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Rozprawa doktorska

Badanie interakcji efektorów peroksydazy tyroidowej oraz wybranych enzymów prooksydacyjnych w układach modelowych

Study of interactions between effectors of thyroid peroxidase and selected prooxidative enzymes in model systems

Rozprawa doktorska wykonana w Katedrze Biochemii i Chemii Żywności

Promotor: prof. dr hab. Urszula Gawlik – Dziki

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Spis treści

1. Streszczenie

Celem rozprawy doktorskiej była ocena wpływu polifenoli występujących w żywności pochodzenia roślinnego na aktywność peroksydazy tyroidowej (TPO) oraz określenie, które z efektorów TPO spełniają również warunek inhibicji enzymów prooksydacyjnych: lipooksygenazy (LOX) i oksydazy ksantynowej (XO). Badania tego typu miały pozwolić na potencjalny dobór składników żywności wspomagających terapię oraz prewencję w przypadku nadczynności i niedoczynności tarczycy.

Pierwszym etapem było wyselekcjonowanie czystych związków o pożądanej, skoordynowanej aktywności biologicznej. Badania obejmowały określenie potencjału antyoksydacyjnego, zdolności do modulowania aktywności TPO oraz inhibicji enzymów prooksydacyjnych (LOX, XO). Na podstawie uzyskanych wyników zostały wybrane surowce pochodzenia roślinnego będące biologicznym źródłem poszukiwanych związków aktywnych.

Ponieważ jednym z kluczowych czynników determinujących bioaktywność produktów żywnościowych są interakcje związków czynnych z matrycą żywności, przeprowadzone zostały badania rodzaju i siły interakcji pomiędzy czystymi związkami, a także pomiędzy ekstraktami sporządzonymi z roślinnych produktów spożywczych, w których znajdują się wyselekcjonowane w toku badań substancje aktywne. O wpływie oddziaływań ze składnikami matrycy żywności na potencjał odżywczy i prozdrowotny zaproponowanych produktów można było wnioskować bazując na wynikach uzyskanych z wykorzystaniem modelu ludzkiego przewodu pokarmowego.

Innowacyjność rozprawy doktorskiej polega na kompleksowym ujęciu równoczesnego (skoordynowanego) wpływu składników fitochemicznych wchodzących w skład surowców roślinnych na aktywność peroksydazy tarczycowej (TPO) oraz enzymów prooksydacyjnych (LOX, XO). Szczegółowe badania pozwoliły na uzyskanie obrazu potencjalnego wpływu składników pożywienia na profilaktykę schorzeń związanych z zaburzeniami funkcjonowania tarczycy. Pozwoliło to na dobór takich, które wykazywały działanie przeciwzapalne oraz w ukierunkowany sposób modulowały aktywność peroksydazy tyroidowej. Wiedza ta może być przydatna przy doborze odpowiedniej diety w zależności od występującej dysfunkcji tarczycy (nadczynność lubniedoczynność). Rozprawa doktorska obejmuje również analizę interakcji składników aktywnych, co było przydatne w ocenie finalnej aktywności mieszanek.

Podjęte badania wypełniły istotną lukę w aktualnej wiedzy, tym bardziej, że wiele publikacji dedykowanych osobom z zaburzeniami funkcjonowania tarczycy zawiera informacje wzajemnie się wykluczające. Wielu autorów sugerowało spożywanie pokarmów, które przez innych były odradzane. Zebrane informacje mogą stanowić cenny wkład w doborze odpowiedniej diety dla pacjentów z dysfunkcjami tarczycy.

Słowa kluczowe: peroksydaza tyroidowa, stres oksydacyjny, interakcje.

Summary

The aim of the doctoral dissertation was to evaluate the influence of plant-derived food polyphenols on the activity of thyroid peroxidase (TPO) and to determine which TPO effectors also meet the criteria for inhibiting prooxidative enzymes: lipoxygenase (LOX) and xanthine oxidase (XO). Such studies aimed to facilitate the potential selection of food components that could support therapy and prevention in cases of thyroid hyperfunction and hypofunction.

The first stage involved the selection of pure compounds with desired and coordinated biological activity. The studies encompassed the determination of antioxidant potential, the ability to modulate TPO activity, and the inhibition of prooxidative enzymes (LOX, XO). Based on the obtained results, plant-derived sources containing active compounds were selected.

Considering that one of the key factors determining the bioactivity of food products is the interaction between active compounds and the food matrix, investigations were conducted on the type and strength of interactions between pure compounds, as well as between extracts prepared from plant food products containing the selected active substances. The impact of these interactions with food matrix components on the nutritional and health potential of the proposed products could be inferred from the results obtained using a human gastrointestinal model.

The innovation of the doctoral dissertation lies in the comprehensive approach to the simultaneous (coordinated) impact of phytochemical components present in plant materials on the activity of thyroid peroxidase (TPO) and prooxidative enzymes (LOX, XO). Detailed studies provided insights into the potential influence of food components on the prevention of disorders related to thyroid dysfunction, exhibiting anti-inflammatory properties, and selectively modulating thyroid peroxidase activity. This knowledge can be valuable in selecting an appropriate diet based on the specific thyroid dysfunction present (hyperfunction or hypofunction). The doctoral dissertation also includes an analysis of interactions between active components, which was useful in assessing the final activity of mixtures.

The conducted research fills a significant gap in current knowledge, especially considering that many publications dedicated to individuals with thyroid disorders contain mutually exclusive information. Numerous authors have suggested consuming foods that

others have discouraged. Obtained results can provide a valuable contribution to the selection of an appropriate diet for patients with thyroid dysfunctions.
Keywords: thyroid peroxidase, oxidative stress, interactions.

2. Wykaz publikacji wchodzących w skład rozprawy doktorskiej

Publikacja I

Habza–Kowalska E., Kaczor A. A., Żuk J., Matosiuk D., Gawlik–Dziki U., 2019, Thyroid Peroxidase Activity is Inhibited by Phenolic Compounds—Impact of Interaction, Molecules, 24(15):2766

Punkty MEiN: 100 IF (2019): 3,267 Liczba cytowań wg Web of Science (11)/Scopus (11)

Udział własny – 50%

Publikacja II

Habza–Kowalska E., Gawlik–Dziki U., Dziki D., 2019, Mechanism of action and interactions between thyroid peroxidase and lipoxygenase inhibitorsderived from plant sources, Biomolecules, 9(11), 1-16.

Punkty MEiN: 100 IF (2019): 4,082 Liczba cytowań wg Web of Science (6)/Scopus (6)

Udział własny – 60%

Publikacja III

Habza–Kowalska E., Kaczor A. A., Bartuzi D., Piłat J., Gawlik–Dziki U., 2021, Some Dietary Phenolic Compounds Can Activate Thyroid Peroxidase and Inhibit Lipoxygenase-Preliminary Study in the Model Systems, International Journal of Molecular Sciences, 22(10), 5108.

Punkty MEiN: 140 IF (2019): 6,208 Liczba cytowań wg Web of Science (2)/Scopus (3)

Udział własny – 50%

Publikacja IV (preprint)

Habza–Kowalska E.,Piwowarczyk K., Czyż J., Gawlik–Dziki U., Oatmeal and wheat flour ase the sources of thyroid peroxidase (TPO), Lipoxygenase (LOX) and xantine oxidase (XO) modulators potentially applicable in the prevention of inflammatory thyroid diseases, BioRxiv, DOI: 10.1101/2023.06.05.543703

Udział własny – 60%

Sumaryczny IF publikacji wchodzących w skład dysertacji (zgodnie z rokiem opublikowania): 13,557

Sumaryczna liczba cytowań publikacji wchodzących w skład dysertacji wg Web of Science (19) oraz Scopus (20)

3. Wstęp

W czasach współczesnych zdrowie często jest określane przez pryzmat występowania tzw. przewlekłych chorób niezakaźnych, zwanych również chorobami cywilizacyjnymi. Są to choroby wynikające z nowoczesnego stylu życia, który charakteryzuje się stresem, zbyt małą ilością ruchu, nieodpowiednio zbilansowaną dietą dostarczającą organizmowi zbyt dużo kalorii, a także zanieczyszczeniem środowiska. Wspomniane czynniki sprzyjają powstawaniu reaktywnych form tlenu (RFT), czyli tzw. wolnych rodników, które stanowią zagrożenie dla organizmu człowieka poprzez swoje niekorzystne oddziaływanie na komórki i tkanki. Jednym z efektów może być rozwój przewlekłych stanów zapalnych oraz nadmiernej reaktywności układu odpornościowego. Warto zaznaczyć, że te czynniki często stanowią podstawę dla chorób tarczycy, zarówno w przypadku nadczynności, jak i niedoczynności. Kluczowe dla obu, sukcesu terapii i zapobiegania chorobom tarczycy, jest właściwy styl życia. Należy zwrócić szczególną uwagę na unikanie stresu oraz odpowiedni model żywieniowy. Dieta w chorobach tarczycy powinna mieć wysoki potencjał przeciwzapalny i przeciwutleniający.

Zapalenie tarczycy Hashimoto (HT) (czyli przewlekłe limfocytarne zapalenie tarczycy) to choroba autoimmunologiczna, w której układ odpornościowy atakuje i niszczy ten gruczoł. Wynikające z tego zapalenie często prowadzi do niedoczynności tarczycy. W krajach zachodnich choroba ta dotyka od 0,1% do 5% dorosłej populacji (Chen, Lin, Cheng, Sung, & Kao, 2013). Głównym antygenem w ludzkiej chorobie Hashimoto jest peroksydaza tyroidowa (TPO, EC 1.11.1.1-14), enzym biorący udział w syntezie hormonów tarczycy. Przeciwciała przeciwko TPO indukują cytotoksyczność zależną od dopełniacza. Ponadto, u pacjentów z chorobą Hashimoto wykrywane są przeciwciała przeciwko dopełniaczowi (przeciwko C1q), które korelują z poziomem hormonu tyreotropowego (TSH). W związku z tym, wiele przypadków wrodzonej niedoczynności tarczycy wiąże się z problemami związanymi z syntezą lub jodowaniem tyreoglobuliny (TG), co z kolei związane jest z niedoborem TPO (Carocho & Ferreira, 2013).

TPO, zwane także peroksydazą jodową, jest enzymem biorącym udział w syntezie hormonów tarczycy. W ludzkim organizmie jest kodowany przez gen TPO zlokalizowany na chromosomie 2p25 (Kimura et al., 1987). TPO katalizuje utlenianie jonów jodkowych, tworząc atomy jodu, które są dodawane do reszt tyrozyny w tyreoglobulinie, w celu produkcji tyroksyny lub trójjodotyroniny, czyli hormonów tarczycy (Ruf & Carayon, 2006). Opisany powyżej mechanizm może zostać przedstawiony przez reakcje zachodzące

w następującej kolejności: TPO jest utleniany przez nadtlenek wodoru (H_2O_2), a następnie TPO może utleniać jony jodkowe. Utlenione jony jodkowe łączą się z resztami tyrozyny w tyreoglobulinie (TG). Powstawanie tyroksyny (T4) i trójjodotyroniny (T3) jest efektem utleniania i sprzęgania hormonogennych jodotyrozyn (Carvalho & Dupuy, 2017). Ponieważ enzym TPO jest peroksydazą hemową, nie może utleniać substratu bez uprzedniego utlenienia. Aby utlenić TPO, potrzebna jest cząsteczka H_2O_2. Cząsteczka H_2O_2 jest generowana tylko na powierzchni apikalnej tyreocytów, a cząsteczki TPO obecne na tej powierzchni są aktywowane (Leonard, Tan, Gilbert, Isaacs, & El-masri, 2016). TPO jest zorganizowany jako homodimer o 933 resztach. N-końcowa propeptyda (reszty 1-108) jest usuwana w dojrzałym białku. Trzy domeny w zewnątrzkomórkowej części TPO (reszty 109-846) wykazują wysoki stopień podobieństwa sekwencji do domen o znanym trójwymiarowym układzie: domena podobna do mieloperoksydazy (MPO) (reszty 142-738), domena podobna do białka kontrolującego dopełniacz (CCP) (reszty 740-795) i domena podobna do czynnika wzrostu naskórka (EGF) (reszty 796-846) (Le et al., 2015). Domena transbłonowa składa się z reszt 847-871, a domena wewnątrzkomórkowa z reszt 872-933. Le i in. 2015 (Le et al., 2015) rozważał również alternatywną orientację *cis*, z cząsteczkami hemu zwróconymi w stronę błony komórkowej tarczycy. Zgodnie z powyższymi badaniami orientacja *cis* jest nieco bardziej energetycznie stabilna. Jednak inne dane sugerują, że w celu przeprowadzenia utleniania i dalszego jodowania tyroglobuliny, aktywna katalitycznie część enzymu musi wystawać do światła pęcherzyka tarczycy (Yokoyama & Taurog, 1988), co wymaga orientacji *trans* dimerycznej domeny podobnej do mieloperoksydazy (MPO).

Zaburzenia w funkcjonowaniu tarczycy prowadzą do licznych konsekwencji dla zdrowia, w tym stanów zapalnych oraz narażenia na stres oksydacyjny (OS) (Shahbaz et al., 2018).

Stan zapalny jest naturalnym mechanizmem obronnym związanym z wieloma chorobami wywoływanymi przez drobnoustroje patogenne, chorobami autoimmunologicznymi i przewlekłymi, narażeniem na alergeny, promieniowanie i toksyczne substancje chemiczne, otyłością, nadmiernym spożyciem alkoholu, paleniem tytoniu oraz wysokokaloryczną dietą. Wiele chorób związanych jest z wyższą produkcją RFT, co prowadzi do stresu oksydacyjnego. Główne enzymy, których aktywność powoduje stres oksydacyjny to lipooksygenaza (LOX), cyklooksygenaza (COX) oraz oksydaza ksantynowa (XO) (Zhang et al., 2015).

Istnieją różne możliwe mechanizmy działania przeciwzapalnego związków bioaktywnych, w tym hamowanie aktywności lipooksygenaz (LOXs EC 1.13.11.12), które katalizują utlenianie kwasów tłuszczowych o wielu wiązaniach podwójnych do potężnych cząsteczek sygnałowych biorących udział w procesach zapalnych (Chen et al., 2013). LOXs uczestniczą w syntezie eikozanoidów, takich jak prostaglandyny lub nieklasyczne eikozanoidy. Szlak LOX metabolizmu kwasu arachidonowego generuje RFT, które wraz z innymi metabolitami kwasu arachidonowego odgrywają rolę w zapaleniu i wzroście nowotworów. Aktywność LOX jest związana ze stresem oksydacyjnym w organizmie człowieka. Stres oksydacyjny odnosi się do stanu, w którym równowaga między systemem ochrony antyoksydacyjnej a produkcją RFT jest zaburzona. W literaturze opisano związki o potwierdzonej aktywności dualnego inhibitora 5-LOX/COX jako potencjalne leki do leczenia stanów zapalnych. Działają one poprzez blokowanie tworzenia zarówno prostaglandyn, jak i leukotrienów, ale nie wpływają na tworzenie lipoksyny (Martel-Pelletier, Lajeunesse, Reboul, & Pelletier, 2003).

Oksydaza ksantynowa (XO, EC 1.17.3.2) to enzym ułatwiający produkcję kwasu moczowego i rodników ponadtlenkowych z zasad purynowych hipoksantyny i ksantyny. XO jest również obecna w komórkach nabłonka oddechowego, a jej aktywność może prowadzić do przekształcenia rodników ponadtlenkowych w rodniki hydroksylowe. Ten proces może dalej zaostrzać odpowiedź zapalną i przyczynić się do rozwoju hiperzapalenia, które powszechnie znane jest jako zespół burzy cytokinowej (CSS) (Pratomo et al., 2021).

W przypadku wzmożonej aktywności XO oraz występującego często w chorobach naczyniowych stresu oksydacyjnego, organizm może odpowiedzieć poprzez wzrost stężenia kwasu moczowego (Nieto, Iribarren, Gross, Comstock, & Cutler, 2000). Skutkiem nadaktywności oksydazy ksantynowej jest stan znany jako dna moczanowa oraz ostre zapalenie stawów (Yang, Choi, Chen, & Dionysiou, 2008). Istnieją badania naukowe sugerujące związek między stężeniem kwasu moczowego a występowaniem niektórych chorób przewlekłych, takich jak nadciśnienie tętnicze czy cukrzyca (Kivity et al., 2013). Aby skutecznie leczyć wymienione choroby, należy wpłynąć na homeostazę kwasu moczowego poprzez zwiększenie jego wydalania lub redukcję produkcji. W tym celu stosuje się inhibitory oksydazy ksantynowej. Redukują one naczyniowy stres oksydacyjny oraz stężenie kwasu moczowego. Badania naukowe łączą aktywność XO z różnymi formami niedokrwienia oraz innymi rodzajami urazów tkanek i naczyń oraz przewlekłą niewydolnością serca. Inhibitor oksydazy ksantynowej - allopurinol wykazał skuteczność

w leczeniu tych stanów w badaniach eksperymentalnych na zwierzętach oraz w niewielkich badaniach klinicznych na ludziach (Umamaheswari et al., 2007). Badania wykorzystujące ekstrakty roślinne do hamowania aktywności XO przeprowadzone przez Gawlik-Dziki (2012) (Gawlik-Dziki, 2012) potwierdzają, że roślinne formuły mogą stanowić skuteczną alternatywę dla leków syntetycznych. Należy zwrócić uwagę na fakt, że preparaty roślinne, w przeciwieństwie do syntetycznych leków, nie wywołują negatywnych skutków ubocznych dla organizmu.

Stres oksydacyjny wynika przede wszystkim z nadmiernej produkcji RFT, które nie są usuwane przez naturalne mechanizmy naprawcze i mogą być wspomagane przez dostarczanie organizmowi substancji o aktywności przeciwutleniającej, charakterystycznej dla związków fitochemicznych. Grupa metabolitów wtórnych roślin o udokumentowanej aktywności antyoksydacyjnej to związki fenolowe. Ich skuteczność jako przeciwutleniaczy wynika z wielokierunkowej aktywności: mogą hamować reakcje wolnorodnikowe poprzez zablokowanie tworzenia się wolnych rodników lipidowych, zakłócanie propagacji łańcuchowych reakcji autooksydacji, tłumienie singletowego tlenu, mogą działać jako czynniki redukujące nadtlenki wodoru do stabilnych związków, jako związki chelatujące jony metali przejściowych oraz jako inhibitory prooksydacyjnych enzymów (Carocho & Ferreira, 2013). Uważa się zatem, że dieta bogata w produkty pochodzenia roślinnego stanowi ważny element zapobiegania chorobom cywilizacyjnym. Wiele badań epidemiologicznych wykazało, że spożycie żywności i napojów o wysokiej zawartości związków fenolowych wiąże się z zapobieganiem chorobie wieńcowej, nowotworom itp. (Michael G. L. Hertog, Daan Kromhout, Christ Aravanis, 2015; Scalbert & Williamson, 2000).

Aby polifenole wykazywały swoje biologiczne właściwości, konieczne jest, by były one w wystarczającym stopniu dostępne w docelowej tkance. Dlatego właściwości biologiczne polifenoli zawartych żywności pochodzenia roślinnego mogą zależeć od ich biodostępności i bioprzyswajalności. Ilość bioaktywnych polifenoli w pożywieniu może różnić się ilościowo i jakościowo od polifenoli zawartych w bazach danych żywnościowych. Ponadto większość badań dotyczących biodostępności polifenoli wykorzystuje głównie pojedyncze czyste molekuły (izolowane z żywności lub syntetyzowane chemicznie), chociaż ich biodostępność z całych produktów spożywczych może być różna (Saura-Calixto, Serrano, & Goñi, 2007). Aktywność związków fenolowych badanych *in vitro* (po ich izolacji z żywności) nie musi być zgodna z aktywnością wykazaną u człowieka. Proste, tanie i powtarzalne narzędzia do badania

biodostępności składników żywności to modele *in vitro* oparte na fizjologii człowieka. Są one szeroko stosowane do badania zmian strukturalnych, strawności i uwalniania składników żywności w symulowanych warunkach przewodu pokarmowego (Oomen et al., 2002). W przeciwieństwie do syntetycznych leków, opartych na pojedynczych substancjach chemicznych, wiele fitoleków wywiera swoje korzystne działanie przez dodatkowe lub synergistyczne działanie kilku związków chemicznych działających na pojedyncze lub wiele miejsc docelowych związanych z procesem fizjologicznym (Williamson, 2001). Jednak, mimo że synergizm związków bioaktywnych odgrywa znaczącą rolę, jest niewiele badań dotyczących tego zagadnienia w tak skomplikowanym systemie jakim jest żywność. Ta idea znalazła zastosowanie w farmakologii podczas badań nad kombinacjami kilku metabolitów w terapii wielokierunkowej (Gawlik-Dziki, 2012).

Jak już wspomniano, czynnik, który istotnie wpływa na końcowy efekt związków biologicznie aktywnych, zwłaszcza pochodzących z systemów żywnościowych, to ich interakcje. Zatem mogą one istotnie modyfikować ich działanie jako efektorów TPO, LOX i XO. Metoda wykorzystywana do identyfikacji interakcji między związkami aktywnymi to analiza izobolograficzna. Metoda ta jest niezależna od mechanizmu działania. Analiza izobolograficzna jest użytecznym narzędziem do określania interakcji między składnikami dwuskładnikowych mieszanek, jak również tych składających się z ekstraktów roślinnych będących mieszaniną licznych związków aktywnych (w przypadku liniowej zależności między aktywnością a stężeniem próbki umożliwiającej określenie wartości IC_{50}). Analiza izobolograficzna to naukowe podejście, które graficznie przedstawia interakcje antyoksydacyjne, ułatwiając ich wizualną ocenę. Pozwala na zwięzłe i klarowne przedstawienie interakcji kombinacji próbek i została szeroko uznana jako złoty standard do testowania interakcji farmakologicznych z różnymi kombinacjami ustalonych frakcji (Chou, 2006). Stąd też istotne jest oszacowanie, czy i w jaki sposób związki polifenolowe, zdolne do hamowania aktywności enzymów prozapalnych i prooksydacyjnych oraz wykazujące potencjał antyoksydacyjny modyfikują aktywność TPO.

Rozprawa doktorska koncentruje się na badaniu wpływu polifenoli zawartych w żywności pochodzenia roślinnego na aktywność TPO, LOX i XO oraz zdolności do zapobiegania stresowi oksydacyjnemu. Zebrane informacje mogą stanowić cenny wkład w dobieraniu odpowiedniej diety dla pacjentów z dysfunkcjami tarczycy.

4. Hipotezy badawcze i cel pracy

Na podstawie analizy danych literaturowych oraz badań wstępnych postawiono następujące hipotezy badawcze:

- Związki fenolowe, będące głównymi metabolitami wtórnymi roślin, są efektorami peroksydazy tyroidowej. Dodatkowo wywierany przez nie efekt może być jednocześnie skoordynowany z wpływem na enzymy związane z występowaniem stanów zapalnych oraz na stres oksydacyjny towarzyszący dysfunkcjom tarczycy.
- W przypadku żywności pochodzenia roślinnego ostateczny efekt zależy od interakcji między poszczególnymi związkami fenolowymi oraz między nimi a składnikami matrycy żywności. Ponadto, może być on determinowany przez zmiany zachodzące podczas trawienia (aktywność enzymów i warunki pH).

Celem pracy było określenie wpływu wybranych związków fenolowych, powszechnie występujących w żywności pochodzenia roślinnego, na aktywność TPO skoordynowaną z aktywnością inhibitorową skierowaną przeciwko enzymom prozapalnym i prooksydacyjnym (LOX i XO) oraz zdolnością przeciwrodnikową. Kolejnym aspektem badań było określenie rodzaju i siły interakcji pomiędzy związkami czynnymi oraz wpływu matrycy żywności i przemian podczas procesu trawienia *in vitro* na oczekiwaną, wielokierunkową aktywność badanych związków.

Cele szczegółowe:

1. Określenie wpływu i mechanizmu działania czystych związków fenolowych (wzorców) na aktywność peroksydazy tyroidowej oraz wybranych enzymów prozapalnych i prooksydacyjnych (etap I).
2. Określenie wpływu interakcji pomiędzy efektorami badanych enzymów na skuteczność i mechanizm działania przy zastosowaniu analizy izobolograficznej (etap II).
3. Określenie wpływu matrycy żywności i przemian zachodzących podczas trawienia *in vitro* na aktywność związków (wybranych w etapie I) zawartych w żywności pochodzenia roślinnego (etap III).
5. Określenie wpływu interakcji pomiędzy ekstraktami zawierającymi efektory badanych enzymów na skuteczność i mechanizm działania przy zastosowaniu analizy izobolograficznej (etap IV).

5. Etapy badań

1. Wirtualne przeszukiwanie. Baza danych naturalnych polifenoli (http://phenol-explorer.eu) została przetestowana w oparciu o farmakofory. Na tej podstawie wybrano najbardziej obiecujące związki do dalszych badań. Po weryfikacji *in vitro* przeprowadzono dokowanie efektorów do TPO i LOX. Hipotetyczne miejsca wiązania polifenoli do białka enzymu zostały zidentyfikowane poprzez analizę modelu homologicznego białka przy użyciu serwerów PASS i AlloSite. Następnie, miejsca sugerowane przez wspomniane algorytmy zostały zdefiniowane jako miejsce docelowe dla dokowania w programie Surflex (część pakietu SybylX). Ligandy zostały dokowane do potencjalnych kieszeni wiążących. Najlepsze lokalizacje zostały wybrane jako cel wirtualnego przesiewu w programie LigandScout 4.3.
2. Analiza *in vitro* oddziaływań związków wyselekcjonowanych podczas badan *in silico* na TPO. Wybór aktywatorów i inhibitorów TPO. Określenie mechanizmu hamowania (analiza metodą Lineweavera-Burka, L-B). W przypadku występowania liniowej zależności aktywności od stężenia wyznaczenie wartości $EC_{50.}$
3. Określenie interakcji między efektorami TPO przy zastosowaniu analizy izobolograficznej.
4. Analiza *in vitro* oddziaływań na enzymy prooksydacyjne (LOX i XO). Określenie mechanizmu hamowania (analiza Lineweavera-Burka, L-B). W przypadku występowania liniowej zależności aktywności od stężenia wyznaczenie wartości $EC_{50.}$
5. Określenie rodzaju i siły interakcji między efektorami LOX i XO przy zastosowaniu analizy izobolograficznej.
6. Wybór żywności pochodzenia roślinnego będącej zasobnym źródłem związków wyselekcjonowanych w trakcie wcześniejszych badań (punkty 1-5)
7. Określenie wpływu na TPO, LOX i XO ekstraktów kontrolnych (otrzymanych przy zastosowaniu 50% etanolu) oraz ekstraktów otrzymanych po trawieniu *in vitro* (w celu określenia wpływu matrycy żywności i warunków panujących podczas trawienia na aktywność zawartych w nich związków czynnych).
8. Określenie rodzaju i siły interakcji między ekstraktami zawierającymi efektory badanych enzymów przy zastosowaniu analizy izobolograficznej.
9. Analiza *in vitro* potencjału przeciwnowotworowego wybranych pokarmów roślinnych na przykładzie płatków owsianych i mąki pszennej.

6. Materiały i metody

6.1. Materiał

Tarczyce wieprzowe zostały pozyskane z lokalnej rzeźni (Lublin) i przechowywane w temperaturze −20°C do momentu użycia. Płatki owsiane (Plony Natury, Polska), natka pietruszki, zielona kawa (Targroch, Polska), zielona herbata (Herbapol, Polska), kminek (Prymat, Polska), gorczyca (Prymat, Polska) zostały zakupione w lokalnym supermarkecie w Lublinie. Mąka pszenna typ 650 (z pszenicy cv. Batuta) została zakupiona w lokalnym młynie (Lublin).

6.2. Odczynniki chemiczne

Sacharoza (α-d-glukopiranozylo-(1→4)-β-d-fruktofuranozyd), Tris (1,3-propanodiol-2-amino-2-hydroksymetylo), KCl, NaCl, $MgCl_2$, etanol 90%, NaOH, gwajakol (2-metoksyfenol), H_2O_2 (nadtlenek wodoru), odczynnik Bradforda, ABTS (2,20-azinobis-(3-etylobenzotiazolin-6-sulfonian), kwercetyna, kwas rozmarynowy, kwas chlorogenowy, rutyna i Trolox (6-hydroksy-2,5,7,8-tetrametylochroman-2-karboksylowy), lipoksygenaza (LOX), oksydaza ksantynowa (XO), ksantyna, pankreatyna, pepsyna, wyciąg żółciowy, kwas linolowy, α-amylaza, kwas synapinowy, apigenina, katechina, kempferol, kwas trans-cynamonowy, kwas syryngowy, kwas ferulowy bufor fosforanowo-wodorowęglanowy o pH 7,2 (PBS), EDTA, ludzkie komórki raka tarczycy B-CPAP (ACC 273, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) oraz 8505C,środowisko RPMI 1640 i DMEM/F12 HAM, roztwór trypsyny/EDTA/PBS bez obecności $Ca^{2+/}Mg^{2+}$, zostały zakupione w firmie Sigma-Aldrich (Poznań, Polska)., 10% termicznie inaktywowana surowica krwi płodowej została zakupiona w firmie Thermo Fisher Scientific (Warszawa, Polska), 1% roztwór antybiotykowo-antymykotycznego zakupiono w firmie Merck (Warszawa, Polska). Wszystkie pozostałe odczynniki były w klasie czystości laboratoryjnej.

6.3. Metodologia badań *in silico*:

Ligandy zostały przygotowane do dokowania przy użyciu narzędzia LigPrep z pakietu Schrödinger. Dokowanie molekularne zostało przeprowadzone za pomocą narzędzia Glide z pakietu Schrödinger lub Surflex wchodzącego w skład Sybyl-X. Wirtualne przeszukiwanie oparte zostało na dokowaniu za pomocą Glide oraz na

farmakoforach przy użyciu narzędzia Ligand Scout. Wstępne badania przeprowadzono w celu wyszukiwania efektorów TPO, LOX i XO. Metodologia badan *in silico* została szczegółowo opisana w **pracach I i III**.

6.4. Wybór surowców roślinnych bogatych w wyselekcjonowane polifenole

Wyboru surowców dokonano wykorzystując bazę Phenol Explorer 9 (http://phenol-explorer.eu). uwzględniając zawartość polifenoli, częstotliwość spożycia oraz dostępność produktu na rynku.

6.5. Przygotowanie prób do badań

W przypadku związków wzorcowych przygotowano roztwory w 50% etanolu. Szczegółowa metodyka została opisana w **pracach I, II i III.**

W przypadku surowców roślinnych przygotowano dwa rodzaje prób – kontrolne (ekstrakcja przy zastosowaniu 50% etanolu) oraz próby po trawieniu *in vitro* (Minekus et al., 2014) z pewnymi modyfikacjami. Szczegółową metodykę przedstawiono w **pracach II i IV**.

6.6. Badanie zdolności do neutralizowania wolnych rodników ABTS

Aktywność przeciwutleniająca wobec wolnych rodników ABTS została oznaczona zgodnie z metodyką opisaną przez Re et al. (Re et al., 1999) z pewnymi modyfikacjami. Szczegółowy opis zawarto w **pracach I, II, III i IV**.

6.7. Oznaczanie całkowitej zawartości związków fenolowych (TPC)

Analizy TPC przeprowadzono zgodnie z protokołem Singletona i Rossiego (Singleton & Rossi, 1965) dostosowanym do czytnika mikropłytek (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Winooski, Vermont, USA). Stężenie związków fenolowych odczytano z krzywej kalibracyjnej ustalonej dla kwasu galusowego i wyrażono jako odpowiednik kwasu galusowego (GAE) w mg/g sm. Szczegółowy opis zawarto w **pracy IV**.

6.8. Przygotowanie preparatu peroksydazy tyroidowej (TPO)

Preparat enzymatyczny przygotowano zgodnie z procedura opisaną przez Jomaa (Jomaa, 2015) z niewielkimi modyfikacjami. Zamrożoną tarczycę pokrojono na plasterki i zhomogenizowano w buforze zawierającym 0,25 M sacharozy, 2 mM Tris—HCl, 100 mM KCl, 40 mM NaCl, 10 mM $MgCl_2$ (pH 7,4).. Homogenat został odwirowany przy 4000 obr./min przez 15 minut w temperaturze +4 °C. Białko zostało następnie wysolone do 60% siarczanem amonu ($(NH_4)_2SO_4$. Frakcja ta została użyta do dalszych analiz. Zawartość białka została oznaczona zgodnie z Bradford (Bradford, 1976) z pewnymi modyfikacjami. Pomiar wykonano przy użyciu spektrofotometru płytkowego (BioTek, Model Epoch2TC, Winooski, Vermont, VT, USA) w płytach 96-dołkowych przy długości fali 595 nm. Aktywność enzymu została oznaczona za pomocą testu z gwajakolem (Jomaa, 2015). Szczegółowa procedura otrzymywania preparatu enzymatycznego została przedstawiona w **pracach I, II, III i IV.**

6.9. Ocena wpływu badanych prób na aktywność peroksydazy tyroidowej (TPO)

Badanie przeprowadzono zgodnie z metodyką opisaną przez Jomaa (Jomaa, 2015) z pewnymi modyfikacjami. Pomiar wykonano za pomocą spektrofotometru płytkowego (BioTek, Model Epoch2TC, Winooski, Vermont, VT, USA) na płytach 96-dołkowych przy długości fali 470 nm. Do 50 µL buforu dodano 40 µL badanej próby, 50 µL gwajakolu, 20 µL preparatu TPO i 50 µL H_2O_2. Całkowita objętość mieszaniny wynosiła 210 µL. Odczyty absorbancji były rejestrowane co minutę przez łącznie 3 minuty w temperaturze 37°C. Jako jednostka aktywności TPO definiowana jest zmiana absorbancji o 0,001 na minutę. Szczegółowy opis zawarto w **pracy I.**

6.10. Hamowanie aktywności lipoksygenazy (LOX)

Zdolność do hamowania aktywnosci LOX została określona zgodnie z metodyką podaną przez Axelrod i in. (Axelrod, Cheesbrough, & Laakso, 1981) i dostosowane do czytnika mikropłytek (Spektrofotometr mikropłytek Epoch 2, BioTek Instruments). Sposób hamowania enzymu został określony przy użyciu wykresów Lineweaver-Burka. Jako jednostkę aktywności LOX zdefiniowano zmianę absorbancji na minutę przy długości fali 234 nm. Wartości IC_{50} obliczono na podstawie dopasowanych modeli jako stężenie

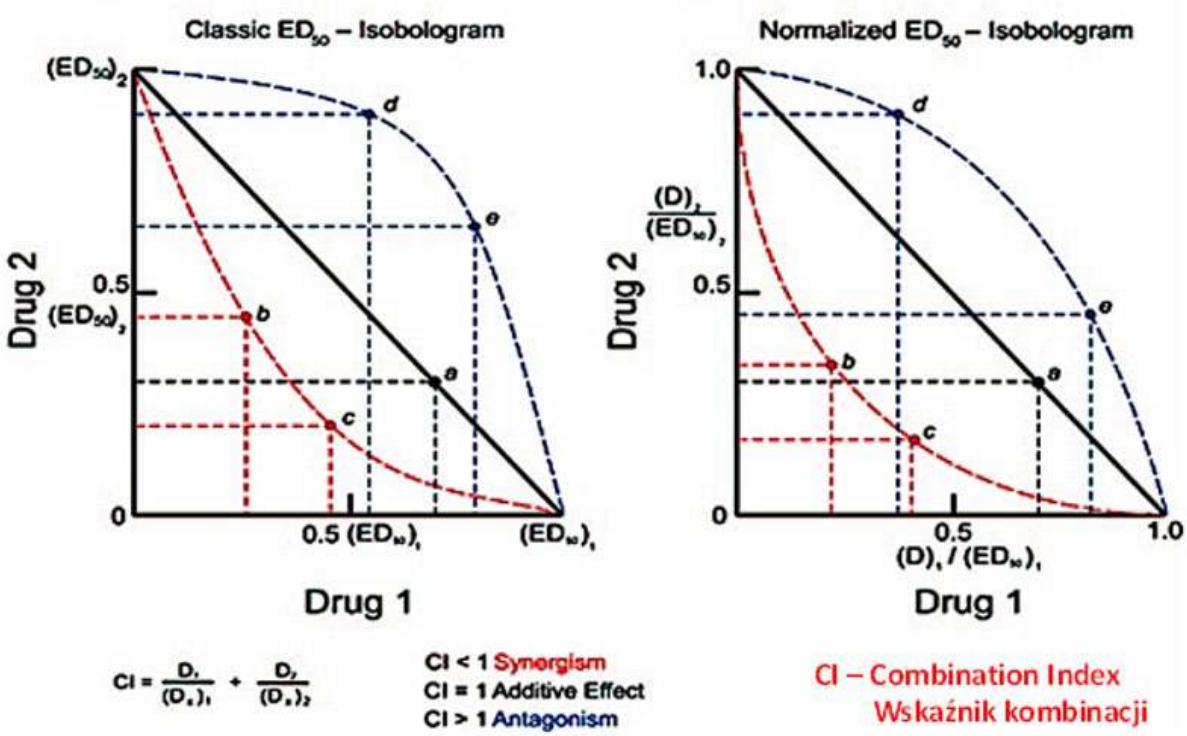
testowanego związku, które powoduje 50% maksymalnego hamowania w zależności od dawki. Szczegółowy opis zawarto w **pracach II, III i IV.**

6.11. Hamowanie aktywności oksydazy ksantynowej (XO)

Zdolność do hamowania aktywnosci XO została określona zgodnie z metodą opisaną przez Sweeney et al. (Sweeney, Wyllie, Shalliker, & Markham, 2001) z pewnymi modyfikacjami. Jako jednostkę aktywności XO zdefiniowano zmianę absorbancji na minutę przy długości fali 295 nm. Szczegółowy opis zawarto w **pracy IV.**

6.12. Analiza interakcji

Do określenia rodzaju i siły interakcji między substancjami aktywnymi wykorzystano metodę analizy izobolograficznej. Metoda ta jest niezależna od mechanizmu działania substancji aktywnych i umożliwia określenie rodzaju interakcji między składnikami mieszanek dwuskładnikowych oraz złożonych ekstraktów roślinnych. Warunkiem koniecznym jest występowanie liniowej zależności między aktywnością a stężeniem próbki. Warto podkreślić, że analiza izobolograficzna jest dość skomplikowana i wymaga dużego nakładu pracy, ale stanowi przydatne narzędzie w badaniach nad interakcjami między składnikami mieszanek oraz substancjami aktywnymi. Kształt izoboli daje informacje o rodzaju interakcji i wartości indeksu kombinacji, CI. Gdy CI jest mniejsze niż jeden wskazuje na synergizm; gdy CI jest równe jeden wskazuje na addycję; gdy CI jest większe niż jeden wskazuje na antagonizm. Izobologramy zostały wykonane zgodnie z metodą Chou (Chou, 2006).



Rysunek 1. Klasyczny oraz znormalizowany izobologram dla dwóch leków. (Chou, 2006).

Roztwory czystych polifenoli zostały wymieszane w różnych stosunkach objętościowych: 1:4, 4:1, 3:2, 2:3, 1:1, natomiast ekstrakty w proporcjach 3:2, 2:3, 1:1. Wszystkie mieszaniny zostały wykonane jako kombinacje dwóch roztworów/ekstraktów. Ocena interakcji została przeprowadzona za pomocą wzoru na wkaźnik kombinacji (CI) dla kombinacji n-składnikowej przy x% hamowaniu, zgodnie z poniższym równaniem:

$$\boldsymbol{CI} = \frac{(\boldsymbol{D})_1}{(\boldsymbol{D_x})_1} + \frac{(\boldsymbol{D})_2}{(\boldsymbol{D_x})_2}$$

Gdzie:

CI – wskaźnik kombinacji;

D- stężenie składnika (w mieszaninie) dla którego osiągnięto zmierzoną aktywność;

D_X - wartość IC_{50} dla pojedynczego składnika

6.13. Badanie właściwości przeciwnowotworowych ekstraktów z mąki pszennej i płatków owsianych

6.13.1. Hodowla komórek

Ludzkie komórki raka tarczycy B-CPAP (ACC 273, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) oraz 8505C (Sigma No. 94090184) były

hodowane w standardowych warunkach w pożywce RPMI 1640 i DMEM/F12 HAM (Sigma No. D8437) z dodatkiem 10% płodowej surowicy cielęcej (FBS; Gibco, No. A3840402) oraz antybiotyków (Merck, No. A5955). Komórki były pasażowane z wykorzystaniem trypsyny/EDTA/PBS bez Ca^{2+}/Mg^{2+} (Gibco No. 25200072), liczone w liczniku cząsteczek Z2 (Beckman Coulter), wysiewane do wielostudzienkowych naczyń hodowlanych (Falcon®), a następnie traktowane ekstraktami podanymi w stężeniu 0,01, 0,05, 0,1, 1 i 3% w pożywce hodowlanej (odpowiadające 1,5, 7,5, 15, 150 i 450 g produktu/75 kg masy ciała) przez 48 godzin.

6.13.2. Testy dotyczące proliferacji i przeżywalności komórek

Do oceny dobrostanu i proliferacji komórek B-CPAP i 8505C, komórki wysiewano do naczyń 12-dołkowych (Corning®Costar®) na 24 godziny przed podaniem ekstraktów. Przeżywalność komórek była oceniana za pomocą testu EtBr/FDA przy użyciu mikroskopu fluorescencyjnego Leica DMI6000B, a proliferacja za pomocą licznika Coulter 48 godzin po podaniu ekstraktów (Ryszawy et al., 2019).

6.13.3. Test gojenia się rany

Komórki B-CPAP i 8505C były wysiewane do naczyń 12-dołkowych. Po 24 godzinach, dodawano ekstrakty (wraz z nową pożywką; 0,1 i 1%). Po kolejnych 48 godzinach, w centralnej części każdego dołka wykonywano ranę za pomocą pipety. Następnie rejestrowano 16 zdjęć rany bezpośrednio po jej wykonaniu oraz 24 godziny później, aby obliczyć postęp zarastania rany przez komórki przy użyciu mikroskopu fluorescencyjnego Leica DMI6000B.

