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Sveučilište u Zagrebu

Farmaceutsko-biokemijski fakultet

Kristina Radić

**BIODOSTUPNOST, PERMEABILNOST I
ANTIOKSIDACIJSKA AKTIVNOST
NUTRACEUTIKA IZ KOMINE MASLINE**

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Mentorica: prof. dr. sc. Dubravka Vitali Čepo

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**BIOACCESSIBILITY, PERMEABILITY
AND ANTIOXIDATIVE ACTIVITY OF
OLIVE POMACE-BASED
NUTRACEUTICALS**

DOCTORAL DISSERTATION

Supervisor: Prof. Dubravka Vitali Čepo, PhD

Zagreb, 2021

Rad je predan na ocjenu Fakultetskom vijeću Farmaceutsko-biokemijskog fakulteta Sveučilišta u Zagrebu radi stjecanja akademskog stupnja doktora znanosti iz područja biomedicine i zdravstva, polje farmacija, grana farmacija.

Rad je izrađen na Zavodu za kemiju prehrane Farmaceutsko-biokemijskog fakulteta Sveučilišta u Zagrebu pod mentorstvom prof. dr. sc. Dubravke Vitali Čepo u sklopu poslijediplomskog doktorskog studija „Farmaceutsko-biokemijske znanosti“.

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Kristalna kocka vedrine.

Tin Ujević

Sažetak

Tijekom proizvodnje maslinovog ulja iz ploda masline (*Olea europaea* L.) nastaju velike količine otpada u obliku otpadnih voda i komine (krutog ostatka). Njihov kemijski sastav karakterizira značajan udio fenolnih spojeva koje nalazimo i u maslinovom ulju. Kako bi se povećale mogućnosti korištenja komine masline kao sekundarne sirovine u razvoju novih kategorija proizvoda dodane vrijednosti, u fazi preliminarnih istraživanja razvijen je „zeleni” proces ekstrakcije antioksidansa iz komine masline i formulacije suhog produkta ekstrakta komine masline (EKM) zadovoljavajućih fizikalno-kemijskih karakteristika. Osnovni cilj ovog istraživanja bio je funkcionalna karakterizacija EKM-a i to u kontekstu istraživanja biodostupnosti i intestinalne permeabilnosti karakterističnih aktivnih sastavnica EKM-a: hidroksitirosola (HTS), tirosola (TS) i oleuropeina (OLE) te istraživanje lokalnog antioksidacijskog učinka neprobavljive frakcije EKM-a na stanice crijevnog epitela. Biodostupnost i permeabilnost navedenih biološki aktivnih spojeva istražena je primjenom optimiranih *in vitro* postupaka s posebnim naglaskom na određivanje utjecaja matriksa EKM-a, korištenih ciklodekstrina i različitih matrica hrane na navedene parametre. Neprobavljiva frakcija EKM-a dodatno je istražena u kontekstu ostvarivanja protektivnih učinaka na stanice crijevnog epitela s posebnim naglaskom na utvrđivanje mehanizama opaženih antioksidacijskih učinaka. Na temelju svih dobivenih podataka, EKM je uspoređen s maslinovim uljem kako bi ga se vrednovalo kao alternativni nutritivni izvor karakterističnih biološki aktivnih fenolnih sastavnica. Zaključak ove studije je da je EKM bogat izvor biološki aktivnih i biodostupnih fenola HTS-a, TS-a i OLE-a, a njihov sadržaj i biodostupnost usporedivi su ili bolji u odnosu na maslinovo ulje. Također, zaključeno je da navedeni fenoli međusobno ne interferiraju na razini permeabilnosti što je važan preduvjet za daljnji razvoj kompleksnih nutraceutika iz komine masline. EKM ostvaruje značajne antioksidacijske učinke na stanice crijevnog epitela koji su posljedica direktnog antiradikalnog učinka, ali i učinaka na produkciju endogenih antioksidansa stanice. Matriks EKM-a, prisutnost i vrsta ciklodekstrina u formulaciji te prisutnost hrane u probavnom sustavu značajno utječu na sve promatrane parametre. Stoga rezultati studije ukazuju na važnost usmjerene, ali sveobuhvatne funkcionalne karakterizacije u procesu oblikovanja visoko vrijednih nutraceutika komine masline te naglašavaju mogućnosti uvođenja jednostavnih intervencija tijekom formulacije koje će rezultirati poboljšanom funkcionalnošću završnog proizvoda.

Ključne riječi: fenoli komine masline; antioksidansi; bioraspoloživost; interakcije fenol-hrana

Summary

Introduction

Production of olive oil from olive fruit (*Olea europaea* L.) results in large amount of waste, which is in the form of wastewater and pomace (solid residue). Their chemical composition is characterized by a significant proportion of phenolic compounds that are also found in olive oil and are known for their powerful antioxidative properties and protective effects on human health. In order to increase the possibilities for olive pomace usage as a secondary raw material in the development of value-added products, we developed a “green” process of antioxidants’ extraction from olive pomace in the preliminary phase of the research which resulted in formulation of dry olive pomace extract (OPE) with adequate physico-chemical characteristics. Given the high content of biologically active phenols characteristic for olive oil, OPE could be considered as a high-value nutraceutical with the spectrum of biological effects similar to those of olive oil: pronounced antioxidative, immunomodulatory, chemoprotective and anticancerogenic. However, the potential biological effects of OPE are significantly conditioned by the bioavailability of all active components. Although the bioavailability of a compound is primarily conditioned by physico-chemical properties of a compound, it is also significantly influenced by the matrix of the food/extract and functional carrier used during the formulation and the presence of food in the digestive system. All of them can change both the bioaccessibility and the intestinal permeability of certain active components to a large extent. Therefore, given the large differences in the physico-chemical properties of olive oil and OPE, it is not possible to assume the same bioavailability or biological effectiveness of OPE and olive oil phenols because of similar physico-chemical profile of bioactive components.

The main goal of this study was a functional characterization of OPE in the context of bioaccessibility and intestinal permeability of active components specific for OPE: hydroxytyrosol (HTS), tyrosol (TS) and oleuropein (OLE) and investigation of local antioxidant effect of indigestible OPE fraction on intestinal epithelium cells. Namely, systemic effects of OPE phenols will be conditioned by the fraction of phenols that are available in the small intestine for the absorption and their active metabolites, while the main local effects in intestine will be achieved by the unabsorbed fraction of OPE phenols. Bioaccessibility and intestinal permeability were investigated particularly in the context of the influence of OPE and cyclodextrin (functional carrier) matrix and food/meal presence in the gastrointestinal tract (GIT).

Methods

Olive pomace was collected from two-phase mills in the Republic of Croatia. Phenolic compounds were extracted from defatted olive pomace without addition (native sample) or with the addition of the following cyclodextrins: β (bCD), hydroxypropyl β (hpbCD), randomly methylated β (ramCD) and γ (gCD), according to a previously optimized procedure. The influence of OPE matrix on bioavailability of HTS, TS and OLE was investigated by comparing their share before and after simulation of gastrointestinal digestion and intestinal permeability of pure compounds and those from OPE. *In vitro* testing of phenol bioaccessibility from OPE was conducted by using a standardized procedure of static model of gastrointestinal digestion. Intestinal permeability of HTS, TS and OLE was investigated by using Caco-2 cell model with prior determination of non-toxic concentrations of samples which did not reduce cell viability, what is a prerequisite for preserving the integrity of cell monolayer and simulation of intestinal barrier. The study of food presence in GIT on the bioavailability of HTS and TS was conducted by using biorelevant media and OPE co-digestion with different groups of foods (grouped according to the origin of certain macronutrients and dietary fibers). Identification and quantification of HTS, TS and OLE was carried out by using high-performance liquid chromatography (HPLC system with multi-fluorescent detector) according to the previously optimized method. The share of the total antioxidants and anti-radical potential of OPE was determined with the Trolox equivalents antioxidant capacity method, while the total phenolic content was determined by using the Folin-Ciocalteu method. The antioxidant activity of OPE, HTS, TS and OLE was investigated on HepG2 and Caco-2 cell line models with induction of oxidative stress by prooxidant tert butyl hydroperoxide. Parameters used for monitoring the antioxidant effects were: cell viability (MTT assay); the amount of reactive oxidative species (ROS) (dichlorofluorescein diacetate (DCF-DA) fluorescent dye); the amount of glutathione (GSH) (monochlorobimane (mBCl) fluorescent dye); activity of enzymes that participate physiologically in the elimination of prooxidative agent (glutathione peroxidase (GPx), superoxide dismutase (SOD)).

Results

The results of the study showed that HTS, TS and OLE as well as other OPE antioxidants have good gastrointestinal stability and remain active in digestive tract's conditions. Total phenol content and their bioaccessibility from OPE were comparable, or significantly better than from olive oil.

In all OPE samples, we observed a slight increase of HTS content in the bioaccessible fraction and a significant increase of TS content (129 % – 178 % of the initial amount of TS). TS bioaccessibility was further increased by the presence of bCD and hpbCD in the OPE. The quantity of the main olive secoiridoid OLE after the simulation of digestion was unchanged when compared to the quantity found in OPE before digestion, which is consistent with the available literature data and the fact that glycosylated secoiridoids are not subjected to acid hydrolysis in the stomach.

The change in pH, ion strength, enzyme activity or presence/amount of bile salts that occur because of the presence of food in the digestive system does not significantly affect the bioaccessibility of the observed phenolic components (total phenols, HTS, TS and OLE). The study in which OPE was directly co-digested with different types of foods showed that, depending on the type of food, the bioavailability of OPE phenol either do not change significantly or is significantly reduced. Foods with a relatively high protein content (52.0 %, 12.4 %, 10.5 %) reduced the OPE phenols' bioaccessibility by 51.8 %, 60.6 % and 71.9 %, respectively, what was characterized as very negative effect on phenols' bioaccessibility. Bioaccessibility of HTS and TS was also negatively affected by dietary fibres and by foods with a high dietary fiber content. Namely, relative TS bioaccessibility was reduced by 67.0 % – 89.0 %, while HTS bioaccessibility was decreased by 54.8 % – 84.2 %. Based on the obtained results, it was concluded that the influence of dietary fiber on the bioavailability of phenols cannot be extrapolated from data obtained by *in vitro* simulation of phenol digestion with isolated dietary fiber, but only by co-digestion with complete foods, due to the significant influence of other food components.

The results of the intestinal permeability study of HTS and TS indicate predominantly passive absorption of HTS and TS, while the values of apparent permeability coefficients obtained during our study are in line with the values available in the literature ($1 \times 10^{-5} \text{ cm}^{-1}$ for HTS and $3 \times 10^{-5} \text{ cm}^{-1}$ for TS). The apparent OLE permeability coefficient was very low ($1 \times 10^{-7} \text{ cm}^{-1}$), which indicates poor permeability or intensive metabolism of OLE in Caco-2 cells. Further research found no significant differences in the permeability coefficients obtained by examining each individual compound (HTS; TS; OLE) and mixtures of compounds (HTS + TS + OLE) and we concluded that they do not interfere with each other at the level of absorption, which is an important prerequisite for the further development of complex nutraceuticals from olive pomace. The study revealed enzymatic hydrolysis of OLE during digestion and transepithelial transport which is mediated by glycosides, but also absorption and further

metabolism of oleuropein aglycone, whose metabolites retain antioxidative activity. OPE matrix had a strong negative impact on the permeability of OPE's total antioxidants, HTS, TS and OLE. Namely, the increase of antioxidant potential in the basolateral compartment which was observed with pure analytes was lacking when OPE was applied to the cell monolayer, possibly due to the saturation of membrane and cytosol glycosides by other components present in the complex matrix of OPE. The above-mentioned negative influence of OPE on permeability of HTS, TS and OLE could be explained by non-covalent interactions between HTS, TS and OLE and macromolecules present in OPE which occurs mainly due to the weak associations (combination of hydrogen bonds and hydrophobic interactions) and may reduce the passive diffusion rate through the intestinal epithelium (which is the main mechanism of HTS and TS transport). The influence of CD on phenol permeability was generally negative (but to a much lesser extent comparing to the OPE matrix) and the observed effects are specific to the type of CD and the specific phenolic component. The presence of CD in the formulation negatively affected the permeability of TS and OLE, however bCD and ramCD significantly increased the transmembrane transport of HTS (from 5 % to 17 % and 12 % respectively). The study of food impact on the permeability of HTS, TS and OLE revealed positive effect of glucose on the intestinal permeability of HTS and TS possibly as a result of decreased transepithelial electrical resistance (TEER) of Caco-2 cell monolayer and increased possibility of paracellular and transcellular passive transport. That was in accordance with the results of TEER cell monolayer measurements, where incubation with a glucose solution led to a 20 % reduction in TEER. Cellulose also had a significant negative effect on TEER; however, this did not result in increased permeability of HTS and TS. This is probably due to the formation of insoluble complexes between HTS/TS and cellulose, which is in accordance with our previous results which showed negative impact of dietary fiber on the bioaccessibility of HTS and TS.

Reducing the oxidative stress in the GIT is especially important since numerous studies have shown that oxidative stress is directly related to the development of numerous GIT diseases. The obtained results showed that OPE effectively protected Caco-2 cells from oxidative stress and that antioxidative activity was positively influenced by the presence of bCD, hpbCD and ramCD in the formulation. The hypothesized mechanism of the observed effect was the ability of CD to increase the bioaccessibility of OPE's phenol in the small intestine, which was supported by the previously presented results. Furthermore, results showed that the incubation with OPE lead to an increase in intracellular glutathione levels, the most important non-enzymatic antioxidant, and does not affect the activity of antioxidative enzymes glutathione

peroxidase and superoxide dismutase. OPE was proven to be a more potent antioxidant compared to equivalent amounts of HTS and TS (12 % higher viability of cells treated with OPE), confirming the presence of several other components with antioxidative potential in OPE. hpbCD showed a positive impact on the antioxidative potential of OPE reducing the accumulation of intracellular reactive oxygen compounds (ROS) by 16 %. As well as in the study on the Caco-2 cell model, the hypothesized mechanism by which hpbCD affects antioxidative potential is to improve the solubility of antioxidants from OPE in gastrointestinal fluids. However, given the observed effect of hpbCD of increasing TS bioavailability, the removal of ROS could also be due to the accumulation of precisely this powerful intracellular antioxidant.

Conclusion

OPE is a rich source of biologically active and bioaccessible phenols HTS, TS and OLE, and their content and bioaccessibility are comparable or higher than from olive oil. This study revealed that these phenols do not interfere at the permeability level, which is an important prerequisite for the further development of complex nutraceuticals from olive pomace. OPE achieves significant antioxidative effects on intestinal epithelium cells, which are the result of direct antiradical effect but also of the endogenous antioxidative molecules production. The OPE matrix, the presence and type of CD in the formulation and the presence of food in the digestive tract significantly affect all observed parameters. Therefore, the results of the study highlight the importance of directed but comprehensive functional characterization in the process of high-value nutraceuticals' development and emphasize the possibilities of simple interventions during the formulation that could result in improved functionality of the final product.

