]^-$	471 $[M+Na]^+$	151, 107, 133, 161			4.01		
40	8.44	feruloyltartaric acid	290sh, 325	325, 649 $[2M-H]^-$	327	161					0.16
41	8.45	HCA	290sh, 325	463	-	123, 149, 167, 311				0.15	
42	8.47	SL derivative (*m/z* 261-proline conjugate)	215	376	378	114, 217, 173, 261	14.46				
43	8.60	HCA	295, 320	195	-	108, 107, 136			2.07		
44	8.63	di-*O*-feruloyltartaric acid	290sh, 325	501	525 $[M+Na]^+$, 503	133, 113, 165, 175					0.29
45	8.64	HCA	290sh, 325	463	-	123, 149, 167, 311				0.32	
46	8.64	SL derivative (SL-proline conjugate)	215	524	526	114, 161	2.61				
47	8.68	HPA derivative	220, 275, 325sh	609	633 $[M+Na]^+$	193, 175, 337, 151			0.48		
48	8.82	di-*O*-feruloyltartaric acid	290sh, 325	501	503, 525	133, 177, 113, 165					0.12
49	8.84	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447	471 $[M+Na]^+$	151, 107, 133, 161			14.27	0.26	
50	8.96	HPA derivative	220, 275, 325sh	609	-	151, 107, 193, 175, 323, 417			0.99		
51	9.11	L-chicoric acid[a]	290sh, 327	473, 947 $[2M-H]^-$	497 $[M+Na]^+$	179, 149, 135, 219					63.04
52	9.17	di-hydroxyphenylacetate	220, 275	447, 895 $[2M-H]^-$	449, 471	151, 107, 133, 161			3.87		

(*continued on next page*)

Table 1 (*continued*)

		inositol ester (di-PIE)[a]			[M+Na]$^+$						
53	9.30	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447	471 [M+Na]$^+$	151, 107, 133, 161			0.43		
54	9.30	SL derivative (*m/z* 261-*O*-D-malonylglucoside-proline conjugate)[c]	220	624	626	376, 217, 261, 114, 173	0.92				
55	9.43	feruloylquinic acid	290sh, 327	367	369, 391 [M+Na]$^+$	135, 179, 161			0.82	7.71	
56	9.53	SL derivative	220	598 [M-H+HCO$_2$H]$^-$, 552	554	390, 217, 346, 124, 286, 270	0.98				
57	9.56	SL derivative	215	730	732	568, 306, 272, 550, 254		0.94			
58	9.58	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447	471 [M+Na]$^+$	151, 107, 133, 161			0.66		
59	9.88	unidentified	220, 267	321	-	181, 151	0.77				
60	9.90	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447, 895 [2M-H]$^-$	449, 471 [M+Na]$^+$	151, 107, 133, 161			3.30		
61	9.91	HPA derivative	220, 275	277, 415, 530	-	-		2.39			
62	10.01	HPA derivative	220, 275	477	501 [M+Na]$^+$	151, 107			0.29		
63	10.09	*meso*-chicoric acid[a]	290sh, 327	473, 947 [2M-H]$^-$	497 [M+Na]$^+$	149, 179, 135, 219					1.72
64	10.35	HPA derivative	220, 275, 325sh	609	633 [M+Na]$^+$	151, 107, 179, 217			1.24		
65	10.48	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447, 895 [2M-H]$^-$	449, 471 [M+Na]$^+$	151, 107, 133, 161			0.81		
66	10.79	HPA derivative	220, 275	609	633 [M+Na]$^+$	151, 107, 179, 217			2.30		
67	11.28	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447, 895 [2M-H]$^-$	449, 471 [M+Na]$^+$	175, 151, 165, 193, 133			3.80		
68	11.31	SL derivative	220	568	570	143, 272, 306, 254, 128, 179, 210		0.36			
69	11.45	HPA derivative	220, 275, 325sh	549	573 [M+Na]$^+$	151, 175, 193, 113, 281			0.48		
70	11.58	di-hydroxyphenylacetate inositol ester (di-PIE)[a]	220, 275	447	471 [M+Na]$^+$	175, 151, 165, 193, 133			2.55		
71	11.61	4-methoxycinnamoyltartaric acid	290sh, 327	309	311, 333 [M+Na]$^+$	161, 129, 133, 135				4.84	
72	11.75	SL (*m/z* 293-*O*-D-glucoside)	215	455	479 [M+Na]$^+$	249, 293, 217, 173		0.79			
73	11.95	di-*O*-caffeoylquinic acid[a]	290sh, 327	515, 1031 [2M-H]$^-$	517	191, 179, 353, 135					7.03
74	11.99	HCA	220, 275, 325sh	693	717 [M+Na]$^+$	179, 161, 151, 175, 263			0.21		
75	12.05	di-*O*-caffeoylquinic acid[a]	290sh, 325	515	517	191, 179, 353, 135					0.90
76	12.07	ferulic acid[a]	290sh, 325	193	-	133, 134, 161			0.72		
77	12.07	4-methoxycinnamoyltartaric acid	290sh, 330	309	311, 333 [M+Na]$^+$	161, 133, 135				4.16	
78	12.77	unidentified	220	579	603 [M+Na]$^+$	417, 181, 402			0.47		
79	12.87	ainslioside[a]	215	469 [M-H+HCO$_2$H]$^-$, 423	447 [M+Na]$^+$	261, 217, 173	0.59	3.39			
80	12.89	SL	215	455 [M-H+HCO$_2$H]$^-$, 409	433 [M+Na]$^+$	101, 119, 185, 113, 229, 149	1.79	2.71			
81	13.15	di-*O*-caffeoylquinic acid[a]	290sh, 327	515	517	173, 179, 191, 353, 135					0.55
82	13.23	taraxinic acid-*O*-β-D-glucoside[a]	220	469 [M-H+HCO$_2$H]$^-$, 423	447 [M+Na]$^+$, 425	217, 261, 173, 111	4.14	58.54			
83	13.52	HPA derivative	220, 275, 325sh	531	555 [M+Na]$^+$	175, 193, 151, 107			0.20		
84	13.65	11β,13-dihydrotaraxinic acid-*O*-D-glucoside[a]	215	471 [M-H+HCO$_2$H]$^-$, 425	449 [M+Na]$^+$, 427	263, 219, 175	1.54	4.98			
85	13.93	HPA derivative	220, 275	531, 1063 [2M-H]$^-$	555 [M+Na]$^+$	151, 175, 107, 193, 113			7.10		
86	14.05	HPA derivative	220, 275	795	797	481, 287, 175, 151, 225			0.33		
87	14.14	tri-hydroxyphenylacetate inositol ester (tri-PIE)[a]	220, 275	581	605 [M+Na]$^+$	151, 295, 107, 161, 133, 429			0.56		
88	14.37	tri-hydroxyphenylacetate inositol ester (tri-PIE)[a]	220, 275	581	605 [M+Na]$^+$	151, 295, 107, 161, 133, 429			0.21		
89	14.65	HPA derivative	220, 275	563	587 [M+Na]$^+$	151, 175, 193, 107, 113		0.86	0.94		
90	15.13	tri-hydroxyphenylacetate inositol ester (tri-PIE)[a]	220, 275	581	605 [M+Na]$^+$	151, 295, 107, 133, 161, 429			0.69	0.89	
91	15.21	tri-hydroxyphenylacetate inositol ester (tri-PIE)[a]	220, 275	581	605 [M+Na]$^+$	151, 295, 107, 161, 133, 429			0.36		
92	15.26	caffeoyl-*O*-feruloylquinic acid	290sh, 327	529	531	161, 179, 367, 135					2.16
93	15.46	tri-hydroxyphenylacetate inositol ester (tri-PIE)[a]	220, 275	581	605 [M+Na]$^+$	151, 295, 107, 133, 161, 429			3.00	0.96	
94	15.53	taraxinic acid-*O*-D-malonylglucoside[c]	220	1019 [2M-H]$^-$, 509	533 [M+Na]$^+$	261, 303, 275		0.59			
95	15.61	tri-hydroxyphenylacetate inositol ester (tri-PIE)[a]	220, 275	581	605 [M+Na]$^+$	151, 295, 107, 161, 133, 429			2.82		
96	15.65	caffeoyl-*O*-feruloyltartaric acid	290sh, 327	487	511 [M+Na]$^+$	163, 161, 179					0.30
97	15.93	11β,13-dihydrotaraxinic acid-*O*-D-malonylglucoside[c]	220	1023 [2M-H]$^-$, 511	535 [M+Na]$^+$, 513	263, 305, 277, 335, 407		0.72			
98	16.53	caffeoyl-*O*-feruloylquinic acid	290sh, 327	529	531, 553 [M+Na]$^+$	179, 161, 367, 135, 349					0.40
99	16.55	tri-hydroxyphenylacetate inositol ester (tri-PIE)[a]	220, 275	581	605 [M+Na]$^+$	151, 295, 107, 133, 161, 429			0.52	0.75	
100	16.69	caffeoyl-*O*-feruloyltartaric acid	290sh, 330	487, 973 [2M-H]$^-$	511 [M+Na]$^+$	163, 161, 145, 179					20.82
101	16.82	HPA derivative	220, 275	743	767 [M+Na]$^+$	151, 393, 217, 179, 197				0.19	
102	17.04	HPA derivative	220, 277	633	635	151, 225, 243, 193,			2.36		

(*continued on next page*)

Table 1 (*continued*)

						267, 175, 287, 481					
103	17.05	ainsliolide (desgluco ainslioside)	220	261	285 $[M+Na]^+$	not observed		0.43			
104	17.48	taraxinic acid[a]	217	261	285 $[M+Na]^+$	175, 163, 111, 151, 147	0.93	7.75			
105	18.02	11β,13-dihydrotaraxinic acid[a]	220	263	287 $[M+Na]^+$	139, 245	3.64	5.12			
106	18.05	caffeoyl-*O*-feruloylquinic acid	290sh, 323	529	-	235, 219, 179, 349, 367					0.20
107	19.09	HPA derivative	220, 275	515, 561 $[M-H+HCO_2H]^-$	571, 539 $[M+Na]^+$	151, 159, 175, 135, 193, 113		1.21			
108	19.67	caffeoyl-*O*-feruloylquinic acid	290sh, 323	529	553 $[M+Na]^+$	179, 235, 219					0.17
109	20.34	unidentified	220	327	351 $[M+Na]^+$	211, 171, 183, 229	1.44				
110	22.06	unidentified	220	329	353 $[M+Na]^+$	211, 183, 171, 229	1.50				
111	22.46	di-*O*-feruloyltartaric acid	290sh, 335	501	525 $[M+Na]^+$	161, 179, 135, 339					0.50
112	23.16	caffeoyl-*O*-feruloylquinic acid	290sh, 330	529	553 $[M+Na]^+$	205, 161, 179, 135, 367					0.69
Legend:							Total (%Area)				
hydroxycinnamic acid (HCA)									16.74	96.95	100.00
hydroxyphenylacetic acid (HPA) derivative and hydroxyphenylacetate inositol ester (PIE)								11.47	80.61	3.05	
sesquiterpene lactone (SL)							95.04	86.32			
unidentified							4.96	2.21	2.65		

[a] – compound already confirmed in *T. officinale* organs (roots, leaves, flowers, latex)
[b] – base peak ions highlighted by underlining
[c] – presumably new compound

[a] – compound already confirmed in *T. officinale* organs (roots, leaves, flowers, latex).
[b] – base peak ions highlighted by underlining.
[c] – presumably new compound.

spectroscopic data and information on the relative content of major phytochemicals in dandelion root preparations are presented in Table 1.

As shown in Table 1, in total, about 100 different compounds were tentatively identified in the five dandelion fractions (A-E), which then could be classified into three different groups of secondary metabolites, i.e. HCA derivatives, hydroxyphenylacetic acid (HPA) derivatives, and SL derivatives. Among them, the most abundant were HCA esters (42 compounds) and HPA derivatives (39 compounds), while 20 compounds were assigned to SLs, and other 11 minor compounds could not be identified and classified. As shown by UPLC-UV-MS analysis, the five dandelion root fractions A-E differed significantly in the final qualitative chemical composition; corresponding UPLC-UV (max plot) chromatograms of the five dandelion root fractions are demonstrated in Fig. 2.

Different sesquiterpene lactones and SL derivatives, including glycosides and conjugates with amino acids, were the dominant constituents of the two fractions A and B, where they constituted 95% and 86% of the total compounds, respectively (Fig. 2, Table 1). As revealed

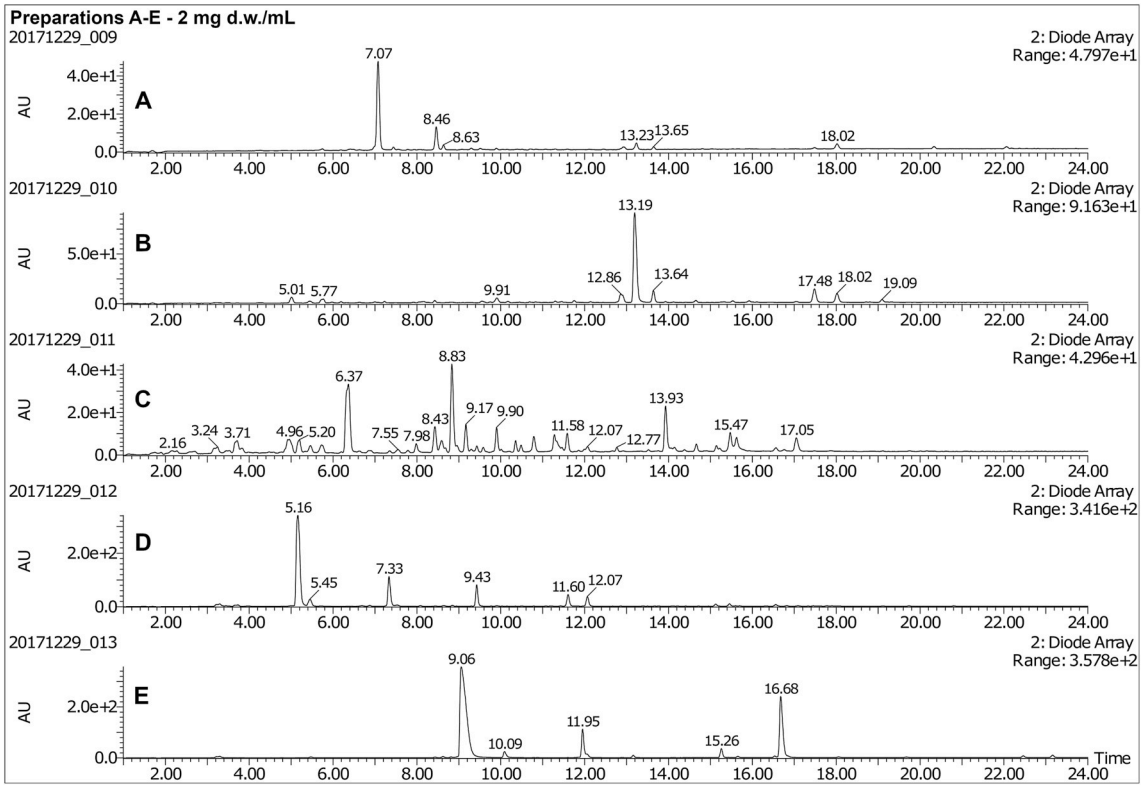

Fig. 2. LC-UV (max plot) chromatograms of dandelion root preparations (A–E).

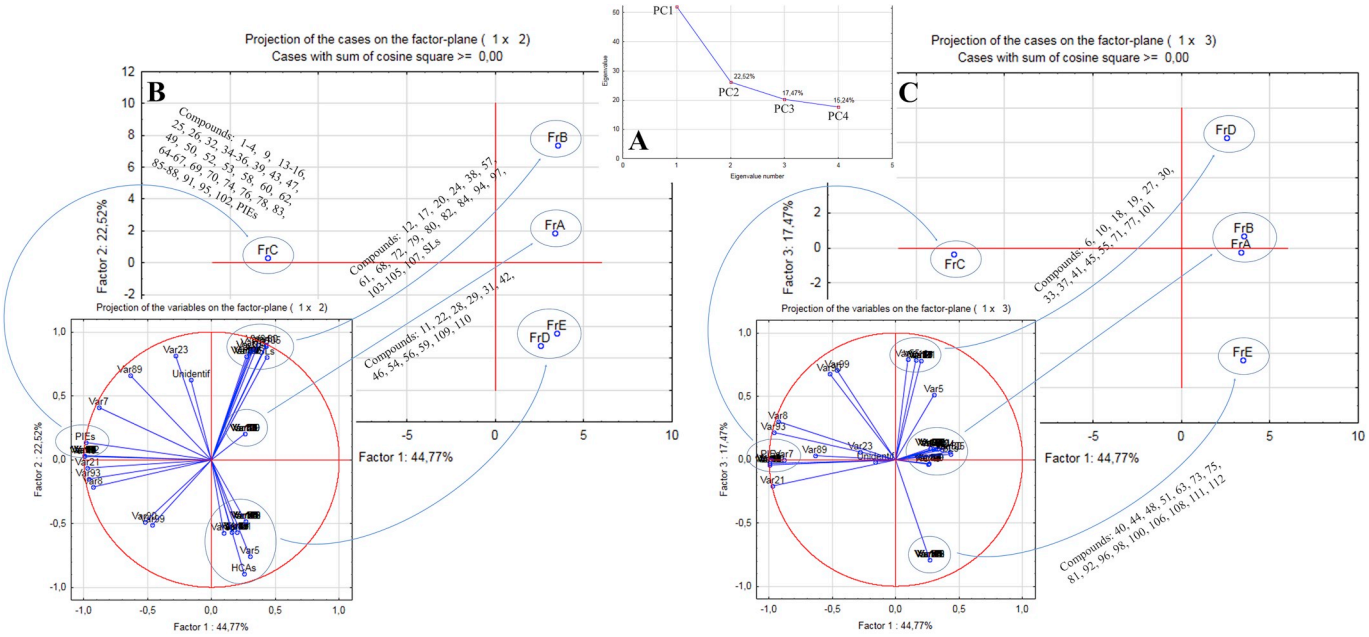

Fig. 3. Results of PCA analysis for dandelion root fractions: the four derived PCs with eigenvalues explaining the percentage of total variation (A); representation of dandelion fractions and variables as functions of the PC1 vs PC2, with marked relationships between compounds and dandelion preparation (B); representation of dandelion fractions and variables as functions of the PC1 vs PC3, with marked relationships between compounds and dandelion preparation (C).

by acid hydrolysis and subsequent LC-UV-MS/MS analysis of the cleavage products, the hexose attached to several present SL compounds was identified as D-glucose, while the analysis of amino acids confirmed the presence of proline (Pro), glutamic acid (Glu) and glycine (Gly) in the preparation A. The amino acid-sesquiterpene adducts were present only in the preparation A, where the two compounds 29 and 42 ($[M-H]^-$ at *m/z* 538 and 376; Table 1), tentatively identified as *O*-D-glucoside SL ($[M-H]^-$ at *m/z* 261)-proline adduct and its desgluco derivative, were the dominant components (together about 75% of the total compounds). Moreover, the presence of at least seven other SL-amino acid adducts (compounds 11, 28, 46, 54, 56, 57 and 68; Table 1) was confirmed in fraction A. In turn, the major constituent of the preparation B (about 60% of the total compounds) was identified as taraxinic acid-*O*-β-D-glucoside (compd. 82; Table 1) on the basis of comparison (retention time and *m/z* value) with isolated standard compound. Additionally, other eleven SLs and their glucosides were found in this fraction, including taraxinic acid (compd. 104), 11β,13-dihydrotaraxinic acid and its glucoside (compd. 105 and 84), as well as two malonylated derivatives of both (compd. 94 and 97; Table 1). Furthermore, the preparation B contained also smaller amounts of hydroxyphenylacetic acid esters (seven compounds comprising over 10% of the total) in addition to the predominant SLs (Fig. 2, Table 1).

Preparation C contained mainly HPA derivatives (overall 33 compounds), such as PIEs, which together accounted for about 80% of all compounds; and lacked by far the dominant component, as in the case of other fractions (Fig. 2). Moreover, among hydroxyphenylacetic acid esters in this fraction, the di-hydroxyphenylacetate inositol esters (di-PIEs; $[M-H]^-$ at *m/z* 447) were present in the largest amount (11 compounds comprising about 50% of the total compounds), whereas tri-hydroxyphenylacetate inositol esters (tri-PIEs; $[M-H]^-$ at *m/z* 581) and other HPA derivatives accounted for 8% (7 compounds) and 18% (15 compounds) of all compounds, respectively. Furthermore, the fraction contained also smaller amounts of HCA derivatives (14 compounds comprising over 15% of all compounds), which were mainly caffeic and ferulic acid esters (Table 1).

The hydroxycinnamic acids, mainly caffeic, coumaric and ferulic acid esters, were the dominant constituents of the preparations D (15 compounds) and E (18 compounds) where they constituted over 95% and 100% of the total compounds, respectively (Fig. 2, Table 1). The major constituent of the fraction D (about 60% of all compounds) was identified as 5-*O*-caffeoylquinic acid (compd. 18; Table 1) on the basis of comparison (retention time and *m/z* value) with the standard. Moreover, in this preparation, in addition to chlorogenic acids (caffeoyl- and feruloylquinic acids) occurred also several HCA esters of tartaric acid (compd. 5, 30, 37, 71, 77; Table 1) which comprised together about 25% of all compounds. Additionally, the five minor PIEs were identified in fraction D, which together constituted about 3% of total compounds. In turn, preparation E contained exclusively caffeic and ferulic acid esters of tartaric (9 compounds comprising together about 90% of all compounds) or quinic acid (chlorogenic acids; 8 compounds comprising together about 10% of all compounds). The major constituent of this fraction (over 60% of all compounds) was identified as L-cichoric acid (compd. 51; Table 1) on the basis of comparison (retention time and *m/z* value) with the isolated standard compound.

The LC-UV-MS data on the relative content of individual compounds in dandelion root preparations were used, after standardization, to perform the principal component analysis. Hence, the PCA permitted a reduction of 116 variables (including 112 identified compounds, total HCAs, PIEs, SLs, and unidentified compounds) to four principal components (PC1-PC4) which explained the entire tested variation (100%) (Fig. 3A). Fig. 3B and C shows the associations obtained between the tested 116 variables and the five dandelion root fractions (PC1 vs PC2, and PC1 vs PC3). The inspection of Fig. 3 reveals that each dandelion preparation creates its own group that is formed by distinct and relevant variables i.e. compounds present only or in large quantities in a given fraction (Table 1).

3.2. DPPH free radical scavenging activity

The antiradical capacity of the five dandelion root fractions (A-E) was expressed as TEAC and IC_{50}. The results are shown in Table 2. Only the two tested preparations D and E possessed good DPPH radical scavenging activity. The estimated TEAC values varied from 0.02 to 0.75 of Trolox equivalents. Fraction E presented the highest scavenging activity (0.75 of TE), followed by fraction D (0.51 of TE), fraction C

Table 2
Antioxidant activity estimated by DPPH● method.

Sample	Trolox equivalents (TE)	IC_{50} (mg DW/mL)
Fraction A	0.02 ± 0.00a[a]	> 2
Fraction B	0.02 ± 0.00a	> 2
Fraction C	0.06 ± 0.00b	1.901 ± 0.033c
Fraction D	0.51 ± 0.01c	0.215 ± 0.003b
Fraction E	0.75 ± 0.01d	0.134 ± 0.002a
Trolox	1.00e	0.120 ± 0.003a

Values are means (n = 3) ± standard deviation.
[a] Different letter within a column indicates significant differences ($p < 0.05$).

(0.06 of TE), and finally fractions A and B (0.02 of TE). With regard to the IC_{50} values, it was not possible to determine it for preparations A and B at the range of concentrations tested in the study. At the highest concentration (2 mg/mL), only about 15 and 15.5% of DPPH radicals were scavenged by fractions A and B, respectively. Summarizing, the DPPH scavenging activity was in the following order: Trolox > Fraction E > Fraction D > Fraction C > Fraction B ≥ Fraction A.

3.3. Effects on oxidative stress markers in human plasma

The effect of the five dandelion root preparations (A-E; concentration range 0.5–50 μg/mL; incubation time 30 min) on the level of selected biomarkers of oxidative stress in plasma was studied *in vitro*. First of all, the tested plant preparations did not exert any significant effect on the level of biomarkers of oxidative stress in plasma not treated with H_2O_2/Fe (data not shown). Exposure of plasma to a strong physiological oxidant – H_2O_2/Fe resulted in a significantly enhanced level of lipid peroxidation, and oxidation of protein thiols and protein carbonylation (Fig. 4). As shown in Fig. 4a, all tested dandelion root preparations (A-E) significantly inhibited plasma lipid peroxidation induced by H_2O_2/Fe starting from the dose 5 μg/mL, and the strongest effect was observed at the concentration of 50 μg/mL. Nevertheless, the best results were obtained for the three fractions C, A, and E (reduction by more than 30% in comparison to control positive). On the other hand, no positive effect was observed in the five tested fractions in the lowest tested concentration (0.5 μg/mL). Additionally, the preparation's activity (A-E) was not concentration-dependent for 30 min of the incubation time (Fig. 4a).

As shown in Fig. 4b, all tested dandelion root preparations (A-E) were found to protect human plasma against H_2O_2/Fe-induced oxidation of protein thiols, however, some fractions, such as B, C, and D, did not exert activity within the whole range of tested concentrations (0.5–50 μg/mL). The effect for a given fraction depended largely on the dose used. Fractions A, B, and D showed the highest activity at higher tested concentrations (5–50 μg/mL). Whereas, fraction C exerted the strongest effect at lower doses (0.5–5 μg/mL), and fraction E showed significant activity both at the lowest (0.5 μg/mL) and highest doses (10 and 50 μg/mL). In general, the presence of preparations A and C had the strongest effect on the protection of thiol groups (more than a three-fold increase in the level of thiol groups in comparison to control positive). Moreover, all tested dandelion fractions inhibited the increase of plasma protein carbonylation induced by H_2O_2/Fe (Fig. 4c).

3.4. Effects on hemostatic parameters of human plasma and blood platelets

Analysis of the effect on the coagulation activity of plasma showed that none of dandelion root preparations (A-E; concentration range 0.5–50 μg/mL; incubation time 30 min) changed the activated partial thromboplastin time (APTT) and the prothrombin time (PT) (data not shown). On the other hand, the five dandelion fractions (A-E) significantly increased the thrombin time (TT), throughout the investigated range, when plasma was pre-incubated with tested fractions (Fig. 5a). However, the preparation's activity was not concentration-dependent for 30 min of incubation time. In general, the weakest results were observed in the presence of fraction A (increase below 10% in comparison to control), while the strongest effect was recorded for the two fractions D and E (increase above 15% in comparison to control) (Fig. 5). At the same time, the five dandelion preparations (A-E) were not as active in prolongation of the thrombin time when plasma was incubated with the mixture of pre-incubated thrombin and tested fraction, because only the two fractions D and E, and only at the highest dose 50 μg/mL, significantly increased the TT. The effect of the other three preparations A-C was not significant throughout the investigated range (Fig. 5b). In conclusion, the best overall anticoagulant effect was obtained in the presence of the two polyphenol preparations D (chlorogenic acids enriched fraction) and E (chicoric acid enriched fraction), whereas it was clearly the weakest in the case of fraction A (SL-amino acid adducts enriched fraction).

In order to verify the effect of root preparations on thrombin-induced platelet aggregation, a light transmission aggregometry method was used. To ensure full blood platelet aggregation, 1 U/mL of thrombin was added. We observed that none of the root preparations changed the platelet aggregation stimulated by thrombin (Fig. 6).

3.5. Cytotoxicity against blood platelets

The cytotoxicity of root preparations A-E on human blood platelets was evaluated by measuring the extracellular LDH activity. We observed that none of the tested fractions caused the lysis of platelets, determined as leakage of lactate dehydrogenase into the extracellular medium (Fig. 7).

4. Discussion

Our findings showing the presence of three major classes of secondary metabolites in *T. officinale* roots i.e. hydroxycinnamic acids, sesquiterpene lactones, and hydroxyphenylacetic acid derivatives, are generally consistent with the results obtained by other authors (Kenny et al., 2014b; Schütz et al., 2005; Williams et al., 1996; Xue et al., 2017). To date, more than 10 HCAs and their esters have been isolated and identified in dandelion roots, including L- and *meso*-chicoric, caftaric, coutaric, chlorogenic, cryptochlorogenic, neochlorogenic, caffeic, ferulic and *o*- and *p*-coumaric acids (European Medicines Agency, 2009). Additionally, several other hydroxycinnamic acid derivatives, such as caffeoyl hexosides, caffeoyl-cumaroyltartaric acid, and other caffeic acid derivatives, have been reported in an alcoholic extracts from dandelion herb (Jedrejek et al., 2017) or the whole plant (Schütz et al., 2005), therefore their presence in the roots has not been entirely clear. Thanks to the applied extract's purification and fractionation process we were able to detect the much larger number of HCAs (42 compounds), including 16 known components of the dandelion plant, and other 26 HCAs that are reported here for the first time as *T. officinale* root constituents (Table 1).

Sesquiterpene lactones are common in plants of the Asteraceae family. They are generally enriched in the plant root and are often present as glycosides. To date, nine SLs have been isolated and identified in *T. officinale* root and leaf, including two eudesmanolides (4α,15,11β,13-tetrahydroridentin B and taraxacolide-1′—*O*-β-D-glucopyranoside), five germacranolides (taraxinic and 11β,13-dihydrotaraxinic acids and their glucosyl esters, and ainslioside) and two guaianolides (ixerin D and 11β, 13-dihydrolactucin) (Esatbeyoglu et al., 2017; Kisiel and Barszcz, 2000). Moreover, many other terpene lactones and their glycosides have been reported in other dandelion species (Martinez et al., 2015). We detected 20 different SLs and their derivatives, including several glucosides and amino acid adducts, in our five dandelion root preparations (Table 1). The five of them could be assigned to the already known guaianolide type terpene lactones described in *T. officinale* (Table 1). Of the remaining fifteen tentatively identified SL derivatives,

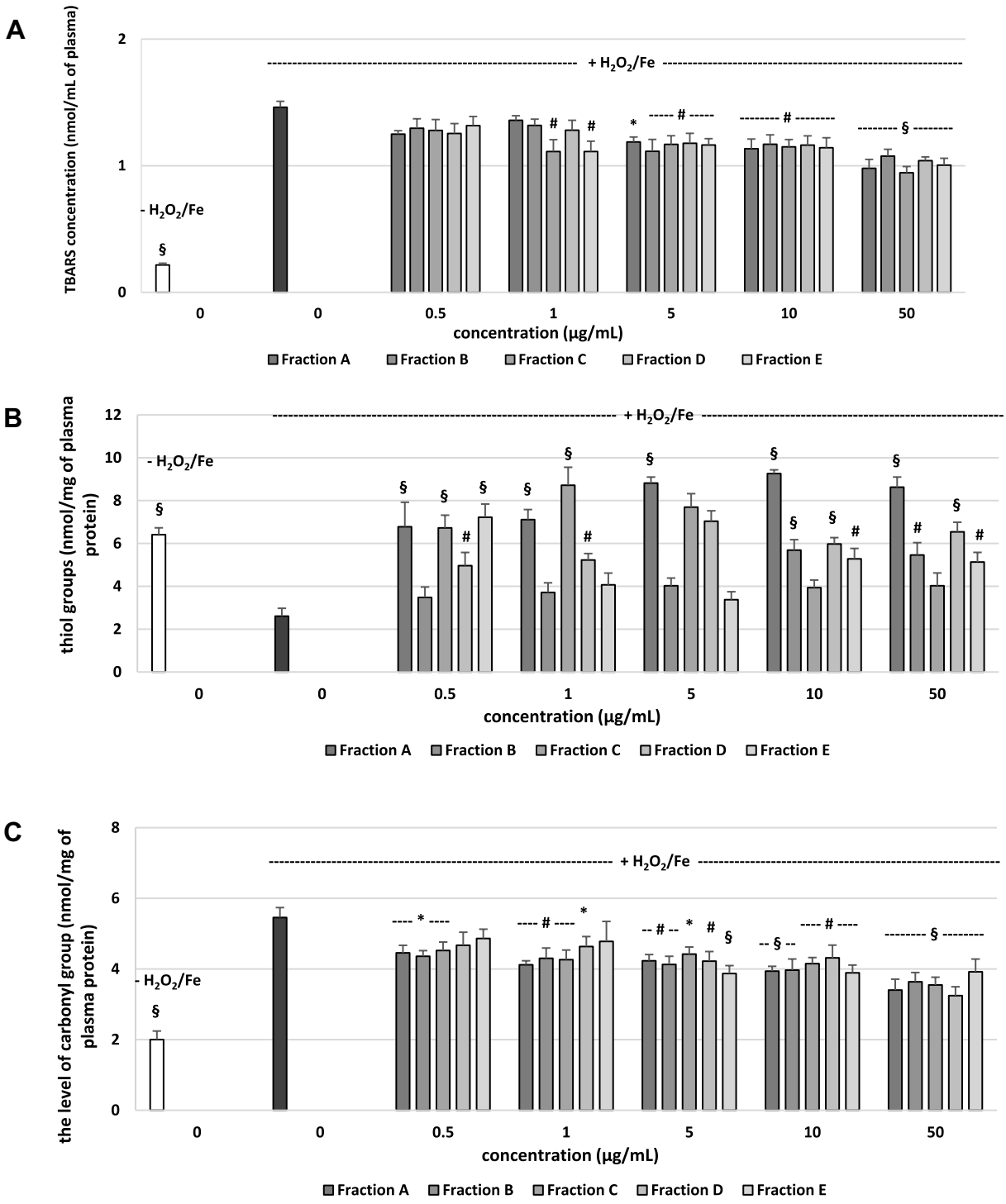

Fig. 4. Effects of the five dandelion root preparations (A-E, concentration range 0.5–50 µg/mL, pre-incubation time – 5 min) on lipid peroxidation in plasma treated with H_2O_2/Fe (incubation time – 25 min) (a), and on the oxidative damages of plasma protein treated with H_2O_2/Fe: the level of thiol groups (b) and protein carbonylation (c). Results are given as mean ± SEM (n = 5–8). Control negative (white bar) refers to plasma not treated with H_2O_2/Fe, whereas control positive (black bar) to plasma treated with H_2O_2/Fe. One-way ANOVA followed by a multicomparison Duncan's test: *p < 0.05, #p < 0.01, §p < 0.001, compared with positive control (treated with H_2O_2/Fe).

a group of several amino acid-sesquiterpene lactone conjugates (mainly containing proline) deserves special attention as they have never been described as constituents of any dandelion species. Nevertheless, compounds of this type have been isolated and identified in two other plant species of the Asteraceae family i.e. *Saussurea lappa* (Yoshikawa et al., 1993) and *Ixeris dentata* (Cha et al., 2012). The amino acid-sesquiterpene lactone conjugates are supposed to be produced biogenetically in plants *via* Michael type attack of endogenous amino acids, such as L-proline and L-alanine (Cha et al., 2012). Moreover, to the best of our knowledge, the presence of malonylglucoside (indicated by MS/MS product ion $[M\text{-}H]^-$ at *m/z* 248) attached to the terpene lactone has also never been reported in *Taraxacum* genus and most probably in the plant kingdom, thus the three detected SL malonylglucosides are presumably new compounds (Table 1).

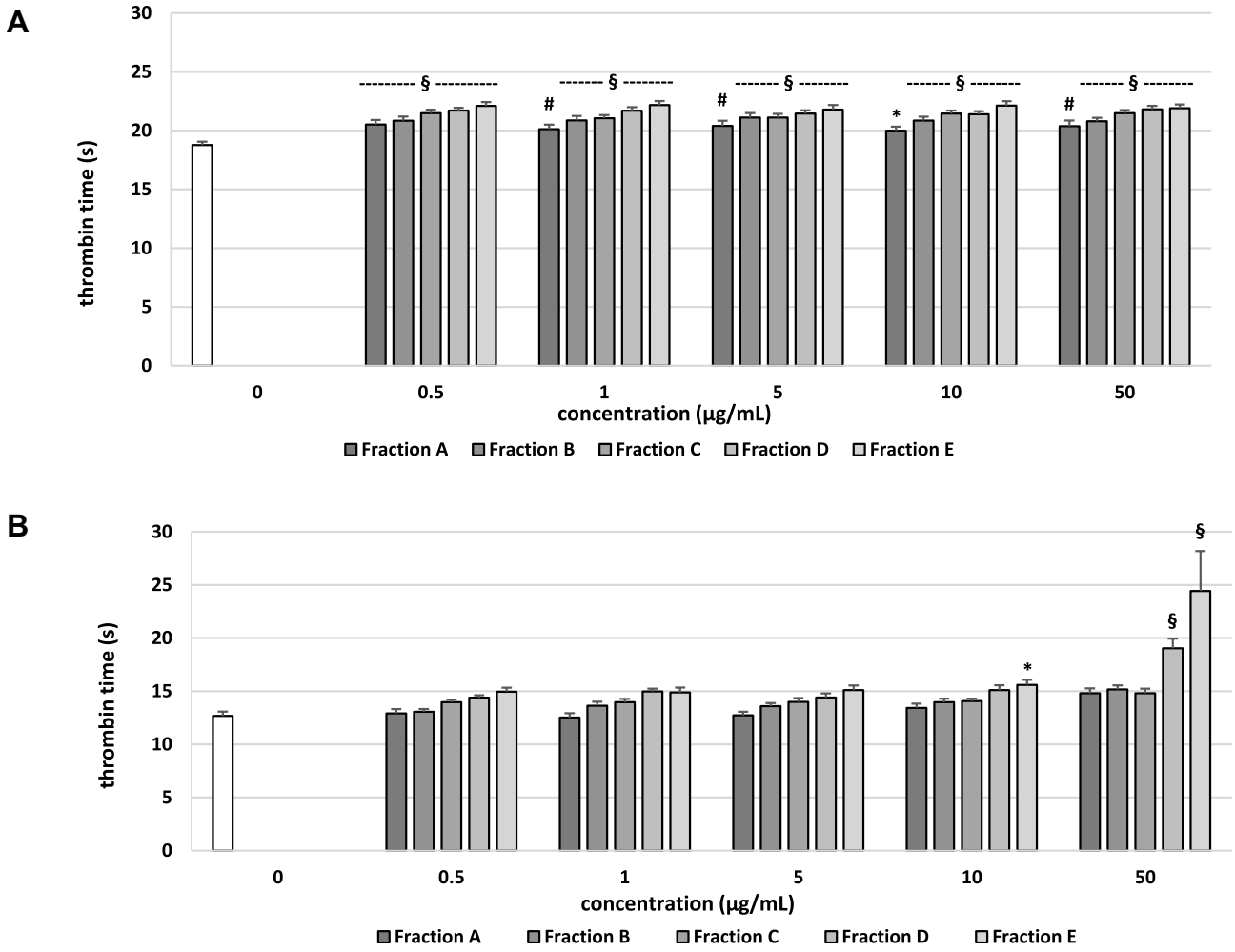

Fig. 5. Effects of the five dandelion root preparations (A-E, concentration range 0.5–50 µg/mL, incubation time – 30 min) on the hemostatic parameters (thrombin time – TT) of human plasma: the plasma was incubated with tested fractions and then with thrombin (a), and plasma was incubated with the mixture of pre-incubated thrombin and tested fractions (b). Data are expressed as mean ± SEM (n = 8–13). One-way ANOVA followed by a multicomparison Duncan's test: *p < 0.05, #p < 0.01, §p < 0.001, compared with control.

In the previous studies by Rauwald and Huang (1985), Kenny et al. (2014b) and Huber et al. (2015) a number of 4-hydroxyphenylacetic acid esters were identified in *T. officinale* organs, including 4-hydroxyphenylacetic acid, taraxacoside, and the series of isomeric di- and trihydroxyphenylacetic acid inositol esters (five different di-PIEs and four tri-PIEs). We were able to detect 39 different HPA derivatives, including 11 di-PIEs, 7 tri-PIEs, 3 mono-PIEs, taraxacoside and 16 other unidentified HPA derivatives having a common UV spectrum (220, 275 nm) and MS/MS product ion $[M\text{-}H]^-$ at *m/z* 151 (Table 1). Considering that only about 10 hydroxyphenylacetic acid esters have been described so far as constituents of *T. officinale*, most of recognized by us the HPA derivatives seem to be new for this plant species.

In the end, in some phytochemical studies, the presence of minor flavonoids was reported in dandelion roots, mainly luteolin and apigenin (Huber et al., 2015; Kenny et al., 2014a). However, we did not detect any flavonoid components in the five tested *T. officinale* root preparations, and these results are also in agreement with previous reports (Williams et al., 1996; Xue et al., 2017).

Oxidative stress is known as one of the major contributors to diverse human pathological processes (Lobo et al., 2010; Xue et al., 2017), therefore, search for potent natural antioxidants has been conducted for reducing harmful oxidative stress and further health-promoting effect. Several studies conducted both *in vitro* and *in vivo* have demonstrated the anti-oxidative effect of either dandelion extracts or individual compounds extracted from dandelion herb or root (Esatbeyoglu et al., 2017; Kenny et al., 2014a; Mingarro et al., 2015; Xue et al., 2017; You et al., 2010). The responsibility for the antioxidant activity of dandelion was almost exclusively attributed to polyphenols, and among them mainly flavonoids and hydroxycinnamic acids (Mingarro et al., 2015; You et al., 2010), and sometimes even without a detailed analysis of the chemical composition of the tested plant material, as the properties of phenolic compounds to efficiently scavenge reactive oxygen and nitrogen species are well documented (Cai et al., 2006).

In the DPPH free radical scavenging activity test, all studied *T. officinale* root preparations (A-E), that significantly differed in the chemical composition, demonstrated antiradical activity, estimated as Trolox equivalents (TEAC) or IC_{50} values (Table 2). However, under the conditions used (methanol as reaction medium), the two fractions E and D, characterized as hydroxycinnamic acid enriched fractions, showed significantly higher radical scavenging effect than other tested fractions: fraction C - moderate action, and fractions A and B – very weak action. Cinnamic acid derivatives have been previously reported as compounds primarily responsible for *in vitro* antioxidant activity of many plant extracts, including dandelion flower, leaf and root extract (Mingarro et al., 2015; Kenny et al., 2014a,b; Xue et al., 2017). Additionally, the clear presence of some HCA in fraction C (in addition to dominant hydroxyphenylacetic acid inositol esters; Table 1) could have a significant impact on demonstrated moderate $DPPH^{\bullet}$ scavenging activity (Table 2), as the results of recent study of Mo et al. (2017) have shown very weak antiradical effect in $DPPH^{\bullet}$ test of several HPA

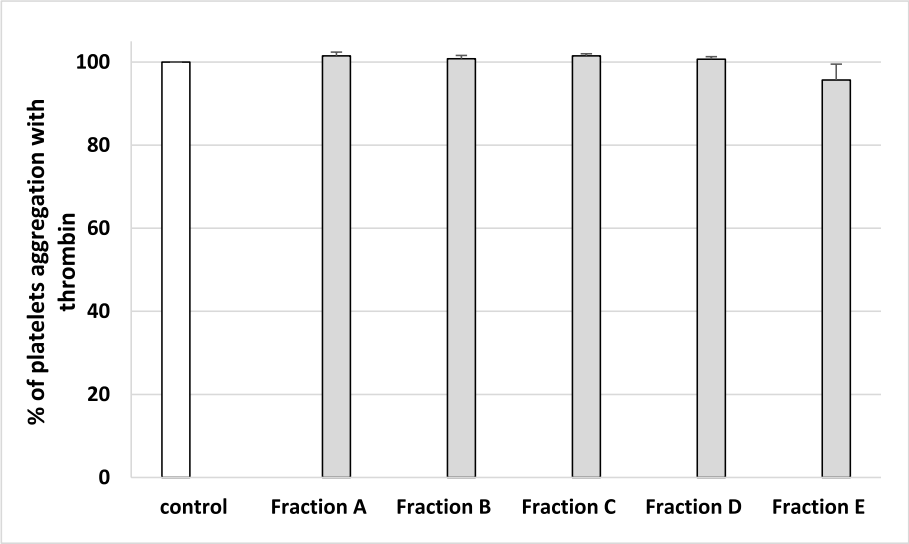

Fig. 6. Effects of the five dandelion root preparations (A-E, concentration - 50 μg/mL, incubation time – 30 min) on blood platelet aggregation, stimulated by thrombin. Data are expressed as mean ± SEM (n = 4). One-way ANOVA followed by a multicomparison Duncan's test.

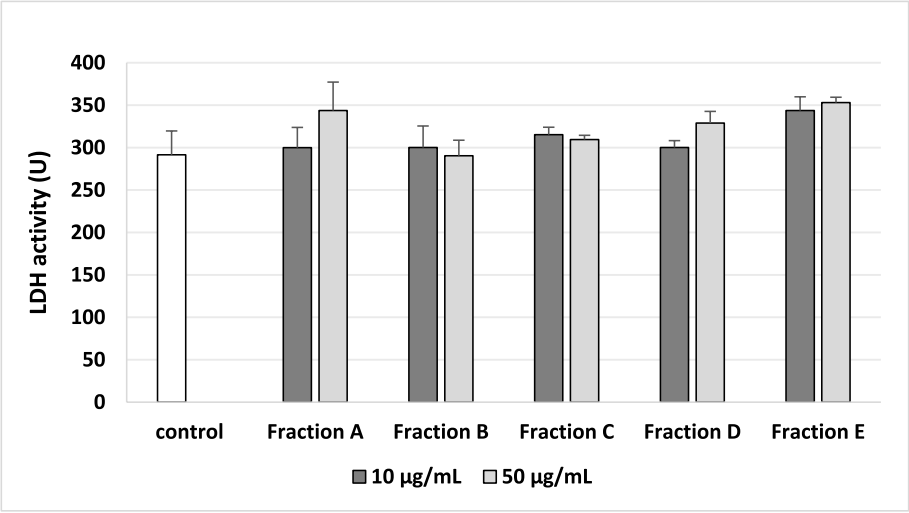

Fig. 7. Effects of the five dandelion root preparations (A-E, concentration range 10 and 50 μg/mL, incubation time – 30 min) on the damage of human blood platelets. Data are expressed as mean ± SEM (n = 6). One-way ANOVA followed by a multicomparison Duncan's test.

inositol esters isolated from *T. coreanum*. Moreover, two dandelion preparations that showed the weakest DPPH$^{\bullet}$ scavenging action (preparations A and B) have been characterized by high content of sesquiterpene lactones which have been found as compounds lacking direct anti-oxidative activity (Chadwick et al., 2013). In this way, our results are generally consistent with the published data.