6.13.4. Immunofluorescencja

Komórki B-CPAP i 8505C były wysiewane do naczyń wielodołkowych z umieszczonymi w nich szkiełkami nakrywkowymi, poddane działaniu ekstraktów, utrwalane 3,7% formaldehydem, a następnie permeabilizowane 0,1% Tritonem X-100 (Pudełek et al., 2020). Barwienie cytoszkieletu przeprowadzano z wykorzystaniem przeciwciał przeciw winkulinie (Sigma no. V9131), przeciwciała II-rzędowego skoniugowanego z AlexaFluor488 (ThermoScientific No. A-11029), falloidyny skoniugowanej z AlexaFluor546 (Invitrogen, No. A22283) oraz barwnika Hoechst 33258 (Sigma). Zdjęcia zostały wykonane za pomocą mikroskopu fluorescencyjnego Leica DMI6000B wyposażonego w kamerę CCD DFC360FX i poddano obróbce liniowej przy użyciu oprogramowania ImageJ. Metodykę szczegółowo opisano w **pracy IV**.

7. Omówienie wyników badań

7.1. Wpływ czystych związków polifenolowych (wzorców chemicznych) na aktywność peroksydazy tyroidowej (TPO, EC 1.11.1.1-14) oraz lipooksygenazy (LOX, EC. 1.13.11.12)

Polifenole to grupa związków organicznych występujących w roślinach, zawierające przynajmniej dwie grupy hydroksylowe przyłączone do pierścienia aromatycznego. Polifenole występują naturalnie w roślinach. Badania naukowe wskazują na potencjalne korzyści zdrowotne wynikające z ich spożycia, w tym działanie przeciwutleniające, przeciwzapalne oraz antyproliferacyjne (Hossen et al., 2017; Rathod et al., 2023). W niniejszej części pracy przedstawiono wyniki badań *in vitro* dotyczące wpływu czystych polifenoli na aktywność enzymów TPO i LOX. Wyniki zostały zawarte w **pracy I, II oraz III**. Związki polifenolowe: apigeninę, kwercetynę, katechinę, rutynę, kwas chlorogenowy, kwas rozmarynowy, kwas ferulowy, kwas *trans* – cynamonowy, kempferol, kwas synapinowy oraz kwas syryngowy wytypowano na podstawie wirtualnego przeszukiwania (badania *in silico*) i badań wstępnych. Określono ich wpływ na aktywność TPO i stwierdzono, iż apigenina, kwercetyna, katechina, rutyna, kwas chlorogenowy, kwas rozmarynowy, kempferol i kwas synapinowy hamowały działanie enzymu (**Praca I i II**), podczas gdy kwas ferulowy, kwas *trans*-cynamonowy oraz kwas syryngowy okazały się być jego aktywatorami **(Praca III).**

Rutyna i kwas rozmarynowy działały jako inhibitory kompetencyjne. Wybrane pozycje wiązania tych inhibitorów przedstawiono na **rysunku 2 w Pracy I.** Rutyna **(Rysunek 2A)** tworzy wiązania wodorowe z Gln 235, His 239, Phe 243, Thr 244, Gln 246, Ser 247 i Glu 399. Kwas rozmarynowy **(Rysunek 2B)** tworzy wiązania wodorowe z Gln 235, Asp 238, His 239, Thr 244, Gln 246, Arg 396, Glu 399 i Arg 582. Porównanie **rysunku 2** i **rysunku 1** pozwala wywnioskować, że oba konkurencyjne ligandy oddziałują z resztami zaangażowanymi w wiązanie hemu. Ponadto, blokują dostęp hemu do His 239 w miejscu katalitycznym, uniemożliwiając proces katalityczny. W przypadku kwercetyny i kwasu chlorogenowego stwierdzono niekompetyncyjny sposób hamowania. **Rysunek 4 w Pracy I** przedstawia wyniki dokowania kwasu chlorogenowego **(Rysunek 4A i 4B)** i kwercetyny **(Rysunek 4C i 4D)** do TPO. Miejsce wiązania kwasu chlorogenowego znaleziono w kieszeni wiążącej pomiędzy podjednostkami białka. Inhibitor ten tworzy wiązania wodorowe z Ala 172, Arg 175 i Thr 480 z jednej podjednostki oraz z Ser 309, Asn 312 i Gln 315 z drugiej podjednostki. Kwercetyna zajmuje miejsce peryferyjne w obrębie

jednej podjednostki i tworzy wiązania wodorowe z Phe 195, Leu 202, Pro 271, Gln 581 i Arg 584. (**Praca 1, Rysunek 4D**). Katechina, kwas sinapinowy i kempferol działały jako inhibitory kompetycyjne, podczas gdy apigenina wykazywała aktywność inhibitora akompetycyjnego (**Praca II, Rysunek 1**). Te wyniki sugerują, że polifenole mogą potencjalnie hamować syntezę hormonów tarczycy przez hamowanie aktywności TPO.

W dostępnej literaturze opisano potencjał hamowania TPO przez różne flawony, takie jak apigenina, chryzyna, witexyna i baicaleina, obecnych w natce pietruszki, czereśniach, tymianku, oliwkach, herbacie i brokułach (Paunkov et al., 2019). Flawanole, takie jak kempferol, kwercetyna, fisetyna, morina, mirycetyna i rutyna, obecne w jarmużu, cebuli, pomidorach, czereśniach, jabłkach i czerwonym winie, wraz z flawanonami, narynginą i naryngeniną, również wykazują zdolność do hamowania jodowania tyrozyny przez TPO (Cai, Luo, Sun, & Corke, 2004; Liu et al., 2019). W przypadku hamowania aktywności TPO, co prowadzi do zmniejszenia syntezy hormonów tarczycy, może wystąpić kompensacyjny wzrost TSH, co może prowadzić do powstania charakterystycznego wola, zwłaszcza przy znacznym spożyciu tych związków (Paunkov et al., 2019). Jednakże, według naszej najlepszej wiedzy, w dostępnej literaturze brak jest danych dotyczących aktywatorów fenolowych TPO.

Po weryfikacji *in vitro* wyników otrzymanych w badaniach *in silico* stwierdzono, że kwas ferulowy, kwas syryngowy oraz kwas *trans*-cynamonowy aktywują TPO. Dla wszystkich związków określono wartości AC_{50} - stężenia efektora, w którym uzyskano 50% aktywacji. Biorąc pod uwagę ten parametr, przetestowane kwasy fenolowe można sklasyfikować następująco: kwas trans-cynamonowy> kwas ferulowy> kwas syryngowy. Aktywujące działanie wszystkich badanych związków może wynikać z interakcji z miejscem allosterycznym TPO. Zaproponowano zmianę konformacyjną wynikającą z wiązania aktywatora z kieszenią allosteryczną TPO będącej skutkiem elastyczności pobliskiej pętli utworzonej przez reszty Val352-Tyr363. (**Praca III, Rysunek 2 i 4).**

W związku z tym, że choroby tarczycy są związane z występowaniem stanu zapalnego, kolejnym etapem badań było określenie, czy te same polifenole wykazują zdolność do hamowania LOX,. Dane opublikowane w **Pracy III** potwierdziły, że polifenole wykazują zdolność do hamowania aktywności tego enzymu. W przypadku kwasu *trans* – cynamonowego, kwasu syryngowego jak i kwasu ferulowego, stwierdzono akompetycyjny mechanizm hamowania LOX. Wartość IC_{50} była najwyższa dla kwasu ferulowego ($0{,}027 \pm 0{,}0013$ mM), natomiast plasowała się na zbliżonym poziomie w przypadku dwóch pozostałych kwasów – *trans* - cynamonowego oraz syryngowego ($0{,}009 \pm 0{,}0004$

mM). We wszystkich przypadkach sugerowana jest interakcja między inhibitorami grup karboksylowych i atomami łańcucha bocznego Arg102 i Arg139 w allosterycznej kieszeni LOX. (**Praca III, Rysunek 3, Tabela 2**).

Dodatkowo przeprowadzono badanie właściwości antyoksydacyjnych badanych związków. Wyniki jednoznacznie wskazują na ich wysoki potencjał przeciwrodnikowy. Biorąc pod uwagę wartość EC_{50} badane związki można uszeregować następująco: katechina (0,078 ± 0,008 mM) > kwas synapinowy (0,084 ± 0,001 mM) > kwas rozmarynowy (0,151 ±0,006 mM) > kwas syryngowy (0,22 ± 0,011 mM) > kwas chlorogenowy (0,267 ±0,008 mM) > rutyna (0,292 ± 0,010 mM) > kempferol (0,337 ± 0,002mM) > apigenina (0,406 ± 0,003 mM) > kwas ferulowy (0,41 ± 0,02 mM) > kwercetyna (0,447 ± 0,018 mM) > kwas *trans* – cynamonowy (2,12 ± 0,106) (**Praca I, II i III**).

7.1.1. Wpływ interakcji związków wzorcowych na zdolność do modulowania aktywności TPO i LOX

Polifenole występują w żywności w bardziej złożonych kombinacjach, co stanowi wyzwanie dla wyodrębnienia ich właściwości biologicznych. Jednakże badania wykazują, że leki zawierające więcej niż jedną substancję aktywną są bardziej skuteczne (Leonard et al., 2016). Z tego powodu, kolejnym etapem badań nad wpływem polifenoli na aktywność TPO oraz LOX było oszacowanie rodzaju interakcji między badanymi czystymi polifenolami (wzorcami), które działają jako efektory TPO.

Analiza izobolograficzna inhibitorów TPO wykazała, że rutyna i kwas chlorogenowy działały synergistycznie, podczas gdy interakcja addytywna została stwierdzona w przypadku rutyny i kwercetyny oraz rutyny i kwasu rozmarynowego. Antagonizm stwierdzono natomiast w przypadku kwasu chlorogenowego i kwas rozmarynowego, kwasu chlorogenowego i kwercetyny oraz kwasu rozmarynowego i kwercetyny (**Praca I, Rysunek 5**). Wszystkie rodzaje interakcji zostały wyrażone jako wartość CI zgodnie z interpretacją Chou (Chou, 2006), która wyjaśnia siłę interakcji. Jak wykazały wartości CI, czyste substancje wykazywały umiarkowany antagonizm (kwas rozmarynowy/kwercetyna oraz kwas rozmarynowy/kwas chlorogenowy), antagonizm (kwas chlorogenowy/kwercetyna), addycję (rutyna/kwas rozmarynowy oraz rutyna/kwercetyna) i lekki synergizm (kwas chlorogenowy/rutyna) (**Praca I, Tabela 5).**

W wyniku przeprowadzonej analizy izobolograficznej aktywatorów TPO stwierdzono, że kwas *trans* – cynamonowy oraz kwas ferulowy działają addytywnie (CI = 0,98 ± 0,06).

Z kolei kwas syryngowy i kwas ferulowy wykazują silną synergię (CI = 0,24± 0,05), a kwas syryngowy i kwas *trans* - cynamonowy działają synergistycznie (CI = 0,46 ± 0,04) **(Praca III, Rysunek 5, Tabela 4).**

W dostępnej literaturze brak jest danych na temat wpływu interakcji związków polifenolowych na aktywność TPO.

Wyniki analizy izobolograficznej wykazały, że w przypadku hamowania aktywności LOX kwas *trans* –cynamonowy/kwas syryngowy działają antagonistycznie. Z kolei kwas ferulowy/*trans*-cynamonowy oraz kwas ferulowy/kwas syryngowy działają synergistycznie **(Praca III, Rysunek 6).**

7.2. Wpływ matrycy pokarmowej oraz przemian zachodzących podczas trawienia *in vitro* na fenolowe efektory enzymów TPO, LOX oraz XO zawarte w żywności pochodzenia roślinnego

W badaniach nad wpływem substancji bioaktywnych zawartych w żywności na organizm ludzki istotne znaczenie ma nie tylko zrozumienie właściwości poszczególnych związków czynnych, ale również zjawiska interakcji między nimi w bardziej złożonych układach jakimi są ekstrakty oraz wpływu samej matrycy żywności. Dlatego też w kolejnym etapie badań wybrano żywność pochodzenia roślinnego zasobną w związki, których badania przeprowadzono w poprzednich etapach. Porównano aktywność ekstraktów etanolowych (będących ekstraktami kontrolnymi) oraz ekstraktów po symulowanym trawieniu na aktywność TPO oraz enzymów prooksydacyjnych LOX i XO. Poznanie skomplikowanej wzajemnej zależności między składnikami pokarmowymi oraz pomiędzy składnikami a matrycą żywności jest kluczowe dla pełnego zrozumienia wpływu substancji bioaktywnych na zdrowie człowieka. Wyniki badań do tej części zawarto w **Pracach II i IV**.

Choć w niektórych przypadkach stwierdzono występowanie analogii pomiędzy aktywnością i mechanizmem działania zwiazków wzorcowych i zawierających je ekstraktów otrzymanych z surowców roślinnych, zależność taka nie jest oczywista. I tak, czysta katechina działała jako inhibitor kompetycyjny TPO, podobnie jak etanolowe ekstrakty z zielonej herbaty i zielonej kawy **(Praca II, Rysunek 1B, 2B i 3B).**Ten sam mechanizm inhibicji TPO stwierdzono w przypadku kempferolu i bogatego w ten związek ekstraktu z kminku (**Praca II, Rysunek 1C, 2D i 3D** oraz kwasu synapinowego i ekstraktu z gorczycy **(Praca II, Rysunek 1D i 2E).** Wpływ matrycy zywności zaobserwowano porównując działanie apigeniny i ekstraktu z pietruszki. Apigenina działała jako inhibitor

akompetycyjny, podczas gdy ekstrakt etanolowy z wykazywał kompetycyjny mechanizm hamowania **(Praca II, Rysunek 1Ai 2A).**

W przypadku dietorapii i dietoprewencji kluczowa jest biodostepność związków czynnych, fundamentalną rolę odgrywają również zmiany zachodzące pod wpływem warunków panujących w przewodzie pokarmowym. Z badań przedstawionych w **Pracy II** wynika, iż mogą one drastycznie zmienić ostateczny efekt. Podczas gdy ekstrakty etanolowe z badanych roślin hamowały aktywność TPO, warunki zachodzące podczas trawienia *in vitro* skutkowały zdolnością aktywacji tego enzymu. Największą zdolność do aktywacji TPO stwierdzono w przypadku potencjalnie biodostępnych związków pochodzących z gorczycy (24,66%) i kminku (19,85%).

W przypadku analogicznych badań przeprowadzonych dla enzymu LOX (**Praca II**) najwyższy potencjał inhibicyjny LOX zaobserwowano dla etanolowych ekstraktów zielonej herbaty i kawy (IC_{50} = 13,74 i 15,96 mg DW/mL, odpowiednio), a najniższy dla ekstraktu z gorczycy (29,01 mg DW/mL) **(Praca II, Tabela 5).** W przypadku większości etanolowych ekstraktów uzyskano mieszany typ inhibicji LOX. Jedynie w przypadku etanolowego ekstraktu z gorczycy zaobserwowano kompetycyjny model inhibicji **(Praca II, rysunek 4).** Po przeprowadzeniu symulowanego trawienia, sposób inhibicji LOX zmienił się w większości ekstraktów, z wyjątkiem gorczycy, nastąpił także spadek zdolnosci do hamowania LOX, o czym świadczy wzrost wartosci IC_{50} (w porównaniu do ekstraktów etanolowych) (**Praca II, Rysunek 5, Tabela 5**).

Jako dietetyczne źródła kwasu ferulowego – aktywatora TPO (**Praca III, Rysunek 1A**) wybrano makę pszenną i płatki owsiane (**Praca IV**), W przeciwieństwie do wyników uzyskanych dla czystego kwasu ferulowego **(Praca III, Rysunek 1A)**, zaobserwowano hamowanie dla kontrolnego (etanolowego) ekstraktu z płatków owsianych, co wskazuje na wpływ matrycy żywności. Zależności takiej nie stwierdzono w przypadku mąki pszennej **(Praca III rysunek 1A).** Co ważne, w obu przypadkach po trawieniu *in vitro* otrzymano próby działajace jako aktywatory TPO, co dla płatków owsianych jasno wskazuje na kluczową rolę tego procesu w kształtowaniu bioaktywności (**Praca IV, Rysunek 1A).** Można przypuszczać, że w trakcie trawienia *in vitro* kwas ferulowy jest uwalniany z jego nieaktywnych połączeń **(Praca IV, Rysunek 1B).**

Aktywacja została zaobserwowana w przypadku obydwu ekstraktów z mąki pszennej (zarówno kontrolnych jak i po symulowanym trawieniu), co wykazało podobieństwo do wyniku uzyskanego dla czystego kwasu ferulowego **(Praca III rysunek 1A).** W tym wypadku matryca żywnościowa oraz warunki panujące podczas symulowanego trawienia

nie miały znaczącego wpływu na zmianę mechanizmu działania substancji aktywnej Dla kontrolnych ekstraktów owsianych stwierdzono mieszany rodzaj hamowania **(Praca IV, Rysunek 1A).**

Kolejnym krokiem było sprawdzenie wpływu ekstraktów kontrolnych i trawionych na aktywność LOX. Stwierdzono, że zarówno ekstrakty z mąki pszenniej, jak i płatków owsianych hamowały aktywność LOX. W przypadku mąki pszennej zaobserwowano niekompetycyjny mechanizm inhibicji dla ektraktu kontrolnego oraz mieszany rodzaj inhibicji dla ekstraktu po trawieniu *in vitro* **(Praca IV, Rysunek 1D)**. Natomiast dla czystego kwasu ferulowego zaobserwowano akompetycyjny mechanizm hamowania LOX **(Praca III, Rysunek 3A)**,. Różnice w mechanizmie inhibicji sugerują, że mógł on ulec zmianie pod wpływem matrycy żywności oraz warunków panujących podczas symulowanego trawienia. W przypadku płatków owsianych obserwowano mechanizm hamowania akompetycyjnego dla obydwu rodzajów ekstraktów **(Praca IV, Rysunek 1C, Tabela 3),** czyli analogiczny do zaobserwowanego w przypadku czystego związku referencyjnego **(Praca III, Rysunek 3A)**.

Dodatkowo wykonano badania dotyczące hamowania aktywności oksydazy ksantynowej ze względu na coraz większą liczbę doniesień naukowych wskazujących na rolę tego enzymu w procesach chorobowych, w tym procesach zapalnych. Ponadto, XO jest głównym endogennym źródłem RFT.

Wszystkie rodzaje ekstraktów z obydwu surowców działały jako inhibitory akompetycyjne XO, co może sugerować, że zmiany zachodzące podczas symulowanego trawienia nie miały decydującego wpływu na mechanizm oddziaływania na enzym **(Praca IV, Rysunek 1E i 1F).**

7.2.1. Badanie interakcji ekstraktów zawierających efektory TPO, LOX i XO

Wiadomo, że polifenole zawarte w roślinnych składnikach żywności występują w bardziej złożonych kombinacjach. Potencjalnie bioaktywne związki z testowanych roślin: kminek, zielona herbata, zielona kawa, gorczyca, płatki owsiane oraz mąka pszenna aktywowały TPO. Jest to bardzo ważne zwłaszcza dla osób cierpiących na chorobę Hashimoto. Jednak przyjmowanie ilości dającej znaczący efekt jest w praktyce bardzo trudne, dlatego następnym krokiem było określenie rodzaju interakcji między testowanymi ekstraktami. Jak już wcześniej wspomnianio, analiza interakcji jest metodą stosowaną do

identyfikacji interakcji między związkami aktywnymi. Metoda ta jest niezależna od mechanizmu działania; jest przydatnym narzędziem do określania interakcji między składnikami mieszanek dwuskładnikowych oraz tych składających się z ekstraktów roślinnych będących mieszaninami wielu aktywnych związków (Chou, 2006). W **pracy II**, w której przebadano interakcje pomiędzy ekstraktami trawionymi w układach: zielona herbata/zielona kawa, kminek/zielona kawa/gorczyca/zielona herbata, kminek/zielona herbata gorczyca/kminek w przypadku wszstkich wymienionych kombinacji stwierdzono oddziaływania synergistyczne **(Praca II, Rysunek 6)** .

Kolejnym etapem prowadzonych badań było określenie rodzaju interakcji między potencjalnie biodostępnymi ekstraktami, które pełniły rolę aktywatorów TPO, i równocześnie inhibitorów LOX. Analiza interakcji wykazała, że w przypadku mieszanek wszystkich ekstraktów uzyskano synergistyczny rodzaj oddziaływań **(Praca II, Rysunek 5).** Biorąc pod uwagę charakter oddziaływań, jak i fakt, że wszystkie z komponentów były aktywatorami TPO, połączenia te są obiecujące dla ludzi zmagających się z chorobą Hashimoto.

W kolejnej cześci badań przeprowadzono analizę interakcji pomiędzy aktywatorami TPO oraz inhibitorami LOX i XO.zawartymi w kontrolnych (etanolowych) i trawionych ekstraktach z płatków owsianych i mąki pszennej. Siła interakcji została określona za pomocą wartości indeksu kombinacji (CI) (Chou, 2006). W przypadku kontrolnych ekstraktów (etanolowych) stwierdzono, że próbki z mąki pszennej aktywowały TPO, a z płatków owsianych – hamowały; nie przeprowadzono więc w tym wypadku analizy izobolograficznej **(Praca IV, Rysunek 1A i 1B)**. Wynika to z faktu, że połączenie aktywatora i inhibitora może prowadzić do problemów z interpretacją wyników. Z drugiej strony, jeśli chodzi o trawione ekstrakty, uzyskano silny efekt synergizmu w stosunku do aktywacji TPO **(Praca IV, Rysunek 2A),** co potwierdza wartość indeksu kombinacji (CI) wynosząca 0,18 ± 0,02 **(Praca IV, Rysunek 2A).** W przypadku inhibitorów enzymu LOX obserwowano silne synergiczne interakcje dla obu rodzajów ekstraktów z płatków owsianych i mąki pszennej, czyli kontrolnych oraz po symulowanym trawieniu (wartości CI wynosiły odpowiednio 0,23 ± 0,03 i 0,25 ± 0,01) **(Praca IV, Rysunek 2B i 2C).** W przypadku inhibitorów enzymu XO również zaobserwowano oddziaływania synergiczne. Wartości CI dla ekstraktu wyjściowego i ekstraktu po trawieniu wynosiły odpowiednio 0,26 ± 0,04 i 0,45 ± 0,02 **(Praca IV, Rysunek 2D i 2E).**

Jak wspomniano wcześniej, naukowcy zwracają uwagę głównie na interakcje między lekami, ale istnieją również badania skupiające się na interakcjach między dodatkami do

żywności lub źródłami żywności. Dane te są stosunkowo nowe. Badania przeprowadzone przez Lau i in. (Lau, McLean, Williams, & Howard, 2006) wykazały synergistyczny rodzaj interakcji między popularnymi dodatkami do żywności, takimi jak aspartam i Quinoline Yellow i między Brilliant Blue a L-glutaminianem. Wiedza na temat interakcji substancji innych niż leki jest wciąż niewielka. Brak informacji generuje potrzebę takich badań.

7.2.2. Badania potencjału przeciwnowotworowego *in vitro*

Ponieważ zdolność do hamowania aktywnosci LOX jest często skorelowana z aktywnością przeciwnowotworową badania poszerzono o część dotyczącą właściwości przeciwnowotworowych ekstraktów pochodzących z mąki pszennej oraz płatków owsianych.

W badaniach *in vitro* wykorzystano model eksperymentalny oparty na komórkach linii B-CPAP wykazujących ekspresję peroksydazy tarczycowej (ang. thyroperoxidase; TPO+) izolowanych ze słabo zróżnicowanego raka tarczycy (PDTC)(Patel & Shaha, 2006) oraz na komórkach niewykazujących ekspresji peroksydazy tarczycowej (TPO-) linii 8505C pochodzących z anaplastycznego (niezróżnicowanego) raka tarczycy (Meireles et al., 2007). Oba te typy nowotworów tarczycy związane są ze złym rokowaniem i śmiertelnością pacjentów obciążonych tą choroba. Model ten umożliwił oszacowanie wpływu statusu zróżnicowania komórek raka tarczycy i/lub ekspresji TPO na ich wrażliwość na działanie substancji zawartych w ekstraktach otrzymanych z płatków owsianych i mąki pszennej.

Testy żywotności komórek B-CPAP wykazały podobną (zależną od dawki) aktywność cytotoksyczną obu ekstraktów poddanych trawieniu *in vitro*. Po podaniu w stężeniu od 0,01% do 3% (odpowiadającemu odpowiednio od 1,5 do 450 g produktu natywnego/75 kg masy ciała), oba te ekstrakty zmniejszały frakcję żywych komórek do ok. 85% **(Praca IV, Rysunek 3A)**. Z kolei wyraźniejszą aktywność cytostatyczną ekstraktu z płatków owsianych ilustruje wolniejsza proliferacja komórek B-CPAP hodowanych w obecności ekstraktu z płatków owsianych do ok. 60%, w porównaniu do 75% dla ekstraktu z mąki pszennej **(Praca IV Rysunek 3B).** Ta obiecująca obserwacja potwierdza wcześniejsze dane dotyczące bioaktywności obu produktów w modelach nowotworowych (Meireles et al., 2007) i rozszerza je na raka tarczycy.

Od dawna sugeruje się, że kwas ferulowy hamuje proliferację komórek nowotworowych, jednak jego stężenia w bardziej aktywnym ekstrakcie z płatków owsianych poddanych trawieniu *in vitro* były niższe niż w analogicznym ekstrakcie z mąki

pszennej **(Praca IV Tabela 1).** Wskazuje to na udział innych substancji w cytostatycznej aktywności badanych ekstraktów. Aktywność ta korelowała z ich hamującym wpływem na aktywność LOX, co sugeruje, że różna wrażliwość komórek B-CPAP na ekstrakty z płatków owsianych i mąki pszennej po trawieniu *in vitro* może być częściowo związana z różną zawartością modulatorów LOX (w tym kwasu ferulowego).

Co zaskakujące, oczekiwanym efektom cytostatycznym obu badanych ekstraktów towarzyszył ich nieoczekiwany stymulujący wpływ na ruchliwość komórek B-CPAP **(Praca IV, Rysunek 3C).** Jednocześnie zaobserwowano wyraźne rearanżacje cytoszkieletu aktynowego komórek B-CPAP hodowanych w obecności obu ekstraktów, w tym tworzenie wiązek mikrofilamentów (tzw. włókien naprężeniowych; ang. stress fibers) i dojrzewanie kontaktów zogniskowanych (ang. focal contacts) **(Praca IV Rysunek 3D).** Najwyraźniej silnemu efektowi cytostatycznemu ekstraktów z mąki pszennej i płatków owsianych po trawieniu *in vitro* stosowanych w dawkach fizjologicznych towarzyszy indukcja ruchliwości B-CPAP. Jednocześnie nie zaobserwowaliśmy żadnych wyraźnych oznak apoptotycznej odpowiedzi B-CPAP na substancje zawarte w ekstraktach. Potwierdza to, że za ten efekt odpowiada indukcja ruchliwości (części?) komórek, a nie negatywna selekcja komórek o niskiej ruchliwości i wysokiej wrażliwości na cytotoksyczne działanie ekstraktów z natury rzeczy heterogennych populacji komórek nowotworowych. Dane te mogą wskazywać na cytoprotekcyjną aktywność ekstraktów względem komórek (na przykład związaną z wygaszaniem wolnych rodników) i/lub aktywację proinwazyjnych szlaków sygnałowych w komórkach B-CPAP).

Ponadto nie zaobserwowano znaczących różnic w jakości reakcji komórek 8505C i B-CPAP na obecność obu ekstraktów **(Praca IV Rysunek. 5A, 3C 4C).** Stosunkowo wysoka wrażliwość komórek 8505C, które nie wykazują ekspresji TPO, na związki zawarte w badanych ekstraktach wskazuje na marginalną rolę aktywacji TPO w reaktywności komórkowej na substancje w nich zawarte. Guzy anaplastyczne, których "reprezentantem" jest linia 8505C, stanowią podgrupę guzów tarczycy związaną z najgorszym rokowaniem, a komórki 8505C wykazują wysoką inwazyjność. Dlategoobserwacje potwierdzają również, że ekstrakty z płatków owsianych/ mąki pszennej mogą wywoływać działania niepożądane u pacjentów z zaawansowanymi nowotworami tarczycy.

8. Wnioski

1. Związki fenolowe wykazują wielokierunkową, skoordynowaną aktywność biologiczną *in vitro* działając równocześnie jako efektory TPO, inhibitory LOX i XO oraz przeciwutleniacze drobnocząsteczkowe. Aktywność ta w istotny sposób modyfikowana jest poprzez interakcje związków czynnych.
2. Z uwagi na zdolność do równoczesnego aktywowania TPO, hamowania aktywności LOX i XO oraz działania przeciwrodnikowego kwasy syryngowy, *trans* - cynamonowy oraz ferulowy mogą być wskazane w przypadku niedoczynności tarczycy.
3. Kwas chlorogenowy, kwercetyna, kwas rozmarynowy i rutyna okazały się inhibitorami wszystkich badanych enzymów, co wskazuje na ich potencjalne korzystne oddziaływania w przypadku nadczynności tarczycy.
4. W przypadku związków fenolowych pochodzących z żywności kluczową rolę w kreowaniu aktywności odgrywają interakcje z matrycą żywności i przemiany zachodzące podczas trawienia.
5. Próby otrzymane po trawieniu *in vitro* pietruszki, zielonej kawy, zielonej herbaty, kminku i gorczycy aktywowały TPO, podczas gdy ekstrakty etanolowe, podobnie jak związki referencyjne, hamowały aktywność tego enzymu. Fakt ten wskazuje na złożoność przemian i konieczność dalszych, dogłębnych badań.
6. W przypadku ekstraktów otrzymanych po trawieniu *in vitro* płatków owsianych i mąki pszennej stwierdzono zdolność do aktywowania TPO oraz hamowania LOX i XO podobnie jak w przypadku związku referencyjnego - kwasu ferulowego. Co ważne, w przypadku tych produktów potwierdzono synergizm działania. Wyniki te wskazują na potencjalne korzystne działanie tych produktów w przypadku osób cierpiących na niedoczynność tarczycy.
7. Wstępne badania potencjału przeciwnowotworowego ekstraktów z płatków owsianych i mąki pszennej wskazują raczej na ich potencjał prewencyjny, co stanowi kolejną przesłankę sugerującą zasadność wprowadzenia ich do codziennej diety osób cierpiacych na niedoczynność tarczycy.
8. Wnioskowanie o aktywnosci biologicznej pokarmów bogatych w związki czynne jedynie na podstawie znajomości zawartości, aktywności i mechanizmów działania czystych wzorców może prowadzić do istotnych błędów. W celu potwierdzenia prozdrowotnego działania składników

pokarmowych zawierających związki polifenolowe i opracowania konkretnych zaleceń dotyczących suplementacji lub stosowania tych składników w celach prewencyjnych i/lub wspomagania farmakoterapii niezbędne są dalsze badania, zarówno *in vitro* jak i *in vivo*.

9. Dalsze perspektywy badawcze

1. Poszerzenie badań o enzym cykooksygenazę (COX, EC 1.14.99.1), którego aktywność, podobnie jak w przypadku LOX, może być przyczyną powstania stanu zapalnego w organizmie ludzkim.
2. Poszerzenie badań o kolejne źródła pokarmowe i określenie wpływu zawartych w nich związków potencjalnie biodostepnych na aktywność TPO, LOX, COX oraz XO.
3. Stworzenie bazy wiedzy dotyczącej optymalnych połączeń składników żywności o określonym działaniu na TPO, LOX, COX i XO, dobrane pod kątem konkretnego zaburzenia funkcjonowania tarczycy

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10. Publikacje wchodzące w skład rozprawy doktorskiej

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Thyroid Peroxidase Activity is Inhibited by Phenolic Compounds—Impact of Interaction

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Abstract: The aim of this study was to estimate the mode of thyroid peroxidase (TPO) inhibition by polyphenols: Chlorogenic acid, rosmarinic acid, quercetin, and rutin. All the tested polyphenols inhibited TPO; the IC_{50} values ranged from 0.004 mM to 1.44 mM (for rosmarinic acid and rutin, respectively). All these pure phytochemical substances exhibited different modes of TPO inhibition. Rutin and rosmarinic acid showed competitive, quercetin—uncompetitive and chlorogenic acid—noncompetitive inhibition effect on TPO. Homology modeling was used to gain insight into the 3D structure of TPO and molecular docking was applied to study the interactions of the inhibitors with their target at the molecular level. Moreover, the type and strength of mutual interactions between the inhibitors (expressed as the combination index, CI) were analyzed. Slight synergism, antagonism, and moderate antagonism were found in the case of the combined addition of the pure polyphenols. Rutin and quercetin as well as rutin and rosmarinic acid acted additively (CI = 0.096 and 1.06, respectively), while rutin and chlorogenic acid demonstrated slight synergism (CI = 0.88) and rosmarinic acid with quercetin and rosmarinic acid with chlorogenic acid showed moderate antagonism (CI = 1.45 and 1.25, respectively). The mixture of chlorogenic acid and quercetin demonstrated antagonism (CI = 1.79). All the polyphenols showed in vitro antiradical ability against 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), ABTS. The highest ability (expressed as IC_{50}) was exhibited by rosmarinic acid (0.12 mM) and the lowest value was ascribed to quercetin (0.45 mM).

Keywords: thyroid peroxidase (TPO); dietary polyphenols; molecular modeling; interactions

1. Introduction

Thyroid peroxidase, also called thyroperoxidase (TPO, EC 1.11.1.1-14) or iodide peroxidase, is an enzyme participating in the synthesis of thyroid hormones. In the human body, it is encoded by the TPO gene located on chromosome *2p25* [1]. TPO catalyzes iodide oxidation to form iodine atoms, which are added onto tyrosine residues on thyroglobulin for the production of thyroxine or triiodothyronine, i.e., thyroid hormones [2]. The mechanism mentioned above can be described by reactions occurring in the following order: TPO is oxidized by H_2O_2 and then TPO can oxidize iodide ions. Oxidized iodide ions bind to tyrosyl residues of thyroglobulin (TG). Formation of T4 and T3 iodothyronines is an effect of oxidation and coupling of hormonogenic iodotyrosines [3].

As the TPO enzyme is a heme peroxidase, it cannot oxidize the substrate without having been oxidized. To oxidize TPO, the H_2O_2 molecule is necessary. The H_2O_2 molecule is generated only at the apical surface of thyrocytes, and TPO molecules present at this surface are activated [4].

TPO is organized as a 933-residues homodimer. The N-terminal propeptide (residues 1–108) is cleaved in the mature protein. Three domains in the TPO extracellular region (residues 109–846) display a high degree of sequence similarity to domains of known 3D structure: Myeloperoxidase (MPO)-like domain (residues 142–738), complement control protein (CCP)-like domain (residues 740–795), and epidermal growth factor (EGF)-like domain (residues 796–846), [5]. The transmembrane domain is constituted by residues 847–871 and intracellular domain by residues 872–933. Figure 1 presents the dimeric MPO-like domain in trans orientation with heme molecules exposed to thyroid follicular lumen. Le [5] considered also an alternative *cis* orientation, with heme molecules facing the thyrocyte membrane. According to the above studies cis orientation is slightly more energetically stable. However, other reported data suggested that in order to perform the oxidation and further iodination of thyroglobulin the catalytically active portion of the enzyme projects into the follicular lumen [6], which requires trans orientation of the dimeric MPO-like domain.

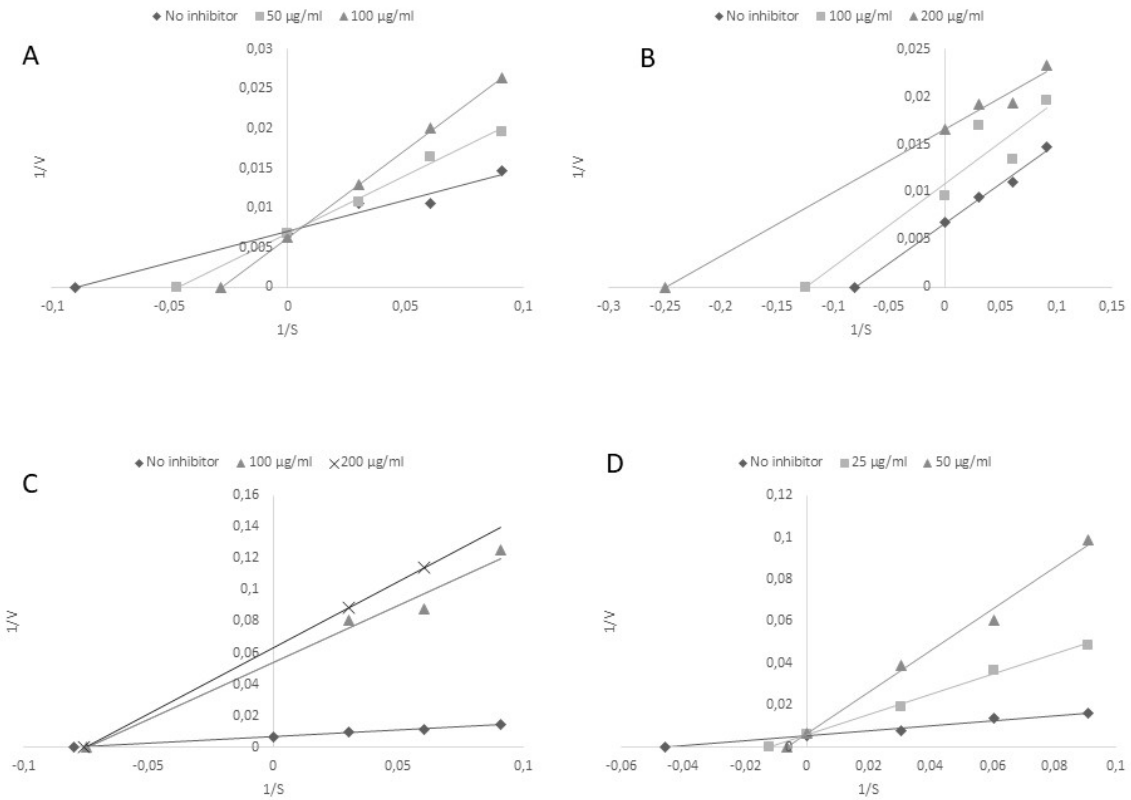


Figure 1. Lineweaver—Burk double reciprocal plots for the inhibition of TPO by rutin (**A**), quercetin (**B**), chlorogenic acid (**C**) and rosmarinic acid (**D**). Plots are expressed 1/velocity versus 1/guaiacol [µg/mL] without or with inhibitors in reaction solution.

Thyroid peroxidase is the major antigen in human Hashimoto's disease, and anti-TPO antibodies induce complement-dependent cytotoxicity. Furthermore, antibodies against complement (anti-C1q) are detected in patients with Hashimoto's disease. They are correlated with thyroid-stimulating hormone (TSH) levels. Many patients with congenital hypothyroidism have problems related to synthesis or iodination of TG that are connected to TPO deficiency. When TPO activity is not normal or is totally absent, thyroid iodide organification may occur giving rise to congenital hypothyroidism. Another autoimmune disease correlated with pathological thyroid gland functioning is Grave's disease. It is characterized by the presence of antibodies against the TSH receptor, thyroid peroxidase enzyme (TPO), and thyroglobulin (TG). TSH receptor antibodies play a crucial role in the development of hyperthyroidism. The increase in thyroid hormone synthesis is associated with the onset of Grave's disease, showing T3 dominance [7]. Pathological conditions of the thyroid are associated with oxidative

stress. Oxidative stress results mainly from excessive production of ROS, which are not removed by natural repair mechanisms. These mechanisms may be supported by substances with antioxidative activity, e.g., phytochemical compounds, especially phenolics, provided to the organism [8]. Thus, diet rich in food of plant origin is considered an important element of prevention of so called lifestyle diseases (for example Alzheimer's disease, arteriosclerosis, cancer, cirrhosis, chronic obstructive pulmonary disease, diabetes, hypertension, heart disease, stroke). Many epidemiological studies have found that the consumption of foods and drinks with high phenolic content is associated with the prevention of coronary disease, cancer, etc. [9,10]. It is known that plants are a rich source of secondary metabolites (i.e., antioxidants) such as polyphenols with documented biological activity.

Combinations of different pure bioactive compounds or their extracts from food sources can increase the benefits of individual bioactive compounds. This type of interaction is referred to as synergism [11]. In some cases, mixtures of two compounds may lower the biological effects, e.g., they can decrease the capability to scavenge free radicals in comparison with this ability exhibited by single substances [12]. The available studies of the influence of food compounds on TPO activity are not sufficient and sometimes inconsistent, hence the need for additional, more complex investigation. The first step of the investigation should be to carry out an analysis in simple model systems; therefore, studies of pure chemical substances: Quercetin, chlorogenic acid, rutin and rosmarinic acid were carried out. Quercetin is a natural flavonoid present in many plants such as berries, onions, tea, or apples. It has anti-inflammatory, antioxidant, anti-apoptotic, and anticancer properties [13]. Studies reported by Cheng et al. [14] showed that quercetin could be used in the treatment of human retinal inflammatory diseases. Chlorogenic acid is present mainly in coffee and tea, as well as in grape wines, tropical fruits, cabbages, and root vegetables [15]. This polyphenolic acid plays a role in inducing apoptosis in chronic myeloid leukemia cells [16], glucose and lipid metabolism, inhibition of DNA methylation. It also has anti-obesity and anti-inflammatory activity, attenuates hypertension, and modifies the concentrations of cholesterol, triacylglycerols, and minerals [15]. Rutin is a non–toxic biologically active flavonoid. It is present in many plant products, such as tea, coffee, red wine, oil [17], and linden and heather honeys [15]. Rutin plays a role as an antioxidant mostly in free radical scavenging and in the treatment of hypertension, hemorrhoids, and spontaneous bleeding. It also protects vascular walls and shows antiviral and antimicrobial activity. Moreover, rutin has anti-inflammatory and anti-allergic properties [17]. Plants from the Lamiaceae family comprising many herbs: Rosemary, oregano, marjoram, sage, basil, thyme, and chia seeds are a source of rosmarinic acid [18]. Rosmarinic acid has a high antioxidant potential as well as anti-inflammatory, antiviral, antimicrobial, and antimutagenic activity [19].