Keywords: olive pomace phenols; antioxidant; bioavailability; food-phenol interaction

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1. UVOD

1.1. Komina masline kao izvor bioaktivnih fenolnih spojeva

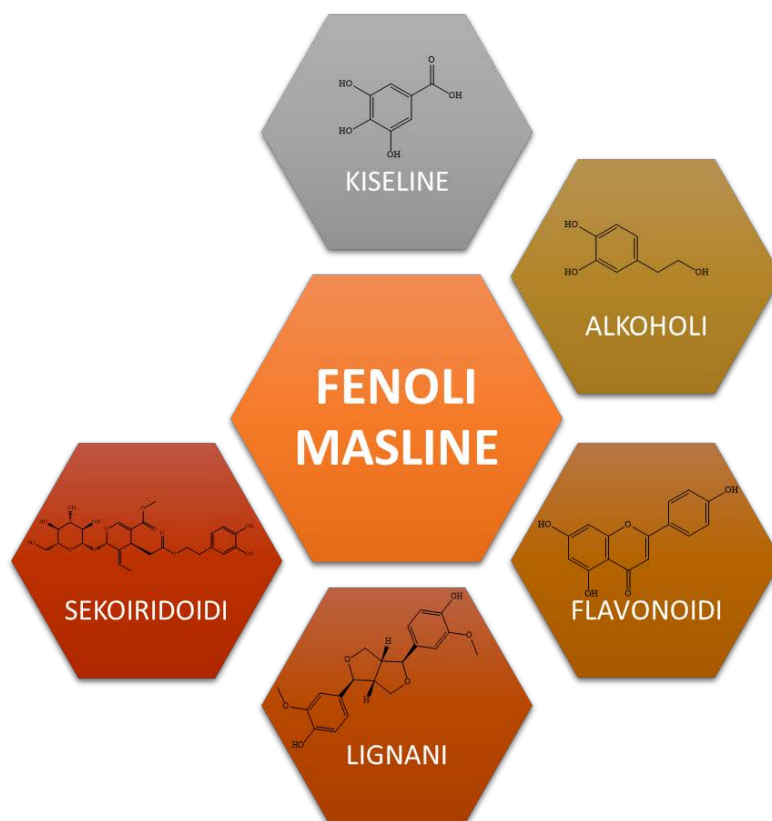
Tijekom proizvodnje maslinovog ulja iz ploda masline (*Olea europaea* L.) nastaju velike količine otpada u obliku otpadnih voda i komine (krutog ostatka). Preciznije, u mediteranskoj regiji gdje se i proizvodi većina maslinovog ulja, godišnje se generira 30 milijuna kubičnih metara otpada iz mlinova. Nekontrolirano odlaganje takvog otpada dovodi do pojave fitotoksičnih učinaka kao što su: povećanje hidrofobnosti tla i posljedično smanjenje razine zadržavanja vode te promjene kiselosti, saliniteta, koncentracija dušika, lipida, organskih kiselina i prirodno prisutnih fenola što direktno narušava biosferu područja, a osobito mikrobiom tla. Upravo zbog značajnog sezonalnog generiranja fitotoksičnog otpada, industrija proizvodnje maslinovog ulja jedan je od najvećih agroindustrijskih zagađivača okoliša, a nastali otpad zbog svojih karakteristika zahtjeva adekvatnu obradu prije odlaganja što za proizvođače predstavlja dodatan trošak [1].

Potrošnja i proizvodnja maslinovog ulja imaju stalnu tendenciju rasta, između ostalog i zbog činjenice da je maslinovo ulje prepoznato kao važan nositelj pozitivnih učinaka mediteranske prehrane na ljudsko zdravlje. Razlog tome su visoki sadržaj hidrofilnih i lipofilnih antioksidansa te značajan udio oleinske kiseline [2]. Posljedično raste interes za pronalaženje održivih i učinkovitih načina obrade nastalog otpada koji bi omogućili njegovo sigurno odlaganje ali i uporabu biološki aktivnih sastavnica koje zaostaju u otpadu pomoću učinkovitih ekstrakcijskih metoda. Do danas je razvijen niz postupaka koji za cilj imaju smanjiti negativne učinke otpada masline na okoliš, a temelje se na primjeni postupaka ekstrakcije ulja koji generiraju manje količine otpada (npr. zamjena dvofaznog centrifugalnog sustava s trofaznim), detoksikacije otpada te u konačnici recikliranja visokovrijednih sastavnica, u prvom redu fenolnih spojeva koji su glavni nositelji antioksidacijske aktivnosti masline. Intenziviranje iskorištavanja otpada masline kao sekundarne sirovine imalo bi značajan pozitivan odjek prvenstveno zbog smanjenja ekološkog otiska proizvodnje maslinovog ulja, smanjenja troškova proizvodnje maslinovog ulja, bolje dostupnosti jeftine sekundarne sirovine za primjenu u poljoprivredi i proizvodnji energije te povećane dostupnosti biološki aktivnih spojeva masline u hrani i dodacima prehrani. Nažalost, trenutno se iskorištava vrlo mali dio otpada koji nastaje proizvodnjom maslinovog ulja; nastali otpad uglavnom se koristi kao gnojivo, biomasa ili aditiv u stočnoj hrani, a najveći dio zaostaje neiskorišten [3].

Komina masline se sastoji od pulpe ploda, koštica i otpadne vode čiji udio uvelike varira o postupku korištenom za ekstrakciju maslinovog ulja. U tradicionalnom postupku ekstrakcije prešanjem zaostane oko 30 %, u trofaznom centrifugalnom postupku oko 50 %, dok u

dvofaznom centrifugalnom postupku zaostane i do 60 % vode. Sastav suhog ostatka komine je karakteriziran velikim udjelom polimernih neprobavljivih ugljikohidrata (lignin, celuloza i hemiceluloze), lipidnom (zaostalo maslinovo ulje) i proteinskom frakcijom, dok su minerali, šećeri i fenolne sastavnice prisutne u manjem udjelu. Fenolni spojevi čine kominu kiselom (pH 5) i iznimno rezistentnom na biodegradaciju te njezino nekontrolirano odlaganje u okoliš dovodi do inhibicije rasta mikroorganizama i biljaka. Međutim, ti spojevi imaju i širok spektar bioloških učinaka i dokazane pozitivne učinke na ljudsko zdravlje [4].

Široko rasprostranjeni u biljkama, fenolni spojevi su prirodni antioksidansi koji se intenzivno istražuju zbog brojnih pozitivnih bioloških učinaka. Njihovu kemijsku strukturu karakterizira prisutnost jedne ili više hidroksilnih grupa (polarni dio) koje su vezane izravno na ugljikov atom aromatskog prstena (apolarni dio) i češće se u biljkama nalaze u obliku glikozida nego kao slobodne molekule (Slika 1). Recentne epidemiološke studije su pokazale povezanost prehrane bogate fenolima sa smanjenjem rizika od pobolijevanja od različitih vrsta karcinoma, kardiovaskularnih bolesti, dijabetesa i kroničnih bolesti povezanih sa starenjem [5]. Fenolni spojevi masline se mogu podijeliti na lipofilne i hidrofilne. Dok se lipofilni fenoli, uključujući tokoferole, sterole i triterpene, mogu naći i u drugim vrstama biljnih ulja, hidrofilni fenoli (hidroksitirosol (HTS), tirosol (TS) i oleuropein (OLE)) karakteristični su upravo za maslinovo ulje te ih ne nalazimo u drugim vrstama ulja i masti. S obzirom na to da moderne tehnike proizvodnje maslinovog ulja uključuju dodatak vode u postupku ekstrakcije, hidrofilni fenolni spojevi (particijski koeficijent u rasponu $6 \times 10^{-4} - 1,5$) u najvećem dijelu zaostaju u otpadu (~98 %) [6]. Njih uglavnom čine: fenolne kiseline (kumarinska, cimetna, kofeinska, ferulična, galna, vanilinska, elenoična, sinapska, klorogenska, protokatehuidna, siringinska), flavonoidi (apigenin, hesperidin, cijanidin flavon, antocijan, kvercetin, luteolin), lignani (acetoksipinoresinol, pinoresinol) i sekoiridoidi (oleuropein, oleokantal, oleacin, demetiloleuropein, oleuropein aglikon, ligstrozid), a glavnim nositeljima bioloških učinaka masline smatraju se fenolni alkoholi HTS i TS.



Slika 1. Kategorizacija hidrofilnih fenolnih spojeva masline i strukturni prikaz sljedećih predstavnika: galne kiseline, alkohola hidroksitirosola, flavonoida apigenina, lignana pinoresinola, sekoiridoida oleuropeina (strukture prikazane pomoću *ChemDraw Professional*).

HTS je snažan antioksidans sa širokim spektrom bioloških učinaka [7] čiji su pozitivni utjecaji na zdravlje prepoznati i priznati od strane službenih regulatornih tijela. Jedno od njih je Europska agencija za sigurnost hrane (engl. *European Food Safety Authority*, EFSA) koja je 2011. godine odobrila zdravstvenu tvrdnju o učincima maslinovog ulja. Zdravstvenom tvrdnjom se potvrđuje da fenoli maslinovog ulja standardizirani na udio HTS-a i njegovih derivata štite čestice lipoproteina male gustoće (LDL) od oksidacijskog oštećenja [8]. Trenutno se razmatraju i druge zdravstvene tvrdnje o učincima fenola masline, a neke od njih su: održavanje normalne koncentracije lipoproteina velike gustoće (HDL), LDL-a, triglicerida i glukoze u krvi te protuupalni, antimikrobni, fotoprotektivni učinci. Također, u usporedbi s vitaminima C i E, standardnim antioksidansima koji se unose prehranom, HTS je pokazao bolju sposobnost uklanjanja slobodnih radikala [9].

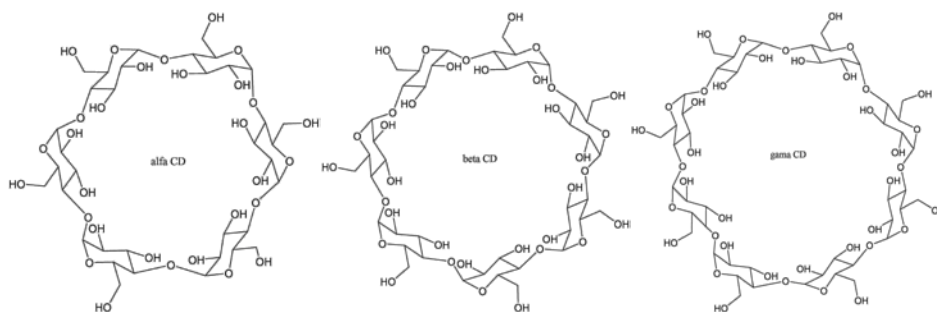
1.2. Upotreba ciklodekstrina u razvoju nutraceutika iz komine masline

Po svom fenolnom sastavu, komina masline je vrlo slična maslinovom ulju te predstavlja jeftin i lako dostupan izvor HTS-a i derivata. U posljednjih desetak godina, potencijal komine masline kao bogatog izvora biološki aktivnih spojeva prepoznat je u znanstvenoj i stručnoj literaturi. Također, primijećeno je da su antioksidacijski učinci HTS-a u ekstraktu komine masline (EKM) snažniji od učinaka ekvivalentne količine čistog spoja. To je potaknulo razvoj brojnih učinkovitih i održivih postupaka ekstrakcije koji su za cilj imali dobivanje nutraceutika iz komine masline tj. izolaciju fitokompleksa čija primjena, u odgovarajućem farmaceutskom obliku, može rezultirati korisnim učincima na zdravlje [10].

Izdvajanje fenola iz komine masline uključuje niz postupaka: makroskopsku predobradu, razdvajanje sastavnica velike i male molekularne mase, ekstrakciju, pročišćavanje/izolaciju i inkapsulaciju. Pri tome predobrada komine masline uključuje postupke uklanjanja vodene i lipidne komponente dok se makromolekule najčešće uklanjaju precipitacijom s alkoholom. Najčešće korišteni postupci ekstrakcije fenola iz komine masline uključuju ekstrakcije otapalima, kromatografske metode, ekstrakcije superkričnim otapalima, ekstrakcije potpomognute ultrazvukom ili mikrovalovima i ekstrakcije subkričnom vodom. Otapalo izbora za ekstrakciju fenola iz komine masline (uzimajući u obzir njihovu polarnost) često je etanol zbog brojnih prednosti koje omogućuju direktnu primjenu etanolnih ekstrakata u farmaceutskoj i prehrambenoj industriji kao što su: cijena, mogućnost ponovne upotrebe, netoksičnost te sposobnost precipitacije makromolekula (npr. prehrambenih vlakana). Ekstrahirane fenolne sastavnice su najčešće pročišćene od nečistoća pomoću aktivnog ugljena, kromatografskih smola ili polisaharida. Posljednji korak izdvajanja fenola iz komine masline uključuje njihovu inkapsulaciju što omogućuje produljenu stabilnost spojeva, maskiranje nepoželjnih organoleptičkih karakteristika te zaštitu od negativnih utjecaja okoliša. Fenoli komine masline se najčešće uklapaju u polisaharide kao što su ciklodekstrini (CD) i maltodekstrini. Završni korak u razvoju nutraceutika dobivenog iz otpada masline uključuje sušenje smrzavanjem ili raspršivanjem [11].

Inkapsulacija fenolnih spojeva iz komine masline je neophodna zbog iznimno loših tehnoloških svojstava sirovih ekstrakata kao što su izrazita higroskopnost, intenzivan miris i nestabilnost bioaktivnih sastavnica tijekom skladištenja čime se njihova šira primjena uvelike ograničava [12]. U okviru preliminarnih istraživanja, suhi ekstrakti poboljšanih fizikalno-kemijskih svojstava dobiveni su uklapanjem bioaktivnih sastavnica u odgovarajuće nosače [13] i to upotrebom različitih vrsta CD-a u procesu ekstrakcije. CD su ciklički oligosaharidi koji se

sastoje od α -1,4 povezanih D-glukopiranoznih jedinica, a nastaju enzimskom razgradnjom škroba [14]. Ubrajamo ih u GRAS skupinu spojeva (engl. *Generally Recognized as Safe*), a karakterizira ih amfifilna struktura, s hidrofilnim vanjskim dijelom i unutarnjom hidrofobnom šupljinom (Slika 2). To omogućuje, na molekularnoj razini, inkluziju lipofilnih molekula što rezultira poboljšavanjem njihove topljivosti i/ili stabilnosti. Osim navedenog, CD se često primjenjuju u svrhu modifikacije organoleptičkih svojstava nutraceutika (konzistencije, boje, okusa ili mirisa).



Slika 2. Strukturni prikaz različitih vrsta ciklodekstrina (strukture prikazane pomoću *ChemDraw Professional*).

Upravo su CD uspješno primijenjeni kao funkcionalni nosači u procesima ultrazvukom i mikrovalovima potpomognute ekstrakcije što je rezultiralo povećanjem prinosa ukupnih fenola, smanjenjem higroskopsnosti i značajnim povećanjem stabilnosti EKM-a te poboljšanjem antioksidacijske učinkovitosti EKM-a u model-sustavima hrane [14–16]. Pri tome su na promatrane parametre učinkovitosti značajno utjecali i vrsta i količine korištenih CD-a. Korištenjem naprednih ekstrakcijskih tehnika i netoksičnih otapala zadovoljeni su principi „zelenih“ kemije što je u skladu s idejom održivog i ekološki prihvatljivog iskorištavanja otpada masline. Optimizacija učinkovitih „zelenih“ postupaka ekstrakcije te postizanje zadovoljavajućih fizikalno-kemijskih karakteristika suhih ostataka rezultirali su povećanim mogućnostima primjene EKM-a kao prirodnog aditiva (antioksidansa) u hrani. Međutim, da bi se kritički procijenila mogućnost korištenja EKM-a kao biološki aktivne sastavnice dodatka prehrani ili funkcionalne hrane, nužno je istražiti bioraspoloživost i biološku aktivnost glavnih biološki aktivnih sastavnica, u prvom redu HTS-a i njegovih derivata, TS-a i OLE-a.

1.3. Biološka aktivnost nutraceutika iz komine masline

Biološka aktivnost fenolnih spojeva maslinovog ulja je istraživana u brojnim pretkliničkim (*in vitro*, *ex vivo* i *in vivo*) studijama te u nekoliko kliničkih studija. Rezultati studija sugeriraju njihov velik potencijal u prevenciji i liječenju bolesti i promicanju ljudskog zdravlja. S obzirom na mnogostruko veći udio fenolnih sastavnica, EKM bi mogao ostvarivati bolje učinke na zdravlje od maslinovog ulja [17]. No, biološki učinci EKM-a bit će, osim sastavom, uvjetovani bioraspoloživošću aktivnih komponenata, u prvom redu HTS-a, TS-a i OLE-a. Ljudski organizam prepoznaje fenolne spojeve kao ksenobiotike stoga su brojni primjeri fenolnih spojeva koji su, iako u hrani prisutni u visokim koncentracijama, lošeg profila bioraspoloživosti i posljedično smanjenog učinka na zdravlje [18]. Na bioraspoloživost, tj. na količinu bioaktivnih sastavnica koja nakon oralne primjene dospije u sistemsku cirkulaciju, utječe njihova gastrointestinalna biodostupnost, intestinalna permeabilnost, metabolizam i fermentacija neapsorbiranih fenola crijevnom mikrobiotom [19].