Subsequently, dandelion fractions have been evaluated for *in vitro* antioxidant action in blood plasma. In our present study, the five *T. officinale* root preparations (A-E) showed anti-oxidative action against harmful to plasma lipid and protein oxidation induced by a strong oxidant – H_2O_2/Fe, nevertheless, they differed in the demonstrated antioxidant potential. In agreement with the previous findings (Kenny et al., 2014a; Mingarro et al., 2015; Xue et al., 2017) and earlier DPPH$^{\bullet}$ test, preparations D and E (hydroxycinnamic acid enriched fractions) showed potent antioxidant activity, however, the best overall protective effect was observed for the other two preparations A (SL-amino acid adducts enriched fraction) and C (PIEs enriched fraction). Thus, the results of the preliminary assessment of the anti-oxidative action of the tested fractions using an inorganic experimental system (DPPH$^{\bullet}$ test) did not coincide with those obtained in the biological experimental system (blood plasma). In this way, our study revealed the possible contribution of other groups of compounds, such as sesquiterpene lactone derivatives and hydroxyphenylacetic acid inositol esters, to the anti-oxidative action reported for dandelion root extract.

It was found that SLs do not exert direct anti-oxidative action, which is attributable to their structure, but the presence of additional components attached to them, such as allyl alcohol, can affect the activity (Chadwick et al., 2013). Nevertheless, in our *in vitro* experiments, both SL enriched fractions (A and B) exerted protective action in plasma lipids and proteins against oxidation by H_2O_2/Fe. However, preparation B, containing a significant amount of taraxinic acid-*O*-β-D-glucoside, showed weaker anti-oxidative potential than preparation A, characterized by high content of SL-amino acid adducts, and other tested preparations C-E. In a few biological studies on amino acid-sesquiterpene lactone conjugates, some compounds have been found to exert potent anti-inflammatory action by inhibiting iNOS enzyme activity, iNOS, and HSP 72 proteins, and NF-κB activation (Matsuda et al., 2003), even though they had reduced the α-methylene-γ-lactone group, considered as chemical mediator of SLs. In turn, Cha et al. (2012) reported the weak inhibitory effect of SL-amino acid derivatives on the

proliferation of cultured human cancer cell lines, which was explained by the reduction of the *exo*-methylene group of the tested compounds. In our present experiment, the extremely high antioxidant potential of preparation A possibly linked to the presence of amino acid fragment, such as proline, attached to sesquiterpene lactone, because as has been discovered all 20 amino acids have the potential to interact with free radicals (e.g. hydroxyl radical) (Elias et al., 2008). Interestingly, preparation C, characterized by high content of hydroxyphenylacetate inositol esters, also showed overall antioxidant action greater than HCA rich preparations (D and E). Moreover, it seems to be important that this fraction showed high activity even at low concentration (0.5–5 μg/mL), as for thiol groups marker. In the recent study by Mo et al. (2017), six HPA derivatives isolated from *Taraxacum coreanum* demonstrated weak activity in DPPH radical scavenging test. Therefore, the explanation of the mechanism of the protective action of PIEs against oxidation of plasma lipids and proteins needs further investigation. Additionally, inositol derivatives have been found to possess anti-inflammatory and anti-diabetic activity (Garayev et al., 2017; Worawalai et al., 2015).

Changes in hemostasis, including coagulation process or blood platelet activation, are often associated with oxidative stress and can lead to the development of cardiovascular disorders. In our previous study on the four *T. officinale* leaf and petal fractions, characterized as HCA or flavonoid-rich preparations, we found that the two petal fractions showed higher anticoagulant potential than corresponding leaf fractions (Lis et al., 2018). For example, in the test when human plasma was incubated with dandelion preparations followed by thrombin addition, both petal preparations prolonged the thrombin time in the entire range of tested concentrations (0.5–50 μg/mL). We concluded that the anticoagulant activity of tested dandelion fractions can be correlated with modulation of thrombin's activity, which is plasma enzyme acting a vital function in hemostasis (Lis et al., 2018). In several studies, it has been demonstrated that inhibition of this enzyme activity may be due to the presence of polyphenols, especially flavonoids (Olas et al., 2018; Sikora et al., 2014).

In our present study, the five dandelion preparations (A-E) significantly prolonged the thrombin time when plasma was treated with tested fraction followed by enzyme addition (throughout tested concentration range 0.5–50 μg/mL), however, only the two preparations D and E (hydroxycinnamic acids enriched preparations) were active when plasma was treated with pre-incubated dandelion fraction and thrombin. The differences in chemical profiles of tested dandelion fractions may possibly explain the various effect on coagulation activity of plasma. The observed anticoagulant potential of polyphenol-rich fractions (D and E) stays in agreement with previous findings, while for other fractions (A-C) the explanation of the mechanism responsible for this process seems to need further investigation.

The uncontrolled platelet aggregation is one of the most important risk factors of the cardiovascular system disturbance. Moreover, in different cardiovascular diseases the presence of activated platelets in the systemic circulation was observed (Stakos et al., 2012). However, the observed action of five root preparations (at the highest used concentration – 50 μg/mL) on aggregation stimulated by thrombin was not statistically significant, and one of the possibilities is that tested fractions, and compounds contained in them, do not interact with thrombin receptors on platelet membrane. In addition, none of the tested dandelion root preparations caused the lysis of blood platelets, at all tested range. Thus, we can confirm dandelion roots as safe plant material.

Noteworthy is also the fact that despite demonstrating the anticancer potential of dandelion roots in several different studies (Ovadje et al., 2011, 2012; 2016; Sigstedt et al., 2008), it has not yet been possible to identify the appropriate component responsible for this activity.

5. Conclusion

In this work, the dandelion root was separated, applying different chromatographic techniques, into five preparations differing in phytochemical content. UPLC-ESI-MS/MS system and other chemical analyzes (hydrolysis) were used to identify in total about 100 different compounds in the test fractions, including mainly hydroxycinnamic acids, hydroxyphenylacetic acid inositol esters, and sesquiterpene lactone derivatives. The results of a detailed phytochemical study of root preparations revealed the presence of numerous presumably new compounds, both for the genus *Taraxacum* and the plant kingdom, such as amino acid-sesquiterpene adducts. The five dandelion fractions were evaluated for *in vitro* antioxidant and anticoagulant properties. In an inorganic experimental system ($DPPH^{\bullet}$ test) two preparations characterized by high content of hydroxycinnamic acids (D and E) showed the highest radical scavenging activity, while the other three preparations demonstrated weaker action. In turn, in the biological experimental system (blood plasma) the best overall protective effect against oxidation by H_2O_2/Fe was obtained in the presence of preparations A (SL-amino acid adducts enriched fraction) and C (PIEs enriched fraction). However, the mechanism of their antioxidant activity remains unclear and requires further studies. On the other hand, a stronger anticoagulant effect was demonstrated for *T. officinale* preparations enriched with hydroxycinnamic acids (fractions D and E), therefore these kinds of compounds seem to be primarily responsible for this activity of the plant. It was proposed that the anticoagulant activity of tested dandelion fractions can be correlated with modulation of thrombin's activity.

Thus, our previous and present studies demonstrate that *T. officinale* herb and roots are a valuable source of different classes of secondary metabolites (without cytotoxicity) possessing various biological potential, including antioxidant and anticoagulant properties. Consequently, the use of the dandelion plant as nutritious food or component of medical preparations may bring potentially a range of benefits for human health.

Declaration of interest statement

None to declare.

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Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study

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ABSTRACT

The effect of dandelion root and its various ingredients on the biological functions of blood platelets has not been well investigated. Thus, the objective of our *in vitro* study was to examine the five root fractions (A–E), differing in chemical composition, for changes induced in the human platelets' model using selected hemostatic parameters. As a result, the best anti-platelet potential was shown by preparation enriched with hydroxyphenylacaetate inositol esters – PIEs (fraction C). On the other hand, the best overall protective effect against oxidation of lipids and proteins of platelets, and the best inhibitory effect on the generation of $O_2^{-\cdot}$ were observed in the presence of fraction A (SL-amino acid adducts enriched fraction). Thus, PIEs and SLs rich plant organs, such as dandelion root, may be considered as potentially suitable additives for functional food products used in prophylaxis and treatment of cardiovascular diseases associated with hyperactivation of blood platelets.

1. Introduction

The blood of human adults contains in circulation about one trillion of small morphotic elements called blood platelets, which are turned over every 8–10 days. Platelets are anucleate discoid cells, roughly 2–3 μm in diameter, which are produced from bone marrow megakaryocytes. They play an important role as regulators of hemostasis, serving as the "band-aids" of the bloodstream, responding to blood vessel injury by changing shape, secreting their granule contents, and adhesing and aggregating (Thon & Italiano, 2012). Moreover, platelets bear their pivotal role in both health and disease states, such as cardiovascular disorders (Bradley, 2014). For the proper functioning, the cells need to be activated, which is caused by the interaction of the activating agent, such as thrombin, collagen or adenosine diphosphate (ADP), with the appropriate receptor on the surface of their membrane.

The occurrence of oxidative stress associated with excess free radicals may increase the reactivity of platelets (Olas & Wachowicz, 2007; Wachowicz, Olas, Żbikowska, & Buczynski, 2002), which further can contribute to the development of pathological processes of the cardiovascular system. In turn, it is well known that a diet rich in fruits, vegetables, and herbs, which are the source of natural antioxidants, such as polyphenols, brings many health benefits, especially for the proper functioning of the blood circulatory system.

Dandelion (*Taraxacum officinale* L.) has a long history as medicinal plant, presently, all of its organs (roots, leaves, and flowers) are used to produce commercially available dietary supplements and pharmacological preparations (capsules, syrups, herbal teas and alcoholic juices) that are mainly recommended to treat liver, gallbladder and kidney disorders, digestive ailments, and arthritic and rheumatic diseases (cleansing effect) (Schütz, Carle, & Schieber, 2006). In addition, the strengthening and protective effect against free radicals of the entire dandelion is indicated, as well as the anti-cancer effect of the dandelion roots (Mingarro et al., 2015).

Our previous studies showed that dandelion roots contain an array of different secondary metabolites, including hydroxycinnamic acids (HCAs), sesquiterpene lactones (SLs) and hydroxyphenylacetate inositol esters (PIEs), among which amino acid-SL derivatives and PIEs demonstrated the best overall protective effect against oxidation by H_2O_2/Fe in blood plasma, while HCAs had a stronger anticoagulant effect (Jedrejek, Lis, Rolnik, Stochmal, & Olas, 2019). However, the effect of dandelion root components on the biological function of blood platelets is not well known. Therefore, in the continuation of our study, the same five root fractions were examined for anti-platelet (anti-adhesive and anti-aggregatory) action. Additionally, the aim of the presented study was to determine the activity of these fractions on nonenzymatic lipid peroxidation in resting platelets and enzymatic lipid peroxidation (arachidonic acid metabolism) in platelets stimulated by thrombin, by measuring the level of thiobarbituric acid reactive substances (TBARS).

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Table 1
Simplified phytochemical characteristics of five tested dandelion root fractions A-E on the basis of UPLC-PDA-MS/MS analyses.

Major groups and individual secondary metabolites	Relative content in fraction [%, UVmaxplot]				
	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E
Total hydroxycinnamic acids (HCAs), specifying:	–	–	**16.74**	**96.95**	**100.00**
– 5-*O*-caffeoylquinic acid			2.32	58.21	–
– Coumaroyltartaric acid			–	12.54	–
– L-chicoric acid			–	–	63.04
– Caffeoyl-*O*-feruloyltartaric acid			–	–	20.82
Total hydroxyphenylacetic acid (HPA) derivatives, specifying:	–	**11.47**	**80.61**	**3.05**	–
– Di-hydroxyphenylacetate inositol esters (di-PIEs)		–	50.69	0.26	
– Tri-hydroxyphenylacetate inositol esters (tri-PIEs)		–	8.16	2.60	
Total sesquiterpene lactones (SLs), specifying:	**95.04**	**86.32**	–	–	–
– Taraxinic acid-*O*-β-D-glucoside-proline conjugate	60.12	–			
– Taraxinic acid-proline conjugate	14.46	–			
– Taraxinic acid-*O*-β-D-glucoside	4.14	58.54			
– Taraxinic acid	0.93	7.75			

Moreover, the protective effect of the five dandelion root preparations against oxidation of blood platelets was also measured by using various biomarkers of oxidative stress, such as generation of superoxide anion ($O_2^{-\cdot}$) in resting platelets and platelets activated by thrombin, protein carbonylation and oxidation of protein thiol groups in platelets treated with the donor of hydroxyl radicals – H_2O_2/Fe.

2. Materials and methods

2.1. Chemicals

5,5′-Dithio-bis(2-nitro-benzoic acid), dimethylsulfoxide (DMSO), cytochrome *c*, thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), collagen type I, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Adenosine diphosphate was obtained from Chrono-Log (Havertown, USA). Thrombin was purchased from BioMed Lublin, Poland. All other reagents represented analytical grade and were provided by commercial suppliers. Fibrinogen was isolated from pooled human plasma, according to Doolittle, Schubert, and Schwartz (1967). The concentration was determined spectrophotometrically at 280 nm using an extinction coefficient 1.55 for 1 mg/mL solution. The final concentration of purified fibrinogen was 2 mg/mL.

2.2. Plant material

The dandelion roots were collected in September 2016 from a natural habitat (south-eastern Poland) and identified by prof. Krzysztof Oklejewicz (Department of Botany, University of Rzeszow, Poland). A voucher specimen (TR0916) has been deposited at the Department of Biochemistry and Crop Quality of the Institute of Soil Science and Plant Cultivation – State Research Institute in Pulawy (Poland). Plant material was thoroughly washed with tap water, then cut into small pieces and freeze-dried (Gamma 2-16 LSC, Christ, Osterode am Harz, Germany). Subsequently, dried roots were pulverized (Grindomix GM200, Retsch, Haan, Germany) and stored in a refrigerator before extraction.

2.3. Extraction and preparation of dandelion root fractions

T. officinale L. root fractions were prepared as previously described (Jedrejek et al., 2019). Briefly, the finely ground dandelion roots (560 g) were thrice extracted with 80% methanol at room temperature (36 h), with eighteen cycles of sonication (18 × 10 min). The extracts were combined, concentrated with a vacuum evaporator (40 °C) and deffated with *n*-hexane. The deffated extract was further purified by SPE on a short column (6 × 4 cm, Cosmosil C18-PREP, 140 μm, Nacalai Tesque Inc., Kyoto, Japan). The column was first washed with 4% methanol containing 0.1% formic acid (v/v), and then with 80% methanol to elute the compounds of interest. The evaporation residue was finally fractionated on Sephadex LH-20 (Sigma-Aldrich) column (80 × 2.8 cm i.d.) using the isocratic flow of methanol/tert-butanol (3:1, v/v). Altogether, five fractions (A–E) were collected and subsequently freeze-dried, which yielded 0.80 g (A), 1.90 g (B), 1.07 g (C), 0.18 g (D) and 0.96 g (E) of dry weight, respectively. The detailed information on the extraction and fractionation of plant material can be found in our previous publication (Jedrejek et al., 2019).

2.4. Chemical characteristics of dandelion root fractions

The composition of the analyzed dandelion root fractions (A–E) was determined using an ACQUITY UPLC system (Waters), equipped with a photodiode array detector (PDA) and a tandem quadrupole mass spectrometer (TQD) using electrospray ionization (ESI) source. The conditions of chromatographic separations and MS detector settings are described in our previous publication (Jedrejek et al., 2019). Components of the dandelion root fractions (A–E) were identified on the basis of their MS and UV spectra and available literature data. In addition, the presence and nature of the amino acid and/or monosaccharide fragment in a number of compounds in fractions A and B was confirmed by acid hydrolysis of the fraction followed by the derivation of the compounds and LC-PDA-MS analyses (Jedrejek et al., 2019). The relative content of individual compounds was determined on the basis of UV max plot chromatogram (full wavelength scan chromatogram from 190 to 490 nm) and expressed as a percentage of the total peak area (Table 1).

The chromatographic analyses showed the presence of about 100 different compounds in total in the five tested *T. officinale* fractions (A–E). All identified compounds were classified into three major groups of secondary metabolites, already known from dandelion roots, i.e. hydroxycinnamic acids (HCAs), hydroxyphenylacetic acid (HPA) derivatives, and sesquiterpene lactones (SLs) As can be seen in Table 1, fractions A and B were enriched with SLs where they constituted 95% and 86% of the total compounds, respectively. Among the sesquiterpene lactones present the most important were derivatives of taraxinic acid, such as its glucoside and/or proline conjugate. Preparation C contained mainly HPA derivatives, such as 4-hydroxyphenylacaetate inositol esters (PIEs), which together accounted for about 80% of all compounds. The hydroxycinnamic acids, mainly caffeic, coumaric and ferulic acid esters, were the dominant constituents of the preparations D and E where they constituted over 95% and 100% of the total compounds, respectively. The more detailed characterization of the above fractions is described in our previous paper (Jedrejek et al., 2019).

Stock solutions of dandelion root fractions A-E were prepared with 50% (v/v) aq. DMSO. The final concentration of DMSO in tested

samples was lower than 0.05%, and its effect was determined in all experiments.

2.5. Blood platelets isolation

Blood was obtained from regular, medication- free donors at a blood bank and a Medical Center (Lodz, Poland) collected into CPDA solution (citrate/phosphate/dextrose/adenine; 8.5:1; v/v; blood/CPDA). They had not taken any medications or addictive substances. The protocol was approved by the Committee for Research on Human Subjects of the University of Lodz number 2/KBBN-UŁ/II/2016. Platelet-rich plasma (PRP) was prepared by centrifugation of fresh human blood at 1200*g* for 12 min at room temperature. Platelets were then sedimented by centrifugation at 2300*g* for 15 min at room temperature. The platelet pellet was suspended in the modified Tyrode's buffer (pH 7.4). The concentration of platelets in suspensions was estimated spectrophotometrically at 800 nm (Gruppy, Whisson, McConnell, & Abas, 1995; Walkowiak, Michalak, Koziołkiewicz, & Cierniewski, 1989), and amounted to 2×10^8/mL.

Each sample of blood platelets taken for analysis came from another patient. In our experiment, suspensions of blood platelets were either incubated at 37 °C for 30 min with the five dandelion fractions A-E (tested concentrations: 10 and 50 μg/mL), and if necessary treated further with thrombin (5 U/mL, 37 °C, 5 min), or incubated at 37 °C for 5 min with the five dandelion fractions A-E (tested concentrations: 10 and 50 μg/mL) and then treated with 4.7 mM H_2O_2/3.8 mM Fe_2SO_4/2.5 mM EDTA (37 °C, 25 min).

2.6. Platelet adhesion to collagen and fibrinogen

Adhesion of blood platelets to collagen and fibrinogen was measured using a statistic method with the determination of acid phosphatase activity (Bellavite et al., 1994). To the wells of a 96-well microtiter dish, a 100 μL of fibrinogen (final concentration of 2 mg/mL) or collagen solution (final concentration of 0.04 μg/mL) was added, and the plate was incubated at 4 °C for 24 h. Non-adherent proteins were removed by aspiration and the wells were washed 3 times with 250 μL of Tris-buffered saline (TBS). Afterward, a 200 μL of 1% albumin (BSA) was added and the plate was incubated at 37 °C for 2 h. After incubation BSA was removed and wells were washed 3 times with 250 μL of TBS. Subsequently, a 100 μL of a mixture of platelet suspension and tested dandelion fraction (A–E, two final concentrations of 10 and 50 μg/mL), previously incubated at 37 °C for 30 min, was added to each well. Then the wells were supplemented with 50 μL of thrombin or ADP (final concentration of 0.2 U/mL or 30 μM, respectively), and the plate was incubated at 37 °C for 1 h. Nonadherent cells were removed by aspiration and the wells were washed 3 times with 250 μL of phosphate buffered saline (PBS). Immediately after washing, the citrate buffer containing 0,1% Triton and 5 mM p-nitrophenyl phosphate was added to the wells, and the plate was incubated at room temperature for 1 h. Afterward, a 100 μL of 2 M NaOH was added and the absorbance was determined at 405 nm with a microtiter plate reader (SPECTROstar Nano Microplate Reader, BMG LABTECH, Germany). The adhesion of platelets in the control samples (without tested dandelion fractions) was normalized to 100%.

2.7. Platelet aggregation

Blood platelet aggregation was determined by measuring platelet turbidity, with 0% aggregation calibrated as the absorbance of platelet poor plasma (PPP), and 100% aggregation as the absorbance of platelet-rich plasma (PRP). The turbidity of PRP, preincubated with tested dandelion fractions (A–E), in response to 10 μM ADP or 2 μg/mL collagen was recorded using an aggregometer (Chrono-log, Model 490) during 10 min (Born, 1962).

2.8. Carbonyl groups measurement

The procedure was carried out according to Levine et al. (1990) and Bartosz (2013). The carbonyl groups of the proteins react with 2,4-dinitrophenylhydrazine (DNPH) and as a result, the yellow complex (DNP) is formed. Carbonyl content was determined by measuring the absorbance at 375 nm with SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). More details on the method are described in our earlier papers (Jedrejek, Kontek, Lis, Stochmal, & Olas, 2017; Lis, Jedrejek, Stochmal, & Olas, 2018).

2.9. Thiol groups measurement

The reaction of Ellman's reagent: 5,5′-dithio-bis-(2-nitrobenzoic acid) with thiol groups leads to the formation of the disulphide mixture accompanied by the release of a colored thiol or a 5-thio-2-nitrobenzoic acid anion (TNB) of intense yellow color. The content of the colored compound was measured spectrophotometrically at 412 nm (Ando & Steiner, 1973a, 1973b) using SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). More details on the method are described in our earlier papers (Jedrejek et al., 2017; Lis et al., 2018).

2.10. Lipid peroxidation measurement

Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid reactive substances (TBARS). Blood platelets were incubated with tested dandelion fractions A-E at 37 °C for 30 min, then the mixture was cooled in an ice bath. Afterward, blood platelets were transferred to an equal volume of a mixture of cold 15% (v/v) trichloroacetic acid in 0.25 M HCl and 0.37% (v/v) thiobarbituric acid in 0.25 M HCl, then immersed in boiling water bath for 10 min and centrifuged at 10,000*g* for 15 min at 18 °C. Absorbance was measured at 535 nm using the SpectroStar Nano Microplate Reader (BMG LABTECH, Germany) (Bartosz, 2013). The TBARS concentration was calculated using the molar extinction coefficient ($\mathcal{E} = 156{,}000\ M^{-1}\ cm^{-1}$) and expressed as nmol TBARS/10^8 blood platelets.

2.11. Superoxide anion measurement

Cytochrome *c* reduction was used to measure $O_2^{-\cdot}$ generation in blood platelets. Absorbance was measured spectrophotometrically at 595 nm. More details on the method are described in our earlier paper (Lis et al., 2018).

2.12. Data analysis

Statistical analysis was performed using Statistica 10 (StatSoft). Normal distribution of data was checked by normal probability plots, and the homogeneity of variance by Levene and Brown-Forsythe tests. Differences within and between groups were assessed by applying one-way ANOVA followed by a multicomparison Dunnett's test; for clarity reason, only the differences between the tested fractions and the control/control positive were marked. Results are presented as means ± S.E. Significance was considered at $p < 0.05$. In order to eliminate uncertain data, the Q-Dixon test was performed.

3. Results

The anti-platelet (anti-adhesive and anti-aggregatory) and antioxidant properties of the five fractions, together phenolic and non-phenolic, from dandelion roots (fractions A-E), were studied *in vitro*. We found that adhesion of resting blood platelets to collagen was significantly inhibited after preincubation with the five tested preparations (fractions A-E, tested doses 10 and 50 μg/mL) (Fig. 1A). While in the case of thrombin-activated platelets only phenolic fractions (C, D and E) confirmed the above inhibitory activity (Fig. 1B). Moreover, the

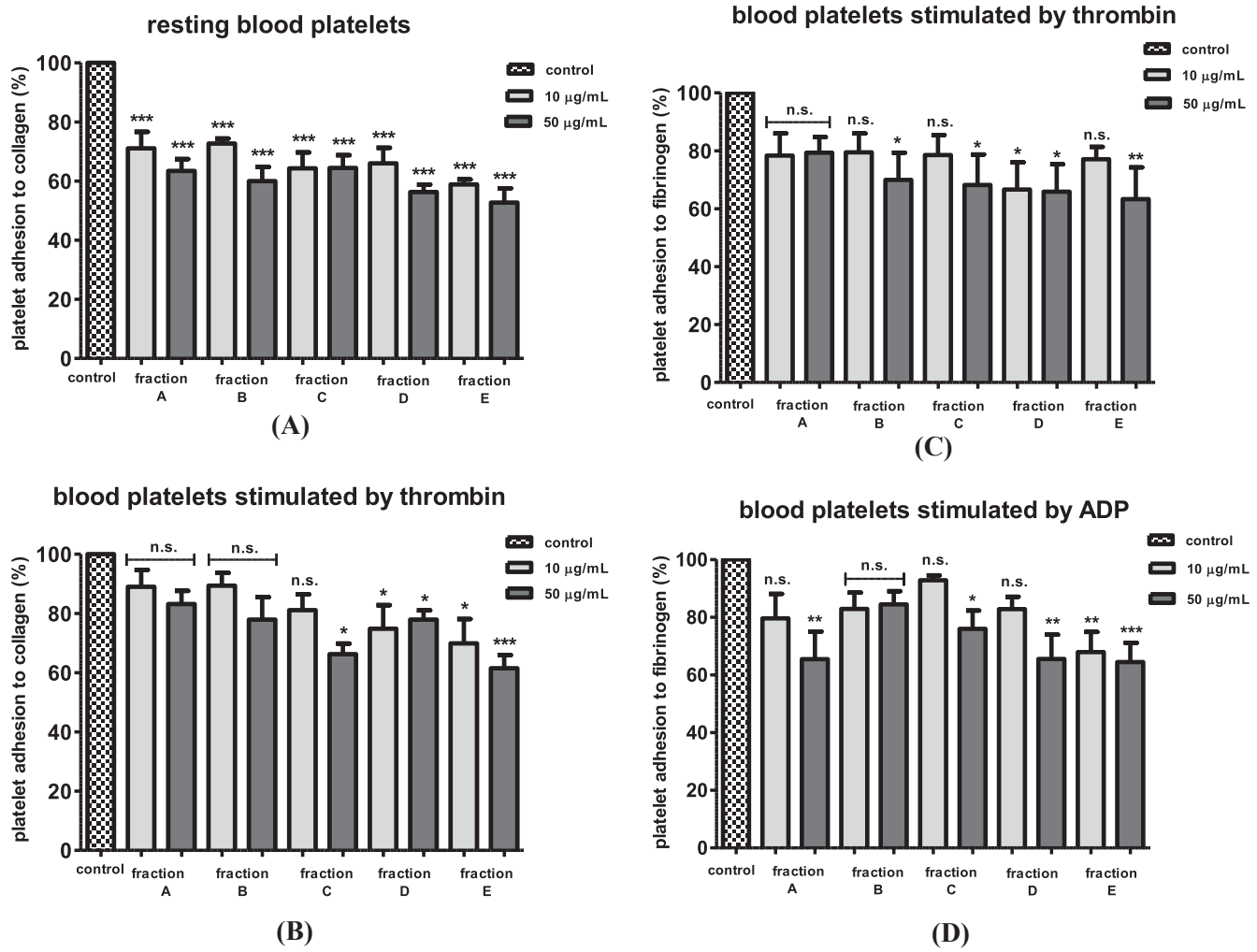

Fig. 1. Effects of fractions from dandelion roots (10 and 50 μg/mL; 30 min) on adhesion to collagen of resting blood platelets (A) and thrombin-activated platelets (B), and adhesion to fibrinogen of thrombin or ADP-activated platelets (C and D). In the graphs, the adhesion of test samples is expressed as a percentage of the control samples (without tested fractions), which have been normalized to 100%. Data represent mean ± S.E. of 5–8 healthy volunteers. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s. $p > 0.05$.

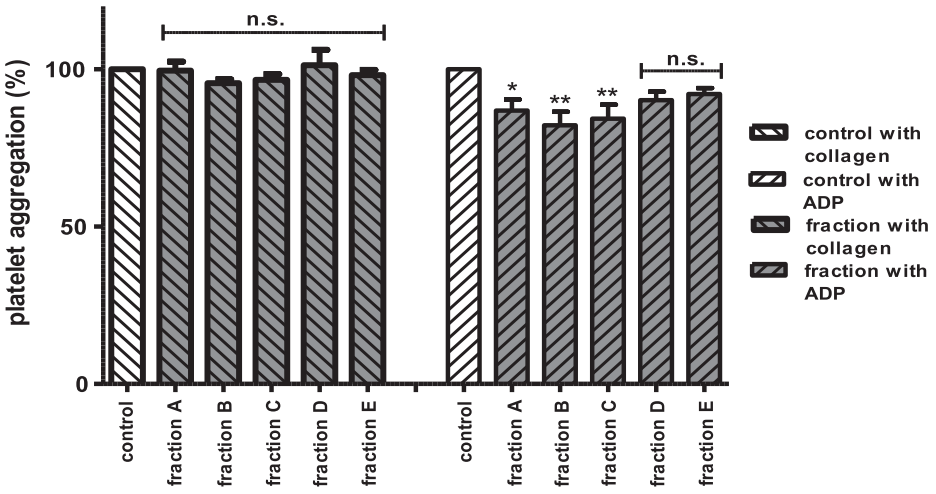

Fig. 2. Effects of fractions from dandelion roots (50 μg/mL; 30 min) on blood platelet aggregation stimulated by two different agonists: ADP and collagen. The percentage of platelet aggregation in ADP and collagen control samples (without tested plant fractions) was 71.8 ± 4.5% and 80.0 ± 2.4%, respectively. In the graph, the aggregation of test samples is expressed as a percentage of the control samples (ADP or collagen control sample), which have been normalized to 100%. Data represent mean ± S.E. of 6–8 healthy volunteers. $^{*}p < 0.05$, $^{**}p < 0.01$, n.s. $p > 0.05$.

adhesion of thrombin-activated platelets to fibrinogen was significantly inhibited by preparation D (both tested doses 10 and 50 μg/mL) and higher tested concentration (50 μg/mL) of other three preparations (B, C and E) (Fig. 1C). While, in the case of ADP-activated platelets fraction E showed the highest inhibitory action and for both tested doses (10 and 50 μg/mL), additionally higher tested concentration (50 μg/mL) of other three preparations (A, C and D) exhibited the above activity (Fig. 1D).

None of the tested dandelion root fractions (A–E, tested concentration 50 μg/mL) showed anti-aggregatory action when platelets

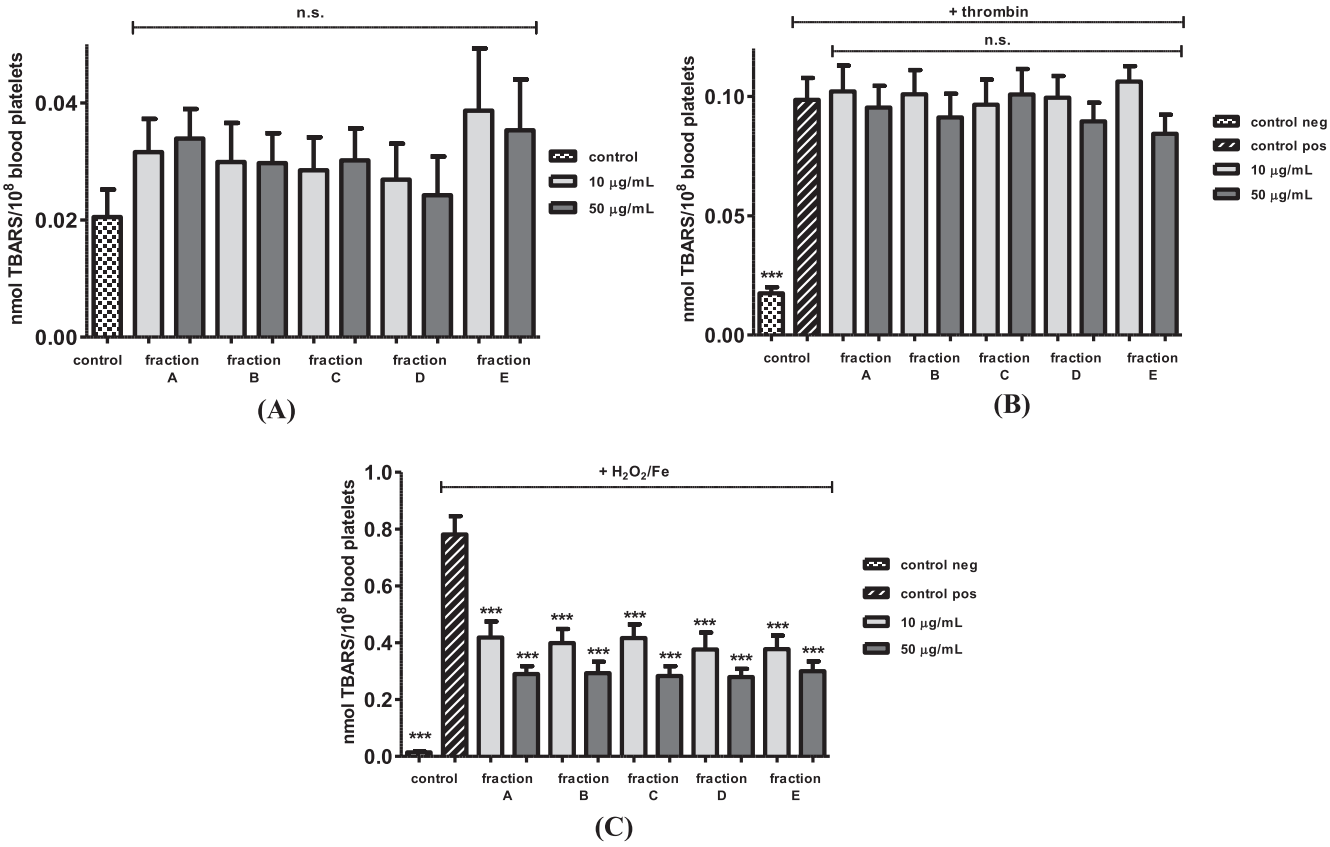

Fig. 3. Effects of fractions from dandelion roots (10 and 50 μg/mL) on lipid peroxidation in resting blood platelets (A), platelets activated by thrombin (B), and platelets treated with H_2O_2/Fe (C). Data represent mean ± S.E. of 8–10 healthy volunteers. Control negative (neg) refers to platelets not treated with thrombin (B) or H_2O_2/Fe (C), whereas control positive (pos) to platelets treated with thrombin (B) or H_2O_2/Fe (C). $^{***}p < 0.001$, n.s. $p > 0.05$.

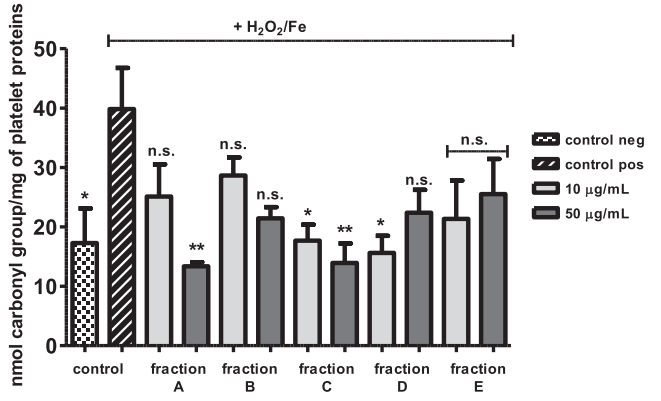

Fig. 4. Effects of fractions from dandelion roots (10 and 50 μg/mL) on protein carbonylation in blood platelets treated with H_2O_2/Fe. Data represent mean ± S.E. of 8 healthy volunteers. Control negative (neg) refers to platelets not treated with H_2O_2/Fe, whereas control positive (pos) to platelets treated with H_2O_2/Fe. $^{*}p < 0.05$ (*vs.* control negative); $^{*}p < 0.05$, $^{**}p < 0.01$, n.s. $p > 0.05$ (*vs.* control positive).

were stimulated by collagen (Fig. 2). On the other hand, three preparations A, B and C (tested concentration 50 μg/mL) reduced the aggregation of platelets stimulated by ADP (about 20% inhibition in comparison with control for three fractions) (Fig. 2).

As shown in Fig. 3A and B, all tested dandelion root fractions (A–E; tested concentrations 10 and 50 μg/mL) did not change the level of TBARS in resting platelets and platelets activated by thrombin. On the other hand, the same preparations (A–E; tested concentrations 10 and 50 μg/mL) significantly inhibited platelet lipid peroxidation induced by H_2O_2/Fe, and at the higher concentration, the inhibition was about 70% in comparison with control positive for the five fractions (Fig. 3C).

As demonstrated in Fig. 4, fraction C inhibited protein carbonylation in platelets treated with H_2O_2/Fe at two tested concentration levels (10 and 50 μg/mL), while fraction A was active only at higher (50 μg/mL) and fraction D at lower (10 μg/mL) concentration; other two fractions B and E remained inactive.

After 30 min incubation of resting blood platelets with the five tested fractions (A–E, tested doses 10 and 50 μg/mL), the number of thiol groups in blood platelet proteins did not significantly change, compared to control, for the four fractions A, C, D and E (Fig. 5A). Only fraction B, at the higher concentration (50 μg/mL), significantly increased the level of thiol groups (Fig. 5A). Moreover, as shown in Fig. 5B, only the two tested preparations A and E (B–D) reduced the oxidation of protein thiol groups in blood platelets treated with H_2O_2/Fe (lower tested concentration 10 μg/mL).

In another set of experiment, the superoxide anion production was measured in resting platelets and platelets activated by thrombin, in the presence tested fractions (A–E) at two concentrations: 10 and 50 μg/mL. Fig. 6A shows that only fraction A (at a lower concentration) significantly reduced the amount of $O_2^{-\cdot}$ in resting platelets in comparison with control, whereas other fractions (B–E) remained inactive at both tested concentration levels. Moreover, the three tested preparations – A, B and C (both tested concentrations 10 and 50 μg/mL) did not significantly change the level of $O_2^{-\cdot}$ in thrombin-activated platelets, whereas two hydroxycinnamic acids enriched fractions – D and E (at the doses 10 and 50 μg/mL) significantly stimulated $O_2^{-\cdot}$ production in platelets, at to tested doses (10 and 50 μg/mL) (Fig. 6B).

Table 2 shows the comparative effects of phenolic and non-phenolic fractions from dandelion roots (A–E, tested concentration of 50 μg/mL) on the biological activity of blood platelets *in vitro*. Summarizing, the hydroxyphenylacetate inositol esters enriched preparation (fraction C)

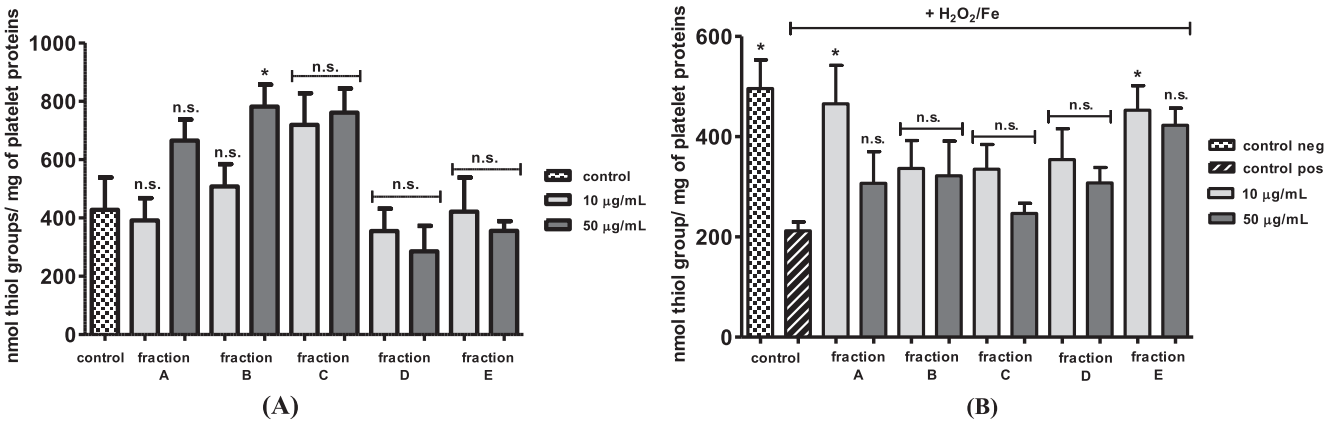

Fig. 5. Effects of fractions from dandelion roots (10 and 50 μg/mL) on the level of thiol groups in resting blood platelets (A) and platelets' protein treated with H_2O_2/Fe (B). Results are given as means ± S.E. (n = 5–8). Control negative (neg) refers to platelets not treated with H_2O_2/Fe, whereas control positive (pos) to platelets treated with H_2O_2/Fe (B). $^{*}p < 0.05$ (*vs.* control negative); $^{*}p < 0.05$, n.s. $p > 0.05$ (*vs.* control positive).

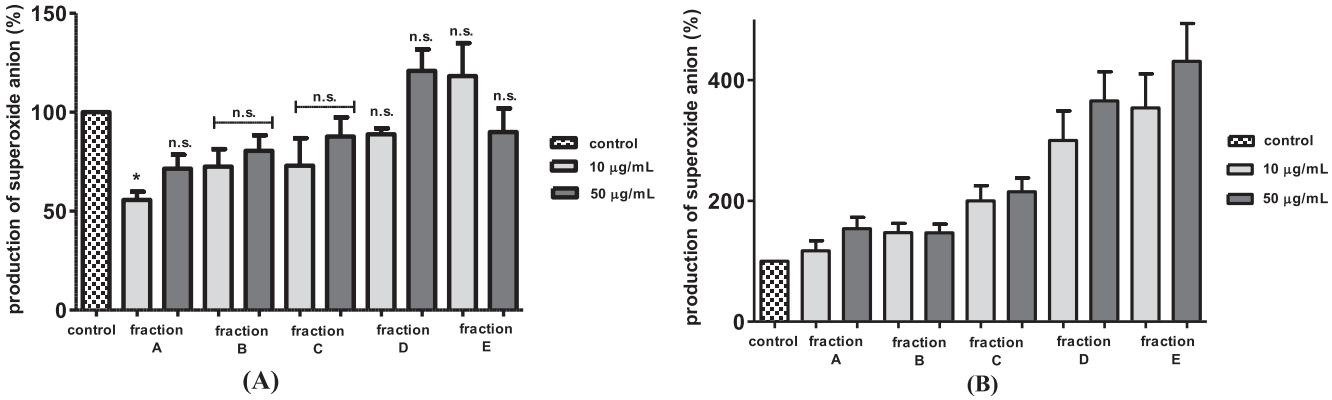

Fig. 6. Effects of fractions from dandelion roots (10 and 50 μg/mL; 30 min) on $O_2^{-\cdot}$ production in resting blood platelets (A) and platelets activated by thrombin (B). In the experiment, the $O_2^{-\cdot}$ level of control samples (platelets without tested fractions) was 0.678 ± 0.267 nmol/mg of platelet protein (resting platelets) and 1.534 ± 0.555 nmol/mg of platelet protein (platelets activated by thrombin). In the graphs, the $O_2^{-\cdot}$ production is expressed as a percentage of control samples (100%). Data represent means ± S.E. (n = 4–5). $^{*}p < 0.05$, $^{***}p < 0.001$, n.s. $p > 0.05$.

had stronger overall anti-platelet (anti-adhesive and anti-aggregatory) property than other tested fractions (A, B, D, and E). Whereas, the two preparations – A (sesquiterpene lactone-amino acid conjugates enriched fraction) and C (hydroxyphenylacetate inositol esters enriched fraction) exerted stronger overall antioxidant action (inhibition of lipid peroxidation and protein carbonylation in platelets treated with H_2O_2/Fe) than other tested fractions (B, D and E).

4. Discussion

It has been proved that the long-term inhibition of platelets activation is an effective strategy to treat different cardiovascular disorders. However, some anti-platelet drugs, such as aspirin and clopidogrel, in addition to positive action can induce serious adverse effects. The most common side effects of aspirin include indigestion, stomach aches, and bleeding. Based on the above, new active substances that do not have or have limited adverse effects are still being sought for.

Some food products, such as garlic, ginger, dark chocolate, onion, tomato, and red wine have been demonstrated to possess the ability to reduce platelet aggregation (Bradley, 2014; Olas, 2017). Their anti-platelet property has been linked to the high content of bioactive compounds, mainly polyphenols, and poly-unsaturated fatty acids. The ability of phenolic compounds to reduce platelet aggregation has been explained by a range of inhibitory effects on platelet activation, signal transduction pathways and receptors (thromboxane A_2, TXA_2), and also an enhancement of nitric oxide (NO) production (Goszcz, Duthie, Stewart, Leslie, & Megson, 2017).