In the natural environment, all substances appear not as single compounds but in connection with other substances. Due to this fact, our study includes the isobolographic analysis. This method is widely used for analysis of drug combinations and is also applied in the analysis of food compounds

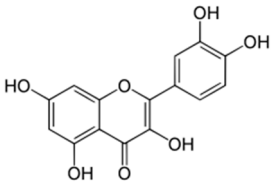
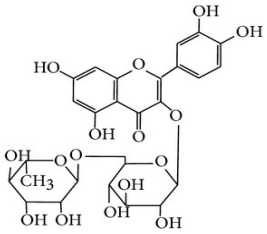
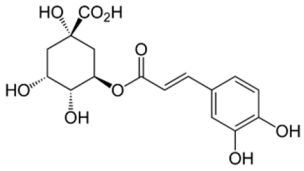
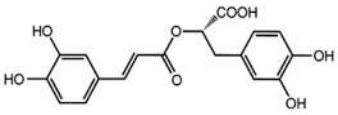
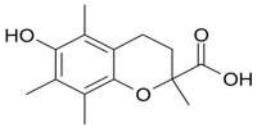
The study is a part of more complex investigations, which focus on the influence of biologically active substances contained in food components on TPO activity. The obtained data can be useful in choosing an appropriate diet coordinated with the type of thyroid disorder (hyperthyroidism, hypothyroidism). The present study determines the effect of four most common dietary polyphenols (chlorogenic acid, rosmarinic acid, quercetin, and rutin) on TPO activity to elucidate these mechanisms.

2. Results and Discussion

2.1. Antioxidant Capacity

The antioxidant capacities of pure polyphenols (quercetin, rutin, rosmarinic acid, chlorogenic acid) were evaluated by the most commonly used antioxidant assay, i.e., ABTS method. The results clearly indicated that the polyphenols significantly scavenged free radicals in this assay. The ABTS radical scavenging ability of rosmarinic acid was found to be the highest. The lowest antiradical activity was observed for the quercetin (Table 1).

Table 1. Chemical structure and ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging properties of chosen polyphenols (quercetin, rutin, chlorogenic acid, rosmarinic acid) expressed in IC_{50} (Efficient Concentration), $n = 9$.

Compound	Chemical Formula	IC_{50} [mM]
quercetin		0.447 ± 0.018 e *
rutin		0.292 ± 0.010 c
chlorogenic acid		0.267 ± 0.008 b
rosmarinic acid		0.151 ± 0.006 a
Trolox (positive control)		0.407 ± 0.014 d

* The values designated by the different letters are statistically significantly different.

The results obtained by Grzesik et al. [20] showed antiradical properties in grapes (which are a source of quercetin) [21] for a solution concentration of 50 µg/mL at the level of 43.06% in the case of skin, 16.75%—seeds, and 7.01%—flesh. Chlorogenic acid exhibited ABTS radical scavenging activity at a IC_{50} value of 0.267 mM, which was higher than the results obtained for quercetin (0.447 mM) and rutin (0.292 mM) but lower than for rosmarinic acid (0.151 mM).

2.2. TPO Inhibitory Studies

The biochemical characteristic of TPO isolated from the thyroid gland is shown in Table 2.

Table 2. Km value, Vmax and content of proteins in thyroid peroxidase (TPO) enzyme solution prepared from porcine thyroid gland, ($n = 9$).

Parameter	Value
Proteins [mg/mL]	0.67 ± 0.02
Km [µg/mL]	11.07 ± 0.47
Vmax [ΔAU/min]	140.85 ± 5.22

Polyphenols showing inhibitory effect on TPO activity used in the study and IC_{50} value, which was calculated at fitted models as the concentration of the tested compound that gave 50% of the maximum response based on a dose-dependent mode of action were presented in Table 3.

Table 3. IC_{50} [μM] value of polyphenols showing inhibitory effect on TPO activity used in the study, $n = 9$.

Compound	IC_{50} [mM]
Rutin	0.122 ± 0.004 c **
Rosmarinic acid	0.004 ± 0.00014 a
Quercetin	0.199 ± 0.008 d
Chlorogenic acid	1.439 ± 0.041 e
Propylthiouracil (PTU, positive control) *	0.004 a
Methimazole (MMI, positive control) *	0.0107 b

* According to literature data [22]; ** The values designated by the different letters are statistically significantly different.

All the tested polyphenols inhibited TPO, but with quite different potencies (Table 3). The EC50 values ranged from 0.004 mM to 1.44 mM with the following order of potency: Rosmarinic acid > rutin > quercetin > chlorogenic acid. Studies conducted by Divi and Doerge [23] showed a similar relationship between quercetin and rutin, where quercetin had a higher TPO inhibitory effect than rutin (IC_{50}: 2.4 μM for quercetin and 40.6 μM for rutin).

Polyphenols are not the only substances, which can inhibit TPO activity. Amino acids are TPO inhibitors as well [24]. Scientists found that different amino acids inhibited TPO with different strength. Similar results were observed in the case of polyphenols.

It has been demonstrated that TPO inhibitors are potential therapeutic agents in hyperthyroidism. Pure substances were chosen on the basis of preliminary studies (unpublished data) and the main reason for choosing the polyphenols was their presence in plant food sources and the high antioxidant potential. The antioxidant potential is the main property of polyphenols, which show anticarcinogenic, antimutagenic, and antiaging activity, contained in medicinal plants [25]. The next step of our investigation was to determine the inhibitory potential of the tested polyphenols against TPO activity. As expected, the pure chemical solutions demonstrated high TPO inhibitory activity. The chosen polyphenols showed different modes of TPO inhibition. Rutin and rosmarinic acid acted as competitive inhibitors (Figure 1A,D). The uncompetitive mode of inhibition was determined for quercetin with respect to the guaiacol concentration, since Km and Vmax were affected (Figure 1B). The noncompetitive type of inhibition was indicated in the case of chlorogenic acid, where Vmax, but not Km, was affected (Figure 1C).

In our study, the IC_{50} value for quercetin was 0.199 mM, whereas data obtained by Divi and Doerge [23] showed that the IC_{50} value was 2.4 μM in relation to TPO. In our study, the IC_{50} value for rutin was 0.121 mM, whereas data obtained by Divi and Doerge [22] showed an IC_{50} value of 40.6 μM. The differences in these data can be associated with the differences in the methodology, solvents, and the source of TPO. Elucidation of the mechanisms observed in the analyzed polyphenols may provide important insight in the development and possibly prevention of inflammation.

Data obtained in this study indicate the ability of dietary polyphenols to inhibit TPO activity (Table 3). Of course, their ability to inhibit the activity of TPO is definitely lower than the commonly used drugs propylthiouracil (PPT) and methimazole (MMI) [22]. The major role in creating the biological activity of phenolic compounds plays their bioavailability, however it is still not fully understood. The intestinal absorption and metabolism of chlorogenic acids (385 μmol) following a single intake of 200 mL of instant coffee by human with an ileostomy was investigated by Stalmach et al. [26]. The HPLC–MS3 analysis of 0–24 h post-ingestion ileal effluent revealed the presence of 274 μmol of chlorogenic acids and their metabolites accounting for 71 of intake [26]. Quercetin is widely distributed in edible plants, mainly as glycosides such as rutin. It has been reported to be absorbed in mammals, but its metabolism

needs further investigation to evaluate its possible physiological effects [27]. Hollman et al. [28] have found a human plasma concentration of about 0.65 umol/l quercetin after consumption of a meal containing 150 g of fried onions (equivalent to 64 mg of pure quercetin). Rutin was absorbed more slowly than quercetin because it must be hydrolysed by the cecal microflora. Following six weeks supplementation with rutin, significant changes in the plasma levels of quercetin, kaempferol and isorhamnetin were measured in the rutin-treated volunteers. The increase of 2.5 fold in plasma quercetin, 3-fold in plasma kaempferol and 10 fold in plasma isorhamnetin was found [29]. Rosmarinic acid (RA) exhibits diverse pharmacological effects, however, its oral absolute bioavailability and dose proportionality in vivo have not been comprehensively studied. The absolute bioavailability of RA in rats was estimated as 1.69%, 1.28% and 0.91% after oral administration of RA at the doses of 12.5, 25 and 50 mg/kg, respectively [30]. The serum concentration of total rosmarinic acid peaked at 1 h after administration of *Melissa officinalis* extract containing 500 mg rosmarinic acid in a fasted state, with a maximum serum concentration 162.20 nM [31]. In the light of the cited works, it seems difficult to achieve bioavailability at the IC_{50} level obtained in our study, therefore, the further part of the work is to determine of interaction between these compounds as a factor that can increase their effectiveness.

There are not many data about the influence of pure chemicals on TPO activity. The first data reports the influence of extracts from peanut seed coats [32]. The study showed the total polyphenol content in the plant material, but did not focus on the interactions between the substances. Attention was also paid to the inhibitory influence of the polyphenols contained in the peanut seed coat on the TPO activity. As shown by literature studies, peanuts are a rich source of dietary flavonoids [33]. In another study, the influence of the green tea extract on TPO activity was investigated. Green tea is a rich source of dietary polyphenols such as catechin, quercetin, kaemferol, and chlorogenic acid. It was found that green tea extracts inhibited TPO in a dose-dependent manner. Therefore, it was concluded that most of the polyphenols contained in tea extracts are potential inhibitors of TPO [32].

Many studies aimed to explore the influence of particular drug components on TPO activity and interactions between these components. Data concerning the influence of pure polyphenols on in vitro TPO activity are sparse. There are data on the influence of polyphenols on other enzymes e.g., xanthine oxidase, and lipoxygenase. Data obtained by Gawlik–Dziki et al. [34] showed the influence of pure chlorogenic and ferulic acid extracts on LOX activity. Lin [35] showed the ability of pure polyphenolic substances to inhibit xanthine oxidase and their mode of action. As described above, there are literature data confirming that polyphenolic compounds have an impact on enzymatic activity, but there are very limited data about the influence of pure polyphenolic substances on TPO activity.

To characterize further the binding region of TPO, the Lineweaver—Burk double reciprocal plots are shown in Figure 1; kinetic parameters were presented in Table 4.

Table 4. Mode of inhibition, K_i and V_{max} values of chosen polyphenols on TPO activity, $n = 9$.

Compound	Mode of Inhibition	Ki [mM]	V_{max} [ΔAU/min]
Chlorogenic acid	noncompetitive	0.17 ± 0.005 d *	149.25 ± 5.97 a *
Quercetin	uncompetitive	0.02 ± 0.001 b	149.25 ± 4.35 a
Rosmarinic acid	competitive	0.001 ± 0.00 a	175.44 ± 7.01 b
Rutin	competitive	0.13 ± 0.003 c	149.25 ± 5.01 a

* The values designated by the different letters are statistically significantly different.

2.3. Molecular Modelling

In order to study the interactions of the considered inhibitors with TPO at the molecular level, the homology model of TPO was built. The homodimeric model with two molecules of heme bound in the MPO-domain is presented in Figure S1A.

Competitive inhibitors, i.e., rutin and rosmarinic acid were docked to the catalytic site of TPO. In general, rutin was scored higher than rosmarinic acid in molecular docking simulations, which is in agreement with experimental data. The selected binding poses of these inhibitors are shown in

Figure 2. Rutin (Figure 2A) forms hydrogen bonds with Gln 235, His 239, Phe 243, Thr 244, Gln 246, Ser 247 and Glu 399. Rosmarinic acid (Figure 2B) forms hydrogen bonds with Gln 235, Asp 238, His 239, Thr 244, Gln 246, Arg 396, Glu 399 and Arg 582. Comparison of Figure 2 and Figure S1 enables to conclude that both competitive ligands interact with residues involved in heme binding. Moreover, they block access of heme to His 239 in the catalytic site, making the catalytic process impossible.

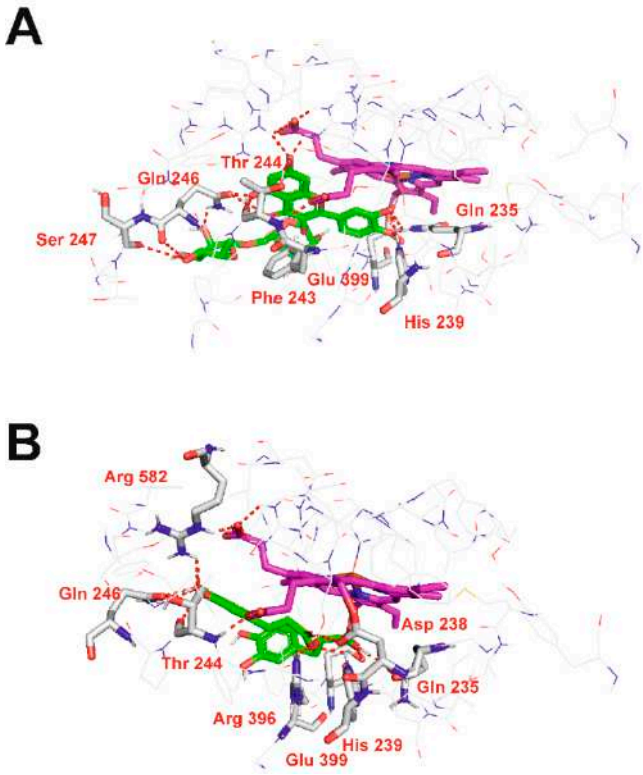


Figure 2. 3D view of rutin (**A**) and rosmarinic acid (**B**) in the catalytic site of TPO. Inhibitor molecules shown as sticks with green carbon atoms. Heme shown as sticks with magenta carbon atoms. Protein presented in wire representation with grey carbon atoms. Most important residues shown as sticks. Hydrogen bonds depicted as red dashed lines. Non-polar hydrogen atoms omitted for clarity.

In order to find possible binding sites for binding of a non-competitive inhibitor chlorogenic acid and uncompetitive inhibitor quercetin AlloPred, PARS and Achilles on-line tools were used. Figure 3 presents possible allosteric binding sites of TPO predicted with AlloPred (Figure 3A,B) and PARS (Figure 3C) using the normal mode analysis approach. In particular both tools predicted that allosterism at TPO might be connected with ligand binding in the domain between protein subunits. Moreover, all allosteric sites predicted by PARS may affect protein flexibility and lead to a conformational change upon ligand binding but none of them was found to be structurally conserved.

Non-competitive inhibitor chlorogenic acid and uncompetitive inhibitor quercetin were docked using the blind docking approach so the whole protein was considered as a potential site for interaction. Uncompetitive inhibitors bind to the enzyme simultaneously as the substrate of the enzyme. The binding of the inhibitor influences the binding of the substrate, and vice-versa. The uncompetitive inhibitor usually binds to an allosteric binding site and exerts the allosteric effect on the active site by changing its conformation so that the affinity of the substrate for the active site is reduced. Non-competitive inhibitors bind to an allosteric site and change the conformation of the enzyme's active site making it unsuitable for the substrate binding.

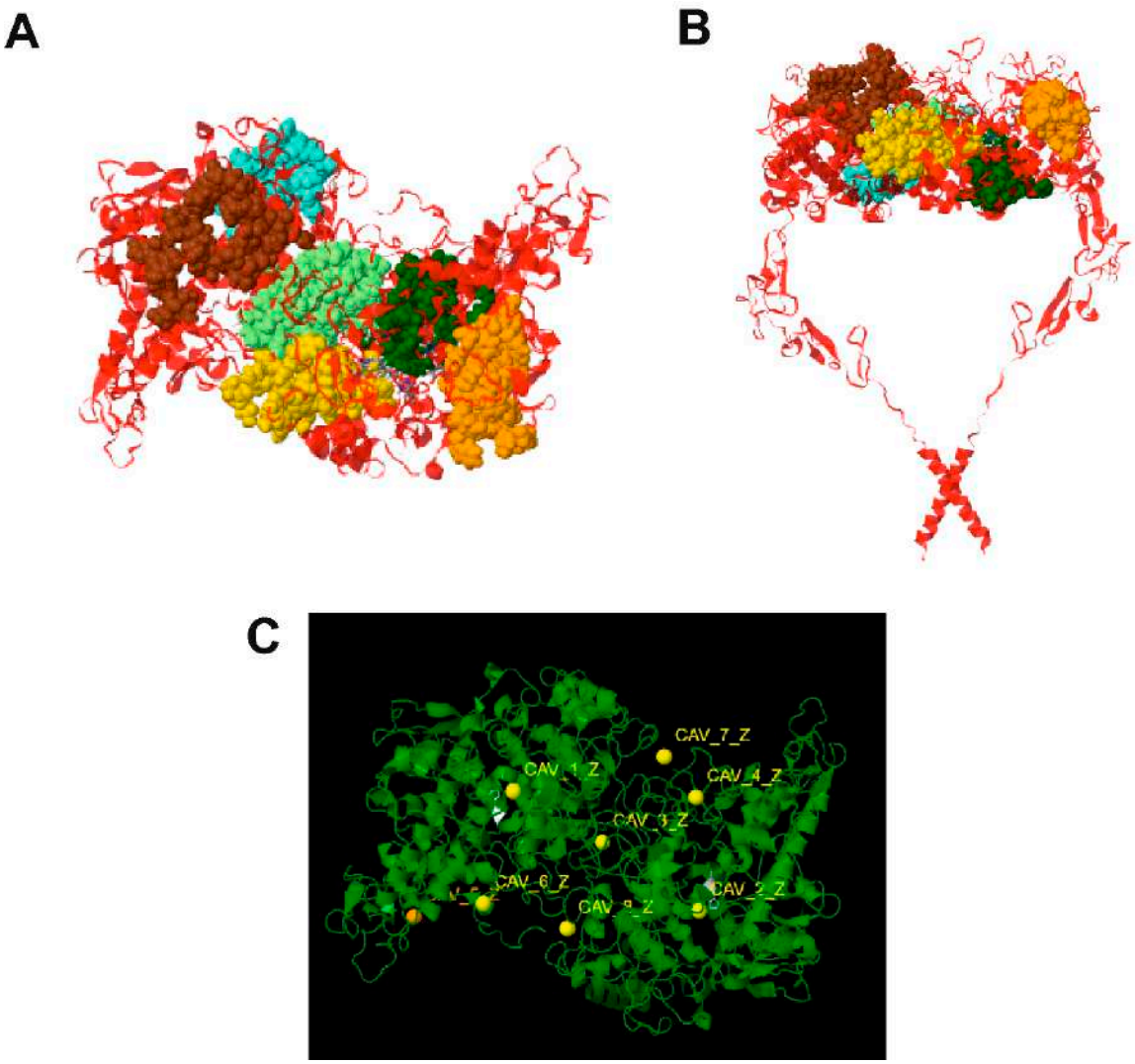


Figure 3. Prediction of allosteric sites at TPO using the normal mode analysis approach. (**A**) and (**B**) allosteric sites predicted using AlloPred on-line tool, top view and side view, respectively; (**C**) allosteric site predicted using PARS; all sites were found to affect protein flexibility but none of them was predicted as structurally conserved.

Figure 4 presents the results of blind docking of chlorogenic acid (Figure 4A,B) and quercetin (Figure 4C,D) to TPO. The identified binding pockets of both ligands were also found by AlloPred and PARS. Chlorogenic acid binding site was found in the binding pocket between the protein subunits. This inhibitor forms hydrogen bonds with Ala 172, Arg 175 and Thr 480 from one subunit and with Ser 309, Asn 312 and Gln 315 from the other subunit. Quercetin occupies a peripherial site within one subunit and forms hydrogen bonds with Phe 195, Leu 202, Pro 271, Gln 581 and Arg 584.

In order to find how binding chlorogenic acid in this pocket allosterically prevents TPO from heme binding, molecular dynamics simulations should be performed, which will be the subject of our future work.

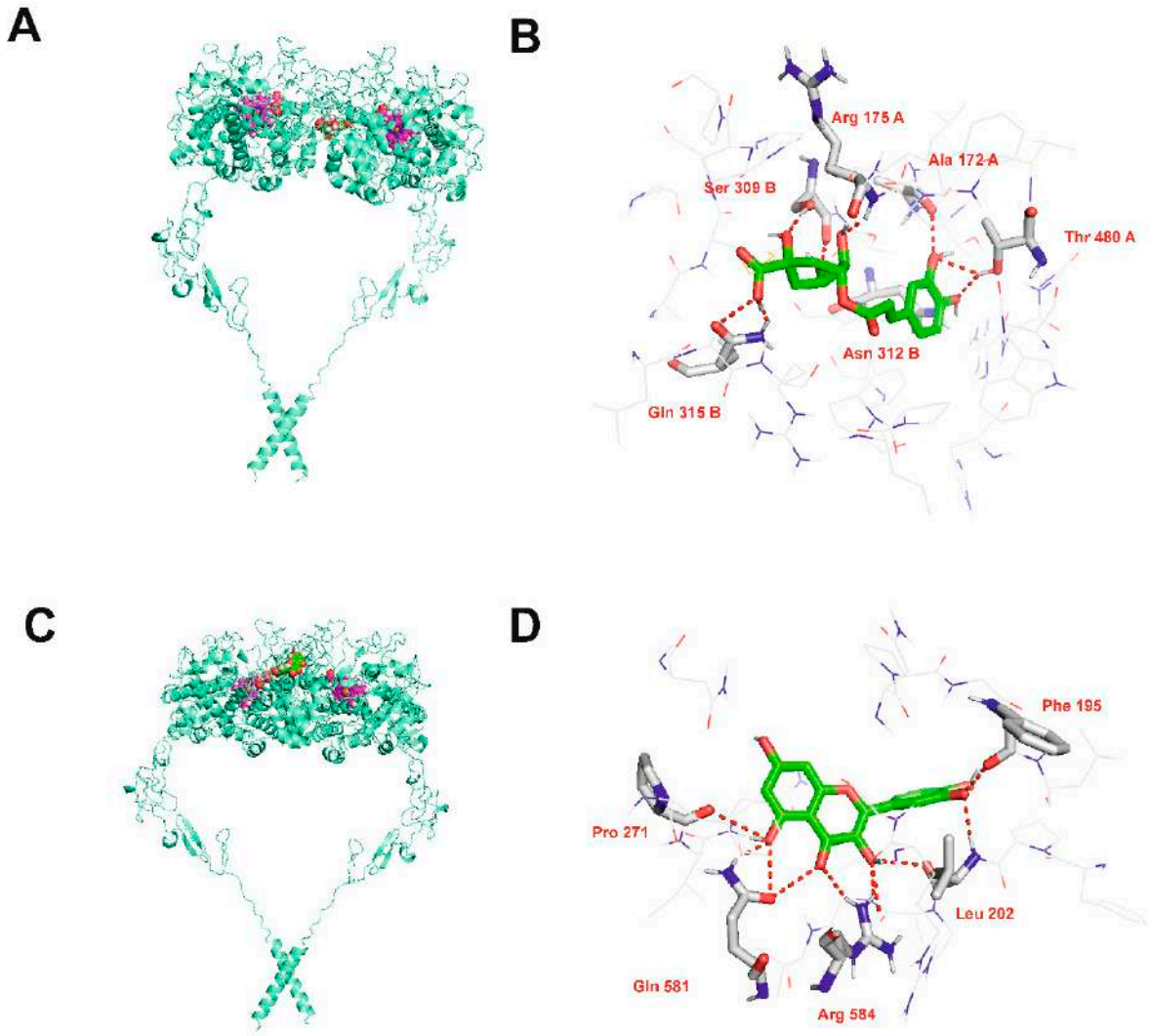


Figure 4. Results of blind docking of chlorogenic acid (**A**,**B**) and quercetin (**C**,**D**) to TPO. Parts A and C show general position of the binding site. Protein shown in cartoon representation. Heme and inhibitors shown as spheres with magenta and green carbon atoms, respectively. Parts B and D show details of the binding site. Inhibitor molecules shown as sticks with green carbon atoms. Protein presented in wire representation with grey carbon atoms. Most important residues shown as sticks. Hydrogen bonds depicted as red dashed lines. Non-polar hydrogen atoms omitted for clarity.

2.4. Interactions Assay

It is known that polyphenolic substances contained in food sources appear in more complex combinations. Additionally, medicines with more than one active substance are more effective [4]. Therefore, the next step of the study was to estimate the type of interaction between the studied pure chemical standards (acting as TPO inhibitors). A method used for identification of the interactions between active compounds is the isobolographic analysis. This method is independent of the mechanism of activity; however, it should be emphasized that this analysis is quite complicated and labor consuming. The isobolographic analysis is a useful tool for determination of interactions between components of two-component mixtures, as well as those composed of plant extracts being mixtures of numerous active compounds (in the case of a linear relationship between the activity and sample concentration enabling determination of the IC_{50} value). The shape of the isobole gives information about the type of interaction and the CI value, i.e., the strength of interaction.

As presented in Figure 5, the interactions between the polyphenols used in the study were synergistic, antagonistic, and additive. Synergism means that two components mutually enhance their

activities (concave isobole), antagonism means that two components decrease the activity of the single component (convex isobole), and additive interaction (the isobole is a straight line) [36].

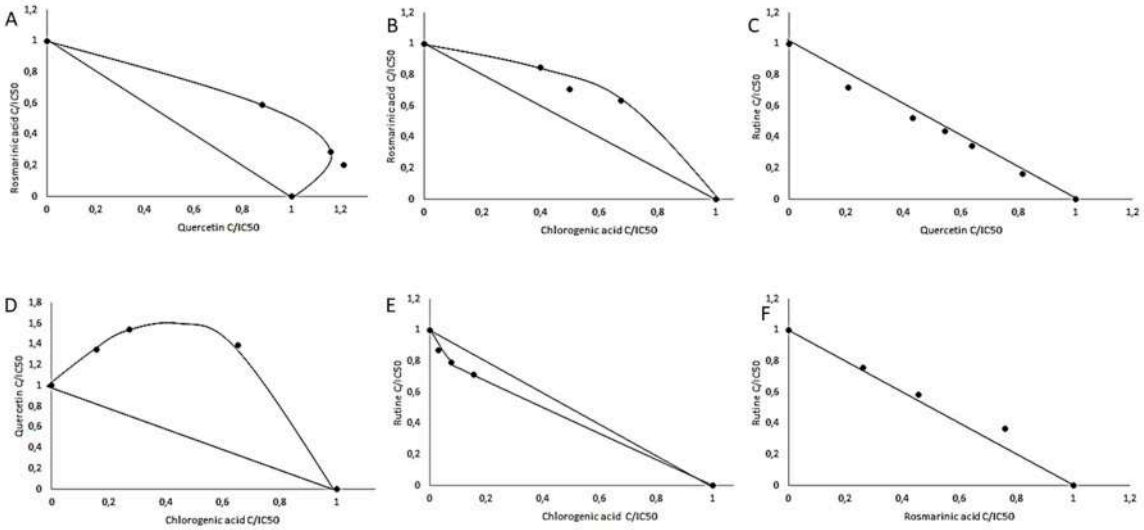


Figure 5. Dose-normalized isobolograms for chosen polyphenols with TPO inhibitory activity.

The isobolographic analysis showed that the rutin and chlorogenic acid acted synergistically (Figure 5E), whereas additive interaction was found in the case of rutin and quercetin or rutin and rosmarinic acid (Figure 5C,F). Chlorogenic acid and rosmarinic acid showed antagonistic interaction (Figure 5B) as well as chlorogenic acid with quercetin and rosmarinic acid with quercetin (Figure 5D,A). All types of interactions were expressed as a CI (Combination index) value in accordance with the interpretation by Chou [36], which explains the strength of the interactions. As shown by the CI values, the pure chemicals showed moderate antagonism, antagonism, nearly additive action, and slight synergism (Table 5).

The isobolographic analysis is used for characterization of pharmaceuticals. There are no data on the influence of polyphenolic combinations on TPO activity. Some studies have shown the influence of phytochemical compounds on the activity of other enzymes, e.g., xanthine oxidase (XO) and lipoxygenase (LOX). Studies carried out by Gawlik-Dziki [37] demonstrated inhibitory activity of pure and dietary polyphenols on XO activity and interactions between these inhibitors. Other studies focused on the interactions of LOX and OX inhibitors derived from natural sources of dietary polyphenols [38].

Table 5. CI (Combination Index) value between polyphenolic mixtures. Scale according to Chou, 2006 [39], $n = 9$.

Compound	Quercetin	Rutin	Chlorogenic Acid	Rosmarinic Acid
Quercetin	-	0.096 ± 0.05 Nearly additive	1.79 ± 0.2 Antagonism	1.45 ± 0.03 Moderate antagonism
Rutin	0.096 ± 0.05 Nearly additive	-	0.88 ± 0.02 Slight synergism	1.06 ± 0.05 Nearly additive
Chlorogenic acid	1.79 ± 0.2 Antagonism	0.88±0.02 Slight synergism	-	1.25 ± 0.05 Moderate antagonism
Rosmarinic acid	1.45 ± 0.03 Moderate antagonism	1.06 ± 0.05 Nearly additive	1.25 ± 0.05 Moderate antagonism	-

3. Materials and Methods

3.1. Chemicals

Sucrose (α-D-glucopyranosyl-(1→4)-β-D-fructofuranoside), Tris (1,3-Propanediol-2-amino-2-hydroxymethyl), KCl, NaCl, $MgCl_2$, 90% ethanol, NaOH, guaiacol (2-methoxyphenol), H_2O_2 (hydrogen peroxide), Bradford reagent, ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), quercetin, rosmarinic acid, chlorogenic acid, rutin and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich Company (Poznan, Poland). All other chemicals were of analytical grade.

3.2. Material

Porcine thyroid glands were obtained at a local slaughterhouse (Lublin, Poland) and stored in −20 °C until used.

3.3. Preparation of Pure Substance Solutions

Chlorogenic acid, quercetin, rosmarinic acid, and rutin were diluted to concentrations of 25 μg/mL (only for rosmarinic acid), 50 μg/mL, 100 μg/mL, 200 μg/mL and used for further assay.

3.4. In Vitro Antioxidant Capacity Assay

The ABTS radical scavenging activity was determined according to Re et al. [39] with some modifications. 250 μL of ABTS solution was mixed with 10 μL of each pure polyphenols solutions (concentration 50 μg/mL) and measured at the wavelength 724 nm using a UV/Vis spectrophotometer (BioTek, Model Epoch2TC, Winooski, Vermont, VT, USA) after 15 min of incubation in room temperature. The inhibition percentage of ABTS discoloration was calculated using the following equation:

$$AA = \frac{A_c - A_p}{A_c} \times 100\%$$

Where A_c—the absorbance of control, A_p—the absorbance of pure polyphenols solutions.

3.5. TPO Preparation

The assay was prepared according to Jomaa [22] with some modifications. The frozen thyroid gland was cut into slices and homogenized in a buffer containing 0.25 M sucrose, 2 mM Tris—HCl, 100 mM KCl, 40 mM NaCl, 10 mM $MgCl_2$ (pH 7.4) with a Philips homogenizer. The thyroid gland was than centrifuged two times at 4000 RPM per 15 min in temperature +4 °C. The enzyme protein was then salted-out to 60%. The supernatant was used for further analysis and was stored in a −20 °C freezer.

Protein content was determined according to the Bradford [40] with some modification. The measurement was made using a plate spectrophotometer (BioTek, Model Epoch2TC, Winooski, Vermont, VT, USA) in 96–well plates at a wavelength of 595 nm. In the single well 10 μL of the sample probe and 250 μl of Bradford reagent were mixed. The measurement was made with three repetitions.

The activity of the enzyme was assayed using a guaiacol assay. The reaction mixture contained: 33 mM guaiacol, 0.27 mM H_2O_2, and 33 mM of prepared sucrose buffer. The reaction components were incubated in 37 °C before the assignment. The absorbance was determined using a Shimadzu spectrophotometer (Model UV-1280, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 470 nm. The assay was conducted as follows: To a cuvette, 180 μL of buffer, 100 μL of guaiacol and 40 μL of TPO were mixed to the final volume of 420 μL. The cuvette was then placed into the spectrophotometer and the reaction was started by the addition of 100 μL H_2O_2. Absorbance readings were recorded every minute for a total of 3 min. Verification of the TPO activity was accomplished by linearly correlating the TPO concentration with absorbance readings.

3.6. TPO Inhibitory Assay

The assay was used according to Jomaa [22] with some modification. The measurement was made using a plate spectrophotometer (BioTek, Model Epoch2TC, Winooski, Vermont, VT, USA) in 96–well plates at a wavelength of 470 nm. The assay was conducted as follows: 50 µL of buffer, 40 µL of pure substance solution, 50 µL of guaiacol, 20 µL of TPO enzyme and 50 µL H_2O_2. The total volume of the mixture was 210 µL. In the sample probe, extracts were replaced by the buffer. Absorbance readings were recorded every minute for a total of 3 min in 37º. As a unit of TPO activity is defined as the change of absorbation per minute.

TPO inhibitory activity was calculated as follows:

$$\%inhibition = \left(1 - \frac{\frac{\Delta A}{\min_{test}}}{\Delta A \min_{blank}}\right) \times 100$$

Where: ΔA/min test is the linear absorbance change per minute of the test material and ΔA min blank is the linear change in absorbance per minute of the blank.

The IC_{50} value was determined by the interpolation of dose response curves. The IC_{50} values were calculated at fitted models as the concentration of the tested compound that gave 50% of the maximum inhibition based on a dose-dependent mode of action. The mode of inhibition of the enzyme was performed using the Lineweaver–Burk plot.

3.7. Molecular Modelling

The protein sequence of human TPO was extracted in a FASTA format from the UniProt database (Entry ID: P07202) [41]. Searching for templates was performed using the NCBI BLAST web server (https://blast.ncbi.nlm.nih.gov/). The MPO-like, CCP-like and EGF-like domains as well as a transmembrane domain were modelled with the homology modelling approach using five different templates. The MPO-like domain was modelled applying X-ray crystal structures of human myeloperoxidase at a 1.8 A resolution (PDB ID: 1CXP as a template [42] (residues 142–738 of TPO modelled using residues 167–744 of the template, sequence identity: 47%). The CCP-like domain was modelled applying X-ray structures of N-terminal domain of complement factor H-related protein at a 1.99 Å resolution (PDB ID: 3ZD2) as a template [43] (residues 730–795 of TPO were modelled using residues 57–123 of a template, sequence identity: 38%). The EGF-like domain was modelled applying X-ray structures of EGF-like module containing mucin-like hormone receptor-like 2 precursor at a 2.6 Å resolution (PDB ID: 2BO2) [44] and venom prothrombin activator pseutarin-C non-catalytic subunit at a 3.32 Å resolution (PDB ID: 4BXS) [45] as templates (residues 794–830 of TPO were modelled using residues 41–77 of 2BO2, sequence identity: 41%; residues 814–844 of TPO were modelled using residues 100–130 of 4BXS, sequence identity: 42%). The transmembrane domain was modelled applying a solution NMR structure of receptor tyrosine-protein kinase erbB-4 dimeric membrane domain (PDB ID: 2L2T) as a template [46] (residues 851–871 of TPO were modelled using residues 647–667 of a template as previously reported [5]. A population of 150 homology models of TPO was generated using Modeller 9.19 [47]. The symmetry of the homodimer was maintained by appropriate constraints in the Modeller software. Constraints were also used to model the inter-subunit disulfide bridge (at Cys 296). Residues 841–847 were modelled applying the appropriate loop modelling protocol of the Modeller software. Secondary structure constraints were applied to assure the α-helical structure of the protein transmembrane part. Heme molecule was modelled using Modeller.

The final models were evaluated based on their Discrete Optimized Protein Energy (DOPE) profiles obtained from the Modeller software. Twenty models with lowest DOPE values were further validated using Verify3D [48], ANOLEA (Atomic Non-Local Environment Assessment) [49] and ProCheck [50]. The best model was properly protonated with H++ server [51]. Next, the final TPO model was minimized using 500 steps minimization with Gromacs v. 5.0.7 [52] applying the Amber03 force field.

The 3D structures of quercetin, rutin, rosmarinic acid and chlorogenic acid were modelled with the Hartree-Fock approach and 6-31G* basis set of Spartan v. 10 VI.0.1 (Spartan 10 VI.01 (2016) Wavefunction, Inc., Irvine). Molecular docking of competitive ligands was carried out using the Molegro Virtual Docker 6.0 software for docking simulations of flexible ligands into the rigid TPO model. The docking space was limited and centered around the heme molecule for competitive inhibitors. The actual docking simulations were performed using the following settings: Number of runs = 100; maximal number of iterations = 10,000; maximal number of poses = 50, and the poses representing the lowest value of the scoring function (MolDockScore) were further analyzed as previously reported [53]. In order to identify the potential binding sites for a non-competitive ligand and an uncompetitive ligand, AlloPred [54] and PARS [41] on-line tools were used. AlloPred uses a novel method of normal mode analysis and pocket features to predict allosteric pockets on proteins. PARS is also based on the normal mode analysis and constitutes a simple and fast method that queries protein dynamics and structural conservation to identify pockets on a protein structure that may exert a regulatory effect upon the binding of a small-molecule ligand. The achilles blind docking on-line tool [54] was used to find possible binding poses of a non-competitive and an uncompetitive ligand.

Visualization of molecular modelling results was achieved with the Maestro Release 2019.1 (Small-Molecule Drug Discovery Suite 2019-1, Schrödinger, LLC, New York, NY, USA, 2019) and PyMol 2.0.4 (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC, New York, NY, USA) software.

3.8. Effect of Pure Substances Combinations on TPO Activity (Isobolographic Analysis)

Results (type and strength of interactions) can be shown on the isobolograms and described by the combination Index (CI). When CI is lower than one, indicates synergy; when CI is equal to one, indicates addition; when CI is higher than one, indicates antagonism [36]. Isobolograms were performed according to Chou [36]. For this assay only substances with 100 µg/mL concentrations were used (according to previous results). Pure substances were mixed in various volume ratios: 1:4, 4:1, 3:2, 2:3, 1:1. All of the mixtures were made in combinations of two substances. The inhibitory assay was made using the same proportions as with single solutions. The evaluation of the interaction was done using the combination index (CI) equation for n-drug combination at an x% inhibition as follows:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{1}{(DRI)_1} + \frac{1}{(DRI)_2}$$

Where CI—sum of the dose of drugs that exerts x% when combined; D_x—for D as a single substance that inhibits a system ×%. CI value shows the type of interactions, which can be synergistic, antagonistic or additive, when its value is smaller, greater or equal to one.

3.9. Statistics

Experimental data were presented as a means ± S.D. For biochemical analyses and means ± SEM for anticancer activity assays. In biochemical analyses, statistical significance was estimated with Tukey's test (for the data obtained from three independent samples of each extract in three parallel experiments; $n = 9$).

4. Conclusions

Our investigation was mainly focused on the interactions between pure polyphenolic inhibitors of TPO in simple models where single polyphenols were applied.

In this study, simple combinations of substances were used to visualize the type of interactions between the food components and their inhibitory influence on the TPO enzyme and thus on changes in biological properties. The most effective TPO inhibitor as well as the compound capable of scavenging free radicals is the rosmarinic acid.

We performed molecular modeling studies of ligand-inhibitor interactions to illustrate and clarify their competitive, uncompetitive, or non-competitive modes of action at the molecular level. In the study, we found nearly additive interactions between rutin and quercetin and between rosmarinic acid and rutin. Slight synergism was observed between the chlorogenic acid and rutin, whereas moderate antagonism was detected between rosmarinic acid and quercetin and between rosmarinic acid and chlorogenic acid. There was antagonism between chlorogenic acid and quercetin. More research should be undertaken to understand completely the mechanism of interactions between bioactive compounds and their influence on TPO activity. It seems difficult to achieve bioavailability at the IC_{50} level obtained in our work (Table 3), more importantly is the knowledge about the interactions of these compounds. As has been shown, they substantially affect their potential inhibitory activity (Figure 5, therefore the effect of the mixture of inhibitors can be substantially different from the predicted and even lower concentrations can be effective. An explanation of these relationships is extremely difficult and requires further, extensive and interdisciplinary research.

Supplementary Materials: The following are available online, Figure S1: A—Homology model of TPO. Protein shown in cartoon representation with subunits colored light and dark blue. Disulfide bridges depicted as spheres with yellow sulfur atoms. Heme molecules in the MPO domain presented as spheres with magenta carbon atoms. B—The Ramachandran plot for the homology model of TPO. Glycine residues shown as triangles and proline residues as squares. C—3D view of heme binding site at TPO. Heme molecule shown as sticks with magenta carbon atoms. Protein presented in wire representation with grey carbon atoms. Most important residues shown as sticks. Hydrogen bonds depicted as red dashed lines. Non-polar hydrogen atoms omitted for clarity.

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Sample Availability: Samples of the compounds are available from the authors.

biomolecules

MDPI

Article

Mechanism of Action and Interactions between Thyroid Peroxidase and Lipoxygenase Inhibitors Derived from Plant Sources

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Abstract: This study focused on the effect of kaempferol, catechin, apigenin, sinapinic acid, and extracts from plants (i.e., parsley, cumin, mustard, green tea, and green coffee) on thyroid peroxidase (TPO) and lipoxygenase (LOX) activity, antiradical potential, as well as the result of interactions among them. Catechin, sinapinic acid, and kaempferol acted as a competitive TPO inhibitors, while apigenin demonstrated an uncompetitive mode of inhibitory action. Ethanol extracts from all plants acted as competitive TPO inhibitors, while, after in vitro digestion, TPO activation was found especially in the case of mustard (24%) and cumin (19.85%). Most importantly, TPO activators acted synergistically. The TPO effectors acted as LOX inhibitors. The most effective were potentially bioaccessible compounds from green tea and green coffee (IC_{50} = 29.73 mg DW/mL and 30.43 mg DW/mL, respectively). The highest free radical scavenging ability was determined for catechin and sinapinic acid (IC_{50} = 78.37 μg/mL and 84.33 μg/mL, respectively) and potentially bioaccessible compounds from mustard (0.42 mg DW/mL) and green coffee (0.87 mg DW/mL). Green coffee, green tea, cumin, and mustard contain potentially bioaccessible TPO activators that also act as effective LOX inhibitors, which indicate their potentially health-promoting effects for people suffering from Hashimoto's disease.