1.3.1. Gastrointestinalna biodostupnost i intestinalna permeabilnost nutraceutika iz komine masline

U kompleksnim matricama (kao što su hrana ili biljni ekstrakti) na bioraspoloživost aktivnih sastavnica značajno utječe gastrointestinalna biodostupnost odnosno količina spoja dostupna za apsorpciju u tankom crijevu. Biodostupnost je uvjetovana brojnim čimbenicima: stupnjem oslobađanja aktivne sastavnice iz matriksa namirnice (npr. iz kompleksa s prehrambenim vlaknima) ili stupnjem razgradnje kompleksnijih spojeva (oslobađanje HTS-a iz OLE-a; hidroliza glikozida); topljivošću u gastrointestinalnim tekućinama; interakcijama s probavnim enzimima i žučnim kiselinama i solima te drugim sastavnicama kompleksnog matriksa; te u konačnici kemijskom ili enzimskom razgradnjom u uvjetima gastrointestinalnog trakta (GIT) [20]. Upravo stoga, realno je očekivati da bioraspoloživost derivata HTS-a iz maslinovog ulja neće biti jednaka onoj iz EKM-a [21]. Bioraspoloživost HTS-a i TS-a iz maslinovog ulja istražena je u više *in vivo* studija i utvrđeno je da je zadovoljavajuća, pri čemu koncentracije HTS-a i TS-a u plazmi rastu kratko nakon unošenja djevičanskog maslinovog ulja i dosežu vršnu koncentraciju nakon otprilike 1 h u plazmi i 0 – 2 h u mokraći [22]. Također, primijećeno je da su složeni sekoiridoidi iz maslinovog ulja slabo bioraspoloživi te podložni biotransformaciji djelovanjem crijevne mikrobiote, što rezultira porastom postprandijalnih koncentracija HTS-a i TS-a [23,24].

Slijedom svega navedenog razvidno je da na biodostupnost i intestinalnu permeabilnost fenola EKM mogu bitno utjecati i farmaceutski ekscipijensi koji se koriste u razvoju suhih ekstrakata. Stvaranje inkluzijskih kompleksa HTS-a i TS-a s CD-om [25,26] može bitno utjecati na njihovu biodostupnost u prvom redu zbog modificiranja stabilnosti u uvjetima probave i to na nekoliko načina: smanjenjem stupnja hidrolize, oksidacije, steričkih promjena, racemizacije i enzimske razgradnje; modificiranjem interakcija s drugim sastavnicama matriksa, druge hrane ili probavnih enzima / žučnih soli u lumenu GIT-a. Do sada su CD uspješno primijenjeni za poboljšanje biodostupnosti različitih fenolnih spojeva i to: apigenina [27], resveratrola [28], fenola kore nara [29], elagične kiseline [30] i ferulične kiseline [31].

Pretpostavlja se da bi CD mogli utjecati i na permeabilnost HTS-a, TS-a i OLE-a kroz crijevnu stijenu. Mehanizam kojim fenoli masline prolaze epitelnu barijeru još uvijek nije u potpunosti razjašnjen, no iz dosadašnjih istraživanja se zna da on ovisi o polarosti i veličini pojedinog fenolnog spoja [32]. Također, nije poznato interferiraju li međusobno fenoli masline prilikom transporta. S obzirom na svoje $\log P$ vrijednosti, HTS, TS i OLE se svrstavaju u spojeve dobro topljive u gastrointestinalnim tekućinama i dobre permeabilnosti [33,34]. Manna i suradnici u svom radu postuliraju da se polarni TS i HTS apsorbiraju pasivnom difuzijom dok se OLE, također polaran, ali veći spoj, vjerojatno prenosi aktivnim transportom uz pomoć transportera za glukozu [35]. Utjecaj CD-a na intestinalnu permeabilnost HTS-a, TS-a i ostalih antioksidanasa EKM-a slabo je istražen, međutim dosadašnja istraživanja ukazuju na moguć pozitivan učinak, a pretpostavljeni mehanizmi opaženih učinaka su povećavanje paracelularne permeabilnosti, inhibiranje efluksnih pumpi te omogućavanje prelaska epitelne barijere endocitozom [36]. Uzimajući u obzir različite učinke CD-a i kompleksnost matriksa EKM-a, vrlo je teško predvidjeti biodostupnost i intestinalnu permeabilnost HTS-a i TS-a, najvažnije čimbenike u ostvarivanju bioloških učinaka nutraceutika iz EKM-a.

Idealan pristup za određivanje bioraspoloživosti bioaktivnih molekula, obzirom na brojne čimbenike koji utječu na procese u ljudskom organizmu, bio bi provođenje *in vivo* studije bioraspoloživosti. No, *in vivo* studije su dugotrajne, skupe i komplicirane iz etičke perspektive. *In vitro* metode su jeftinije i jednostavnije, a u prethodnim desetljećima su razvijeni modeli čiji rezultati dobro koreliraju s onima u *in vivo* studijama. Oni uključuju simulaciju transporta molekula uz prethodnu *in vitro* simulaciju gastrointestinalne digestije.

Jedan od najjednostavnijih načina simuliranja gastrointestinalne digestije je primjena statičkog modela koji podrazumijeva simulaciju uvjeta procesa probave u fazama: salivarne gastrične i

intestinalne faze. U literaturi je dostupan velik broj protokola za statičku simulaciju gastrointestinalne digestije koji se međusobno razlikuju po nekim ključnim karakteristikama (trajanje i brzina miješanja, osmolalnost, pH, koncentracije probavnih enzima i žučnih soli, prisutnost fosfolipida, omjer bolus : probavna tekućina) čime je znatno otežana usporedba rezultata različitih studija. Ti su parametri kritički evaluirani u odnosu na podatke iz *in vivo* studija što je rezultiralo donošenjem internacionalnog konsenzusa i razvojem standardiziranog postupka simulacije gastrointestinalne digestije [37]. U tom postupku se koriste konstantni omjeri bolusa i probavnih tekućina te se održava konstantan pH u svakom koraku digestije. To metodu čini jednostavnom za upotrebu, ali nepogodnom za kinetičku simulaciju digestije. Koristeći ovu metodu, uzorci hrane podvrgavaju se sekvencijalno oralnoj, gastičnoj i intestinalnoj digestiji dok se parametri poput elektrolita, enzima, žuči, razrjeđenja, pH i vremena digestije temelje na dostupnim fiziološkim podacima.

Među brojnim modelima razvijenim za simulaciju transporta molekula kroz epitel tankog crijeva, Caco-2 stanični monosloj se još uvijek smatra zlatnim standardom. Caco-2 stanice su stanična linija ljudskog epitelnog karcinoma debelog crijeva koje se, kultivirane u monosloju, diferenciraju tako da tvore čvrste spojeve (engl. *tight junctions*) između stanica te mogu poslužiti kao model paracelularnog transporta spojeva [38]. Nadalje Caco-2 stanice eksprimiraju transportne (efluksne i inluksne) proteine, te enzime druge faze metabolizma zbog čega su primjenjive za simulaciju raznih transcelularnih transportnih puteva, i metabolizma ispitivanih tvari. Iako, zbog svega navedenog Caco-2 stanični monosloj u mnogim aspektima oponaša ljudski crijevni epitel bitno je naglasiti i postojanje funkcionalnih razlika. Za razliku od stanica crijevnog epitela, Caco-2 stanice ne eksprimiraju izoenzim citokroma P450, CYP3A4, inače visoko eksprimiran u crijevnom epitelu i posebno važan za metabolizam ksenobiotika. Ostali nedostaci ovog modela su manjak mukusa, slaba permeabilnost za hidrofilne spojeve koji se transportiraju paracelularno te prevelika ekspresija nekih proteina, primjerice P-glikoproteina [39]. Usprkos nedostacima, Caco-2 stanični model predstavlja pouzdan, primjenjiv i široko korišten model kojim se može predvidjeti i pasivni i aktivni transport bioaktivnih molekula.

1.3.2. Utjecaj hrane na gastrointestinalnu biodostupnost i intestinalnu permeabilnost nutraceutika iz komine masline

Kao što je prethodno spomenuto, na bioraspoloživost fenola uvelike utječu njegove fizikalno-kemijske karakteristike. Međutim, ako ga unosimo kao dio namirnice ili kompleksnog ekstrakta onda je njegova bioraspoloživost u velikoj mjeri određena i njegovim interakcijama s

kompleksnom matricom namirnice/ekstrakta. Utjecaj kompleksnih matrica na bioraspoloživost nutraceutika posebno je važan u slučajevima kada je potrebno usporediti različite namirnice/ekstrakte kao izvore nekog biološki aktivnog spoja (na primjer usporediti maslinovo ulje i EKM kao izvora HTS-a i TS-a). Procjena interakcija fenola i hrane također je od velike važnosti jer fenoli mogu biti izolirani iz svog prirodnog okoliša, pročišćeni, a zatim dodani hrani s ciljem poboljšavanja njezinih funkcionalnih karakteristika [40]. Osim toga, saznanja o značajnosti i vrsti interakcija fenolnih spojeva i hrane (nutrijenata) omogućuju promicanje onih interakcija koje djeluju pozitivno na bioraspoloživost fenola te usmjerenu formulaciju matriksa hrane koja za cilj ima postizanje bolje bioraspoloživosti biološki aktivnih sastavnica [41].

Interakcije glavnih fenolnih spojeva EKM-a s hranom do danas su slabo istražene. Iz studija koje su u fokusu imale slične fenolne spojeve razvidno je da hrana može imati negativan, ali i pozitivan učinak na njihovu bioraspoloživost te da učinak ovisi o brojnim čimbenicima poput omjera mase fenola i matrice hrane, temperature, pH i lipofilnosti istraživanog spoja. Pritom, prisutnost hrane mijenja fiziološke uvjete u ljudskom GIT-u (volumen tekućine, gastričnu i intestinalnu pokretljivost, pražnjenje želuca, pH vrijednosti, kapacitet enzima, osmolalnost, sastav žučnih soli). Sve zajedno za posljedicu ima modifikaciju stabilnosti i topljivosti pojedinih fenolnih spojeva. No, na biodostupnost fenolnih spojeva možda u najvećoj mjeri utječe stvaranje kompleksa fenola s različitim komponentama obroka kao što su prehrambena vlakna, ugljikohidrati, lipidi i proteini. Kompleksacija može zaštititi fenole od oksidacijske razgradnje u GIT-u, ali i onemogućiti njihovu apsorpciju u tankom crijevu. S obzirom na poznati afinitet fenolnih spojeva da se vežu na makromolekule prisutne u hrani, u prvom redu na proteine i polisaharide, za konačnu procjenu bioraspoloživosti fenolnog nutraceutika izrazito je važno istražiti njegove interakcije s hranom/pojedinim sastavnicama namirnice i posljedični utjecaj na biodostupnost i bioraspoloživost [42]. Osim toga, prisustvo hrane u GIT-u može utjecati na proces apsorpcije fenola i indirektnim mehanizmima, putem interakcija s inluksnim i efluksnim crijevnim transporterima, metabolizirajućim crijevnim enzimima ili sa samim enterocitima modificiranjem integriteta stanica i/ili čvrstih spojeva [43–46].

Istraživanja interakcija fenola s hranom ili pojedinim kategorijama nutrijenata pokazuju da, ovisno o vrsti fenola, te interakcije mogu značajno utjecati na gastrointestinalnu dostupnost i bioraspoloživost promatranog spoja. Većina dostupnih istraživanja tiče se interakcija fenolnih spojeva s proteinima te njihovi zaključci ukazuju na činjenicu da proteini uglavnom smanjuju biodostupnost i bioraspoloživost fenolnih spojeva. S druge strane, utjecaj prehrambenih vlakana na bioraspoloživost fenola može biti pozitivan ili negativan, ovisno o fizikalno-

kemijskim karakteristikama vlakna. Prisutnost lipida u matriksu hrane, općenito pozitivno utječe na bioraspoloživost fenola [47].

Iako je broj provedenih studija kao i raspon analiziranih fenolnih nutraceutika relativno malen, na temelju do danas provedenih istraživanja može se zaključiti da učinci kompleksnih matrica na bioraspoloživost fenola mogu biti značajni, a njihova priroda i značajnost određeni su fizikalno-kemijskim karakteristikama matriksa i samog promatranog spoja. Dakle, zbog kompleksnosti mogućih interakcija, nije moguće izvlačiti generalne zaključke već se svaki sustav mora vrednovati posebno [47].

Kao što je već spomenuto, biljni ekstrakti bogati bioaktivnim sastavnicama, kao što je EKM, sve se više koriste kao funkcionalne sastavnice dodataka prehrani i hrane. Stoga je od velike važnosti razumjeti prirodu i značaj interakcija fenola i hrane kako bi se osigurale učinkovite i točne upute za doziranje i omogućila ciljana formulacija dodataka prehrani ili matriksa hrane koja će rezultirati optimiziranom bioaktivnošću spojeva koji imaju pozitivan učinak na ljudsko zdravlje.

1.3.3. Antioksidacijska aktivnost nutraceutika iz komine masline

Dosadašnja istraživanja antioksidacijske aktivnosti EKM-a ukazuju da su HTS i TS najjači prisutni antioksidansi. No, imajući na umu već navedene moguće interakcije HTS-a i TS-a s ostalim sastavnicama EKM-a, jasno je da njihova antioksidacijska aktivnost može biti modulirana. Stoga je u ovom istraživanju uspoređena antioksidacijska aktivnost HTS-a i TS-a primijenjenih kao dio kompleksne matrice s onom ekvivalentne količine čistih spojeva u *in vitro* staničnom modelu.

Antioksidacijska aktivnost fenolnih spojeva obično se pripisuje njihovoj sposobnosti gašenja slobodnih radikala, kompleksaciji kelirajućih metala i općenito njihovoj redukcijskoj aktivnosti. Međutim, literaturni podaci pokazuju da fenoli također aktivno sudjeluju u antioksidacijskoj obrani organizma putem neizravnih mehanizama, primarno aktivacijom endogenih obrambenih sustava u stanici (stimulacija transkripcije glavnih antioksidacijskih i detoksikacijskih obrambenih sustava u stanici) [48].

Kombinacija *in vitro* modela simulacije digestije sa staničnim modelima epitela crijeva se može koristiti za procjenu biološke aktivnosti nutraceutika u smislu istraživanja njihovog lokalnog antioksidacijskog, protuupalnog ili protutumorskog učinka [49]. Smanjenje oksidacijskog stresa u GIT-u je osobito važno s obzirom na to da su brojne studije pokazale da je oksidacijski

stres izravno povezan s razvojem bolesti GIT-a kao što su upalna bolest crijeva i karcinom kolona koji je trenutno drugi najsmrtonosniji oblik karcinoma [50]. Crijevno tkivo se pokazalo kao izuzetno osjetljivo na oksidacijski stres zbog peroksidacije lipida (lančane reakcije koja dovodi do štetnog oštećenja stanične membrane) koja se može pokrenuti nutritivnim mastima. Prema rezultatima dostupnih studija, bioaktivni fenoli masline koncentriraju se u lumenu GIT-a upravo zbog ograničene apsorpcije u tankom crijevu te ostvaruju lokalne protektivne učinke na sluznicu crijeva putem različitih mehanizama: izravnog antioksidacijskog učinka, utjecaja na staničnu signalizaciju te utjecaja na stanični ciklus. Opaženi učinci ovise o fizikalno-kemijskim karakteristikama najzastupljenijih fenola te o mogućim sinergističkim učincima, ako su primijenjeni u obliku kompleksnih ekstrakata (kao što je EKM).