Dandelion belongs to *Asteraceae* family and is a widespread perennial inhabiting fields, roadsides and ruderal sites (Schütz et al., 2006). Although very often considered a troublesome weed, the plant is also recognized as a nontoxic herb with multidirectional activity. Due to demonstrated choleretic, diuretic, antirheumatic, anti-inflammatory, hepatoprotective, antioxidant and anticancer action dandelion has been used for centuries in traditional medicine in China, Turkey and Mexico (Hu & Kitts, 2005; Rodriguez-Fragoso, Reyes-Esparza, Burchiel, Herrera-Ruiz, & Torres, 2008; Jedrejek et al., 2017, 2019; Lis et al., 2018). The main components of *T. officinale* root include phenolic acids (such as chicoric, caffeic and chlorogenic acids), terpenes (such as taraxacoside, ainslioside, and taraxinic acid) and storage carbohydrates (inulin), and they are given the health-related properties of this plant (Schütz et al., 2006). The unique phytochemical composition of dandelion roots and aerial parts has made this plant the subject of a number of both *in vitro* and *in vivo* studies addressing the impact of dried dandelion organs and/or plant extracts on the animal and human body. For example, Choi et al. (2010) observed hypolipidemic and antioxidant effects of dried dandelion root (1% w/w addition) in rabbits fed a high-cholesterol diet. Similarly, results of Arafa, Massoudel-Said, Abdel-Rahman, and Abdel-Megeid (2010) showed that supplementation of the diet of rats with dried dandelion roots and leaves (10 g of plant material/200 g of bread) induced changes in their bodies' lipid profile, including an increase of high-density lipoprotein (HDL) fraction in blood plasma.

Table 2
Comparative effects of the five fractions from dandelion roots on biological functions of blood platelets (tested concentration 50 μg/mL).

Experiment	Tested dandelion root fraction at the level of 50 μg/mL				
	Fraction A – sesquiterpene lactone-amino acid adducts enriched fraction	Fraction B – sesquiterpene lactone-glucose derivatives enriched fraction	Fraction C – hydroxyphenylacetate inositol esters enriched fraction	Fraction D – chlorogenic acids enriched fraction	Fraction E – hydroxycinnamate tartaric acid esters enriched fraction
Adhesion of resting platelets to collagen	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)
Adhesion of thrombin-activated platelets to collagen	No effect	No effect	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)
Adhesion of thrombin-activated platelets to fibrinogen	No effect	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)
Adhesion of ADP-activated platelets to fibrinogen	Positive action – inhibition of this process (anti-adhesive potential)	No effect	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)	Positive action – inhibition of this process (anti-adhesive potential)
Aggregation of ADP-stimulated platelets	Positive action – inhibition of this process (anti-aggregatory potential)	Positive action – inhibition of this process (anti-aggregatory potential)	Positive action – inhibition of this process (anti-aggregatory potential)	No effect	No effect
Aggregation of collagen-stimulated platelets	No effect	No effect	No effect	No effect	No effect
Lipid peroxidation in resting platelets	No effect	No effect	No effect	No effect	No effect
Lipid peroxidation in thrombin-activated platelets	No effect	No effect	No effect	No effect	No effect
Lipid peroxidation in platelets treated with H_2O_2/Fe	Positive action – inhibition of this process (antioxidative potential)	Positive action – inhibition of this process (antioxidative potential)	Positive action – inhibition of this process (antioxidative potential)	Positive action – inhibition of this process (antioxidative potential)	Positive action – inhibition of this process (antioxidative potential)
Protein carbonylation in platelets treated with H_2O_2/Fe	Positive action – inhibition of this process (antioxidative potential)	No effect	Positive action – inhibition of this process (antioxidative potential)	No effect	No effect
Protein thiol groups in resting platelets	No effect	Positive action – inhibition of this process (antioxidative potential)	No effect	No effect	No effect
Oxidation of protein thiols in platelets treated with H_2O_2/Fe	No effect	No effect	No effect	No effect	No effect
$O_2^{-\cdot}$ production in resting platelets	No effect	No effect	No effect	No effect	No effect
$O_2^{-\cdot}$ production in thrombin-activated platelets	No effect	No effect	No effect	Negative action – stimulation of this process (prooxidative potential)	Negative action – stimulation of this process (prooxidative potential)

Nevertheless, the antioxidant and hemostatic effects of dandelion root and its constituents on aggregation of blood platelets and platelets' proteins and lipids so far remained unknown. Our previous study (Jedrejek et al., 2019) demonstrated the presence in *T. officinale* root of about 100 different compounds, which have been classified into the three groups of plant secondary metabolites: sesquiterpene lactones (SLs), hydroxycinnamic acids (HCAs), and hydroxyphenylacetic acid inositol esters (PIEs). All five dandelion preparations (A-E) were found to be safe for the blood platelets because they did not cause their damage (determined as leakage of lactate dehydrogenase into the extracellular medium) within the tested concentration range (5–50 μg/mL). In addition, two fractions A and C, characterized by high content of SL-amino acid adducts (fraction A) and PIEs (fraction C), presented the highest anti-oxidative action in human plasma, while other two preparations D and E, both enriched with HCAs, demonstrated the strongest anticoagulant effect (Jedrejek et al., 2019). The results of our present study confirm the anti-oxidative potential of dandelion root and its secondary metabolites in the *in vitro* human blood model because each of the five tested fractions showed such activity in at least one test (Figs. 3–6, Table 2). Thus, it can be stated that all three groups of secondary metabolites (HCAs, SLs, and PIEs) recognized in *T. officinale* root contribute to the anti-oxidative action reported for this plant, including constituents presenting very weak direct antiradical *in vitro* activity, such as sesquiterpene lactone derivatives and hydroxyphenylacetic acid inositol esters (Jedrejek et al., 2019). However, the antioxidant action of each of the preparations varied in different experiments. For example, none of the tested fractions inhibited lipid autoperoxidation in resting platelets and enzymatic lipid peroxidation in platelets stimulated by thrombin (Fig. 3A and B). It may suggest that dandelion constituents, both phenolic and non-phenolic, do not possess the ability to modulate platelets' activity by interfering with the metabolism of arachidonic acid. Moreover, surprisingly, the two hydroxycinnamic acids enriched fractions (D and E) presented in addition to the protective anti-oxidative effect (against lipid peroxidation by H_2O_2/Fe) also an adverse pro-oxidative action, consisting in increasing the production of $O_2^{-\cdot}$ in platelets activated by thrombin, instead of its inhibition. Current results are in agreement with our earlier findings that showed that the two above polyphenol fractions D–E possess weaker antioxidant potential than other fractions isolated from dandelion roots (A, B and C) using an *in vitro* blood platelets model (Jedrejek et al., 2019). In the current research the best overall protective effect against oxidation of lipids and proteins of platelets (induced by the donor of hydroxyl radical – H_2O_2/Fe), and the best inhibitory effect on the generation of $O_2^{-\cdot}$ were observed in the presence of fraction A (SL-amino acid adducts enriched fraction). Very similar results were obtained previously in studies of an *in vitro* human plasma model, where the same preparation A, together with preparation C (hydroxyphenylacetate inositol esters enriched fraction), showed the highest antioxidant activity (Jedrejek et al., 2019). This is interesting due to the lack of confirmed direct antiradical activity for sesquiterpene lactones (Mo et al., 2017). Concluding with the antioxidant potential of non-phenolic fractions from dandelion root (fractions A and B), which was clearly higher for amino acid-SL conjugates compared to sugar derivatives, it can be assumed that the presence of amino acid fragment, such as proline, attached to sesquiterpene lactone has decided on greater anti-oxidative action of fraction A.

One of the key findings of our study is a demonstration of the anti-adhesive property of dandelion constituents, both phenolic and non-phenolic, in an experimental system of isolated washed blood platelets stimulated by agonists (ADP and thrombin) in the presence of two adhesive proteins – collagen and fibrinogen. The tested fractions differed in the demonstrated anti-adhesive potential, that was broadly stronger for fractions containing polyphenols – C, D, and E, which inhibited platelet adhesion in all used models (resting blood platelets, thrombin-activated platelets, and ADP-activated platelets). Additionally, anti-aggregative action of dandelion root preparations was estimated by measuring platelet aggregation in platelet-rich plasma in the presence of the cells agonists – ADP and collagen. In contrast to the anti-adhesive action, the two HCAs enriched fractions (D and E) did not exert antiplatelet activity, while two SL derivatives (A and B) and PIEs (C) enriched fractions caused significant inhibition of cell aggregation stimulated by ADP. One of the possible mechanisms of anti-aggregative action of these preparations is the interaction of their constituents with ADP receptors on blood platelet membrane. Our results are consistent with the previous study of Neef, Cilli, Declerck, and Laekeman (1996) who reported inhibitory activity of ethanolic extracts from dandelion root, characterized by high content of either low-molecular polysaccharides or terpenes, on platelet aggregation stimulated by ADP. Summarizing, the best overall antiplatelet action was observed in the presence of fraction C (hydroxyphenylacetate inositol esters enriched fraction), in our biological experimental model (human blood platelets). However, the mechanism of the antiplatelet activity of PIE compounds is not entirely clear and requires further research, so far we can only assume that the sugar part (inositol) of PIEs plays an important role here as other phenolic constituents of dandelion root, such as hydroxycinnamic acids in fractions D and E, have not shown such a high activity.

Our current research on dandelion roots indicates them as a valuable source of secondary metabolites, such as hydroxyphenylacetate inositol esters, with simultaneous antioxidant, anticoagulant, and antiplatelet properties. Consequently, further investigation of dandelion organs and their constituents in terms of their use in the prophylaxis and treatment of cardiovascular diseases associated with hyperactivity of blood platelets and oxidative stress should be conducted, including other *in vitro* and *in vivo* models, and detailed phytochemical identification of active compounds.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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The anti-oxidative and hemostasis-related multifunctionality of L-chicoric acid, the main component of dandelion: An *in vitro* study of its cellular safety, antioxidant and anti-platelet properties, and effect on coagulation

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ABSTRACT

All dandelion (*Taraxacum officinale*) organs, i.e. the roots, leaves and flowers, are rich in L-chicoric acid, and nowadays are commonly included in dietary products and pharmaceutical preparations. However, little is known of the effects of dandelion leaves and petals, or L-chicoric acid itself, on the biological activity hemostasis elements such as blood platelets and plasma. Therefore, the aim of the present study was to examine the effect of chicoric acid extracted from dandelion on the biological properties of human plasma and blood platelets *in vitro*. Briefly, the study evaluated the effects of four phenolic fractions, obtained from leaves (fraction A and B) and petals (fraction C and D), with different concentrations of chicoric acid, as well as L-chicoric acid isolated from dandelion leaves, on biomarkers of oxidative stress, parameters of coagulation and blood platelet activation. Chicoric acid offers novel antioxidant and anti-adhesive potential without cytotoxicity.

1. Introduction

Of the many defensive mechanisms developed in living organisms over the course of evolution, one of the most important is hemostasis: a complex sequence of reactions intended, on the one hand, to maintain the fluidity of blood circulating in blood vessels, and on the other, to stop blood loss when the blood vessels are damaged. While hemostasis is regulated primarily by blood platelets, important roles are also played by the blood vessel wall, plasma factors (or pro-coagulatory factors), which participate in the transformation of fibrinogen into fibrin, and the fibrinolytic system, which serves to liquidate clots that may prevent proper blood flow (Gale, 2011; Kluft & Burggraaf, 2011).

Blood platelets are the smallest, morphotic elements of the blood; they are responsible for the formation of the primary hemostatic pivot and may interact with blood vessels. Platelets are characterized by the presence of centrally-located α-grains and osmophilic grains of high electron density, where such compounds as adenosine diphosphate (ADP), serotonin (5-HT), fibrinogen (Fg), von Willebrand factor (vWf), thrombospondin (Tsp), platelet factor 4 (PF-4), platelet-derived growth factor (PDGF) and selectin P are stored. However, the role of platelets is not limited to participation in the hemostasis process, they can also be involved in thrombosis, inflammatory processes and metastasis; atherosclerotic changes can also develop in the walls of blood vessels as a result of excessive platelet activation. Under physiological conditions, various factors, such as thrombin, collagen, ADP, thromboxane A_2 (TXA_2) and serotonin, serve as platelet activators, inducing shape change, aggregation or adhesion among platelets, as well as the secretion of other factors (Gale, 2011).

It has been proven that reactive oxygen species (ROS) are also produced by platelets and that these can encourage platelet activation; they can also originate from the external environment, e.g. from drugs, physical exercise, cigarettes and alcohol, and from endogenous sources,

Abbreviations: ADP, adenosine diphosphate; APTT, activated partial thromboplastin time; BSA, bovine serum albumin; CPDA, citrate/phosphate/dextrose/adenine; CVDs, cardiovascular diseases; DHBV, duck hepatitis B virus; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; Fg, fibrinogen; fraction A, leaf 50% MeOH fraction; fraction B, leaf 85% MeOH fraction; fraction C, petal 50% MeOH fraction; fraction D, petal 85% MeOH fraction; GSH, glutathione; HBV, Hepatitis B virus; HIV, Human Immunodeficiency Virus; 5-HT, 5-hydroxytryptamine; LDH, lactate dehydrogenase; NADH, nicotinamide-adenine-dinucleotide; O_2^-, superoxide anion; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PF4, platelet factor 4; PRP, platelet-rich plasma; ROS, reactive oxygen species; PT, prothrombin time; TBARS, thiobarbituric acid reactive substances; Tsp, thrombospondin; TT, thrombin time; TXA_2, thromboxane A_2; vWf, von Willebrand factor; WHO, World Health Organization

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such as aerobically respiring cells. Whether they are generated endogenously or exogenously, ROS induce oxidative modification of all major cellular macromolecules such as carbohydrates, lipids, proteins and DNA. Any imbalance between ROS production and antioxidant defenses increases oxidative stress, which contributes to many pathological processes, including cardiovascular diseases (CVDs) (Liguori et al., 2018).

CVDs are a leading cause of morbidity and mortality, particularly among the elderly, and interest in non-pharmacologic nutraceutical-based treatments is continually growing. It has been proven that antiplatelet drugs such as aspirin, ibuprofen and clopidogrel can reduce blood platelet activation and oxidative stress, and may have an important impact on the treatment of CVD, both in atherosclerosis and in the development of acute thrombotic events; similar beneficial effects can also be obtained by certain dietary components and supplements obtained from plants, which also demonstrate antiplatelet and antioxidant potential (Dias, Urban, & Roessner, 2012).

This use of natural products represents an interesting alternative approach to antiplatelet therapy, as conventional drugs are often display side effects. It is estimated that less than 10% of the world's biodiversity has been evaluated for potential biological activity, and many more useful natural compounds await discovery (Dias et al., 2012). One promising source of natural chemicals is dandelion (*Taraxacum officinale* L.). The plant is a widespread perennial belonging to the *Asteraceae* family with a long history of use (Lis & Olas, 2019; Schutz, Carle, & Schieber, 2006). Phenolic and non-phenolic fractions obtained from various dandelion organs, including the leaves, roots and petals, have anti-platelet, antioxidant and anticoagulant properties (Jedrejek, Lis, Rolnik, Stochmal, & Olas, 2019; Lis, Jedrejek, Stochmal, & Olas, 2018). In addition, these organs, especially the leaves, are also good sources of chicoric acid (Jedrejek, Kontek, Lis, Stochmal, & Olas, 2017; Lis et al., 2018). LC–MS analysis has confirmed that chicoric acid constitutes about 350 mg/g d.w. (about 80% of total polyphenols) for fraction A; about 195 mg/g d.w. (about 45% of total polyphenols) for fraction B; about 115 mg/g d.w. (about 40% of total polyphenols) for fraction C (Jedrejek et al., 2017; Lis et al., 2018). Moreover, *in vitro* studies have demonstrated that phenolic fractions from dandelion leaves and petals reduce oxidative stress in human plasma and human blood platelets: two key elements of hemostasis (Jedrejek et al., 2017; Lis et al., 2018); in addition, fraction A obtained from dandelion leaves also displayed anti-platelet and anticoagulant properties (Lis et al., 2018).

Cichoric acid exhibit also anti-obesity activity *in vivo*, following by reducing body weight and lowering serum lipid parameters. Researchers from the University of China observed that chicory acid alleviates liver damage in mice caused by a high-fat diet, i.e. decreased liver steatosis, fatty acid synthase, alanine aminotransferase and aspartate aminotransferase levels in serum. Also, the positive effect was visible by reducing tumor necrosis factor-α, interleukin-6 levels as well as expression of cyclooxygenase-2 and p-JNK proteins. In addition, chicoric acid regulated the levels of leptin and adiponectin. Further, also inhibited the protein expressions of peroxisome proliferator-activated receptor γ and CCAAT/enhancer-binding protein α (Xiao, Xie, et al., 2013).

Hepatitis B virus (HBV) and Human Immunodeficiency Virus (HIV) are global serious public health problems. Worldwide, the number of chronically infected HBV is over 370 million, while the HIV infection is affected by nearly 40 million people (Boroń-Kaczmarska, 2014). Zhang et al. (2014) confirmed that chicoric acid (1–100 μg/mL) significantly inhibited duck hepatitis B virus (DHBV) DNA replication in infected duck fetal hepatocytes. Further, it inhibited HBV DNA replication of HepG2.2.15 cells at concentrations of 50–100 μg/mL. Another study demonstrated that chicoric acid inhibited integration at concentrations from 500 nM to 10 μM, as well as inhibits entry at concentrations above 1 μM, using quantitative real-time polymerase chain reaction (PCR). Also, it is a noncompetitive but reversible inhibitor of integrase *in vitro* and of HIV integration *in vivo* (Reinke et al., 2004).

The present study examines the biological properties of pure chicoric acid isolated from dandelion leaves (fraction A). It also compares its biological effects to those of the entire fraction A, as well as with other dandelion leaf and petal phenolic fractions (fraction B and C), which were both good sources of chicoric acid; and petal phenolic fraction (fraction D without chicoric acid). Biological activity was determined in human plasma and human blood platelets (*in vitro*) according to selected biomarkers of oxidative stress, coagulation parameters and blood platelet activation. Additionally, the effect of chicoric acid on blood platelet damage was evaluated by measuring the extracellular lactate dehydrogenase (LDH) activity.

2. Materials and methods

Adenosine diphosphate (ADP) was obtained from Chrono-Log Corporation (Havertown, USA). Thrombin was purchased from BioMed Lublin, Poland. Collagen type I, bovine serum albumin (BSA), Folin-Ciocalteu reagent, gallic acid, 2-diphenyl-1-picrylhydrazyl radical (DPPH), sodium carbonate and Trolox were purchased from Sigma (St. Louis, MO., USA). Fibrinogen was isolated from pooled citrated human plasma by cold ethanol precipitation followed by ammonium sulphate fractionation at 26% saturation at 4 °C, according to Doolittle, Schubert, and Schwartz (1967). Its concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 1.55 for 1 mg/mL solution. The concentration of purified fibrinogen in the reaction system was 2 mg/mL.

2.1. Plant material

Leaves and petals of dandelion (*Taraxacum officinale* L.) were obtained from a farm in Rzeszow, Poland (50.114175°N, 21.911738°E) in 2015 (Jedrejek et al., 2017).

2.2. Preparation and quantification of phenolic fractions from dandelion leaves and petals

The four phenolic fractions from *T. officinale* L. leaves and petals were prepared as previously described (Jedrejek et al., 2017). Briefly, the defatted plant material (leaves – 420 g, and petals – 250 g) was extracted with 80% MeOH under reflux, and the obtained extracts were separated on a Cosmosil 140 C18-PREP (60 × 100 mm, Nacalai Tesque Inc., Japan). Altogether, four phenolic fractions were obtained: leaf 50% MeOH fraction (fraction **A**, 7.16 g), leaf 85% MeOH fraction (fraction **B**, 0.52 g), petal 50% MeOH fraction (fraction **C**, 7.66 g) and petal 85% MeOH fraction (fraction **D**, 2.05 g). The polyphenols present in each fraction were tentatively characterized (25 phenolic acids and their derivatives and 27 flavonoids) and quantified using UPLC-PDA, as described by Jedrejek et al. (2017). The predominant phenolic compound in fraction A (351.58 mg/g d.w.), B (196.07 mg/g d.w.), and C (117.17 mg/g d.w.) was L-chicoric acid, whereas fraction D did not contain any phenolic acids.

More detailed information on the extraction, fractionation, and chemical analysis is given by Jedrejek et al. (2017).

2.3. Isolation and identification of L-chicoric acid from dandelion leaf 50% fraction

The compound of interest (L-chicoric acid) was isolated from previously-obtained fraction A (Jedrejek et al., 2017), in which it was the dominant component. The purification process comprised two stages and utilized the semi-preparative HPLC chromatographic system (Gilson Inc., Middleton, WI, USA) combined with an evaporative light scattering detector (ELSD, Gilson prepELS II). In the first step, the leaf fraction was purified on a column filled with Cosmosil 40 C18–PREP (10 × 120 mm, Nacalai Tesque Inc.). The chromatographic separation was performed at room temperature, using a linear gradient (from 15 to

30%) of solvent B (methanol with 0.1% HCOOH) in solvent A (water acidified with 0.1% HCOOH) at a flow rate of 8 mL/min within 30 min.

The sub-fraction thus obtained was further purified on a Kromasil C18 column (10 × 250 mm, 5 µm, AkzoNobel, Bohus, Sweden). The chromatographic separation was performed at 40 °C, using an isocratic flow of aqueous 11% acetonitrile (containing 0.1% HCOOH) at a flow rate of 3.2 mL/min within 40 min. In both stages, the ELS detector drift tube was set up at 65 °C, and the pressure of the nebulizer gas (nitrogen) was adjusted to 47 psi. The effluent from the HPLC system was divided before the ELS detector with a split ratio of 1:100.

The structure of the isolated compound was confirmed by NMR (Bruker Avance III HD Ascend™-500 spectrometer), UHPLC-PDA-MS (Waters ACQUITY Tandem Quadrupole Mass Spectrometer) and published data (Nuissier, Rezzonico, & Grignon-Dubois, 2010). The MS and NMR data for the obtained L-chicoric acid are given in the supplementary information (Fig. 1S, 2S and 3S, Table S1), as are the antioxidant properties and total phenolic content of isolated L-chicoric acid (Table S2).

LC-PDA-ESI-MS analysis found the purity of the isolated phenolic acid to be about 95%. Briefly, the lyophilizate of the compound (5 mg) was dissolved in aqueous 20% acetonitrile (v/v) and separated on an HSS C18 column, at a concentration of 250 µg/mL; the conditions used for the chromatography and MS are described in '*UHPLC-PDA-MS analysis*'. Peak purity was then calculated by normalization. The result of the UPLC-PDA analysis was also compared to the corresponding MS and ^{1}H NMR spectra for the compound. The main impurity present in the chromatogram of the L-chicoric acid lyophilizate was tentatively identified as *meso*-chicoric acid (an isomer of L-chicoric acid), which represented about 2% of total peak area (see supplementary information, Fig. 1S).

2.4. UHPLC-PDA-MS analysis

The LC-MS analysis was performed with an ACQUITY UPLC system (Waters, Milford, MA, USA), equipped with a photodiode array detector (PDA) and a tandem quadrupole mass spectrometer (TQD). The isolated compound was separated on an HSS C18 column (2.1 × 100 mm, 1.7 µm, Waters) at 40 °C. The procedure used a linear gradient from 2 to 40% of solvent B (acetonitrile containing 0.1% (v/v) formic acid) in solvent A (water containing 0.1% (v/v) formic acid) for 12 min, at a flow rate of 0.4 mL/min. The UV spectrum was recorded within the range of 190–490 nm (3.6 nm resolution). The MS analysis was performed in negative ion mode, using the following MS settings: scan range 100–1500 *m/z*; capillary voltage 2.8 kV; cone voltage 35 V; source temperature 150 °C; desolvation temperature 450 °C; desolvation gas flow 900 L/h, and cone gas flow 100 L/h. Data acquisition and processing were performed using Waters MassLynx 4.1 software.

2.5. NMR spectroscopy

The 1D and 2D NMR spectra (^{1}H, ^{13}C DEPTQ, ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC, ^{1}H–^{13}C HSQC-TOCSY, ^{1}H–^{1}H COSY DQF, ^{1}H–^{1}H TROESY) were performed using an Avance III HD Ascend™-500 spectrometer (^{1}H, 500.20 MHz; ^{13}C, 125.80 MHz; Bruker BioSpin, Rheinstetten, Germany) in MeOH-d_4 at 30 °C. Chemical shifts are given in ppm and the *J* values in Hz, and were referenced to residual methanol (δ_H 3.31 and δ_C 49.0). Data processing was performed with the Topspin software (version 3.5pl2, Bruker BioSpin).

2.6. Total phenolic content (TPC)

TPC in the freeze-dried sample of isolated L-chicoric acid was determined with the Folin-Ciocalteu assay with slight modifications using gallic acid (Sigma-Aldrich) as a standard compound. Briefly, 0.1 mL of Folin-Ciocalteu reagent (Sigma-Aldrich) solution was added to 1.6 mL of appropriately diluted compound or standard solution (six different concentrations ranging from 0.5 to 8 µg/mL). The mixture was shaken vigorously and left for 5 min. Subsequently, 0.3 mL of Na_2CO_3 (20% w/v) was added, shaken vigorously and incubated at water bath (40 °C) for 30 min. After cooling, the absorbance at 765 nm was measured against blank sample (containing aqueous 5% methanol (v/v)) using Thermo Scientific Evolution 260 Bio spectrophotometer. TPC value of L-chicoric acid sample was read from the curve for the standard and expressed as milligrams of gallic acid equivalents (mg GAE/g DW).

2.7. DPPH test

Antiradical activity of isolated L-chicoric acid was determined using the method of Brand-Williams (1995), with slight modifications as follows: 0.1 mL of the compound solution (four different concentrations ranging from 30 to 150 µg/mL) or Trolox (Sigma-Aldrich) solution (six different concentrations ranging from 8 to 190 µg/mL) was added to 1.9 mL of DPPH solution (100 µM in methanol) in a cuvette. The mixture was shaken gently and left in the dark for 30 min. Subsequently, the absorbance at 517 nm was recorded against methanol (control sample) using Thermo Scientific Evolution 260 Bio spectrophotometer.

The percentage of absorbance inhibition was calculated from the equation:

$$\text{Inhibition } (\%) = 100 \times [(A_{control} - A_{sample})/A_{control}],$$

where $A_{control}$ and A_{sample} are the absorbance values of the control and the test samples at t = 30 min, respectively.

To obtain Trolox Equivalent (TE) of L-chicoric acid a straight line curves (absorbance inhibition (%) vs. concentration (µg/mL)) were prepared for test compound and Trolox, and then slope of compound curve was divided by slope of standard curve. The IC_{50} value of L-chicoric acid (defined as the concentration of sample necessary to cause 50% inhibition) was determined from the same curve, and Trolox was used as positive control.

2.8. Blood platelets and plasma isolation

Fresh human blood was obtained from regular, medication-free donors at a blood bank and a medical center in Lodz, Poland. In addition, peripheral blood was also obtained from non-smoking men and women: the blood was collected into CPD solution (citrate/phosphate/dextrose; 9:1; v/v blood/CPD) or CPDA solution (citrate/phosphate/dextrose/adenine; 8.5:1; v/v; blood/CPDA). None of the subjects had taken any medication or addictive substances such as tobacco, alcohol, antioxidant supplementation or aspirin, or any other anti-platelet drugs. The protocol was approved by the Committee for Research on Human Subjects of the University of Lodz (number 2/KBBN-UŁ/II/2016).

Platelet-rich plasma (PRP) was prepared by centrifugation of fresh human blood at 1200*g* for 12 min at room temperature. Blood platelets were then sedimented by centrifugation at 2300*g* for 15 min at room temperature. The platelet pellet was washed with modified Tyrode's buffer (pH 7.4). The concentration of the platelets in the suspensions was estimated spectrophotometrically (Walkowiak, Michalak, Koziołkiewicz, & Cierniewski, 1989) to be 1.5–2.5 × 10^8/mL.

2.9. Preparation of samples (plasma, PRP and washed blood platelets) with chicoric acid and plant fraction

A stock solution of L-chicoric acid was prepared with aqueous 50% DMSO (v/v). The final concentration of DMSO in tested samples was lower than 0.05% and its effect was determined in all experiments.

The plasma was pre-incubated (5 min, at 37 °C) with chicoric acid at final concentrations of 0.5, 1, 5, 10 and 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).

PRP was incubated (30 min, at 37 °C) with the following four treatments:

Table 1
Effects of chicoric acid (concentration range 0.5–50 μg/mL, incubation time – 30 min) on the coagulation times of human plasma (APTT, PT, and TT). Data are expressed as mean ± SD (n = 5). Kruskal-Wallis test: n.s. $p > 0.05$, compared with control.

Concentration of chicoric acid (μg/mL)	Coagulation time (s)		
	Thrombin time (TT)	Prothrombin time (PT)	Activated partial thromboplastin time (APTT)
0	17.11 ± 1.4	15.11 ± 2.2	47.15 ± 4.2
0.5	17.10 ± 1.3 ($p > 0.05$)	15.24 ± 2.3 ($p > 0.05$)	46.67 ± 3.0 ($p > 0.05$)
1	16.96 ± 1.1 ($p > 0.05$)	15.03 ± 2.4 ($p > 0.05$)	46.21 ± 4.4 ($p > 0.05$)
5	17.08 ± 1.0 ($p > 0.05$)	14.86 ± 2.4 ($p > 0.05$)	46.17 ± 3.4 ($p > 0.05$)
10	16.82 ± 1.0 ($p > 0.05$)	14.87 ± 2.3 ($p > 0.05$)	46.36 ± 3.7 ($p > 0.05$)
50	17.03 ± 1.0 ($p > 0.05$)	14.89 ± 2.3 ($p > 0.05$)	46.2 ± 3.3 ($p > 0.05$)

– chicoric acid at a final concentration of 50 μg/mL
– fraction A–D at a final concentration of 50 μg/mL.

Washed blood platelets were incubated (30 min, at 37 °C) with the following four treatments:

– chicoric acid at final concentrations of 0.5, 10 and 50 μg/mL
– fraction A–D at final concentrations of 10 and 50 μg/mL.

The washed blood platelets were pre-incubated (5 min, at 37 °C) with chicoric acid at final concentrations of 0.5, 10 and 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).

Washed blood platelets were pre-incubated (25 min, at 37 °C) with chicoric acid at final concentrations of 0.5, 10 and 50 μg/mL, and then treated with thrombin at a final concentration of 5 U/mL (5 min, at 37 °C).

2.10. Parameters of blood platelet activation

2.10.1. Blood platelet adhesion

The adhesion of blood platelets to collagen and fibrinogen was analysed using the static method evaluating acid phosphatase activity (Bellavite et al., 1994). Absorbance was measured at 405 nm with a SPECTROstar Nano Microplate Reader (BMG LABTECH Germany); the absorbance of the control platelets, i.e. those without the tested dandelion fractions or chicoric acid, was assumed to be 100%. The method is described in more detail by Lis et al. (2019).

2.10.2. Blood platelet aggregation

Human blood platelet aggregation was measured by platelet turbidity, with the absorbance of platelet-poor plasma assumed to be 0% aggregation and the absorbance of PRP as 100% aggregation. The PRP samples preincubated with the dandelion fractions or chicoric acid were treated with 10 μM ADP and their aggregation was recorded using an optical Chrono-Log aggregometer (Chrono-Log, Havertown, PA, USA). The method is described in more detail in Skalski et al. (2019).

2.11. Parameters of coagulation process

2.11.1. The measurement of prothrombin time (PT)

PT measurement was carried out coagulometrically using a K-3002 Optic Coagulation Analyser (Kselmed, Poland). After 30 min of treatment with chicoric acid or tested plant fractions, the human plasma (50 μL) was added to a measuring cuvette and incubated for two minutes at 37 °C on a block heater. A 100 μL of Dia-PT liquid was then added (commercial preparation – Kselmed, Poland) and the PT value was measured. More details on the method are described in our earlier paper (Jedrejek et al., 2019).

2.11.2. The measurement of thrombin time (TT)

TT measurement was carried out coagulometrically using a K-3002 Optic Coagulation Analyser (Kselmed, Poland). After 30 min of treatment with chicoric acid or the tested plant fractions, the human plasma (50 μL) was added to measuring cuvette and incubated for one minute at 37 °C on a block heater. Following this, 100 μL of thrombin was added to a final concentration of 5 U/mL and TT was measured. The method is described in more detail by Jedrejek et al. (2019).

2.11.3. The measurement of activated partial thromboplastin time (APTT)

APTT measurement was carried out coagulometrically using a K-3002 Optic Coagulation Analyser (Kselmed, Poland). After 30 min of treatment with chicoric acid or tested plant fractions, the human plasma (50 μL) was added to a measuring cuvette and incubated with 50 μL of Dia-PTT liquid (commercial thromboplastin – Kselmed, Poland) for three minutes at 37 °C on a block heater. Following this, 50 μL of 25 mM $CaCl_2$ was added and the partial thromboplastin time was measured. The method is described in more detail in Jedrejek et al. (2019).

2.11.4. Cytotoxicity

Extracellular LDH activity was determined by following the decrease in absorbance at 340 nm resulting from NADH oxidation. The absorbance was measured using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany) according to Wroblewski and Ladue (1955).

2.12. Parameters of oxidative stress

2.12.1. Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring the concentration of thiobarbituric acid reactive substances (TBARS). More details on the method are given in our earlier papers (Jedrejek et al., 2017, 2019; Lis et al., 2018).

2.12.2. Carbonyl group measurement

The detection of carbonyl groups in proteins was carried out according to Levine et al. (1990) and Bartosz (2008). More details on the method are given in our earlier papers (Jedrejek et al., 2019; Lis et al., 2018).

2.12.3. Thiol group determination

The thiol group content was measured spectrophotometrically at 412 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany) with Ellman's reagent – 5,5′-dithio-bis-(2-nitrobenzoic acid). More details on the method are given in our earlier papers (Jedrejek et al., 2017, 2019; Lis et al., 2018).

2.13. Data analysis

Statistical analysis was performed using Statistica 10 (StatSoft). The normal distribution of data was checked by normal probability plots, and the homogeneity of variance by Levene's test and the Brown-Forsythe test. Differences within and between groups were assessed by

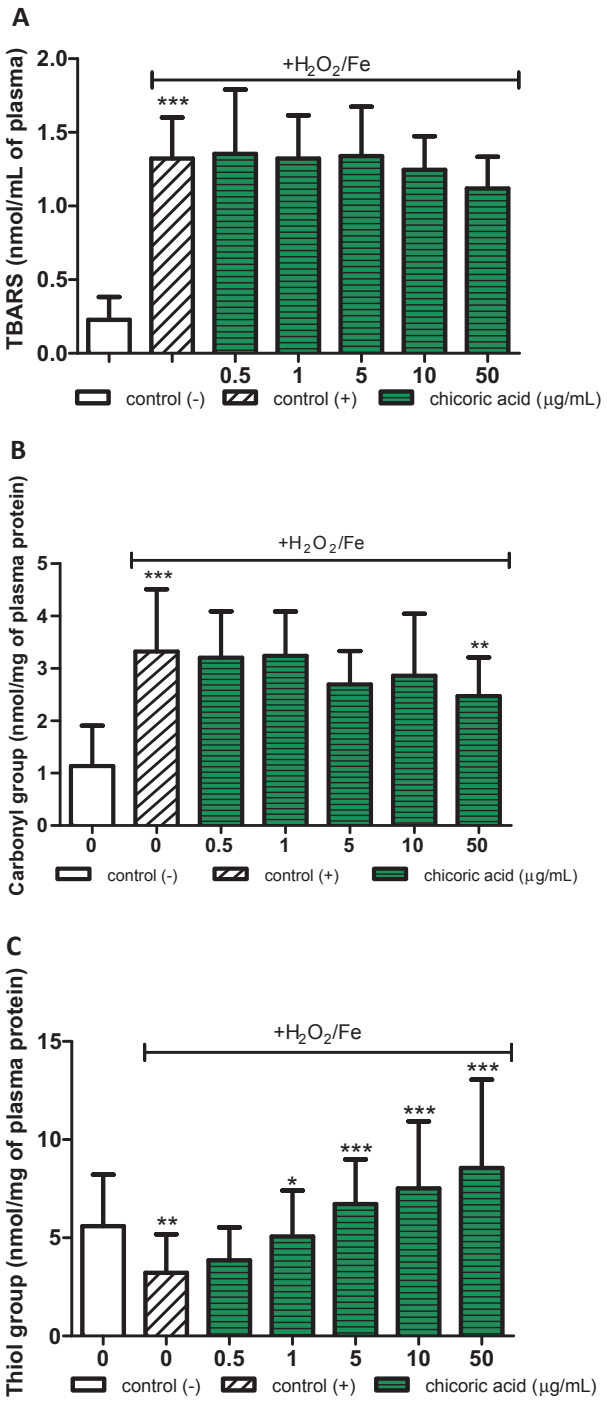

Fig. 1. Effects of chicoric acid (concentration range 0.5–50 μg/mL, pre-incubation time – 5 min) on lipid peroxidation in plasma treated with H_2O_2/Fe (incubation time – 25 min) (A), and on the oxidative damage to plasma protein treated with H_2O_2/Fe: the level of carbonyl groups (B) and the level of thiol groups (C). Results are given as mean ± SD (n = 10). Control negative refers to plasma not treated with H_2O_2/Fe, whereas control positive to plasma treated with H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with positive control (treated with H_2O_2/Fe).

applying Kruskal-Wallis test. For the sake of clarity, only the differences between the tested fractions and the control/control positive were marked. Results are presented as means ± SD. Significance was considered at $p < 0.05$. In order to eliminate uncertain data, the Q-Dixon test was performed.

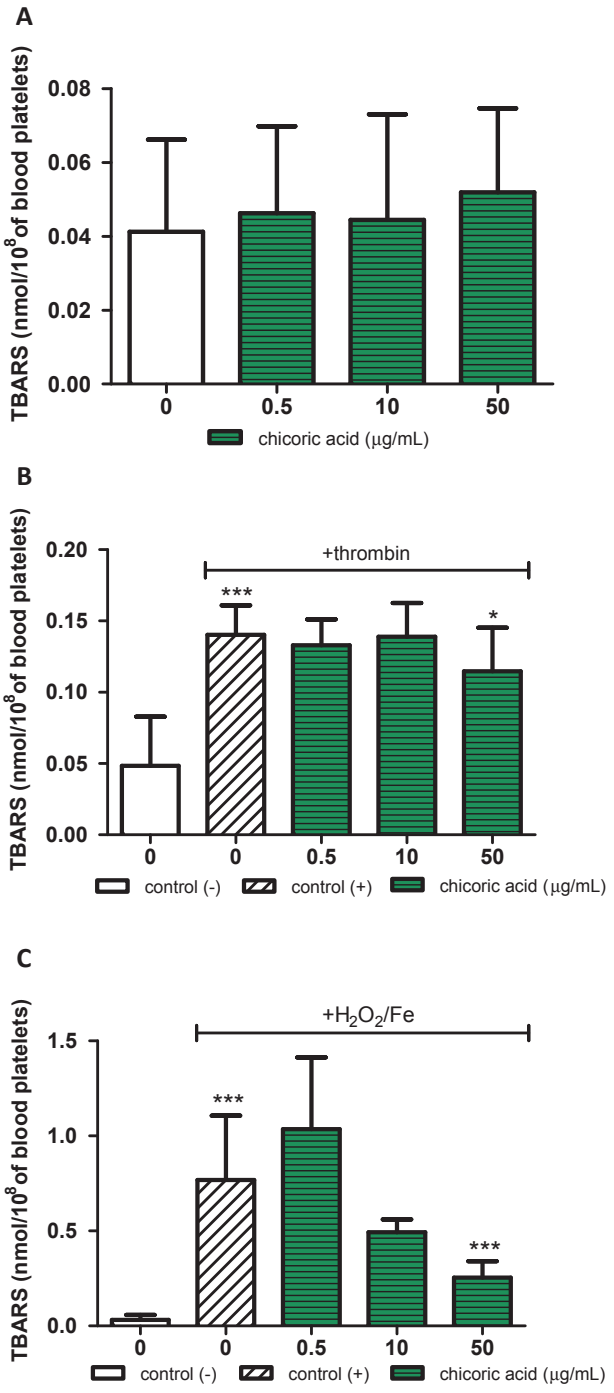

Fig. 2. Effects of chicoric acid (concentration range 0.5–50 μg/mL, incubation time – 30 min) on lipid peroxidation in resting blood platelets (A), platelets activated by 5 U/mL thrombin (pre-incubation time with chicoric acid – 25 min. and incubation time with thrombin – 5 min) (B), and platelets treated with H_2O_2/Fe (incubation time with chicoric acid– 5 min. and incubation time with H_2O_2/Fe – 25 min) (C). Results are given as mean ± SD (n = 4). Control negative refers to platelets not treated with thrombin or H_2O_2/Fe, whereas control positive to platelets treated with thrombin or H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, *$p < 0.05$, ***$p < 0.001$.

3. Results

The antioxidant, anticoagulant and anti-platelet activities of L-chicoric acid isolated from the dandelion leaves were studied *in vitro*. Chicoric acid administration did not influence the coagulation times of human plasma (APTT, PT or TT) at any of the tested concentrations, i.e.

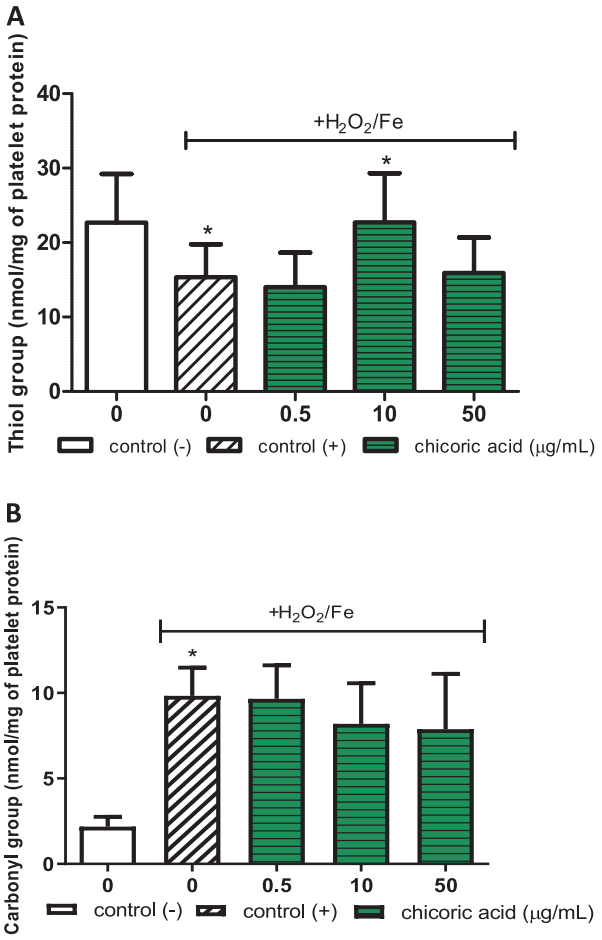

Fig. 3. Effects of chicoric acid (concentration range 0.5–50 μg/mL, pre-incubation time – 5 min) on the oxidative damages of platelet protein treated with H_2O_2/Fe (incubation time – 25 min): the level of thiol groups (A), and the level of carbonyl groups (B). Results are given as mean ± SD (n = 4). Control negative refers to platelets not treated with H_2O_2/Fe, whereas control positive refers to platelets treated with H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, *$p < 0.05$, compared with positive control (treated with H_2O_2/Fe).

0.5–50 μg/mL (Table 1), nor did it demonstrate any antioxidant action in plasma samples treated with H_2O_2/Fe (Fig. 1A). However, at the highest concentration (50 μg/mL) chicoric acid reduced H_2O_2/Fe-induced carbonylation of plasma proteins by about 25% in comparison with control (Fig. 1B). In addition, the oxidation of thiol groups in plasma proteins treated with H_2O_2/Fe was significantly inhibited at all tested concentrations (1, 5, 10 and 50 μg/mL) (Fig. 1C).

None of the tested concentrations of chicoric acid (0.5, 10 and 50 μg/mL) changed the level of TBARS in resting blood platelets (Fig. 2A); however, the highest concentration (50 μg/mL) significantly inhibited enzymatic lipid peroxidation by 20% compared to positive controls in thrombin-activated platelets (Fig. 2B) and platelet lipid peroxidation by about 70% in H_2O_2/Fe-induced platelets (Fig. 2C). In the platelets treated with H_2O_2/Fe, only 10 μg/mL chicoric acid increased the level of thiol groups (Fig. 3A); however, none of the tested concentrations (0.5, 10 and 50 μg/mL) changed the levels of carbonyl groups (Fig. 3B).

Regarding adhesion to collagen, chicoric acid did not inhibit adhesion of resting platelets at any tested concentration (0.5, 10 and 50 μg/mL), but it did inhibit that of thrombin-activated platelets at doses of 10 and 50 μg/mL (Fig. 4A and B). The reduction of adhesion to collagen of resting platelets and adhesion to collagen of thrombin-activated platelets in the presence of tested fractions from leaves and petals of dandelion fractions was also observed *in vitro* (Fig. 4), but the inhibition of adhesion to collagen was not always statistically significant (Fig. 4).

Adhesion of thrombin- or ADP-activated platelets to fibrinogen was significantly inhibited only by the highest tested concentration of chicoric acid (50 μg/mL) (Fig. 5A and B). Adhesion to fibrinogen by ADP-activated platelets was about 40% (Fig. 5B).

The same process was observed when we measured adhesion to fibrinogen of thrombin- or ADP-activated platelets in the presence of tested fractions (Fig. 5).

None of the tested concentrations of chicoric acid (0.5, 10 and 50 μg/mL) demonstrated anti-aggregatory properties against ADP-activated platelets (Fig. 6). Fractions B-D reduced platelet aggregation stimulated by ADP *in vitro* when administered at 50 μg/mL. For example, fraction C inhibited the process by about 30% (Fig. 6).

The platelet lysis test, based on measuring extracellular LDH activity, found that none of the tested concentrations of chicoric acid (0.5, 10 and 50 μg/mL) were cytotoxic (Fig. 7).

Table 2 compares the *in vitro* anti-platelet activity (anti-adhesive and anti-aggregatory activity) of pure chicoric acid with that of four dandelion phenolic fractions (A–D, 50 μg/mL) with different concentrations of chicoric acid. Of these, fraction A had the greatest anti-platelet potential. All five preparations demonstrated anti-platelet potential. In addition, fraction A exerted stronger anti-adhesive and anti-aggregatory properties than chicoric acid.