Keywords: thyroid peroxidase; lipoxygenase; inhibition; dietary polyphenols; antioxidant activity

1. Introduction

Thyroid peroxidase, also called thyroperoxidase (TPO, EC 1.11.1.1-14) or iodide peroxidase, catalyzes iodide oxidation to form iodine atoms which are added onto tyrosine residues on thyroglobulin for the production of thyroxine or triiodothyronine, i.e., thyroid hormones [1]. Thyroid peroxidase is the major antigen in human Hashimoto's disease, and anti-TPO antibodies induce complement-dependent cytotoxicity. Furthermore, antibodies against complement (anti-C1q) are detected in patients with Hashimoto's disease. They are correlated with thyroid-stimulating hormone (TSH) levels [2]. During common thyroid disorders (hyper- and hypothyroidism) the activity level of TPO is changed. Thyroiditis is the inflammation of the thyroid gland due to the numerous etiologies [3]. Graves' disease accounts for 50–80% of cases of hyperthyroidism. The most commonly used group of anti-thyroid drugs in patients with Graves' disease are thionamides: methimazole (MMI), propylthiouracil (PTU), and carbimazole (CBZ). Their main effect is to inhibit the synthesis of thyroid hormone by blocking the action of TPO [4].

Hashimoto's thyroiditis (HT) (i.e., chronic lymphocytic thyroiditis) is an autoimmune disease that causes the immune system to attack and destroy the thyroid gland. The resulting inflammation often leads to an underactive thyroid gland (i.e., hypothyroidism). The disease affects between 0.1% and 5%

of all adults in Western countries. Hashimoto's thyroiditis has been associated with thyroid carcinoma and malignant lymphoma of the thyroid. In addition, patients with HT were at higher risk for breast and lung cancer than those in the control group. However, a number of studies did not confirm such an association with thyroid, breast, and lung cancers [5]

Inflammation is a natural defense mechanism against pathogens and is associated with many pathogenic diseases. Many of them are linked with higher production of reactive oxygen species (ROS) resulting in oxidative stress and a variety of protein oxidation events. One of the main enzymes whose activity induces oxidative stress are lipoxygenases (LOXs) [6]. In animal and cell culture studies, activation of the lipid-oxidizing enzyme 12/15-lipoxygenase (12/15-LOX) plays a central role as an inflammatory mediator in the pathology of oxidative stress. The mechanism of 12/15-LOX involves the production of reactive oxygen species through the metabolism of arachidonic acid as well as direct detrimental effects on organelle membranes [7].

Lipoxygenases (LOXs, EC. 1.13.11.12) are widely known for their presence in plants and animals. They are known as nonheme iron-containing dioxygenases. Lipoxygenase isozymes take part in the metabolism of eicosanoids, e.g. prostaglandins, or non-classic eicosanoids [8]. Human lipoxygenases are located on chromosome *17.p13* with the exception of the *5*-LOX gene (located on chromosome *10q11.2*) [5]. The described human lipoxygenases along with their products are associated with such conditions as inflammatory and allergic diseases, atherosclerosis, and several types of cancers [9]. Lipoxygenase is an enzyme with activity that is related with oxidative stress in the human body. The activity of ROS influence the cells' death.

Under normal physiological conditions, the thyroid gland participates in the autoregulation of the redox balance. Excessive ROS activity can disturb this balance, which can affect thyroid enzyme activity. This mechanism is still unclear and some studies should be undertaken to explore this occurrence [10]. Inflammation and oxidative stress (OS) are closely related processes. Among the various hormonal influences that operate on the antioxidant balance, thyroid hormones play particularly important roles, since both hyperthyroidism and hypothyroidism have been shown to be associated with OS in animals and humans [11].

It is known that plants are a rich source of secondary metabolites (i.e., antioxidants) such as polyphenols with documented biological activity. Many polyphenols are known as bioactive substances with antioxidative, antimutagenic, antibacterial, and antiviral activity. To exhibit their biological properties, polyphenols have to be available to some extent in the target tissue. Therefore, the biological properties of dietary polyphenols may depend on their absorption in the gut and bioavailability. The amount of bioaccessible food-related polyphenols may differ quantitatively and qualitatively from polyphenols included in food databases. Moreover, most studies on polyphenol bioavailability used mainly pure single molecules (isolated from food or chemically synthesized), although their bioavailability from whole foods may be substantially different [12]. Furthermore, the activity of phenolic compounds studied in vitro (after isolation thereof from food) does not have to coincide with the activity demonstrated in the human organism. In vitro models based on human physiology are simple, cheap, and repeatable tools for studying the bioaccessibility of food components. They are widely used to investigate structural changes, digestibility, and release of food components in simulated conditions of the alimentary tract [13].

In contrast to synthetic pharmaceuticals, based upon single chemicals, many phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process [14]. However, the synergism of bioactive compounds plays a considerable role; however, there are only a few studies of this issue in such a complicated system as whole foods.

The method used for identification of the interactions among active compounds is referred to as isobolographic analysis. isobolographic analysis is a useful tool for determination of the interactions among components of two-component mixtures as well as those composed of plant extracts being mixtures of many active compounds [15].

This study focused on the influence of biological active substances contained in food components on TPO and LOX activity, as well as the impact of interactions among active substances contained in plant food sources on TPO activity. The results were compared with those obtained for pure chemical compounds which helped draw conclusions about the impact of the food matrix on the biological activity of phytochemicals. The present study determined the effect of four frequently occurring dietary polyphenols (kaempferol, catechin, apigenin, and sinapinic acid) and extracts from five plants rich in such polyphenols (i.e., parsley, cumin, mustard, green tea, and green coffee) on TPO and LOX activity in order to explain these mechanisms. Polyphenols were chosen on the basis of the database of natural polyphenols [16] and preliminary studies of the influence of pure polyphenols and selection of those which simultaneously act as TPO and LOX inhibitors (data unpublished). Plants were selected based on a database of natural polyphenols [16].

2. Materials and Methods

2.1. Chemicals

Sucrose (α-D-glucopyranosyl-(1→4)-β-D-fructofuranoside), tris (1,3-propanediol-2-amino-2-hydroxymethyl), KCl, NaCl, MgCl2, 90% ethanol, NaOH, guaiacol (2-methoxyphenol), H_2O_2 (hydrogen peroxide), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), lipoxygenase (LOX), xanthine oxidase (XO), xanthine, pancreatin, pepsin, bile extract, linoleic acid, α-amylase, sinapinic acid, apigenin, catechin, and kaempferol were purchased from Sigma–Aldrich (Poznan, Poland). All other chemicals were of analytical grade.

2.2. Material

Porcine thyroid glands were obtained at a local slaughterhouse (Lubmeat S.A., Lublin, Poland) and stored at −20 °C until used. The experimental material consisted of lyophilized parsley leaves, green coffee, green tea, cumin, and mustard which were bought from a local supermarket (TESCO, Lublin, Poland).

2.3. Preparation of Pure Substance Solutions

Sinapinic acid, catechin, apigenin, and kaempferol were diluted in 50% ethanol to concentrations 0.125 μg/mL, 0.25 μg/mL, 0.5 μg/mL, 1.0 μg/mL, 2.0 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL and used for further assay.

2.4. Raw Extracts Preparation

For extraction 1.5 g of individual vegetables were homogenized with 15 mL of 50% ethanol and, subsequently, the samples were shaken for 30 min at room temperature. After centrifuging (15 min, 4000 rpm), the extraction procedure was repeated. The final volume was brought to 50 mL with 50% ethanol. The final extract concentration was 30 mg/mL. Extracts were then diluted to a concentration 0.3 mg/mL and 3 mg/mL.

2.5. High-Performance Liquid Chromatography-Diode-Array Detector Analysis

The high-performance liquid chromatography-diode-array detector (HPLC-DAD) analysis was carried out on a Shimadzu Model SPD-M20A 230V equipped with a column (COSMOSIL 5Diol-20-II Packed Column 7.5 mm ID × 300 mm, UVISON TECHNOLOGIES LIMITED Wrotham, England), city, state (if U, country). For analysis, a 100 μL sample of the extract was injected onto a column. Acetic acid (1%) with formic acid (0.1%, *v*/*v*) and acetonitrile with 0.1% formic acid were used as mobile phases A and B, respectively. The column temperature was maintained at 30 °C. Polyphenols were monitored at 280 nm [17]. Pure phenolic peaks (apigenin, catechin, kaempferol, sinapinic acid) in four concentrations (25 μL, 50 μL, 100 μL, 200 μL) were used to prepare the standard curve. The number of phenolic compounds (μg/g dry weight) was calculated by comparison of the peak areas of the samples with those of standards.

2.6. In Vitro Digestion

In vitro digestion of plant materials was performed according to Minekus et al. [18] with some modifications. The amount of 0.5 g of plant source was mixed with 6 mL of simulated saliva fluid (SSF). 22.75 mL of SSF, 0.163 mL $CaCl_2$, and 3.25 mL of amylase were added to sample probe. The mixture was incubated for 2 min at 37 °C. The gastric phase was performed as follows: 48.75 mL of simulated gastric fluid SGF, 4.518 mL of H_2O, 1.3 mL of HCl, and 10.4 mL of pepsin were mixed with a sample probe after a simulated oral phase, brought to pH 2.0, and incubated for two hours. The intestinal phase was performed as described in the protocol. A blank sample was prepared with 0.5 mL of distilled water. In the end, the mixture was centrifuged at 4500 rpm for 15 min. The supernatant was then stored at -20 °C until further analysis.

2.7. In Vitro Antioxidant Capacity Assay

The ABTS radical scavenging activity was determined according to Re et al. [19], with some modifications. A 250 µL ABTS solution was mixed with 10 µL of each pure polyphenols solutions (concentration 50 µg/mL), ethanol extracts, and digested extracts (concentrations: 0.3 mg/mL), and then was measured at the wavelength 724 nm using a UV/Vis spectrophotometer (BioTek, Model Epoch2TC, Winooski, VT, USA) after 15 min of incubation at room temperature. The inhibition percentage of ABTS discoloration was calculated using the fallowing equation:

$$AA = [(Ac - Ap)/(Ac)] \times 100\% \quad (1)$$

where Ac is the absorbance of control, Ap is the absorbance of pure polyphenols solutions

The half maximal inhibitory concentration IC_{50} value was determined by interpolation of the dose–response curves. The IC_{50} values were calculated at fitted models as, the concentration of the tested compound gave 50% of the maximum inhibition based on a dose-dependent mode of action.

2.8. Thyroid Peroxidase Preparation

The assay was prepared according to Jomaa et al. [20] with some modifications. Frozen thyroid gland was cut into slices and homogenized in buffer containing 0.25 M sucrose, 2 mM Tris-HCl, 100 mM KCl, 40 mM NaCl, and 10 mM MgCl2 (pH 7.4) with Philips homogenizer. The thyroid gland was than centrifuged two times at 4000 RPM for 15 min at 4 °C. The enzyme protein was then salted-out to 60%. The supernatant was used for further analysis and stored at −20 °C.

The activity of the enzyme was assayed using guaiacol assay. The reaction mixture contained: 33 mM guaiacol, 0.27 mM H_2O_2, and 33 mM prepared sucrose buffer. The reaction components were incubated in 37 °C before assignment. The absorbance was determined using a Shimadzu spectrophotometer (Model UV-1280, Shimadzu Corporation, Kyoto, Japan) at a wavelength 470 nm. The assay was conducted as follows: to a cuvette, 180 µL of buffer, 100 µL of guaiacol, and 40 µL of TPO were mixed to the final volume of 420 µL. The cuvette was then placed into the spectrophotometer and the reaction was started by the addition of 100 µL H_2O_2. Absorbance readings were recorded every minute for a total of 3 min. Verification of the TPO activity was accomplished by linearly correlating TPO concentration with absorbance readings.

2.9. Thyroid Peroxidase Inhibitory Assay

The assay was used according to Jomaa et al. [20] with some modification. The measurement was made using a plate spectrophotometer (BioTek) in 96 well plates at a wavelength of 470 nm. The assay was conducted as follows: 50 µL of buffer, 40 µL of pure substance solution or ethanol extracts solution or in vitro digested solution, 50 µL of guaiacol, 20 µL of TPO enzyme, and 50 µL H_2O_2. The total volume of the mixture was 210 µL. In the sample probe, extracts were replaced by buffer. Absorbance readings were recorded every minute for a total of 3 min at 37 °C, as a unit of TPO activity is defined as the change in the absorbance per minute. All measurements were performed in three replicates.

The TPO inhibitory activity was calculated as follows:

$$\%\text{inhibition} = (1 - (\Delta A/[\![\min]\!]\_\text{test})/(\Delta A[\![\min]\!]\_\text{blank})) \times 100\% \quad (2)$$

where ΔA/min test is the linear absorbance change per minute of the test material and ΔA min blank is the linear change in absorbance per minute of blank.

The IC_{50} value was determined by interpolation of dose–response curves. The IC_{50} values were calculated at fitted models as the concentration of the tested compound that gave 50% of the maximum inhibition based on a dose-dependent mode of action. The mode of inhibition of the enzyme was performed using the Lineweaver–Burk plot.

2.10. Inhibition of Lipoxygenase Activity

The inhibition of LOX with linoleic acid as a substrate was measured spectrophotometrically, based on Axelrod et al. [21] and adopted for a microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek Instruments). The mixture contained 240 µL 0.066 M phosphate buffer, 10 µL LOX solution, 10 µL pure substance solution, ethanol extract solution or digested extract. The reaction was started by adding 40 µL 2.5 mmol/L linoleic acid. For a unit of LOX activity, the change in the absorbance per minute at the wavelength 234 nm was defined. All measurements were performed in three replicates.

The IC_{50} values were calculated at fitted models as the concentration of the tested compound that gave 50% of the maximum inhibition based on a dose-dependent mode of action. The mode of inhibition of the enzyme was performed using the Lineweaver–Burk plots.

2.11. Isobolographic Analysis

The results (type of interactions) can be shown on isobolograms as per Chou's method [15]. Synergism means that two components mutually enhance their activities (concave isobole) antagonism means that two components decrease the effect of the single component (convex isobole), and additive interaction (straight line) [15].

For this assay, only substances with a 100 µg/mL concentration were used (according to previous results). Pure substances were mixed in various volume ratio: 3:2, 2:3, and 1:1. All of mixtures were made in combinations of two pure substances or two extracts (digested in concentrations of 0.3 mg/mL).

2.12. Statistical Analysis

All tests were performed in triplicate. The results were statistically analyzed in the Statistica. One-way analysis of variance (ANOVA) was made with the significance level $\alpha = 0.05$. Tukey's test was used to evaluate the differences among means.

3. Results and Discussion

3.1. High-Performance Liquid Chromatography-Diode-Array Detector Phenolic Analysis

As previously mentioned, fruits and vegetables are rich sources of polyphenols. In present study, five plant extracts were used as a source of different polyphenols. Green tea and green coffee are a rich source of catechin; however, a significantly higher content was found in green coffee beans (Table 1). Apigenin can be found in parsley leaves, kaempferol in cumin, and sinapinic acid in mustard. The content of each polyphenol is shown in Table 1.

Table 1. Concentration of chosen polyphenols in ethanol extracts (n = 9).

Plant Source	Polyphenol	Concentration (mg/g DW)
Green coffee	Catechin	47.7 ± 0.23 [a]
Green tea	Catechin	11.5 ± 0.58 [b]
Parsley	Apigenin	0.69 ± 0.034
Cumin	Kaempferol	0.09 ± 0.004
Mustard	Sinapinic acid	1.25 ± 0.627

The values are expressed as the mean ± SD; means with different letter superscripts ([a],[b]) are significantly different (α = 0.05).

The data concerning kaempferol content in cumin were in accordance with those provided by Shan et al. [22]. Studies conducted by Ani et al. [23] showed a higher amount of kaempferol (94.70 µg/g DW) detected in *Cumin nigrum* seeds.

The content of apigenin in parsley depends on the place where it was bought. Such conclusions were drawn by Głowacki et al. [24]. In our study, the lower content of apigenin was determined (0.69 mg/g DW). On the other hand, it was higher than that given by Yashin et al. [25]. The concentration of sinapinic acid in mustard detected by Engels et al. [26] (2.66 mg/g) was higher than that obtained in our study; however, no alkaline hydrolysis was carried out in our studies. The content of catechin depends on the source of the tea or coffee. Our results are in agreement with those obtained by Henning at al. [27]. Of course, plant extracts contain the entire spectrum of polyphenols, and the activity depends on their composition and interactions. Stan et al. [28] identified flavones apigenin and luteolin and the flavonols quercetin and kaempferol in ethanolic extracts from parsley leaves using an HPLC Shimadzu apparatus equipped with PDA and MS detectors. In mustard cotyledons and hulls, the major phenolics were sinapine (SP), with small amounts of sinapoyl glucose (SG), and sinapinic acid (SA) with a significant difference in phenolic contents among the two seed fractions. Cotyledons showed a relatively high content of SP, SA, SG, and total phenolics in comparison to hulls [29]. In the study by Acimovic at al. [30], hydroxybenzoic and hydroxycinnamic acids, as well as glycosides of flavonones and flavonoles were most abundant in the cumin samples from Serbia. The phenolic content of green tea is widely diverse, although catechins are the major constituents; however, other flavonoids and phenolic acids have also been identified [31].

Thus, an additional goal of our work was to show that predicting the activity of plant extracts based only on the content of one specific compound (as it happens in the standardization of plant extracts) can lead to errors.

3.2. Antioxidant Analysis

The antioxidant capacity of pure polyphenols (i.e., kaempferol, apigenin, catechin, and sinapinic acid), ethanolic, and digested extracts was evaluated by the most commonly used antioxidant assay—the ABTS method. The ABTS radical scavenging abilities of catechin and sinapinic acid were found to be the highest (78.37 µg/mL and 84.33 µg/mL, respectively). The lowest antiradical activity was observed for apigenin (405.93 µg/mL) (Table 2).

Table 2. Comparison of ABTS radical scavenging ability (expressed as IC_{50} values) of pure chemicals, ethanol plant extracts (EEs), and digested plant extracts (DEs) (n = 9).

Pure chemical standards	**IC_{50} (mg/mL)**	
Sinapinic acid	0.084 ± 0. 001 ^{a}	
Apigenin	0.406 ± 0.003 ^{c}	
Catechin	0.078 ± 0.008 ^{a}	
Kaempferol	0.337 ± 0.002 ^{b}	
Plant extracts	**IC_{50} (mg DW/mL)**	
Plant	**EE**	**DE**
Parsley	3.47 ± 0.17 ^{e}	1.09 ± 0.05 ^{c}
Green coffee	0.78 ± 0.04 ^{d}	0.87 ± 0.04 ^{b}
Green tea	0.06 ± 0.0003 ^{a}	1.33 ± 0.06 ^{d}
Cumin	0.32 ± 0.01 ^{b}	6.52 ± 0.32 ^{e}
Mustard	0.41 ± 0.02 ^{c}	0.42 ± 0.02 ^{a}

Values are expressed as the mean ± SD; means with different letter superscripts (^{a}–^{e}) in the columns are significantly different (α = 0.05). ABTS -(2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, IC_{50}—The half maximal inhibitory concentration.

The present results show the mutual dependence between pure polyphenolic solutions (Table 2) and plant extracts, which are a source of the investigated polyphenols (Table 1). As expected, the antioxidant activities of plant extracts were significantly lower than the activity of the pure compounds; however, the antiradical activity of the tested extracts was relatively high. The lowest antiradical activity (IC_{50} = 3.47 mg DW/mL) was determined for parsley ethanolic extract. Hinneburg et al. [32] reported that the parsley hydro-distilled extract showed an IC_{50} value of 12.0 ± 0.10 mg/mL in the DPPH scavenging assay. This shows that phenolic compounds of parsley could be responsible for the observed DPPH radical scavenging activity, since these compounds can readily donate hydrogen atoms to the radical. On the other hand, Tang et al. [33] indicated the lack of antiradical activity of methanol extract from parsley. The highest antiradical activity was determined for ethanol extract from green tea. Interestingly, the simulated digestion caused a decrease in antiradical activity in all the raw materials tested (with the exception of mustard). These types of impacts are documented in the literature [34].

3.3. Thyroid Peroxidase Inhibitory Potential

The next step of our investigation was to determine the inhibitory potential of the tested polyphenols and prepare ethanol and digested extracts with anti-TPO activity. As expected, the pure chemical solutions demonstrated high TPO inhibitory activity. Pure substances showed a dose-independent inhibitory effect, thus effective concentrations (EFC_{50}) inhibiting TPO in 50%, of pure polyphenolic substances were used in the study. The highest TPO-inhibitory activity was found for sinapinic acid and catechin, whereas the lowest was for apigenin (EFC_{50} = 25.42, 29.76, and 116.28 µg/mL, respectively). The chosen polyphenols showed a different mode of TPO inhibition (Table 3). Catechin, sinapinic acid, and kaempferol acted as competitive inhibitors, while apigenin demonstrated an uncompetitive mode of inhibitory action (Figure 1).

Table 3. The EFC_{50} (effective concentration), Ki, and V_{max} values and mode of thyroid peroxidase inhibition of catechin, kaempferol, sinapinic acid, and apigenin solutions (n = 9).

Substance	**Mode of Inhibition**	**EFC_{50} (µg/mL)**	**Ki (µg/mL)**	**V_{max} (ΔAU/min)**
Sinapinic acid	Competitive	25.42 ± 1.13 ^{a}	45.92 ± 3.22 ^{b}	149.25 ± 6.26 ^{a}
Apigenin	Uncompetitive	116.28 ± 5.38 ^{d}	338.73 ± 12.31 ^{d}	149.25 ± 5.12 ^{a}
Catechin	Competitive	29.76 ± 2.05 ^{b}	33.42 ± 1.35 ^{a}	144.93 ± 4.33 ^{a}
Kaempferol	Competitive	61.68 ± 3.12 ^{c}	186.95 ± 6.89 ^{c}	192.31 ± 8.34 ^{b}

The values are expressed as the mean ± SD; means with different letter superscripts (^{a}–^{d}) in the columns are significantly different (α = 0.05).

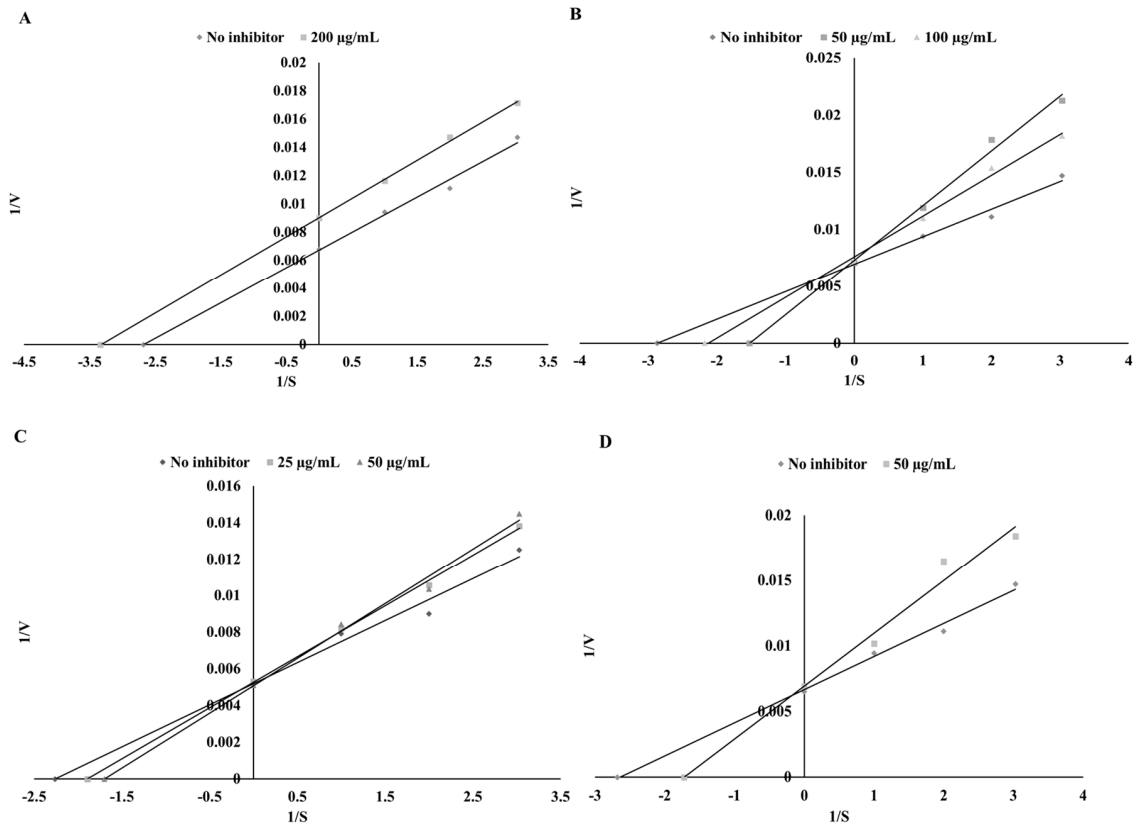

Figure 1. Mode of TPO inhibition by pure polyphenols: apigenin (**A**), catechin (**B**), kaempferol (**C**), and sinapinic acid (**D**).

Based on preliminary studies two concentrations of the extracts were chosen, i.e., 0.3 mg DW/mL and 3 mg DW/mL. Depending on the content of each polyphenol in the tested extracts, some interrelations were observed. As a rich source of catechin, green tea showed a competitive mode of inhibition in the case of the ethanol extracts and activation in the case of the digested extracts (Figures 2 and 3). The same result was observed for cumin and kaempferol (Figure 1) as well as for green coffee and catechin (Figures 2 and 3). In the case of sinapinic acid, a competitive mode of inhibition was observed for the pure substance and for mustard ethanol extract (at a concentration of 0.3 mg DW/mL). For higher concentrations, a competitive mode of inhibition was found (Figure 3). In the case of digested extract from mustard, slight activation was observed for concentrations of 0.3 mg DW/mL (Figure 3). Different modes of inhibition were observed, depending on the concentration of the extract used. Apigenin showed an uncompetitive mode of inhibition, whereas the ethanol extract of parsley (a rich source of apigenin) exhibited a competitive mode of inhibition (Figure 2). The digested parsley extract showed activation at the lower concentration and mixed type of inhibition for the higher concentration. The possible cause of these differences may be the influence of the food matrix. As shown, simulated digestion can strongly affect the final mode of action against the TPO enzyme. While the ethanol extracts from the tested plants inhibited the activity of TPO, conditions occurring during in vitro digestion resulted in the loss of the ability to inhibit this enzyme. Most importantly, in all cases, the ability to activate the TPO enzyme was observed. The highest ability to activate TPO was found for potentially bioaccessible compounds from mustard (24.66%) and cumin (19.85%) (Table 4). Similar dependencies were reported by Gawlik-Dziki et al. [35] during studies on the effect of plant extracts on LOX activity.

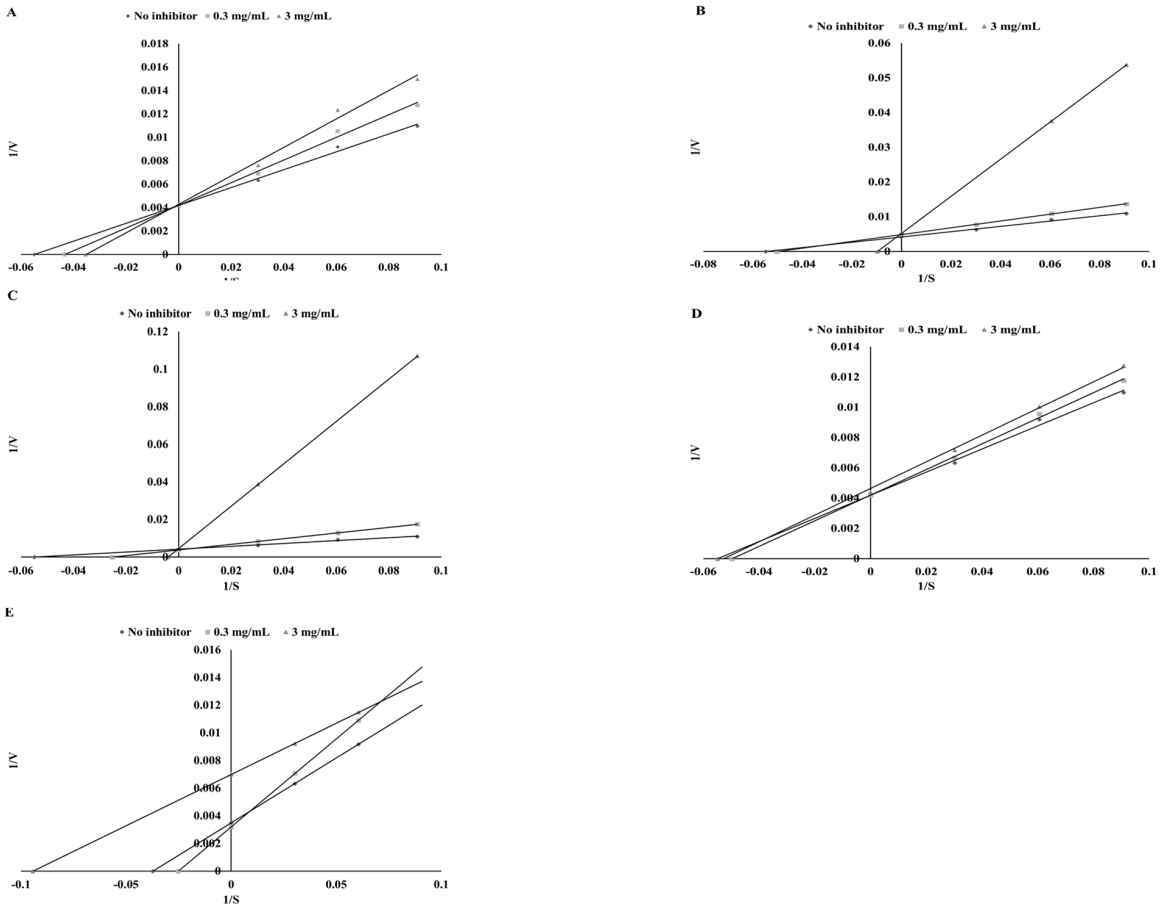

Figure 2. Mode of TPO inhibition by ethanol extracts from parsley (**A**), green coffee (**B**), green tea (**C**), cumin (**D**), and mustard (**E**).

In our investigation, unexpected results were obtained using classic Lineweaver–Burk analysis. As shown in the graph below (Figure 3), the line without an inhibitor runs higher than that with the inhibitor, which indicates activation.

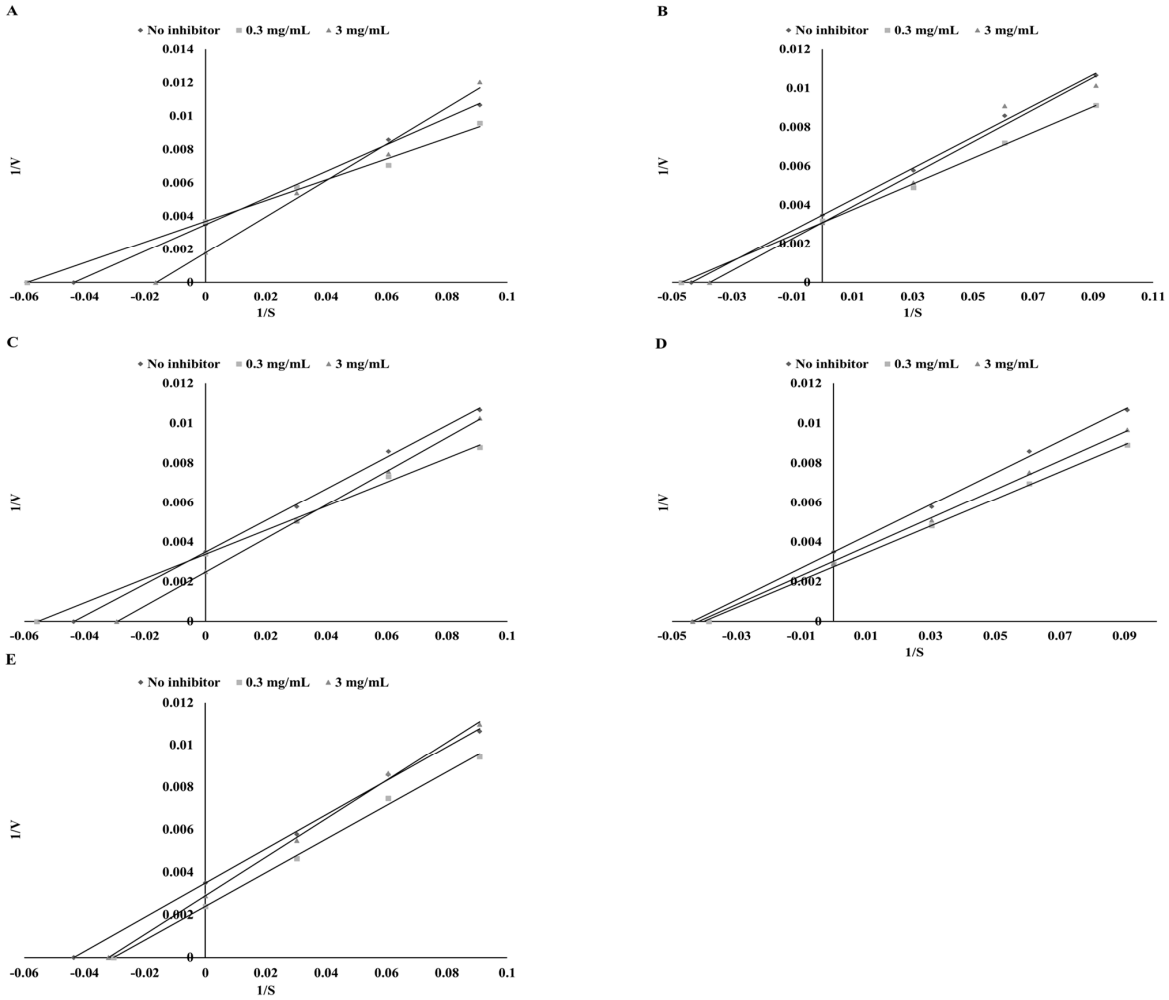

Figure 3. Mode of TPO influence by digested extracts of parsley (**A**), green coffee (**B**), green tea (**C**), cumin (**D**), and mustard (**E**).

Table 4. IC_{50} value, Ki value, V_{max} value and impact on TPO of ethanol and digested extracts from parsley, cumin, green tea, green coffee, and mustard (n = 9).

Plant	Ethanol Extracts				Extracts after Digestion in Vitro	
	Mode of Inhibition	IC_{50} (mg DW/mL)	Ki (mg DW/mL)	V_{max} (Δ AU/min)	Action	% of Activation
Parsley	competitive	103.45 ± 3.12 ^{ab}	387.42 ± 11.53 ^{c}	238.1	activation	0.39 ± 0.06 ^{a}
Green coffee	competitive	185.01 ± 6.32 ^{d}	1971.0 ± 53.5 ^{d}	238.1	activation	17.73 ± 1.12 ^{c}
Green tea	competitive	126.26 ± 4.15 ^{c}	107.12 ± 3.15 ^{a}	238.1	activation	14.07 ± 0.92 ^{b}
Cumin	competitive	100.00 ± 3.21 ^{a}	1891.5 ± 42.6 ^{d}	238.1	activation	19.85 ± 1.30 ^{c}
Mustard	competitive	106.13 ± 4.15 ^{b}	218.4 ± 5.39 ^{b}	285.7	activation	24.66 ± 1.42 ^{d}

The values are expressed as the mean ± SD; means with different letter superscripts (^{a}–^{d}) in the columns are significantly different (α = 0.05).

3.4. Lipoxigenase Inhibitory Potential

Another very interesting question was whether there was some dependency among the modes of action of TPO-effective plant extracts with LOX. As presented in Table 5, the highest LOX inhibitory potential was found for green tea and green coffee ethanol extracts (IC_{50} = 13.74 and 15.96 mg DW/mL), whereas the lowest was in the case of mustard extract (29.01 mg DW/mL). As presented in Table 5 and Figure 4, a mixed type of LOX inhibition was obtained in most of the ethanol extracts. Only in the case

of mustard ethanol extract was a competitive mode of inhibition observed. After simulated digestion, the mode of LOX inhibition changed in most of the extracts except for mustard (Figure 5).

Table 5. IC_{50} value, Ki value, Vmax value, and mode of LOX inhibition by ethanol and digested extracts from parsley, cumin, green tea, green coffee, and mustard (n = 9).

Plant	Ethanol Extracts			Extracts after Digestion in Vitro		
	Mode of Inhibition	IC_{50} (mg DW/mL)	Ki (mg DW/mL)	Mode of Inhibition	IC_{50} (mg DW/mL)	Ki (mg DW/mL)
Parsley	mixed	24.39 ± 1.18^{b}	32.85 ± 1.43^{d}	uncompetitive	45.05 ± 1.98^{c}	6.44 ± 0.53^{b}
Green coffee	mixed	15.96 ± 1.12^{a}	2.82 ± 0.16^{a}	noncompetitive	30.43 ± 1.52^{a}	30.43 ± 1.16^{d}
Green tea	mixed	13.74 ± 0.92^{a}	2.84 ± 0.21^{a}	uncompetitive	29.73 ± 1.12^{a}	4.96 ± 0.19^{a}
Cumin	mixed	21.54 ± 1.23^{b}	12.61 ± 0.96^{b}	competitive	34.13 ± 0.85^{b}	26.94 ± 0.97^{c}
Mustard	competitive	29.01 ± 1.41^{c}	22.46 ± 1.18^{c}	competitive	32.01 ± 1.02^{ab}	72.74 ± 2.16^{e}

The values are expressed as the mean ± SD; means with different letter superscripts (^{a}–^{e}) in the columns are significantly different (α = 0.05).

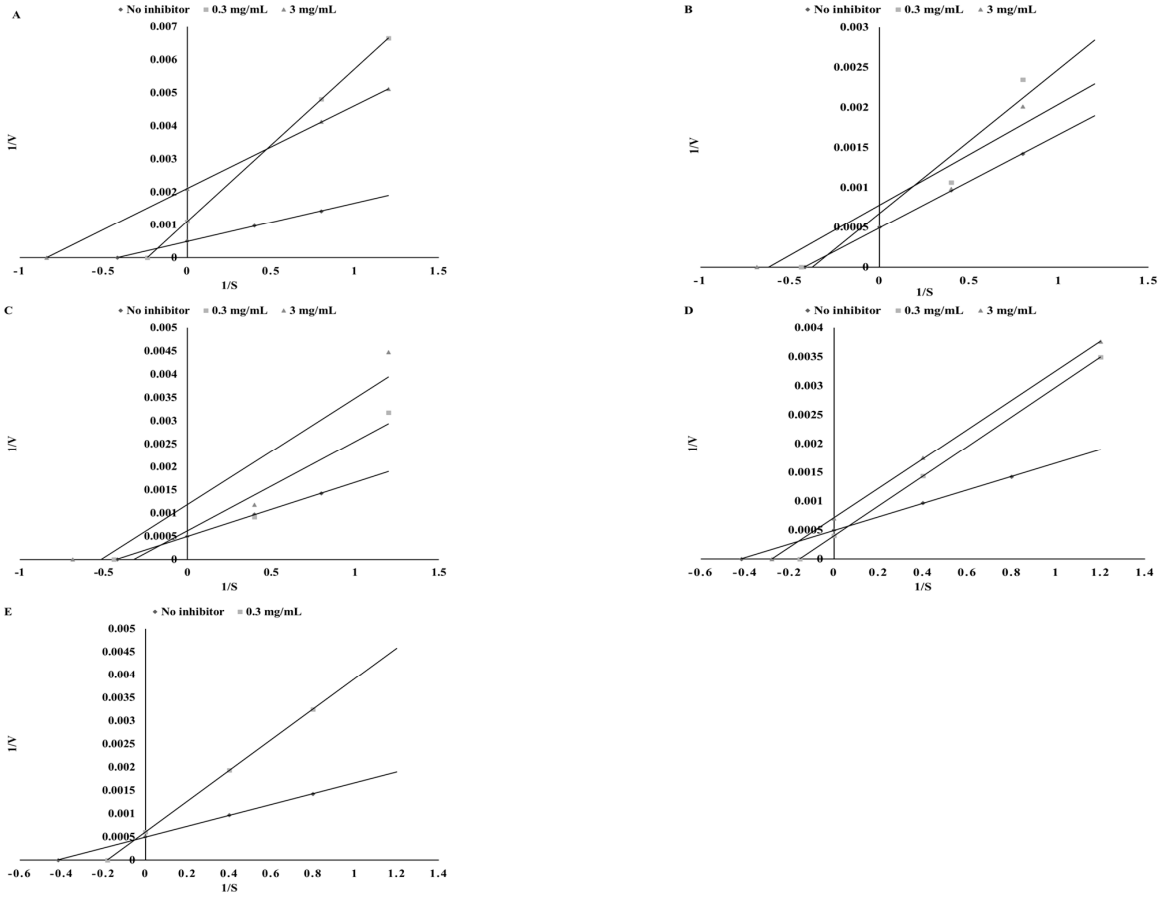

Figure 4. Mode of LOX inhibition by ethanol extracts from parsley (**A**), green coffee (**B**), green tea (**C**), cumin (**D**), and mustard (**E**).