Antioksidacijski učinak na staničnim linijama se ispituje tretiranjem ispitivanim ekstraktima uz prethodno induciranje oksidacijskog stresa. Za konačnu potvrdu učinka i razumijevanje mehanizma djelovanja potrebno je provesti istraživanje na nekoliko staničnih linija i koristiti nekoliko različitih testova. U tom smislu najčešće se koriste HepG2 stanične kulture uglavnom zbog relativno jednostavnog, brzog i jeftinog uzgoja uz istovremeno adekvatno predstavljanje složenosti bioloških sustava [51].

2. Influence of Pomace Matrix and Cyclodextrin Encapsulation on Olive Pomace Polyphenols' Bioaccessibility and Intestinal Permeability

Article

Influence of Pomace Matrix and Cyclodextrin Encapsulation on Olive Pomace Polyphenols' Bioaccessibility and Intestinal Permeability

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Abstract: Olive pomace is a rich source of biologically active compounds, mainly polyphenols. Recently, an efficient and sustainable cyclodextrin (CD)-enhanced extraction was developed. It enabled a relatively simple formulation of high-quality olive pomace extracts (OPEs) that can be used as alternative sources of olive-derived polyphenols in the nutrition and pharma industries. However, biological effects and nutraceutical potential of OPEs are primarily limited by generally low oral bioavailability of major polyphenols (hydroxytyrosol and its derivatives) that can be significantly influenced by OPE matrix and the presence of CDs in formulation. The major goal of this research was to investigate the impact of complex matrix and different types of CDs on gastrointestinal stability and intestinal permeability of major OPE polyphenols, and provide additional data about mechanisms of absorption and antioxidant activity in gut lumen. Obtained results showed high bioaccessibility but relatively low permeability of OPE polyphenols, which was negatively affected by OPE matrix. CDs improved antioxidant efficiency of tested OPEs and tyrosol gastrointestinal stability. Effects of CDs on permeability and the metabolism of particular OPE polyphenols were CD- and polyphenol-specific.

Keywords: olive pomace extract; cyclodextrin; bioavailability; in vitro gastrointestinal digestion; antioxidant

1. Introduction

Olive pomace is the residue remaining after olive oil production, consisting mainly of lignin, cellulose, hemicelluloses, residual olive oil, minerals, and phenolic constituents. Polyphenols are meritorious for the negative effects of the olive pomace on both aquatic and terrestrial ecosystems, which is why such waste must be adequately processed before disposal. It has been shown that the polyphenols inhibit plant and bacterial growth, what directly brings to reduction of biodiversity in surface waters and soil [1,2]. On the other hand, olive pomace polyphenols have been in focus of recent research due to possible positive effects on human health, given the wide spectrum of their biological effects [3]. They are mostly phenolic acids, phenolic alcohols, flavonoids, lignans, and secoiridoids. The main compounds related with positive pharmacological effects of the olive pomace are considered to be hydroxytyrosol (HTS) and its derivatives. HTS is a powerful antioxidant with a broad spectrum of biological effects [4] whose potential has been recognized by the European Food Safety Authority (EFSA), which approved a health claim on the effects of olive oil in the year 2011. EFSA confirmed that olive oil polyphenols (standardized to the proportion of HTS and its derivatives) protect LDL particles from oxidative damage [5]. Other health claims concerning effects of olive's polyphenols are currently being considered, including maintaining the normal concentration of HDL, LDL, triglycerides, and blood glucose, as well as anti-inflammatory, antimicrobial, and photoprotective effects. The reduction of

oxidative stress in the intestine is particularly important given that numerous studies have shown that oxidative stress is directly linked to the development of diseases of the GI tract, such as inflammatory bowel disease and tumor colon. The intestine is the site that was shown to be extremely susceptible to oxidative stress. Namely, lipid peroxidation (as a chain reaction that lead to deleterious cell membrane damage) can be initiated by dietary fats.

Considering its phenolic composition, olive pomace is very similar to olive oil and represents an inexpensive and readily available source of HTS derivatives. In the last decade, the potential of the olive pomace as a rich source of biologically active compounds has been recognized in scientific and professional literature, resulting in the development of a large number of effective extraction procedures [6,7]. However, the poor technological properties of raw olive pomace extracts (OPE) limit their wider application as nutraceuticals and food additives [7]. The main prerequisites for obtaining usable OPE are high yield of HTS derivatives, stability, and satisfactory technological properties of dry extracts. In this regard, cyclodextrins (CDs) were successfully applied for achieving higher polyphenol yields and the formulation of stable and organoleptically acceptable olive pomace extracts (OPEs) [7]. Moreover, α -CD, β -CD, and γ -CD are listed on the generally regarded as safe (GRAS) list of the U.S. Food and Drug Administration (FDA) for use as a food additive [8–10].

In addition to demonstrating favorable chemical composition and technological properties, the quality of OPE is significantly determined by bioavailability and biological activity of its main active components. Considering OPE's very similar polyphenolic composition to olive oil, achieving similar pharmacological effects can be expected [11].

However, the biological effects of OPE will be significantly affected by generally reduced bioavailability of olive polyphenols, mostly due to their incomplete intestinal absorption and to rapid biotransformation favoring urinary excretion of olive polyphenols [12]. Some of the major processes that significantly affect their intestinal absorption are matrix depended, and include releasing the active component from the matrix during the digestion process or creating soluble/insoluble complexes with other components during digestion [13,14]. Therefore, data on intestinal absorption of olive polyphenols from oil cannot be extrapolated to significantly different food matrices, such as OPE.

The bioaccessibility of HTS and its derivatives from OPE has rarely been investigated. Recently, Malapert and co-workers [15] investigated the bioaccessibility of HTS from Alperujo in Caco-2 cells, showing generally low availability of HTS, additionally reduced by the Alperujo matrix or concomitant food intake.

Such strong matrix effects on bioaccessibility of biologically active nutraceuticals can be reduced by significant extent by innovative formulation approaches; therefore, the use of pharmaceutical excipients and advanced formulations for oral administration of nutraceuticals has recently been intensified [16].

Among relatively simple but effective approaches is the formulation of nutraceutical-cyclodextrin inclusive complexes. This approach has recently been successfully applied in formulation of OPE-derived extracts and resulted in improved physical-chemical properties of dry extracts, increased shelf life, and higher antioxidant activity in different food- and biological model systems [7,17].

However, the presence of particular CD in OPE can have significant impact on the bioaccessibility and intestinal permeability of OPE polyphenols. CDs are cyclic oligosaccharides used for the improvement of water-solubility and bioavailability of medicinal products. Their key feature is the hydrophobic internal cavity, that provides the ability to form complexes with a variety of guest molecules resulting with the formation of encapsulation complexes [18]. The encapsulation can influence the stability of nutraceutical in gastrointestinal tract by reducing the rate of hydrolysis, oxidation, steric rearrangement, racemization, enzymatic decomposition, or formation of complexes with other food components which, all together, can have significant impact on nutraceutical bioaccessibility. CDs can also form aggregates with small molecules, influencing their solubility but also their interactions with other molecules in complex matrices [19]. CDs have been successfully applied for enhancement of bioaccessibility of different polyphenol-type nutraceuticals: apigenin [20], resveratrol [21], pomegranate

peel polyphenols [22], ellagic acid [23], and ferulic acid [24]. In addition, CDs can influence intestinal permeability of biologically active compounds through their direct impact on biological membranes.

Taking into account various CD effects and the complexity of OPE matrix, it is very hard to predict the exact effect of CDs on polyphenol bioavailability from OPE. Having in mind already proven efficiency of particular CDs on extraction efficiency and functional characteristics of OPE [7,17], the major goal of this research was to investigate the effects of OPE matrix and different types of CDs on gastrointestinal stability and intestinal permeability of OPE-derived hydroxytyrosol, tyrosol, and oleuropein, and provide novel insight into possibilities of CD utilization in formulation of advanced OPE derived nutraceuticals.

2. Materials and Methods

2.1. Samples

Olive pomace (OP) was collected from several two-phase mills in Croatia during autumn 2018. OP was kept at $-20\text{ }^{\circ}\text{C}$ (Beko CN161220X, Istanbul, Turkey) in polypropylene bags until use. Pre-treatment included drying at $60\text{ }^{\circ}\text{C}$ for 24 h in an incubator (INKO, Zagreb, Croatia), shredding and sieving on Φ 0.8 mm sieve (Prüfsieb DIN 4188, Kassel, Germany), defatting (\sim 5 g of the sample was defatting for 2 h with petrol ether) using the Soxhlet apparatus (INKO SK6ESS, Zagreb, Croatia). Polyphenols were extracted from defatted OP without (native sample) or with the addition of the following cyclodextrins: β (bCD), hydroxypropyl β (hpbCD), randomly methylated β (ramCD), and γ (gCD), according to previously optimized procedure [6]. Briefly, OP was mixed with 20% ethanol (20 g/L) and CDs were added in samples as noted above (8 g/L). The extraction was performed on 700 W of microwave power in high performance microwave digestion unit (Milestone 1200 mega, Sorisole, Italy) for 10 min. The extracts were cooled on ice and filtered to remove the crude parts. The listed CDs were chosen according to the number of successful applications of CDs for olive polyphenols encapsulation in literature data. α CD was not included in this study because it was suggested that, regarding its smaller cavity diameter, it would not provide desired complexation of polyphenols. Obtained extracts were dried for 48 h in a lyophilizator (Alpha 1-4 LOC-1, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). In addition, pure main OP polyphenols (hydroxytyrosol (HTS), tyrosol (TS), oleuropein (OLE)) were included in this study in concentration of $40\text{ }\mu\text{g/mL}$, both as one compound or in a mix. That concentration was chosen to mimic approximately the concentrations in native sample (nat). Stock solutions of pure compounds were prepared in DMSO and diluted with ultrapure water.

2.2. Reagents

Petrol ether and dimethyl sulfoxide (DMSO) were purchased from Carlo Erba Reagents while ethanol was from Gram Mol (Zagreb, Croatia). β cyclodextrin (bCD), hydroxypropyl β cyclodextrin (hpbCD), randomly methylated β cyclodextrin (ramCD), and γ cyclodextrin (gCD) were purchased from Wacker-Chemie GmbH (Burghausen, Germany). Methanol (\geq 99.9%) and sodium acetate used for the preparation of chromatographic analysis were from Sigma-Aldrich (St. Louis, MO, USA) while acetonitrile (\geq 99.9%) was from Honeywell (Charlotte, NC, USA), and acetic acid from Kemika (Zagreb, Croatia). Reference standards of phenolic compounds 3-hydroxytyrosol (HTS), tyrosol (TS), and oleuropein (OLE) were of analytical grade (\geq 98%) and purchased from Sigma-Aldrich. 2,2'-azino-bis [3-ethylbenzothiazoline-6-sulphonic acid] (ABTS), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Dulbecco's Phosphate Buffered Saline (PBS liquid, sterile-filtered, without calcium, without magnesium, suitable for cell culture), and tert-butyl hydroperoxide (tBOOH) were also purchased from Sigma-Aldrich. Bile salts, pancreatin from porcine pancreas ($4\times$ USP), and Dulbecco's Modified Eagle's Medium (DMEM with 4500 mg/L glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate, liquid, sterile-filtered, suitable for cell culture) were purchased from Sigma Aldrich. Rabbit gastric extract (RGE $>$ 25 U/mg) was from

Lipolytech (Marseille, France). Heat inactivated fetal bovine serum (FBS), nonessential amino acids (NEAA), penicillin/streptomycin/amphotericin B (A/A), and trypsin were purchased from Capricorn Scientific (Ebsdorfergrund, Germany). 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Panreac AppliChem (Darmstadt, Germany). Ultrapure water (18 M Ω) was obtained from SG Reinstwassersystem Ultra Clear UV Plus coupled with SG Wasservollentsalzer-Patrone SG-2800 (Günzburg, Germany). Acqua pro injectione was obtained from Croatian Institute of Transfusion Medicine. Hank's balanced salt solution (HBSS) pH 6.0 was prepared by dissolving KCl (0.4 mg/mL), NaHCO₃ (0.35 mg/mL), NaCl (8.0 mg/mL), D-glucose monohydrate (1.1 mg/mL), KH₂PO₄ (0.06 mg/mL), Na₂HPO₄×2H₂O (0.06 mg/mL), CaCl₂×2H₂O (0.185 mg/mL), MgCl₂×6H₂O (0.1 mg/mL), MgSO₄×7H₂O (0.1 mg/mL), and HEPES (7.15 mg/mL) in ultrapure water.

All the solvents needed for chromatographic separation were degassed before analysis with Branson 1210 Ultrasonic Cleaner (Danbury, CT, USA). Acetate buffer was prepared by mixing sodium acetate 0.1 M: acetic acid 0.1 M (2:1 *v/v*) and adjusting the pH to 5 with pH meter (702 SM Titrino, Metrohm, Herisau, Switzerland).

2.3. Quantification of Phenolic Components by HPLC-FLD and Determination of the Radical Scavenging Capacity by TEAC

The main polyphenols in olive pomace (HTS, TS, and OLE) were identified and quantified by HPLC system (Waters Alliance 2695, Milford, MA, USA) coupled with a 2475 Multi λ detector (FLD) with Xenon lamp, according to slightly modified method of Tzabopoulos and co-workers [25]. Samples were prepared by filtration through 0.45 μ m PES syringe filters (Macherey–Nagel, Düren, Germany). Chromatographic separation was conducted by injecting 20 μ L of sample on a reversed phase column (250 × 4.6 mm, 5 μ m) (Agilent Zorbax Eclipse Plus C18, Santa Clara, CA, USA). Mobile phases were 0.05 M sodium acetate buffer pH 5 and acetonitrile with the flow rate of 1 mL/min. Elution was conducted over 20 min at 25 °C. Identification was performed with FLD set at the excitation wavelength of 280 nm, and emission wavelength of 316 nm. Polyphenols were identified by comparing the retention times of the eluting peaks with those of the standards. Peaks were quantified by using the Empower2 software (Waters, Milford, MA, USA) and compared to external standard calibration. Standard stock solutions were prepared by dissolving reference compounds in DMSO. Aliquots of these solutions were further diluted with ultrapure water to obtain calibration curve (1–81 mg/L).

Radical scavenging capacity was measured following the procedure for determining Trolox equivalents antioxidant capacity (TEAC) described by Re and co-workers [26]. In brief, ABTS chromophore was generated a day before the experiment through the reaction between ABTS and potassium persulfate. The ABTS^{•+} was diluted 10× and the initial absorbance was ~ 0.7. Samples were mixed with diluted ABTS^{•+} and incubated 3 min at 30 °C. Discoloration of the radical was measured at 750 nm using the multimode plate reader (Perkin Elmer Victor X3, Waltham, MA, USA). The percentage of the absorbance decrease was compared to Trolox by using the calibration curve (% Δ A vs. Trolox concentration).

2.4. In Vitro Simulated Gastrointestinal Digestion

Bioaccessibility of the OP polyphenols was assessed by in vitro static simulation of gastrointestinal digestion in the upper tract following the standardized protocol described by Brodkorb and co-workers [27]. The procedure was consisted of two sequential incubations; initially in simulated gastric fluid (SGF)/pepsin/gastric lipase to simulated gastric conditions followed by simulated intestinal fluid (SIF)/bile salts/pancreatin to simulate duodenal digestion. Briefly, 500 mg of OPE were dissolved in 2 mL of ultrapure water and mixed with SGF. The samples were incubated at 37 °C for 2 h in a water bath (Büchi B-490, Flawil, Switzerland) with uniform shake at 110 rpm. Then SIF was added and incubated under the same conditions for 2 h. Enzyme solutions were prepared just before use. Samples were put on ice for 10 min and centrifuged (Heraeus Biofuge Stratos, Hanau, Germany) for 20 min at 4 °C and 4100× rpm in order to remove the crude parts of the sample. Supernatants were collected and

the enzyme inactivation was done by a heat shock for 5 min at 100 °C in Thermomixer R (Eppendorf, Hamburg, Germany). Then, the samples were cooled in an ice bath for 10 min and centrifuged again under the same conditions. Supernatants, that represent bioaccessible fraction (bf) of the samples, were collected and stored at −80 °C until analyses. Blank sample contained only digestive solutions and was analyzed to discard interferences due to the reagents. The stability of polyphenols during gastrointestinal digestion was monitored by calculating the percentage (%) according to Equation (1).

$$\%_{GI} = (\text{amount in bioaccessible fraction}/\text{amount in undigested extract}) \times 100 \quad (1)$$

2.5. In Vitro Study of Transepithelial Transport

For investigation of transepithelial transport human epithelial colorectal adenocarcinoma cell line (Caco-2) was used. Caco-2 cells (American Type Culture Collection (ATCC)) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% NEAA, and 1% A/A. Cell cultures were maintained at 37 °C, in a humidity saturated atmosphere consisted of 5% CO₂ (Sanyo MCO-20AIC CO₂ Incubator, Osaka, Japan). Medium was changed every 2 days. Cells were passaged at 80–90% confluence.