4. Discussion

Our findings indicate that the dietary intake of phenolic compounds may play an important role in the prophylaxis and treatment of chronic diseases, including cardiovascular diseases, which are the leading cause of mortality globally. In 2016, about 17.9 million CVD-related deaths were recorded worldwide, accounting for almost one-third of all deaths (WHO, 2017). As the most significant behavioral factor affecting health is dietary risk, it would appear to be the best target in the fight against CVDs. Key dietary components, such as fruits, vegetables and medicinal plants have all demonstrated positive effects on health. Recent studies have confirmed that dietary polyphenols have antioxidant, anti-inflammatory and antithrombotic properties, and can affect nitric oxide bioavailability (Godos et al., 2017).

One rich source of such biologically-active ingredients comprises the leaves, petals and other organs of dandelions. Of the phenolic compounds present in dandelion, those with the greatest beneficial impact on cardiovascular disorders and blood platelet functions may well be the flavonoids and phenolic acids (Ivanov, 2014; Treml & Smejkal, 2017). The predominant group of polyphenols are the hydroxycinnamic acids; of these, the key representative is chicoric acid, also known as chicoric acid or dicaffeoyltartaric acid. It was first isolated from chicory leaves and identified as a phenolic compound in 1958. Chicoric acid is now known to be present in at least 63 genera and species, including seagrass, horsetail, fern, lettuce and basil (Lee & Scagel, 2013; Liu et al., 2017). In addition, many *in vitro* and *in vivo* studies have found it to display antidiabetic (Azay-Milhau et al., 2013), anti-obesity (Xiao, Wang, et al., 2013), anti-inflammatory (Abd El-Twab, Hussein, Hozayen, Bin-Jumah, & Mahmoud, 2019), anticancer (Tsai, Chiu, Chen, Chan, & Lin, 2012) and antiviral properties (Lee, Yoon, & Lee, 2004).

LC-MS studies of the phenolic compounds from dandelion leaves and petals (Jedrejek et al., 2017, 2019; Lis et al., 2018) confirmed the presence of chicoric acid (Jedrejek et al., 2017). As it is unknown which of its chemical components is responsible for the antiplatelet and anticoagulation properties of the phenolic fraction, our study performed a broad examination of the influence of chicoric acid on both hemostasis and oxidative stress using blood platelets and plasma.

Our results confirm those of previous *in vitro* studies indicating that chicoric acid has antioxidant properties (Schlernitzauer et al., 2013; Thygesen, Thulin, Mortensen, Skibsted, & Molgaard, 2007). In addition,

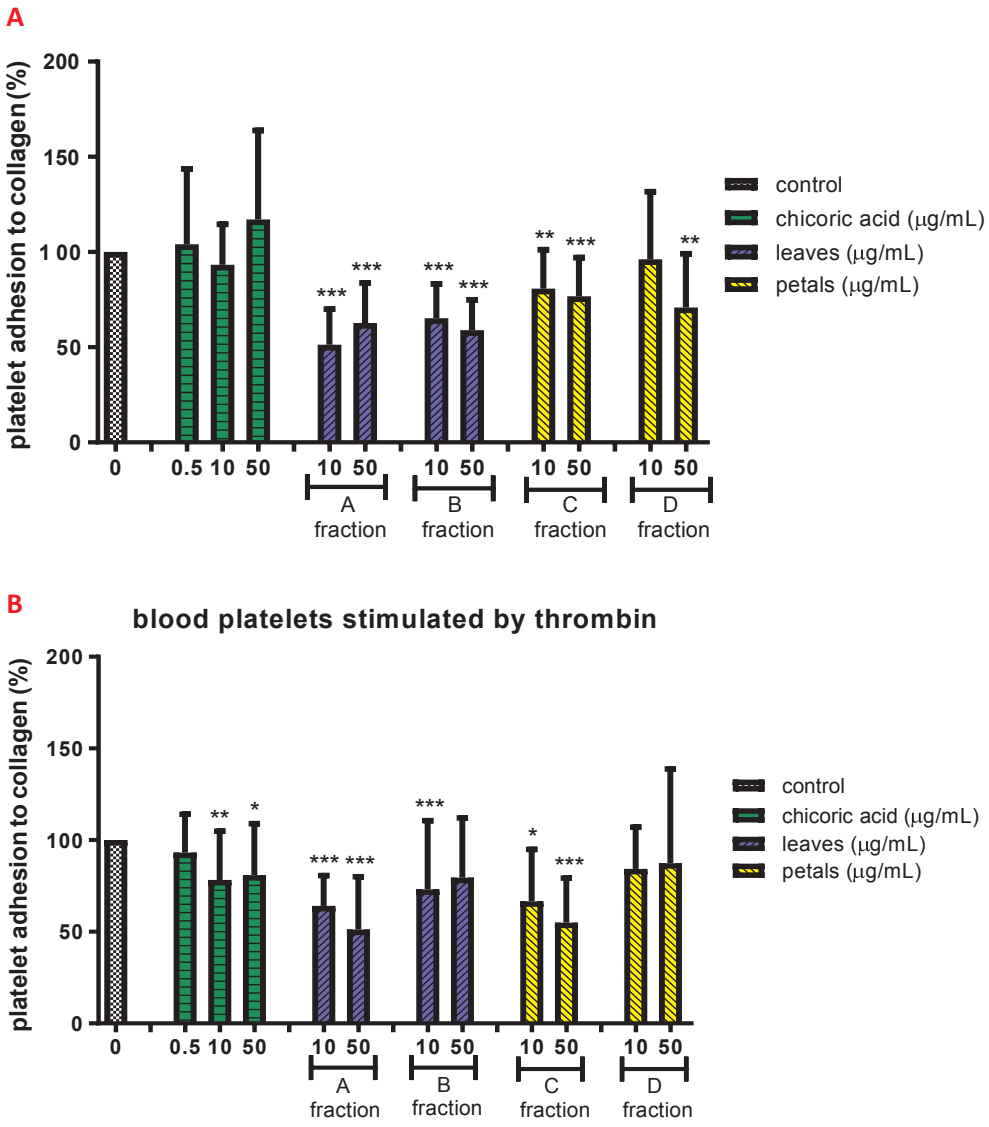

Fig. 4. Effects of chicoric acid (concentration range 0.5–50 μg/mL, incubation time – 30 min), and dandelion phenolic fractions (A–D, 10 and 50 μg/mL, incubation time – 30 min) on adhesion to collagen of resting blood platelets (A) and thrombin (final concentration 0.2 U/mL) – activated platelets (B). In the graphs, the adhesion is expressed as a percentage of the control samples (platelets without chicoric acid or phenolic fraction). Results are given as mean ± SD (n = 4–8). Kruskal-Wallis test: n.s. $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with positive control (treated with H_2O_2/Fe).

apart from the cytotoxicity measured by LDH activity, pure chicoric acid appears to have different biological properties to that of the entire fraction A; for example, the fraction reduces the oxidation of thiol groups in plasma proteins and platelet lipid peroxidation induced by a hydroxyl radical donor (H_2O_2/Fe^{2+}). The inhibition of lipid peroxidation was about 70% for 50 μg/mL chicoric acid. Similarly, *in vitro* studies found fraction A inhibited platelet lipid peroxidation by about 40% (Lis et al., 2018). It is interesting that in our model, chicoric acid has stronger antioxidant properties than the entire fraction A.

Blood platelet activation correlates with arachidonate metabolism. A new finding of the present study, using TBARS concentration as an indicator, is that 50 μg/mL chicoric acid (the highest concentration) inhibits enzymatic lipid peroxidation in thrombin-stimulated platelets. This finding may suggest that chicoric acid could modulate platelet activation by interfering with the metabolism of arachidonate; however, no such change was observed for the four tested dandelion fractions (A–D), of which three also contained the above compound (A–C) (Lis et al., 2018).

One of the key findings of our study was that chicoric acid displays anti-adhesive activity against isolated washed platelets activated by thrombin or ADP in the presence of two adhesive proteins: collagen and fibrinogen. In addition, dandelion leaf fraction A, from which the chicoric acid used in the present study was isolated, demonstrated the best anti-platelet activity for all five used models: adhesion of resting platelets to collagen (1), adhesion of thrombin-activated platelets to collagen (2), adhesion of thrombin-activated platelets to fibrinogen (3), adhesion of ADP-activated platelets to fibrinogen (4), and platelet aggregation stimulated by ADP (5). Interestingly, fraction A also exerted stronger anti-platelet properties than pure chicoric acid, even though chicoric acid represented about 80% of its total polyphenol content.

These results may suggest that the phenolics within the dandelion fraction may act synergistically, and this may affect their physiological properties. In addition, in contrast to the phenolic dandelion leaf and petal fractions (Lis et al., 2018), chicoric acid did not influence the coagulatory properties of human plasma *in vitro*.

The concentration of the tested chicoric acid and phenolic fractions (≤ 50 μg/mL) used in our study with plasma and blood platelets corresponds with the physiological concentrations of phenolic compounds available after oral administration (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005; Manach, Scalbert, Morand, Rémésy, &

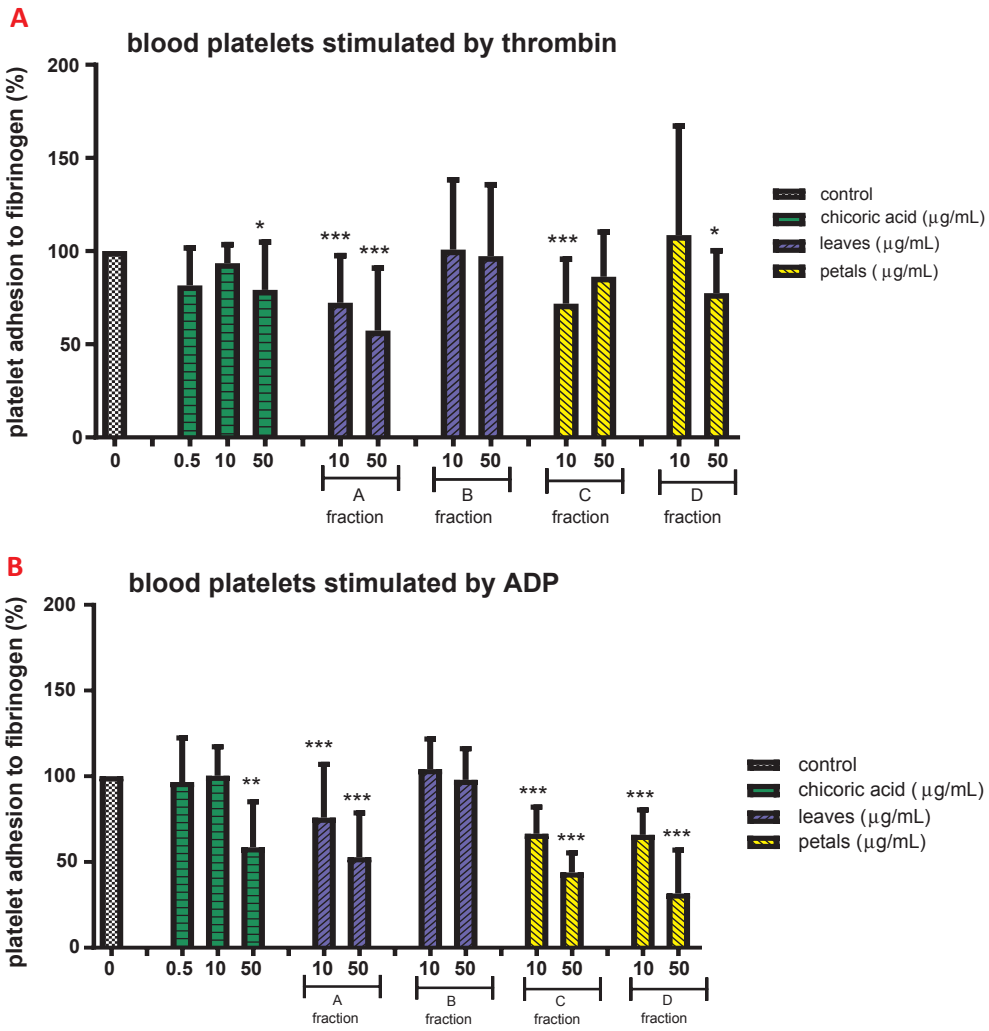

Fig. 5. Effects of chicoric acid (concentration range 0.5–50 µg/mL, incubation time – 30 min), and dandelion phenolic fractions (A–D, 10 and 50 µg/mL, incubation time – 30 min) on adhesion to fibrinogen of and thrombin (final concentration 0.2 U/mL) – activated platelets (A), and ADP (final concentration 30 µM) – activated platelets (B). In the graphs, the adhesion is expressed as a percentage of the control sample (platelets without chicoric acid or phenolic fraction). Results are given as mean ± SD (n = 4–8). Kruskal-Wallis test: n.s. $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with positive control (treated with H_2O_2/Fe).

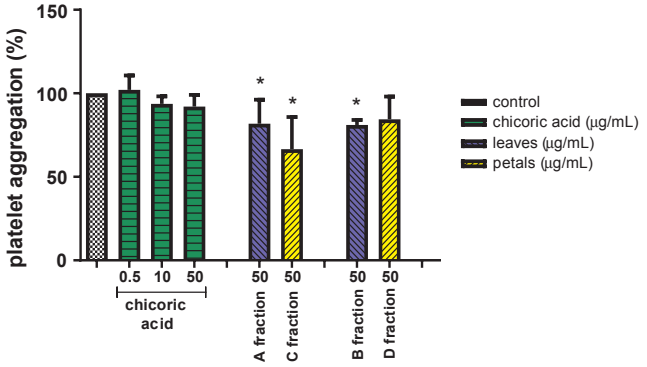

Fig. 6. Effects of chicoric acid (concentration range 0.5–50 µg/mL, incubation time – 30 min), and dandelion phenolic fractions (A–D, 50 µg/mL, incubation time – 30 min) on blood platelet aggregation stimulated by 10 µM ADP. In the graph, the aggregation is expressed as a percentage of the controls sample (PRP without chicoric acid or phenolic fraction). Results are given as mean ± SD (n = 3–5). Kruskal-Wallis test: n.s. $p > 0.05$, *$p < 0.05$, compared with control.

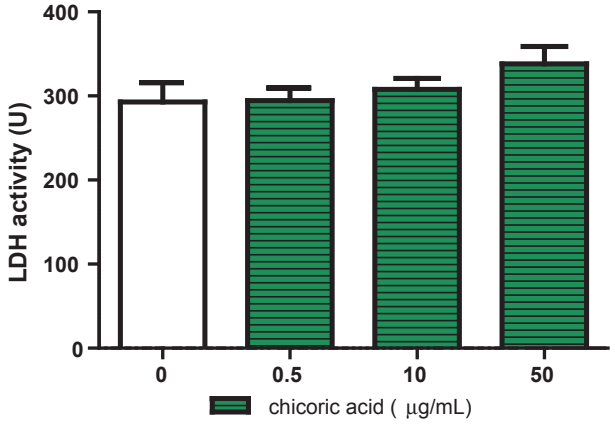

Fig. 7. Effects of chicoric acid (concentration range 0.5–50 µg/mL, incubation time – 30 min) on the damage of human blood platelets. Results are given as mean ± SD (n = 4). Kruskal-Wallis test: n.s. $p > 0.05$, compared with control.

Table 2
Comparison of the anti-platelet activity (anti-adhesive and anti-aggregatory action) of chicoric acid (50 μg/mL, incubation time – 30 min) isolated from fraction A, with those of the phenolic fractions from dandelion leaves and petals (A–D, 50 μg/mL, incubation time – 30 min).

	Inhibition of adhesion of resting platelets to collagen (%)	Inhibition of adhesion of thrombin-activated platelets to collagen (%)	Inhibition of adhesion of thrombin-activated platelets to fibrinogen (%)	Inhibition of adhesion of ADP-activated platelets to fibrinogen (%)	Inhibition of platelet aggregation stimulated by ADP (%)
Chicoric acid	No effect	21.5 ± 12.4	21.4 ± 5.7	44.7 ± 9.9	No effect
Leaf fraction A (chicoric acid represents about 80% of total polyphenols)	40.8 ± 12.2	49.4 ± 13.4	42.5 ± 10.2	52.4 ± 11.1	19.9 ± 4.5
Leaf fraction B (chicoric acid represents about 45% of total polyphenols)	45.7 ± 9.9	No effect	No effect	No effect	20.4 ± 5.5
Petal fraction C (chicoric acid represents about 40% of total polyphenols)	25.9 ± 11.1	44.6 ± 8.4	No effect	61.4 ± 12.4	37.4 ± 8.8
Petal fraction D (fraction without chicoric acid)	30.2 ± 9.4	No effect	23.4 ± 7.9	69.8 ± 11.5	No effect

Jiménez, 2004).

Phenolic compounds are metabolized by the body: following consumption, chicoric acid may circulate in various forms, including caffeic, dihydrocaffeic and dihydroferulic acid conjugates (Azay-Milhau et al., 2013; Liu et al., 2017). Interestingly, these dihydrocaffeic and dihydroferulic acids (0.01–100 μg/mL; *in vitro*) have been found to be stronger inhibitors of blood platelet activation than their phenolic precursors (Baeza et al., 2017). A significant new finding from our present study is that chicoric acid did not induce platelet damage across the entire tested range of concentrations.

To conclude, this is the first paper to present the multifunctionality of chicoric acid isolated from dandelion leaves. Our results show that chicoric acid has antioxidant and anti-adhesive properties and does not display any cytotoxic side effects towards platelets, indicating that it offers potential as a novel dietary component and pharmaceutical. Interestingly, the tested fraction richest in chicoric acid, i.e. leaf fraction A, was found to display not only anti-coagulant properties but also stronger anti-platelet activity than pure chicoric acid, manifested as anti-adhesive and anti-aggregatory properties. It may be that the chicoric acid component may only partly be responsible for the overall biological activity of the fraction, and that the properties of the dandelion fraction may derive from the synergistic relationships between its phenolic components. Moreover, the A fraction contains compounds other than chicoric acid (i.e. derivatives of phenolic acids and flavonoids) which may have stronger effect than this compound.

5. Ethics statement

The protocol was approved by the Committee for Research on Human Subjects of the University of Lodz (number 2/KBBN-UŁ/II/2016).

Declaration of Competing Interest

None.

Acknowledgment

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103524.

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Supplementary Materials

The anti-oxidative and hemostasis-related multifunctionality of L-chicoric acid, the main component of dandelion: an *in vitro* study of its cellular safety, antioxidant and anti-platelet properties, and effect on coagulation

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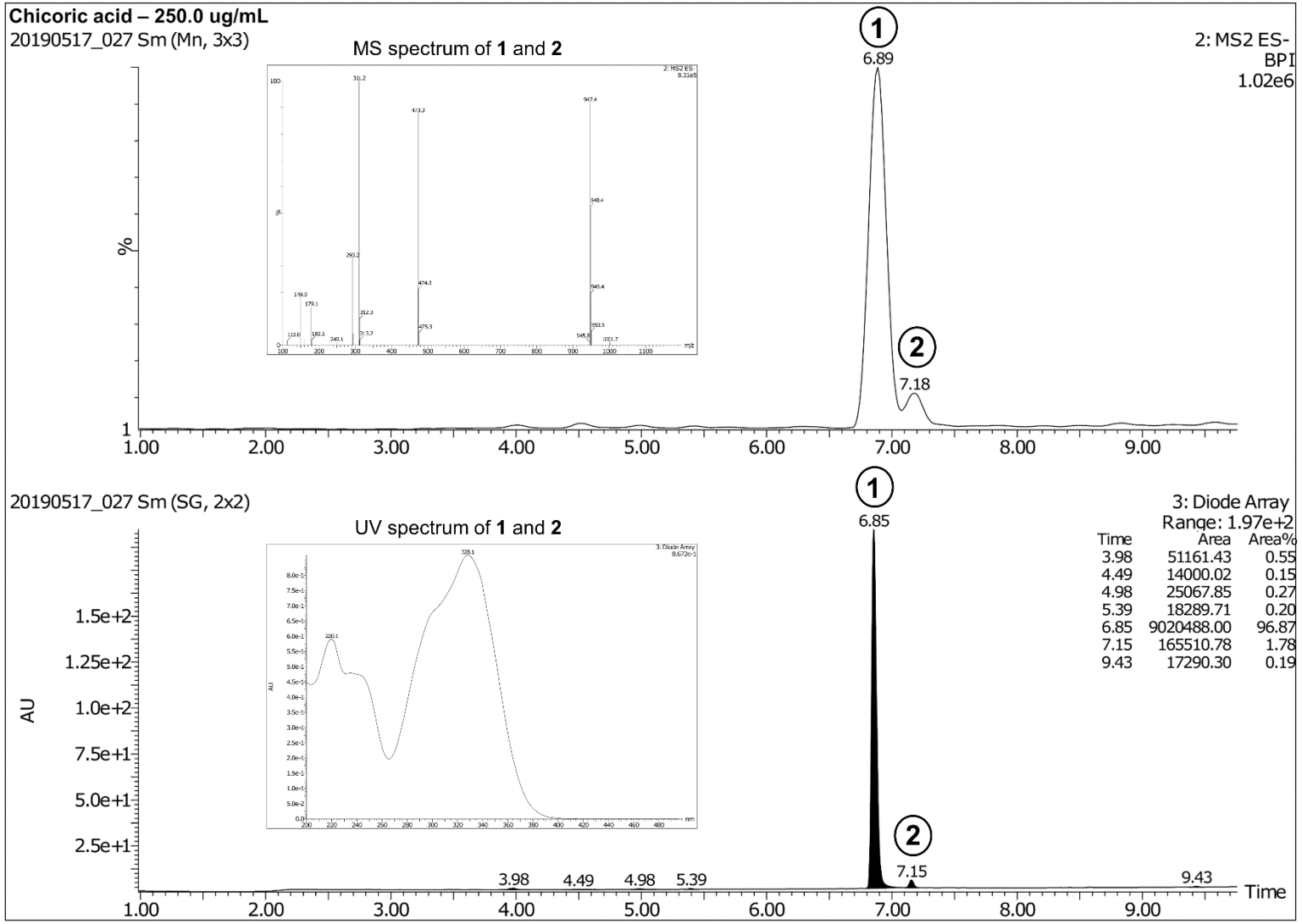

Figure 1S. MS and UV spectrum of isolated chicoric acid: L-chicoric acid – **1**; *meso*-chicoric acid – **2**.

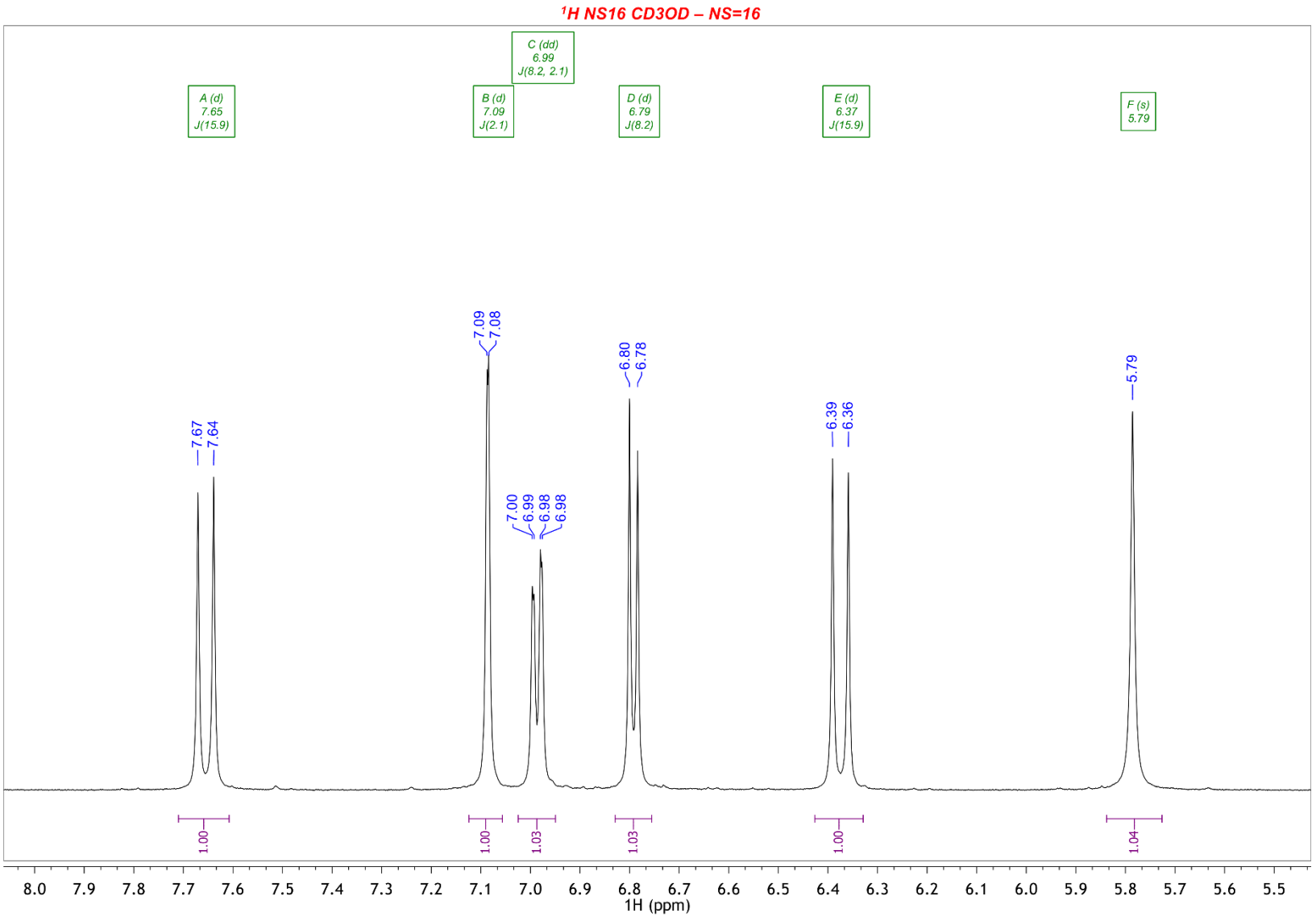

Figure 2S. ^{1}H NMR spectrum of chicoric acid.

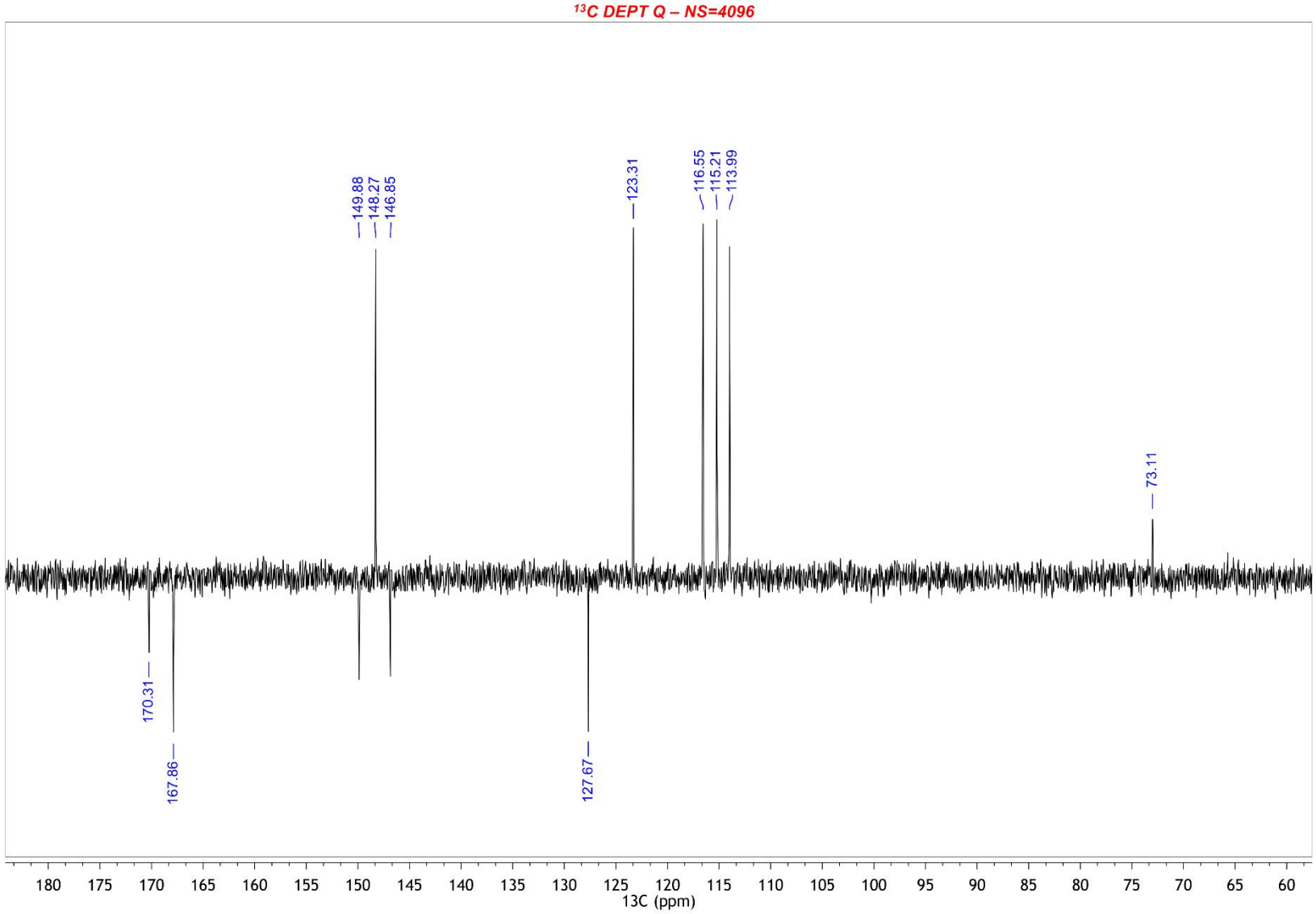

Figure 3S. ^{13}C NMR spectrum of chicoric acid.

Table S1. ^{1}H and ^{13}C-NMR data (MeOH-d_4, 500/125 MHz, at 30 °C) of isolated chicoric acid.

Position	δ_H (J in Hz)	δ_C
Caffeic acid (both subunits)		
1	-	127.7
2	7.09 d (2.1)	115.2
3	-	146.8
4	-	149.9
5	6.79 d (8.2)	116.6
6	6.70 dd (8.2, 2.1)	123.3
7	7.65 d (15.9)	148.3
8	6.37 d (15.9)	114.0
C=O	-	167.9
Tartaric acid		
C1=O	-	170.3
C4=O	-	170.3
2, 3	5.79 s	73.1

Table S2. Antioxidant activity and total phenolic content (TPC) of isolated chicoric acid.

Compound	Antioxidant activity		Total phenolic content (mg GAE/g DW)
	Trolox Equivalents (TE)	IC_{50} (mg DW/mL)	
chicoric acid	1.07 ± 0.01	0.104 ± 0.001	985.63 ± 4.22
Trolox	1.00	0.120 ± 0.003	-

molecules

MDPI

Article

Flavonoid Preparations from *Taraxacum officinale* L. Fruits—A Phytochemical, Antioxidant and Hemostasis Studies

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Abstract: Dandelion (*Taraxacum officinale* L.) roots, leaves, and flowers have a long history of use in traditional medicine. Compared to the above organs, dandelion fruits are the least known and used. Hence, the present paper was aimed at the phytochemical analysis of *T. officinale* fruit extract and estimating its antiradical, antiplatelet, and antioxidant properties related to hemostasis. Methanolic extract of fruits (E1), enriched with polyphenols (188 mg gallic acid equivalents (GAE)/g), was successfully separated into cinnamic acids (E2; 448 mg GAE/g) and flavonoids (E3; 377 mg GAE/g) extracts. Flavonoid extract was further divided into four fractions characterized by individual content: A (luteolin fraction; 880 mg GAE/g), B (philonotisflavone fraction; 516 mg GAE/g), C (flavonolignans fraction; 384 mg GAE/g), and D (flavone aglycones fraction; 632 mg GAE/g). High DPPH radical scavenging activity was evaluated for fractions A and B (A > B > Trolox), medium for extracts (Trolox > E3 > E2 > E1), and low for fractions C and D. No simple correlation between polyphenol content and antiradical activity was observed, indicating a significant influence of qualitative factor, including higher anti-oxidative effect of flavonoids with B-ring catechol system compared to hydroxycinnamic acids. No cytotoxic effect on platelets was observed for any dandelion preparation tested. In experiments on plasma and platelets, using several different parameters (lipid peroxidation, protein carbonylation, oxidation of thiols, and platelet adhesion), the highest antioxidant and antiplatelet potential was demonstrated by three fruit preparations–hydroxycinnamic acids extract (E2), flavonoid extract (E3), and luteolin fraction (A). The results of this paper provide new information on dandelion metabolites, as well as their biological potential and possible use concerning cardiovascular diseases.

Keywords: dandelion fruits; HR-QTOF-MS; flavones; biflavones; flavonolignans; antiplatelet activity; adhesion; blood platelets; oxidative stress

1. Introduction

The occurrence of some civilization diseases, such as atherosclerosis, diabetes, heart attack, and some cancers, has been associated with elevated levels of oxidative stress and dysfunctional hemostasis. Hemostasis is defined as a sequence of reactions that maintain the fluidity of circulating

blood, while also preventing its outflow in the event of a break in the vessel [1,2]. However, the structure and functioning of the biomolecules involved in hemostasis can be impaired by the effects of reactive oxygen species (ROS) [3]. ROS concentrations can be increased in response to biotic factors such as enzymatic reactions, oxidation of respiratory proteins or oxidation of xenobiotics, as well as abiotic factors such as ionizing radiation, environmental pollution, toxins, drugs, and an unhealthy diet. In contrast, many studies indicate that the consumption of products rich in natural antioxidants, i.e., certain foods and nutraceuticals, has a positive effect on maintaining the oxidant-antioxidant balance in the body [4,5]. Many such substances with antiradical and antioxidant properties have been identified so far, and some, such as ascorbic acid, masoprocol, pramipexole, allopurinol, vitamin A or vitamin E [6], are currently used as components of drugs or dietary supplements. Nevertheless, new compounds with such properties are constantly being sought.

Medical plants and their extracts are also particularly well-exploited sources of natural antioxidants. Of these, the last few decades have seen a growth in interest in the dandelion, particularly *T. officinale*, as a source of bioactive compounds bestowing anti-inflammatory, antioxidative, choleretic, diuretic, hepatoprotective, and immunostimulatory effects [7]. In particular, the antioxidant and anti-inflammatory activities of dandelion extracts and preparations have been mainly attributed to their polyphenolic components [8,9]. The most abundant phenolic compounds found in either *Taraxacum* root or aerial parts are hydroxycinnamic acid derivatives (HCAs), such as chicoric (dicaffeoyltartaric), caftaric (monocaffeoyltartaric), and chlorogenic acids [10,11]. Additionally, many flavonoids, such as free aglycones and flavonoid glycosides, have been identified in both flower and leaf extracts, with the most commonly-reported examples being the flavones luteolin, chrysoeriol and apigenin, and various flavonols, such as quercetin, and their *O*-glycosides glucoside and rutinoside [12,13].

Dandelion fruits are straw- to brown-colored achenes and lanceolate in shape. Moreover, the fruits are 3–5 mm long and equipped with characteristic silky pappi to allow wind dispersion. However, very few, if any, studies on *T. officinale* have examined the phytochemical composition and biological properties of its fruit, most likely because they are not regarded as herbal material, unlike the more widely-used roots, leaves and flowers. In the only study, the ethanol extract of *T. officinale* fruits showed free radical scavenging activity and protective effect against oxidation of rat brain cells (neuroprotective activity). The positive action was finally correlated with an elevated phenolic content in the plant extract, however, more detailed information on its chemical composition was lacking [14].

The current study was aimed at the phytochemical analysis of *T. officinale* fruit extract and estimating its antiradical, antiplatelet, and antioxidant properties related to hemostasis. Efforts were also made to estimate the contribution of individual polyphenols on the reported biological activity of dandelion fruits.

2. Results

2.1. Phytochemical Characteristics of Plant Extracts and Fractions

Preliminary UHPLC-PDA-CAD-ESI-QTOF-MS/MS analysis revealed the presence of numerous compounds in methanol extract (E1) of dandelion fruits. The peaks were tentatively identified and classified based on MS and UV spectra and using the SIRIUS 4 tool [15] and the literature; while most appeared to be phenolic derivatives, several unidentified nitrogen-containing metabolites were also detected (Figure 1 and Table 1, peaks were assigned by retention time). Next, the full identity of 14 metabolites was confirmed by comparison with possessed authentic standard compounds (Table 1); these included four caffeic acid esters (5-*O*-caffeoylquinic, 3,5-di-caffeoylquinic, caffeic and L-chicoric acid), nine flavonoids (7-, 4′-, and 3′-*O*-glucosides of luteolin, apigenin, apometzgerin, chrysoeriol, luteolin, philonotisflavone, and tricin) and a sesquiterpene lactone (taraxinic acid-1′-*O*-glucoside). Structures of known described compounds are shown in Figure S2. Consequently, the dominant phenolic constituents in extract E1 of dandelion fruits were found to be hydroxycinnamic acid derivatives (HCAs) and flavone derivatives, which are known constituents of leaves and flowers,

the most widely-studied dandelion aerial organs. Besides, several metabolites not yet reported in *T. officinale*, such as biflavones and some flavonolignans, were also detected (Table 1).

Subsequent multistep fractionation of methanolic extract of dandelion fruits (E1) allowed its successful separation into phenolic acid extract (E2) and flavonoid extract (E3), then extract E3 was further divided into four flavonoid fractions A–D (Figure S1). The phytochemical profiles of all dandelion preparations were examined in detail using UHPLC-PDA-CAD-ESI-QTOF-MS/MS analyses. As can be seen in Figure 1, showing CAD chromatograms of dandelion extracts and fractions (peak numbering according to Table 1), each preparation has an individual composition. Consequently, prepared flavonoid fractions A–D can easily be described according to their composition: A (luteolin fraction), B (philonotisflavone fraction), C (flavonolignan fraction), and D (flavone aglycone fraction).

The contents of hydroxycinnamic acids and flavonoids in dandelion fruit extracts (E1–E3) and fractions (A–D) were evaluated by UHPLC-PDA and expressed as mg L-chicoric acid or luteolin equivalents per gram DW of extract/fraction (Table 2). Caffeic acid derivatives, mainly L-chicoric acid, were the dominant polyphenols in the extract E1 (about 150 mg/g DW) and E2 (about 530 mg/g DW); in turn, the HCAs were a minor component of extract E3 (about 30 mg/g DW) and were completely absent from fractions A–D. The major phenolic compounds in the flavonoid preparations were flavone derivatives (E3 and Fr A–D, in the range between 280–995 mg/g DW); these were sometimes the only polyphenols present.

Additionally, the total phenolic content (TPC) in extracts and fractions of dandelion fruits was estimated by the Folin-Ciocalteu assay (Table 2). In general, the values obtained by TPC were comparable with those of the UHPLC assay, as indicated by the color-coded results given in Table 2. The highest TPC values were found in fractions A and D (880 and 632 mg GAE/g, respectively), and the lowest in extracts E1 and E3 (188 and 377 mg GAE/g, respectively).

2.2. DPPH Free Radical Scavenging Activity

The DPPH radical scavenging activity of dandelion fruits phenolic preparations (E1–E3, Fr A–D) was expressed as Trolox Equivalents (TE) and IC_{50} values (Table 3). The estimated TE values varied from 0.05 to 2.01 of Trolox activity, with 1.00 indicating equivalence to Trolox. Fraction A (luteolin) and B (philonotisflavone) demonstrated the highest scavenging activity (2.01 and 1.09 TE, respectively), followed by the three extracts E1–E3 (0.26–0.55 TE), and the fractions D and C (0.05 and 0.06 TE, respectively). The antioxidant capacity of the tested samples (dandelion preparations and Trolox) proceeded in the following order: Fr A (luteolin) > Fr B (philonotisflavone) > Trolox > E3 (flavonoid extract) > E2 (phenolic acid extract) > E1 (total extract) > Fr C (flavonolignans) > Fr D (flavone aglycones).

Table 1. UHPLC-QTOF-MS/MS data of metabolites identified in the methanol extract (E1) of *Taraxacum officinale* L. fruits.

No	Identity	RT (min)	Formula	UV_{max} (nm)	Error (ppm)	mσ	Observed $[M-H]^-$	Major Fragments (%)
1	unidentified	1.10	$C_{13}H_9N_4O_6$	260	−5.2	28.6	317.0544	225.0072 (23), 164.9851 (3)
2	di-hexose (sucrose)	1.27	$C_{13}H_{23}O_{13}$	220	1.6	2.3	387.1138 $[M + HCO_2H - H]^-$, 341.1085	179.0563 (17), 161.0449 (5)
3	tri-hexose (raffinose)	1.27	$C_{19}H_{33}O_{18}$	220	1.1	7.1	549.1666 $[M + HCO_2H - H]^-$, 503.1610	179.0563 (8), 341.1085 (5)
4	unidentified	1.63	$C_9H_{11}N_2O_6$	275	0.6	5.7	243.0621	-
5	unidentified	3.39	$C_{10}H_{11}N_4O_6$	262	1.2	9.9	283.0681	151.0256 (100)
6	caffeoyl-di-*O*-hexoside	4.08	$C_{22}H_{29}O_{16}$	290br	1.8	6.7	549.1455 $[M + HCO_2H - H]^-$	341.0875 (100), 179.0349 (10)
7	di-hydroxy-benzoic acid hexoside	4.52	$C_{13}H_{15}O_9$	305	0.5	15.2	315.0720	153.0186 (100)
8	caftaric acid	4.78	$C_{13}H_{11}O_9$	328, 295sh	−0.3	15.7	311.0410	179.0348 (100), 135.0438 (51), 149.0079 (15)
9	unidentified	4.97	$C_{15}H_{20}NO_6$	328, 295sh	2.6	16.1	310.1288 $[M + HCO_2H - H]^-$	250.1084 (100), 161.0237 (4), 179.0356 (2)
10	caffeoyl-*O*-hexoside	5.48	$C_{15}H_{17}O_9$	320, 290sh	−0.3	15.8	341.0879	179.0351 (100), 135.0444 (16)
11	5-*O*-caffeoylquinic acid *	5.59	$C_{16}H_{17}O_9$	325, 295sh	0.3	5.1	353.0877	191.0562 (100)
12	caffeic acid	6.11	$C_9H_7O_4$	323, 295sh	−0.8	7.6	179.0351	135.0440 (100)
13	unidentified	6.37	$C_{18}H_{27}O_9$	308br	−0.3	28.9	387.1664	207.1029 (15)
14	luteolin-*O*-di-hexoside	6.95	$C_{27}H_{29}O_{16}$	255, 345	2.7	6.8	609.1445	285.0394 (12)
15	L-chicoric acid	7.38	$C_{22}H_{17}O_{12}$	329, 295sh	1.8	5.8	473.0727	179.0354 (100), 293.0306 (49), 311.0412 (44), 149.0088 (33), 219.0301 (26)
16	luteolin-7-*O*-glucoside	7.76	$C_{21}H_{19}O_{11}$	255, 267, 345	−0.9	19.7	447.0937	285.0408 (96)
17	3,5-di-caffeoylquinic acid	8.26	$C_{25}H_{23}O_{12}$	328, 295sh	1.2	4.5	515.1189	353.0871 (100), 191.0557 (26), 179.0347 (12)
18	luteolin-4′-*O*-glucoside	8.50	$C_{21}H_{19}O_{11}$	269, 337	−1.6	18.1	447.0937	285.0408 (100)
19	luteolin-3′-*O*-glucoside #	8.85	$C_{21}H_{19}O_{11}$	267, 337	−2.0	27.4	447.0942	285.0407 (100)
20	taraxinic acid-1′-*O*-glucoside	9.01	$C_{22}H_{29}O_{11}$	220	−0.5	4.0	469.1718 $[M + HCO_2H - H]^-$	261.1135 (100), 217.1235 (59)
21	luteolin	10.23	$C_{15}H_9O_6$	255, 267, 345	0.3	3.1	285.0404	199.0399 (4), 217.0506 (3), 241.0505 (3)
22	philonotisflavone #	10.23	$C_{30}H_{17}O_{12}$	255, 342	−1.5	5.7	569.0734	391.0469 (8), 433.0574 (4), 459.0371 (3)
23	methyltricetin	10.27	$C_{16}H_{11}O_7$	267, 347	0.4	15.0	315.0509	300.0274 (100), 272.0322 (7)

Table 1. *Cont.*

No	Identity	RT (min)	Formula	UV_{max} (nm)	Error (ppm)	mσ	Observed $[M - H]^-$	Major Fragments (%)
24	bi-flavone (luteolin-luteolin) #	10.52	$C_{30}H_{17}O_{12}$	255, 345	−0.2	20.7	569.0727	417.0610 (10), 285.0411 (2)
25	bi-flavone (luteolin-apigenin) #	10.93	$C_{30}H_{17}O_{11}$	257, 345	−2.3	25.2	553.0789	391.0478 (13), 433.0572 (3), 459.0370 (3)
26	bi-flavone (luteolin-apigenin) #	11.09	$C_{30}H_{17}O_{11}$	257, 345	−1.4	6.4	553.0791	401.0682 (19)
27	bi-flavone (luteolin-chrysoeriol) #	11.28	$C_{31}H_{19}O_{12}$	257, 343	−0.9	9.3	583.0891	431.0781 (11)
28	apigenin	11.32	$C_{15}H_9O_5$	267, 337	−1.6	9.7	269.0460	225.0562 (10)
29	bi-flavone (luteolin-chrysoeriol) #	11.38	$C_{31}H_{19}O_{12}$	257, 343	−1.0	7.6	583.0892	431.0779 (11)
30	flavonolignan (tricin-lignan (*m/z* 170) conjugate) #	11.50	$C_{26}H_{23}O_{10}$	269, 338	2.7	1.7	495.1283, 541.1340 $[M + HCO_2H - H]^-$	329.0658 (100), 447.1076 (8)
31	tricin	11.54	$C_{17}H_{13}O_7$	255, 267, 351	−1.7	0.5	329.0672	299.0204 (100), 314.0438 (71), 271.0256 (10)
32	chrysoeriol	11.62	$C_{16}H_{11}O_6$	251, 267, 345	−2.2	7.0	299.0568	284.0333 (100), 256.0384 (13)
33	flavonolignan (salcolin A/B)	11.62	$C_{27}H_{25}O_{11}$	271, 337	2.7	3.4	525.1388, 571.1445 $[M + HCO_2H - H]^-$	329.0659 (100), 314.0426 (14), 195.0659 (10), 165.0551 (7)
34	flavonolignan (tricin-lignan (*m/z* 170) conjugate) #	11.77	$C_{26}H_{23}O_{10}$	271, 337	2.4	4.3	495.1285, 541.1342 $[M + HCO_2H - H]^-$	329.0659 (100), 314.0425 (24), 135.0441 (4)
35	flavonolignan (salcolin A/B)	11.94	$C_{27}H_{25}O_{11}$	271, 337	2.1	1.4	525.1391, 571.1448 $[M + HCO_2H - H]^-$	329.0660 (100), 314.0425 (14), 195.0659 (9), 165.0551 (6)
36	flavonolignan (tricin derivative) #	12.07	$C_{37}H_{35}O_{14}$	271, 340	2.7	18.1	703.2013, 749.2069 $[M + HCO_2H - H]^-$	329.0657 (100), 373.1282 (41), 673.1911 (30), 343.1178 (18), 685.1909 (16)
37	apometzgerin	12.12	$C_{17}H_{13}O_7$	269, 335	−2.5	2.2	329.0675	314.0441 (100), 299.0206 (95), 271.0257 (11)
38	flavonolignan (tricin-lignan (*m/z* 194) conjugate) #	12.33	$C_{27}H_{23}O_{11}$	271, 327	1.9	11.2	523.1236, 569.1291 $[M + HCO_2H - H]^-$	329.0659 (100), 314.0422 (6)
39	flavonolignan (tricin derivative) #	12.43	$C_{37}H_{35}O_{14}$	271, 340	2.8	14.1	703.2013, 749.2069 $[M + HCO_2H - H]^-$	329.0658 (100), 373.1286 (53), 673.1913 (20), 343.1178 (17), 685.1911 (5)

* the identity of the underlined metabolites was confirmed with authentic isolated compounds. # metabolite reported for the first time in dandelion (*Taraxacum officinale* L.).