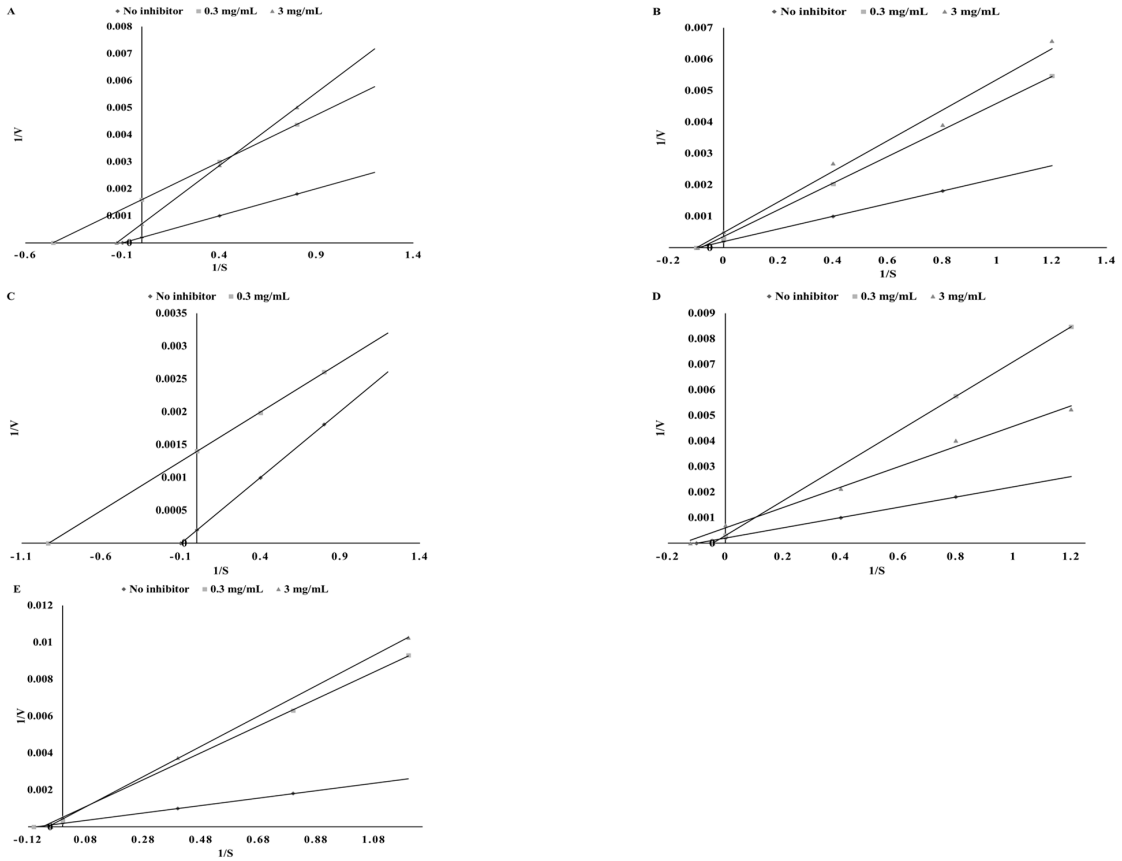

Figure 5. Mode of LOX inhibition by digested extracts from parsley (**A**), green coffee (**B**), green tea (**C**), cumin (**D**), and mustard (**E**).

3.5. Interaction Assay

Many studies have been undertaken to explore the influence of particular drug components on TPO activity and interactions among these components. The data on the influence of plant extracts on in vitro TPO activity are sparse. As described above, there are literature data confirming that polyphenolic compounds have an impact on enzymatic activity, but there are very limited data about the influence of polyphenolic compounds on TPO activity. It is known that polyphenolics contained in food sources appear in more complex combinations. Additionally, medicines with more than one active substance are more effective [36].

As shown in Figure 3, potentially bioaccessible compounds from tested plants activate TPO. This is very important especially for people suffering from Hashimoto's disease. However, the intake of the amount giving a significant effect is in practice very difficult, which is why the next step was to determine the type of interaction among the tested extracts. Isobolographic analysis is a method used for identification of interactions among active compounds. This method is independent of the mechanism of activity; it is a useful tool for determination of interactions between the components of two-component mixtures, as well as those composed of plant extracts being mixtures of many active compounds [15].

As shown in the graphs (Figure 6), the digested extract acted synergistically in all mixtures.

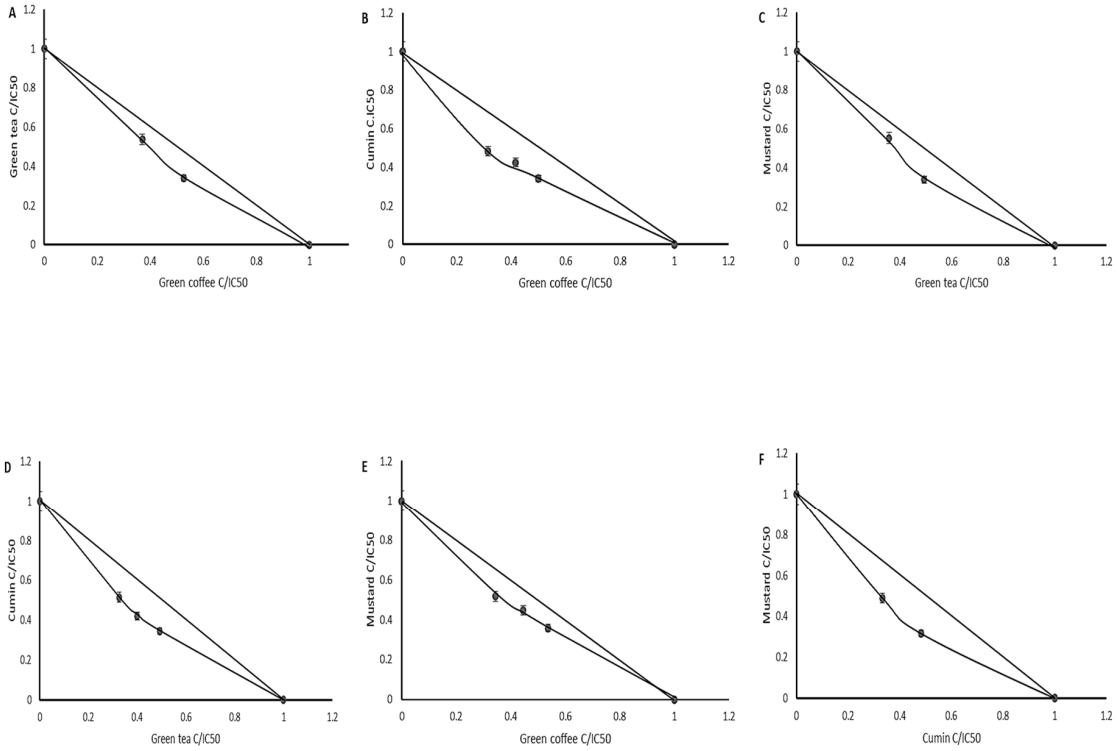

Figure 6. Dose-normalized isobolograms for digested extracts from green tea and green coffee (**A**), cumin and green coffee (**B**), mustard and green tea (**C**), cumin and green tea (**D**), mustard and green coffee(**E**), mustard and cumin (**F**) affecting TPO activity.

Isobolographic analysis is mainly used for characterization of pharmaceuticals. There are not much data about the influence of polyphenolic combinations on TPO activity. As mentioned above, scientists pay attention mainly for the interactions among pharmaceuticals, but there are some studies focused also on the interactions between food additives or food sources. These data are relatively new. Studies provided by Lau et al. [37] show the synergistic type of interaction among popular food additives such as aspartame, Quinoline Yellow, and between Brilliant Blue and L-glutamic acid. Synergism means that the occurrence of both substances in consumed food has a higher impact on humans' health than the occurrence of each substance separately. Used combinations of additives showed higher neurotoxicity by reducing the length of neurite outgrowth. Knowledge about the interactions of substances different than pharmaceuticals is still scarce. The lack of information generates a need for these kinds of studies.

The influence of pure phenolic substances on TPO activity has been shown by Divi and Doerge [38]. There are some studies showing the influence of phytochemical compounds on the activity of other enzymes, e.g., lipoxygenase. Studies conducted by Durak et al. [39] demonstrated inhibitory activity of pure and dietary polyphenols on LOX activity and interactions among these inhibitors. The biological advantages of polyphenols included in plant sources of food appear useful for the development of bioactive functional food. Potent TPO effectors (inhibitors and activators) and potent inhibitors of LOX could be dedicated for subjects with some health diseases, e.g., hyperthyroidism and hypothyroidism. The occurrence of these types of diseases is associated with inflammation caused by the activity of prooxidative enzymes. Selection of foods with documented desirable features may provide dual benefits to human health. The raw material and the proposed functional combinations may be helpful in dietary therapy and prevention of thyroid dysfunctions correlated with an increase in the activity of LOX. This study highlights the need for the testing of interactions between active ingredients of designed functional products.

4. Conclusions

Green coffee, green tea, cumin, and mustard contain potentially bioaccessible TPO activators that also act as effective LOX inhibitors, which may point to their potentially health-promoting effects for people suffering from Hashimoto's disease. It should be emphasized, however, that these are preliminary tests carried out in model systems and require further verification. Additionally, the results presented in this paper indicate a number of factors that complicate the biochemical studies that have been used for years. First is the dose-independent mode of action, which makes it impossible to set IC_{50} values. In addition, in many cases, the mechanism of inhibition varies with the concentration of the compound/extract.

In the case of activators, it is also difficult to determine classic kinetic parameters based on the classic Lineweaver–Burk analysis. The obtained results also indicate clearly that the prediction of biological activity of plant extracts based only on their chemical composition may lead to erroneous inference, because the key role in their case is played by changes occurring during digestion and/or interactions with other components of the food matrix.

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Some Dietary Phenolic Compounds Can Activate Thyroid Peroxidase and Inhibit Lipoxygenase-Preliminary Study in the Model Systems

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Abstract: The presented research concerns the triple activity of *trans*-cinnamic (tCA), ferulic (FA) and syringic acids (SA). They act as thyroid peroxidase (TPO) activators, lipoxygenase (LOX) inhibitors and show antiradical activity. All compounds showed a dose-dependent TPO activatory effect, thus the AC_{50} value (the concentration resulting in 50% activation) was determined. The tested compounds can be ranked as follows: tCA > FA > SA with AC_{50} = 0.10, 0.39, 0.69 mM, respectively. Strong synergism was found between FA and SA. The activatory effects of all tested compounds may result from interaction with the TPO allosteric site. It was proposed that conformational change resulting from activator binding to TPO allosteric pocket results from the flexibility of a nearby loop formed by residues Val352-Tyr363. All compounds act as uncompetitive LOX inhibitors. The most effective were tCA and SA, whereas the weakest was FA (IC_{50} = 0.009 mM and IC_{50} 0.027 mM, respectively). In all cases, an interaction between the inhibitors carboxylic groups and side-chain atoms of Arg102 and Arg139 in an allosteric pocket of LOX was suggested. FA/tCA and FA/SA acted synergistically, whereas tCA/SA demonstrated antagonism. The highest antiradical activity was found in the case of SA (IC_{50} = 0.22 mM). FA/tCA and tCA/SA acted synergistically, whereas antagonism was found for the SA/FA mixture.

Keywords: thyroid peroxidase (TPO); lipoxygenase (LOX); inhibition; dietary polyphenols; antioxidant activity; interactions; isobolographic analysis

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1. Introduction

Hashimoto's thyroiditis (HT) (i.e., chronic lymphocytic thyroiditis) is an autoimmune disease, in the course of which the thyroid gland is attacked and destroyed by the immune system. The resulting inflammation often leads to an underactive thyroid gland (i.e., hypothyroidism). The disease affects between 0.1% and 5% of the adult population in Western countries [1]. Unfortunately, the major antigen in human Hashimoto's disease is thyroid peroxidase (thyroperoxidase, TPO, EC 1.11.1.1–14), an enzyme that participates in the synthesis of thyroid hormones. Anti-TPO antibodies induce complement-dependent cytotoxicity. Furthermore, antibodies against complement (anti-C1q) are detected in patients with Hashimoto's disease. They are correlated with thyroid-stimulating hormone (TSH) levels. Thus, many patients with congenital hypothyroidism have problems related to the synthesis or iodination of thyroglobulin (TG), which is connected to TPO deficiency [2].

Disturbances in the functioning of the thyroid have many effects on human health, such as inflammation and oxidative stress [3]. Our previous studies showed that the selected pure polyphenolic substances can affect TPO activity [4].

Inflammation is a natural defense mechanism against pathogens, and it is associated with many disorders, such as microbial and viral infections and exposure to allergens, radiation and toxic chemicals, as well as autoimmune and chronic diseases. There are various possible mechanisms for the anti-inflammatory effects of bioactive compounds, including the inhibition of lipoxygenases (LOX) that catalyze the oxygenation of polyunsaturated fatty acids into potent signal molecules involved in inflammatory processes [5]. LOXs participate in eicosanoid syntheses, such as prostaglandins or nonclassic eicosanoids. The LOX pathway of arachidonic acid metabolism generates reactive oxygen species (ROS), which, together with other arachidonic acid metabolites, play a role in inflammation and tumor growth [1]. LOX activity is related to oxidative stress (OS) in the human body. OS refers to a condition in which the balance between the antioxidant protective system and the production of ROS is disturbed. The oxidative protection system can be effectively supported by various types of exogenous compounds with antioxidant activity. Some of the most effective antioxidants are phenolic compounds, the group of secondary plant metabolites with documented antioxidant activity [6]. The antioxidant effects of polyphenols are mainly due to their redox potential, which enables them to act as reducing agents, donors of hydrogen and quenchers of single oxygen. They also may be inhibitors of free-radical reaction through the inhibition of lipid radical formation and disruption of the propagation of chain auto-oxidation reactions [2].

Our previous studies showed that polyphenols can affect TPO activity [4]. During in vitro and in silico screening tests, some phenolic acids commonly found in food have been found to activate TPO. Ferulic acid can be found, especially in cereals, fruits and vegetables. For human health, it effectively scavenges free radicals and inhibits lipid peroxidation [7]. Some studies mention its cardioprotective effect and its inhibition of tumor promotion [8]. Sources of cinnamic acid are vegetable oils, berries and citrus juices. This compound shows mainly gastroprotective effects [9]. Syringic acid shows hepatoprotective, antihyperglycemic and antimicrobial activity and can be found in several types of alcohols: brandy, rum, whisky, nut liquors and fortified wines. Good sources of these polyphenols also include cereals, dried fruits and vegetable oils [8].

Since phenolic compounds are widely known for their anti-inflammatory and antioxidant properties, it has been hypothesized that TPO activators can inhibit LOX activity and have antioxidant effects.

A factor that essentially influences the final effect of biologically active compounds, especially from food systems, is their interaction. Therefore, another hypothesis has been made, that the interactions of the test compounds may substantially modulate their action as TPO and LOX effectors. To investigate possible interactions, the isobolographic method was used. Isobolographic analysis is a scientific approach that graphically represents antioxidant interactions, thereby facilitating their visual evaluation. It makes the interactions of sample combinations succinct and clear and has been widely established as a gold standard for testing pharmacological interactions with various combinations of fixed fractions [10].

Thus, this research aimed to estimate TPO-activatory and LOX-inhibitory effects, as well as the antiradical potential of ferulic, syringic and *trans*-cinnamic acids. To elucidate the mechanism of the effectors' action, in silico studies were carried out. Another part of this paper consists of the analysis of the kind and strength of possible interactions between tested compounds.

2. Results and Discussion

2.1. TPO Assay

A previous study [4] contained the first part of the investigation, which showed the TPO-inhibitory potential of pure polyphenols. During the next stage of investigation, activatory effects were observed for some other polyphenols. This paper contains the

second part, which contains the TPO activatory effect of the following phenolic compounds: ferulic (FA), syringic (SA) and *trans*-cinnamic acids (tCA). To present this effect, Lineweaver–Burk plots were prepared. A plot without the polyphenol addition, which is above the plots with addition of polyphenols, shows activation (Figure 1).

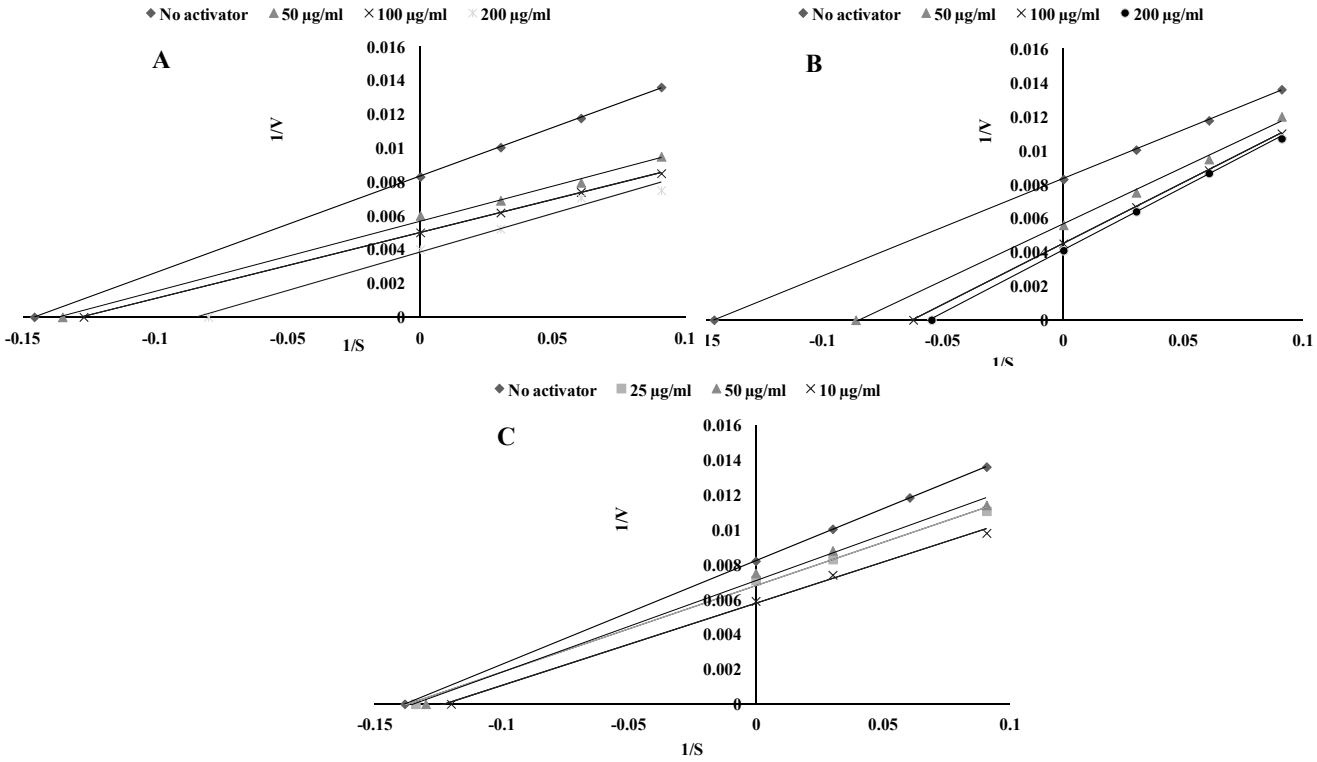

Figure 1. Activatory effect of ferulic acid (**A**), syringic acid (**B**) and *trans*-cinnamic acid (**C**) on thyroid peroxidase TPO activity. Plots are expressed 1/velocity versus 1/guaiacol [µg/mL] without or with activators in a reaction solution.

Tested compounds show a dose-dependent activatory effect, which allowed the determination of the AC_{50} value—the concentration of effector at which 50% activation was obtained. Taking into account this parameter, the tested phenolic acids can be ranked as follows: tCA > FA > SA. The kinetic parameters of TPO activation were presented in Table 1.

Table 1. K_m, V_{max} and AC_{50} values of the chosen phenolic acids on thyroid peroxidase (TPO) activity, $n = 9$.

Compound	K_m [mM]	V_{max} [ΔAU/min]	AC_{50} [mM]
Guaiacol [1]	0.06 ± 0.003	120.5 ± 5.38 [d]	-
trans–cinnamic acid	-	128.2 ± 4.12 [c]	0.10 ± 0.005 [c]
Syringic acid	-	243.9 ± 9.54 [b]	0.69 ± 0.034 [a]
Ferulic acid	-	294.1 ± 9.12 [a]	0.39 ± 0.019 [b]

[1] TPO substrate. Values are expressed as the mean ± SD; means with different letter superscripts (a–d) in the columns are significantly different ($\alpha = 0.05$).

The data showing the influence of phenolic compounds on TPO activity are still incomplete. Most of the studies available concern the inhibitory effect of phenolic compounds on the activity of TPO. TPO-inhibitory potential was described for flavones apigenin, chrysin, vitexin and baicalein, present in parsley, cherries, thyme, olives, tea and broccoli [11]. The flavanols kaempferol, quercetin, fisetin, morin, myricetin and rutin, present in a wide range of food sources such as kale, onions, tomatoes, cherries, apples and red wine, together with the flavanones naringin and naringenin, can also inhibit tyrosine iodination by TPO with varying potencies [12,13]. The antithyroid potential of dihydroxybenzoic acid is also

described [14]. When TPO activity is inhibited, reducing thyroid hormone synthesis, a compensatory increase in TSH may be observed; this may lead to goiter, especially when these compounds are consumed in high quantities [11]. To the best of our knowledge, there are no data on phenolic TPO activators in the available literature.

2.2. Molecular Modeling of TPO Activation

To study TPO activation, a new model of the protein was elaborated. The new model was significantly improved compared to the previous version [4].

The activatory activity of the studied compounds on TPO may be nonspecific and result from their antioxidant properties. In this manner, they may neutralize reactive oxygen species and protect the enzyme against damage. On the other hand, the activatory effect can be specific and result from small-molecule interaction with an allosteric or regulatory site of the enzyme, leading to protein conformational change and enhancing its catalytic activity. Here, such binding sites were identified using PARS and Fpocket online tools and molecular docking with Glide was used to discriminate between the pockets.

Three potential binding cavities were considered most likely to act as regulatory sites for TPO activation: (i) in the vicinity of the heme-binding site, centered around Pro245, Arg370 and Phe523; (ii) on the enzyme dimer interface, centered around Arg175 and Trp176 from one subunit and Trp176, Asp474, Asn478, Lys662 and Asp66 from the other subunit; and (iii) at the binding site centered around Arg412 and Asp536, as shown in Figure 2A. Molecular docking indicated that the highest Glide scores were obtained for all compounds at the binding site involving Arg412 and Asp536. Moreover, docking scores to this site only corresponded to the order of Vmax values. Thus, this pocket, marked in a red square in Figure 2A, was selected for further analysis. Figure 2B–D shows the interactions of the studied compounds with the chosen binding cavity. In the case of all activators, there is an interaction between their carboxylic groups and the main chain atoms of Leu535 and their orientation in the binding pocket is similar. Ferulic acid displays additional interactions between its carboxylic group and the main chain atoms of Asp536 and between its methoxy and hydroxy groups and the main chain atoms of Leu391 (Figure 2B). Syringic acid has its methoxy and hydroxy moieties involved in hydrogen-bond interactions with the side-chain atoms of Arg412 (Figure 2C), while *trans*-cinnamic acid does not form any additional contacts (Figure 2D).

It can be hypothesized that a conformational change resulting from activators binding to this TPO binding pocket results from the flexibility of a nearby loop formed by residues Val352-Tyr363.

2.3. LOX Assay

As inflammation occurs in HT, the next step was to determine the effect of TPO activators on the activity of LOX, one of the main enzymes associated with the occurrence of inflammation and oxidative stress. The similarity in inhibition behavior between soybean LOX-1 and human 5-LOX has been observed, and soybean LOX (sLOX) type 1b has been used for the evaluation of LOX inhibition in drug screening for years [15].

To illustrate this influence, Lineweaver–Burk plots were prepared. As presented in Figure 3, all of the used phenolic acids showed an inhibitory effect. All tested compounds act as uncompetitive LOX inhibitors (Figure 3). The kinetic parameters of LOX inhibition were presented in Table 2. The most effective LOX inhibitors were *trans*-cinnamic and syringic acids with IC_{50} = 0.009 mM, whereas ferulic acid was the weakest LOX inhibitor (IC_{50} = 0.027 mM).

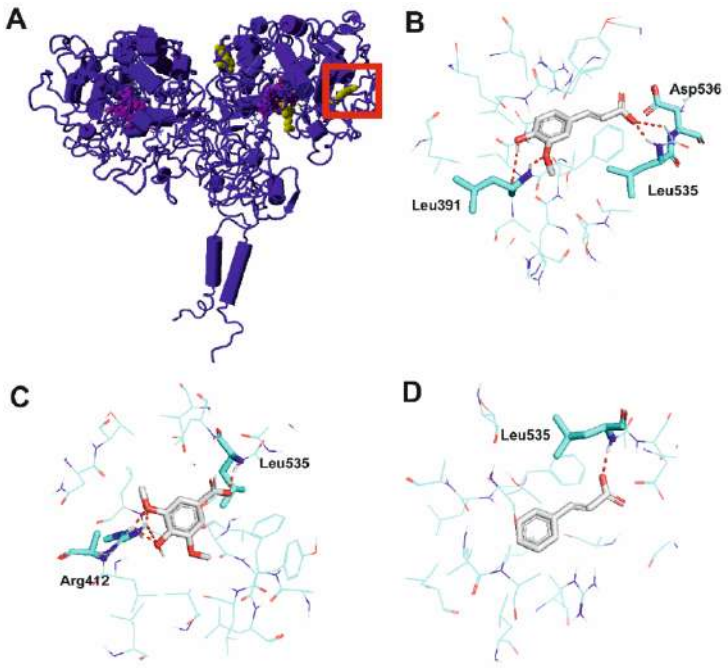

Figure 2. Molecular interactions of activators with thyroid peroxidase (TPO). (**A**) Ferulic acid bound to three considered binding pockets. The protein is shown in dark blue cartoon representation, ferulic acid in yellow ball representation and heme in magenta ball representation. The selected pocket is marked with a red square. (**B**) Ferulic acid, (**C**) syringic acid and (**D**) *trans*-cinnamic acid in a binding pocket of TPO. The protein is shown with cyan carbon atoms in wire representation with the most important residues shown as sticks. Activators depicted with gray carbon atoms in stick representation. Polar bonds are shown as red dashes. Nonpolar hydrogen atoms omitted for clarity.

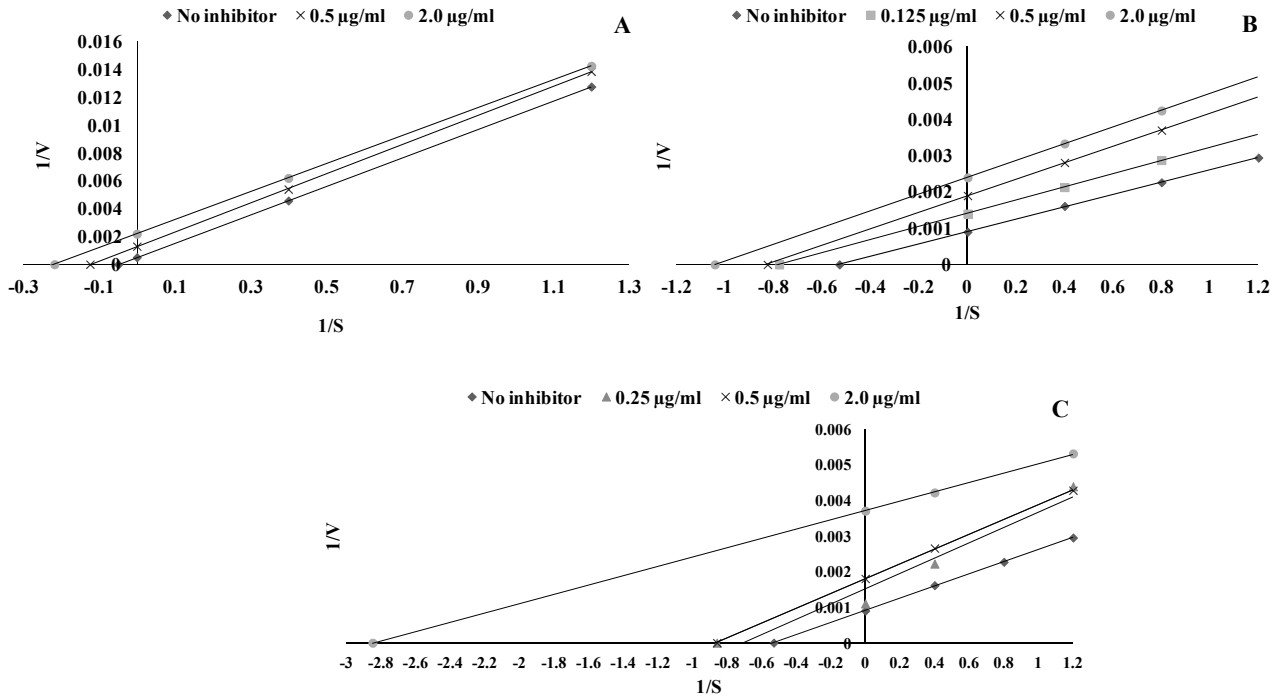

Figure 3. Lineweaver—Burk double reciprocal plots for the inhibition of lipoxygenase (LOX) by ferulic acid (**A**), syringic acid (**B**) and *trans*-cinnamic acid (**C**). Plots are expressed 1/velocity versus 1/linoleic acid [µg/mL] without or with inhibitors in a reaction solution.

Table 2. Mode of inhibition, K_i (K_m without inhibitor), V_{max} and IC_{50} values of chosen phenolic acids on lipoxygenase (LOX) activity.

Compound	Mode of Inhibition	K_i [mM] (K_m without Inhibitor)	V_{max} [ΔAU/min]	IC_{50} [mM]
Linoleic acid *	-	0.007 ± 0.0003 [a]	1111 ± 45.1 [a]	-
trans–cinnamic acid	uncompetitive	0.009 ± 0.0004 [c]	556 ± 10.13 [b]	0.009 ± 0.0004 [b]
Syringic acid	uncompetitive	0.005 ± 0.0003 [d]	526.3 ± 11.02 [c]	0.009 ± 0.0004 [b]
Ferulic acid	uncompetitive	0.008 ± 0.0004 [b]	455 ± 9.9 [d]	0.027 ± 0.0013 [a]

* LOX substrate values are expressed as the mean ± SD; means with different letter superscripts (a–d) in the columns are significantly different (α = 0.05).

In our previous studies [7], it was found that ferulic acid acts as a competitive inhibitor of LOX. The difference may be due to the type of enzyme used for the test and the concentration of the reacting components. The obtained inhibitory potential of pure ferulic acid and *trans*-cinnamic acid are in accordance with Devi et al. [16], where these polyphenols showed 74.4 and 66.6% of inhibition respectively but in different concentration than in the presented study. The weak activity of ferulic acid as a LOX inhibitor is confirmed by the studies by Landberg et al. [5], which showed that low or no inhibition was observed with avenanthramides containing ferulic or *para*-coumaric acid. Another study provided by Saleem et al. [17] showed the LOX inhibitory activity (34.8% of inhibition) of extracts from *Filago germanica*, wherein syringic acid was detected in the amount of 2.23 µg/mL.

2.4. Molecular Modeling of LOX Inhibition

As shown in the experimental part, all tested phenolic acids display an uncompetitive type of inhibition. Uncompetitive inhibitors bind to the enzyme–substrate complex only. The binding of the substrate can result in a conformational change allowing the inhibitor to bind, or the inhibitor binds to the enzyme-bound substrate directly. In all cases, the inhibitor binds in an allosteric pocket and does not compete with the substrate for a binding site.

Similarly as in the case of TPO, searching for potential binding pockets was performed using PARS and Fpocket. A number of potential binding sites were identified, including one situated between the membrane-binding and catalytic domains of 5-LOX also found in an X-ray structure of 5-LOX in complex with an allosteric modulator AKBA, a pentacyclic triterpene acid (PDB ID: 6NCF [18]. According to the PARS online tool, this pocket affects protein flexibility. This binding pocket is shown in Figure 4A and was used for further analysis.

Figure 4B–D shows the details of the interactions of the studied inhibitors with the allosteric pocket of 5-LOX. In all compounds, there is an interaction between their carboxylic groups and the side-chain atoms of Arg102 and Arg139. The mechanism of inhibition may be similar to that reported for AKBA, in which AKBA is wedged in a deep groove between the amino-terminal and catalytic domains and induces a conformational change of the protein which results in enzyme inhibition [18].

2.5. Antiradical Analysis

Under normal physiological conditions, oxidative homeostasis is autoregulated by the thyroid gland. Excessive ROS level can disrupt this balance, which can influence thyroid enzyme activity. However, this mechanism is still unclear [19]. Therefore, it is justified to determine the antioxidant potential of the tested compounds.

For the antioxidant activity of tested phenolic compounds evaluation, the ABTS radical scavenging assay was used. ABTS may be used to determine the activity of both hydrophilic and hydrophobic antioxidants; it is not affected by ionic strength, and it reacts with most antiradical compounds [20]. The lowest radical scavenging ability was shown by *trans*-cinnamic acid (2.12 mM). The highest value of this parameter showed syringic acid (0.22 mM) (Table 3).

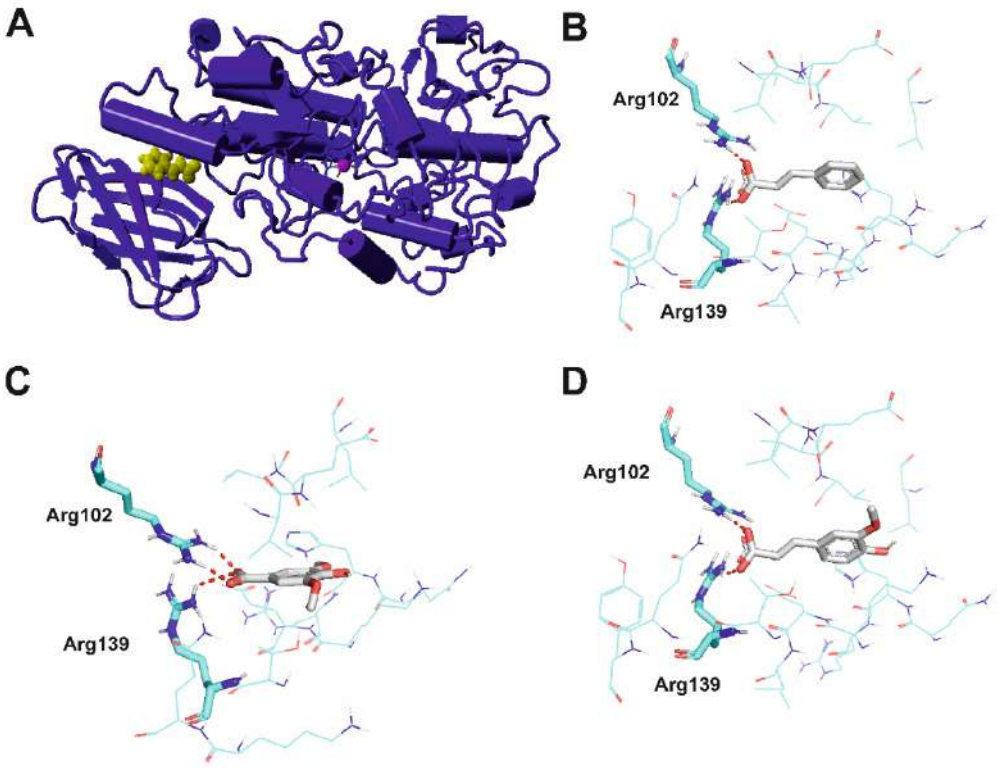

Figure 4. Molecular interactions of inhibitors with 5-lipoxygenase (5-LOX). (**A**) *trans*-cinnamic acid bound to an allosteric binding pocket (general view). The protein is shown in dark blue cartoon representation, *trans*-cinnamic acid in yellow ball representation and iron ion in magenta ball representation. (**B**) *trans*-cinnamic acid, (**C**) syringic acid and (**D**) ferulic acid in a binding pocket of 5-LOX. The protein is shown with cyan carbon atoms in wire representation with the most important residues shown as sticks. Inhibitors are depicted with gray carbon atoms in stick representation. Polar bonds are shown as red dashes. Nonpolar hydrogen atoms omitted for clarity.

Table 3. ABTS radical scavenging ability of chosen phenolic compounds, $n = 9$.

Compound	IC_{50} [mM]
trans-cinnamic acid	2.12 ± 0.106^{a}
ferulic acid	0.41 ± 0.020^{b}
syringic acid	0.22 ± 0.011^{c}

Values are expressed as the mean ± SD; means with different letter superscripts (a–c) in the columns are significantly different ($\alpha = 0.05$).

As expected, tested compounds showed high radical scavenging potential. The obtained results are significantly higher than those obtained by Samsonowicz et al. [21], who showed the antioxidant properties of coffee substitutes containing polyphenols investigated in our study (*trans*-cinnamic acid, ferulic acid and syringic acid). The paper showed that the ABTS radical scavenging activity values were in the range of 0.021–0.066 mg dw/mL. On this basis, we can conclude that pure polyphenolic substances show higher ABTS radical scavenging potential than substances contained in plant food sources.

2.6. Interaction Assay

The next step of the investigation was to estimate the type of mixed-polyphenol interactions and their influence on TPO and LOX enzymatic activity.

Isobolographic analysis of the TPO activators showed that tCA and FA (Figure 5A) acted additively; SA and FA (Figure 5B) showed strong synergism, whereas SA and tCA (Figure 5C) acted synergistically. All types of interactions were expressed as a CI value [10],

which explains the strength of the interactions. As the CI values reveal, pure chemicals exhibited synergism and strong synergism for TPO (Table 4).

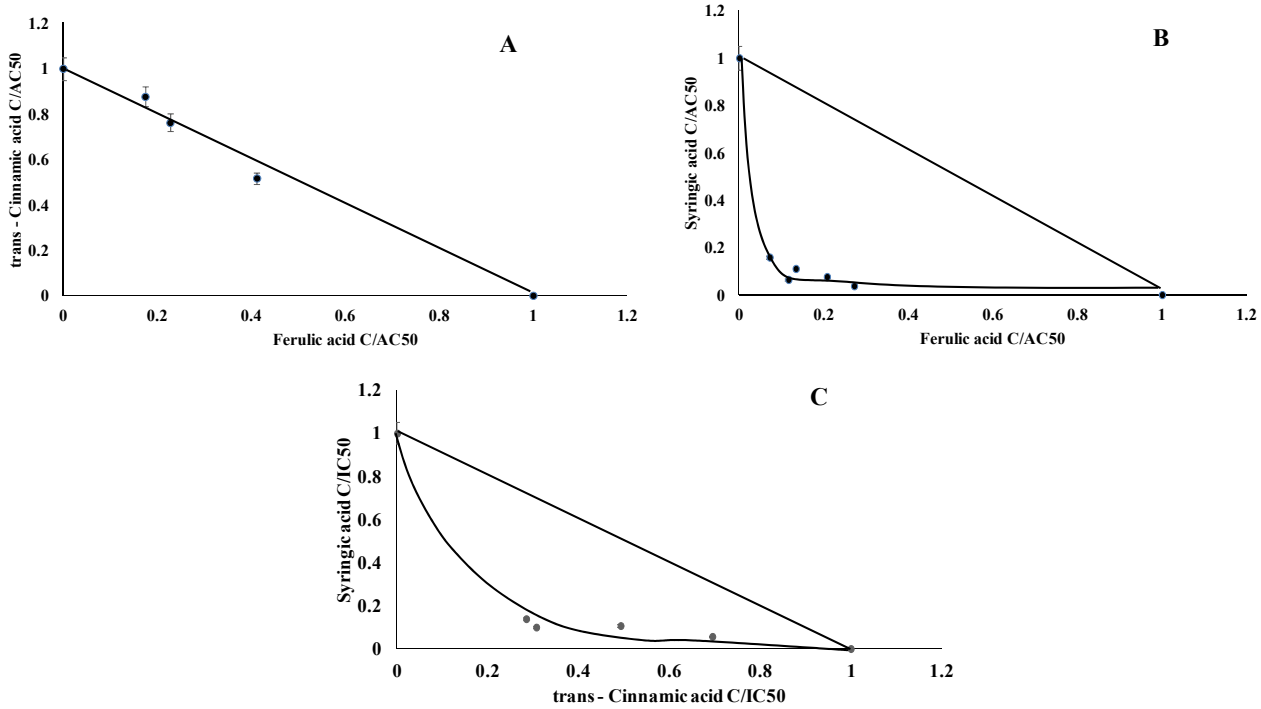

Figure 5. Dose-normalized isobolograms for chosen phenolic acids with thyroid peroxidase activation activity. (**A**) *trans*-cinnamic acid and ferulic acid, (**B**) syryngic acid and ferulic acid, (**C**) syringic acid and *trans*-cinnamic acid).

Table 4. Combination index (CI) value between mixtures consisting of two polyphenols on thyroid peroxidase activity, $n = 9$.

Compound	Ferulic Acid	*trans*–Cinnamic Acid	Syringic Acid
Ferulic acid	-	0.98 ± 0.06^{a} Nearly additive	0.24 ± 0.05^{c} Strong synergism
trans–cinnamic acid	0.98 ± 0.06^{a} Nearly additive	-	0.46 ± 0.04^{b} Synergism
Syringic acid	0.24 ± 0.05^{c} Strong synergism	0.46 ± 0.04^{b} Synergism	-

Values are expressed as the mean ± SD; means with different letter superscripts (a–c) in the columns are significantly different ($\alpha = 0.05$).

Isobolographic analysis of the LOX enzyme showed that *trans*-cinnamic acid and syringic acid acted antagonistically (Figure 6A), and FA/tCA and FA/SA acted synergistically, (Figure 6B,C). The CI values are presented in Table 5.