The highest non-toxic concentration of bioaccessible OPE fractions and pure polyphenols was determined by MTT assay [28]. 3×10^5 cells/well were seeded in 96-well plates (Thermo Fisher Scientific 130188, Rochester, NY, USA) and grown until reaching confluence (approximately 48 h). The medium was aspirated, and the cells washed with 100 µL PBS/well. Cells were incubated for 2 h with either 100 µL of samples or Hank's balanced salt solution (HBSS) for positive control. Samples were applied undiluted or diluted (2×, 5×, 10×) with HBSS. Initial concentration of OPE in bioaccessible fractions were 28 mg/mL while HTS, TS, OLE, mix (HTS + TS + OLE) were applied in initial concentration of 40 µg/mL. Samples were then removed, and cells were washed with 100 µL PBS/well. Cell viability was assessed by the addition of 40 µL of MTT 0.5 mg/mL (diluted in PBS) and incubation for 3 h at 37 °C, followed by dissolution of the formazan crystals in 170 µL of DMSO. Absorbance (A) was measured at 490 nm and cell viability was expressed as fold change (FC_v) relative to the positive control according to Equation (2). Blank absorbance was measured in wells containing MTT without cells.

$$FC_v = (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \quad (2)$$

Transepithelial transport was investigated in 12-well plate with Transwell® permeable supports (Costar 3401, Corning Incorporated, Kennebunk, ME, USA) [29]. The 3×10^5 cells were seeded per well and maintained at 37 °C, in a humidity saturated atmosphere consisted of 5% CO₂. Cells were grown for 23 days in order to form a differentiated monolayer on filters. The medium was warily aspirated (Gilson Safe Aspiration Station, Middleton, WI, USA) and replaced with fresh one every 2 days. The added volume was 0.5 mL in the apical and 1.5 mL in the basolateral compartment. Monolayer integrity was routinely checked by determining transepithelial electrical resistance (TEER) during the cell growth, before and after the transport experiment. Electrical resistance (ER) was measured with STX2 and EVOM resistance meter (World Precision Instruments Inc, Sarasota, FL, USA). TEER defined as ER per area was calculated according to Equation (3).

$$TEER = (R - 120 \Omega) \times 1.12 \text{ cm}^2 \quad (3)$$

where TEER is transepithelial electrical resistance, 120 Ω is resistance of the cell free-well (blank), 1.12 cm² is the filter surface.

On the day of the experiment, the lowest TEER was 228 Ω cm². Day after the experiment, the lowest TEER was 345 Ω cm².

To evaluate transepithelial permeability, medium was aspirated from both apical and basolateral chambers and washed twice with pre-warmed PBS. Then, 0.5 mL of the samples were added to the apical chamber and 1.5 mL of the HBSS to the basolateral chamber of each well. Bioaccessible fractions obtained after the gastrointestinal simulation and pure polyphenols were applied on a cell monolayer

in triplicate and incubated at 100 rpm and 37 °C for 2 h in a shaker (Biosan Incubator ES-20/60, Riga, Latvia). The pure analytes (HTS, TS, OLE) were included in this study to examine the potential interference in transepithelial transport among these three polyphenols but also among the other compounds that are present in the OPE. Polyphenols' content determined by HPLC-FLD and TEAC were determined in apical and basolateral compartment.

After the 2-h incubation of Caco-2 cells with the OPEs and pure polyphenols, transport through the cell monolayer was studied. The content of polyphenols and TEAC were determined in both, apical and basolateral chamber, and expressed as % of the amount applied on cell monolayer according to Equation (4). The difference between the total amounts found in the apical and basolateral chambers at the end of experiment and the initial amount was expressed as metabolized fraction representing the amount that is either metabolized or accumulated inside cells.

$$\% = (\text{amount in apical or basolateral compartment}) / \text{initial amount} \times 100 \quad (4)$$

2.6. Determination of Antioxidative Activity

To assess antioxidant activity of the bioaccessible fractions of OPE and pure polyphenols Caco-2 cell viability was determined. For that purpose, cells were seeded in 96-well microtiter plates at a density of 3×10^5 cells/well in 100 μ L of medium and maintained until reaching confluence (approximately 48 h) at 37 °C in a humidity saturated atmosphere consisted of 5% CO₂. Cells were treated with 20 μ L of analyzed OPEs/pure polyphenols for 2 h and then with 20 μ L of prooxidant tBOOH for the following 2 h. Final tBOOH concentration was 100 μ M. Positive control cells were treated with HBSS only, while negative control cells received HBSS instead of samples and tBOOH. Previously described MTT assay was performed to detect cell viability.

2.7. Statistical Analysis

All experiments were run in quadruplicate unless otherwise stated. Data from the bioavailability and antioxidant assays were statistically tested by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. Results were expressed as average value and standard deviation. $p < 0.05$ was considered statistically significant unless otherwise noted. GraphPad® Prism 6 Software (San Diego, CA, USA) was used for statistical analysis.

3. Results and Discussion

3.1. Composition of Olive Pomace Extracts

The amount of main polyphenols in olive pomace (HTS, TS, and OLE) and TEAC are noted in Table 1. Polyphenols were identified by comparing the retention times of the eluting peaks (Figure 1) with those of the standards. Peaks were quantified by comparison to external standard calibration curve. TEAC was calculated as the percentage of the absorbance decrease compared to Trolox by using the calibration curve (% Δ A vs. Trolox concentration).

Table 1. Olive pomace extracts' composition.

OPE	HTS (μ g/100 mg)	TS (μ g/100 mg)	OLE (μ g/100 mg)	TEAC (μ g/100 mg)	% CD (<i>w/w</i>)
nat	71.7	23.5	31.9	6107	0
bCD	47.3	9.3	12.8	4114	51
hpbCD	36.0	8.9	13.6	3695	52
ramCD	35.9	8.4	14.0	5226	50
gCD	33.1	8.6	19.5	4550	53

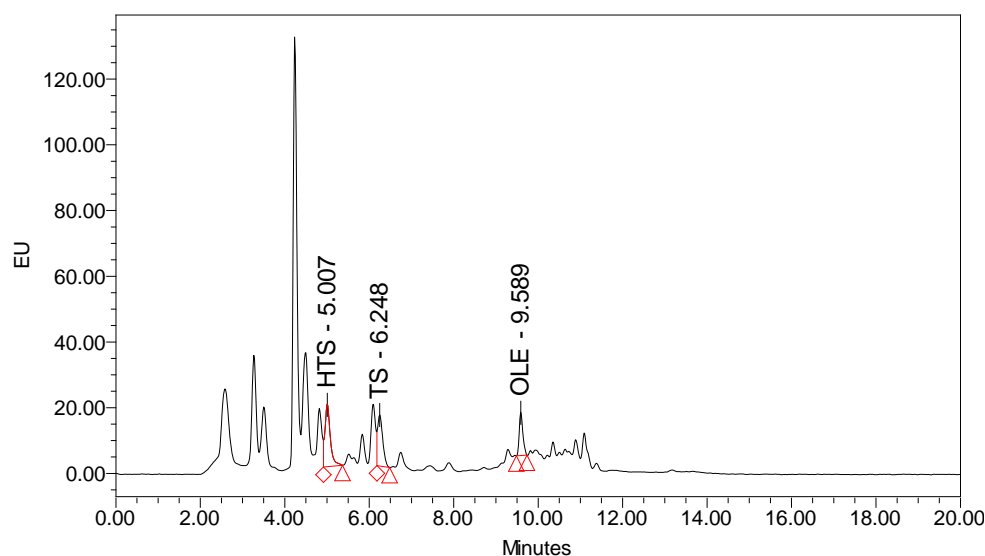


Figure 1. Chromatogram of the native olive pomace extract.

3.2. Bioaccessibility of Olive Pomace Polyphenols

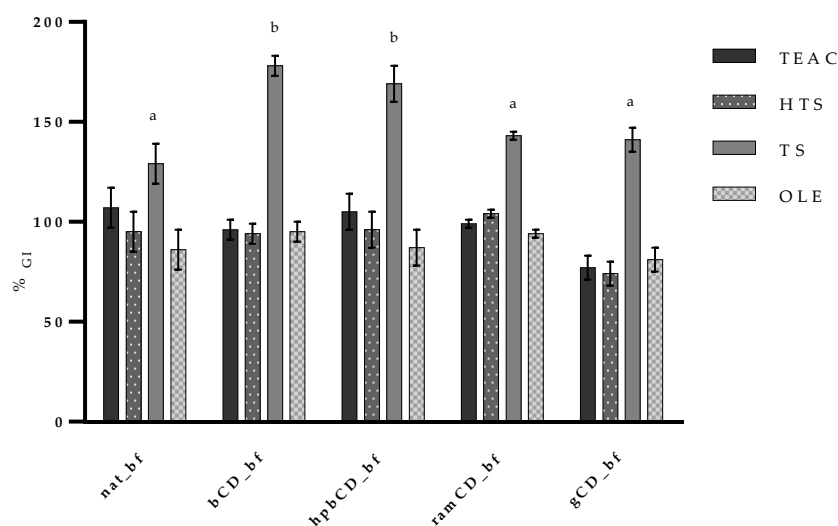
Bioaccessibility is defined as the quantity of a compound that is released from food matrix in gastrointestinal tract, becoming available for absorption [30]. Since it is known that it can be greatly dependent on the type of food matrix [31], we took into account that the presence of different cyclodextrins in analyzed OPE formulations might influence the bioaccessibility of target compounds.

The impact of gastrointestinal conditions on the bioaccessibility of total antioxidants and particular phenolic compounds is presented in Figure 2. Results showed that both, antioxidative capacity and the amounts of HTS, TS, and OLE remain constant during the gastrointestinal digestion. The only change that was observed was an increase of TS amount in all samples' bioaccessible fractions. Considerable increase of TS amount in bioaccessible fraction was also noted by Corona and co-workers [13] after 2-h incubation of olive oil in HCl. Interestingly, in the same work, that phenomenon was observed also for HTS unlike in our work indicating specific matrix effect, which is in agreement with available literature data [32]. Good GI stability of HTS, shown in our work, has already been reported by several authors [15,33,34].

It is important to emphasize that both, HTS and TS can be products of disintegration of more complex polyphenols present in olive pomace in acidic conditions, i.e., secoiridoids or verbascosides [35]. The increase of the amount of TS was probably due to the liberation from verbascosides [36] and it was additionally modified by the presence of particular CDs in the formulation. Our results showed that ramCD and gCD did not influence the release of TS in OPE while the presence of bCD and hpbCD caused significant increase of TS release rate during the GI digestion. More precisely, after digestion of nat, ramCD, and gCD, relative change of TS was 129–143%, while TS amount in bCD_bf and hpbCD_bf increased to 169–178% comparing to the undigested sample. Observed changes might be explained by the strong binding capacity of bile salts that can interact with phenolic hydroxyl groups to form hydrogen/ionic bonds [37]. Formation of inclusion complexes or aggregates of TS with bCD and hpbCD [38] might prevent adsorption resulting in increased bioavailability. Despite the significant increase of TS amount in all samples, their antioxidative potential did not change significantly which could be a confirmation of its weak antioxidant potential. However, TS was shown to be an effective cellular antioxidant, probably due to its intracellular accumulation [39].

The amount of the main olive secoiridoid (OLE) after gastrointestinal digestion was the same as in undigested extracts. This is in accordance to available literature data [40] and the fact that glycosylated secoiridoids are not to be subjected to gastric hydrolysis [34].

Our results clearly show that the main polyphenols and antioxidants from OPE do not degrade under conditions of gastrointestinal digestion and that TS bioaccessibility varies depending on the type of CD used for encapsulation. In comparison to data obtained for olive oil [11,41], gastrointestinal availability of HTS, TS, and OLE from OPE is comparable or higher, indicating that olive pomace matrix doesn't impair bioaccessibility of target phenolic compounds, confirming the applicability of OPE as potential alternative food source of olive polyphenols.



(a)

Examined parameters	nat	bCD	hpbCD	ramCD	gCD
TEAC (µg/100 mg)					
undigested extract	6107	4114	3695	5226	4550
bioaccessible fraction	6543	3968	3889	5166	3511
HTS (µg/100 mg)					
undigested extract	71.7	47.3	36.0	35.9	33.1
bioaccessible fraction	67.8	44.4	34.4	37.2	24.6
TS (µg/100 mg)					
undigested extract	23.5	9.3	8.9	8.4	8.6
bioaccessible fraction	30.4 ^a	16.5 ^b	15.0 ^b	12.1 ^a	12.1 ^a
OLE (µg/100 mg)					
undigested extract	31.9	12.8	13.6	14.0	19.5
bioaccessible fraction	27.4	12.2	11.8	13.2	15.8
% CD (w/w)	0	51	52	50	53

(b)

Figure 2. Bioaccessibility of total antioxidants and main polyphenols present in OPEs. (a) Relative change expressed as percentage of each component remained after the in vitro simulated GI digestion. Data are presented as mean ± standard deviation (n = 3). (b) Absolute values of concentrations (w/w) in undigested samples and bioaccessible fractions; ^a indicate the significant difference of percentage among all samples in each group (p < 0.004); ^b indicates the significant difference of percentage among all samples in each group (p < 0.0001); TEAC (Trolox equivalent antioxidant capacity), HTS (hydroxytyrosol), TS (tyrosol), OLE (oleuropein), OP (olive pomace), bf (bioaccessible fraction), CD (cyclodextrin), nat (native OP), bCD (OP + β CD), hpbCD (OP + hydroxypropyl β CD), ramCD (OP + randomly methylated β CD), gCD (OP + γ CD).

3.3. Permeability of OPE Polyphenols on Caco-2 Cell Monolayer

The potential cytotoxic effect of OPEs' bioaccessible fractions and pure polyphenols (HTS, TS, OLE, and their mix) was investigated to ensure the integrity of the cell monolayer. Their influence on the viability of human epithelial colorectal adenocarcinoma cells Caco-2 was determined by using the MTT assay. Cells were treated with either undiluted samples (bioaccessible fraction of OPEs in initial concentration of 28 mg/mL or HTS, TS and OLE in initial concentration of 40 µg/mL) or the same samples diluted 2×, 5× or 10× with HBSS. The results presented in Figure 3 showed that cell viability was significantly decreased when cells were treated with undiluted nat_bf. The possible explanation for this toxic effect is the fact that polyphenols may exert a pro-oxidant effect at high doses [42,43]. Cell viability was increased after the treatment with undiluted bCD_bf and ramCD_bf, and 2× diluted ramCD_bf. Treatments with all the other samples did not affect the Caco-2 viability. Based on these findings, nat_bf was 2× diluted with HBSS for further investigations, so its initial concentration was 14 mg/mL. Accordingly, mix was diluted 2× for comparison with nat and the initial concentration of each polyphenol was 20 µg/mL.