Table 2. Comparison of phytochemical profile and phenolic content in tested extracts (E1–E3) and fractions (A–D) of dandelion fruits.

No	Compound	Content [mg Standard eq/g DW] (Mean ± SD)						
		E1 (Total Extract)	E2 (Phenolic Acid Extract)	E3 (Flavonoid Extract)	Fr A (Luteolin)	Fr B (Philonotisflavone)	Fr C (Flavonolignans)	Fr D (Flavone Aglycones)
1	unidentified	+ [a]	+	ND [b]	ND	ND	ND	ND
2	di-hexose (sucrose)	+	+	+	ND	ND	ND	ND
3	tri-hexose (raffinose)	+	+	+	ND	ND	ND	ND
4	unidentified	+	+	+	ND	ND	ND	ND
5	unidentified	+	+	ND	ND	ND	ND	ND
6	caffeoyl-di-*O*-hexoside	6.02 ± 0.14	27.63 ± 0.20	ND	ND	ND	ND	ND
7	di-hydroxy-benzoic acid hexoside	+	+	ND	ND	ND	ND	ND
8	caftaric acid	4.97 ± 0.33	ND	ND	ND	ND	ND	ND
9	unidentified	+	+	+	ND	ND	ND	ND
10	caffeoyl-*O*-hexoside	1.02 ± 0.03	4.68 ± 0.26	ND	ND	ND	ND	ND
11	5-*O*-caffeoylquinic acid	21.97 ± 0.56	92.50 ± 1.23	1.88 ± 0.06	ND	ND	ND	ND
12	caffeic acid	1.41 ± 0.03	2.60 ± 0.08	9.19 ± 0.14	ND	ND	ND	ND
13	unidentified	+	ND	+	ND	ND	ND	ND
14	luteolin-*O*-di-hexoside	++ [c]	2.42 ± 0.05	ND	ND	ND	ND	ND
15	L-chicoric acid	104.60 ± 3.33	384.40 ± 4.43	1.41 ± 0.10	ND	ND	ND	ND
16	luteolin-7-*O*-glucoside	1.42 ± 0.06	1.81 ± 0.05	6.13 ± 0.09	ND	ND	ND	ND
17	3,5-di-caffeoylquinic acid	7.54 ± 0.26	18.75 ± 0.50	17.19 ± 0.21	ND	ND	ND	ND
18	luteolin-4′-*O*-glucoside	1.00 ± 0.03	0.76 ± 0.04	3.85 ± 0.08	ND	ND	ND	ND
19	luteolin-3′-*O*-glucoside	0.64 ± 0.03	ND	4.46 ± 0.09	ND	ND	ND	ND
20	taraxinic acid-1′-*O*-glucoside	+	ND	+	ND	ND	ND	ND
21	luteolin		ND		985.86 ± 13.50	ND	ND	ND
22	philonotisflavone	36.03 ± 1.12	ND	203.89 ± 2.58	++	472.21 ± 1.51	ND	ND
23	methyltricetin		ND		8.56 ± 0.20	ND	ND	ND
24	bi-flavone (luteolin-luteolin)	0.85 ± 0.02	ND	3.73 ± 0.02	ND	ND	ND	ND
25	bi-flavone (luteolin-apigenin)	++	ND	++	ND	3.80 ± 0.22	ND	ND
26	bi-flavone (luteolin-apigenin)	++	ND	0.35 ± 0.03	ND	2.60 ± 0.09	ND	ND
27	bi-flavone (luteolin-chrysoeriol)	++	ND	1.50 ± 0.05	ND	++	ND	ND
28	apigenin	0.37 ± 0.04	ND	2.88 ± 0.18	ND	ND	ND	69.60 ± 1.10
29	bi-flavone (luteolin-chrysoeriol)	++	ND	1.20 ± 0.08	ND	5.44 ± 0.08	ND	ND
30	flavonolignan (tricin-lignan (*m/z* 170) conjugate)	++	ND	++	ND	ND	87.03 ± 5.18	ND

Table 2. *Cont.*

No	Compound	Content [mg Standard eq/g DW] (Mean ± SD)						
		E1 (Total Extract)	**E2 (Phenolic Acid Extract)**	**E3 (Flavonoid Extract)**	**Fr A (Luteolin)**	**Fr B (Philonotisflavone)**	**Fr C (Flavonolignans)**	**Fr D (Flavone Aglycones)**
31	tricin	6.53 ± 0.17	ND	37.13 ± 0.41	ND	ND	ND	799.09 ± 8.19
32	chrysoeriol		ND		ND	ND	ND	
33	flavonolignan (salcolin A/B)	0.75 ± 0.01	ND	4.97 ± 0.12	ND	ND	107.28 ± 6.60	ND
34	flavonolignan (tricin-lignan (*m/z* 170) conjugate)	0.77 ± 0.01	ND	4.57 ± 0.14	ND	ND	96.93 ± 5.80	ND
35	flavonolignan (salcolin A/B)	0.89 ± 0.03	ND	6.02 ± 0.21	ND	ND	109.89 ± 6.39	ND
36	flavonolignan (tricin derivative)	++	ND	++	ND	ND	4.37 ± 0.42	ND
37	apometzgerin	++	ND	++	ND	ND	ND	18.01 ± 0.80
38	flavonolignan (tricin-lignan (*m/z* 194) conjugate)	++	ND	0.62 ± 0.01	ND	ND	11.74 ± 0.67	ND
39	flavonolignan (tricin derivative)	++	ND	++	ND	ND	++	ND
	Total caffeic acid derivatives	147.53 ± 4.68	530.56 ± 6.70	29.67 ± 0.51	ND	ND	ND	ND
	Total flavonoids	48.48 ± 1.52	4.99 ± 0.14	281.30 ± 4.03	994.42 ± 13.70	484.05 ± 1.90	417.24 ± 25.06	886.71 ± 10.09
	Total phenolic compounds	196.01 ± 6.20	535.55 ± 6.84	310.97 ± 4.60	994.42 ± 13.70	484.05 ± 1.90	417.24 ± 25.06	886.71 ± 10.09
	Total phenolic content (mg GAE/g DW)	187.70 ± 0.22	447.58 ± 2.21	377.42 ± 1.77	879.55 ± 2.76	516.13 ± 3.31	384.27 ± 2.21	631.71 ± 2.95

[a] +, present but not quantified. [b] ND, not detected. [c] ++, concentration below the lower limit of quantification (LLOQ). Color code generated with the Quick Analysis tool (Microsoft Excel) indicates the level of metabolite content in tested preparations (green (higher level)→white (medium)→red (lower)).

Table 3. Antioxidant activity of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits against DPPH free radical (mean ± SD, $n = 3$) [a,b].

Sample	Trolox Equivalents (TE)	IC_{50} (mg DW/mL)
E1 (total extract)	0.26 ± 0.00b	0.424 ± 0.01d
E2 (phenolic acid extract)	0.48 ± 0.00c	0.215 ± 0.01c
E3 (flavonoid extract)	0.55 ± 0.03d	0.202 ± 0.01c
Fr A (luteolin)	2.01 ± 0.01g	0.055 ± 0.00a
Fr B (philonotisflavone)	1.09 ± 0.01f	0.099 ± 0.00b
Fr C (flavonolignans)	0.06 ± 0.00a	1.368 ± 0.04e
Fr D (flavone aglycones)	0.05 ± 0.00a	1.789 ± 0.04f
Trolox	1.00e	0.113 ± 0.00b

[a] Within each column, different letters (a–g) indicate significant differences in means ($p < 0.05$). [b] Color code generated with the Quick Analysis tool (Microsoft Excel) indicates the level of activity of the tested preparations (red (high activity)→white (medium)→ green(low)).

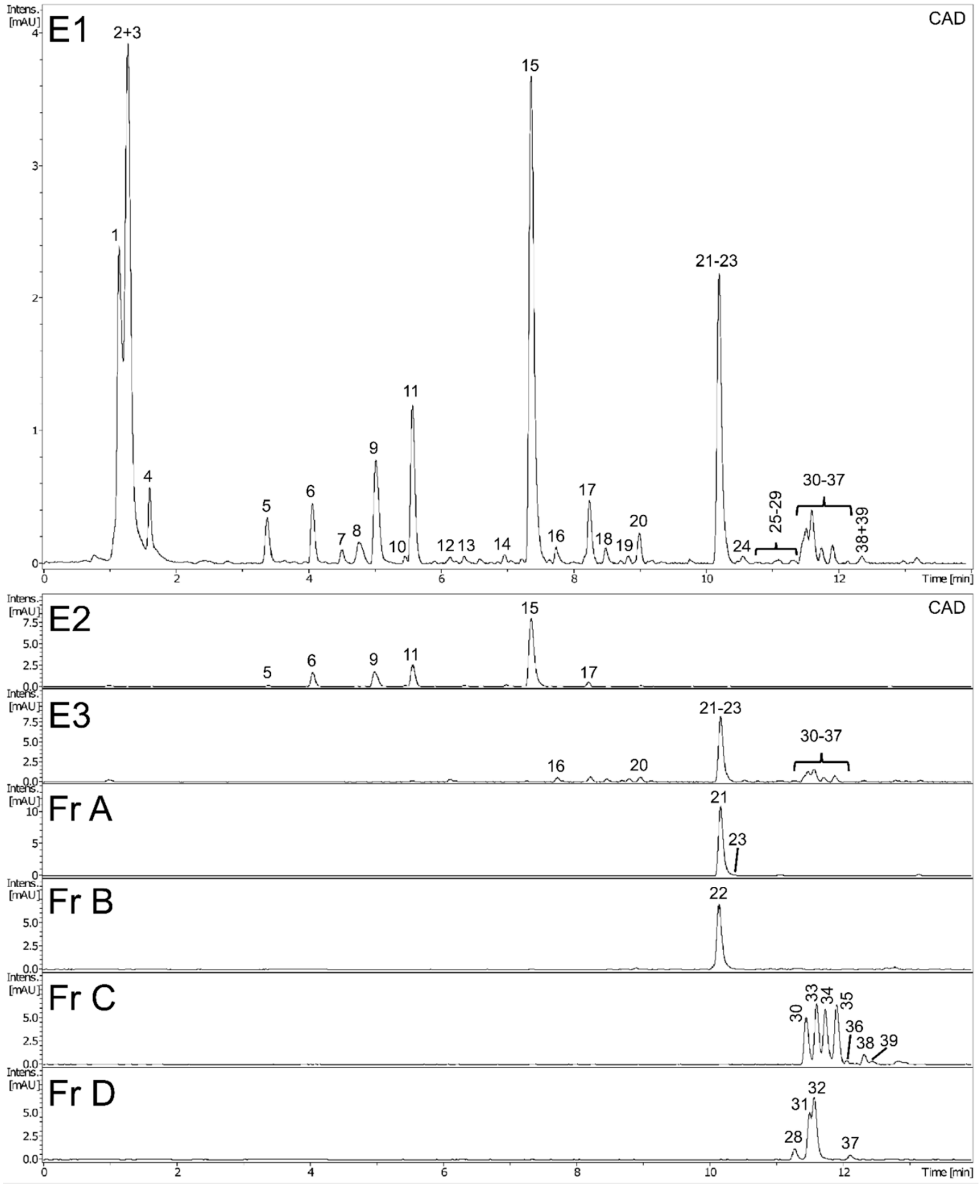


Figure 1. Phytochemical analysis of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits.

2.3. Biomarkers of Oxidative Stress in Plasma and Blood Platelets

Three extracts E1–E3 and two fractions A and D inhibited plasma lipid peroxidation stimulated by H_2O_2/Fe at the highest tested concentration (50 μg/mL); however, fractions B and C remained inactive (Figure 2). In addition, extracts E2 and E3 and fractions B, C, and D reduced protein thiol group oxidation in plasma treated with H_2O_2/Fe (Figure 3); extract E3 reduced this process at 10 and 50 μg/mL (Figure 3).

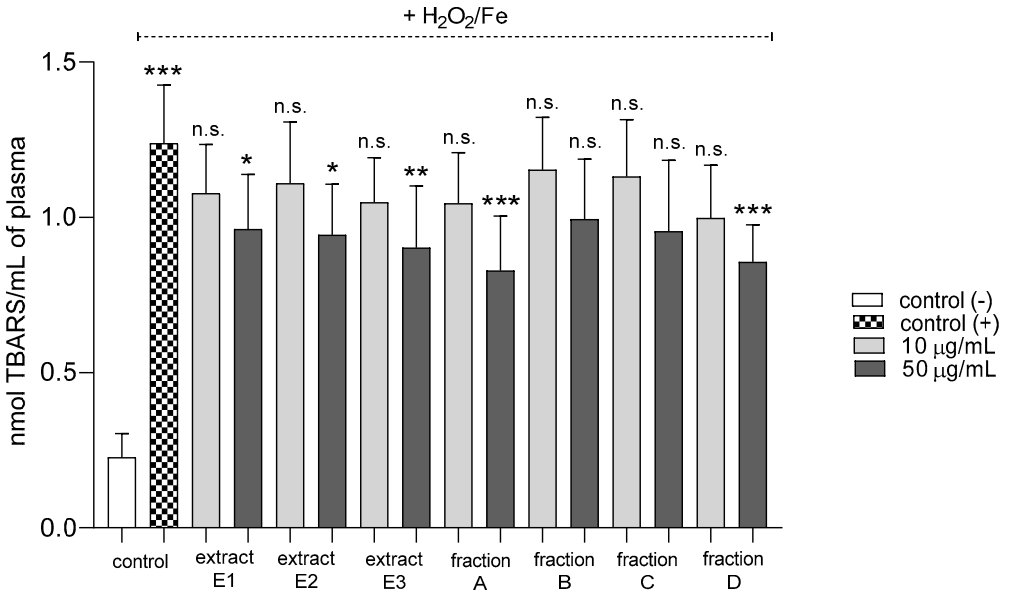


Figure 2. Effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations 10 and 50 μg/mL) on lipid peroxidation in plasma treated with H_2O_2/Fe. The data are presented as means ± SD (n = 10). Control negative refers to plasma not treated with H_2O_2/Fe, and control positive to plasma treated with H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with positive control.

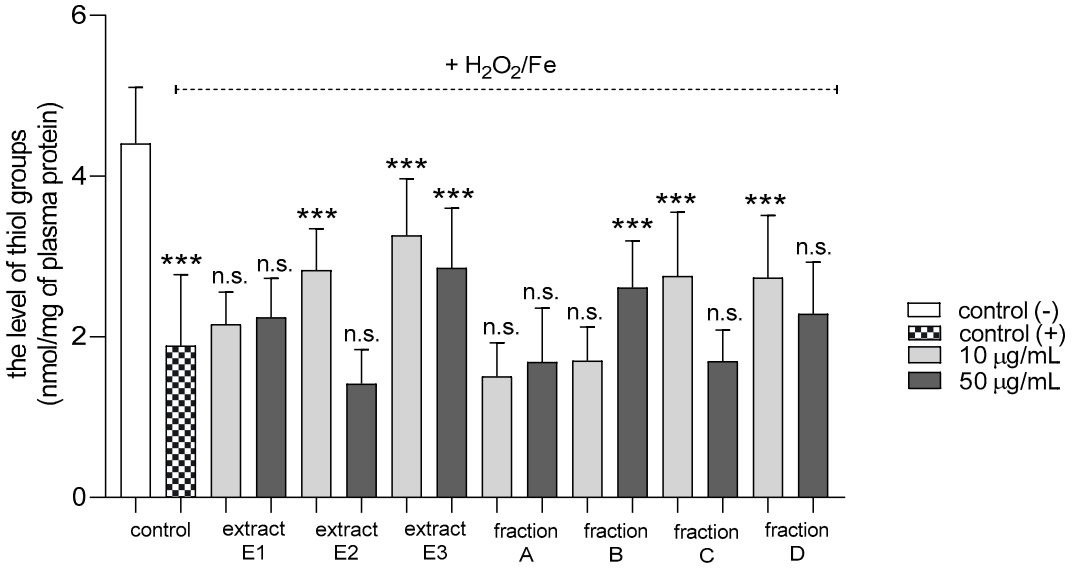


Figure 3. Effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations 10 and 50 μg/mL) on the level of thiol groups in plasma treated with H_2O_2/Fe. The data are presented as means ± SD (n = 8). Control negative refers to plasma not treated with H_2O_2/Fe, and control positive to plasma treated with H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, *** $p < 0.001$, compared with positive control.

All tested dandelion fruit preparations inhibited protein carbonylation in plasma treated with H_2O_2/Fe at the highest tested concentration (50 μg/mL) (Figure 4). In addition, two extracts (E1 and E3) and two fractions (C and D) were also active at the lower tested concentration (10 μg/mL).

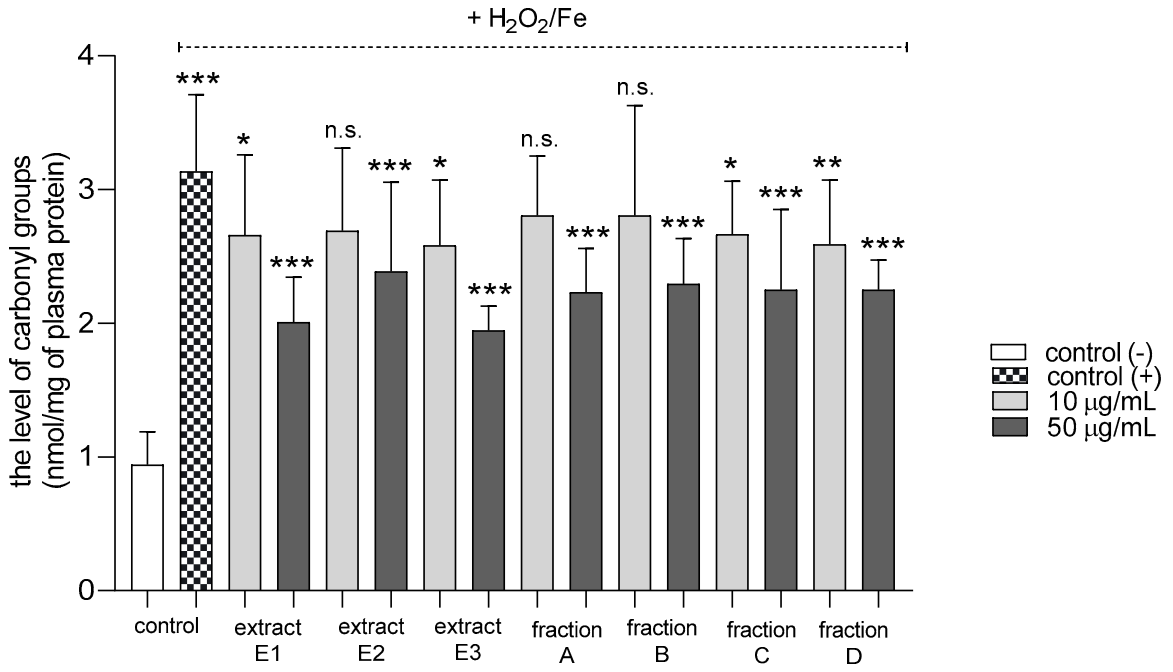


Figure 4. Effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations 10 and 50 μg/mL) on the levels of carbonyl groups in plasma treated with H_2O_2/Fe. The data are presented as means ± SD (n = 8). Control negative refers to plasma not treated with H_2O_2/Fe, and control positive to plasma treated with H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with positive control.

None of the tested preparations changed the TBARS level in resting platelets at either 10 or 50 μg/mL (Figure 5A). However, extract E3 and fractions A–D significantly inhibited thrombin-induced platelet lipid peroxidation when administered at 50 μg/mL. For example, 10 and 50 μg/mL luteolin fraction inhibited this process by about 60% compared with positive controls (Figure 5B). In addition, three flavone fractions (A, B, and D) significantly reduced lipid peroxidation induced by H_2O_2/Fe when administered at 10 and 50 μg/mL; however, two of the extracts (E1 and E2) and fraction C (10 μg/mL) did not appear to exert any activity (Figure 5C). H_2O_2/Fe-induced oxidation of protein thiol functions was ameliorated by preparations E1 and B at 10 μg/mL, and by E3 and A at 50 μg/mL (Figure 6). Moreover, H_2O_2/Fe-induced protein carbonylation was inhibited by extracts E1, E2 and fraction A (Figure 7); for example, phenolic acid extract (E2) demonstrated a protective effect at two used doses (10 and 50 μg/mL) (Figure 7).

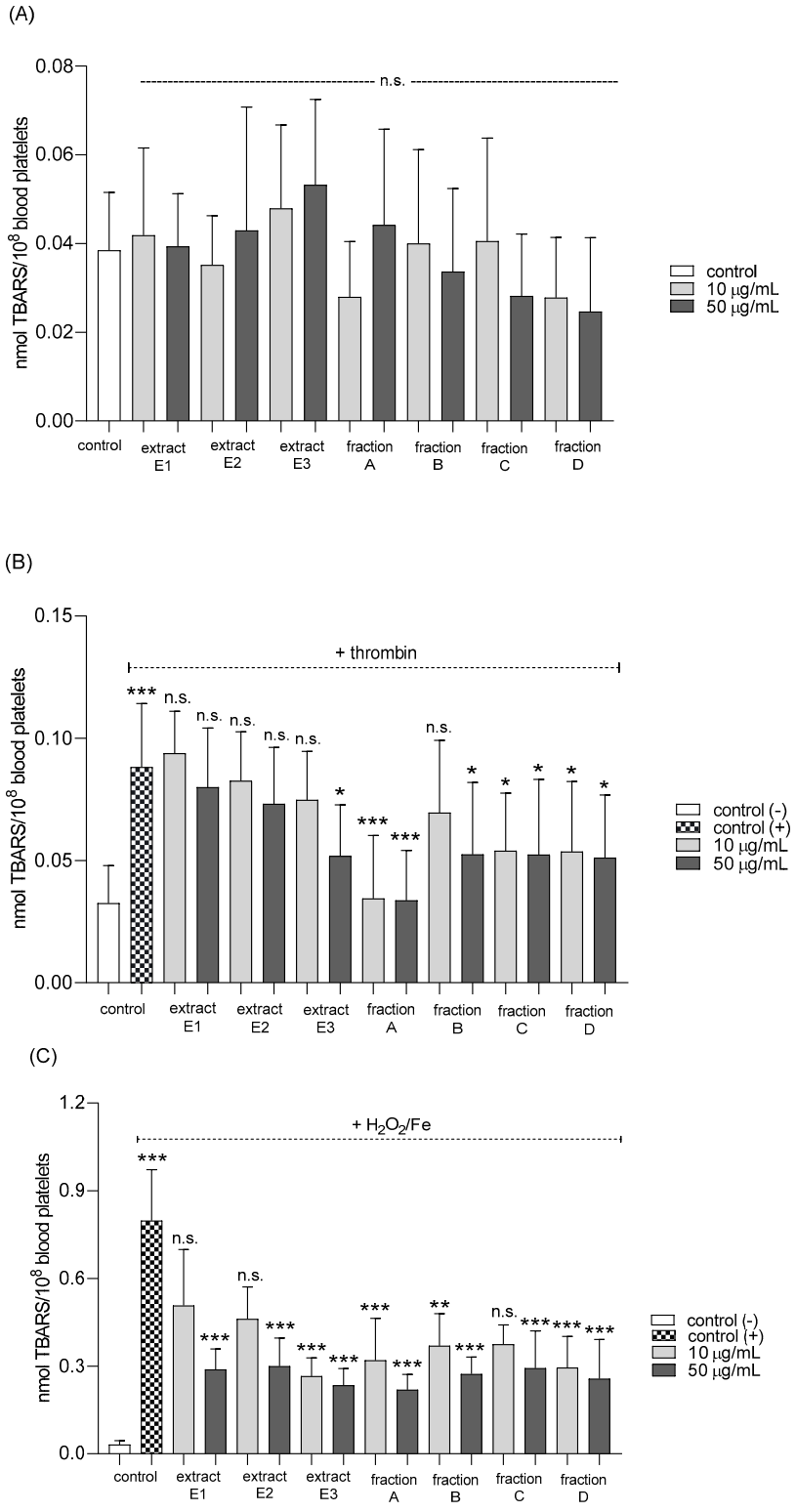


Figure 5. Effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations 10 and 50 μg/mL) on lipid peroxidation in resting blood platelets (**A**), platelets activated by 5 U/mL thrombin (**B**), and platelets treated with H_2O_2/Fe (**C**). The data are presented as means ± SD (n = 5). Control negative refers to platelets not treated with thrombin or H_2O_2/Fe, and control positive to platelets treated with thrombin or H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

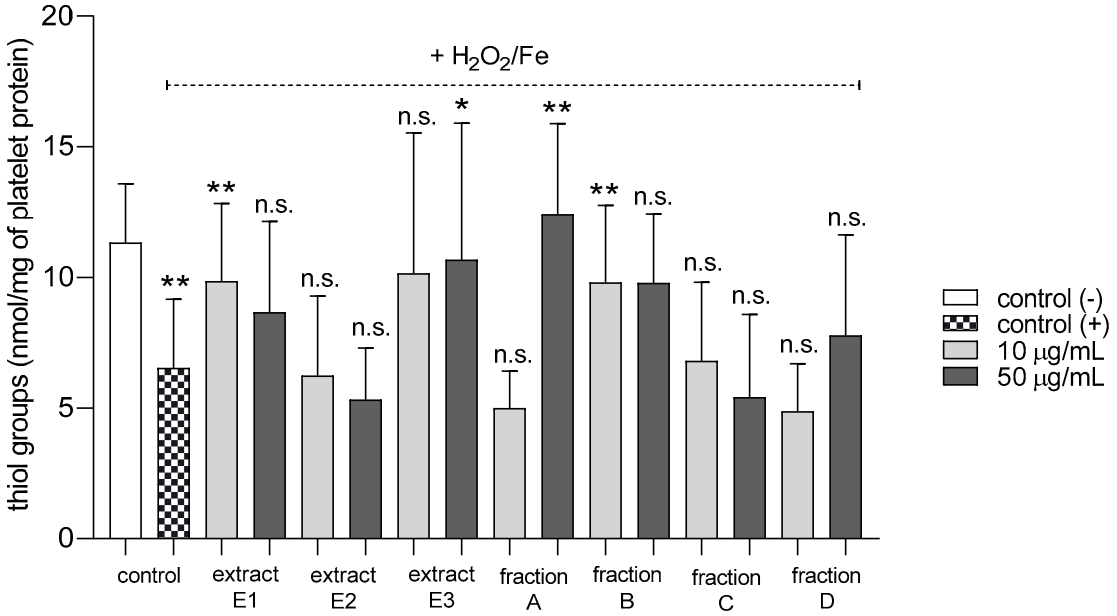


Figure 6. Effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations 10 and 50 μg/mL) on the protein thiol group content of platelets treated with H_2O_2/Fe. The data are presented as means ± SD (n = 5). Control negative refers to platelets not treated with H_2O_2/Fe, and control positive refers to platelets treated with H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, compared with positive control.

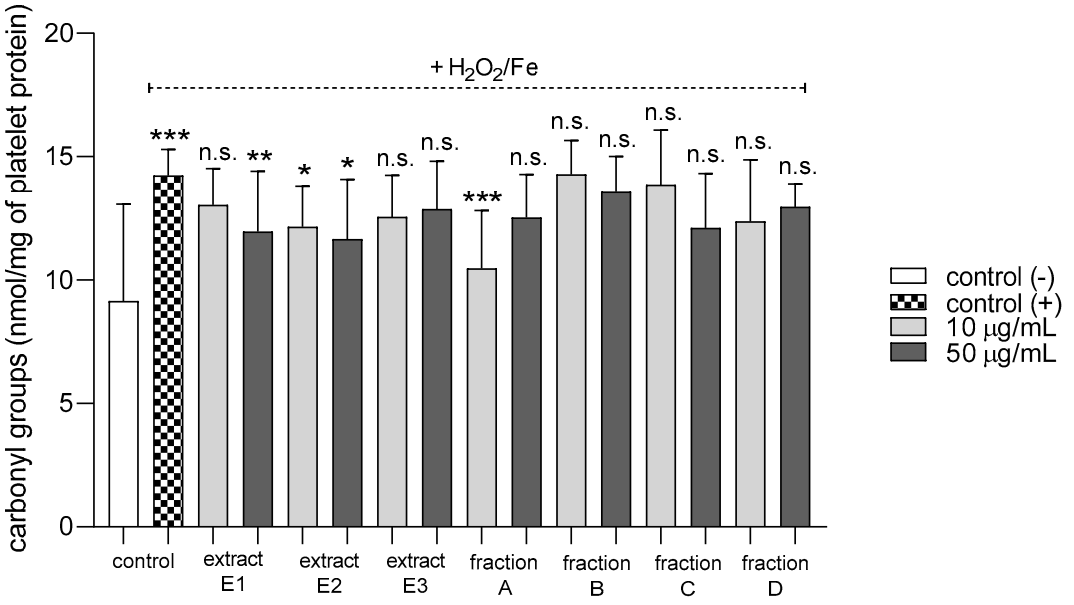


Figure 7. Effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations 10 and 50 μg/mL) on the level of carbonyl groups in platelet protein treated with H2O2/Fe. The data are presented as means ± SD (n = 5). Control negative refers to platelets not treated with H_2O_2/Fe, and control positive to platelets treated with H_2O_2/Fe. Kruskal-Wallis test: n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with positive control.

2.4. Hemostatic Parameters of Blood Platelets and Plasma

Adhesion of thrombin-activated platelets to fibrinogen was significantly inhibited after preincubation with five tested dandelion fruit preparations (E2 and E3, fractions A, B, and C) (Figure 8A). Fraction A was highly active at both concentrations tested—10 and 50 μg/mL, while the

others (E2, E3, B, and C) at the higher concentration (Figure 8A). In the case of ADP-activated blood platelets, all four flavonoid fractions demonstrated inhibitory action, fractions A–C at both tested doses (10 and 50 µg/mL), fraction D only at 10 µg/mL (Figure 8B).

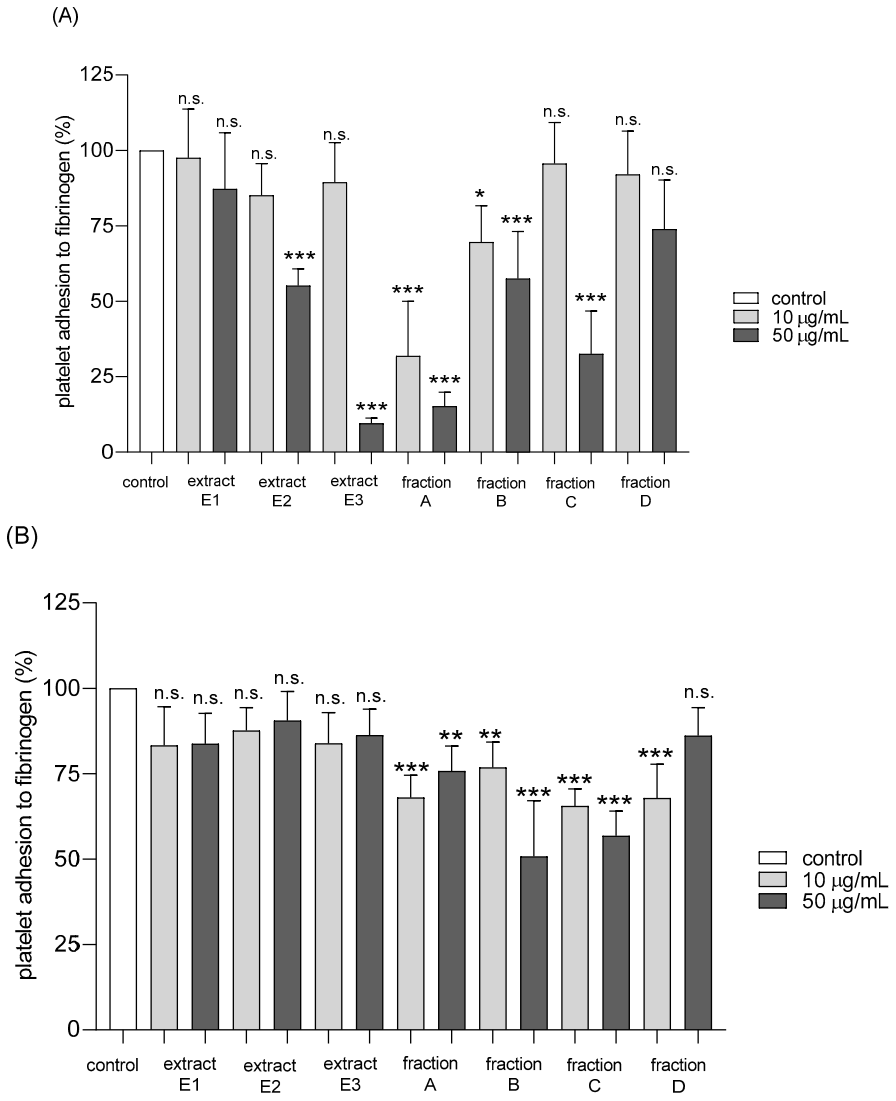
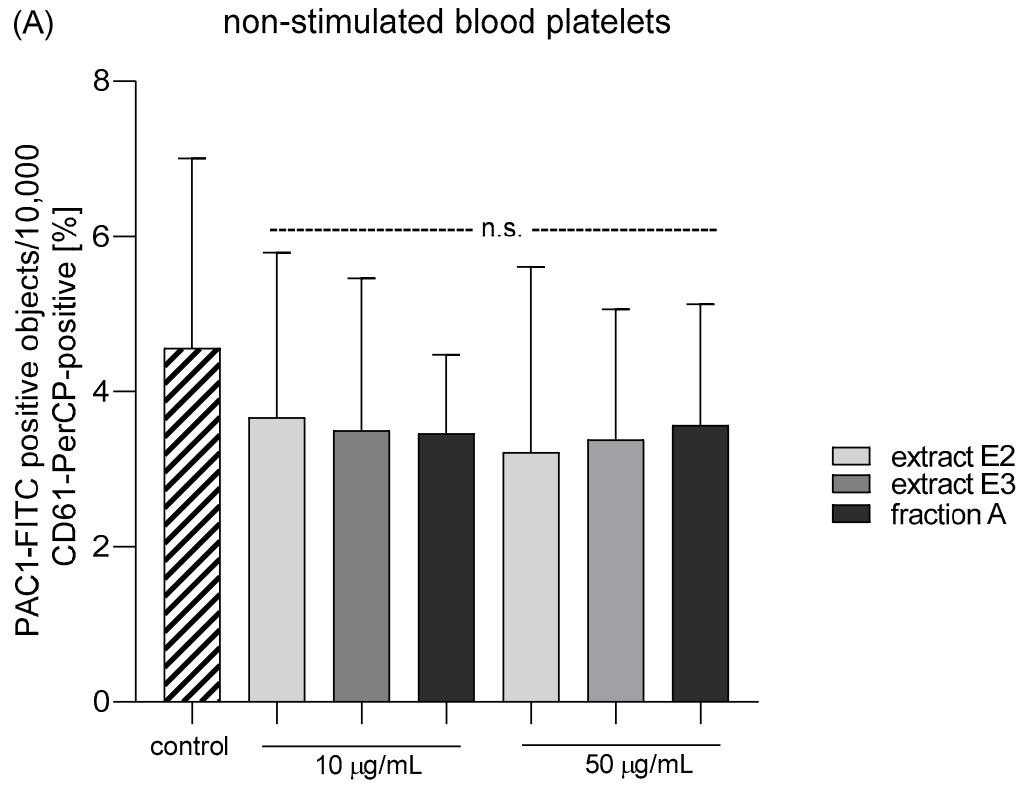
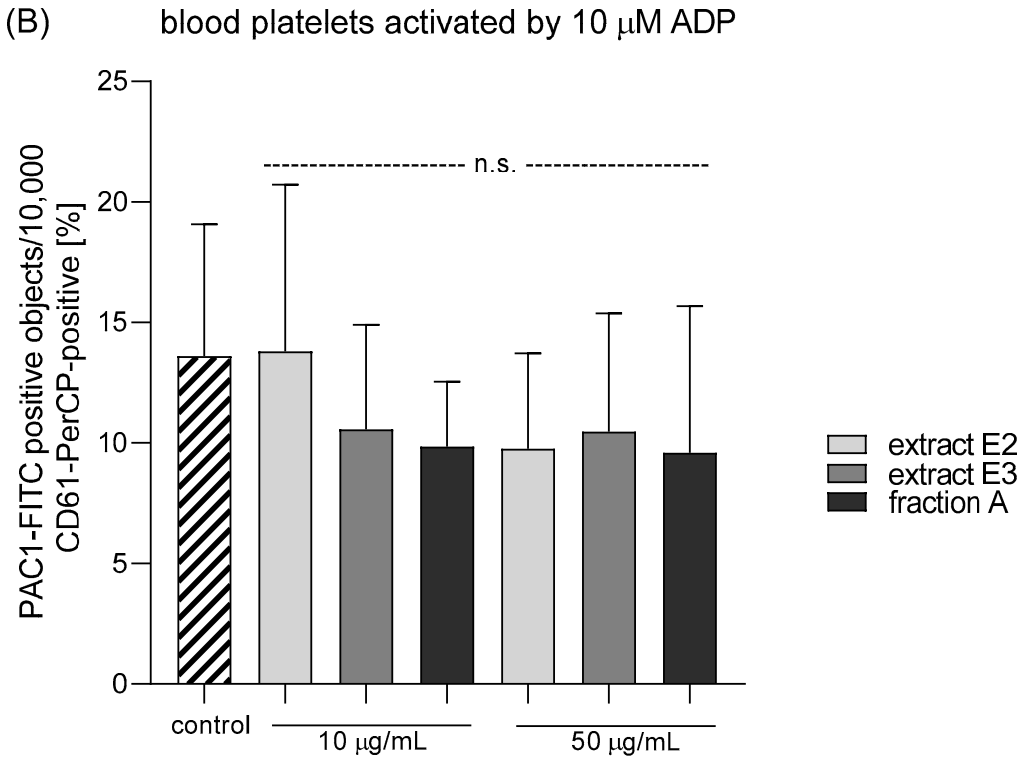


Figure 8. Effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations 10 and 50 µg/mL) on adhesion to fibrinogen and ADP-activated platelets (**A**), and thrombin-activated platelets (**B**). The data are presented as percentages of the control sample (platelets without plant preparation). Results are given as means ± SD (n = 5). Kruskal-Wallis test: n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control.

Three dandelion fruit preparations with the highest antioxidant and antiplatelet potential (extracts E2 and E3, and fraction A) were selected for subsequent flow cytometry analyses. The samples treated with extract E2 or fraction A demonstrated different blood platelet activation states compared with the untreated control samples (Figures 9 and 10). For both above preparations, the effect was only evident at the higher test concentration (50 µg/mL), for example, E2 significantly reduced PAC-1 binding in platelets activated by 20 µM ADP or 10 µg/mL collagen (Figure 9C,D); it also significantly reduced CD62P expression on platelets activated by 10 µg/mL collagen (Figure 10D). In turn, extract E3 did not influence platelet P-selectin expression or GPIIb/IIIa expression for any of the four used models (Figures 9–11).


(A)
non-stimulated blood platelets
PAC1-FITC positive objects/10,000
CD61-PerCP-positive [%]
8
6
4
2
0
n.s.
control
10 μg/mL
50 μg/mL
extract E2
extract E3
fraction A


(B)
blood platelets activated by 10 μM ADP
PAC1-FITC positive objects/10,000
CD61-PerCP-positive [%]
25
20
15
10
5
0
n.s.
control
10 μg/mL
50 μg/mL
extract E2
extract E3
fraction A

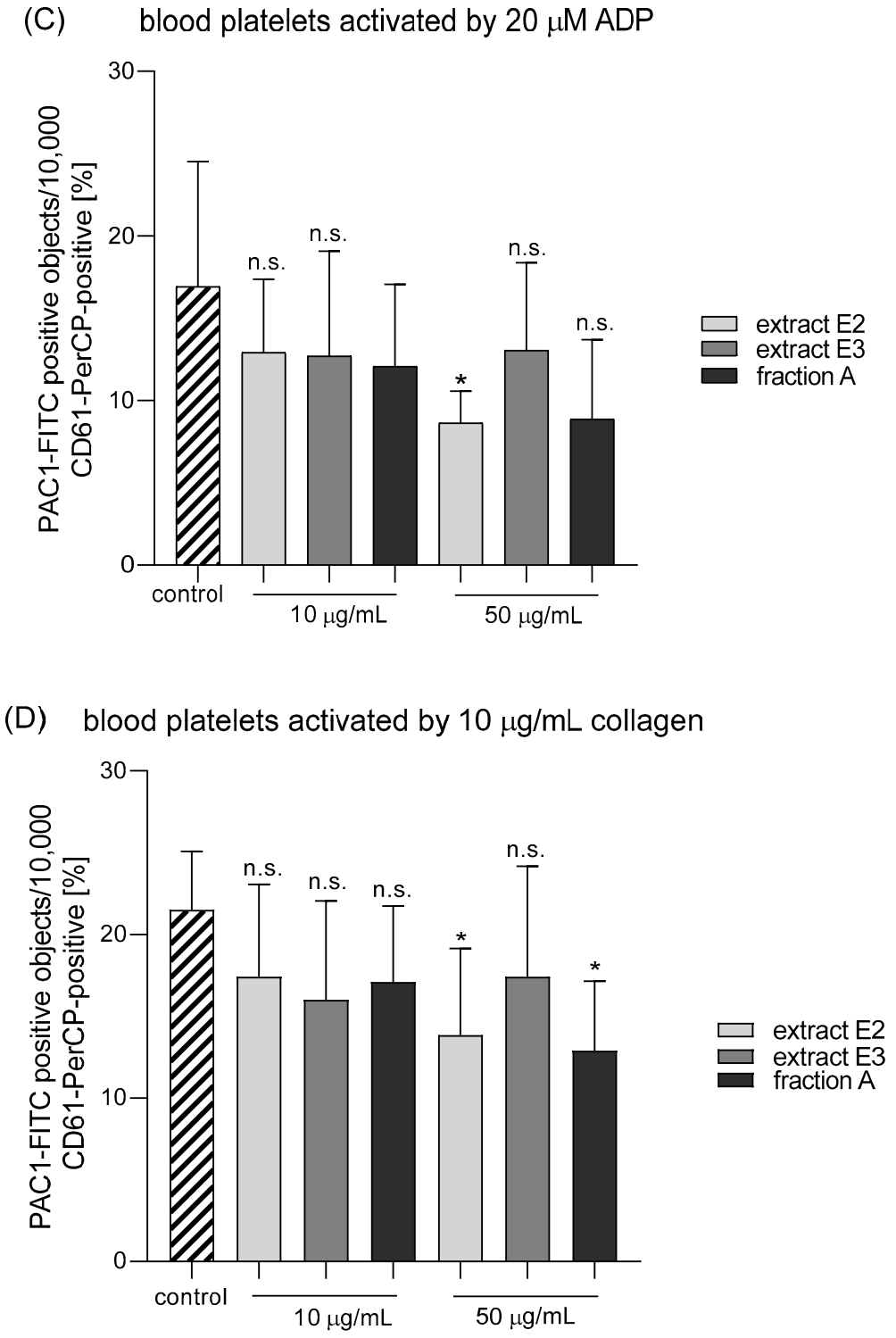
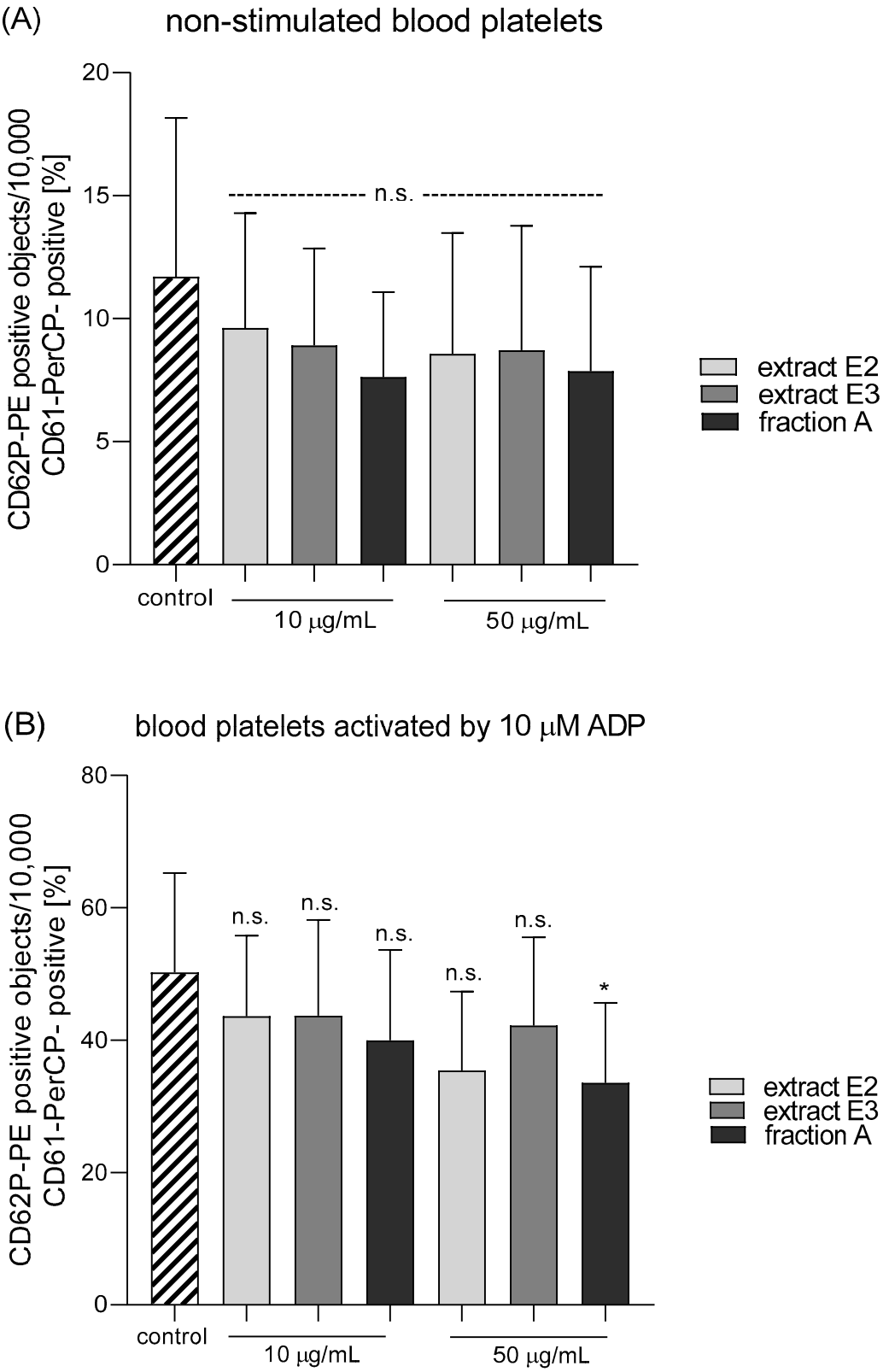


Figure 9. Effects of dandelion fruit preparations (E2 and E3, and fraction A; concentrations 10 and 50 μg/mL) on the expression of the active form of GPIIb/IIIa in resting (**A**) or agonist-stimulated blood platelets: 10 μM ADP (**B**), 20 μM ADP (**C**) and 10 μg/mL collagen (**D**) in whole blood samples. The blood platelets were distinguished based on the expression of CD61. For each sample, 10,000 CD61-positive objects (blood platelets) were acquired. For the assessment of GPIIb/IIIa expression, samples were labeled with fluorescently conjugated monoclonal antibody PAC-1/FITC. Results are expressed as the percentage values of platelets binding PAC-1/FITC. Data represent the means ± SE of six healthy volunteers. n.s. $p > 0.05$; * $p < 0.05$ (vs. control platelets).