There are many scientific reports concerning interactions between drug components. At present, the clinically combined use of drugs is extremely common because the therapeutic effect of combined drugs is often better than that of a single drug [22]. The same effect was observed for the phytochemicals in fruits and vegetables that are responsible for their potent antioxidant and anticancer activities, and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods [12]. One effective method for interactions assay is isobolographic analysis. It is useful to determine the interactions between mixtures consisting of two or three components [22]. It has also been shown to be useful in studying the interactions of bioactive plants and food ingredients [23].

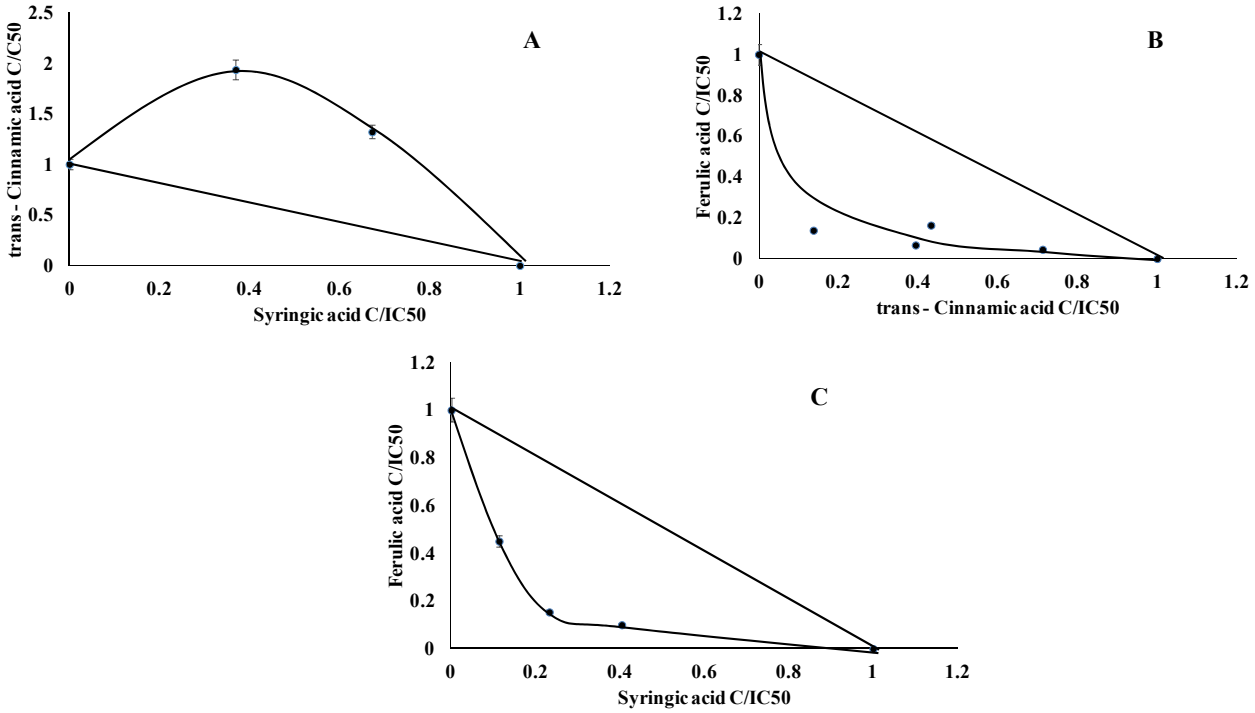

Figure 6. Dose-normalized isobolograms for chosen phenolic acids with lipoxygenase inhibitory activity. (**A**) *trans*-cinnamic acid and syryngic acid, (**B**) ferulic acid and *trans*-cinnamic acid, (**C**) ferulic acid and syryngic acid).

Table 5. Combination index (CI) value between mixtures consisting of two polyphenols on lipoxygenase LOX activity, $n = 9$.

Compound	Ferulic Acid	*trans*–Cinnamic Acid	Syringic Acid
Ferulic acid	-	0.53 ± 0.02^{a} Synergism	0.48 ± 0.01^{a} Synergism
trans–cinnamic acid	0.53 ± 0.02^{a} Synergism	-	2.15 ± 0.08^{b} Antagonism
Syringic acid	0.48 ± 0.01^{a} Synergism	2.15 ± 0.08^{b} Antagonism	-

Values are expressed as the mean ± SD; means with different letter superscripts (a,b) in the columns are significantly different ($\alpha = 0.05$).

Our previous studies used this method on pure polyphenolic compounds and plant extracts against TPO and LOX [4,24]. Studies [7] showed the synergistic action of ferulic and chlorogenic acids as LOX inhibitors. The same kind of interaction was found between chlorogenic and cinnamic acid [25], as well as chlorogenic and vanillic acid [26]. So far there is no information on TPO activators and their interactions in the literature.

The antiradical potential of phenolic compounds is well known [27], however, investigations concerning the interactions between them are still quite rare. In our study synergism was found in FA/ tCA and tCA/SA mixtures (CI = 0.63 and 0.69, respectively), while FA and SA acted antagonistically (CI = 2.21) (Figure 7, Table 6).

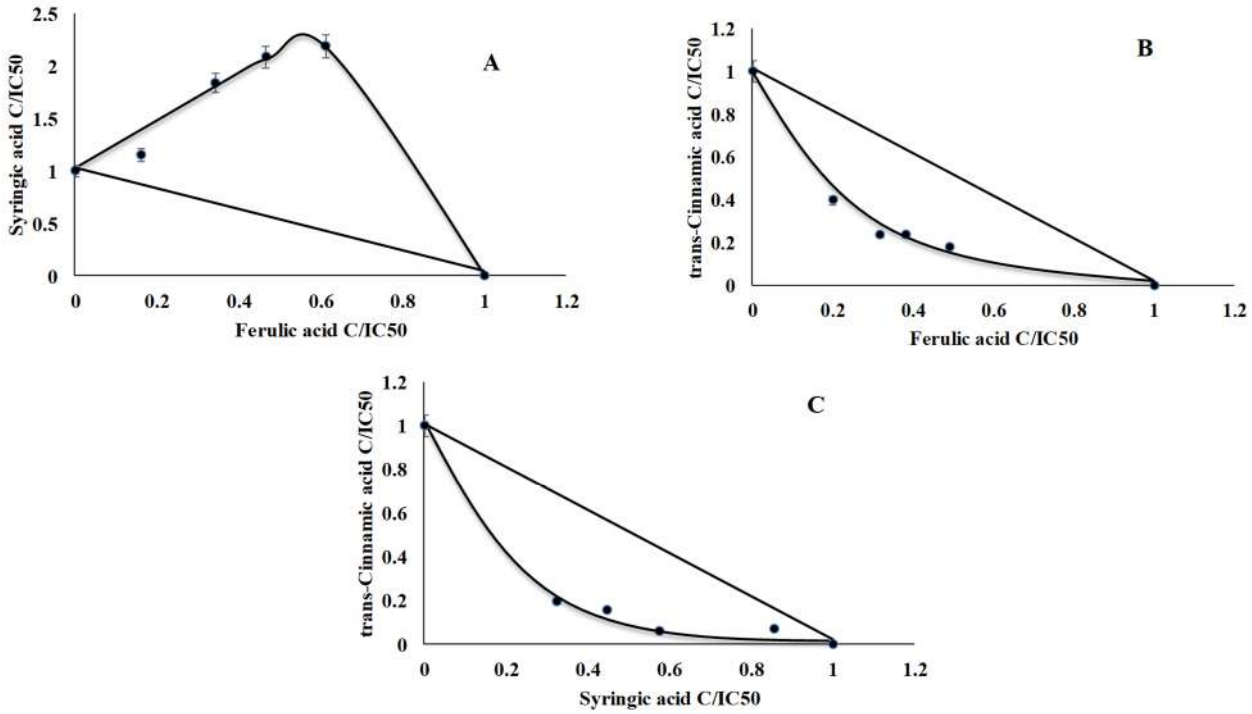

Figure 7. Dose-normalized isobolograms for chosen polyphenols with antiradical activity. (**A**) syryngic acid and ferulic acid, (**B**) *trans*-cinnamic acid and ferulic acid, (**C**) *trans*-cinnamic acid and syryngic acid).

Table 6. Combination index (CI) value between mixtures consisting of two polyphenols on antiradical activity, $n = 9$.

Compound	Ferulic Acid	*trans*–Cinnamic Acid	Syringic Acid
Ferulic acid	-	0.63 ± 0.02^{a} Synergism	2.21 ± 0.06^{b} Antagonism
trans–cinnamic acid	0.63 ± 0.02^{a} Synergism	-	0.69 ± 0.03^{a} Synergism
Syringic acid	2.21 ± 0.06^{b} Antagonism	0.69 ± 0.03^{a} Synergism	-

Values are expressed as the mean ± SD; means with different letter superscripts (a,b) in the columns are significantly different ($\alpha = 0.05$).

During research on antiradical activity, antagonism was found between chlorogenic acid and cinnamic acid [25], chlorogenic acid and vanillic acid [26], and chlorogenic acid and caffeic acid [28]. On the other hand, chlorogenic acid and ferulic acid acted synergistically as hydroxyl radical scavengers [29].

As shown in the present investigation, all tested phenolic acids show triple activity. They effectively activate TPO and inhibit LOX activity, demonstrating at the same time antioxidant potential. To the best of our knowledge, there are no reports of phenolic TPO activators with correlated anti-inflammatory and antioxidant potential in the literature to date. Most importantly, all TPO activators mentioned in the paper belong to the group of dietary polyphenols, commonly found in food of plant origin. However, the key issue here is bioavailability. Bioavailability measures the degree of an active compound/drug that reaches blood circulation and is therefore available at the site of action. As a natural antioxidant, tCA plays an important role in reducing the risk of chronic diseases, delaying ageing and improving immunity. However, the application of tCA is reduced by its poor water solubility and low oral bioavailability [30]. Previous reports indicate tCA is absorbed by all the gastrointestinal organs of the rat digestive tract and recovered in urine; however, the lowest rate of absorption was in the stomach [31]. Low plasma

concentrations are most likely due to limited absorption, intensive metabolism and/or fast elimination of tCA and its derivatives from circulation. These effects may not be sufficient to produce significant in vivo biological effects. To increase the bioavailability of tCA, new formulations have been prepared in which tCA is entrapped into solid and liquid particles [30,32]. High bioavailability of FA (about 50%) was found when FA was perfused as a free pure compound in the rat intestine. The urinary excretion of FA in humans was also high (19–98%) when FA is present in its free form, for example from beer [33]. Although the main source of ferulic acid is cereals, the limited bioavailability of FA from the cereal matrix is due to the embedding of FA in the indigestible polysaccharides of the cell walls [34]. Recent pharmacological studies demonstrated that SA possesses various activities, including antitumor, chemoprevention against skin cancer and antithrombotic activity [35]. Nevertheless, the lipophilicity of SA coupled with its rapid excretion in vivo results in low bioavailability and poor therapeutic effect. On the other hand, the absolute bioavailability of SA in blood samples of rabbits was found to be 86.27% [35].

Food-derived phytochemicals with multidirectional biological properties have already been tested for potential use in a wide range of diseases, including diabetes; viral, microbial and parasitic infections; inflammation; cardiac and psychiatric disorders, as well as cancer [11]. However, little is known about the impact of phytochemicals on thyroid function.

3. Materials and Methods

3.1. Chemicals

Sucrose (α-D-glucopyranosyl-(1–4)-β-D-fructofuranoside), tris (1,3-propanediol-2-amino-2-hydroxymethyl), KCl, NaCl, $MgCl_2$, 90% ethanol, NaOH, guaiacol (2-methoxyphenol), H_2O_2 (hydrogen peroxide), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), soybean lipoxygenase type 1-B (LOX), *trans*-cinnamic acid, syringic acid and ferulic acid were purchased from Sigma-Aldrich Company (Poznan, Poland). All other chemicals were of analytical grade.

3.2. Preparation of Phenolic Acids Solutions

Syringic acid, *trans*-cinnamic acid and ferulic acid were diluted in ethanol to the concentrations 0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1.0 µg/mL, 2.0 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL and were used for further assay.

3.3. Molecular Modeling

3.3.1. Small Molecules Modeling

The studied compounds were downloaded from PubChem (syringic acid [36], trans-ferulic acid [37] and *trans*-cinnamic acid [38]) and modelled using LigPrep [39] from the Schrödinger suite of software (Release 2020–4). To determine protonation states at the physiological pH, the Epik [40] module of the Schrödinger suite of software was applied.

3.3.2. Protein Models

Due to the lack of plausible templates for the complete TPO structure, the construction of a homology model of TPO including its transmembrane domain had to be performed in several steps, which included using preliminary models for positioning templates in space.

In the first step, preliminary homology models of the catalytic, MPO-like domain and the transmembrane domain were prepared with Modeller 9.23 [41]. Human promyeloperoxidase (proMPO, PDB ID: 5MFA) was used as a template for the dimer of the MPO-like domain, due to the high sequence similarity and high resolution of the X-ray structure (1.2 Å). For the transmembrane region, no obvious template was available. Therefore, a search for distant homologs was performed with the MPI Bioinformatics toolkit (https://toolkit.tuebingen.mpg.de accessed on 21 April 2021) [42]. After several HHpred runs, three structures were identified as possible templates (PDB IDs: 6HUM, 6ITH, 6NBY). All these structures allowed the modeling of the monomer of the transmembrane

domain. To align these models in a dimer, a 2L2T PDB structure was used, based on literature data [43].

In the next step, preliminary models of the complete monomer were created with trRosetta [44]. These preparatory models were intended to serve as a scaffold for connecting the MPO-like domain and transmembrane domain. Candidate trRosetta models were superimposed with the model of the catalytic domain dimer, and the trRosetta model that allowed undisturbed dimerization was selected. Two copies of the selected trRosetta model were superimposed to two monomers of the MPO-like domain dimer, and the resulting structure was used as a spatial scaffold for further modelling.

In the final step, the trRosetta model of the whole TPO dimer was used to orient all the necessary templates in space. A homology model of TPO with its transmembrane domain was created with Modeller, using the 5MFA structure as a template for the MPO-like domain, 6HUM, 6ITH, 6NBY and 2L2T structures for the transmembrane domain, and the trRosetta model as a template for the region connecting the MPO-like domain and the transmembrane domain. Four hundred models were generated. The best model was chosen based on the DOPE score and its orientation relative to the membrane—the model with the highest DOPE score, not hindering the membrane plane obtained from the Orientations of Proteins in Membranes (OPM) database record for a 2L2T structure [45] was chosen for further studies.

After the homology modelling stage, the obtained model was refined with a 100 ns molecular-dynamics simulation using Gromacs 2019 [46]. The model was immersed in a membrane composed of POPC, POPE and cholesterol, using the orientation of a 2L2T structure from the OPM database as a template. The simulation box was prepared with CHARMM-GUI [47]. A CHARMM36m force field was used. An NPT ensemble with a timestep of 2 fs was used for the production run.

Regarding 5-lipoxygenase (5-LOX), an X-ray structure of the human enzyme in complex with allosteric modulator AKBA, a pentacyclic triterpene acid (PDB ID: 6NCF [18], was used after applying the necessary mutations.

The structures of the biomolecules were preprocessed using the Protein Preparation Wizard of Maestro Release of the Schrödinger software [48] to optimize the hydrogen bonding network and to remove any possible artifacts as reported previously [49].

3.3.3. Binding Site Identification and Molecular Docking

Two online tools, i.e., PARS [50] and Fpocket [51], were used for putative binding site detection. PARS relies on normal mode analysis and is a simple and fast tool, which queries protein dynamics and structural conservation to find pockets on a protein structure that may exert a regulatory effect upon the binding of a small-molecule ligand. Fpocket is a binding site detection package based on Voronoi tessellation and alpha spheres [52] built on top of the publicly available package Qhull. Binding pockets identified simultaneously by both tools were used for molecular docking. Molecular docking was performed using the standard precision (SP) approach of Glide [53] from the Schrödinger suite of software. Grid files were generated at default settings. Fifty poses were generated for each binding site and each ligand. The final poses were selected by visual inspection. Discrimination between binding sites was carried out based on scoring values. PyMol v. 2.3.3 [53] and Yasara Structure [54] were used for visualization of the results.

3.4. Enzymatic Assay

3.4.1. TPO Preparation

The assay was prepared according to Jomaa et al. [55], with some modifications. Porcine thyroid glands were purchased at a slaughterhouse (Lublin, Poland) and stored at −20 °C until used. The frozen thyroid gland was minced with a fork. The mince was suspended in a buffer containing 0.25 M sucrose, 2 mM tris–HCl, 100 mM KCl, 40 mM NaCl and 10 mM $MgCl_2$ (pH 7.4) and homogenized using Philips homogenizer. The thyroid gland was centrifuged two times at 4000 RPM per 15 min at a temperature + 4 °C.

The enzyme protein was then salted out to 60%. The supernatant was stored at −20 °C until used.

3.4.2. TPO Assay

The assay was used according to Jomaa et al. [55], with some modification. The measurement was made using a plate spectrophotometer (BioTek, Model Epoch2TC, Winooski, VT, USA) in 96–well plates at a wavelength of 470 nm. Absorbance readings were recorded every minute for a total of 3 min at 37 °C. TPO activity is expressed as a change of absorption per minute. All measurements were performed in three replicates. The AC_{50} (activator concentration) values were calculated at fitted models as the concentration of the tested compound providing 50% of the activation based on a dose-dependent mode of action.

3.4.3. Inhibition of Lipoxygenase Activity (LOXI)

The inhibition of LOX with linoleic acid as a substrate was measured spectrophotometrically, based on Axelrod et al. [56] adopted for microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Winooski, VT, USA). Measurement was made at a wavelength of 234 nm. One unit of LOX activity was defined as an increase in absorbance of 0.001 per minute at 234 nm. All measurements were performed in four replicates.

The IC_{50} (inhibitor concentration) values were calculated at fitted models as the concentration providing 50% of activity was based on a dose-dependent mode of action.

3.5. In Vitro Antiradical Capacity Assay

ABTS radical scavenging activity was determined according to Re et al. [57] with slight modifications using a microplate spectrophotometer (BioTek, Model Epoch2TC, Winooski, VT, USA) after 15 min of incubation at room temperature.

The IC_{50} (inhibitor concentration) values were calculated at fitted models as the concentration providing 50% of activity was based on a dose-dependent mode of action.

3.6. Isobolographic Analysis

Dose-normalized isobolograms were performed according to Chou [10]. Tested solutions were mixed in various volume ratios: 1:4, 4:1, 3:2, 2:3, 1:1. Results (type and strength of interactions) were showed as normalized isobolograms and described by combination index (CI). The quantification of interaction was done by the general Equation (1) for n-drug combination at x% inhibition using the CI for interaction interpretation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{1}{(DRI)_1} + \frac{1}{(DRI)_2} \quad (1)$$

where: CI is the sum of the dose of drugs that exerts x% inhibition in a combination. In the denominator, (Dx) is for D "alone" that inhibits a system x%. When CI is lower than 1, it indicates synergy; when CI is equal to 1, it indicates addition; when CI is higher than 1, it indicates antagonism.

3.7. Statistical Analysis

All experimental results were the mean ± SD of four parallel measurements, and data were evaluated by a two-way analysis of variance (Tukey test) using Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA). The statistical tests were carried out at a significance level of $\alpha = 0.05$.

4. Conclusions

The use of phytochemicals can be a widely accepted and inexpensive way to support the treatment and prevention of many diseases, including thyroid disorders. Importantly, this solution is available to both poor societies that do not have access to expensive drugs and developed societies that struggle with the problem of ageing societies and rising health care costs. Our in silico results indicate that both activatory and inhibitory effects on TPO

and LOX, respectively, are mediated by compounds' interaction with the allosteric site of the enzymes. Further studies will be performed to address the mechanism of synergistic, additive or antagonistic interactions between the studied dietary phenolic compounds.

However, it should be kept in mind that the results obtained during in vitro tests in model systems may, to a varying degree, translate into actual effects obtained during in vivo tests, therefore, the results presented in this paper are treated as preliminary and will be verified during further extensive research.

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Oatmeal and wheat flour as the sources of thyroid peroxidase (TPO), lipoxygenase (LOX) and xanthine oxidase (XO) modulators potentially applicable in the prevention of inflammatory thyroid diseases

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Abstract: Despite the widespread potential pro-health effects of ferulic acid (FA), their interference in the progression of thyroid dysfunction has mainly remained unresolved. Here, we combined in vitro enzyme studies with the in vitro cellular approach to investigate the potential of main dietary sources of FA - the oatmeal (OM) and wheat flour (WF) compounds for the prophylactics of inflammatory thyroid diseases. Potentially bioaccessible OM and WF compounds activated thyroid peroxidase (TPO), while inhibiting the activity of lipoxygenase (LOX) and xanthine oxidase (XO). Isobolographic studies revealed cooperation between them. Relatively strong inhibitory activity of bioaccessible OM compounds on LOX activity correlated with their cytostatic and pro-invasive effects in thyroid cancer model in vitro. These data indicate the potential of OM and WF products for the prophylactics of inflammatory thyroid diseases (incl. hypothyroidism). However, it should be considered with care, especially in the context of the oncological status of the patient.

Keywords: thyroid peroxidase (TPO), lipoxygenase (LOX), xanthine oxidase (XO), dietary polyphenols, antioxidant activity, hypothyroidism, cancer

1. Introduction

Inflammation is a natural process that occurs in tissues as a defensive mechanism against tissue injury or microbial infection. However, chronic inflammation is often associated with the pathogenesis and progression of autoimmune, cardiovascular and neurological diseases (Jaismy et al., 2018). It also contributes to the autoimmune destruction of the thyroid gland (Danailova et al., 2022). Development of thyroid diseases, like of many other autoimmune disorders, is believed to result from the combinations of versatile stimuli, including environmental, lifestyle, and genetic factors. For instance, regular consumption of ”proinflammatory” food can result in the intestinal inflammation that spreads to other organs in the body, eventually contributing to the development of thyroid diseases, incl. hypothyroidism and cancer (Kochman et al., 2021).

Thyroid malfunctions prevalently occur in women. Overt hyperthyroidism results from an overproduction of thyroid hormones and is primarily related to Graves' disease. In turn, hypothyroidism results from a deficiency of thyroid hormones, and its most prevalent syndrome is Hashimoto's thyroiditis (Xu et al., 2019). Several clues indicate the involvement of redox enzymes in its etiopathology. For instance, a deficiency or abnormal function of TPO can lead to impaired thyroid hormone synthesis, resulting in congenital hypothyroidism. TPO plays a key role in catalyzing the oxidation of iodide, which is necessary for the iodination of tyrosyl residues in thyroglobulin (TG) - a process known as organification. Additionally, TPO is responsible for the oxidative coupling of iodothyronine residues to form the hormones T4 and T3. In hypothyroidism, TPO acts as an autoantigen, prompting the generation of circulating autoantibodies and thyroid inflammation in patients with Hashimoto's thyroiditis (Williams, 2008).

Inflammatory etiopathology of thyroid diseases implies the involvement of inflammatory mediators/enzymes in their development. For instance, cyclooxygenase (COX) and lipoxygenase (LOX) are

responsible for a wide range of physiological and pathophysiological responses (Yao et al., 2015). Lipoxygenases (LOXs) are responsible for the oxygenation of polyunsaturated fatty acids, such as arachidonic acid, to bioactive lipids, including leukotrienes and hydroxyeicosatetraenoic acids (HETEs). LOX products are involved in a wide range of physiological processes, including inflammation, immune responses, and the proliferation of cancer cells, thus linking the inflammation with carcinogenesis (Yao et al., 2015; Zabiulla et al., 2022). While the participation of COXs and LOXs in inflammatory processes and in oxidative stress induction is well documented, the participation of xanthine oxidase (XO) in these processes has recently gained an increasing interest. Its activity can result in the conversion of superoxide radicals into hydroxyl radicals, which exacerbate inflammatory responses and contribute to the development of the cytokine storm syndrome (CSS) (Pratomo et al., 2021). Whereas several studies reported on the inhibitors that can concomitantly affect the COX and LOX activity (Jaismy et al., 2018), only few studies have been focused on the substances that concomitantly affect XO/LOX activity. Given the possible interrelations between the inflammation and oxidative stress in hypothyroidism, there is also a need to identify and isolate LOX/XO inhibitors that would concomitantly activate TPO (Zabiulla et al., 2022).

We have previously shown that pure polyphenolic substances, such as ferulic acid (FA), can enhance TPO activity *in silico* (Habza-Kowalska, Kaczor, Żuk, Matosiuk, & Gawlik-Dziki, 2019; Habza-Kowalska, Kaczor, Bartuzi, Piłat, & Gawlik-Dziki, 2021). On the other hand, the interference of FA-rich plant products with the activity of LOX and XO has not been addressed so far. In the current study, we (i) identified wheat flour (WF) and oat flakes (oatmeal; OM) as the products relatively rich in FA. Then, we estimated the interference of bioaccessible OM and WF compounds with TPO, LOX and XO activities to assess their potential in diet supplementation for the patients with hypothyroidism. Finally, (iii) using an in vitro cellular approach, we addressed the potential consequences of their activity for the patients with thyroid carcinoma. To the best of our knowledge, this work is the first to consider the activity of diet TPO/LOX/XO modulators in the context of their side effects.

2. Materials and Methods

2.1. Chemicals

Sucrose (α -D-glucopyranosyl-(1→4)- β -D-fructofuranoside), Tris (1,3-Propanediol-2-amino-2-hydroxymethyl), KCl, NaCl, $MgCl_2$, 90% ethanol, NaOH, guaiacol (2-Methoxyphenol), H_2O_2 (Hydrogen Peroxide), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), lipoxygenase (LOX), linoleic and ferulic acids, xanthine oxidase (XO), xanthine, pancreatin, pepsin, bile extract, phosphate buffered saline - pH 7.2 (PBS) were purchased from Sigma-Aldrich Company (Poznan, Poland). All other chemicals were of analytical grade.

2.2. Material

Porcine thyroid glands were purchased at a local slaughterhouse (Lublin, Poland) and stored in -20°C until used. Oatmeal (Plony Natury, Poland) was purchased in local supermarket in Lublin, Poland. Flour from common wheat (cv. Batuta) was purchased in the local mill (Lublin, Poland).

2.3. Preparation of EtOH, raw and digested extracts of polyphenolic plant sources

For ethanol extraction, 1.5 g of individual raw materials were homogenized with 15 mL of 50 % ethanol, samples were shaken for 30 min in the room temperature and then centrifuged for 15 min at 4000 RPM. The extraction procedure was repeated twice. The final samples were brought to 50 ml with 50% ethanol to reach the extract concentration of 30 mg/mL. Extracts were diluted to concentration 0.3 mg/mL. 50% ethanol extracts were used as a control for enzymatic tests and determination of antiradical potential. For *in vitro* tests, PBS extracts (containing potentially bioavailable free compounds) were used to eliminate the potential effect of ethanol. It was prepared analogously to the ethanol extract. *In vitro* digestion of raw materials was performed according to the modified procedure proposed by Minekus et al. (Minekus et al., 2014). 0.5 g of row material

was mixed with 5 ml of simulated saliva fluid (SSF) containing 0.22 mg of α-amylase and 200 µl of 5M HCl) and incubated for 2 min in 37°C. Then, 250 µL of SGF (750 µL of pepsin mixed with 50 mL SGF) was added and incubated for 2 hours in temperature 37°C to simulate gastric digestion, followed by the application of 1.2 ml of 0.1M $NaHCO_3$ (pH 6.0) and 750 µL of 1M $NaHCO_3$. Finally, 600 µL of SIF (0.05 g of pancreatin, 0.3 g of bile extract in 35 ml of SIF; pH 6,0) as applied, the sample was incubated for 1 hour in 37°C and centrifuged at 4500 rpm for 15 minutes. The supernatant was stored at -20°C until further analyses. 0.5 ml of distilled water was used instead of plant source to obtain the control sample.

2.4. Determination of total phenolics content (TPC)

TPC analyses were carried out with the protocol of Singleton and Rossi (Singleton & Rossi, 1965) adopted for microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Winooski, Vermont, USA). Ten microliters of extract, 10 µL of water and 40 µL of Folin- Ciocalteau reagent were diluted in water at the ratio of 1:5. After 3 min, 250 µL of 10% sodium carbonate was added and the solution was thoroughly mixed. 50% ethanol or digested control (H_2O) sample was used as the standard. The absorbance was measured at 725 nm after 30 minutes of incubation and normalized against the standard. The concentration of phenolic compounds was read from the standard curve determined for gallic acid and expressed as gallic acid equivalent (GAE) in mg/g DW.

2.5. *In vitro* antioxidant capacity assay

ABTS radical scavenging activity was prepared according to Re et al. (1999) (Re et al., 1999) with some modifications. 250 µl ABTS was mixed with 10 µl of the sample (ethanol and GD extracts) and measured at the wavelength 724 nm using UV/Vis microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Winooski, Vermont, USA). after 15 min of incubation in RT. ABTS discoloration was calculated as follows:

$$AA = (A_c - A_p)/(A_c) \cdot 100\%, \quad (1)$$

where:

A_c – the absorbance of control, A_p – the absorbance of extract

DPPH radical scavenging activity was measured according to (Brand-Williams et al., 1995) with some modifications. 250 µl of DPPH solution was mixed with 10 µl of the extract (3 mg/ml) and measured at 517 nm using UV/Vis microplate spectrophotometer (BioTek, Model Epoch2TC, Winooski, Vermont, USA) after 15 minutes of incubation in RT. The inhibition percentage of DPPH discoloration was calculated as in (1).

2.6. Thyroid peroxidase (TPO) activity assay

The assays was prepared according to (Jomaa, 2015), with some modifications. Detailed description is provided in the publication by Habza-Kowalska et al. (Habza-Kowalska, Gawlik-Dziki, et al., 2019). TPO activity was calculated using the formula below:

$$\%_{activation} = (\Delta A/[min]\_test)/(\Delta A[min]\_blank) \times 100, \quad (2)$$

Where: ΔA/min test is the linear absorbance change per minute of the test material and ΔA min blank is the linear change in absorbance per minute of blank.

The mode of enzyme activation/inhibition estimated with the Lineweaver-Burk plot.

2.8. Inhibition of lipoxygenase (LOX) activity

LOX activity was analyzed using the protocol proposed by Axelrod et al., (1981) adopted for microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Winooski, Vermont, USA). Detailed description can be found in the publication by Habza-Kowalska et al. 2019 (Habza-Kowalska, Gawlik-Dziki, et al., 2019). LOX inhibitory activity was calculated using the formula (3). The mode of inhibition of the enzyme was performed using the Lineweaver-Burk graph. The EC_{50} values were calculated from the fitted models of dose-dependence and given as the concentration of the tested compound that gave 50% of the maximum inhibition.

$$\%_{inhibition}=(1-(\Delta A/⟦min⟧\_test)/(\Delta A⟦min⟧\_blank))\times 100, \quad (3)$$

Where: ΔA/min test is the linear absorbance change per minute of the test material and ΔA min blank is the linear change in absorbance per minute of blank.

2.9. Inhibition of xantine oxidase (XO) activity

XO activity was measured according to Sweeney et al. (Sweeney et al., 2001) with some modifications: 30 µL of the sample was diluted in 110 µL of 1/15 M/L phosphate buffer (pH 7.5), and 20 µL of enzyme solution (0.01 U/ml in M/15 phosphate buffer). After the preincubation at 30°C for 10 min, the reaction was started by adding 140 µL of 0.15 mM/L xanthine solution. The absorbance (295 nm) was measured every minute for 3 min. XO inhibitory activity was calculated using the formula (3). Inhibitory activity was expressed as EC_{50} (efficient concentration) i.e. the amount of sample needed to inhibit XO to 50% of initial activity.

2.10. Isobolographic analysis

Isobolographic analyses were performed according to (Chou, 2006). A detailed description can be found in the publication by Habza-Kowalska et al. (Habza-Kowalska, Gawlik-Dziki, et al., 2019).

2.11. In vitro approach

2.11.1. Cell culture

Human thyroid cancer B-CPAP (ACC 273, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) and 8505C cells (Sigma No. 94090184) were cultured in the standard conditions in RPMI 1640 and DMEM/F12 HAM, respectively (Sigma No. D8437) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, No. A3840402) and 1% Antibiotic-Antimycotic Solution (Merck, No. A5955). For each experiment, the cells were harvested with $Ca^{2+/}Mg^{2+}$-free 0,25% trypsin/EDTA/PBS solution (Gibco No. 25200072), counted in Z2 particle counter (Beckman Coulter) and seeded into multi-well tissue culture plates (Falcon®). Cells were exposed to extracts administered at the concentration of 0.01, 0.05, 0.1, 1 and 3% in culture medium (corresponding to 1.5, 7.5, 15, 150 and 450 g of the product/75 kg body mass) for 48 hours.

2.11.2. Proliferation and viability tests

For the analyses of cell viability and proliferation, B-CPA and 8505C cells were seeded into 12–well cell culture plates (Corning®Costar®) at the density of 3 and $2.5x10^4$ cells/well and cultivated for 24 hours before the administration of the extract in a fresh culture medium. Cell viability was estimated with EtBr/FDA assay 48 hours after extract administration. Proliferation was estimated with Coulter counter 48 hours after the administration of extracts (Ryszawy et al., 2019).

2.11.3. Wound healing assay

B-CPAP and 8505C cells were seeded in 12-well plates at the density of 400 cells/mm^2. After 24 hours, the extracts were added along with the medium at the concentration of 0.1 and 1%. After the next 48 hours, a wound was made in the center of each well with a clean tip and 16 wound pictures were registered immediately

afterwards and 24 hours thereafter to calculate the %age of wound coverage. The analyses were performed at 37°C and 5% CO2 using a Leica DMI6000B fluorescence microscope.

2.11.4. Immunofluorescence

For immunofluorescence studies, B-CPAP and 8505C cells were seeded into 12-well plates on UVC-sterilized coverslips at the density of 3 and 2.5x10^4 cells/well, respectively, cultured for 24 hours and processed in the presence/absence of the extracts administered at the concentrations given in the text. Then, they were fixed with 3.7% formaldehyde followed by 0.1% Triton X-100 permeabilisation (Pudełek et al., 2020) and non-specific binding sites were blocked with 3% BSA (Invitrogen, No. 37525; 30 min. in 37^{o}C). After washing with 2% PBS, the mouse monoclonal anti-vinculin IgG (with 1% BSA, Sigma no. V9131) was applied for 45 min. Then, the specimens were washed before the application of the mixture of AlexaFluor488-conjugated goat anti-mouse IgG (ThermoScientific No. A-11029), AlexaFluor546-conjugated phalloidin (Invitrogen, No. A22283; for F-actin visualization) and Hoechst 33258 (Sigma; for DNA staining) for 45 min. Finally, specimens were mounted in Agilent Dako mounting medium (Agilent Dako; No. S3023). Images were acquired with Leica DMI6000B fluorescence microscope equipped with DFC360FX CCD camera. For better clarity, the raw images were additionally processed (linear contrast adjustment and background subtraction) in ImageJ software.

2.11.5. Statistical analysis.

All data were expressed as mean +/- SEM from at least three independent experiments (n = 3). The statistical significance was tested with one-way ANOVA followed by post-hoc Dunnett's or Tukey's comparison for variables with a non-normal (tested with Levene's comparison) and normal distribution, respectively. Statistical significance was shown at $p < 0.05$.

3. Results and Discussion

3.1. Determining Ferulic Acid Content in Natural Plant-Based Food Sources Using Phenol Explorer Database

Previously, we have shown versatile effects of purified polyphenols and their natural sources (plant extracts) on the activity of TPO and LOX, as the enzymes involved in thyroid diseases (Habza-Kowalska, Kaczor, et al., 2019; Habza-Kowalska, Gawlik-Dziki, et al., 2019). For instance, ferulic acid (FA) has been shown to activate TPO (Habza-Kowalska et al., 2021), which indicates its potential in the prophylactics of hypothyroidism. Ferulic acid is a naturally occurring phenolic compound of plant-based foods, which displays antioxidant, anti-inflammatory, and anticancer properties (Nile et al., 2016). The content of FA varies between food sources, which points to the necessity for the identification of FA-rich products. Based on our preliminary studies on FA activity, we first concentrated on the identification of natural sources of FA (Patel et al., 2013). Phenol Explorer (http://phenol-explorer.eu/) database has been developed to provide comprehensive information on the content of phenolic compounds in foods. Previously, it allowed to identify the sources of FA (grains, fruits, and vegetables), which can easily be incorporated into a healthy diet to increase the intake of this bioactive compound (Ramli et al., 2017). Using this database, we pinpointed oatmeal (OM) and wheat flour (WF) as the rich FA sources.

Table 1. Ferulic acid content*

Source	Mean content [µg/g DW]
Wheat flour	593.94 ± 56.25
Oatmeal	207.4 ± 7

*data based on the papers (Buczek et al., 2023; Soycan et al., 2019)

Detailed studies on the qualitative/quantitative profile of phenolic acids in OM and WF used in this study have recently been published (Soycan et al., 2019; Buczek et al., 2023). These data indicate that WF and OM are rich sources of this compound. As shown in the she summary of these data (Table 1), WF contains almost 3-fold higher FA content than OM. Therefore, in the following experiments we focused on the activity of the OM and WF extracts. These extracts were subjected to simulated gastrointestinal digestion (GD) and their activity was compared to the commonly studied 50% EtOH WF and OM extracts.

3.2. TPC and anti-oxidative activity of WF and OM extracts

Total phenolic content (TPC) is a widely used predictor of the potential antioxidant and anti-inflammatory activity of plant extracts. Our preliminary experiments were performed to estimate the basic parameters of EtOH and GD extracts from wheat flour (WF) and oatmeal (OM). TPC estimated for EtOH WF extracts reached 14.25 ± 0.71 mg GAE/g dry weight (Table 2). It was considerably higher than that estimated for EtOH OM extracts (5.66 ± 0.28 mg GAE/g dry weight), but lower than the values reported by Călinoiu & Vodnar (2020) for MetOH WF and OM extracts (WF: 39.61 ± 0.51 mg GAE/100 g dry weight; OM: 25.15 ± 0.45 mg GAE/100 g dry weight). These differences could be attributed to different sample preparation, and the type and quality of the wheat flour and oatmeal used. For instance, TPC of water extracts from WF can range from 211.55 to 1393.27 µg GAE/g (Tian et al., 2021;Yu & Beta, 2015). The EtOH extraction method can also be less effective than the others, even if it is commonly used in the preparation of para-pharmaceutics. Accordingly, gastrointestinally digested (GD) WF and OM extracts were characterized by higher TPCs (18.60 ± 0.93 and 11.59 ± 0.58 mg GAE/g dry weight, respectively) than their EtOH counterparts. This observation indicates that GD effectively releases phenolic compounds from WF and OM and justifies further focus on the properties of GD extracts.

Table 2. Total phenolic content and antiradical activity against DPPH ans ABTS free radicals of oatmeal and wheat flour extracts (n=9).

Raw material	Total phenolics content (TPC) [mg GAE g^{-1}]	Antioxidant activity EC_{50} [µg ml^{-1}]	
		DPPH	**ABTS**
Control extracts			
Oatmeal	5.66 ± 0.28^{a}	332.18 ± 16.61^{a}	249.75 ± 12.49^{b}
Wheat flour	14.25 ± 0.71^{b}	338.57 ± 16.93^{b}	162.31 ± 8.12^{a}
Digested extracts			
Oatmeal	11.59 ± 0.58^{a}	50.37 ± 2.52^{a}	810.7 ± 40.53^{a}
Wheat flour	18.60 ± 0.93^{b}	66.29 ± 3.31^{b}	930.06 ± 46.5^{b}

Values are expressed as the mean ± SD; means with different letter superscripts (a-b) in the columns are significantly different (α = 0.05).

Generation of reactive oxygen species (ROS) during the synthesis of thyroid hormones and systemic inflammatory responses can evoke oxidative stress and damage to the thyroid gland. Consequently, its inflammation (sometimes also neoplasia) is prompted when the excessive amount of ROS is not satisfactorily managed by the cells (Ramli et al., 2017). These data highlight the need to determine the antioxidant potential of the investigated extracts. Radical scavenging assays were performed to estimate antioxidative potential of GD WF and OM extracts. They demonstrated higher antioxidative efficiency of GD OM extracts than of their GD WF counterparts, as is illustrated by EC_{50} values estimated with ABTS and DPPH assay (50.37±2.52 for OM vs. 66.29±3.31 µg DW/mL for WF). Notably, these values were considerably higher than those obtained for EtOH extracts. They also considerably differ from the data previously obtained for oat and wheat MetOH and acetonic extracts (MetOH/Oat: 510 to 18 µg/ml; (Ihsan et al., 2022), 6.57 ± 0.023 mg DW/mL acetonic/oat flour (Žilić et al., 2011). Again, the differences in ABTS results for the given product may be due to the plant species and variety, the growth conditions, the age of the plant, and the storage conditions. These discrepancies may also be attributed to the differences in the extraction methods and solvents used, which can have a significant impact on

the extraction efficiency and antioxidant activity of the samples. The differences between EtOH and GD OM/WF extract activity that we obtained with ABTS and DPPH assay support this notion. As expected, the scavenging activity of the extracts was lower than that obtained for pure FA (Habza-Kowalska et al., 2021; Habza-Kowalska, Kaczor, et al., 2019). However, the data on FA content in both plants (Table 1) suggest that FA is not a primary antioxidative compound in GD OM extract. In conjunction with the differences in TPC, we show relatively high activity and potential nutritional value of OM compounds for the patients with hypothyroidism.