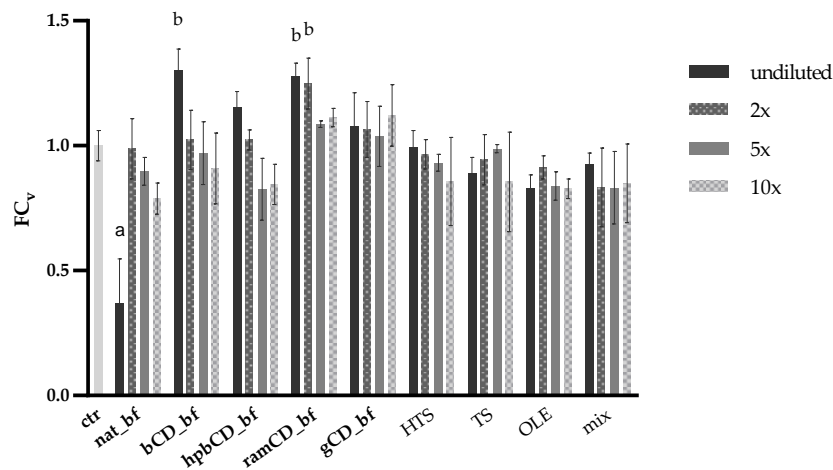


Figure 3. Effect of a 2-h exposure of olive pomace extracts (OPEs') bioaccessible fractions and pure polyphenols on Caco-2 cell viability. Control cells (ctr) were treated with Hank's balanced salt solution (HBSS). Data are presented as mean \pm standard deviation of the fold change calculated according to Equation (2); ^a significant decrease of cell viability compared to ctr ($p < 0.001$); ^b significant increase of cell viability compared to ctr ($p < 0.001$); HTS (hydroxytyrosol), TS (tyrosol), OLE (oleuropein), mix (HTS + TS + OLE) in initial concentration of 40 µg/mL; bf (bioaccessible fraction), CD (cyclodextrin); nat (native OP), bCD (OP + β CD), hpbCD (OP + hydroxypropyl β CD), ramCD (OP + randomly methylated β CD), gCD (OP + γ CD) in initial concentration of 28 mg/mL. 2×, 5×, and 10× are dilutions of the samples.

The first goal of permeability research was to evaluate the potential interference between HTS, TS, and OLE transport through Caco-2 monolayer. For that purpose, tested compounds were applied to Caco-2 monolayer as pure compounds and as a mixture (mix). Both, apical to basolateral (a–b), and basolateral to apical (b–a) transport was investigated in order to elucidate the transport mechanisms of each tested compound. To determine the amount of the compounds transported per time, the apparent permeability coefficient (P_{app}) was calculated according to the following general equation that does not require sink conditions:

$$C_R(t) = \left(\frac{M}{V_A + V_B} \right) + \left(C_{R,0} - \frac{M}{V_A + V_B} \right) e^{-P_{app}A \left(\frac{1}{V_A} + \frac{1}{V_B} \right) t} \quad (5)$$

where V_A is the volume in the apical compartment, V_B is the volume of the basolateral compartment, A is the area of the filter (1.12 cm²), M is the total amount of substance in the system (mol), $C_{R,0}$ is the

concentration of the substance in the basolateral compartment at the start of the time interval and $C_{R(t)}$ is the concentration of the substance at time t measured from the start of the time interval (s).

P_{app} of hydroxytyrosol was approximately $1 \times 10^{-5} \text{ cm s}^{-1}$ while P_{app} of tyrosol was three times higher (approximately $3 \times 10^{-5} \text{ cm s}^{-1}$). There was no difference in P_{app} when TS was added to the apical or basolateral chamber indicating a passive transport through the monolayer. The efflux ratio (defined as the quotient of the secretory permeability and the absorptive permeability ($P_{app_{b-a}}/P_{app_{a-b}}$)), and the uptake ratio (the inverse of the efflux ratio, $P_{app_{a-b}}/P_{app_{b-a}}$) results are available in Table S1. There are two studies in which P_{app} of TS was determined [13,44]. Interestingly, these two studies obtained significantly different results. Our was in accordance to work from Corona and co-workers [13] and the P_{app} value was approximately 20 times higher than the one from D'Antuono and co-workers [44]. The possible reason for that significant difference is that in the mentioned study, Caco-2/TC7 cells were used for permeability examination. Caco/TC7 are characterized with more developed intercellular junctions. Those junctions act as diffusion barriers within the lipid bilayer of the plasma membrane which can decrease the rate of passive transport. HTS permeability, on the other hand, was studied by several authors [13,45,46]. P_{app} of approximately $1 \times 10^{-5} \text{ cm s}^{-1}$ we obtained for HTS was in accordance with all these studies. However, we also observed that HTS is effluxed back to the apical chamber with the ratio of 1.47 ± 0.07 (data available in supplementary material). P_{app} of OLE was determined only when present along with HTS and TS (mix). However, the value was $1 \times 10^{-7} \text{ cm s}^{-1}$ indicating very low permeability of OLE what indicates its intensive metabolism in the Caco-2 cells.

Obtained results also showed that there was no difference between P_{app} of HTS and TS obtained by testing one-compound solutions or the mix (Figure 4) indicating no interference in their absorption. OLE, on the other hand, showed slightly lower metabolism rate when present along with HTS and TS. As far as we know, there are no published studies of the potential permeability interference of olive polyphenols and therefore we find this result an important prerequisite for the development of olive-derived nutraceuticals.

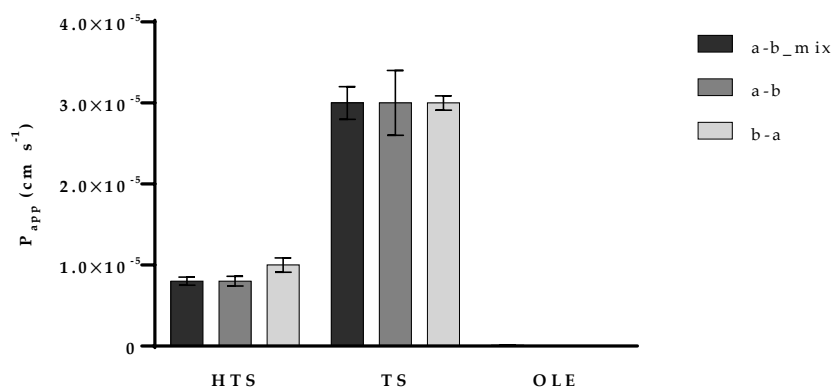


Figure 4. Determination of the hydroxytyrosol (HTS) olive pomace polyphenols' transport mechanism. P_{app} (apparent permeability coefficient) was determined for HTS (hydroxytyrosol), TS (tyrosol), and OLE (oleuropein) transport from apical (a) to basolateral (b) chamber either in their mix (a-b_mix) or as one-compound (a-b), and when transported from basolateral to apical chamber (b-a). Data are presented as mean \pm standard deviation. All the experiments were done in triplicates.

In the second phase of investigation we determined the permeability of total antioxidants, HTS, TS, OLE, and from OPEs bioaccessible fractions (Figure 5). Permeability of total antioxidants was assessed based on investigation of TEAC and it revealed a significant decrease of the antioxidative potential in the apical chamber of all investigated OPEs while TEAC in the basolateral chambers was rather low, from 1% to 3% of the value applied to cell monolayer (Figure 5a). Obtained results indicate high permeability, but also extensive metabolism of OPE-derived antioxidants.

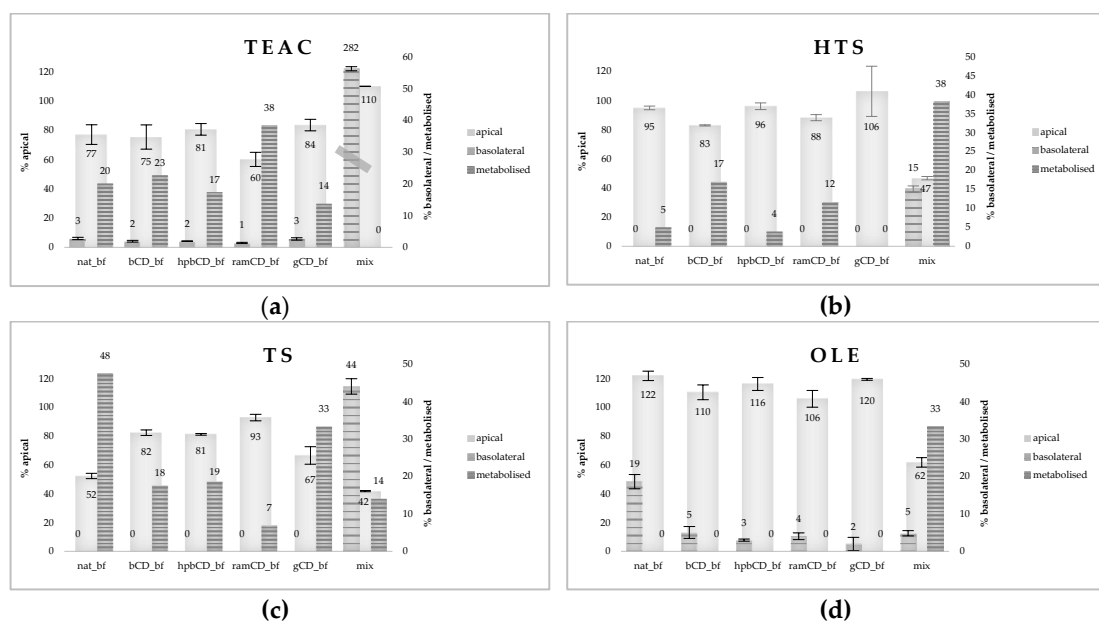


Figure 5. Permeability of total antioxidants (a) and specific polyphenols (HTS (b), TS (c), and OLE (d)) from OPEs bioaccessible fractions and mix. Polyphenols' amount and TEAC (Trolox equivalent antioxidant capacity) were determined in both, apical and basolateral chamber and expressed as percentage of the amount applied on cell monolayer. The difference between the amount found in the apical and basolateral chamber and the initial amount was expressed as metabolized fraction representing the amount that is either metabolized or accumulated inside cells. Initial concentrations were: 14 mg/mL for nat (native OP); 28 mg/mL for bCD (OP + β CD), hpbCD (OP + hydroxypropyl β CD), ramCD (OP + randomly methylated β CD), gCD (OP + γ CD); 20 μ g/mL for mix (hydroxytyrosol, tyrosol, oleuropein); data are presented as mean \pm standard deviation. All the experiments were done in triplicate; OPE (olive pomace extract), bf (bioaccessible fraction), CD (cyclodextrin).

However, results were significantly different for mix (pure HTS+TS+OLE) where we noticed the slight increase in TEAC in the apical but also 2.82 times increase in TEAC in the basolateral compartment, indicating high bioavailability. Obtained results indicate that OPE matrix decreases bioavailability of antioxidants and that bioavailability of HTS, TS, and OLE is increased when they are applied as the mixture of pure compounds. Additionally, results indicate "formation" of antioxidants during transport through Caco-2 monolayer, when they are applied as mixture of pure compounds. The explanation of this phenomena could be the fact that glucoside residue of OLE in mix is removed by the small intestine brush border or cytosolic enzymes resulting with increased antioxidant activity and increased permeability of the aglycone compound. The deglycosylation of dietary flavonoid glycosides was studied in detail by Németh and co-workers [30], where two β -glucosidases were isolated from human small intestine mucosa: lactase-phlorizin hydrolase (localized to the apical membrane of small intestinal epithelial cells) and cytosolic β -glucosidase; they significantly affected flavonoids' absorption and metabolism. Permeability of OLE from mix is low, and metabolism extensive (as shown in Figure 4). Therefore, we can assume that in Caco-2 cells OLE and OLE aglycone were metabolized into potent antioxidants, such as HTS, resulting in significant increase in TEAC in basolateral chamber. In OPE samples the effect is absent probably due to saturation of brush border and cytosolic enzymes by other substrates present in complex matrix.

The explanation of TEAC results is confirmed by data obtained for OLE permeability (Figure 5d). OLE content in apical chambers increased during 2 h of incubation, particularly in nat_bf. Since it was shown that lactase-phlorizin hydrolase has low but significant ability to hydrolyze several natural β -glycosides (also cellulose), it could be that the part at the OLE entrapped in the OP matrix was liberated during incubation and that this reaction was inhibited by the presence of CDs in reaction

mixture. In contrast, in mix (pure HTS+TS+OLE), OLE apical content was decreased to 62% of initial concentration indicating formation of OLE aglycone, as previously explained. However, extensive metabolism of mix resulted in low basolateral OLE, which was comparable to that of CD-encapsulated OPEs. The highest bioavailability of OLE was from native sample, indicating negative effect of CDs on OLE permeability.

HTS and TS were not found in basolateral chambers, except when present as a mix of pure analytes (15% and 44% of the initial amount respectively) indicating strong negative matrix effect on their in vitro bioavailability. On the other hand, significant decrease of their amount in apical chambers indicates extensive metabolism. Figure 5b shows that amount of metabolized/accumulated HTS fraction was increased in bCD_bf and ramCD_bf (17%, 12%, respectively) signaling, enhancing effect of bCD and ramCD on HTS metabolism rate and/or permeation in the cell.

According to available literature data, HTS is transported by passive diffusion, and the transport is bi-directional [46]. Negative effect of OPE matrix on HTS permeability might be explained by non-covalent polyphenol/macromolecule interactions that are largely due to weak associations (combination of hydrogen bonds and hydrophobic interactions) and can impair the possibility of passive diffusion through intestinal epithelium [47]. On the other hand, presence of bCD and ramCD in reaction mixture increased the permeability of HTS, probably due to their previously reported ability to increase the permeability of Caco-2 monolayer by removing cholesterol from the membranes changing their structure and resulting in higher permeability [48]. Unchanged amount of HTS in apical compartments of analyzed OPEs can be explained by the fact that most polyphenols found in natural matrices exist as esters, glycosides or polymers that can be hydrolyzed and release aglycons at the brush border of small intestine epithelial cells due to the activity of brush-border hydrolases [49].

Permeation of TS inside the cell was significantly higher in comparison to HTS, resulting in decreased apical fraction in all samples except ramCD_bf (Figure 5c). Obtained results are consistent with available literature data indicating higher relative transport rate (apical to basolateral) of TS in comparison to HTS [11]. Generally, permeation of TS was negatively influenced by the presence of all investigated CDs, resulting in lower metabolized fraction in comparison to nat_bf. ramCD_bf showed particularly strong effect and completely blocked the permeation of TS through apical membrane. TS was not detected in basolateral departments of analyzed OPEs probably due to intensive metabolism in Caco-2 cells, which is consistent with available literature data [50]. The amount of TS that might have remained inside the Caco-2 cells was the highest in nat_bf and gCD_bf (48% and 33%, respectively). Since TS is known as an effective cellular antioxidant [39], nat_bf and gCD_bf could be proposed as potent sources of TS which exert its effect after the accumulation in the cells. Data obtained for mix show much higher permeability of TS in comparison to OPE indicating negative effect of matrix on TS bioavailability. Observation is consistent to available literature data and can be explained by the same mechanisms as in the case of HTS [47].

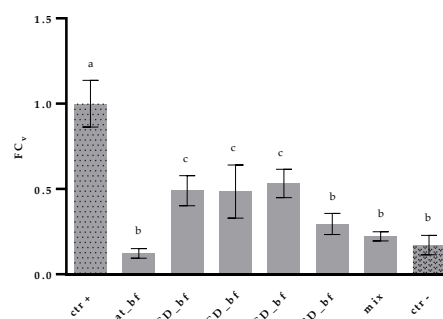
3.4. Antioxidative Effect of OPE on Caco-2 Cells

To assess the antioxidative effect of OPEs we determined their effect in decreasing oxidative stress in small intestine epithelial cells. Cells were treated with OPE in concentrations noted in Figure 6a. Mix was prepared with HTS, TS, and OPE in the same amounts as in nat_bf. All of the samples contained similar amounts of these polyphenols, i.e., HTS 1.2–2.1, TS 0.6–0.8, and OLE 0.6–0.9 µg/mL. The treatment of Caco-2 cells with 100 µM tBOOH significantly reduced cell viability clearly showing the toxic effect of peroxide. Pre-incubation with mix did not show protective effect against prooxidant in this high concentration as well as nat_bf and gCD_bf. However, we observed a significant increase in viability at cells treated with bCD_bf, hpbCD_bf, and ramCD_bf (Figure 6b). The capability of CDs to improve the biological, chemical and physical properties of plant bioactive molecules was observed by many authors [51]. Particularly their ability to improve the solubility of the bioactive compounds is important since it is directly connected to the exertion of biological activities. It could be that other

compounds in OPE that possess antioxidative activity can exert their potential when encapsulated by bCD, hpbCD, and ramCD.