(A)
non-stimulated blood platelets
CD62P-PE positive objects/10,000
CD61-PerCP- positive [%]
20
15
10
5
0
n.s.
control
10 μg/mL
50 μg/mL
extract E2
extract E3
fraction A
(B)
blood platelets activated by 10 μM ADP
CD62P-PE positive objects/10,000
CD61-PerCP- positive [%]
80
60
40
20
0
n.s.
n.s.
n.s.
n.s.
n.s.
*
control
10 μg/mL
50 μg/mL
extract E2
extract E3
fraction A

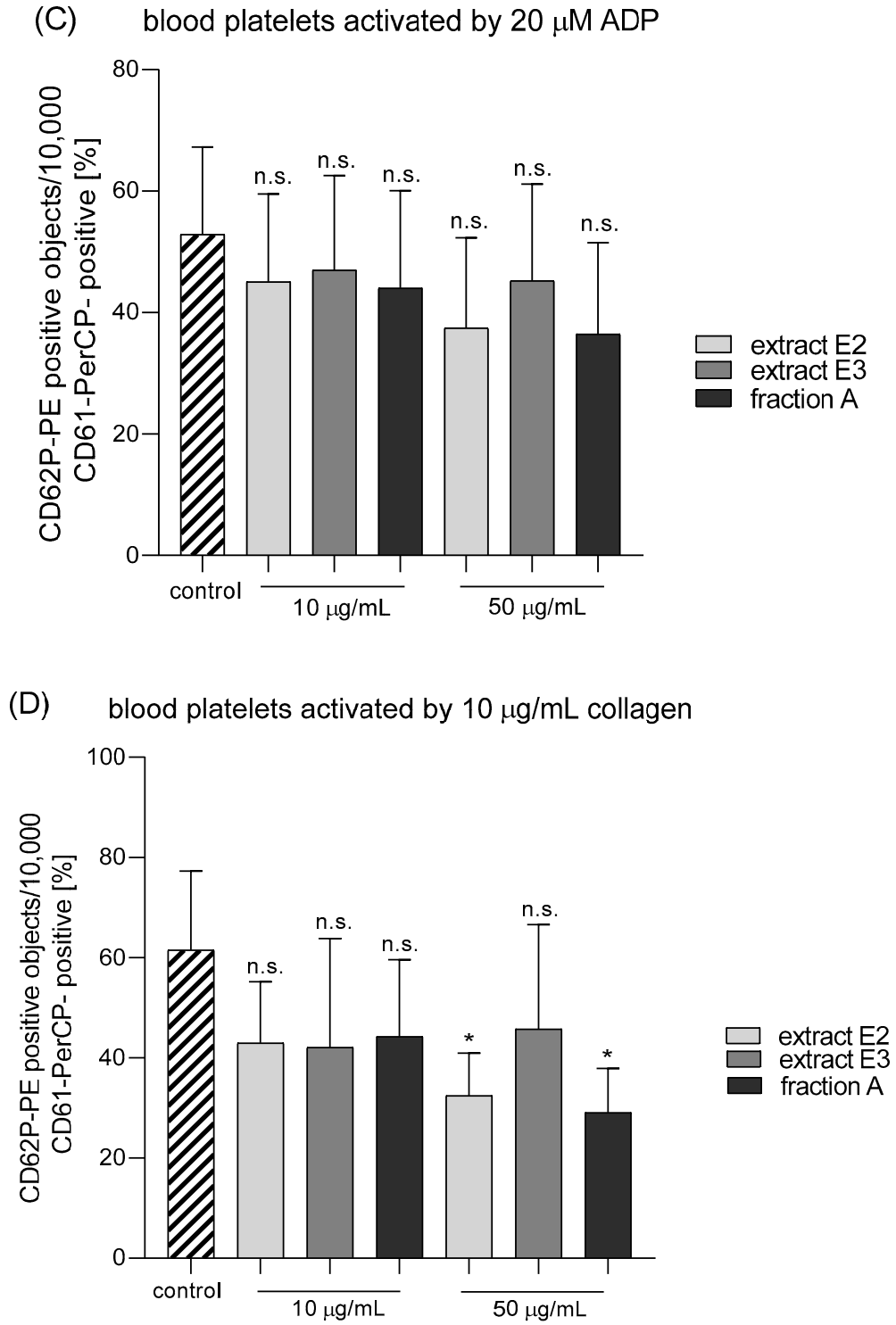


Figure 10. Effects of dandelion fruit preparations (E2, E3, and fraction A; concentrations 10 and 50 μg/mL) on the expression of P-selectin in resting (**A**) or agonist-stimulated blood platelets: 10 μM ADP (**B**), 20 μM ADP (**C**) and 10 μg/mL collagen (**D**) in whole blood samples. The blood platelets were distinguished based on the expression of CD61/PerCP. For each sample, 10,000 CD61-positive objects (blood platelets) were acquired. For the assessment of P-selectin expression, samples were labeled with fluorescently conjugated monoclonal antibody CD62P. Results are expressed as the percentage values of platelets expressing CD62P. Data are presented as the means ± SE of six healthy volunteers. n.s. $p > 0.05$; * $p < 0.05$ (vs. control platelets).

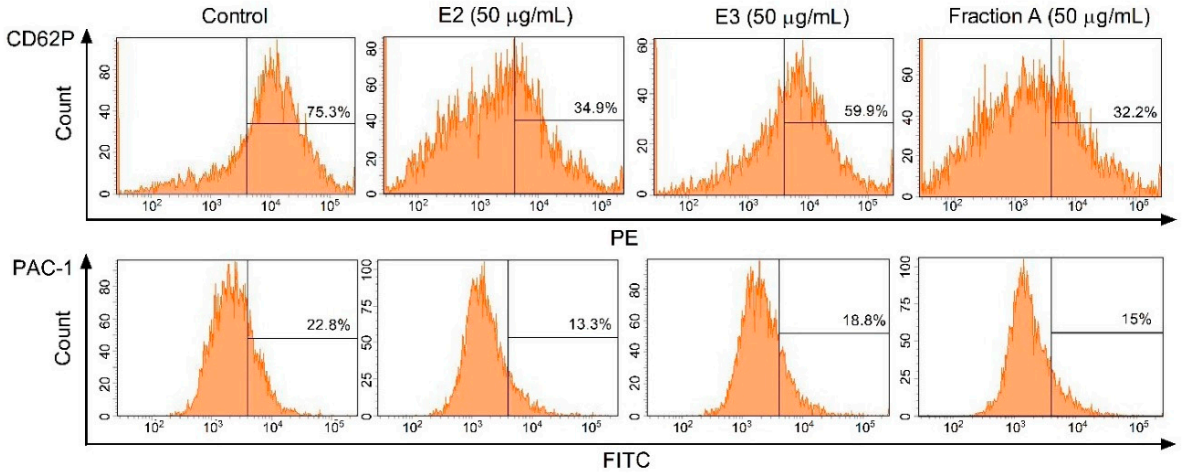


Figure 11. Effects of dandelion fruit preparations (E2, E3, and fraction A; concentration 50 μg/mL) on the expression of P-selectin and the active form of GPIIb/IIIa in platelets stimulated by 10 μg/mL collagen in whole blood samples. Figure demonstrates selected diagrams.

None of the dandelion fruit preparations changed the APTT, PT, TT, or AUC_{10} values measured by T-TAS for either human plasma or whole blood (Figure 12, Table 4).

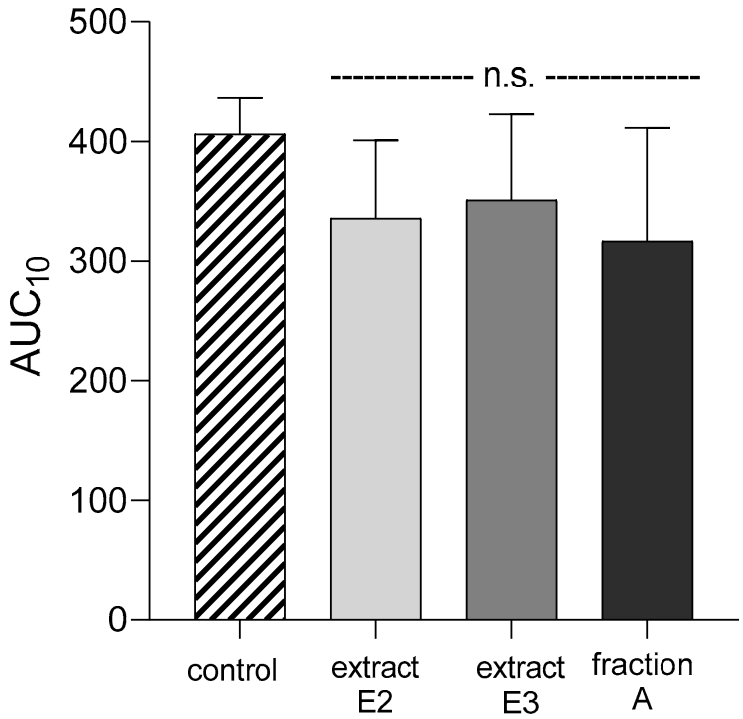


Figure 12. Effects of dandelion fruit preparations (E2, E3, and fraction A; concentration 50 μg/mL) on the T-TAS using the PL-chip in whole blood samples. Whole blood samples were analyzed by the T-TAS at the shear rates of 1000 s^{-1} on the PL-chips. The area under the curve (AUC_{10}) in PL are shown as closed circles. Data represent the means ± SE of six healthy volunteers; n.s. $p > 0.05$, compared with control.

Table 4. Effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations 10 and 50 μg/mL) on the coagulation times of human plasma (APTT, PT, and TT). Data are presented as means ± SD (n = 10).

Sample Name	Tested Concentration (μg/mL)	(TT)	(PT)	(APTT)
		Mean ± SD		
control	0	15.5 ± 1.2 $^{n.s.}$	15.0 ± 0.6 $^{n.s.}$	43.1 ± 5.2 $^{n.s.}$
E1 (total extract)	10	15.5 ± 1.6 $^{n.s.}$	14.9 ± 0.5 $^{n.s.}$	43.5 ± 4.8 $^{n.s.}$
	50	15.6 ± 1.6 $^{n.s.}$	14.8 ± 0.5 $^{n.s.}$	43.4 ± 5.2 $^{n.s.}$
E2 (phenolic acid extract)	10	15.3 ± 1.5 $^{n.s.}$	14.8 ± 0.6 $^{n.s.}$	42.6 ± 4.4 $^{n.s.}$
	50	15.4 ± 1.5 $^{n.s.}$	14.9 ± 0.6 $^{n.s.}$	42.9 ± 4.7 $^{n.s.}$
E3 (flavonoid extract)	10	15.5 ± 1.4 $^{n.s.}$	14.7 ± 0.6 $^{n.s.}$	43.1 ± 4.5 $^{n.s.}$
	50	15.4 ± 1.5 $^{n.s.}$	15.0 ± 0.4 $^{n.s.}$	43.3 ± 4.5 $^{n.s.}$
Fr A (luteolin)	10	15.5 ± 1.3 $^{n.s.}$	14.9 ± 0.4 $^{n.s.}$	42.8 ± 4.3 $^{n.s.}$
	50	15.3 ± 1.5 $^{n.s.}$	14.9 ± 0.5 $^{n.s.}$	42.5 ± 4.4 $^{n.s.}$
Fr B (philonotisflavone)	10	15.5 ± 1.4 $^{n.s.}$	15.1 ± 0.7 $^{n.s.}$	42.5 ± 4.3 $^{n.s.}$
	50	15.3 ± 1.3 $^{n.s.}$	14.9 ± 0.7 $^{n.s.}$	43.1 ± 4.4 $^{n.s.}$
Fr C (flavonolignans)	10	15.4 ± 1.3 $^{n.s.}$	15.0 ± 0.6 $^{n.s.}$	42.3 ± 4.5 $^{n.s.}$
	50	15.7 ± 1.3 $^{n.s.}$	15.1 ± 0.7 $^{n.s.}$	42.6 ± 4.4 $^{n.s.}$
Fr D (flavone aglycones)	10	15.6 ± 1.2 $^{n.s.}$	15.1 ± 0.6 $^{n.s.}$	42.3 ± 5.0 $^{n.s.}$
	50	15.7 ± 1.1 $^{n.s.}$	15.2 ± 0.6 $^{n.s.}$	42.7 ± 5.5 $^{n.s.}$

n.s., not statistically significant ($p > 0.05$, compared with control).

2.5. Cytotoxicity against Blood Platelets

To examine the toxicity of all dandelion fruit preparations on platelets, the extracellular LDH activity was measured. Compared to a control group, there was no significant difference in the viability of platelets after exposure to dandelion extracts (E1–E3) and fractions (A–D) at 10 and 50 μg/mL (Figure 13).

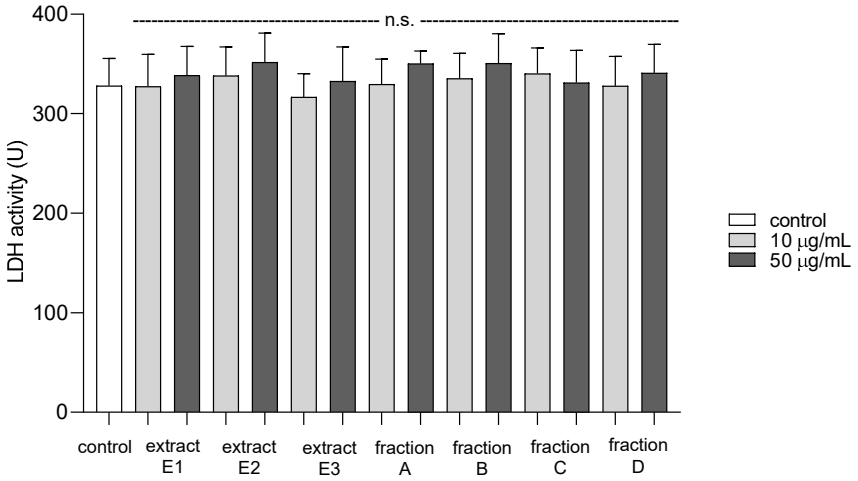


Figure 13. Cytotoxic effect of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (concentrations: 10 and 50 μg/mL) on human blood platelets. Data are presented as means ± SD (n = 4); n.s. $p > 0.05$, compared with control.

Table 5 compares the effects of three extracts (E1–E3) and four fractions (A–D) from dandelion fruits on the biological properties of plasma and blood platelets. It can be seen that extract E2 and fraction A demonstrated stronger antioxidant and antiplatelet properties than the other tested preparations (Table 5).

Table 5. Comparative effects of extracts (E1–E3) and flavonoid fractions (A–D) of dandelion fruits (tested concentration-50 μg/mL) on biological properties of plasma and blood platelets.

Experiment	E1 (Total Extract)	E2 (Phenolic Acid Extract)	E3 (Flavonoid Extract)	Fr A (Luteolin)	Fr B (Philonotisflavone)	Fr C (Flavonolignans)	Fr D (Flavone Aglycones)
Plasma							
Lipid peroxidation induced by H_2O_2/Fe	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	No effect	No effect	Positive action (antioxidative potential)
Oxidation of protein thiols induced by H_2O_2/Fe	No effect	No effect	No effect	No effect	Positive action (antioxidative potential)	No effect	No effect
Protein carbonylation induced by H_2O_2/Fe	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)
Blood platelets							
Lipid peroxidation in resting platelets	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Lipid peroxidation in thrombin-activated platelets	No effect	No effect	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)
Lipid peroxidation in platelets treated with H_2O_2/Fe	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)	Positive action (antioxidative potential)
Oxidation of protein thiols in platelets treated with H_2O_2/Fe	No effect	No effect	Positive action (antioxidative potential)	Positive action (antioxidative potential)	No effect	No effect	No effect
Protein carbonylation in platelets treated with H_2O_2/Fe	Positive action (antioxidative potential)	Positive action (antioxidative potential)	No effect	No effect	No effect	No effect	No effect
Adhesion of thrombin-activated platelets to fibrinogen	No effect	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	No effect
Adhesion of ADP-activated platelets to fibrinogen	No effect	No effect	No effect	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	Positive action (anti-platelet potential)	No effect
GPIIb/IIIa expression–non-stimulated platelets	ND	No effect	No effect	No effect	ND	ND	ND
GPIIb/IIIa expression–platelets activated by 10 μM ADP	ND	No effect	No effect	No effect	ND	ND	ND
GPIIb/IIIa expression–platelets activated by 20 μM ADP	ND	Positive action (anti-platelet potential)	No effect	No effect	ND	ND	ND

Table 5. *Cont.*

Experiment	E1 (Total Extract)	E2 (Phenolic Acid Extract)	E3 (Flavonoid Extract)	Fr A (Luteolin)	Fr B (Philonotisflavone)	Fr C (Flavonolignans)	Fr D (Flavone Aglycones)
GPIIb/IIIa expression–platelets activated by 10 μg/mL collagen	ND	Positive action (anti-platelet potential)	No effect	Positive action (anti-platelet potential)	ND	ND	ND
P-selectin expression–non-stimulated platelets	ND	No effect	No effect	No effect	ND	ND	ND
P-selectin expression–platelets activated by 10 μM ADP	ND	No effect	No effect	Positive action (anti-platelet potential)	ND	ND	ND
P-selectin expression–platelets activated by 20 μM ADP	ND	No effect	No effect	No effect	ND	ND	ND
P-selectin expression–platelets activated by 10 μg/mL collagen	ND	Positive action (anti-platelet potential)	No effect	Positive action (anti-platelet potential)	ND	ND	ND

ND, not determined.

3. Discussion

The fruits of *Taraxacum officinale* are not as widely used in phytopharmacology as its other organs, and little is hence understood of their chemical composition and biological properties. In the continuation of our study on the dandelion, the current paper was aimed at the phytochemical analysis of *T. officinale* fruit extract and estimating its antiradical, antiplatelet, and antioxidant properties related to hemostasis. Efforts were also made to estimate the contribution of individual polyphenols on the reported biological activity of dandelion fruits. The idea behind the separation/fractionation process of methanolic extract of fruits (E1) was, on the one hand, to facilitate and broaden the metabolite identification (preliminary LC-MS analysis showed the presence of some unreported compounds in dandelion), and on the other hand, to test all extracts and fractions for antioxidant and antiplatelet activity, and conclude about the input of individual compounds/groups of compounds into biological potential and exerted effect of dandelion fruits. Previous research on the *T. officinale* plant identified high numbers of hydroxycinnamic acid derivatives [16–18]; therefore, the present study focused more on fractions enriched in flavonoid compounds, which are also an important component of the aerial parts, i.e., the leaves, flowers, and fruits of the dandelion.

With the use of HR-QTOF-MS analysis, about 30 secondary metabolites were identified in *T. officinale* fruit extract. The phytochemical profile was dominated by flavonoids and hydroxycinnamic acid derivatives (Table 1). Most of the detected polyphenols, such as caffeic acid esters and flavones, have already been described in dandelion species [13,19,20]; however, several flavone derivatives previously undescribed in the genus *Taraxacum* and the *Asteraceae* family were also identified. These included a series of flavone-flavone dimers (biflavones), particularly philonotisflavone (2′,8-biluteolin), as well as luteolin 3′-*O*-glucoside (Figure S1).

The C-C biflavones are a diverse group of phenolic compounds characterized by a variety of flavone units and linkage sites and have been found in many fruits, vegetables, and plants [21]. However, philonotisflavone has previously been known only from the gametophyte of a few bryophyte species, such as *Philonotis Fontana* [22] and *Bartramia pomiformis* [23]. Moreover, biflavonoids have so far been found in only one member of the *Asteraceae* family: *Saussurea eopygmaea* Hand.-Mazz. (*Carduoideae* subfamily) [24]. Several studies on biflavonoids, such as amentoflavone and ginkgetin, as well as on plant extracts enriched with these compounds, such as *Gingko biloba* leaf extract, have found them to demonstrate a wide range of cytotoxic, antimicrobial, antiviral, antioxidative, and anti-inflammatory activities [25].

To date, several luteolin glycosides have been identified in the *Taraxacum* genus, including two different isomers of luteolin glucoside (7-*O*- and 4′-*O*-glc) [13]; however, the present findings also indicate the presence of a new compound: luteolin 3′-*O*-glucoside. In addition, several flavonolignans (tricin-lignan conjugates) were observed, such as calquiquelignan D/E and salcolin A/B, which were recently described by Choi et al [26]. in an extract from the whole *T. officinale* plant.

Studies on the biological activity of dandelion fruit preparations began with the radical scavenging DPPH test. Five preparations (E1–E3, and fractions A,B) demonstrated significant antiradical activities (IC_{50} 0.06–0.42 mg/mL), while fractions C-D displayed relatively weak activity (IC_{50} > 1.3 mg/mL). No simple correlation between polyphenol content (TPC) and antiradical activity (TE and IC_{50} values) was observed, indicating a significant influence of qualitative factor. The DPPH test demonstrated the higher free radical scavenging effect of dandelion fruit flavonoids (E3 extract) compared with phenolic acids (E2 extract), even though E3 had significantly lower TPC than E2 (Tables 2 and 3). Similarly, luteolin, the main component of the flavonoid extract (E3), exhibited 2-fold higher TE (2.0 TE, Fraction A, Table 3) than chicoric acid (1.1 TE) [16], the main component of phenolic acid extract. Moreover, the results show that the antiradical potential of the fruit extract is very little influenced by flavonoids lacking an active B-ring catechol moiety, as exemplified by the C-D fractions. The tricin-lignan conjugates and flavone aglycones (chrysoeriol, tricin, and apigenin), the main components of fractions C and D, lack or have modified (methylated or substituted) the catechol system in the B ring of the aglycone, which is known to enhance the antiradical property of phenolics [27].

The anti-oxidative, antiplatelet, and hemostasis related effects of dandelion fruit preparations were investigated using several experiments in vitro. Hemostasis is based on a series of strictly-regulated processes that enable platelet activation, vascular repair, and blood clotting. The ability of blood to clot prevents its excessive loss when the skin or blood vessels are broken. Although a key role in the clotting process is played by fibrinogen, which acts to transform soluble fibrinogen into insoluble fibrin, the process requires a cascade of consecutive biochemical reactions to produce a fibrin clot: each of the factors involved is activated by a previously-activated factor [28]. However, three discrete phases can be distinguished in the formation of a platelet plug: adhesion, activation, and aggregation of platelets. The adhesion phase starts when a blood vessel is damaged, and during this phase, platelets begin to adhere to the exposed elements of the subendothelial layer.

This stage is followed by platelet activation, which is stimulated by contact with an agonist such as ADP, collagen, or thrombin. During this stage, the platelet cytoskeleton undergoes reorganization and the platelets become less regular in shape, thus facilitating easier adhesion of successive layers. After activation by thrombin and then together with diacylglycerol lipase, phospholipase A_2, or phospholipase C, the platelets release arachidonic acid (AA) from their cell membrane phospholipids. AA is converted to cyclic prostaglandin peroxides (PGG_2 and PGH_2) by cyclooxygenase (COX), and then to thromboxane A_2 (TXA_2) by thromboxane A_2 synthase. The prostaglandin peroxides form a range of substances including malondialdehyde (MDA), taking place via a non-enzymatic pathway, whereas pro-aggregation PGF_{2a} and PGE_2 prostaglandins are formed by S-glutathione transfer. The PGG_2 and PGH_2 peroxides are converted to antiaggregatory PGD_2 prostaglandins by PGD_2 isomerase. The AA metabolic pathway leads to the production of mainly hydroxy acids and hepoxylins, a process catalyzed by 12-lipoxygenase. AA is converted into epoxyicatrienic acids by epoxygenase, interacting with cytochrome P450, and the AA in platelet blood may be converted to isoprostanes via a non-enzymatic route [28]. This phase is followed by aggregation, in which successive platelets adhere to a single layer of cells, resulting in the eventual formation of a platelet plug.

Physiological hemostasis is maintained by an equilibrium between the efficiency of the blood vessel wall, platelet, coagulation, and fibrinolysis system and the inhibitors regulating them. Any imbalance leads to excessive bleeding or clotting [29,30]. To prevent the formation of clots and blockages, antiplatelet drugs are typically introduced. Antiplatelet, otherwise known as anti-aggregation, drugs inhibit specific stages of platelet activation, i.e., adhesion or aggregation. Administration of rhodium, for example, may affect the synthesis of thromboxane A_2, and influence the cyclic concentration of AMP (cAMP) or the total conversion of AA. Other antiplatelet drugs may block platelet membrane receptors such as acetylsalicylic acid or clopidogrel [31]; however, their use can cause various side effects.

Our previous publications demonstrated that in vitro tests using plasma and platelets, well reflecting the conditions in a living organism, are very helpful in finding substances with antioxidant and antiplatelet properties. In the current study, dandelion fruit preparations (E1–E3, A–D) showed various effects on oxidative stress in human plasma and blood platelets, as well as hemostatic properties of blood platelets.

The antiplatelet potential of the tested preparations, evaluated as an inhibitory effect on the adhesion of washed blood platelets to fibrinogen, was determined colorimetrically. Fractions A, B, and C demonstrated the greatest anti-adhesive activity in the two used models: (1) adhesion of thrombin-activated platelets to fibrinogen and (2) adhesion of ADP-activated platelets to fibrinogen. In addition, changes in hemostasis, such as the degree of platelet activation, occurring following exposure to the tested dandelion fruit preparation were identified in whole blood by T-TAS. T-TAS is a microchip-based flow chamber system that evaluates thrombogenicity in whole blood and may be used to assess the influence of anti-thrombotic preparations on blood platelet activation and coagulation reactions against a collagen or collagen/tissue thromboplastin-coated surface [32]. All selected dandelion fruit preparations (E2, E3, and fraction A) were found to demonstrate anti-coagulant potential against the platelets bound to collagen; however, this activity was not statistically different from untreated blood samples.

Platelet activation was also assessed using flow cytometry analysis of P-selectin expression (CD62P) and activation of GPIIb/IIIa complex (PAC-1 binding) in whole blood samples containing unstimulated platelets, and platelets stimulated by ADP or collagen. Flow cytometry can be used for both diagnoses and basic research, and in this case, it enabled the biological activity of blood platelets to be measured in the natural environment, i.e., immediately after blood collection. GPIIb/IIIa receptors are a good marker of blood platelet activation as the increasing number and change of their conformation during activation, aggregation, and adhesion [33]. Our findings confirm that the surface expression of the active form of GPIIb/IIIa on blood platelets decreases in the presence of extract E2 and fraction A (luteolin); also indicate significantly lower blood platelet adhesion in the presence of the dandelion fruit preparations, especially fraction A against washed blood platelets: we propose, therefore, that inhibition of platelet adhesion to fibrinogen may be associated with low expression of GPIIb/IIIa.

Another marker of blood platelet activation is P-selectin: a glycoprotein present in the alpha granules of unstimulated platelets. During activation, P-selectin is released with the alpha granules and can be seen on the surface of the platelet. Present findings indicate that P-selectin expression was reduced in whole blood following treatment with luteolin fraction (A) and hydroxycinnamic acids extract (E2). Finally, it appeared that luteolin demonstrated the best anti-platelet properties of the two preparations, which was observed not only in washed blood platelets but also in whole blood. Our observations are consistent with the literature: Benavente-Garcia and Castillo [34] reported that luteolin has antithrombotic activity, while Guerrero et al [35]. and Dell'Agli et al [36]. found it to have anti-platelet potential.

Platelet activation is associated with arachidonic acid metabolism. Our study demonstrates that all fruit flavonoid enriched preparations (E3, and A–D) reduced enzymatic lipid peroxidation in thrombin-activated platelets in vitro, as indicated by TBARS measurements. This finding suggests that dandelion fruit flavonoids can modulate blood platelet activation by interfering with the metabolism of arachidonic acid. No such change was observed for the other tested extracts, *viz.* E1 and E2, even at the highest used concentration, due to different chemical profiles—lack or low level of flavonoids.

In the anti-oxidative experiments, blood platelets and plasma were exposed to a hydroxyl radical donor (H_2O_2/Fe^{2+}) and protective effects of dandelion preparations were measured. Four models were used to evaluate this activity: (1) plasma lipid peroxidation, (2) plasma protein carbonylation, (3) platelet lipid peroxidation, and (4) protein thiol oxidation or protein carbonylation in platelets treated with H_2O_2/Fe^{2+}. It was found that extracts E1–E3 and fraction A (luteolin) demonstrated the best antioxidant properties. The results were therefore largely, but not entirely (an example of fraction B), consistent with the antiradical test with DPPH (E2-E3 extracts and luteolin fraction). In addition, a strong protective effect against the oxidation of plasma lipids and proteins exerted by hydroxycinnamic acids extract (E2), dominated by L-chicoric acid, is consistent with our previous publications [16,17].

The compounds extracted from the dandelion fruits appeared to be safe for use in the blood model, as none of the tested preparations (concentrations 10 and 50 μg/mL) demonstrated the cytotoxicity against platelets, measured as extracellular LDH activity. It is important to note that the concentrations of the tested extracts and fractions correspond with the physiological concentrations of phenolic compounds, including flavonoids, available after oral supplementation [37,38].

Flavonoids are metabolized by a two-phase process: phase I, based on hydroxylation and demethylation by cytochrome P 450 takes place in the liver, and phase II, based on O-methylation and coupling with glucuronic or sulfuric acid, in the intestine. These products are excreted with urine and bile; but must first pass through the intestinal-hepatic circulation, thus prolonging their elimination time and possible activity. The non-absorbent and bile-derived flavonoid metabolites are processed by the intestinal microflora, mainly in the large intestine. Bacterial enzymes can catalyze reactions such as the hydrolysis of glucuronides, sulfates, and glycosides, as well as dehydroxylation, demethylation, double bond reduction, and decomposition of the C-ring with the formation of phenolic acids, followed by their decarboxylation. Phenolic acids can also be absorbed, conjugated,

or *O*-methylated in the liver and then excreted with the urine. Absorption may be relevant to total plasma antioxidant activity, as catecholytic acids show free radical scavenging activity [39]. Interestingly, various metabolites of phenolic compounds have been found as stronger inhibitors of platelet activation than their precursors [40].

In conclusion, the present study provides new information on *Taraxacum* metabolites, such as biflavonoid–philonotisflavone, and luteolin 3′-*O*-glucoside. From biological studies, it can be concluded that both the antioxidant and antiplatelet potential of dandelion fruit, demonstrated in several different in vitro experiments, was mainly due to luteolin (the main component of fraction A) and chicoric acid (the main component of E2 extract). We suggest that inhibition of platelet activation by fraction A may be associated with inhibition of receptor's expression (including GPIIb/IIIa) and inhibition of arachidonic acid metabolism. By inhibiting platelet activation and oxidative stress, dandelion fruits and their polyphenols can be recognized as novel and valuable phytopharmacological agents. However, the mechanism of their antiplatelet and antioxidant properties remains unclear and required further studies. Additional studies, including in vivo experiments, are also needed to investigate the overall antioxidant and antiplatelet effects of dandelion fruits.

4. Materials and Methods

4.1. Chemical Reagents

Acetonitrile (isocratic grade and LC-MS grade), methanol (isocratic grade), and formic acid (98–100% purity) were purchased from Merck (Darmstadt, Germany). Dimethylsulfoxide (DMSO), thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), guanidine hydrochloride, bovine serum albumin (BSA), MS-grade formic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, Folin-Ciocalteu reagent, and Trolox were acquired from Sigma-Aldrich (St. Louis, MO., USA). All other reagents were purchased from commercial suppliers, including POCH (Poland), Kselmed (Poland), Chrono-log (Poland), and Chempur (Poland). Ultrapure water was prepared in-house using a Milli-Q water purification system (Millipore, Milford, MA, USA). Fibrinogen was isolated from pooled human plasma, according to Doolittle [41]. The concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 1.55 for 1 mg/mL solution (final concentration of fibrinogen was 2 mg/mL).

4.2. Plant Material

Dandelion fruits were harvested in May 2017 on a farm located in south-eastern Poland (50°05′ N, 21°57′ E). 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. A voucher specimen (TO-F-2017.05-1) is deposited at the Department of Biochemistry and Crop Quality of the Institute of Soil Science and Plant Cultivation–State Research Institute in Pulawy.

4.3. Preparation of Phenolic Extracts and Fractions from Dandelion Fruits

The finely-ground dandelion fruits (700 g) were defatted by extraction with *n*-hexane (4 L) under reflux (eight hours). The obtained defatted material (565 g) was twice extracted with 80% methanol (*v/v*; 4 L × 2; 12 h × 2) at 30 °C and sonicated (12 × 10 min) to enhance the extraction efficiency. The methanol extracts were filtered and combined, obtaining extract E1, which was then concentrated with a vacuum rotary evaporator (40 °C). The aqueous suspension of E1 (~50 g) was subjected to liquid-liquid extraction with ethyl acetate (0.75 L × 3). After evaporation of the organic solvent, flavonoid extract (E3) was freeze-dried to give 8.1 g of E3. The aqueous residue (~40 g) was found to contain large amounts of water-soluble primary metabolites (carbohydrates and amino acids) in addition to phenolics, as indicated by liquid chromatography-mass spectrometry (LC-MS) analysis. It was further purified by solid-phase extraction (SPE) on a short C18 column (12 × 5 cm, Cosmosil C18-PREP, 140μm, Nacalai Tesque Inc., Kyoto, Japan). The ballast components were washed with 4% methanol (*v/v*),

and the compounds of interest were eluted with 80% methanol (*v/v*). After evaporation of the organic solvent, phenolic acid extract (E2) was lyophilized to give 9.5 g of E2.

The flavonoid extract (E3) was further fractionated isocratically on a Sephadex LH-20 (Sigma–Aldrich) column (80 × 2.8 cm i.d.) using 95% methanol (*v/v*) at a flow rate of 2.4 mL/min. The single sample capacity was 800 mg (dissolved in 10 mL of 95% methanol). The separation was monitored by LC-MS analyses. The five pooled fractions (I–V) were collected, concentrated, and freeze-dried to give 1290 mg of fraction I, 115 mg of fraction II, 850 mg of fraction III, and 480 mg of fraction IV. Due to their complex composition, or the presence of impurities, as indicated by LC-MS analysis (data not shown), the four fractions (I–IV) were further purified by high-performance liquid chromatography (HPLC) (Dionex, Sunnyvale, CA, USA) on a system equipped with a photodiode array detector (PDA-100) and FC 204 fraction collector (Gilson, Middleton, WI, USA). Separations were carried out on an Atlantis T3 C18 semi-preparative column (250 × 19 mm, 5 µm, Waters) at 40 °C with aqueous acetonitrile, containing 0.1% formic acid, at a flow rate of 5.5 mL/min as mobile phase. The conditions of the chromatographic run were individually optimized for each fraction (isocratic or gradient mode between 15–55% acetonitrile). The PDA detector was operated at 210 and 345 nm (5 nm bandwidth). The separation was monitored by LC-MS analyses. In total, four fractions (A–D) were collected, concentrated at 40 °C, and freeze-dried: the total yields were 1150 mg for fraction A, 95 mg for fraction B, 275 mg for fraction C, and 375 mg for fraction D. The extraction and fractionation process are illustrated in Figure S1.

4.4. Phytochemical Profiling

4.4.1. Qualitative High-Resolution LC-MS Analysis

The dandelion fruit extracts (E1–E3) and fractions (A–D) were 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). Chromatographic separations were carried out on an HSS C18 column (100 × 2.1 mm, 1.7 µm, Waters, Milford, MA, USA) at 40 °C. The injection volume was 3 µL. Mobile phase A was 0.1% (*v/v*) formic acid in MilliQ water, and mobile phase B consisted of acetonitrile containing 0.1% (*v/v*) of formic acid. The sample was separated using a linear gradient from 2 to 50% of solvent B in solvent A (0.4 mL/min, 13 min).

The UV absorbance was measured in the range of 200–600 nm (5 nm bandwidth). The acquisition frequency of both the DAD and CAD detector was set to 10 Hz. The MS analysis was performed in both ESI(–) and ESI(+) ion modes, using the following settings: scanning range 50–1800 *m/z*; negative ion capillary voltage 3.0 kV; positive ion capillary voltage 4.5 kV; dry gas flow 6 L/min; dry gas temperature 200 °C; nebulizer pressure 0.7 bar; collision RF 750 Vpp; transfer time 100 µs; prepulse storage time 10 µs. The collision energy was set to 20 eV. The results were calibrated internally with sodium formate injected into the ion source at the beginning of separation. Data processing was performed using Bruker DataAnalysis 4.3 software.

4.4.2. Quantitative LC-UV Analysis of Flavonoids and Phenolic Acids

The flavonoid and phenolic acid contents in the dandelion extracts and fractions were determined using an ACQUITY UPLC system (Waters) equipped with a photodiode array detector (PDA) and a tandem quadrupole (TQD) mass spectrometer. Chromatographic separations were carried out on an HSS C18 column (100 × 2.1 mm, 1.7 µm, Waters), using parameters identical to those described in Section 4.4.1. The injection volume was 2.5 µL. The UV spectra were recorded within the range of 190–490 nm (3.6 nm resolution).

The lyophilized samples were dissolved in 80% methanol at a concentration of 2 mg DW/mL (two replicates were prepared for each sample) and appropriately diluted before chromatographic analyses.

Flavonoids were detected at UV_{345nm} and phenolic acids at UV_{320nm}. Quantitative determinations were based on an external standard method, using two group standards: luteolin and L-chicoric acid (the main flavonoid and phenolic acid of dandelion fruits, respectively). Both reference compounds had been previously isolated by our laboratory and their purity was determined by LC-MS analysis: L-chicoric acid (95%) [16], and luteolin (99%).

Both the luteolin and L-chicoric acid calibration curves were prepared in six concentrations, ranging from 0.5 to 150 μg/mL, and showed good linearity ($R^2 \geq 0.999$). Three injections were performed for each sample/standard working solution. Quantitative results were expressed as mg standard (L-chicoric acid or luteolin) equivalents (eq)/g of extract/fraction.

4.5. Determination of Total Phenolic Content (TPC)

The total phenolic content in all samples was determined with the Folin-Ciocalteu assay, as described previously [16]. Briefly, 100 μL of F-C reagent was added to 1600 μL of an appropriately diluted sample (6–25 μg/mL) or gallic acid standard solution (six concentrations in the range between 0.5–8 μg/mL). After adding 300 μL of Na_2CO_3 (20% *w/v*), the mixture was incubated in a water bath (40 °C) for 30 min. The absorbance was measured at 765 nm against a blank sample using an Evolution 260 Bio spectrophotometer (Thermofisher Scientific). The TPC of samples was read from the linear curve for gallic acid ($R^2 > 0.999$) and expressed as milligrams of gallic acid equivalents (mg GAE/g DW).

4.6. DPPH Free Radical Scavenging Activity

The radical scavenging activity of the extracts and fractions against the DPPH free radical was determined according to Brand-Williams [42], with slight modifications as described by Lis et al [17]. Briefly, 1900 μL of DPPH methanol solution (100 μM) was mixed with 100 μL of the sample (four different concentrations in the range between 10–500 μg/mL) or Trolox solution (Sigma-Aldrich; five different concentrations in the range between 10–200 μg/mL) in a cuvette. After 30 min, the absorbance was measured against methanol (blank) at 517 nm using the Evolution 260 Bio spectrophotometer (Thermofisher Scientific). The percentage of absorbance inhibition was calculated from the equation:

Inhibition (%) = $100 \times [(A_{blank} - A_{sample})/A_{blank}]$, where A_{blank} and A_{sample} are the absorbance values of the blank and test samples at $t = 30$ min, respectively.

To calculate the Trolox Equivalent (TE) of samples on DPPH, the slope of the sample linear curve, i.e., the absorbance inhibition (%) vs. concentration (μg/mL), was divided by the slope of the standard linear curve.

The IC_{50} value of extracts and fractions, defined as the concentration of sample necessary to cause 50% inhibition was determined from the sample linear curves as absorbance inhibition (%) vs. concentration (μg/mL), with Trolox used as a positive control.

4.7. Blood Platelets and Plasma Isolation

Human blood was obtained from a Medical Center in Lodz (Poland), and the biological material came from regular, non-smoking/non-drinking alcohol and medication-free donors. The blood was collected into tubes with citrate/phosphate/dextrose/adenine (CPDA) anticoagulant. All experiments were approved by the University of Lodz Committee for Research on Human Subjects and carried out under permission number 2/KBBN-UŁ/II/2016.

Plasma and blood platelets were isolated from fresh human blood by differential centrifugation as described previously [16]. The platelet pellet was suspended in modified Tyrode's buffer (pH 7.4). The number of platelets in suspensions used in the experiments was determined at 800 nm using a UV-Visible Helios α spectrophotometer (Unicam) according to Walkowiak et al [43].; the amount was found to be $1.5–2.5 \times 10^8$/mL. The protein concentration was calculated by measuring the absorbance of tested samples at 280 nm according to Whitaker and Granum [44]. Each sample (both plasma and blood platelets) taken for testing was obtained from different subjects and could be regarded as an independent trial.

4.8. Incubation of Plasma, Blood Platelets, and Whole Blood with Plant Extracts and Fractions

Stock solutions of the *T. officinale* L. fruit extracts and fractions were prepared with 50% DMSO (*v/v*). The final concentration of DMSO in test samples was lower than 0.05%, and its effects were determined in all experiments. The extracts and fractions were then taken for use in different models to evaluate the antithrombotic properties. Briefly:

The plasma was pre-incubated for five minutes at 37 °C with dandelion extracts (E1–E3) and fractions (A–D) at two concentrations, 10 and 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 blood platelets were incubated for 30 min at 37 °C with dandelion extracts (E1–E3) and fractions (A–D) at final concentrations of 10 and 50 μg/mL.