3.3. Effects of the OM and WF extracts on the activity of TPO, LOX and XO .

To further assess the potential of OM compounds for the prophylactics of hypothyroidism, we investigated the influence OM and WF extracts on TPO activity (Fig. 1A-B, Table 3). Relatively strong activating effects of both GD extracts could be observed. They were corresponding to that observed for purified FA, even if its action was more efficient (Habza-Kowalska et al., 2021). Because we observed rather distinct differences in the impact of the extracts and pure FA on TPO activity, these data might indicate that *in vitro* digestion releases modulators of FA activity from the food matrix. In any case, they confirm relatively strong TPO activating effect of both extracts. Based on the data contained in Table 3, it can be assumed that the activating effect of the tested extracts consists mainly in increasing the affinity of the enzyme for the substrate (decrease in Km value), although in the case of samples obtained after *in vitro* digestion of OM, an increase in Vmax was also observed (compared to the activity of pure TPO) (Table 3). In the available literature, there are few studies on the mechanism and kinetics of enzyme activation. Shabani et al. (Shabani & Sariri, 2010) proved that same saturated and unsaturated fatty acids enhanced tyrosinase activity and affected both of kinetic parameters (decrease of Km and increase of Vmax). This type of kinetic behavior is typical of a mixed-type activator meaning that activators could bind to both the free enzyme and enzyme-substrate complex. The same situation occurred in our research in the case of GD OM samples (Fig1B, Table 3).The current literature lacks data on the mechanisms of TPO activation by bioactive compounds derived from food, so this issue requires further, comprehensive research.

Table 3. Impact of control and digested extracts from oatmeal and wheat flour on thyroid peroxidase, lipoxyhenase and xanthine oxidase activity. The EC_{50} and kinetic parameters values (n = 9)

	Kind of extract	Mode of action	EC_{50} (mg DW/mL)	Km (mM)	Vmax (Δ AU/min)
Thyroid peroxidase					
Control enzyme		-	-	678.75±10.25^{a}	1250±45.27^{a}
Oatmeal	Control	uncompetitive inhibition	6.71±0.34^{a}	152.05±7.42^{b}	256.41±9.98^{b}
	After digestion	activation	8.84±0.44^{b}	170.07±5.38^{c}	2500±32.24^{c}
Wheat flour	Control	activation	0.39±0.004^{c}	192.55±8.33^{d}	500±8.54^{d}
	After digestion	activation	8.31±0.42^{b}	307.60±15.88^{e}	666.67±31.25^{e}
Lipoxygenase					
Control enzyme		-	-	15.13±0.85^{a}	434.78±11.08^{a}
Oatmeal	Control	uncompetitive inhibition	4.80±0.24^{a}	5.91±0.07^{b}	153.85±4.56^{b}
	After digestion	uncompetitive inhibition	3.57±0.17^{b}	5.29±0.09^{b}	103.09±5.27^{c}
Wheat flour	Control	non-competitive inhibition	6.3±0.31^{c}	370.37±12.32^{c}	20.74±0.58^{d}
	After digestion	mixed inhibition	9.38±0.47^{d}	204.08±8.75^{d}	9.67±0.05^{e}
Xanthine oxidase					
Control enzyme		-	-	4.87±0.03^{a}	81.96±3.35^{a}
Oatmeal	Control	uncompetitive inhibition	0.37±0.001^{a}	2.79±0.02^{b}	56.49±2.31^{b}

	After digestion	uncompetitive inhibition	0.33±0.00[a]	3.27±0.01[c]	66.23±3.61[c]
Wheat flour	Control	uncompetitive inhibition	4.07±0.001[b]	3.07±0.01[c]	65.36±3.25[c]
	After digestion	uncompetitive inhibition	0.34±0.002[a]	3.27±0.01[c]	66.24±2.85[c]

Values are expressed as the mean ± SD; means with different letter superscripts (a-e) in the columns (for each enzyme separately) are significantly different ($\alpha = 0.05$).

In turn, the data shown in the Fig. 1 C-F (summarized in the Table 3) again pointed to the higher pro-health value of OM extract. Extraction-dependent differential effects of OM and WF extracts on the LOX activity are illustrated by the differences in the mode of action between WF and OM extracts and differential EC_{50}. Overall, they indicate the strongest inhibitory activity of GD OM compounds on the activity of this enzyme (Table 4). Interestingly, the mode of LOX inhibition by WF extract was dependent on kind of extract; control extract acted as non-competitive inhibitor, whereas GD extract demonstrated mixed mechanism of inhibition. In the case of the OM extracts, uncompetitive mode of LOX inhibition was observed (Fig. 1C and D).

Gastrointestinal digestion also had a strong effect on the inhibitory activity of WF extracts against XO. This is illustrated by a very low EC_{50} values estimated for GD WF extract. In this case, however we did not observe any differences between the activities of GD OM and GD WF extract (Fig. 1 E,F; Table 3). For XO enzyme, uncompetitive inhibition was observed; The interference of wheat compounds with XO activity has already been reported. Studies of Pavia et al. (2013) demonstrated the potential of wheat bran-derived ferulic acid derivatives as XO inhibitors and scavengers of hydroxyl radical with the EC_{50} values ranging from 0.16 to 0.43 mM. Furthermore, FA derivatives had higher antioxidant activity than the parental compound (FA). Our results suggest that FA derivatives may also be responsible for the differences between OM and WF extracts. Even if this notion requires experimental verification, our study is the first to show the combined inhibitory effect of OM and WF extract on TPO, LOX and XO. It also shows the potential of OM compounds for hypothyroidism prophylactics. On the other hand, the activity of analyzed extracts (its strength and mode) non-linearly depended on their composition and concentrations. They may have serious consequences for their potential application in the prophylactics of thyroid diseases.

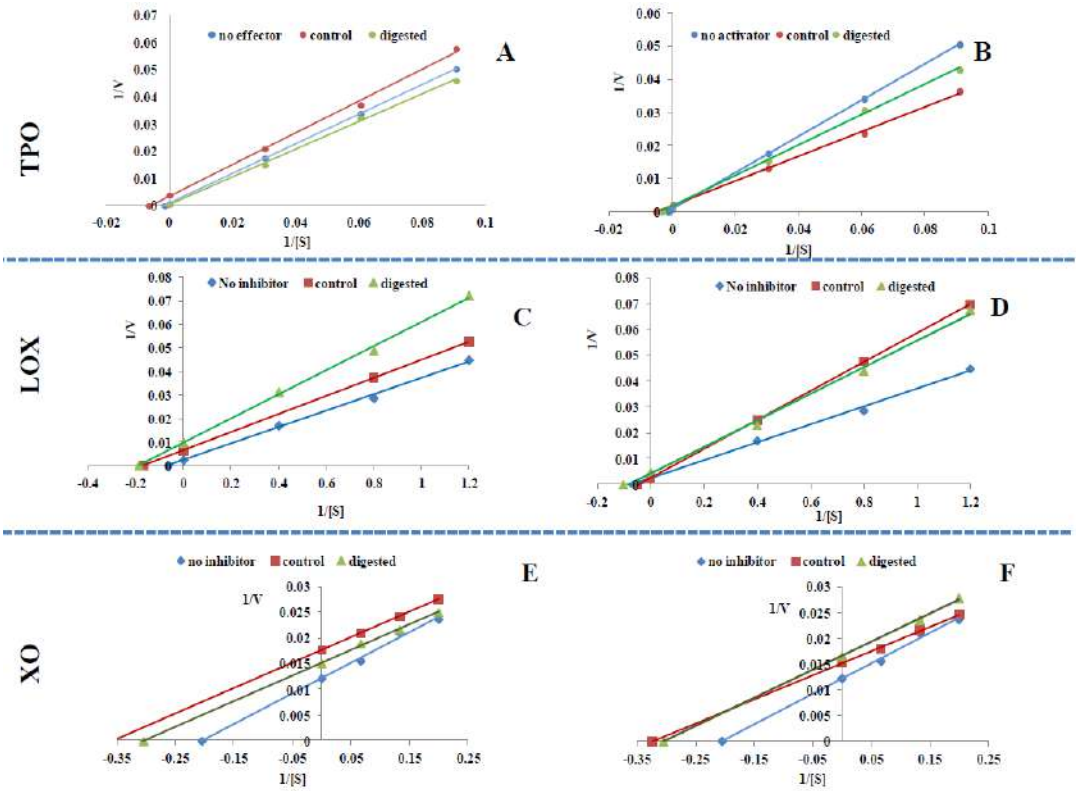

Fig. 1. Mode of thyroid peroxidase (TPO), lipoxygenase (LOX) and xanthine oxidase (XO) affection by control and digested extracts from oatmeal (A, C, E) and wheat flour (B,D,F).
Plots are expressed 1/velocity versus 1/substrate [mM] without or with extracts in a reaction solution. Guaiacol was used as a substrate for TPO, linoleic acid for LOX and xanthine for XO activity estimation.

3.4. Interaction assay

When scrutinizing the bioactivity of the plant compounds, it is important to consider the interactions between food components and their effect on the bioavailability, metabolism, and on overall health outcomes of the product. Numerous reports have documented that the interactions between drug components can enhance their individual effects (Huang et al., 2019). However, our knowledge on the interactions between the modulators of pro-oxidative enzymes and TPO is limited. Our previous study and the data described above suggest the interactions between polyphenolic activators of TPO and LOX inhibitors (Habza-Kowalska et al., 2021) To assess how food matrix affects the interactions between different FA sources, we used isobolographic method, where the combination of two active substances can enhance or reduce the strength of single component influence on human health (Chou, 2006). The interaction strength is then described by the CI (Combination Index) value (Meireles et al., 2007;Chou, 2006).

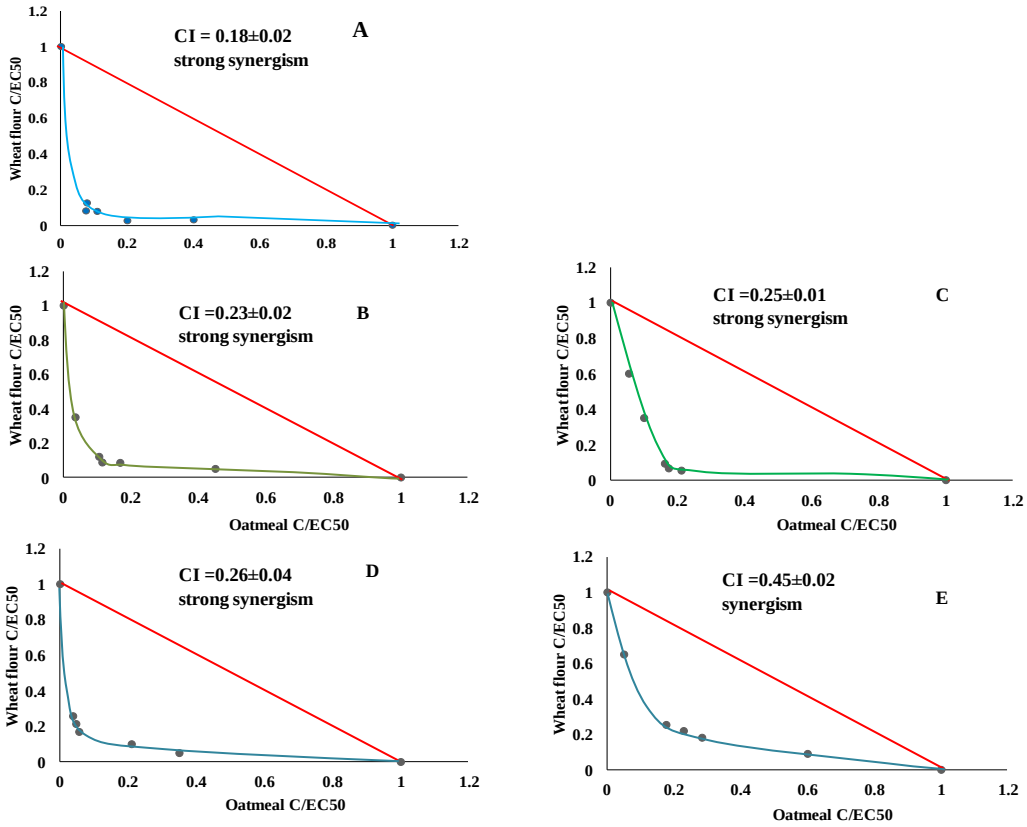

Figure 2. Dose – normalized isobolograms and combination index (CI) values for digested extracts of oatmeal and wheat flour with TPO activatory activity (A), control and digested extracts of oatmeal and wheat flour with LOX inhibitory activity (B and C, respectively), control and digested extracts of oatmeal and wheat flour with XO inhibitory activity (D and E, respectively).

The combination of an activator and an inhibitor can lead to problematic results; therefore, we did not analyze the synergistic effects of EtOH extracts on TPO activity. However, a strong synergy of stimulatory effects of GD OM and WF extracts on TPO is illustrated by relatively low CI value (0.18 ± 0.02; Fig. 2A). Similarly, strong synergy of inhibitory effects of EtOH and GD extracts on LOX activity could be seen (CI values of 0.23 ± 0.03 and 0.25 ± 0.01 respectively). Finally, we found synergistic interactions between OM and WF extracts on the inhibition of XO enzyme (CI=0.26 ± 0.04 and 0.45 ± 0.02 for EtOH and GD extracts, respectively; Fig.2 B-E).

Understanding the complex interplay between food components is crucial for a full comprehension of the impact of bioactive compounds on human health. Studies on the effect of food components on prooxidative enzymes (e.g., LOX and XO) and the analyses of the interactions between different sets of bioactive substances (incl. FA derivatives) may provide a valuable information on their potential in reducing the risk of oxidative stress and inflammation. This may be of special importance for the patients with hypothyroidism or Hashimoto disease. Our data, which show strong synergism between GD OM and WT extracts, also indicate that their phenolic content may differ, even if their basic LOX/XO inhibitory activity is similar. The same substances can affect the activity of different enzymes in diverse ways, depending on the composition of food matrix and the way of extraction. These differences may have consequences for people with thyroid diseases coexisting with oxidative stress and inflammation in the organism. On the other hand, our *in silico* observations need to be confirmed by *in vitro/in vivo* data.

3.5. The activity of OM anf WF extracts in vitro

3.5.1. GD OM extract exerts cytostatic and pro-invasive effects in TPO$^+$ B-CPAP populations

Thyroid peroxidase (TPO)$^+$ B-CPAP thyroid cancer cells represent a biological model of poorly differentiated thyroid carcinomas (PDTC) that, together with anaplastic (undifferentiated) thyroid carcinomas (ATC), are associated with a poor prognosis and mainly account for thyroid cancer-related mortality. PDTC represent an intermediate stage in the progression of well-differentiated thyroid carcinoma towards ATC (K. N. Patel & Shaha, 2006). To correlate the inhibitory effects of OM and WF extracts on the activity of pro-oxidative enzymes in silico with their biological activity, we first analyzed the basic neoplastic traits of GD OM- or GD WF-treated B-CPAP cells (Fig. 3). Cell viability tests demonstrated similar dose-dependent cytotoxic activity of both GD extracts in B-CPAP model. When administered at the concentration between 0,01% to 3% (i.e. between 1.5 and 450 g of the native product, respectively), both extract reduced the fraction of viable cells to ca. 85% (Fig. 3A). In turn, a more pronounced cytostatic activity of GD OM extract (Fig. 3B) is illustrated by attenuated proliferation of B-CPAP cells in the presence of GD OM extract (to ca. 60%, compared to 75% estimated for WF extract). This promising observation confirms previous data on the bioactivity of both products (Meireles et al., 2007) and extend them to thyroid cancer. Actually, FA has long been suggested to inhibit the proliferation of cancer cells, however OM extract was less abundant in FA that WF (cf. Table 1). It suggests the involvement of other factors. Cytostatic activity of the extracts correlated with their inhibitory effects on LOX activity, which indicates that differential sensitivity of B-CPAP cells to GD OM and WF extracts can be partly related to different content of LOX modulators (incl. FA). However, it is also conceivable that the “interactome” of OM and WF compounds comprises a multitude of networked signaling pathways, which implies a necessity of further research on this topic.

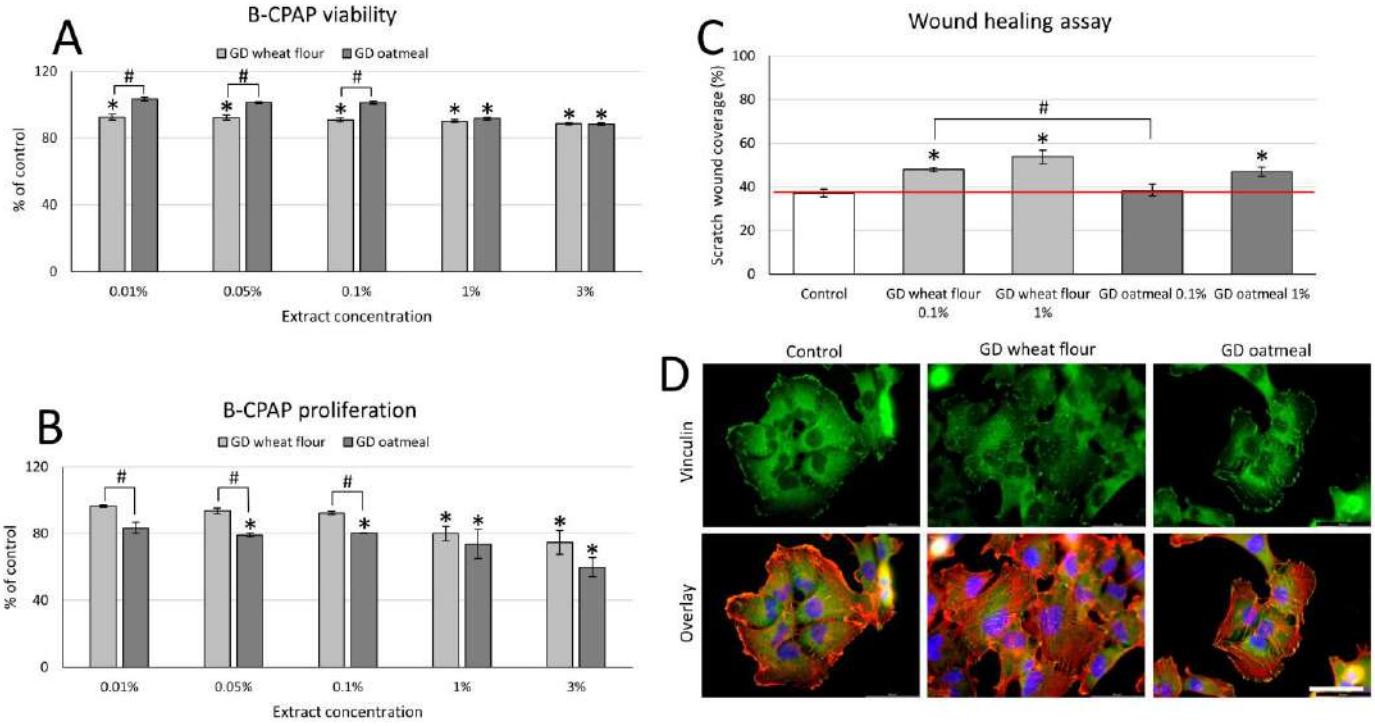

Figure 3. Cytostatic effects of GD WF and OM extracts in thyroid cancer B-CPAP cell populations. (A, B) B-CPAP cells were incubated in the presence of the GD extracts from WF and OM. Their viability (A) and proliferation (B) was estimated with FDA test and Coulter Counter Z2, respectively. (C, D) Effect of GD OM/WF extracts on the wound-healing efficiency (C) and actin cytoskeleton architecture/vinculin localization (D) estimated with time-lapse video microscopy and fluorescence microscopy, respectively. Statistical significance was calculated by one-way ANOVA followed by post hoc Tukey's HSD (A,C) or Dunnet test (B), $^{*,\#}p<0.05$ vs. relevant control. Data representative for 3 independent experiments. Bars represent SEM values. Note the cytostatic effects of the extracts, accompanied by their pro-invasive activity.

Surprisingly, the cytostatic effects of both GD extracts were accompanied by their unexpected effects on B-CPAP motility. It was slightly enhanced after their application, as illustrated by wound healing experiments (Fig. 3C). Concomitantly, we observed distinct actin cytoskeleton rearrangements (stress fibers formation) and the maturation of focal adhesions in B-CPAP cells cultivated in the presence of both extracts (Fig. 3D). Apparently, a strong cytostatic effect of WF and OM extracts applied at physiologic doses is accompanied by the induction of B-CPAP motility. We did not observe any signs of prominent apoptotic B-CPAP response, which indicates that the induction of motile phenotype rather than a selection of motile cells from heterogeneous populations accounts for the observed effects. In any case, these data may indicate the protective effect of ROS scavenging on cancer cells and/or the activation of pro-invasive signaling pathways.

3.5.2. Gastrointestinal processing and anti-cancer activity of the extracts.

To estimate the contribution of gastrointestinal digestion of OM and WF extracts on their activity in thyroid cancer model, we also estimated the activity of raw (PBS) extracts from both products. PBS OM and WF extracts displayed similar cytostatic activity to that of their GD counterparts (Fig. 4A,B). However PBS extract from OM had no “pro-invasive” properties (Fig. 4C). Concomitantly, we observed less pronounced actin cytoskeleton rearrangements in the cells treated with PBS extracts (Fig. 4D). Thus, gastrointestinal digestion augments the bioavailability (by release from food matrix) of protective/pro-invasive compounds of oatmeal, while retaining their cytostatic activity.

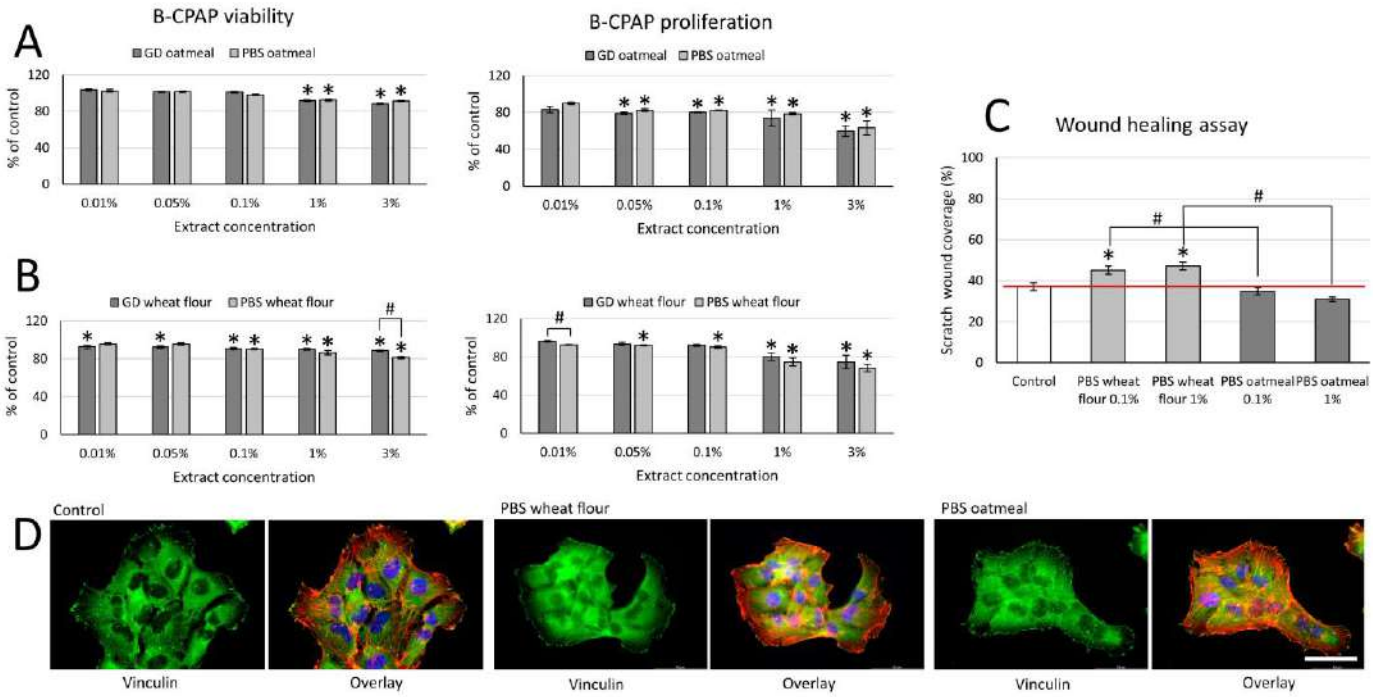

Figure 4. The effect of raw (PBS) extracts from wheat flour (WF) and oatmeal (OM) on the proliferation and invasiveness of B-CPAP cells. (A, B) B-CPAP viability (A) and proliferation (B) in the presence of PBS extracts from WF and OM was estimated with FDA test and Coulter Counter Z2, respectively. (C, D) Effect of PBS WF/OM extracts on the wound-healing efficiency (C) and actin cytoskeleton architecture/vinculin localization estimated with time-lapse videomicroscopy and fluorescence microscopy (GD effect = 100%). Scale bar – 50 μm. Statistical significance was calculated by one-way ANOVA followed by post hoc Tukey's HSD (A) or Dunnet test (B, C), $^{*,\#}p<0.05$ vs. control. Data representative for 3 independent experiments. Bars represent SEM values. Note a less pronounced pro-invasive effects of PBS extracts.

3.5.3. TPO and cell sensitivity to GD extracts.

Finally, we addressed the potential role of TPO activation in the determination of thyroid cancer cell reactivity to GD OM and WF extracts. For this purpose, we used an experimental approach based on TPO^- (8505C) cells, which represent a well-recognized cellular model of human thyroid cancer (Meireles et al., 2007). These "dedifferentiated" cells have lost thyroid-specific markers and display higher malignancy when compared to their TPO^+ B-CPAP counterparts. Because they do not express TPO (TPO^- phenotype), they are also suitable for the analyses of TPO contribution to the reactivity of thyroid cancer cells to extrinsic signals, incl. natural bio-compounds. We estimated the effect of TPO on the sensitivity of 8505C cells to both extracts by comparing their motility (wound healing) in the presence of GD/PBS OM/WF extract with the motility of B-CPAP cells in these conditions. No significant differences in the quality of 8505C and B-CPAP cells to both extracts could be seen (Fig. 5A, cf. Fig. 3C and Fig. 4C). 8505C cells remained sensitive to pro-invasive activity of 1% GD OM extract, whereas PBS extracts again displayed lower pro-invasive activity. Thus, gastrointestinal digestion increases the activity of this extract in both cellular models. Moreover, pro-invasive effects were accompanied by actin cytoskeleton rearrangements, in particular the redistribution of F-actin to cell peripheries (Fig. 5B). Collectively, a relatively high sensitivity of TPO^- cells to GD-released compounds indicates a marginal role of TPO activation in cellular reactivity to OM and WF extracts. Because ATC represent a subset of thyroid tumors that are associated with the worst prognosis, while TPO^- 8505C ATC cells display aggressive behavior and increased locoregional and distant invasion, our observations also confirm that OM/WF extracts may induce adverse effects in the patients with advanced thyroid tumors.

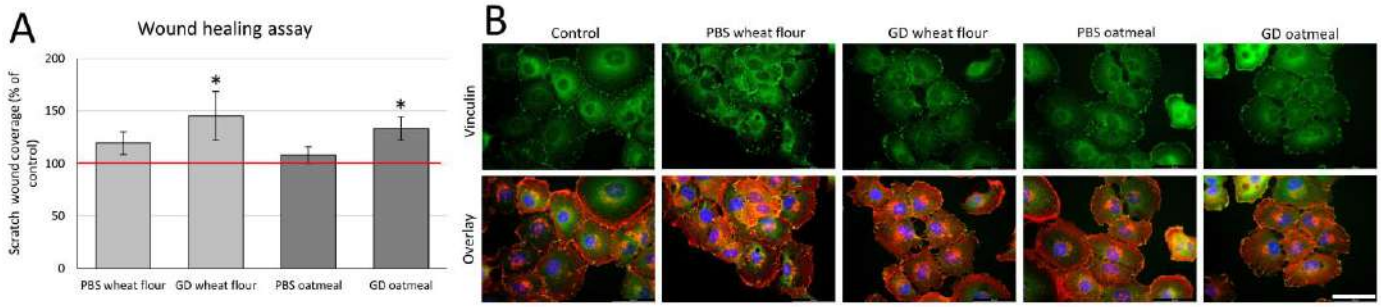

Figure 5. Cytostatic effects of flour and flake extracts in thyroid cancer 8505C cell populations. (A,B) Effect of GD/PBS WF/OM extracts on the wound-healing efficiency (C) and actin cytoskeleton architecture/vinculin localization estimated with time-lapse videomicroscopy and fluorescence microscopy. Statistical significance was calculated by one-way ANOVA followed by post hoc Dunnet test, *p<0.05 vs. control. Data representative for 3 independent experiments. Scale bar – 50 μm. Bars represent SEM values. Data representative for 3 independent experiments. Bars represent SEM values. Note a pro-invasive activity of both extracts in 8505C model.

Collectively, an experimental model based on TPO^+ PDTC B-CPAP cells and their TPO^- ATC counterparts (8505C) cells enabled us to estimate the effect of the malignancy (differentiation status) of thyroid cancer cells' and/or TPO expression on cellular sensitivity to plant extracts. Using this approach, we demonstrated (i) a relatively high cytostatic activity of GD OM extract, (ii) its correlation with the LOX inhibitory activity of this extract accompanied by (iii) its cytoprotective/pro-invasive effects. This points to the role of the balance between cytoprotective and cytostatic effects of compounds in the regulation of cell proliferation/motility. On the other hand, (iv) TPO activation is apparently not involved in these interactions. Cytoprotective activities of OM and WF compounds may improve cellular welfare at low concentrations. A shift from proliferation to invasion state in extract-treated cells may also be related to the "escape" strategy of stress management (Pani et al., 2010), however further research is necessary to fully elucidate mechanisms underlying this phenomenon (Pudełek et al., 2020).

4. Conclusions

Studies on the antiradical potential of oatmeal and wheat flour compounds (selected as a rich ferulic acid sources is based on the polyphenol database (Rothwell et al., 2013)), on their LOX/XO inhibitory activity and TPO activating effects, as well as on their effect on thyroid cancer development, provided important insights into the mechanisms underlying the health-promoting effects of these commonly consumed products. Apparently, oatmeal and wheat flour contain TPO activators and effective LOX and XO inhibitors, which can play an important role in the prophylactics of Hashimoto's disease. To the best of our knowledge, this is the 1st report on the plant products that contain such a set of bioactive compounds. Accordingly, OM and WF (especially OM!) can be used for diet supplementation in the treatment of hypothyroidism. It may have implications for the development of new functional foods and dietary supplements with multiple health benefits. However, the application of extractable hydrophilic compounds of oatmeal and wheat flour extracts as a universal supplement to interfere with thyroid cancer promotion is questionable.

Our study also provides valuable information on the effects of the gastrointestinal processing and food matrix on the bioavailability of TPO, LOX and XO modulators. In conjunction the prominent differences in the phenolic content between OM and WF extracts, we confirm that the synergy/antagonism of individual compounds is decisive for overall bioactivity of the product. Notably, the concentrations that we used in the experiments are relatively low and partly correspond to the physiologic values, which adds to the significance of our data. Therefore, our data provide new and valuable information that opens the way to further scientific investigations. It is obvious that issues related to thyroid cancer need to be confirmed in subsequent experiments. Further research is also needed to fully understand the mechanisms of the interactions between LOX/XO/TPO-related effects and cytostatic/pro-invasive activity of the tested extracts. *In vivo* and cohort studies should help to determine the safety and efficacy of these compounds as supplements or drug candidates.

Author Contributions: Conceptualization, UGD, JC, KP. and EHK; methodology, UGD, JC, KP. and EHK .; validation, UGD, JC, KP; formal analysis, UGD, JC; investigation, EHK. KP, UGD, JC; resources, UGD, JC, KP. and EHK; data curation, EHK, UGD, KP, JC; writing—original draft preparation, EHK, KP ; writing—review and editing UGD, JC.; visualization, EHK, KP supervision, UGD, JC; project administration, UGD; funding acquisition, UGD, JC. All authors have read and agreed to the published version of the manuscript.

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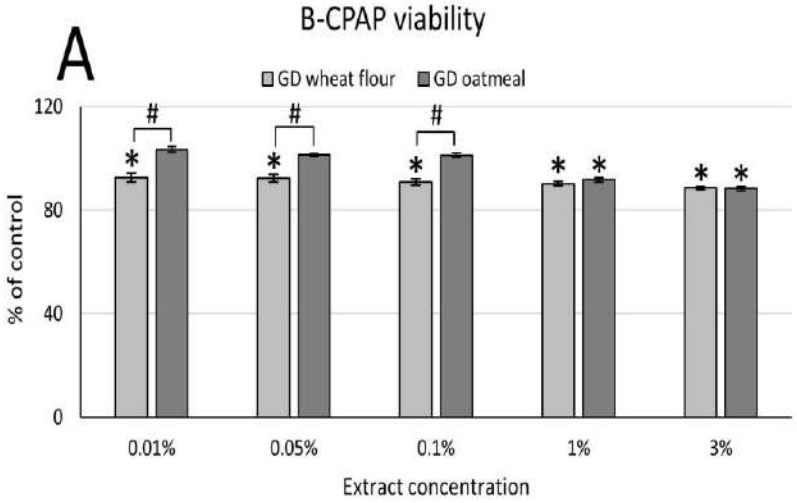
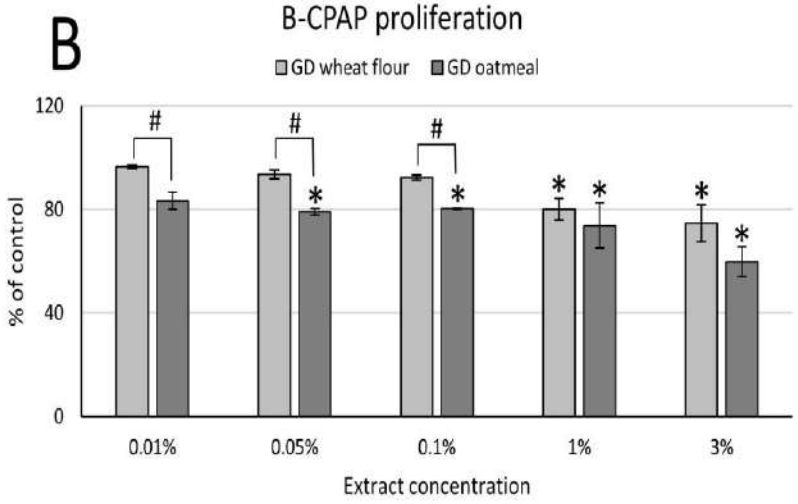
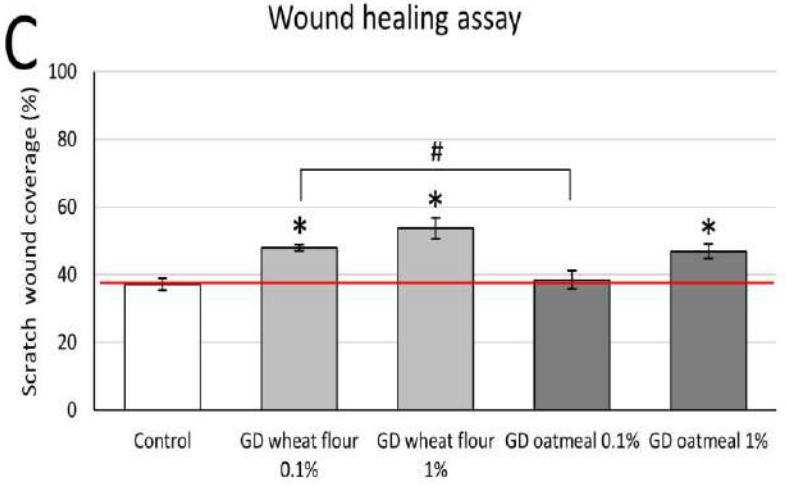
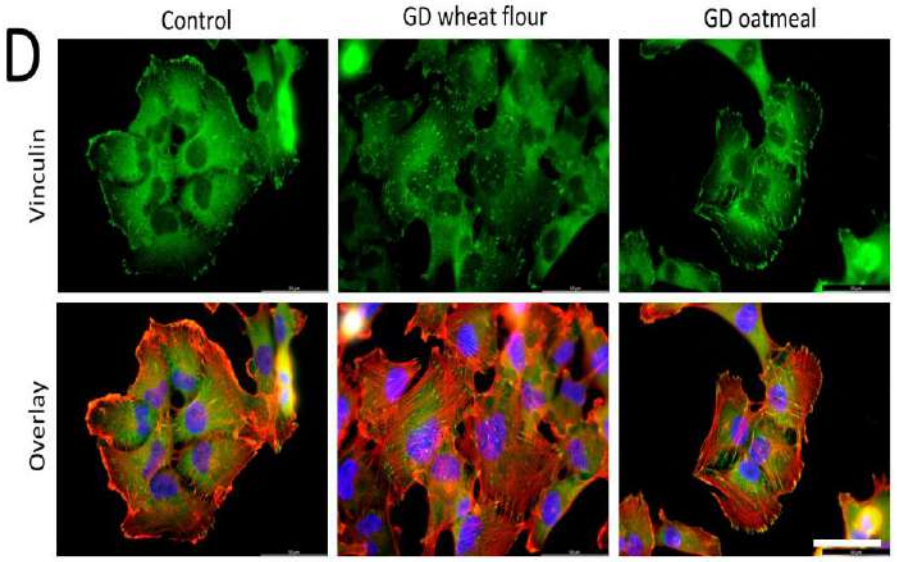
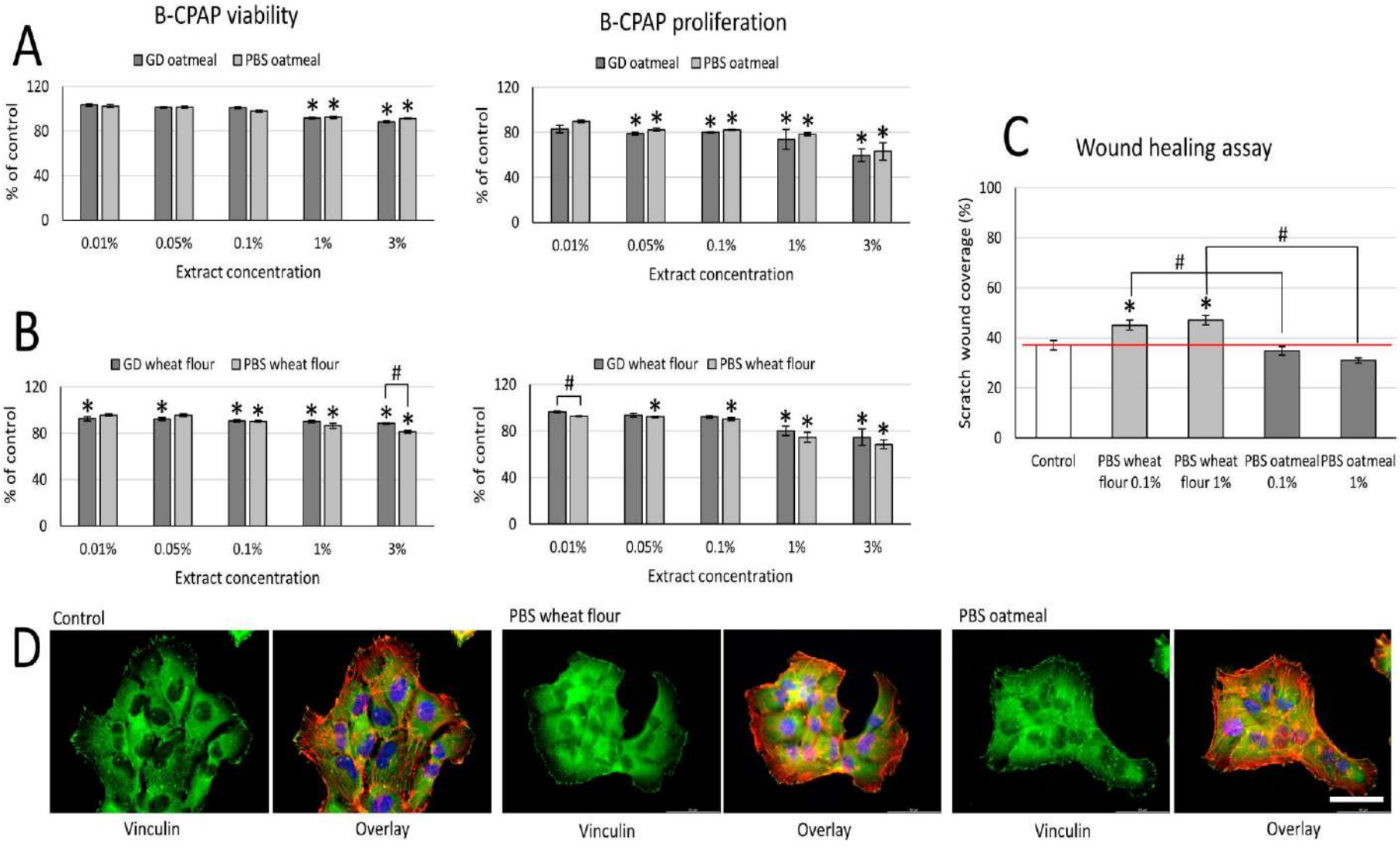
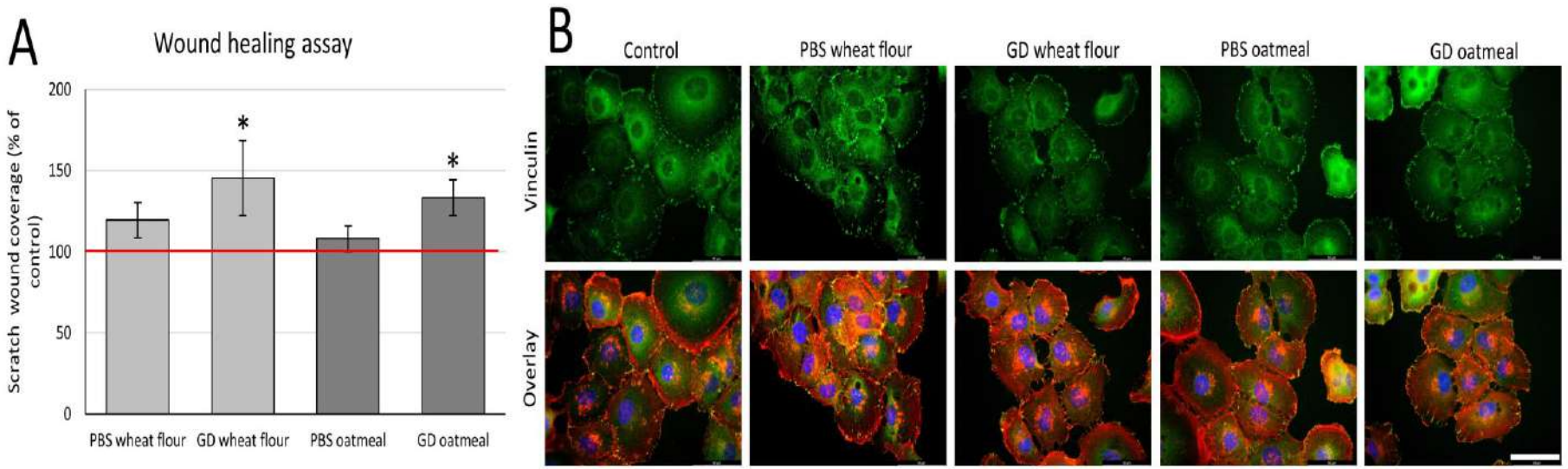