OPE	γ OPE (mg/mL)	γ HTS (μ g/mL)	γ TS (μ g/mL)	γ OLE (μ g/mL)
nat_bf	2.3	1.6	0.7	0.7
bCD_bf	4.7	2.1	0.8	0.6
hpbCD_bf	4.7	1.6	0.7	0.6
ramCD_bf	4.8	1.8	0.6	0.7
gCD_bf	4.7	1.2	0.6	0.9
mix	0	1.6	0.7	0.7

(a)



(b)

Figure 6. Determination of antioxidative effect of OPEs bioaccessible fractions and mix of polyphenols. (a) mass concentration and polyphenol composition of each OPE administered in each well with confluent Caco-2 monolayer. (b) Caco-2 cell viability determined by the MTT assay when treated with HBSS (ctr+); OPE and tBOOH (nat_bf, bCD_bf, hpbCD_bf, ramCD_bf, gCD_bf); HTS, TS, and OLE, and tBOOH (mix); and tBOOH (ctr-). Cell viability was expressed as fold change calculated by Equation (2). Data was expressed as mean \pm standard deviation. All the experiments were done in 3 replicates; HTS (hydroxytyrosol), TS (tyrosol), OLE (oleuropein), mix (HTS + TS + OLE), bf (bioaccessible fraction), CD (cyclodextrin), nat (native OP), bCD (OP + β CD), hpbCD (OP + hydroxypropyl β CD), ramCD (OP + randomly methylated β CD), gCD (OP + γ CD), OPE (olive pomace extract), tBOOH (tert-butyl hydroperoxide).

4. Conclusions

In this work we evaluated the influence of OPE matrix and CDs on bioaccessibility and intestinal permeability of the main olive pomace polyphenols. Obtained results indicate that major olive pomace polyphenols are stable during gastrointestinal digestion. Presence of CDs in formulation significantly increases bioaccessibility of TS, probably by formation of inclusion complexes and prevention of TS adhesion to bile salts or other macromolecules present in reaction mixture during simulation of OPE digestion. Investigation of permeability of pure polyphenols (HTS, TS, and OLE) showed that HTS and TS are primarily absorbed by passive diffusion and confirmed extensive intracellular metabolism of OLE in Caco-2 cells. P_{app} was unaffected when HTS and TS were applied as a mix, indicating the absence of interactions at the absorption level. OPE matrix negatively influenced the permeability of OPE total antioxidants and investigated polyphenols. However, observed effects were comparable to those of olive oil reported by other authors. Presence of CDs in tested formulations significantly affected permeability of particular polyphenols but results were CD- and polyphenol specific. bCD and ramCD increased permeability of HTS, probably through direct effects on Caco-2 cell monolayer. gCD significantly increased intracellular TS concentrations. Impact of CDs on OLE permeability was generally negative, but to a much lesser extent in comparison to matrix effect. Investigation of antioxidative activity showed that OPE can be considered a rich source of highly permeable antioxidants other than HTS, TS and OLE. OPE also provided efficient protection of Caco-2 cells against oxidative stress, and their antioxidative activity was positively affected particularly by bCD, hpbCD and ramCD.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6643/12/3/669/s1>, Table S1: Determination of the HTS olive pomace polyphenols' transport mechanism.

Author Contributions: Conceptualization, K.R., and D.V.Č.; methodology, K.R., D.V.Č., B.J.D.; formal analysis, K.R.; investigation, K.R., D.V.Č., B.J.D.; resources, D.V.Č.; data curation, K.R.; writing—original draft preparation, K.R.; writing—review and editing, B.J.D.; visualization, K.R. and D.V.Č.; supervision, D.V.Č.; project administration, K.R. and D.V.Č.; funding acquisition, D.V.Č. All authors have read and agreed to the published version of the manuscript.

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Supplementary material

Table S1. Determination of the HTS olive pomace polyphenols' transport mechanism.

Transport	P_{app} (cm s ⁻¹)		
	HTS	TS	OLE
a-b_mix	8.1E-06 ± 4.8E-07	3.2E-06 ± 2.4E-06	1.1-07 ± 1.1-08
a-b	7.6E-06 ± 6.3E-07	3.2E-06 ± 3.5E-06	0
b-a	1.2E-05 ± 8.6E-07	3.0E-06 ± 8.9E-07	0
Ratio			
Efflux	1.47 ± 0.07	0.93 ± 0.00	N/A
Uptake	0.67 ± 0.03	1.08 ± 0.00	N/A

P_{app} (apparent permeability coefficient) was determined for HTS (hydroxytyrosol), TS (tyrosol), and OLE (oleuropein) transport from apical (a) to basolateral (b) chamber either in their mix (a-b_mix) or as one-compound (a-b), and when transported from basolateral to apical chamber (b-a). The efflux ratio is defined as the quotient of the secretory permeability and the absorptive permeability (P_{app_b-a}/P_{app_a-b}). Uptake ratio is defined as the inverse of the efflux ratio (P_{app_a-b}/P_{app_b-a}). Data are presented as mean ± standard deviation. All the experiments were done in triplicates.

3. Food (Matrix) Effects on Bioaccessibility and Intestinal Permeability of Major Olive Antioxidants

Article

Food (Matrix) Effects on Bioaccessibility and Intestinal Permeability of Major Olive Antioxidants

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Abstract: Background: olive pomace extract (OPE) is a rich source of health promoting polyphenols (hydroxytyrosol (HTS) and tyrosol (TS)) and can be used as a nutraceutical ingredient of dietary supplements and functional foods. Its adequate bioavailability is a prerequisite for excreting biological activity and can be significantly and specifically affected by different food matrices. Methods: in order to investigate food effects on polyphenol bioaccessibility, OPE was co-digested with different foods according to internationally harmonized in vitro digestibility method. Impact of particular nutrients on HTS and TS permeability was assessed on Caco-2 cell monolayer. Results: HTS and TS bioaccessibility and transepithelial permeability can be significantly affected by foods (nutrients), especially by casein and certain types of dietary fiber. Those effects are polyphenol- and nutrient-specific and are achieved either through complexation in gastrointestinal lumen and/or through direct effects of nutrients on intestinal monolayer. Conclusions: obtained results emphasize the significance and complexity of polyphenol interactions within the food matrix and the necessity of individual investigational approaches with respect to particular food/nutrient and interacting phenolic compounds.

Keywords: olive pomace; hydroxytyrosol; tyrosol; polyphenol-food interaction; Caco-2 monolayer; in vitro digestion

1. Introduction

The well-established health effects of olive oil can be attributed to its high monounsaturated fatty acid content; it is principally made of oleic acid (56% to 84% of the total fraction of fatty acids) and its phenolic compounds are effective at decreasing the risk of cardiovascular disease, mostly due to its ability to reduce the peroxidation of blood lipids [1,2]. The main antioxidants in olive oil are two groups of phenolic compounds: lipophilic and hydrophilic. While lipophilic phenols, including tocopherols and tocotrienols, can be found in other types of vegetable oils, hydrophilic phenols (hydroxytyrosol, tyrosol, and oleuropein) are characteristic of olive oil, and are not found in other oils and fats. They demonstrate unusual sensory properties, exert antioxidant, immunomodulatory, antimicrobial, and anticancer activity, and may decrease the risk of numerous, chronic, non-infectious diseases, such as atherosclerosis, different types of cancer (colorectal, prostate, breast, etc.), chronic inflammation, strokes (e.g., ischemic stroke), and other degenerative diseases (primarily neurodegenerative diseases, such as Alzheimer's disease) [2]. Olive pomace and olive pomace wastewater, the residues left after the production of olive oil, are secondary raw materials that contain significantly higher amounts of olive polyphenols compared to olive oil, particularly hydroxytyrosol (HTS) and tyrosol (TS). Numerous green extraction methods for efficient extraction of polyphenols from olive mill waste were recently developed [3–6], and significant steps forward have been taken in developing functional

formulations of olive pomace extracts (OPEs) that will ensure satisfactory composition, stability, bioavailability, and biological activity of major active compounds, primarily HTS and TS [7,8].

Bioavailability of polyphenols is essential for their biological activity. Since it differs greatly among various phenolic compounds and depends on the characteristics of the food source, polyphenols that are the most abundant in food are not necessarily the ones with the most important health-related impacts. Since the current knowledge on health-effects of polyphenols arise mainly from epidemiological studies, it is necessary to gain better insight into their bioavailability in order to establish conclusive evidence for their effectiveness in disease prevention [9].

Bioavailability can be defined as the fraction of a particular compound available for physiological functions in the body. In the case of polyphenols, it depends on release of polyphenols from the food matrix and/or interactions with meal (1); structural changes that occur during gastro-intestinal digestion (2); cellular uptake of glycoside forms of polyphenols and respective aglycons by enterocytes (3); fermentation of nonabsorbed polyphenols by gut microbiota and formation and absorption of metabolites (4); and metabolism of all absorbed compounds (5) [10].

Impact of food matrix (food components) on bioavailability of nutraceutical is particularly important in cases when it is necessary to compare different food matrices as the sources of the same bioactive compounds (for example olive oil vs. OPE as sources of HTS and TS). Assessing polyphenol-food interactions is also of great importance because nutraceuticals may be isolated from their natural environment, purified, and then used as nutraceutical ingredients in processed (functional) food [11]. Additionally, sufficient knowledge on the significance and the type of polyphenol-food (nutrient) interactions enables targeted use of absorption, promoting interactions and subsequent formulation of excipient foods that would improve bioavailability of particular polyphenols [12].

Mechanisms of polyphenol–food interactions are numerous. Presence of food in the gastrointestinal system changes the physiological conditions in the human gastrointestinal tract (fluid volumes, gastric, and intestinal motility, gastric emptying, luminal pH values, enzyme capacity, osmolality, bile salt content) and, consequently, affects stability and solubility of particular phenolic compounds. Moreover, polyphenols can form complexes with nutrients (components of the meal) such as dietary fiber, carbohydrates, lipids, and proteins, which can significantly affect their bioaccessibility. Additionally, nutrients can also interact with the process of absorption of polyphenols through interactions with influx and efflux intestinal transporters, interactions with intestinal monolayer permeability, or interactions with intestinal metabolizing enzymes bioaccessibility [13–15].

Bioavailability of HTS and TS from olive oil has been investigated in more *in vivo* studies and it was found to be satisfactory, with plasma HTS and TS concentrations rising early after virgin olive oil ingestion and reaching a peak at around 1 h in plasma and 0–2 h in urine [16]. It is important to emphasize that complex secoiridoids from olive oil are biotransformed by gut microflora, giving additional rise to postprandial HTS and TS [17,18]. OPEs are generally richer sources of HTS and TS in comparison to extra-virgin olive oil, but they have rarely been investigated in terms of bioavailability. Recent data obtained by Radić and co-authors [8] show that OPE polyphenols are stable during gastrointestinal digestion (88–187%; due to degradation of secoiridoids and formation of HTS and TS during digestion). Investigation of permeability showed that HTS and TS are primarily absorbed by passive diffusion and that absorbed oleuropein is extensively metabolized in Caco-2 cells. OPE matrix negatively influenced the permeability of HTS and TS, but the negative effect could be partially neutralized by absorption-promotive effects of particular cyclodextrins [8].

This research builds up on the work of Radić and co-authors [8], and its main focus is to investigate the impact of particular foods on gastrointestinal bioaccessibility and permeability of HTS and TS from OPE. As mentioned before, functional extracts, rich in bioactive health promoting compounds, such as OPE, are nowadays being extensively used as nutraceutical ingredients of dietary supplements and functional foods. It is therefore of great importance to understand the nature and significance of polyphenol–food interactions in order to provide efficient and accurate dosing instructions and

enable targeted formulation of dietary supplements or excipient foods that will result in optimized bioavailability of those health-promoting compounds.

2. Materials and Methods

2.1. Chemical and Reagents

Petrol ether, dimethyl sulfoxide (DMSO) ethanol, methanol ($\geq 99.9\%$), sodium acetate, Folin-Ciocalteu reagent, Dulbecco's Phosphate Buffered Saline (PBS liquid, sterile-filtered, without calcium, without magnesium, suitable for cell culture), tert-butyl hydroperoxide (tBOOH), 3-hydroxytyrosol (HTS) and tyrosol (TS) ($\geq 98\%$), bile salts, thermostable α -amylase (A3306), pancreatin from porcine pancreas ($4 \times$ USP), Dulbecco's Modified Eagle's Medium, D-glucose, ethylenediaminetetraacetic acid (EDTA), and sodium caseinate were from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile ($\geq 99.9\%$) was from Honeywell (Charlotte, NC, USA). Acetic acid and Na_2CO_3 were from Kemika (Zagreb, Croatia). Pepsin (from porcine gastric mucosa) 0.7 FIP-U/mg was from Merck (Darmstadt, Germany). Heat inactivated fetal bovine serum (FBS), nonessential amino acids (NEAA), penicillin/streptomycin/amphotericin B (A/A), and trypsin were from Capricorn Scientific (Ebsdorfergrund, Germany). The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Panreac AppliChem (Darmstadt, Germany). Caco-2 cells were from American Type Culture Collection (ATCC, Manassas, VA, SAD). Hydroxypropyl β cyclodextrin (HPB) was purchased from Wacker–Chemie GmbH (Burghausen, Germany), Cellulose Alba Fiber[®]C200 from Mikro-Technik (Bürgstadt, Germany), and Inulin Orafit[®]HSI from Beneo (Mannheim, Germany). Pectin was from Foodchem (Zhangjiang, China). Ultrapure water (18 M Ω) was obtained from SG Reinstwassersystem Ultra Clear UV Plus coupled with SG Wasservollentsalzer-Patrone SG 2800 (Günzburg, Germany). Sunflower oil and full-fat milk and food matrices were obtained from local suppliers, unless noted otherwise. All other chemicals were from Kemika (Zagreb, Croatia).

The exact composition of simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) is presented in Table S1. Fed state simulated gastric fluid (FeSSGF) contained sodium chloride (237 mM), acetic acid (17.1 mM), and sodium acetate (29.8 mM) and was mixed with full fat milk in 1:1 ratio. The pH was adjusted to 3 by using hydrochloric acid. Fed state simulated intestinal fluid (FeSSIF) contained CaCl_2 (1.67 mM), MgSO_4 (0.81 mM), KCl (5.37 mM), KH_2PO_4 (0.44 mM), NaHCO_3 (0.42 mM), NaCl (137 mM), Na_2HPO_4 (0.34 mM), D-glucose (5.55 mM), L-glutamine (2 mM), lecithin (7.5 mM), sodium taurocholate (1 mM) and MES (1 M) to adjust pH to 6. Standardized food model (SFM) was prepared according to the procedure of Zhang and co-workers [19] with slight modifications (Table S2). Briefly, sodium caseinate (1%, *w/w*) was dissolved in phosphate buffer solution (10 mM, pH 7) and then filtered to remove any residual insoluble matter. Sunflower oil was gradually added (7.6%, *w/w*) and vortexed for 5 min. Powdered sodium caseinate was gradually added into the emulsion to reach a final concentration of 7.7% *w/w* protein and continuously stirred for 30 min. An aqueous pectin (1.6% *w/w*) solution was obtained by dispersing pectin powder into distilled water and then mixing at 50 °C for 60 min. The mixture was then cooled to room temperature and stirred continuously to fully dissolve the pectin. Then 44.8 g of the prepared pectin solution was mixed with 44.8 g of emulsion (1:1, *w/w*). At the end, 5.15 g of corn starch was slowly poured into the previous mixture with continuous stirring until it was fully dissolved (around 30 min). Pepsin solution was prepared by dissolving 50 mg of pepsin in 1 ml of SGF (25 000 U/mL). Pancreatin solution was prepared by dissolving 8 mg of pancreatin in 1 mL of SIF (800 U/mL). Bile salt solution was prepared by dispersing 65.37 mg of bile salt in 1 mL of water. The α -amylase solution was prepared by dissolving thermostable α -amylase in SSF (1500 U/mL). Hank's balanced salt solution (HBSS) pH 6.0 was prepared by dissolving KCl (0.4 mg/mL), NaHCO_3 (0.35 mg/mL), NaCl (8.0 mg/mL), D-glucose monohydrate (1.1 mg/mL), KH_2PO_4 (0.06 mg/mL), $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ (0.06 mg/mL), $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ (0.185 mg/mL), $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ (0.1 mg/mL), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (0.1 mg/mL), and HEPES (7.15 mg/mL) in ultrapure water. All of the solvents needed for chromatographic separation were degassed with Branson 1210 Ultrasonic

Cleaner (Danbury, CT, USA) before analysis. Acetate buffer was prepared by mixing sodium acetate 0.1 M:acetic acid 0.1 M (2:1 *v/v*) and adjusting the pH to 5 with pH meter (702 SM Titrino, Metrohm, Herisau, Switzerland).