The blood platelets were pre-incubated for five minutes at 37 °C with dandelion extracts (E1–E3) and fractions (A–D) at two concentrations, 10 and 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 blood platelets were pre-incubated (25 min at 37 °C) with dandelion extracts (E1–E3) and fractions (A–D) at two concentrations, 10 and 50 μg/mL, and then treated with thrombin at a final concentration of 5 U/mL (5 min at 37 °C).

Whole blood was incubated (30 min at 37 °C) with dandelion extracts (E2 and E3) and fraction A at final concentrations of 10 and 50 μg/mL.

4.9. Parameters of Blood Platelet Activation

4.9.1. Platelet Adhesion to Fibrinogen

Adhesion of blood platelets to fibrinogen was determined colorimetrically by measuring acid phosphatase activity [45]. Blood platelets were incubated in microtiter plates that had been precoated with fibrinogen, then nonadherent platelets were washed out and dissolved with Triton X-100. The details of the procedure were described previously [16,17]. Finally, 2M NaOH was added and the *p*-nitrophenol produced was measured at 405 nm, using SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). A control group was platelets without the addition of plant preparations; and its absorbance was expressed as 100%.

4.9.2. Flow Cytometry

Changes in the activation and reactivity of resting and stimulated blood platelets were studied in the whole blood model [46]. Fresh blood samples (150 μL) were incubated with dandelion fruit preparations (30 min., 25 °C), and then stimulated with 10 and 20 μM ADP for 15 min at room temperature (RT), or collagen (10 μg/mL, 15 min, RT). After incubation, the samples were diluted tenfold (1:9) in sterile PBS with Mg^{2+} and stained with anti-CD61/PerCP, anti-CD62/PE, or PAC-1/FITC antibodies (30 min, RT, in the dark). Appropriate isotype controls were then prepared: the resting blood samples were stained with anti-CD61/PE and isotype control antibodies marked with FITC or PE. Finally, all samples were fixed with 1% CellFix (60 min, 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 relative to the total number of platelets (CD61-positive cells) presented in each sample. Non-specific antibody binding was determined using the isotype control antibodies IgG1/PE and IgM/FITC. All results were analyzed using BD FACSDiva software (Becton Dickinson, San Diego, CA, USA).

4.10. Parameters of the Coagulation Process

4.10.1. Thrombin Time (TT) Measurement

Plasma (50 µL) was incubated in a measuring cuvette (1 min., 37 °C), then a 100 µL of thrombin at the final concentration of 5 U/mL was added. The TT was measured using an Optic Coagulation Analyser (Kselmed, Poland). The procedure was carried out as described by Malinowska et al [47]. Time until clot formation was measured, the results are given in seconds. Samples were tested in duplicate.

4.10.2. Prothrombin Time (PT) Measurement

Plasma (50 µL) was incubated in a measuring cuvette (2 min., 37 °C), then a 100 µL of Dia-PT liquid (commercial preparation) was added. The PT was measured using an Optic Coagulation Analyser (Kselmed, Poland). The procedure was carried out as described by Malinowska et al [47]. Time until clot formation was measured, the results are given in seconds. Samples were tested in duplicate.

4.10.3. Activated Partial Thromboplastin Time (APTT) Measurement

Fifty microliters of Dia-PTT liquid (commercial preparation) were added to 50 µL of human plasma, followed by incubation at 37 °C (3 min). Afterward, a 50 µL of 25 mM $CaCl_2$ was added. The APPT was measured using an Optic Coagulation Analyser (Kselmed, Poland). The procedure was carried out as described by Malinowska et al [47]. Time until clot formation was measured, the results are given in seconds. Samples were tested in duplicate.

4.10.4. Total Thrombus Formation Analysis System (T-TAS®)

T-TAS® was used to analyze the thrombus formation process under flow conditions. Platelet thrombus formation was measured using the PL-chip microchip coated with collagen. Whole blood (400 µL) anticoagulated with BAPA (benzylsulfonyl-D-arginyl-prolyl-4-amidinobenzylamide) was incubated with the tested fractions (30 min., 37 °C). Subsequently, samples (340 µL) were transferred to the PL-chip. The results were taken as AUC_{10} i.e., Area Under the Curve [48].

4.11. Cytotoxicity

The cytotoxicity of dandelion fruit preparations against blood platelets was examined by measuring extracellular LDH activity. Firstly, the 270 µL of 0.1 M phosphate buffer, 10 µL of supernatant, and 10 µL of nicotinamide-adenine-dinucleotide (NADH) were added to the microtiter plate and then incubated (20 min., 25 °C). Thereafter, 10 µL of pyruvate was added and the absorbance was immediately measured at 340 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). The measurement was carried out for 10 min, and it was repeated every minute [49].

4.12. Parameters of Oxidative Stress

4.12.1. Lipid Peroxidation Measurement

Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS). The procedure was carried out according to Bartosz [4]. After incubation of plasma with dandelion preparations, an equal volume of cold 15% (*v*/*v*) trichloroacetic acid in 0.25 M HCl and 0.37% (*v*/*v*) thiobarbituric acid in 0.25 M HCl were transferred to the mixture. Then, the samples were incubated in the boiling water bath for 10 min, followed by cooling in an ice bath and centrifugation (10,000× *g*, 15 min, 18 °C) [4,50]. Absorbance of the colored product was measured at 535 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). The TBARS concentration was calculated using the molar extinction coefficient (ε = 156,000 M^{-1} cm^{-1}). The results were expressed as nmol/mL of plasma, or nmol/10^8 blood platelets.

4.12.2. Carbonyl Group Measurement

The level of protein carbonyl groups in plasma was measured by the addition of 2,4-dinitrophenylhydrazine (DNPH), which in the dark binds with this functional group. The resulting colored compound was assayed spectrophotometrically at 375 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). The procedure was carried out according to Levine et al [51]. and Bartosz [4]. Carbonyl group concentration was calculated based on the molar extinction coefficient (ε = 22,000 M^{-1} cm^{-1}). The results were expressed as nmol/mg of plasma protein, or nmol/mg of platelet protein.

4.12.3. Thiol Group Determination

Thiol group level was measured using Ellman's reagent, i.e., 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB). The procedure was carried out according to Ando and Steiner [52,53]. The resulting colored compound was assayed spectrophotometrically at 412 nm using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). Finally, the thiol group concentration was calculated based on the molar extinction coefficient (ε = 136,000 M^{-1} cm^{-1}). The results were expressed as nmol/mg of plasma protein, or nmol/mg of platelet protein.

4.13. Data analysis

Data distribution was checked by normal probability plots, and the homogeneity of variance by Levene's test. Differences within and between groups were assessed by the Kruskal-Wallis test; for the sake of clarity, only the differences between the tested preparations and the control/control positive were marked. Additionally, in Tables 2 and 3, the data was marked with color code, for easy visualization of the differences between the preparations, generated with the use of the Quick Analysis tool (Microsoft Excel). Results are presented as means ± SD. Significance was considered at $p < 0.05$. To eliminate uncertain data, the Q-Dixon test was performed.

Supplementary Materials: The following are available online. Figure S1: Flow diagram of extraction and fractionation of dandelion fruits, Figure S2: Structures of 14 fully identified metabolites in methanol extract and prepared phenolic preparations of *Taraxacum officinale* fruits.

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Abbreviations

AA: arachidonic acid; ADP, adenosine diphosphate; APTT, activated partial thromboplastin time; BAPA, benzylsulfonyl-D -arginyl-prolyl-4-amidinobenzylamide; BSA, bovine serum albumin; CAD, charged aerosol detector; COX, cyclooxygenase; CPDA, citrate/phosphate/dextrose/adenine; DAD, diode array detector; DMSO, dimethylsulfoxide; DNPH, 2,4-dinitrophenylhydrazine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTNB, 5,5′-dithiobis-(2-nitrobenzoic) acid; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; Fg, fibrynogen; GAE, gallic acid equivalents; H_2O_2, hydrogen peroxide; HCAs, hydroxycinnamic acid esters; HPLC, high-performance liquid chromatography; HR-QTOF-MS, high resolution-quadrupole time of flight-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; LDH, lactate dehydrogenase; MDA, malondialdehyde; NADH, nicotinamide-adenine-dinucleotide; PGG_2, prostaglandin peroxide G_2; PGH_2, prostaglandin peroxide H_2; PT, prothrombin time; ROS, reactive oxygen species; SPE, solid phase extraction; TBARS, thiobarbituric acid reactive substances; TE, trolox equivalents; TPC, total phenolic content; TT, thrombin time; T-TAS, total thrombus formation analysis system.

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Sample Availability: Samples of dandelion fruit preparations E1–E3 and A–D are available from the authors.

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Supplementary Materials

Article

Flavonoid Preparations from *Taraxacum officinale* L. Fruits-a Phytochemical, Antioxidant and Hemostasis Studies

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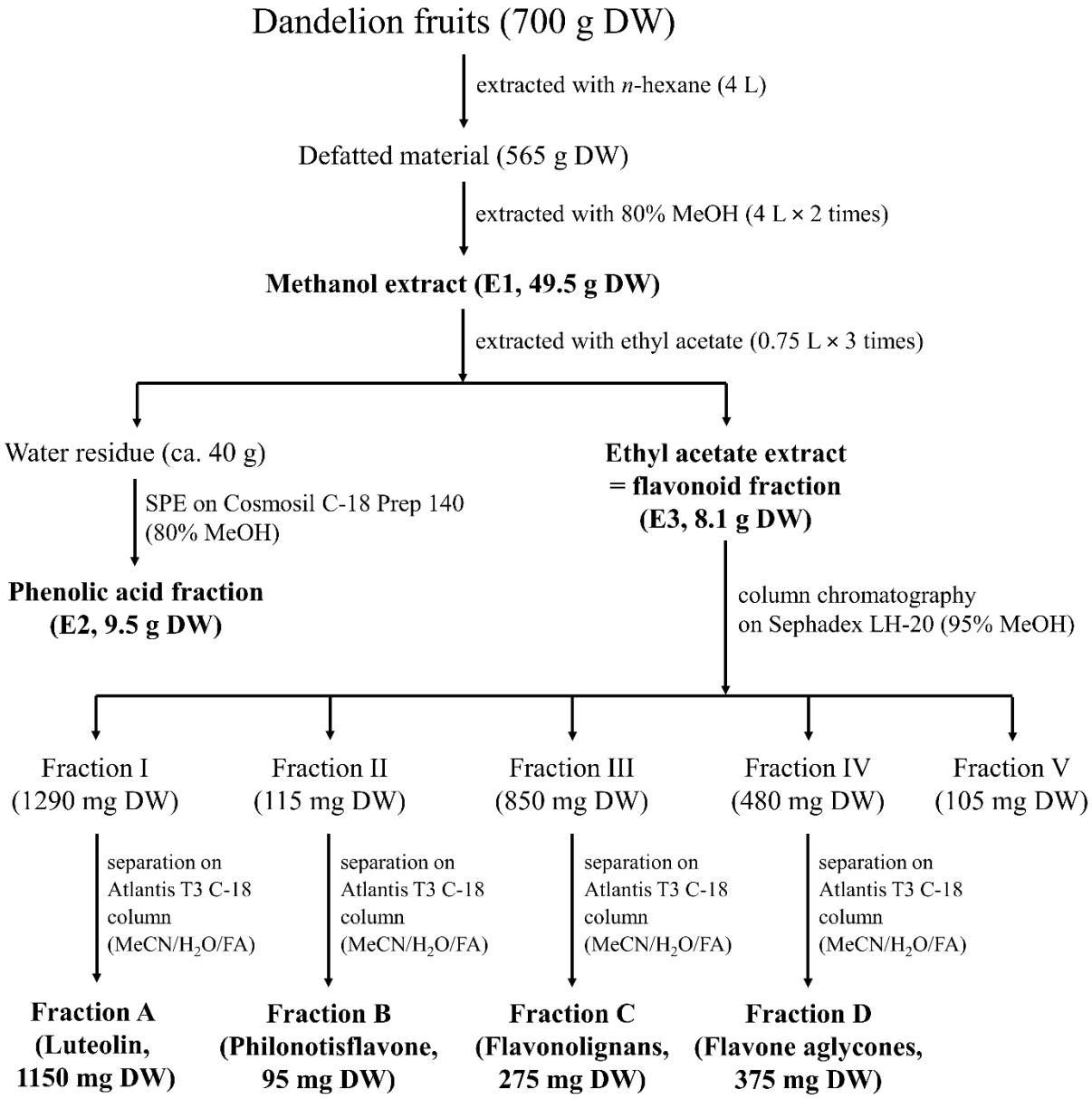
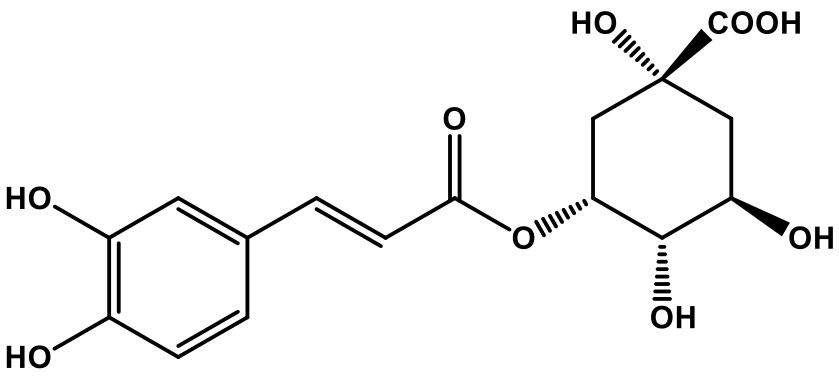
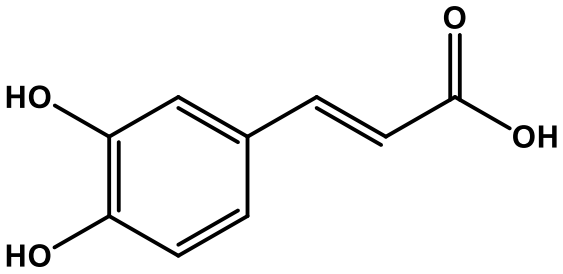


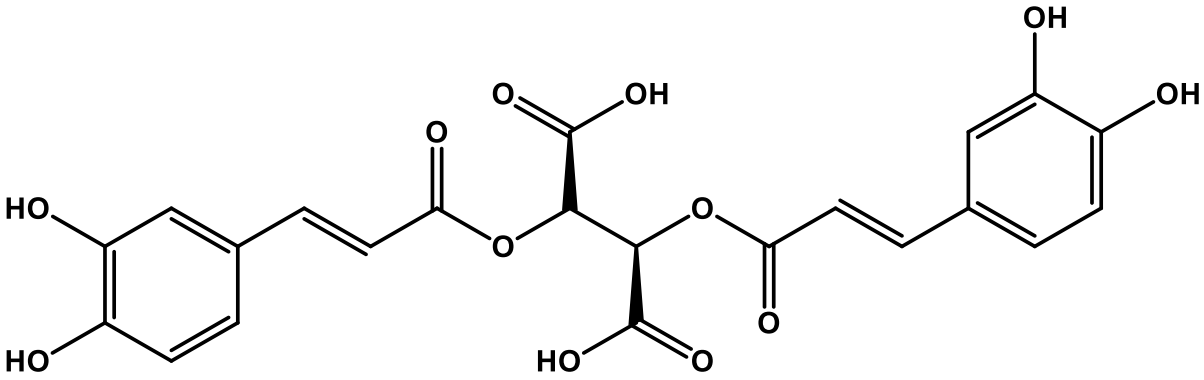
Figure S1. Flow diagram of extraction and fractionation of dandelion fruits



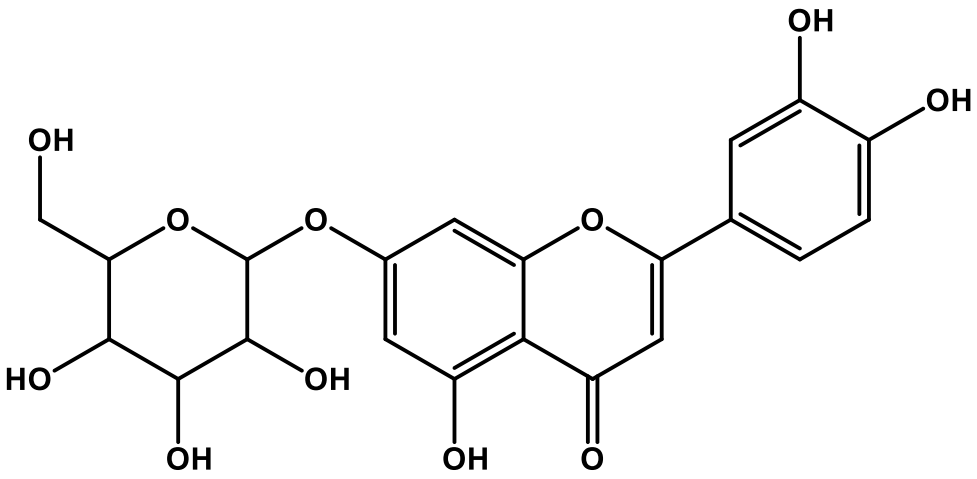
5-*O*-caffeoylquinic acid (MW = 354)



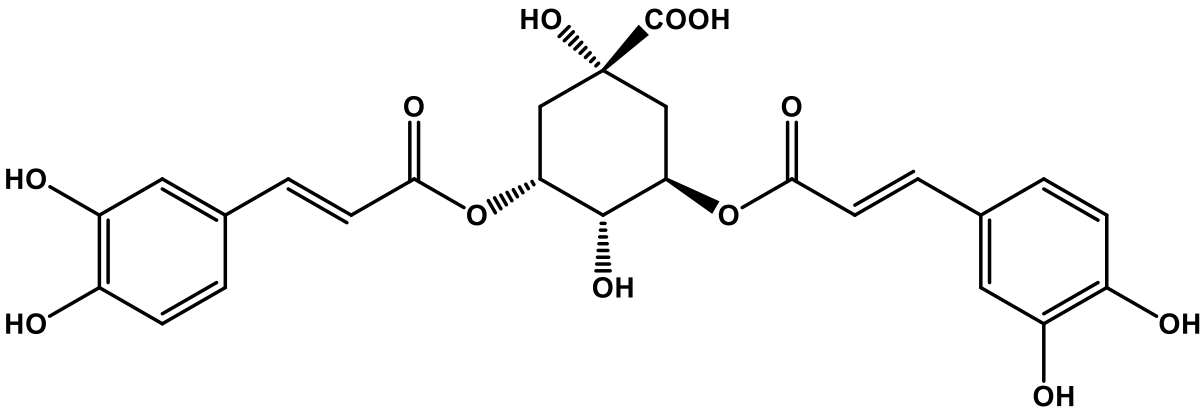
caffeic acid (MW = 180)



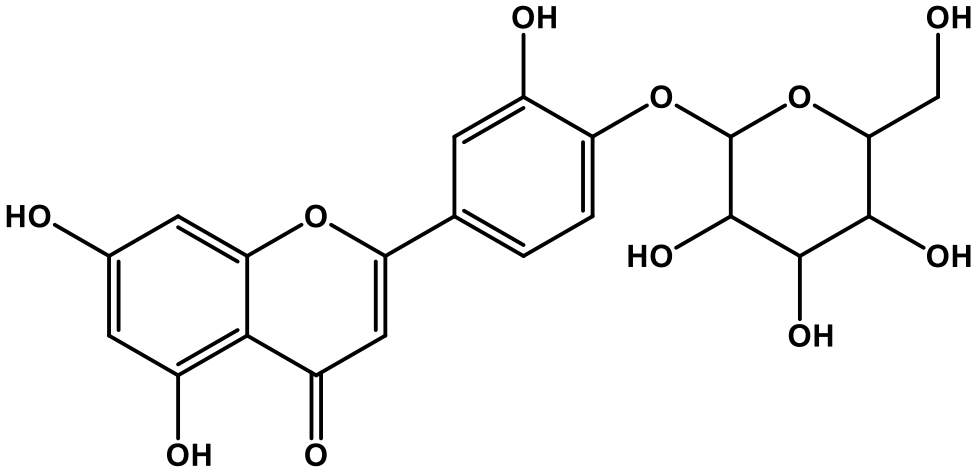
L-chicoric acid (MW = 474)



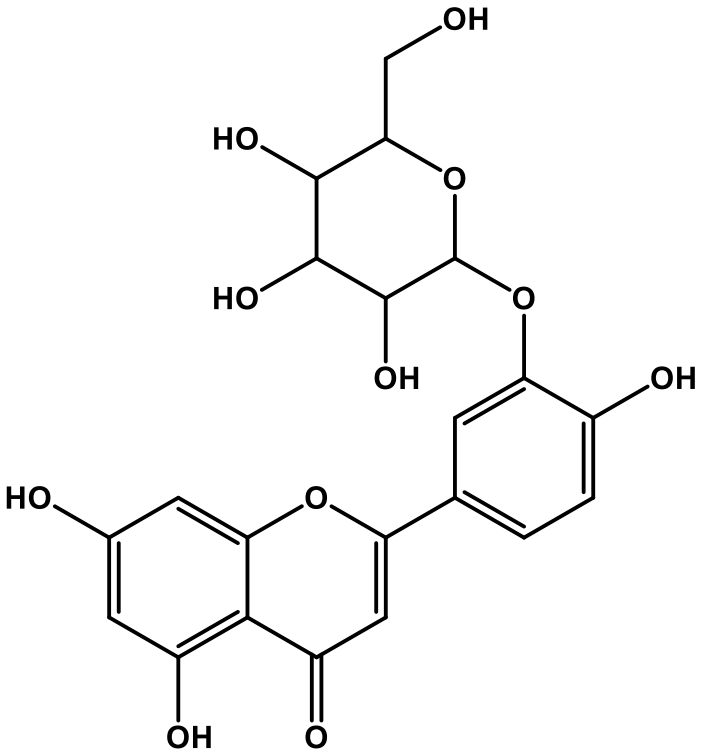
luteolin 7-*O*-glucoside (MW = 448)



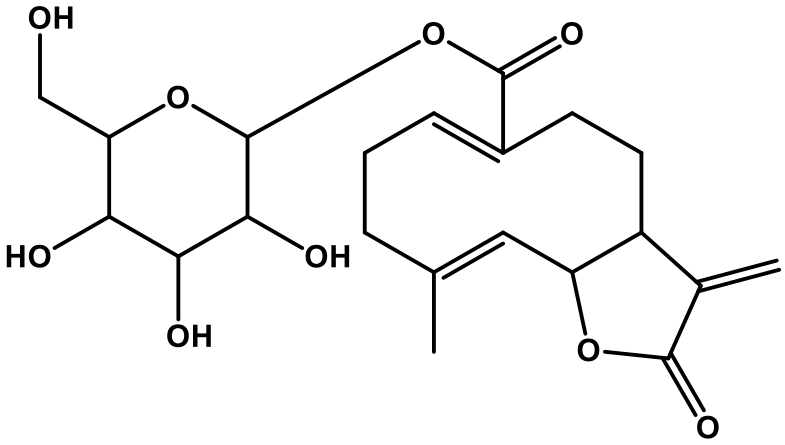
3,5-di-caffeoylquinic acid (MW = 516)



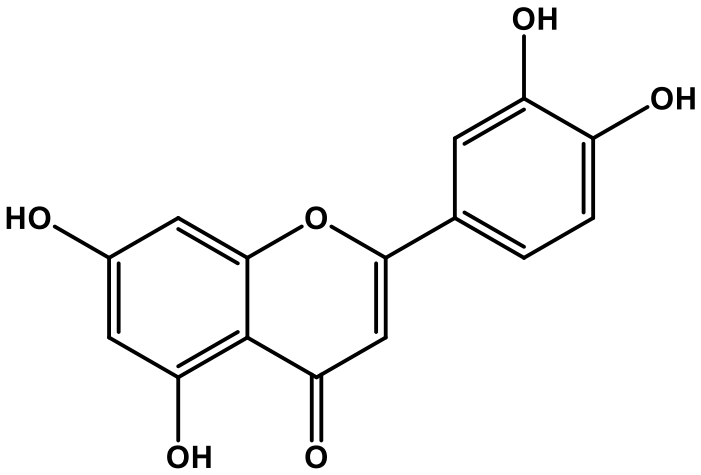
luteolin 4'-*O*-glucoside (MW = 448)



luteolin 3'-*O*-glucoside (MW = 448)



taraxinic acid 1'-*O*-glucoside (MW = 424)



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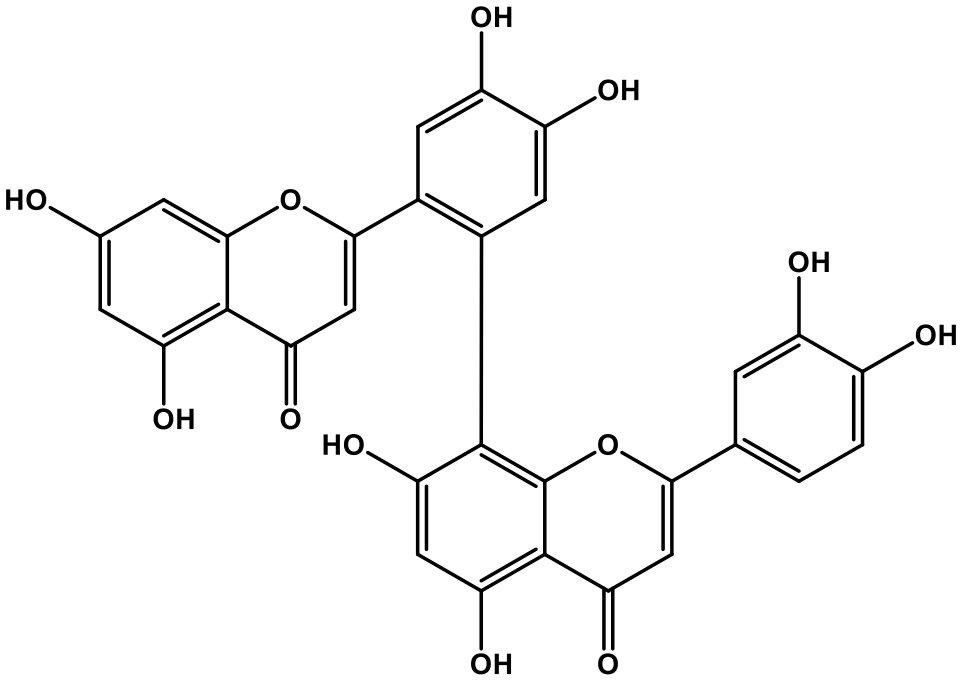
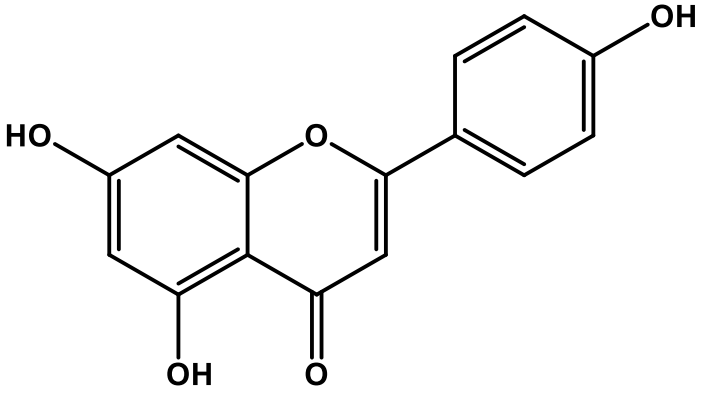
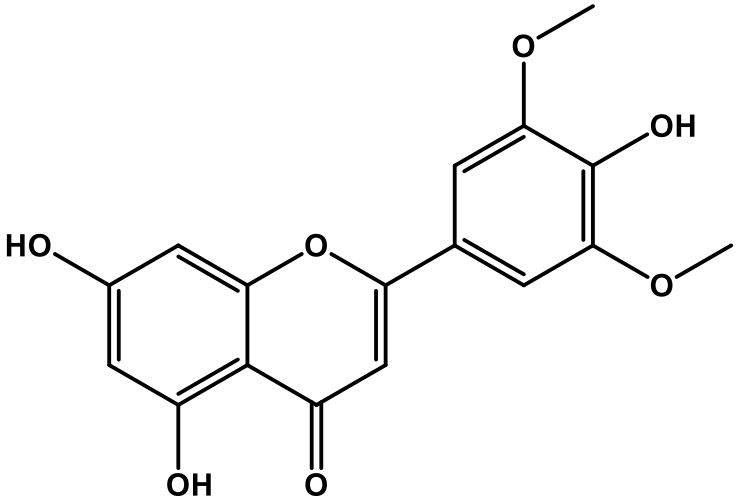


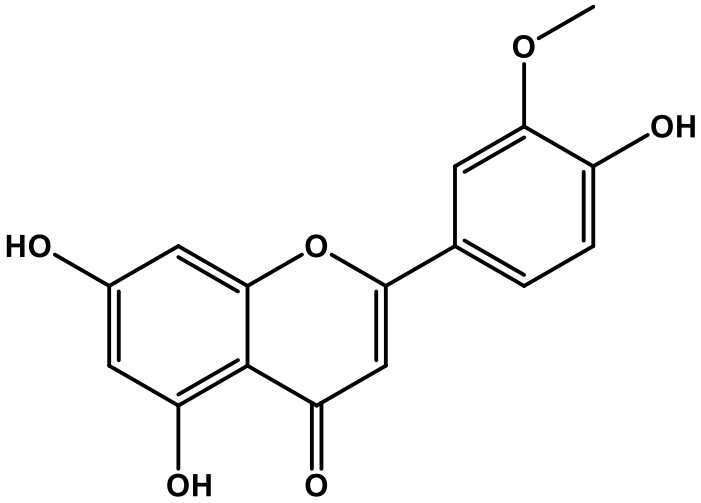
philonotisflavone (MW = 570)



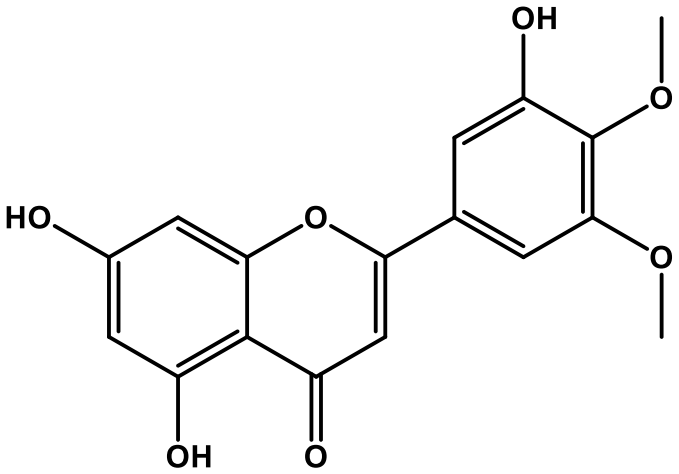
apigenin (MW = 270)



tricin (MW = 330)



chrysoeriol (MW = 300)



apometzgerin (MW = 330)

Figure S2. Structures of 14 fully identified metabolites in methanol extract and prepared phenolic preparations of *Taraxacum officinale* fruit

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Pro-health activity of dandelion (*Taraxacum officinale* L.) and its food products – history and present

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ABSTRACT

Since ancient times, dandelion (*Taraxacum officinale* L.) has been consumed in various forms as a valuable source of nutrients, minerals and vitamins which can also have beneficial effects on various complex diseases. It is described as a nontoxic herb with exceptional biological activity. The aim of this review is to present the multidirectional activity of various dandelion products whose dietary intake may help to prevent, or reduce the risk of, the development of a range of diseases, including cancer, obesity, hepatitis, arthritis and cardiovascular disease. In addition, the paper describes the history of dandelion usage and serves as an overview of current literature based on *in vitro* and *in vivo* studies. However, the correlation between the chemical content and the biological properties of dandelion and its food products are not always clear, and further experiments are required to determine the therapeutic doses of dandelion products for use in future research.

1. Introduction

Recent decades have seen increasing attention to the role of diet in human health. It has been confirmed that alternative therapy based on natural remedies can play a role in the treatment of many ailments; for instance, cardiovascular diseases are the leading cause of death not only in Poland, but around the world (WHO, 2018). It is believed that a diet rich in antioxidants plays a dominant role in preventing these diseases, particularly, the consumption of plant products rich in natural antioxidants, such as polyphenols. These components possess the ability to scavenge harmful free radicals that are capable of attacking the healthy cells of the body, causing them to lose their structure and function. As cell damage caused by free radicals appears to be a major contributor to degenerative diseases such as cancer, cardiovascular disease or osteoporosis (Nimse & Pal, 2015), interest in medicinal plants has been steadily growing, due to their beneficial effects on oxidative stress disorders and health in general (Petkova, Ivanov, Topchieva, Denev, & Pavlov, 2015). In addition, treatment based on supplements or medicines containing natural compounds lack many of the side effects associated with pharmacological treatment.

Plants have served humanity for thousands of years. Medicinal plants are an important source of chemical compounds with varied biological activities, including antioxidant properties. One such plant with multidirectional health effects is dandelion (*Taraxacum officinale* L.), a member of the *Asteraceae* family. It is a non-toxic herbaceous perennial often considered as a weed, and which is widely distributed across the Northern Hemisphere (Jinchun & Jie, 2011), and has spread to all corners of the globe with favorable climatic conditions. The therapeutic use of dandelion has been mentioned by Arabian, Native American, Chinese and Ayurvedic Medicine (Yarnell & Abascal, 2009). The dandelion contains a wide range of phytochemicals with specific biological activities; some examples of which are sesquiterpene lactones with anti-inflammatory and antimicrobial properties; triterpenes/phytosterols with anti-atherosclerotic effects; phenolic acids with antioxidant and immunostimulatory properties; coumarins which demonstrate antitumor, anti-inflammatory, antimicrobial and anticoagulant properties; as well as flavonoids displaying antioxidant activity (Gonzalez-Castejon, Visioli, & Rodriguez-Casado, 2012; Schütz, Carle, & Schieber, 2006; Xu, Wang, Chu, & Jia, 2019). Furthermore, dandelion roots contain a storage carbohydrate with probiotic activity, i.e. inulin

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; B.C., Before Christ; CD, cluster of differentiation; CPC, China Pharmacopeia Commission; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DRE, dandelion root extract; ESCOP, European Scientific Cooperative on Phytotherapy; FAO, Food and Agriculture Organization; FDA, Food and Drug Administration; GAE, gallic acid equivalent; GRAS, generally recognized as safe; HDL, high density lipoprotein; HSP, heat shock proteins; IC, inhibitory concentration; IL, interleukin; IU, international unit; LDL, low density lipoprotein; LPS, lipopolysaccharide; MPEC, 2-methyl-6-p-methoxyphenylethynylimidazopyrazynone; PIEs, phenolic inositol esters; RBC, red blood cells; TA-G, sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester; TMC, Taemyeongcheong; TNF-α, tumor necrosis factor α; TriAC, triterpene acetates; UV, ultraviolet; VLDL, very low density lipoprotein; WHO, World Health Organization

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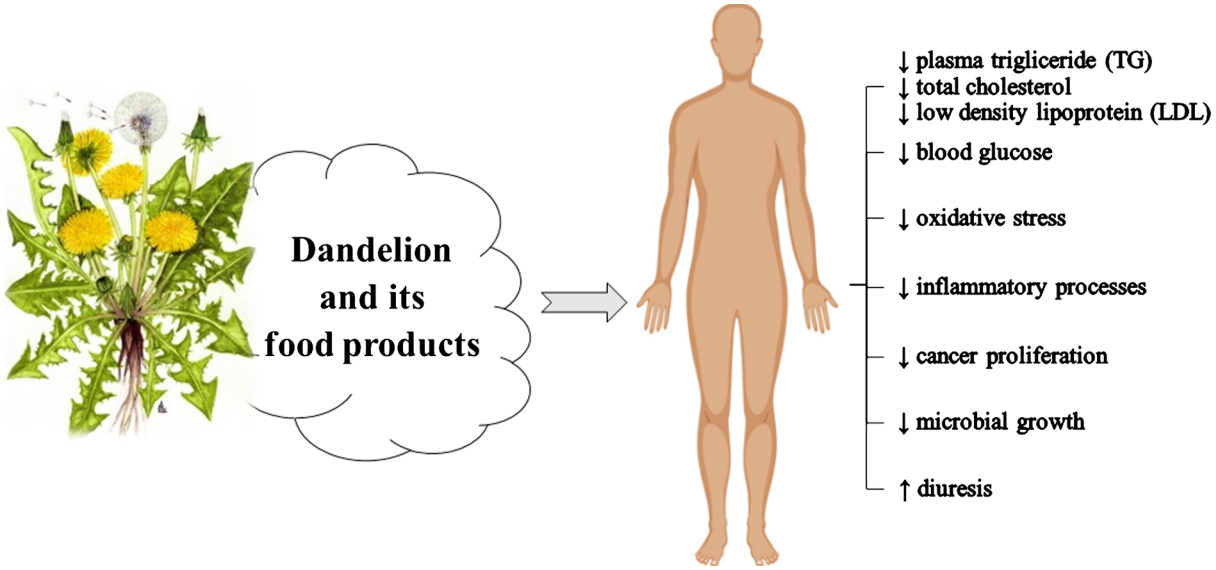

Fig. 1. Pro-health activity of dandelion and its products.

(Gonzalez-Castejon et al., 2012).

Apart from being used as a remedy, dandelion flowers or petals, leaves and roots can also be processed into different food products. For instance, dandelion leaves can be eaten fresh as a salad, the roots can be roasted and used as an additive in the production of coffee; dandelion extract can also be used as a flavouring additive in food products. Due to the presence of a variety of nutrients, including vitamins, minerals and fatty acids, the consumption of dandelion can also have an impact on health (Fig. 1). Although thorough chemical analysis is a vital part of any assessment of biological activity associated with dandelion raw materials, this information is often absent from *in vitro* or *in vivo* studies. However, several experiments have recently indicated an association between the chemical composition and bioactivity of dandelion (Jędrejek, Kontek, Lis, Stochmal, & Olas, 2017; Jędrejek, Lis, Rolnik, Stochmal, & Olas, 2019; Lis, Jędrejek, Stochmal, & Olas, 2018). The aim of this review is to provide an overview of the current knowledge of the pro-health activity of dandelion, obtained from both *in vitro* and *in vivo* experiments, and its use as a food.

2. Botanical characteristic of dandelion

Dandelion (*T. officinale* L.) is a herbaceous perennial belonging to the *Asteraceae* family, *Cichorioideae* subfamily and *Lactuceae* tribe. The name of the genus *Taraxacum* means *bitter herb* in Arabic, while in Greek, it may be derived from the words taraxia (*eye disorder*) and akeomai (*to cure*). The common name 'dandelion' probably comes from the French (dent de lion), meaning *teeth of the lion*, referring to the tooth-like edges of the leaves (Qureshi, Adil, Abd el-Hack, Alagawany, & Farag, 2017; Yarnell & Abascal, 2009). Similar translations can be seen in other languages e.g. *Löwenzahn* in German or *diente de leon* in Spain (Yarnell & Abascal, 2009).

Dandelion is an edible plant regarded as a weed and which is widely distributed in the warmer temperate zones of the Northern Hemisphere (Jinchun & Jie, 2011; Xue, Shuming, Du, & Zhu, 2017); it is also distributed in some Central and South American regions, and in Australia and New Zealand (Mingarro et al., 2015). It is believed that dandelion first arose in Eurasia, but has since been unintentionally moved by humans during historical times. It has been suggested that dandelion first arrived in North America with the Vikings or that it could be transported by hitchhikers in vegetable seed packets (Stewart-Wade, Neumann, Collins, & Boland, 2002). Although dandelions can tolerate a wide range of conditions, they are thought to grow best in areas sheltered from the wind, with a soil rich in humus, calcium and water (Tumbarski, Petkova, & Ivanov, 2016). Dandelion mainly grows on meadows, lawns, roadsides, gardens, orchards and wasteland (Rasool & Sharma, 2014) both in full sun or partial shade (Lim, 2014). It is a hardy plant, which is drought and frost tolerant. Dandelion can also occurs on other areas with moist soil from near sea level to 1000 m elevation. Moreover, it appears in the tropics in the cool highlands at altitudes of 1200–1500 m (Lim, 2014).

Dandelion is a plant with a well-developed tap root whose length has been found to reach up to 0.5 m (Bashmakov, Tserkovnova, Lukatkin, & Teixeira da Silva, 2008), or even 2 m (Ianovici, 2016; Stewart-Wade et al., 2002). The lateral roots are distributed relatively regularly along its length. Moreover, roots have a high capacity for regeneration, and can produce shoots or roots from very small fragments. In winter, when unfavourable conditions occur, the dandelion roots are shorter and come down into the soil, where they are better protected (Ianovici, 2016). The stems are acaulescent (1–2.5 cm of length) with very short internodes, with a radial rosette of leaves at the base. The shape of the leaves themselves is highly variable, ranging from lobeless to tooth-edged to highly incised; their length varies from 5 to 40 cm, and width from 0.7 to 15 cm (Stewart-Wade et al., 2002). The basal rosette gives rise to one to numerous cylindrical scapes (5–50 cm tall) bearing single inflorescences (capitulum) 2–5 cm in diameter at their ends. The flowers are semi-florets, bisexual and bright yellow (Ianovici, 2016). Blooming occurs twice a year, with the first observed at the turn of April and May, and the second in September (Lim, 2014). The fruit resembles light-brown or olive-brown achenes with a white floccus of 10 mm in length that increases the surface area for flight (Bashmakov et al., 2008). Additionally, specialized latimer cells present in almost all of the organs throughout the plant are used to produce latex (Wahler et al., 2009).

3. History and traditional uses of dandelion

The Greek naturalist Theophrastus (371 BCE–287 BCE) recommended that the herb be taken as a tonic, especially against freckles and liver spots on the skin (Sharifi-Rad et al., 2018). Dandelions were also fermented into wine for consumption by the ancient Celts, and the plants were consumed by Anglo-Saxon tribes to prevent scurvy and for use as a diuretic and laxative (Mars, 1999). Dried dandelion roots have found application in traditional Chinese medicine as a means of supporting the treatment of swelling (Saeki et al., 2013). The Persian doctor, philosopher and scientist Abu Ali Sino, known as Avicenna, reported that dandelion milk juice possesses beneficial effects in

reducing the symptoms of glaucoma. Furthermore, the squeezed dandelion juice was recommended as a useful remedy for liver protection and against hydrops, as well as an antidote to scorpion bites. Another physician, Muhammad Husain, reported that dandelion may arrest haemoptysis and that it strengthens the stomach (Sharifi-Rad et al., 2018). Further evidence for therapeutic use of dandelion was mentioned by Arabian physicians of the 10th and 11th centuries to treat liver and spleen ailments (Schütz et al., 2006). Dandelion is also mentioned as a medicinal plant in a European herbal written in 1485 by Ortus Sannitatis (Leyel, 2007). In the 16th century, the German physician and botanist Leonhard Fuchs described dandelion being used to medicate gout, diarrhea, blisters, spleen and liver complaints (Schütz et al., 2006).

Moreover, dandelion has been used for many centuries in the treatment of gastrointestinal ailments, cancer, liver inflammatory diseases, eye diseases, osteoarthritis, eczema and anemia (Petkova et al., 2015). In North American aboriginal medicine, infusions and decoctions of the root and herb were applied to remedy kidney ailments, dyspepsia, heartburn, menstrual cramps, jaundice, chest pains and served to help heal broken bones, bruises, swellings, sores, and fractures (Mars, 1999; Schütz et al., 2006). The Digger Indians of Colorado and the Papago of the Southwest ate both raw and cooked dandelions, while the Iroquois boiled the leaves with fatty meats. Other tribes chewed dandelion stems as a gum to moisten their mouths and observed that the plant possesses mild narcotic properties. In addition, several tribes applied the juice from the stem to bee stings, and used the flowers to make a yellow dye for deerskin. In traditional Chinese medicine dandelion is combined with other herbs to treat hepatitis, to enhance immune response to upper respiratory tract infections, bronchitis or pneumonia (Sweeney, Vora, Ulbricht, & Basch, 2009). More recently, during World Wars I and II, dandelion was a source of food during periods when many people suffered nutritional deficiencies (Mars, 1999). Also, most of the research carried out since 1930 on dandelion has focused on the evaluation of its pharmacological properties and biochemical characterization (Martinez et al., 2015).

Taraxacum has been mentioned in ethnopharmacology for treatment of diseases and their symptoms. For instance, *T. officinale* L. was recommended for tooth ache in Kosovo, as malaria treatment in Venezuela, and for hypertension in Ghana (Martinez et al., 2015). The herb remains of special value in traditional Mexican and Chinese medicine for a variety of health problems such as loss of appetite and dyspepsia, and for use as a blood tonic or laxative (Rodriguez-Fragoso, Reyes-Esparza, Burchiel, Herrera-Ruiz, & Torres, 2008). In Bulgarian traditional herbal medicine, it is used for the treatment of digestive diseases, prevention of renal gravel and loss of appetite (Petkova et al., 2015). In Russia, dandelion root is used to treat tuberculosis and prevent miscarriage (Mars, 1999), and is a popular folk medicine in Turkey due to its laxative, diuretic and hypoglycaemic properties (Schütz et al., 2006). Also, it has been proposed that dandelion may optimize breastfeeding through initiating or increasing milk production by increasing the production of oxytocin or prolactin; however, this has not be supported by any scientifically valid clinical trials (Mars, 1999).

Many other uses have been devised for dandelion. Dandelion is one of the most widespread plant sources of natural latex in Europe, this being the milky juice present throughout the plant, but present in largest amounts in the roots. This is a white secretion with thick and sticky consistency produced in special cells called latimers (Huber et al., 2015). It is composed of proteins, carbohydrates, oils, secondary metabolites and rubbers. Latex is dominated by three classes of compounds, including phenolic inositol esters (PIEs), triterpene acetates (TritAc) and sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G). This mixture of polymers and metabolites gives the latex a bitter taste (Wahler et al., 2009). In addition, the presence of TritAc and TA-G compounds are toxic to certain species of organisms such as beetles from the *Scarabaeidae* family; therefore, dandelion is often used in compost to prevent infestation by pests (Huber et al., 2015) as well as a mosquito repellent (Sohail et al., 2014). Further, its wide spectrum of occurrence in areas modified by humans allow it to be used as an indicator of the level of soil contamination with toxic elements: copper, arsenic, cadmium, chromium, iron, mercury, nickel, antimony, selenium, titanium, vanadium and zinc are accumulated by the plant in proportion to their levels in the environment (Ligocki, Tarasewicz, Zygmunt, & Aniśko, 2011).