A
B-CPAP viability
GD wheat flour
GD oatmeal
% of control
120
80
40
0
0.01%
0.05%
0.1%
1%
3%
Extract concentration


B
B-CPAP proliferation
GD wheat flour
GD oatmeal
% of control
120
80
40
0
0.01%
0.05%
0.1%
1%
3%
Extract concentration


C
Wound healing assay
Scratch wound coverage (%)
100
80
60
40
20
0
Control
GD wheat flour 0.1%
GD wheat flour 1%
GD oatmeal 0.1%
GD oatmeal 1%


D
Control
GD wheat flour
GD oatmeal
Vinculin
Overlay


A
B-CPAP viability
GD oatmeal
PBS oatmeal
% of control
120
80
40
0
0.01%
0.05%
0.1%
1%
3%
Extract concentration
B-CPAP proliferation
GD oatmeal
PBS oatmeal
% of control
Extract concentration
B
GD wheat flour
PBS wheat flour
% of control
Extract concentration
GD wheat flour
PBS wheat flour
% of control
Extract concentration
C
Wound healing assay
Scratch wound coverage (%)
100
80
60
40
20
0
Control
PBS wheat flour 0.1%
PBS wheat flour 1%
PBS oatmeal 0.1%
PBS oatmeal 1%
D
Control
PBS wheat flour
PBS oatmeal
Vinculin
Overlay
Vinculin
Overlay
Vinculin
Overlay

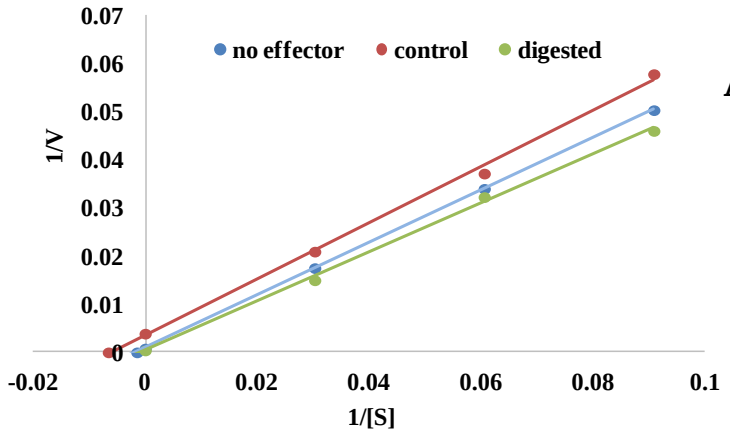

A
Wound healing assay
Scratch wound coverage (% of control)
200
150
100
50
0
*
*
PBS wheat flour
GD wheat flour
PBS oatmeal
GD oatmeal
B
Control
PBS wheat flour
GD wheat flour
PBS oatmeal
GD oatmeal
Vinculin
Overlay

Source	Mean content [µg/g DW]
Wheat flour	593.94 ± 56.25
Oatmeal	207.4 ± 7

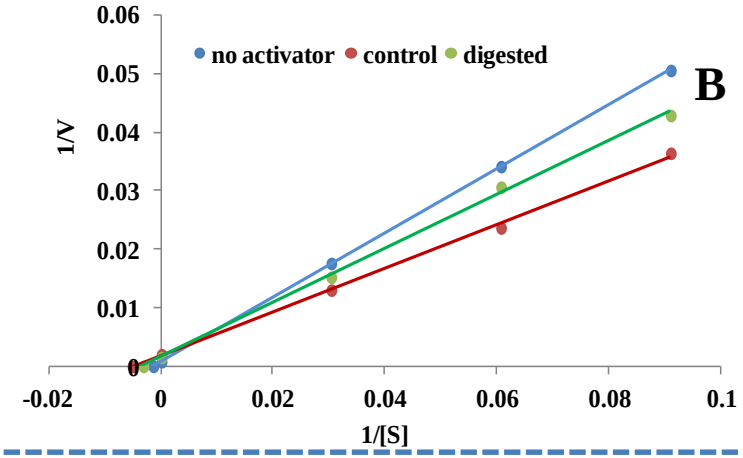
Raw material	Total phenolics content (TPC) [mg GAE g^{-1}]	Antioxidant activity EC_{50} [μg ml^{-1}]	
		DPPH	**ABTS**
Control extracts			
Oatmeal	5.66 ± 0.28[a]	332.18 ± 16.61[a]	249.75 ± 12.49[b]
Wheat flour	14.25 ± 0.71[b]	338.57 ± 16.93[b]	162.31 ± 8.12[a]
Digested extracts			
Oatmeal	11.59 ± 0.58[a]	50.37 ± 2.52[a]	810.7 ± 40.53[a]
Wheat flour	18.60 ± 0.93[b]	66.29 ± 3.31[b]	930.06 ± 46.5[b]

	Kind of extract	Mode of action	EC_{50} (mg DW/mL)	Km (mM)	Vmax (Δ AU/min)
Thyroid peroxidase					
Control enzyme		-	-	678.75±10.25[a]	1250±45.27[a]
Oatmeal	Control	uncompetitive inhibition	6.71±0.34[a]	152.05±7.42[b]	256.41±9.98[b]
	After digestion	activation	8.84±0.44[b]	170.07±5.38[c]	2500±32.24[c]
Wheat flour	Control	activation	0.39±0.004[c]	192.55±8.33[d]	500±8.54[d]
	After digestion	activation	8.31±0.42[b]	307.60±15.88[e]	666.67±31.25[e]
Lipoxygenase					
Control enzyme		-	-	15.13±0.85[a]	434.78±11.08[a]
Oatmeal	Control	uncompetitive inhibition	4.80±0.24[a]	5.91±0.07[b]	153.85±4.56[b]
	After digestion	uncompetitive inhibition	3.57±0.17[b]	5.29±0.09[b]	103.09±5.27[c]
Wheat flour	Control	non-competitive inhibition	6.3±0.31[c]	370.37±12.32[c]	20.74±0.58[d]
	After digestion	mixed inhibition	9.38±0.47[d]	204.08±8.75[d]	9.67±0.05[e]
Xanthine oxidase					
Control enzyme		-	-	4.87±0.03[a]	81.96±3.35[a]
Oatmeal	Control	uncompetitive inhibition	0.37±0.001[a]	2.79±0.02[b]	56.49±2.31[b]
	After digestion	uncompetitive inhibition	0.33±0.00[a]	3.27±0.01[c]	66.23±3.61[c]
Wheat flour	Control	uncompetitive inhibition	4.07±0.001[b]	3.07±0.01[c]	65.36±3.25[c]
	After digestion	uncompetitive inhibition	0.34±0.002[a]	3.27±0.01[c]	66.24±2.85[c]

TPO

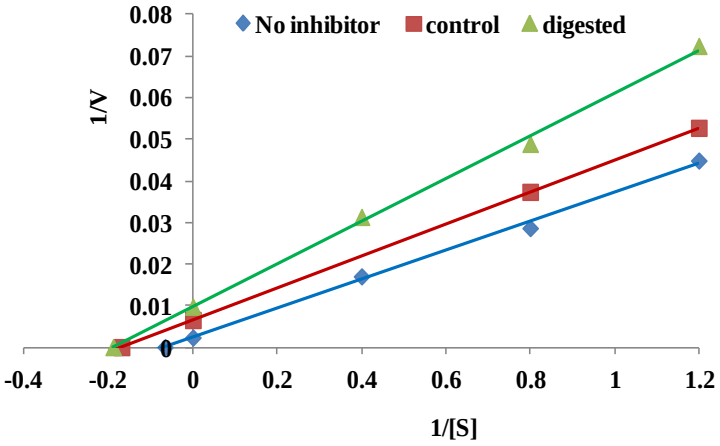


A

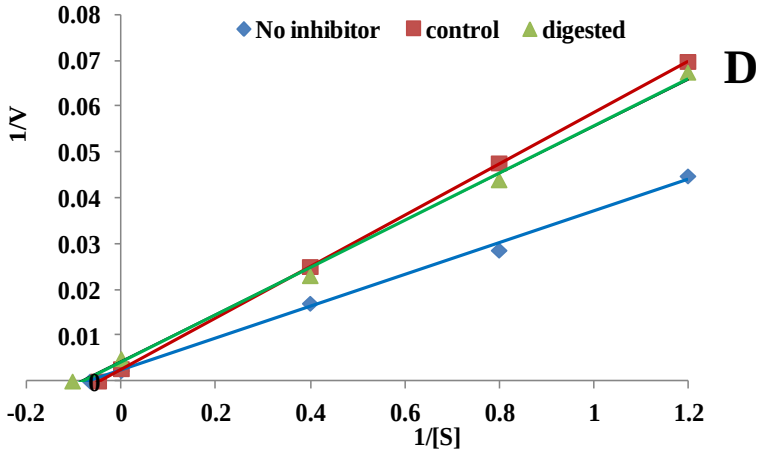


B

LOX

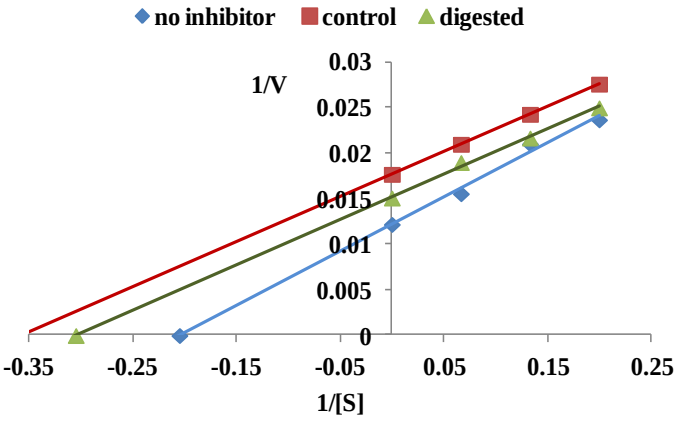


C

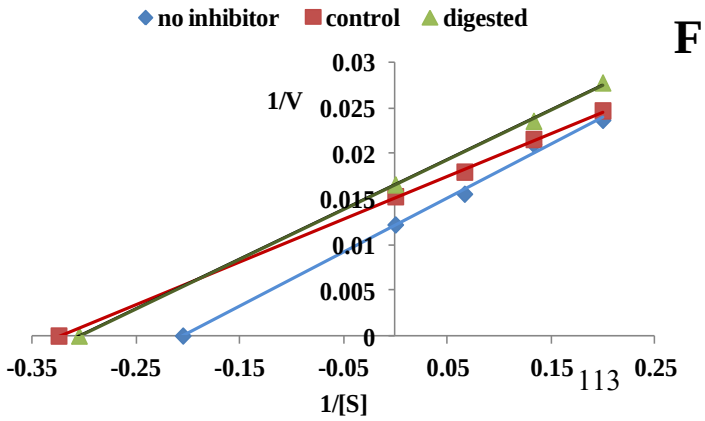


D

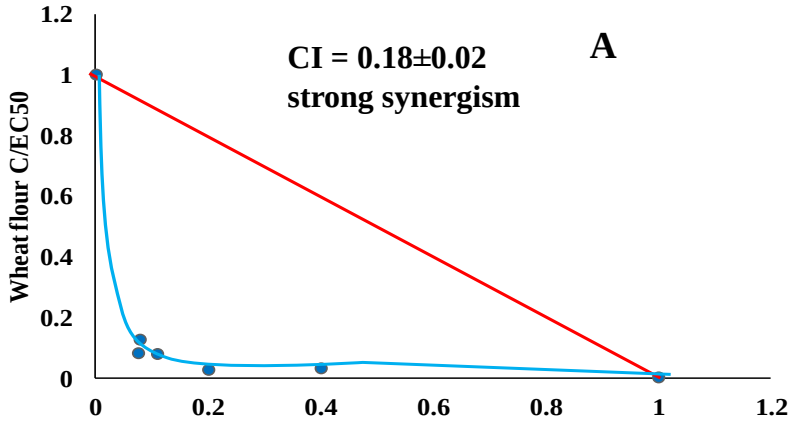
XO

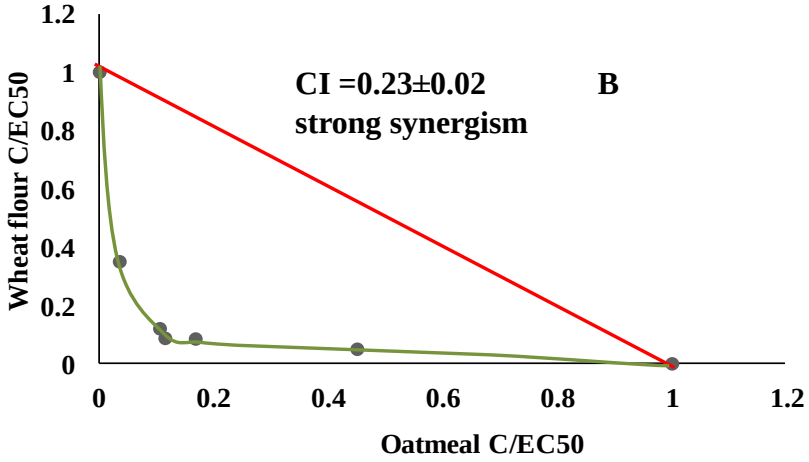


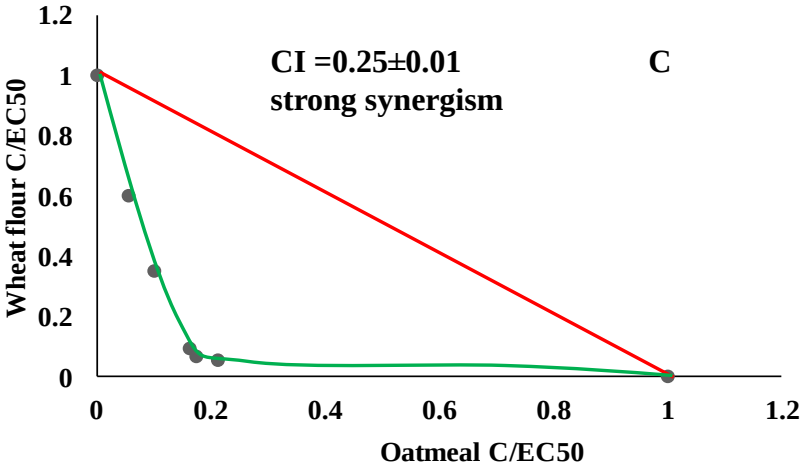
E

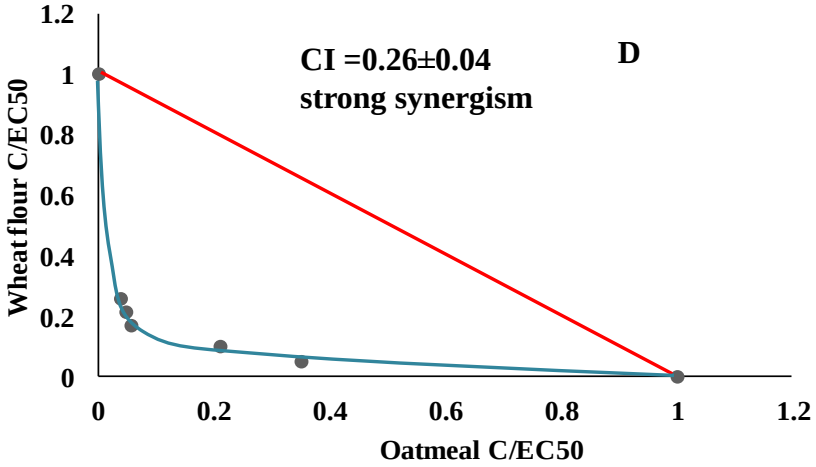


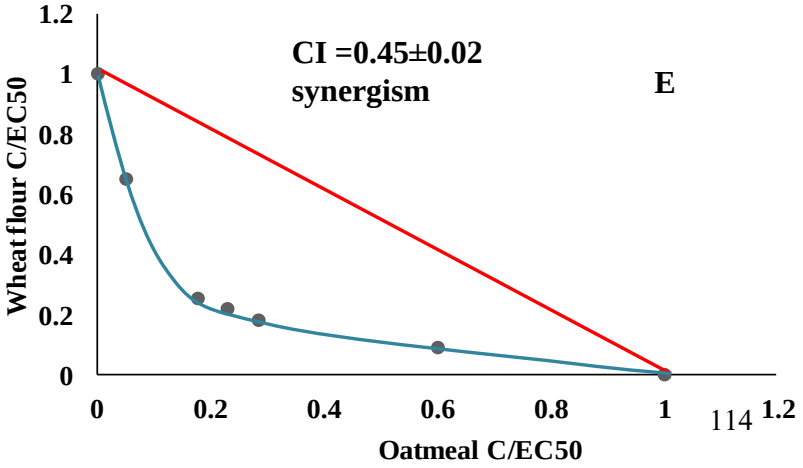
F


A
CI = 0.18±0.02
strong synergism
Wheat flour C/EC50
0
0.2
0.4
0.6
0.8
1
1.2


B
CI =0.23±0.02
strong synergism
Wheat flour C/EC50
Oatmeal C/EC50
0
0.2
0.4
0.6
0.8
1
1.2


C
CI =0.25±0.01
strong synergism
Wheat flour C/EC50
Oatmeal C/EC50
0
0.2
0.4
0.6
0.8
1
1.2


D
CI =0.26±0.04
strong synergism
Wheat flour C/EC50
Oatmeal C/EC50
0
0.2
0.4
0.6
0.8
1
1.2


E
CI =0.45±0.02
synergism
Wheat flour C/EC50
Oatmeal C/EC50
0
0.2
0.4
0.6
0.8
1
1.2

11. Oświadczenia współautorów publikacji stanowiących przedmiot rozprawy doktorskiej

Lublin, 14.06.2023

Oświadczenie autorów publikacji

Niniejszym oświadcza się, że publikacja:

Habza - Kowalska E., Kaczor A. A., Żuk J., Matosiuk D., Gawlik - Dziki U. (2019). Thyroid peroxidase activity is inhibited by phenolic compounds - impact of interaction. Molecules , 24 (15), 2766.

powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Habza – Kowalska E. - współtworzenie koncepcji pracy, dokonanie przeglądu literatury, metodologia, przeprowadzenie doświadczeń laboratoryjnych, opracowanie wyników badań, napisanie manuskryptu, pomoc w przygotowaniu odpowiedzi na recenzje.

mgr inż. Ewa Habza - Kowalska

Ewa Habza - Kowalska

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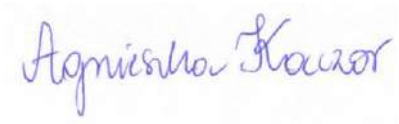
powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Kaczor A. A. - metodologia, współtworzenie manuskryptu, opracowanie wyników badań, pomoc w przygotowaniu manuskryptu nadzór merytoryczny.



dr hab. n. farm. Agnieszka Kaczor prof. uczelni

Lublin, 14.06.2023

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powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Żuk J. – metodologia, współtworzenie manuskryptu, opracowanie wyników badań.

dr n. farm. Justyna Żuk

Lublin, 14.06.2023

Oświadczenie autorów publikacji

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powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Matosiuk D. – nadzór merytoryczny, analiza formalna pracy.

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powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Gawlik – Dziki U. – współtworzenie koncepcji pracy, metodologia, pomoc w przygotowaniu manuskryptu, nadzór merytoryczny, analiza formalna pracy, pełnienie funkcji autora korespondencyjnego, przygotowanie odpowiedzi na recenzje.

Urszula Gawlik - Dziki

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Lublin, 14.06.2023

Oświadczenie autorów publikacji

Niniejszym oświadcza się, że publikacja:

Habza - Kowalska E., Gawlik - Dziki U., Dziki D. (2019). Mechanism of Action and Interactions Between Thyroid Peroxidase and Lipoxygenase Inhibitors Derived from Plant Sources. Biomolecules, 9 (11), 1-16.

powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Habza – Kowalska E. - współtworzenie koncepcji pracy, dokonanie przeglądu literatury, metodologia, przeprowadzenie doświadczeń laboratoryjnych, opracowanie wyników badań, napisanie manuskryptu, pomoc w przygotowaniu odpowiedzi na recenzje.

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Ewa Habza - Kowalska

mgr inż. Ewa Habza - Kowalska

Urszula Gawlik - Dziki

prof. dr hab. Urszula Gawlik – Dziki

Dariusz Dziki

prof. dr hab. Dariusz Dziki

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Habza - Kowalska E., Kaczor A. A., Bartuzi D., Piłat J., Gawlik - Dziki U. (2021). Some dietary phenolic compounds can activate thyroid peroxidase and inhibit lipoxygenase-preliminary study in the model systems. *Int. J. Mol. Sci.*, 22 (10), 5108.

powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Habza – Kowalska E. - współtworzenie koncepcji pracy, dokonanie przeglądu literatury, metodologia, przeprowadzenie doświadczeń laboratoryjnych, opracowanie wyników badań, napisanie manuskryptu, pomoc w przygotowaniu odpowiedzi na recenzje.

mgr inż. Ewa Habza - Kowalska

Ewa Habza - Kowalska

Lublin, 14.06.2023

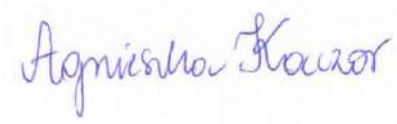
Oświadczenie autorów publikacji

Niniejszym oświadcza się, że publikacja:

Habza - Kowalska E., Kaczor A. A., Bartuzi D., Piłat J., Gawlik - Dziki U. (2021). Some dietary phenolic compounds can activate thyroid peroxidase and inhibit lipoxygenase-preliminary study in the model systems. *Int. J. Mol. Sci.*, 22 (10), 5108.

powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Kaczor A. A. - metodologia, współtworzenie manuskryptu, opracowanie wyników badań, pomoc w przygotowaniu manuskryptu nadzór merytoryczny.



dr hab. n. farm. Agnieszka Kaczor prof. uczelni

Lublin, 14.06.2023

Oświadczenie autorów publikacji

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powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Bartuzi D. – metodologia, współtworzenie manuskryptu, opracowanie wyników badań.

Lublin, 14.06.2023

Oświadczenie autorów publikacji

Niniejszym oświadcza się, że publikacja:

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powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Piłat J. – nadzór merytoryczny, analiza formalna pracy.

Jacek Piłat

dr n. med. Jacek Piłat

Lublin, 14.06.2023

Oświadczenie autorów publikacji

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powstała w wyniku poniżej określonego, indywidualnego wkładu pracy współautorów:

Gawlik – Dziki U. – współtworzenie koncepcji pracy, metodologia, pomoc w przygotowaniu manuskryptu, nadzór merytoryczny, analiza formalna pracy, pełnienie funkcji autora korespondencyjnego, przygotowanie odpowiedzi na recenzje.

Urszula Gawlik - Dziki

prof. dr hab. Urszula Gawlik – Dziki

Lublin, 14.06.2023

Oświadczenie autorów publikacji

Niniejszym oświadcza się, że publikacja:

Habza - Kowalska E., Piwowarczyk K., Czyż J., Gawlik - Dziki U. (2023). Oatmeal and wheat flour as the sources of thyroid peroxidase (TPO), lipoxygenase (LOX) and xanthine oxidase (XO) modulators potentially applicable in the prevention of inflammatory thyroid diseases, *BioRxiv,* DOI. 10.1101/2023.06.05.543703

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Habza – Kowalska E. - współtworzenie koncepcji pracy, dokonanie przeglądu literatury, metodologia, przeprowadzenie doświadczeń laboratoryjnych, opracowanie wyników badań, napisanie manuskryptu, pełnienie roli autora korespondencyjnego.

Piwowarczyk K. - współtworzenie koncepcji pracy, metodologia, współtworzenie manuskryptu, opracowanie wyników badań.

Czyż J. – współtworzenie koncepcji pracy, analiza formalna pracy, pomoc w przygotowaniu manuskryptu, nadzór merytoryczny.

Gawlik – Dziki U. – współtworzenie koncepcji pracy, metodologia, pomoc w przygotowaniu manuskryptu, nadzór merytoryczny, analiza formalna pracy.

Ewa Habza - Kowalska

Lublin. 14.06.2023

Oświadczenie autorów publikacji

Niniejszym oświadcza się, że publikacja:

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Gawlik – Dziki U. – współtworzenie koncepcji pracy, metodologia, pomoc w przygotowaniu manuskryptu, nadzór merytoryczny, analiza formalna pracy.

Katarzyna Piwowarczyk

Kraków, 19.06.2023

Oświadczenie autorów publikacji

Niniejszym oświadcza się, że publikacja:

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Gawlik – Dziki U. – współtworzenie koncepcji pracy, metodologia, pomoc w przygotowaniu manuskryptu, nadzór merytoryczny, analiza formalna pracy.

Jarosław Czyż

Lublin, 14.06.2023

Oświadczenie autorów publikacji

Niniejszym oświadcza się, że publikacja:

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Urszula Gawlik-Dziki

12. Zestawienie dorobku naukowego

19.06.2023, Lublin

Biblioteka Główna UP w Lublinie
Baza publikacji Pracowników Uniwersytetu Przyrodniczego
Raport autora za lata 2017-2021 – mgr inż. Ewa Habza-Kowalska

1. Publikacje w czasopismach naukowych

1.1. Publikacje w czasopiśmie naukowym posiadającym Impact Factor IF

Lp	Opis bibliograficzny	IF	Punkty ministerialne
1.	**Some dietary phenolic compounds can activate thyroid peroxidase and inhibit lipoxygenase-preliminary study in the model systems.** [AUT.] EWA HABZA-KOWALSKA, AGNIESZKA A. KACZOR, DAMIAN BARTUZI, JACEK PIŁAT, [AUT. KORESP.] URSZULA GAWLIK-DZIKI. *Int. J. Mol. Sci.* 2021 Vol. 22 Issue 10 Article number 5108, il., bibliogr., sum. DOI: 10.3390/ijms22105108	6,208	140,00
2.	**Drying kinetics, grinding characteristics, and physicochemical properties of broccoli sprouts.** [AUT. KORESP.] DARIUSZ DZIKI, [AUT.] EWA HABZA-KOWALSKA, URSZULA GAWLIK-DZIKI, ANTONI MIŚ, RENATA RÓŻYŁO, ZBIGNIEW KRZYSIAK, WALEED H.HASSOON. *Processes* 2020 Vol. 8 Iss. 1, Article number 97 s. 1-11, il., bibliogr., sum. DOI: 10.3390/pr8010097	2,847	70,00
3.	**Leaves of white beetroot as a new source of antioxidant and anti-inflammatory compounds.** [AUT. KORESP.] URSZULA GAWLIK-DZIKI, [AUT.] LAURA DZIKI, JAKUB ANISEWICZ, EWA HABZA-KOWALSKA, MAŁGORZATA SIKORA, DARIUSZ DZIKI. *Plants* 2020 Vol. 9 Issue 8 Article number 944 s. 1-14, il., bibliogr., sum. DOI: 10.3390/plants9080944	3,935	70,00
4.	**Impact of interactions between ferulic and chlorogenic acids on enzymatic and non-enzymatic lipids oxidation: An example of bread enriched with green coffee flour.** [AUT. KORESP.] URSZULA GAWLIK-DZIKI, [AUT.] JAROSŁAW BRYDA, DARIUSZ DZIKI, MICHAŁ ŚWIECA, EWA HABZA-KOWALSKA, URSZULA ZŁOTEK. *Appl. Sci.-Basel* 2019 Vol. 9 Iss. 3 s. 1-12 Article number 568, il., bibliogr., sum. DOI: 10.3390/app9030568	2,474	100,00
5.	**Mechanism of action and interactions between thyroid peroxidase and lipoxygenase inhibitors derived from plant sources.** [AUT.] EWA HABZA-KOWALSKA, [AUT. KORESP.] URSZULA GAWLIK-DZIKI, [AUT.] DARIUSZ DZIKI. *Biomolecules* 2019 Vol. 9 Iss. 11 s. 1-16, il., bibliogr. DOI: 10.3390/biom9110663	4,082	100,00
6.	**Molecular techniques for detecting food adulteration.** [AUT.] EWA HABZA-KOWALSKA, MAŁGORZATA GRELA, MAGDALENA	0,281	70,00

	GRYZIŃSKA, [AUT. KORESP.] PIOTR LISTOS. *Med. Weter.* 2019 Vol. 75 nr 7 s. 404-409, il., bibliogr., sum. DOI: 10.21521/mw.6261		
7.	**Thyroid peroxidase activity is inhibited by phenolic compounds - impact of interaction.** [AUT.] EWA HABZA-KOWALSKA, AGNIESZKA A. KACZOR, JUSTYNA ŻUK, DARIUSZ MATOSIUK, [AUT. KORESP.] URSZULA GAWLIK-DZIKI. *Molecules (Basel,Online)* 2019 Vol. 24 Issue 15 Article no. 2766, il., bibliogr., sum. DOI: 10.3390/molecules24152766	3,267	140,00
	Suma:	23,094	690,00

1.2 Publikacja w czasopiśmie naukowym nieposiadającym IF

Lp	Opis bibliograficzny	Punkty ministerialne
1.	**Znaczenie enolazy jako białka „moonlighting (The significance of enolase as a "moonlighting" protein).** [AUT. KORESP.] KLAUDIA GUSTAW, [AUT.] EWA HABZA, ADAM WAŚKO. *Nauki Przyr. i Med.(Lub.)* 2018 nr 3 (21) s. 63-73, bibliogr., streszcz., sum.	2,00
	Suma:	2,00

2. Monografie naukowe

2.1 Autorstwo rozdziału w monografii naukowej

Lp	Opis bibliograficzny	Punkty ministerialne
1.	**Bakterie wykorzystywane w celu tworzenia,biofunkcyjnych i prozdrowotnych produktów mlecznych (Bacteria and yeast used to create biofunctional and health-promoting dairy foods).** [AUT.] JAGODA SZAFRAŃSKA, JAN MAŁECKI, EWA HABZA-KOWALSKA. W: Żywność i żywienie / Redakcja naukowa Jędrzej Nyćkowiak, Jacek Leśny Poznań 2020, Młodzi Naukowcy, s. 98-104, il., bibliogr., streszcz, 978-83-66392-82-3.	5,00
2.	**Charakterystyka enzymu peroksydazy tyroidowej (TPO), wpływ stresu oksydacyjnego na jegofunkcjonowanie oraz zastosowanie metody izobolograficznej do obrazowania interakcji pomiędzy składnikami aktywnymi żywności na jego obniżenie (Characterization of the thyroid peroxidase enzyme (TPO), the impact of oxidative stress on its functioning and the use of the isobolographic method to visualize the interaction between active ingredients of food to reduce the oxidative stress).** [AUT.] EWA HABZA-KOWALSKA, JAGODA SZAFRAŃSKA. W: Żywność i żywienie / Redakcja naukowa Jędrzej Nyćkowiak, Jacek Leśny Poznań 2020, Młodzi Naukowcy, s. 49-53, il., bibliogr., streszcz, 978-83-66392-59-5.	5,00
3.	**Właściwości antyoksydacyjne wybranych surowców roślinnych w zależności od temperatury przechowywania oraz zastosowania obróbki wysokotemperaturowej**	5,00

	(Antioxidant properties of selected plant materials depending on storage temperature and the use of high temperature termination treatment). [AUT.] EWA HABZA-KOWALSKA, LAURA DZIKI. W: Żywność i żywienie / Redakcja naukowa Jędrzej Nyćkowiak, Jacek Leśny Poznań 2020, Młodzi Naukowcy, s. 54-60, il., bibliogr., streszcz, 978-83-66392-59-5.	
4.	**Bakteriocyny w przemyśle mleczarskim (Bacteriocins in dairy industry).** [AUT.] JAGODA SZAFRAŃSKA, MACIEJ NASTAJ, EWA HABZA-KOWALSKA, ILONA MAZURKIEWICZ. W: Żywność i żywienie / Redakcja naukowa Marcin Baran, Jędrzej Nyćkowiak Poznań 2019, Młodzi Naukowcy, s. 89-95, il., bibliogr., streszcz, 978-83-66139-99-2.	5,00
5.	**Charakterystyka enzymów prozapalnych: lipooksygenazy (LOX), cyklooksygenazy (COX) i oksydazy ksantynowej (XO) oraz rola fitozwiązków jako potencjalnych inhibitorów tych enzymów.** [AUT.] EWA HABZA-KOWALSKA, JAGODA SZAFRAŃSKA, JUSTYNA BOCHNAK, ILONA MAZURKIEWICZ. W: Nauki przyrodnicze Część I : Fauna i flora / red. nauk. Marcin Baran, Jędrzej Nyćkowiak, UPP Poznań 2019, Młodzi Naukowcy, s. 40-44, il., bibliogr., streszcz, 978-83-66392-02-1.	5,00
6.	**Hydrokoloidy jako dodatki teksturotwórcze (Hydrocolloids as textural additives).** [AUT.] JAGODA SZAFRAŃSKA, BARTOSZ SOŁOWIEJ, EWA HABZA-KOWALSKA, ILONA MAZURKIEWICZ. W: Żywność i żywienie / Redakcja naukowa Marcin Baran, Jędrzej Nyćkowiak Poznań 2019, Młodzi Naukowcy, s. 83-88, il., bibliogr., streszcz, 978-83-66139-99-2.	5,00
7.	**Możliwości wykorzystania proszków z dyni jako komponentów żywności funkcjonalnej (Pumpkin powders as a component of functional foods).** [AUT.] JUSTYNA BOCHNAK, EWA HABZA, MAŁGORZATA SIKORA, MICHAŁ ŚWIECA. W: Rośliny zielarskie, kosmetyki naturalne i żywność funkcjonalna : nowe nadzieje fitoterapii : monografia naukowa / redakcja naukowa: Grzegorz Bazylak, Henryk Różański Krosno ; Wrocław 2019, Państwowa Wyższa Szkoła Zawodowa im. Stanisława Pigonia w Krośnie, s. 206-214, bibliogr, 978-83-64457-51-7.	5,00
8.	**Białkowo-polifenolowe cząsteczki i ich rola w zapewnianiu funkcjonalności strukturalnej i zdrowotnej żywności (Protein-polyphenol molecules and their role in providing structural and health functionality of food).** [AUT.] EWA HABZA, JAGODA SZAFRAŃSKA. W: Nauki medyczne i nauki o zdrowiu Część IV – Farmacja Poznań 2018, Młodzi Naukowcy, s. 26-31, il., bibliogr, 978-83-66139-22-0.	5,00
9.	**Ocena wybranych właściwości miodów dostępnych w województwie lubelskim.** [AUT.] JAGODA SZAFRAŃSKA, EWA HABZA, BARTOSZ SOŁOWIEJ. W: Procesy technologiczne a jakość żywności / Pod redakcją naukową Karoliny M. Wójciak, Małgorzaty Karwowskiej, XXIII Sesja Naukowa Sekcji Młodej Kadry Naukowej "Żywność – tradycja i nowoczesność" : VI International Session Of Young Scientific Staff "Food - tradition and modernity" Lublin 2018, Towarzystwo Wydawnictw Naukowych LIBROPOLIS, s. 90-104, il., bibliogr, 978-83-63-761-75-2.	5,00
10.	**Rola witaminy C w terapii nowotworowej (The role of vitamin C in cancer therapy).** [AUT.] EWA HABZA, JAGODA SZAFRAŃSKA. W: Nauki medyczne i nauki o zdrowiu Część IV – Farmacja Poznań 2018, Młodzi Naukowcy, s. 20-25, il., bibliogr, 978-83-66139-22-0.	5,00
	Suma:	50,00

3.Materiały konferencyjne

Lp	Opis bibliograficzny
1.	**Zdolność do aktywowania peroksydazy tarczycowej (TPO) i hamowania lipooksygenazy (LOX) przez wybrane związki fenolowe - badania w układzie modelowym.** [AUT.] URSZULA GAWLIK-DZIKI, EWA HABZA-KOWALSKA, AGNIESZKA A. KACZOR, DAMIAN BARTUZI. W: Żywność w strategii Zielonego Ładu : XLV Sesja Naukowa Komitetu Nauk o Żywności i Żywieniu PAN, 1-2 lipca 2021 Gdańsk, Politechnika Gdańska, Wydział Chemiczny : Materiały konferencyjne s. 26. [b.m.] [2021], [b.w.].
2.	**Polyphenols can modulate TPO enzymatic activity.** [AUT.] EWA HABZA-KOWALSKA, URSZULA GAWLIK-DZIKI. W: Badania i rozwój młodych naukowców w Polsce 2020. Materiały konferencyjne- wiosna. Część III. Redakcja naukowa dr. Jędrzej Nyćkowiak i dr. hab Jacek Leśny s. 38. Poznań 2020, Młodzi Naukowcy, 978-83-66392-65-6.
3.	**Rola polifenoli jako naturalnych dodatków do żywności.** [AUT. KORESP.] EWA HABZA-KOWALSKA, [AUT.] URSZULA GAWLIK-DZIKI. W: Badania i rozwój młodych naukowców w Polsce 2020. Materiały konferencyjne- wiosna. Część III. Redakcja naukowa dr. Jędrzej Nyćkowiak i dr. hab Jacek Leśny s. 39. Poznań 2020, Młodzi Naukowcy, 978-83-66392-65-6.
4.	**Impact of chosen polyphenols on thyroid peroxidase (TPO) enzymatic activity.** [AUT.] EWA HABZA-KOWALSKA. W: Badania i rozwój młodych naukowców w Polsce 2019. Materiały konferencyjne - wiosna. Część pierwsza - Lublin / Red. nauk. Jędrzej Nyćkowiak, Jacek Leśny s. 54. Poznań, Młodzi Naukowcy, 978-83-66139-83-1.
5.	**Polifenole –naturalne źródło przeciwutleniaczy w profilaktyce chorób cywilizacyjnych.** [AUT.] EWA HABZA-KOWALSKA. W: Badania i rozwój młodych naukowców w Polsce 2019. Materiały konferencyjne - wiosna. Część pierwsza - Lublin / Red. nauk. Jędrzej Nyćkowiak, Jacek Leśny s. 54. Poznań, Młodzi Naukowcy, 978-83-66139-83-1.
6.	**Analiza i ocena wybranych właściwości miodów dostępnych na rynku w województwie lubelskim.** [AUT.] JAGODA SZAFRAŃSKA, EWA HABZA, BARTOSZ SOŁOWIEJ. W: XXIII Sesja Naukowa Sekcji Młodej Kadry Naukowej" Żywność – tradycja i nowoczesność" : VI International Session Of Young Scientific Staff "Food - tradition and modernity", Materiały konferencji naukowej, Lublin, 24-25 maj 2018 s. 48. Lublin 2018, Towarzystwo Wydawnictw Naukowych "Libropolis" Sp. z o.o, 978-83-63761-74-5.
7.	**Antioxidant potential of acorn flour.** [AUT.] EWA HABZA-KOWALSKA, URSZULA GAWLIK-DZIKI. W: Badania i rozwój młodych naukowców w Polsce 2018. Materiały konferencyjne-jesień. Część trzecia- Lublin / Red.nauk.Jędrzej Nyćkowiak,Jacek Leśny s.52. Poznań 2018, Młodzi Naukowcy.
8.	**Biochemical properties of thermally processed red cabbage and red onion.** [AUT.] EWA HABZA, URSZULA GAWLIK-DZIKI, JAGODA SZAFRAŃSKA. W: Badania i rozwój młodych naukowców w Polsce 2018. Materiały konferencyjne - wiosna. Część czwarta - Lublin / Red. nauk. Jędrzej Nyćkowiak, Jacek Leśny s. 43. Poznań 2018, Młodzi Naukowcy, 978-83-65917-78-2.
9.	**Drying kinetics, color changes and antioxidant activity of broccoli sprouts.** [AUT.] E. HABZA, W. H. HASSOON, D. DZIKI, U. GAWLIK-DZIKI. W: ICA 2018, 12th International Conference on Agrophysics: Soil, Plant&Climate, Book of Abstracts, 17-19 September, 2018 Lublin s.144. Lublin 2018, [b.w.], 978-83-89969-59-0.
10.	**Optymalizacja temperatury przyłączania startera w reakcji multiplex PCR w celu wykrycia zafałszowań.** [AUT.] EWA HABZA, MAGDALENA GRYZIŃSKA. W: XV Międzynarodowe Seminarium Studenckich Kół Naukowych nt. "Środowisko - Zwierzę - Produkt" : V Konferencja Doktorantów, Lublin, 17 kwietnia 2018 r. / Uniwersytet Przyrodniczy w Lublinie s. 123, Tytuł równolegle w języku angielskim. Lublin 2018, Wydawnictwo Uniwersytetu Przyrodniczego w Lublinie.

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