4. Chemical composition of dandelion

The medicinal raw materials are the root (*Taraxaci radix*), root with herb (*Taraxaci radix* cum *herba*), leaves (*Taraxaci folium*) and flowers (*Taraxaci flox*). The harvesting period, environmental conditions and drying method have a huge impact on the chemical composition of the raw materials (Stewart-Wade et al., 2002). Therefore, it is important to determine the concentration of chemical compounds contained in extracts used in studies; however, many experimental works fail to include this information.

Dandelion is a rich source of various phytochemicals including flavonoids, phenolic acids and terpenes. In addition to its range of polyphenol compounds, many others have been found to be present. Of the phenolic compounds with antioxidant and anti-inflammatory activity present in the leaves and petals, the highest concentrations have been noted for hydroxycinnamic acid derivatives, particularly chicoric acid, chlorogenic acid and caffeic acid (Jędrejek et al., 2017). Major sesquiterpene lactones (generally occurring as glycosides) contain taraxacolides, dihydro-lactucin, ixerin, taraxinic acids, and ainslioside (Gonzalez-Castejon et al., 2012; Sharifi-Rad et al., 2018). Furthermore, our experimental work demonstrated that standardized dandelion phenolic fractions from leaves and petals are able to reduce oxidative stress and modulate important processes in blood platelets (Lis et al., 2018). It is also thought to boost the immune system of the body while also cleansing the liver (Tumbarski et al., 2016).

The flavonoids constitute a large group of plant secondary metabolites ingested by humans. They can be divided into several groups, each with a flavone ring as the basic structure. In general, flavonoids possess strong antioxidant activity due to their ability to scavenge radicals and participate in antioxidant reactions (Treml & Smejkal, 2016). For instance, dandelion leaf and flower extracts are rich in luteolin-7-*O*-glucoside, luteolin-7-diglucosides and luteolin; together with other antioxidants, such as phenolic acids and terpenoids, these flavonoids can be used to protect the human body from the pathological effects of free radicals (Gonzalez-Castejon et al., 2012; Xue et al., 2017).

The bitterness of dandelions is caused by the presence of sesquiterpene lactones, mostly eudesmanolides (i.e. tetrahydroridentin B; taraxacolide-O-β-glucopyranoside); guaianolides (i.e. 11β,13-dihydrolactucin; ixerin D); and esterified germacranolides acids (i.e. taraxinic acid β-glucopyranosyl ester, its 11,13-dihydroderivative and ainslioside), which are unique to the plant (Sharifi-Rad et al., 2018). The root is a bitter raw material with mainly choleretic effects. Biologically-active compounds i.e. sesquiterpene lactones stimulate the liver to secrete bile, which facilitates its flow into the duodenum. Furthermore, a glucoside of taraxic acid affect the normalization of the digestive process due to their beneficial effect on the secretory functions of the pancreas and the increased secretion of gastric juice (Wirngo, Lambert, & Jeppesen, 2016).

Dandelion roots also contain inulin, which has various beneficial effects such as eliminating pathogens in the gastrointestinal tract, and repressing obesity, cancer and osteoporosis (Wirngo et al., 2016). The content of inulin in roots is determined by season, e.g. the smallest amount is observed in the spring (2% of total secondary compound content) and the largest in autumn (40%) (Gonzalez-Castejon et al., 2012).

5. Scientific evaluation of dandelion on studied *in vitro*

Various *in vitro* studies have demonstrated that dandelion can act as a natural remedy for cardiovascular diseases, as well as for cancer or obesity. Experimental studies suggest that the consumption of foods supplemented with natural dietary antioxidants may prevent or reduce the risk of oxidative stress- relative diseases. For example, dandelion flowers are rich source of caffeic acid, chlorogenic acid, luteolin and luteolin-7-glucoside. Flower extract, with its abundant polyphenolic content, has been found to exhibit superoxide radical inhibiting activity and reduce the formation of uric acid at concentrations of 166.7 mg/ml. In addition, dandelion flower extract neutralized non-site-specific, hydroxyl-radical induced deoxyribose cleavage in a concentration-dependent manner. Higher concentrations of extract suppressed lipid oxidation. These findings indicate that dandelion flower extract has a scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, and protects living cells from peroxyl-radical-induced intracellular oxidation. In addition, the standardized dandelion extract suppressed nitric oxide in cultured mouse macrophage RAW264.7 cells stimulated by bacterial lipopolysaccharide (LPS) without inducing cytotoxicity. This effect can be related with the presence of luteolin or luteolin-7-glucoside, which acts by reducing the expression of both inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) without a concomitant reduction in enzymatic activities (Hu & Kitts, 2005).

Recently, Jędrejek et al. (2017) and Lis et al. (2018) studied the phytochemical composition of the leaves and petals from dandelion, as well as their anti-platelet properties and antioxidant potential against the effect of a strong biological oxidant: hydrogen peroxide (H_2O_2) or H_2O_2/Fe (the donor of hydroxyl radical). Their findings indicate that four standardized dandelion phenolic fractions from leaves and petals are able to reduce oxidative stress (i.e. lipid peroxidation and protein carbonylation); can protect thiol groups in blood platelet as well as in plasma proteins. Their results show that dandelion petals had stronger antioxidant properties. Especially with regard to the protection of plasma proteins, than the phenolic fractions from dandelion leaves; this difference may be associated with differences in their chemical profiles, as the total concentration of flavonoids is 633.71 mg/g in an 85% fraction of petals and 160.72 mg/g in an 85% fraction of leaves. Additionally, all dandelion fractions prolonged the thrombin time of plasma when they were preincubated with thrombin – a multifunctional plasma serine protease with a central function in controlling hemostasis; this may be associated with inhibition of thrombin activity. Elsewhere, it was found that five preparations taken from dandelion roots were valuable sources of different classes of non-cytotoxic secondary metabolites possessing various biological potential, including antioxidant and anticoagulant properties (Jędrejek et al., 2019). The authors suggest that the natural compounds contained in dandelion display antioxidant, anti-platelet and anticoagulant activities; these can be beneficial in the prevention and treatment of cardiovascular diseases, which often arise in response to changes in hemostasis and oxidative stress.

Nowadays, the fast pace of modern life, together with its associated unbalanced diet and stress, make it impossible to provide all the components needed for proper functioning of the skin. A study of the antioxidant properties of dandelion leaf and stem extract by Xie et al. (2018) found caffeic acid to be the predominant component of dandelion stem extract (86.7 mg/g) and chlorogenic acid to predominate in dandelion leaf extract (2.34 mg/g). Chemically, chlorogenic acid is an ester formed between caffeic acid and quinic acid. Both chlorogenic acid and caffeic acid possess an aromatic residue with a vicinal hydroxyl group, the presence of which determines their antimutagenic, carcinogenic and antioxidant potential (Sato et al., 2011). Both extracts possessed similar reducing power and superoxide anion radical scavenging capacity; however, the stem extract indicated the strongest UVA and UVB absorption and showed the strongest tyrosinase inhibition. In addition, molecular docking simulation results indicated the caffeic acids in the stem extract to inhibit tyrosinase mainly via hydrogen bonding with its Gly165 and Pro160 residues. Dandelion stem extract therefore offers promise as a skin care agents, but further studies are needed. Higher total phenol content, and antioxidant activity have also been demonstrated by the leaf extract (2.019 mg GAE/L) than the root extract (1.997 mg GAE/L) (Ozcan, Paksoy, & Unver, 2012). Sato et al. (2011) evaluated the biological activities of chlorogenic acid and their main metabolite i.e. caffeic acid. The results of *in vitro* studies suggest that caffeic acid has a better antioxidant effect than chlorogenic acid, as indicated by the MPEC test (2-methyl-6-p-methoxyphenylethynylimidazopyrazynone). The results of a subsequent test based on an *in vivo* intestinal ischemia–reperfusion model found caffeic acid to play an important role in the protective effect demonstrated by chlorogenic acid on intestinal ischemia-reperfusion injury, described as a condition that restores the bloodstream by the reoxigenate after temporary hypoxia. These findings indicate that in addition to their known antioxidant activity, these tested acids may also have a protective effect on intestinal injury.

Further, Kim and Baik (2015) studied a probiotic functional fermented beverage from dried dandelion leaves with a high level of caffeic acid, which is also proposed as an effective way of preventing or treating diabetes, a condition that affected about 422 million people in 2014 (WHO, 2016). Although drugs based on sulfonylureas are often used to treat diabetes, their use is associated with adverse effects such as hypoglycemia and secondary insulin secretion failure. A possible alternative is offered by the use of probiotication to produce fermented functional foods, which act by enhancing the intestinal flora (Mustafa, Chua, Enshasy, Majid, & Malek, 2016). One of method for converting chlorogenic acid to caffeic acid in antidiabetic products is through an enzymatic hydrolysis approach using cinnamoyl esterase. The obtained caffeic acid can stimulate insulin secretion from pancreatic β-cells more effectively, thus lowering blood glucose levels. In addition, caffeic acid can be more easily absorbed in the stomach than chlorogenic acid. Interestingly, the authors obtained the functional *lactobacillus* LA-F46 with high-level cinnamoyl esterase activity from human feces (Kim & Baik, 2015). Recent studies have found dandelion extracts to possess potential anti-diabetic activity thanks to their heterogeneous mix of components including phytosterols, sesquiterpene lactones, flavonoids and phenolic acids. Twenty-eight compounds were isolated from the whole plant of *T. officinale,* and their inhibitory effects against a-glucosidase were evaluated. Two of them displayed outstanding inhibitory activities (IC_{50} 61.2 and 39.8 μM, respectively), and the novel isolates showed activities (IC_{50} 145.3–181.3 μM) similar to acarbose (IC_{50} 179.9 μM) which is an anti-diabetic drug used to treat type 2 diabetes mellitus (Choi, Yoon, & Kim, 2018; Wirngo et al., 2016).

Xue et al. (2017) confirmed that extracts from leaves, flowers, roots, and stems are rich in chicoric acid, and that the crude leaf extract possesses the highest total phenolic and flavonoid content and antioxidant activity and the crude root extract the lowest. Moreover, leaf extract suppressed intestinal oxidative stress and inflammation in cultured human colonic cells. Therefore, dandelion extract could provide alternative antioxidative and anti-inflammatory therapeutics for chronic diseases in the gut, such as inflammatory bowel disease or colorectal cancer.

Cancer remains one of the leading causes of deaths, with over 12 million new cancer cases arising annually and over seven million cancer-related deaths worldwide. One approach to reducing the risk of contracting cancer is through the modification of lifestyle factors. For example, enriching the diet with taraxasterol, found in like herbal tea or coffee substitutes based on dandelion root, has been found to potentially reduce the likelihood of contracting cancer (Sharma & Zafar, 2014). Taraxasterol is a pentacyclic-triterpene constituent isolated not only from dandelion, but also from chicory, cardoon or mountain arnica (Yang et al., 2015). Koo et al. (2004) report that aqueous dandelion extracts demonstrated antitumor activities in HepG2 cells by influencing TNF-α and IL-1α secretion. Further, Ovadje, Ammar, Guerrero, Arnason, and Pandey

Table 1
Various biological activity of dandelion (*in vivo* experiments).

Plant extract	Dose	Days/month	Subjects	Effects	References
ANIMAL					
High-cholesterol diet with 1% dandelion leaf (total phenol content: 7.9 ± 0.4%), and a high-cholesterol diet with 1% dandelion root (total phenol content: 9.4 ± 0.3%)	250 g/day	1 month	Rabbits (28 male)	**Hypolipidemic and antioxidant effects.** That treatment with dandelion root and leaf positively changed plasma antioxidant enzyme activities and lipid profiles in cholesterol fed rabbits.	Choi et al., 2010
95% ethanol extract of dandelion leaves (total phenol content: undefined)	400 mg/kg	–	Mice (16 male)	**Anti-obesity effect.** A single oral dose of dandelion extract inhibited increases in plasma triglyceride levels and reduced the areas under the curves of plasma triglyceride response curve.	Zhang et al., 2008
Ethanolic extract of dandelion leaves (total phenol content: undefined)	150 mg/kg, 300 mg/kg	10 weeks	Rats (30 male)	**Anti-obesity effect.** Dandelion extract (150 and 300 mg/kg) added to a high fat diet showed significant activity through a decrease in the body weight, similarly to Orlistat (standard drug). However, oral administration of dandelion extract reduced the levels of blood glucose, serum triglycerides, LDL (low density lipoprotein), VLDL (very low density lipoprotein), total cholesterol; and increased the level of HDL (high density lipoprotein) compared to the high fat diet group.	Rao et al., 2015
Dandelion leaf extract (total phenol content: 8.017 ± 0.130 mg chlorogenic acid equivalents per gram of dried dandelion)	2 g/kg, 5 g/kg	10 weeks	Mice (40 male)	**Antidiabetic effect.** Dandelion leaf extract has been shown to reduce serum glucose, cholesterol, and triglyceride levels, possibly through the elevation of adenosine monophosphate-activated protein kinase (AMPK) in the liver, with a significant fall in lipid accumulation and improvement in insulin sensitivity.	Davaatseren et al., 2013
70% ethanol extract from dandelion leaves (total phenol content: undefined)	100 mg/kg	56 days	Guinea- pigs (20 male)	**Anticholinergic and anti-inflammatory effects.** Dandelion extract application showed significant antagonistic effect on contraction of trachea. Also, reduced monocytes, lymphocytes and neutrophils level in ovalbumin –sensitized guinea-pigs. An extract possesses anticholinergic and reduces neutrophil, eosinophil and basophil counts in ovalbumin-sensitized guinea-pigs.	Awortwe et al., 2011
Dandelion extract from whole plant; prepared by decocting the dried prescription of herbs with boiling distilled water. (total phenol content: undefined)	10 mg/kg	5 days	Rats (5 male/group)	**Anti-inflammatory effect.** Studies showed that dandelion extract decreased the pancreatic weight/body weight ratio; increased the expression of HSP60 and HSP72 (HSP preinduction protected the pancreas from cerulein induced pancreatitis) compared to saline- treated controls. Additionally, IL-6 (a principal mediator of acute phase response) and TNF-α (a predominantly macrophage-derived cytokine) secretion was decreased in the group treated with dandelion extract.	Seo et al., 2005
Dandelion extract from whole plant (total phenol content: undefined)	150 mg/kg, 200 mg/kg, 250 mg/kg	8 days	Rats (30 male)	**Anti-inflammatory effect.** The administration of dandelion extract (250 mg/kg) had beneficial effects upon the reproductive parameters through the protection upon sperm cells (the rate of dead sperm was lower in compared to group treated with paracetamol). Moreover, dandelion extract (200 mg/kg) was found to increase antioxidant level (glutathione); antioxidant enzyme activity (catalase, glutathione peroxidase and superoxide dismutase); and reduce lipid peroxidation.	Omur et al., 2017
Taemyeongcheong (TMC)- traditional healthy drink including 6 herbs such as: lizard's tail, dandelion, ginger, Korean thistle, glasswort, licorice (total phenol content: undefined)	100 mg/kg, 250 mg/kg, 500 mg/kg	14 days	Mice (60 male)	**Hepatoprotective effect.** TMC dose dependently protects against acetaminophen- induced acute liver damage in mice i.e. increased the hepatic levels of catalase, superoxide dismutase, glutathione peroxidase, glutathione; and reduced serum levels of the inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6.	Yi et al., 2015
Dandelion hydro alcoholic extract from whole plant (total phenol content: undefined)	50 mg/kg, 100 mg/kg, 200 mg/kg	20 days	Mice (50 female)	**Effect on blood cells.** That treatment showed increase of red blood cells (RBC) (dose 100 and 200 mg/kg), mean hemoglobin (dose 50, 100 and 200 mg/kg), white blood cells (200 mg/kg), and lymphocyte (dose 50, 100 and 200 mg/kg). Also, there was observed decrease in blood platelet levels (dose 50, 100 and 200 mg/kg). Increase of RBC and hemoglobin could be due to the positive effect of dandelion on the liver and increased secretion of erythropoietin.	Modaresi & Resalatpour, 2012

(*continued on next page*)

Table 1 (*continued*)

Plant extract	Dose	Days/month	Subjects	Effects	References
70% ethanol extract of dandelion leaves (total phenol content: undefined)	500 mg/kg, 1000 mg/kg	–	Rats (25 female)	**Immunostimulant effect.** Treatment of doxorubicin in combination with dandelion extract 1000 mg/kg and 500 mg/kg increased the number of immune cells: leukocytes, lymphocytes, neutrophils, cytotoxic CD8 + T cells.	Kasiamningsih et al., 2011
Dandelion extract from whole plant was prepared by decocting the dried prescription of herbs with boiling distilled water (total phenol content: undefined)	10 mg/kg, 30 mg/kg, 100 mg/kg	42 days	Mice (40 male)	**Anti-physical fatigue effect.** Mice receiving dandelion extract displayed significantly longer swimming time to exhaustion than untreated controls. In addition, dandelion extract could increase fat utilization during the activity, and effectively delay the lowering of glucose in the blood. Also, dandelion extract prevented the increase the lactate and triglyceride concentrations in the blood.	Jinchun & Jie, 2011
HUMAN					
95% ethanol extract of dandelion leaves (total phenol content: undefined)	8 mL (3 doses/day)	4 days	28 volunteers (group of healthy female)	**Diuretic activity.** It increased urination frequency and the excretion ratio.	Clare et al., 2009

(2016) proposes that aqueous dandelion root extracts may have anti-cancer potential, as they allow to stimulate a multiple signaling pathways in cancer cells, with no toxicity to non-cancer cells. Regarding the effectiveness of dandelion roots in at killing colon cancer cells, the authors note that cell death was selectively induced in > 95% of colon cancer cells *in vitro*, which was also confirmed by *in vivo* models. In previous studies, Ovadje et al. (2011) also report that chemical components of aqueous dandelion root extracts can act either alone or in combination with each other to induce formation of the death-inducing signaling complex and subsequent extrinsic apoptosis selectively in human leukemia cell lines. They also note that very early activation of caspase-8 and the subsequent activation of caspase-3 may be responsible for inducing apoptosis; as caspase inhibition rendered this extract ineffective, it appears that dandelion root extract (DRE)- induced apoptosis is caspase-dependent. A recent study by Rehman et al. (2017) clearly demonstrated the potency of methanolic extracts from dandelion root (500 μg/mL) against liver cancer. The authors indicate that dandelion extract enhanced the phosphorylation level of adenosine monophosphate-activated protein kinase (AMPK) of HepG2 cells, which is considered crucial in cancer treatment and other metabolic diseases.

6. *In vivo* studies

Both *in vitro* and animal *in vivo* studies have shown that different parts of dandelion can serve as natural remedies for many diseases, with hypolipidemic, hepatoprotective, antioxidant, anti-obesity, anti-inflammatory, anticholinergic, antidiabetic and anti-physical fatigue effects (Awortwe, Sackeyfio, Osei-Safo, Bugyei, & Asiedu-Gyekye, 2011; Choi et al., 2010; Davaatseren et al., 2013; Rao, Jyothi, & Rabban, 2015; Sharifi-Rad et al., 2018; Yi, Song, Lim, Kim, & Park, 2015; Zhang et al., 2008). The results of animal *in vivo* studies and its therapeutic potential are demonstrated in Table 1. However, only one experiment has demonstrated diuretic activity (Clare, Conroy, & Spelman, 2009).

7. Medicines and food products from dandelion

Various dandelion plant parts have been studied both chemically and nutritionally. Apart from being used as a remedy of illness, dandelion petals, leaves and roots are processed into various food products (Fig. 2). For instance, in popular dishes in France and Vietnam, the young leaves from dandelion are consumed as salads, either alone or in combination with another plants such as lettuce, shallot tops and chives. In addition, the leaves can be boiled, drained and sprinkled with spices (Gonzalez-Castejon et al., 2012; Lim, 2014). The preparation of such products as liquors or marmalades is a common practice in Italy (Martinez et al., 2015). Due to their mineral-rich content, dandelion tea may help prevent osteoporosis and strengthen the teeth. Mineral analysis of ten types of commercially available tea show that 3.5 cups of dandelion infusion and 4.5 cups of *Echinacea* infusion provide a good source of potassium i.e. 10% RDA (Gallaher, Gallaher, Marshall, & Marshall, 2006). In dried form, the leaves are popular components of many digestive, dietary drinks and herb beers common in England and Canada (Gonzalez-Castejon et al., 2012). Both leaves and roots can be consumed as herbal tea, tincture or as capsules. Furthermore, leaves from *T. officinale* L. are a good source of raw material for flour rich in protein and other nutrients (Table 2), which the Food and Agriculture Organization of the United Nations suggest may be used to improve the nutritional condition of areas with poor economic resources (FAO) (Escudero, De Arellano, Fernandez, Albarracin, & Mucciarelli, 2003). The roasted roots can also be drunk as coffee, being first thoroughly cleaned, then dried by artificial heat, and slightly roasted till they are the tint of coffee. Dandelion coffee is especially recommended for diabetics due to the presence of a complex carbohydrate (fructo-oligosaccharides) with many beneficial effects (Wirngo et al., 2016).

It is found that inulin, a fructan naturally occurring in dandelion, can improve glucolipid metabolism in diabetic conditions. When

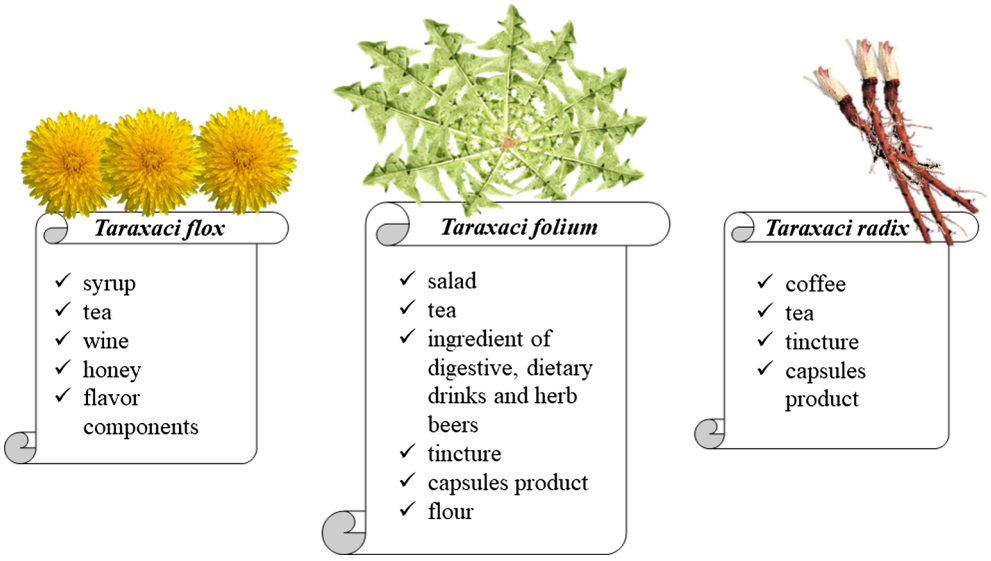

Fig. 2. Food products from different parts of dandelion: flower *(Taraxaci flox),* leaf *(Taraxaci folium)* and root *(Taraxaci radix)*.

Table 2
Nutritional value of flour from dandelion leaves (Escudero et al., 2003- modified).

Component	Value
Protein, carbohydrates and other (g per 100 g of dry matter)	
Protein	15.5
Polyunsaturated fatty acids	–
Saturated fat	–
Carbohydrates	58.4
Ash	14.6
Dietary fiber	47.8
Minerals and vitamins (g per 100 g of dry matter)	
Sodium	–
Potassium	2520.0
Calcium	695.0
Magnesium	470.0
Manganese	–
Iron	–
Phosphorus	–
Zinc	–
Copper	–
Vitamin A	13.8
Vitamin C	53.0
Vitamin E	–
Vitamin K	–
Fatty acids (%)	
Palmitic	27.6
Stearic	4.2
Palmitoleic	6.5
Oleic	8.6
Linoleic	18.5
Linolenic	34.6

supplied with food act as prebiotic, inulin resists digestion in the human small intestine and reaches the colon, where it is fermented by the gut microbiota i.e. bifidobacteria, which constitute a major part of the human intestinal microflora and offer considerable health benefits to the host (Trojanova, Rada, Kokoska, & Vlkova, 2004). However, inulin derived from dandelion roots is used for the microbiological production of high fructose syrup, and hence may play a role in preventing the spread of civilization diseases such as diabetes and obesity (Ning et al., 2017).

Dandelion coffee, without the narcotic effect of normal coffee, has been growing in popularity in Poland, being obtainable at most vegetarian restaurants and stores. However, dandelion root is believed to offer the greatest medicinal properties in its unroasted form and may be taken as a tea, an extract or in capsule form (Escudero et al., 2003). Lydia Pinkham, a popular herbalist in the mid-nineteenth and early twentieth centuries, included dandelion root in the original recipe for tonic intended for women during menstruation and menopause. Hepatichol, an over-the-counter drug made primarily from dandelion, is available for liver and gallbladder problems (Mars, 1999).

In Germany, dandelion juice from the stem and root are used to improve eye health (Mars, 1999). In the USA, dandelion root and leaf preparations are used as choleretic, diuretic and tonic components in a wide range of compound dietary supplements or health food products (Escudero et al., 2003). Also, extracts of dandelion flower are used to flavor frozen dairy desserts, candies, gelatins or even cheese (Schütz et al., 2006). The unopened flower buds are eaten in pancakes, omelettes, schnapps and fritters. Also, they can be preserved in vinegar and consumed as capers, while some petals may be used like confetti in rice dishes. The flowers are one of the ingredients added to cake called 'yublo' in Middle East or can be made into jam (Lim, 2014).

Their use in foodstuffs is justified by their low calorific content (45 kcal per 100 g of raw greens) and rich nutrient density, with carbohydrates being the predominant macroelement. In particular, dandelion leaves contain a wide range of vitamins such as vitamin A (10160 IU ~ 0.3 mg), in a similar amount to baby carrots (13790 IU ~ 0.4 mg), together with a number of other vitamins, including vitamin C, E, K, B_6, B_5, choline, folate, thiamin, riboflavin and niacin (Table 3). Dandelion leaves also contain a number of minerals including calcium, sodium, magnesium, iron, copper, phosphorus, zinc and manganese; of these, potassium is present in the highest amounts, similar to those in banana (397 mg/100 g; and 358 mg/100 g, respectively). Hence, dandelion contains four times the amount of potassium than other botanical diuretics (i.e. cranberries have a 85 mg of potassium) and provides more potassium than that lost from diuresis induced by ingesting (Data, 2018), dandelion supplementation could be a good pharmaceutical strategy for patients receiving diuretics (Clare et al., 2009). In addition, increased potassium intake with food (at least 3510 mg/day for an adult) plays a role in lowering blood pressure and reducing the risk of cardiovascular disease, stroke or ischemic heart disease (Gallaher et al., 2006; WHO, 2012).

8. Toxicology and safety

As with all drugs containing bitter substances, discomfort due to gastric hyperacidity may occur following consumption (Yarnell &

Table 3
Nutritional value of raw dandelion leaves (Data, 2018- modified).

Component	Value
Carbohydrates, protein, fat and other (g per 100 g of product)	
Protein	2.7
Polyunsaturated fatty acids	0.3
Saturated fat	0.2
Cholesterol	0
Sugars	0.7
Dietary fiber	3.5
Water	85.6
Minerals (mg per 100 g of product)	
Sodium	56
Potassium	397
Calcium	138
Magnesium	50
Manganese	0.2
Iron	6.2
Phosphorus	58
Zinc	1.1
Copper	0.1
Vitamins (mg per 100 g of product)	
Thiamin	0.2
Riboflavin	0.3
Niacin	0.8
Vitamin A	0.3
Vitamin C	35
Vitamin E	3.4
Vitamin K	0.8
Vitamin B_6	0.3
Vitamin B_5	0.1
Choline	35.3
Folate	0.3
Riboflavin	0.3
Selenium	0.0005

Abascal, 2009). Nevertheless, consumption of the leaves during pregnancy can help prevent preeclampsia, which manifests as high blood pressure with edema and their high content can help to prevent anemia (Mars, 1999). Contact dermatitis has been observed in cases of frequent contact with dandelion latex (Wirngo et al., 2016; Yarnell & Abascal, 2009); however, the sensitizing potential of dandelion is classified as very weak (Escudero et al., 2003), with no significant or visible signs of toxicity observed in experiments conducted on rabbits, mice, rats and other animals (Wirngo et al., 2016).

Due to its multidirectional effects on health, dandelion was an official herb in the early Pharmacopoeia of the United States. The roots were of great significance and they were included in the *National Formulary* until 1965. Also, dandelion was included in the pharmacopoeias of Hungary, Poland, Russia, and Switzerland (Mars, 1999). The European Scientific Cooperative on Phytotherapy (ESCOP) and the German Commission E authorizes the use of dandelion root for the support of liver function (García-Carrasco, Fernandez-Dacosta, Dávalos, Ordovás, & Rodriguez-Casado, 2015). In addition, dandelion is listed on the U. S. Food and Drug Administration's (FDA) "generally recognized as safe" (GRAS) as a food and supplement (Martinez et al., 2015), also it is officially recognized in the Chinese Pharmacopoeia (China Pharmacopeia Commission (CPC), 2015). It has been recommended that up to 50 g of fresh dandelion organs can be consumed per day, and the recommended dosage of the crude dried leaves and roots is 4–10 g per day (Yarnell & Abascal, 2009). The doses administered by leaf and root tincture range from 9 to 15 mL per day, assuming three doses a day, and that type of consumption is especially popular in the United States. The British Herbal Pharmacopoeia (BPH) recommends 0.5–2 g of root (three times/day) or 4–8 mL of root tincture (three times/day); however, the recommended dosage of dandelion leaf is 3–5 g or 5–10 mL of leaf tincture (both 2 times/day). Moreover, in Germany, dandelion root with herb is licensed as a standard medicinal tea to treat biliary disorders, digestive and gastrointestinal complaints, and to stimulate diuresis. Dandelion herb and dandelion root with herb are approved in the Commission E monographs and recommended dosage is 3–4 g roots (two times/day) or 10–15 drops of tincture (three times/day). Furthermore, the recommended dosage of leaf is 4–10 g and 2–5 mL of tincture (both three times/day) (Wirngo et al., 2016; Yarnell & Abascal, 2009).

9. Conclusions

Growing interest in food, nutraceuticals, and medicinal products from plants and other natural sources that retain beneficial health properties has been observed in developed countries. Dandelion can be used as a nutraceutical, a food product that combines nutritional and healing value, which can also be used as dietary antioxidant prevent various disorders associated with oxidative stress i.e. cardiovascular disorders, cancer and the inflammatory process. Undoubtedly, its healing properties may be attributable to the presence of a variety of nutrients, including vitamins, minerals, fatty acids and a wide range of other chemical substances. Both *in vitro* and especially animal *in vivo* studies on dandelion have found a range of bioactive chemicals in its leaves, roots, flowers and stem; these compounds exhibit a wide range of biological activities.

However, the relationship between the chemical content and the biological properties of dandelion and its food products are not always clear, especially in human models, and further experiments are required to determine the prophylactic and the therapeutic doses of various dandelion products for use in future clinical studies. Moreover, the easy availability of raw material in particular encourages future studies and the search for new possibilities for the use of dandelion in Medicine, especially in an era characterised by an intensified search for new, natural pharmaceuticals.

Ethics statement

Our article is a review and did not include any human subjects and animal experiments.

Declaration of Competing Interest

The author declares no conflict of interest.

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Oświadczenia współautorów publikacji wchodzących w skład rozprawy doktorskiej

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(miejsce, data)

Oświadczenie

Oświadczam, że w pracy **Lis B.**, Jędrejek D., Stochmal A., Olas B. (2018) Assessment of effects of phenolic fractions from leaves and petals of dandelion in selected components of hemostasis, Food Research International 107, 605 – 612 mój udział wynosił 60% i polegał na realizacji części doświadczalnej (oznaczenie parametrów stresu oksydacyjnego tj. peroksydacji lipidów w płytkach krwi metodą z kwasem tiobarbiturowym, grup tiolowych i grup karbonylowych w białkach płytek krwi metodą kolorymetryczną, zawartości anionorodnika ponadtlenkowego w płytkach krwi metodą redukcji cytochromu c; oznaczenie parametrów hemostazy tj. czasów krzepnięcia metodą koagulometryczną, oznaczenie parametrów cytotoksyczności metodą Wróblewskiego i La Due), opracowaniu wyników, analizie statystycznej, wykonaniu rycin i przygotowaniu części manuskryptu.

Oświadczam, że w pracy Jędrejek D., **Lis B.**, Rolnik A., Stochmal A., Olas B. (2019) Comparative phytochemical, cytotoxicity, antioxidant and haemostatic studies of *Taraxacum officinale* root preparations, Food and Chemical Toxicology 126, 233 – 247 mój udział wynosił 32% i polegał na realizacji części doświadczalnej (oznaczenie parametrów stresu oksydacyjnego tj. peroksydacji lipidów w osoczu metodą z kwasem tiobarbiturowym, grup tiolowych i grup karbonylowych w białkach osocza metodą kolorymetryczną; oznaczenie parametrów hemostazy tj. agregacji metodą turbidymetryczną; oznaczenie parametrów cytotoksyczności metodą Wróblewskiego i La Due) opracowaniu wyników, wykonaniu rycin i przygotowaniu części manuskryptu.

Oświadczam, że w pracy **Lis B.**, Rolnik A., Jędrejek D., Soluch A., Stochmal A., Olas B. (2019) Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 52% i polegał na realizacji części doświadczalnej (oznaczenie parametrów stresu oksydacyjnego tj. peroksydacji lipidów w płytkach krwi metodą z kwasem tiobarbiturowym, grup tiolowych i grup karbonylowych w białkach płytek krwi metodą kolorymetryczną, zawartości anionorodnika ponadtlenkowego w płytkach krwi metodą redukcji cytochromu c), opracowaniu wyników, analizie statystycznej, wykonaniu rycin i przygotowaniu części manuskryptu.

Oświadczam, że w pracy **Lis B.**, Jędrejek D., Mołdoch J., Stochmal A., Olas B. (2019) The anti-oxidative and hemostasis-related multifunctionality of L-chicoric acid, the main component of dandelion: an *in vitro* study of its cellular safety, antioxidant and anti-platelet properties, and effect on coagulation, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 57% i polegał na realizacji części doświadczalnej (oznaczenie parametrów stresu oksydacyjnego tj. peroksydacji lipidów w osoczu i płytkach krwi metodą z kwasem tiobarbiturowym, grup tiolowych i grup karbonylowych w białkach osocza i płytek krwi metodą kolorymetryczną; oznaczenie parametrów hemostazy tj. agregacji metodą turbidymetryczną,

adhezji metodą statyczną, czasów krzepnięcia metodą koagulometryczną; oznaczenie parametrów cytotoksyczności metodą Wróblewskiego i La Due), opracowaniu wyników, analizie statystycznej, wykonaniu rycin i przygotowaniu części manuskryptu.

Oświadczam, że w pracy **Lis B.**, Olas B. (2019) Pro-health activity of dandelion (*Taraxacum officinale* L.*)* and its food products - history and present, Journal of Functional Foods 59, 40-48 mój udział wynosił 50% i polegał na przygotowaniu całego manuskryptu oraz wykonaniu rycin i tabel.

Oświadczam, że w pracy **Lis B.**, Jędrejek D., Rywaniak J., Soluch A., Stochmal A., Olas B. (2020) Flavonoid preparations from *Taraxacum offcinale* L. fruits – a phytochemical, antioxidant and hemostasis studies" *Molecules* 25, 1 – 33 mój udział wynosił 52% i polegał na realizacji części doświadczalnej (oznaczenie parametrów stresu oksydacyjnego tj. peroksydacji lipidów w osoczu i płytkach krwi metodą z kwasem tiobarbiturowym, grup tiolowych i grup karbonylowych w białkach osocza i płytek krwi metodą kolorymetryczną; oznaczenie parametrów hemostazy tj. czasów krzepnięcia metodą koagulometryczną adhezji metodą statyczną, oznaczenie powstających skrzeplin w warunkach przepływu krwi z wykorzystaniem techniki microchipów, oznaczenie ekspresji selektyny P i receptora GPIIb/IIIa na powierzchni płytek krwi metodą cytometrii przepływowej; oznaczenie parametrów cytotoksyczności metodą Wróblewskiego i La Due), opracowaniu wyników, analizie statystycznej, wykonaniu rycin i przygotowaniu części manuskryptu.

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.....

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Oświadczenie

Oświadczam, że w pracy Lis B., Jędrejek D., Stochmal A., **Olas B.** (2018) Assessment of effects of phenolic fractions from leaves and petals of dandelion in selected components of hemostasis, Food Research International 107, 605 – 612 mój udział wynosił 15% i obejmował współudział w stworzeniu koncepcji i projektowaniu badań, pomoc w redakcji manuskryptu oraz merytoryczne konsultacje.

Oświadczam, że w pracy Jędrejek D., Lis B., Rolnik A., Stochmal A., **Olas B.** (2019) Comparative phytochemical, cytotoxicity, antioxidant and haemostatic studies of *Taraxacum officinale* root preparations, Food and Chemical Toxicology 126, 233 – 247 mój udział wynosił 10% i obejmował współudział w stworzeniu koncepcji i projektowaniu badań, pomoc w redakcji manuskryptu oraz merytoryczne konsultacje.

Oświadczam, że w pracy Lis B., Rolnik A., Jędrejek D., Soluch A., Stochmal A., **Olas B.** (2019) Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 10% i obejmował współudział w stworzeniu koncepcji i projektowaniu badań, pomoc w redakcji manuskryptu oraz merytoryczne konsultacje.

Oświadczam, że w pracy Lis B., Jędrejek D., Mołdoch J., Stochmal A., **Olas B.** (2019) The anti-oxidative and hemostasis-related multifunctionality of L-chicoric acid, the main component of dandelion: an *in vitro* study of its cellular safety, antioxidant and anti-platelet properties, and effect on coagulation, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 10% i obejmował współudział w stworzeniu koncepcji i projektowaniu badań, pomoc w redakcji manuskryptu oraz merytoryczne konsultacje.

Oświadczam, że w pracy Lis B., **Olas B.** (2019) Pro-health activity of dandelion (*Taraxacum officinale* L.) and its food products - history and present, Journal of Functional Foods 59, 40-48 mój udział wynosił 50% i obejmował współudział w stworzeniu koncepcji oraz ocenę merytoryczną manuskryptu.

Oświadczam, że w pracy Lis B., Jędrejek D., Rywaniak J., Soluch A., Stochmal A., **Olas B.** (2020) Flavonoid preparations from *Taraxacum offcinale* L. fruits – a phytochemical, antioxidant and hemostasis studies" *Molecules* 25, 1 – 33 mój udział wynosił 10% i obejmował współudział w stworzeniu koncepcji i projektowaniu badań, pomoc w redakcji manuskryptu oraz merytoryczne konsultacje.

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Oświadczenie

Oświadczam, że w pracy Lis B., **Jędrejek D.**, Stochmal A., Olas B. (2018) Assessment of effects of phenolic fractions from leaves and petals of dandelion in selected components of hemostasis, Food Research International 107, 605 – 612 mój udział wynosił 20% i obejmował współudział w redakcji i korekcie manuskryptu.

Oświadczam, że w pracy **Jędrejek D.**, Lis B., Rolnik A., Stochmal A., Olas B. (2019) Comparative phytochemical, cytotoxicity, antioxidant and haemostatic studies of *Taraxacum officinale* root preparations, Food and Chemical Toxicology 126, 233 – 247 mój udział wynosił 40% i obejmował współudział w redakcji i korekcie manuskryptu, metodyce, analizie statystycznej wyników oraz przygotowaniu preparatów z korzeni mniszka pospolitego do badań.

Oświadczam, że w pracy Lis B., Rolnik A., **Jędrejek D.**, Soluch A., Stochmal A., Olas B. (2019) Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 25% i obejmował współudział w redakcji i korekcie manuskryptu.

Oświadczam, że w pracy Lis B., **Jędrejek D.**, Mołdoch J., Stochmal A., Olas B. (2019) The anti-oxidative and hemostasis-related multifunctionality of L-chicoric acid, the main component of dandelion: an *in vitro* study of its cellular safety, antioxidant and anti-platelet properties, and effect on coagulation, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 25% i obejmował współudział w redakcji i korekcie manuskryptu, metodyce i przygotowaniu preparatu (kwasu cykoriowego) do badań.

Oświadczam, że w pracy Lis B., **Jędrejek D.**, Rywaniak J., Soluch A., Stochmal A., Olas B. (2020) Flavonoid preparations from *Taraxacum offcinale* L. fruits – a phytochemical, antioxidant and hemostasis studies" *Molecules* 25, 1 – 33 mój udział wynosił 25% i obejmował współudział w redakcji i korekcie manuskryptu, metodyce, przygotowaniu i analizie chemicznej preparatów z owoców mniszka pospolitego.

Dariusz Jędrejek
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Katedra Biochemii Ogólnej

Łódź 1.07.2021
(miejsce, data)

Oświadczenie

Oświadczam, że w pracy Jędrejek D., Lis B., **Rolnik A.**, Stochmal A., Olas B. (2019) Comparative phytochemical, cytotoxicity, antioxidant and haemostatic studies of *Taraxacum officinale* root preparations, Food and Chemical Toxicology 126, 233 – 247 mój udział wynosił 15% i obejmował współudział w wykonywaniu eksperymentów (czasy krzepnięcia) oraz opracowanie wyników.

Oświadczam, że w pracy Lis B., **Rolnik A.**, Jędrejek D., Soluch A., Stochmal A., Olas B. (2019) Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 5% i obejmował współudział w wykonywaniu eksperymentów (adhezji i agregacji) oraz opracowanie wyników.

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Puławy 09.07.2021
(miejsce, data)

Oświadczenie

Oświadczam, że w pracy Lis B., Jędrejek D., **Mołdoch J.**, Stochmal A., Olas B. (2019) The anti-oxidative and hemostasis-related multifunctionality of L-chicoric acid, the main component of dandelion: an *in vitro* study of its cellular safety, antioxidant and anti-platelet properties, and effect on coagulation, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 5% i obejmował współudział w przygotowaniu preparatu (kwasu cykoriowego) do badań.

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Państwowy Instytut Badawczy
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Puławy, 2.07.2021r.
(miejsce, data)

Oświadczenie

Oświadczam, że w pracy Lis B., Rolnik A., Jędrejek D., **Soluch A.**, Stochmal A., Olas B. (2019) Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 5% i obejmował współudział w analizie chemicznej preparatów z korzeni mniszka pospolitego.

Oświadczam, że w pracy Lis B., Jędrejek D., Rywaniak J., **Soluch A.**, Stochmal A., Olas B. (2020) Flavonoid preparations from *Taraxacum offcinale* L. fruits – a phytochemical, antioxidant and hemostasis studies" *Molecules* 25, 1 – 33 mój udział wynosił 5% i obejmował współudział w przygotowaniu preparatów z owoców mniszka pospolitego do badań.

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Katedra Immunologii i Biologii Infekcyjnej

Łódź, 29.06.21r.
(miejsce, data)

Oświadczenie

Oświadczam, że w pracy Lis B., Jędrejek D., **Rywaniak J.**, Soluch A., Stochmal A., Olas B. (2020) Flavonoid preparations from *Taraxacum offcinale* L. fruits – a phytochemical, antioxidant and hemostasis studies" *Molecules* 25, 1 – 33 mój udział wynosił 5% i obejmował współudział w metodologii i opracowaniu wyników cytometrycznych.

J. Rywaniak
(podpis)

prof. dr hab. Anna Stochmal
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Państwowy Instytut Badawczy
Zakład Biochemii i Jakości Plonów

Puławy, 2.07.21r.
(miejsce, data)

Oświadczenie

Oświadczam, że w pracy Lis B., Jędrejek D., **Stochmal A.**, Olas B. (2018) Assessment of effects of phenolic fractions from leaves and petals of dandelion in selected components of hemostasis, Food Research International 107, 605 – 612 mój udział wynosił 3% i obejmował ocenę merytoryczną manuskryptu.

Oświadczam, że w pracy Jędrejek D., Lis B., Rolnik A., **Stochmal A.**, Olas B. (2019) Comparative phytochemical, cytotoxicity, antioxidant and haemostatic studies of *Taraxacum officinale* root preparations, Food and Chemical Toxicology 126, 233 – 247 mój udział wynosił 3% ocenę merytoryczną manuskryptu.

Oświadczam, że w pracy Lis B., Rolnik A., Jędrejek D., Soluch A., **Stochmal A.**, Olas B. (2019) Dandelion (*Taraxacum officinale* L.) root components exhibit anti-oxidative and antiplatelet action in an *in vitro* study, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 3% obejmował ocenę merytoryczną manuskryptu.

Oświadczam, że w pracy Lis B., Jędrejek D., Mołdoch J., **Stochmal A.**, Olas B. (2019) The anti-oxidative and hemostasis-related multifunctionality of L-chicoric acid, the main component of dandelion: an *in vitro* study of its cellular safety, antioxidant and anti-platelet properties, and effect on coagulation, Journal of Functional Foods 59, 16 – 24 mój udział wynosił 3% i obejmował ocenę merytoryczną manuskryptu.

Oświadczam, że w pracy Lis B., Jędrejek D., Rywaniak J., Soluch A., **Stochmal A.**, Olas B. (2020) Flavonoid preparations from *Taraxacum offcinale* L. fruits – a phytochemical, antioxidant and hemostasis studies" *Molecules* 25, 1 – 33 mój udział wynosił 3% i obejmował ocenę merytoryczną manuskryptu.

(podpis)