2.2. Preparation of Samples

Olive pomace extract (OPE) was prepared according to previously published procedure [8] with some modifications. Briefly, olive pomace was dried at 60 °C for 24 h in an incubator (INKO, Zagreb, Croatia), sieved through Φ 0.8 mm sieve (Prüfsieb DIN 4188, Kassel, Germany), and defatted with petrol ether using the Soxhlet apparatus (INKO SK6ESS, Zagreb, Croatia). Pre-treated olive pomace was mixed with 20% ethanol (20 g/L) and the extraction was performed by 2 h maceration in a shaking water bath at 70 °C and 100 rpm (Thermostat, Inko, Zagreb, Croatia). The mixture was filtered to remove the crude parts, ethanol was removed from the filtrate under reduced pressure (Rotavapor R-220 EX, BÜCHI Labortechnik AG, Flawil, Switzerland) and obtained water extracts were dried for 48 h in a lyophilizator (Alpha 1-4 LOC-1, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Dry OPE was used for the simulation of gastrointestinal digestion. Chemical composition of OPE is presented in Table S3. Olive oil (OO) was obtained from the local supplier. Prior to simulation of gastrointestinal digestion OO was diluted with water in 1:10 ratio and vortexed thoroughly (VTY-3000L, Mixer UZUSIO, Tokyo, Japan). For the investigation of polyphenol-food interactions the following materials were used: dietary fiber (cellulose, pectin, inulin), tuna (canned), sour cream (12% milk fat), milk (fresh, 3.2% milk fat), yogurt (2.8% milk fat), milk formula, banana (raw minced), breakfast cereals, soy flakes, fresh cheese, meat sauce, apple (grated, skinless), silver beat (blanched, minced), bread, potato (boiled), honey, and corn starch. Basic nutritive composition was obtained from producers' nutrition data labels or from nutrition data tables [20] and are presented in supplementary materials (Table S4).

2.3. Determination of Total Phenols

Total polyphenols (total reductive capacity) of analyzed samples was assessed by Folin–Ciocalteu method that relies on the transfer of electrons in alkaline medium from phenolic (or other reductive) compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined spectroscopically at 765 nm. Experiments were conducted according to the protocol described by Ainsworth and co-workers [21]. Moreover, 20 μ L of adequately diluted sample/standard/solvent (blank) were added in triplicate to 96-well plate (Thermo Fisher Scientific 130188, Rochester, NY, USA) and incubated at 37 °C with 50 μ L of Folin Ciocalteu reagent (10% (*v/v*)) for 5 min. The 160 μ L of Na₂CO₃ (700 mM) was added into each well, shaken, and incubated at 37 °C for 30 min. Absorbance was read at 765 nm and results were expressed as gallic acid equivalents (GAE).

2.4. HPLC-FLD Determination of Hydroxytyrosol and Tyrosol

HTS and TS were identified and quantified by HPLC system (Waters Alliance 2695, Milford, MA, USA) coupled with a 2475 Multi λ detector (FLD) with Xenon lamp, according to slightly modified method of Tzarbopoulos and co-workers [22]. Samples were prepared by filtration through 0.45 μ m polyethersulfone (PES) syringe filters (Macherey–Nagel, Düren, Germany). Chromatographic separation was conducted by injecting 20 μ L of sample on a reversed phase column (250 \times 4.6 mm, 5 μ m) (Agilent Zorbax Eclipse Plus C18, Santa Clara, CA, USA). Mobile phases were 0.05 M sodium acetate buffer pH 5 and acetonitrile with the flow rate of 1 mL/min. Elution was conducted over 25 min at 25 °C. Identification was performed with FLD set at the excitation wavelength of 280 nm, and emission wavelength of 316 nm. Polyphenols were identified by comparing the retention times of the eluting peaks with those of the standards. Peaks were quantified by using the Empower2 software (Waters, Milford, MA, USA) and compared to external standard calibration. Standard stock solutions were prepared by dissolving reference compounds in DMSO.

2.5. Simulation of Gastrointestinal Digestion

In vitro simulation of gastrointestinal digestion and the assessment of bioaccessibility of hydroxytyrosol and tyrosol from OPE was conducted by standardized static in vitro digestion method suitable for food [23]. Three-phase digestion process was applied consisting of short salivary phase, simulation of gastric digestion and simulation of duodenal digestion. Briefly, 200 mg of OPE was used alone or mixed with 600 mg of food matrix (Table S4) in 50 mL Falcon tube. The 875 μ L of SSF, 125 μ L of salivary α amylase solution, 6.25 μ L of CaCl_2 (0.3 mol/L), and 243.8 μ L of water was added, and the mixture incubated at 37 °C for 2 min with constant shaking. The 2.5 mL of reaction mixture were transferred to another Falcon tube and mixed with 1.875 mL of SGF, 1.25 μ L of CaCl_2 (0.3 mol/L), and 50 μ L of HCl (1 mol/L). The pH of the mixture was adjusted to 3, and total volume was adjusted to 5 mL with water. The content of the Falcon tube was vortexed and incubated for 2 h at 37 °C with constant shaking. Every 15 min pH of the mixture was checked and adjusted to 3 if necessary. At the end of simulation of the gastric digestion step, 5 mL of aliquot were transferred to another 50 mL Falcon tube for the simulation of intestinal digestion. The 2.75 mL of SIF, 1.25 mL of pancreatin solution, 0.625 mL of bile salt (160 mmol/L), 10 μ L of CaCl_2 (0.3 mol/L), and 37.5 μ L of NaOH (1 mol/L) were added. The pH was checked and adjusted to 7. Total volume of reaction mixture was adjusted to 10 with water, mixtures were vortexed and incubated for 2 h at 37 °C with constant shaking. Every 15 min, pH of the mixture was checked and adjusted to 7 if necessary. After digestion, simulation samples were cooled at -20 °C for 10 min and centrifuged (Heraeus Biofuge Stratos, Hanau, Germany) for 20 min at 4 °C and 4100 rpm. For HPLC analysis of hydroxytyrosol and tyrosol, obtained supernatants were additionally filtered through 0.45 μ m polyether sulfone (PES) syringe filters. Supernatants were collected and used for the assessment of bioaccessible HTS and TS fractions. Aliquots of samples to be used on cell monolayers were kept at 100 °C for 5 min in Thermomixer R (Eppendorf, Hamburg, Germany) for enzyme inactivation. For each sample blank was prepared, by omitting the addition of OPE to reaction mixture. Simulations of gastrointestinal digestion were conducted in duplicates. For fed state simulation using biorelevant media FeSSGF and FeSSIF were used instead of SGF and SIF and gastrointestinal digestion of OPE was simulated according to the procedure described above. For simulation of food effects on bioaccessibility of polyphenols using SFM, SFM was mixed with SSF in 1:1 ratio in the beginning of the experiment and gastrointestinal digestion of OPE was simulated according to the procedure described above. Total amount of polyphenols/HTS/TS in OPE was determined by dissolving dry OPE in distilled water. Bioaccessibility of polyphenols was calculated according to Equation (1):

$$\text{Bioaccessibility (\%)} = \frac{\text{bioaccessible amount}}{\text{total amount}} \times 100 \quad (1)$$

Impact of food on bioaccessibility of investigated compounds was expressed as relative bioaccessibility, that compares the bioaccessibility after digestion with food with bioaccessibility from OPE digested without the presence of food using SSF/SGF/SIF, and it was calculated according to Equation (2):

$$\text{Relative bioaccessibility (\%)} = \frac{\text{bioaccessibility with food (\%)}}{\text{bioaccessibility without food (\%)}} \times 100 \quad (2)$$

2.6. Transepithelial Transport of HTS and TS in Caco-2 Monolayers

For investigation of transepithelial transport human epithelial colorectal adenocarcinoma cell line (Caco-2) was used. Caco-2 cells (ATCC) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% NEAA, and 1% A/A. Cell cultures were maintained at 37 °C, in a humidity saturated atmosphere consisted of 5% CO_2 (Sanyo MCO 20AIC CO_2 Incubator, Osaka, Japan). Medium was changed every 2 days. Cells were passaged at 80–90% confluence. The study of transepithelial transport was conducted by using 40 μ g/mL of TS and HTS diluted in HBSS (MIX) in

the presence of one of the following substances: 10 mM glucose (GLU); 0.25% nonessential amino acids (NEAA); 0.125% nonessential amino acids (NEAA2x); 2.4 mg/mL hydroxypropyl β cyclodextrin (HPB); 1% cellulose (alba fiber); 1% inulin (inulin). The highest non-toxic concentration of samples was determined by MTT assay [24]. The 3×10^5 cells/well were seeded in 96-well plates (Thermo Fisher Scientific 130188, Rochester, NY, USA) and grown until reaching confluence (approximately 48 h). The medium was aspirated, and the cells washed with 100 μ L PBS/well. Cells were incubated for 4 h with either 100 μ L of samples or Hank's balanced salt solution (HBSS) for positive control or 350 μ M tBOOH for negative control. Samples were then removed, and cells were washed with 100 μ L PBS/well. Cell viability was assessed by the addition of 40 μ L of MTT 0.5 mg/mL (diluted in PBS) and incubation for 3 h at 37 $^{\circ}$ C, followed by dissolution of the formazan crystals in 170 μ L of DMSO. Absorbance (A) was measured at 490 nm and cell viability was expressed as percentage (%) relative to the positive control according to Equation (3). Blank absorbance was measured in wells containing MTT without cells.

$$\% \text{ viability} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \quad (3)$$

Transepithelial transport was investigated in 12-well plate with Transwell[®] permeable supports (Costar 3401, Corning Incorporated, Kennebunk, ME, USA) [25]. The 3×10^5 cells were seeded per well and maintained at 37 $^{\circ}$ C, in a humidity saturated atmosphere consisted of 5% CO₂. Cells were grown for 21 days in order to form a differentiated monolayer on filters. The medium was warily aspirated (Gilson Safe Aspiration Station, Middleton, WI, USA) and replaced with fresh one every 2 days. The added volume was 0.5 mL in the apical and 1.5 mL in the basolateral compartment. Monolayer integrity was routinely checked by determining transepithelial electrical resistance (TEER) during the cell growth, before and after the transport experiment. Electrical resistance (ER) was measured with STX2 and Epithelial Volt/Ohm Meter (EVOM) (World Precision Instruments Inc., Sarasota, FL, USA). TEER, defined as ER per area, was calculated according to Equation (4).

$$\text{TEER} = (R - 120 \Omega) \times 1.12 \text{ cm}^2 \quad (4)$$

where TEER is transepithelial electrical resistance, 120 Ω is resistance of the cell free-well (blank), 1.12 cm² is the filter surface.

To evaluate transepithelial permeability, medium was aspirated from both apical and basolateral chambers, and washed twice with pre-warmed PBS. Then, 0.5 mL of the sample or MIX (TS and HTS (40 μ g/mL)) was added to the apical chamber and 1.5 mL of the HBSS to the basolateral chamber of each well. Samples were incubated at 100 rpm and 37 $^{\circ}$ C for 2 h in a shaker (Biosan Incubator ES-20/60, Riga, Latvia). The initial amount of HTS and TS added to a monolayer and the amount in basolateral compartment after the 2 h incubation of Caco-2 cells with the samples was determined by HPLC-FLD. The transepithelial transport of HTS and TS was expressed as % of the amount applied on cell monolayer according to Equation (5).

$$\% = \frac{\text{amount in basolateral compartment}}{\text{initial amount}} \times 100 \quad (5)$$

2.7. Statistical Analysis

All experiments were run in triplicate unless otherwise stated. Data were statistically tested by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. Results were expressed as average value and standard deviation. $p \leq 0.05$ was considered statistically significant unless otherwise noted. GraphPad[®] Prism Software (San Diego, CA, USA) was used for statistical analysis.

3. Results and Discussion

3.1. Impact of Food-Induced Physiological Changes in Gastrointestinal System on Bioaccessibility of OPE Polyphenols

In order to assess the gastrointestinal bioaccessibility of particular compound by an in vitro approach it is essential to mimic the conditions in the gastrointestinal tract as closely as possible. There are several types of in vitro digestion methods that are commonly used for food, but static models, which use a constant ratio of food to enzymes and electrolytes, and a constant pH for each digestive phase, are used most often. However, the experimental conditions in static digestion simulation protocols differ significantly, depending on the purpose of the study and type of analyte/food matrix analyzed. This results with the large number of digestion protocols, which differ considering pH, duration of digestion phases, enzyme concentration and activity, and composition of simulated digestive fluids [26], resulting in questionable relevance of obtained data. Therefore, recently, international consensus was reached in terms of determining optimal conditions that should be applied for in vitro digestion simulation in static models. Such optimized and standardized procedure was applied in this investigation [23].

Results presented in Figure 1 show that bioaccessibility of total phenols, HTS and TS is slightly but significantly increased during gastrointestinal digestion, due to liberation of polyphenolic compounds from complex food matrix (281.0 to 357.5 mg/100g; 78.4 to 109.3 mg/100g; and 20.7 to 27.4 mg/100g, respectively). This is consistent with available literature data stating that during the gastric phase of digestion the majority of polyphenols is released from food matrix where they originally formed covalent bonds with carbohydrates of the cell wall, proteins, or one with another [10]. Our previous investigation that focused on the impact of cyclodextrin encapsulation on bioaccessibility of HTS and TS also showed that both, HTS and TS are stable in the gastrointestinal tract, and that their concentrations remain the same or increase during gastrointestinal digestion [8].

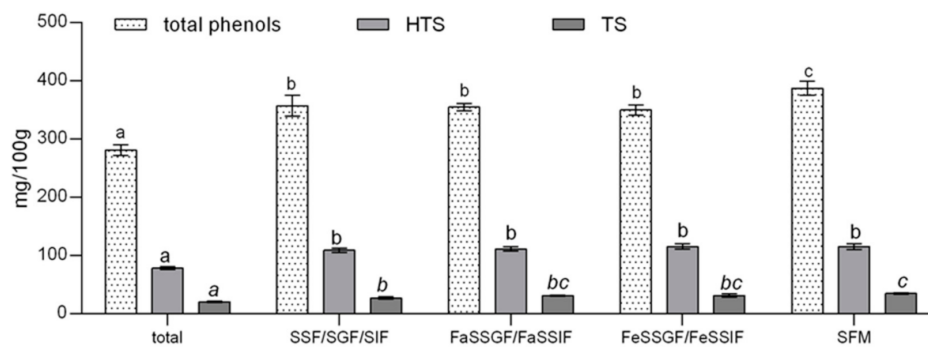


Figure 1. Impact of fed-state on gastrointestinal bioaccessibility of total polyphenols, hydroxytyrosol, and tyrosol from olive pomace extract. SSF—simulated salivary fluid; SGF—simulated gastric fluid; SIF—simulated intestinal fluid; FaSSGF—fasted state simulated gastric fluid; FaSSIF—fasted state simulated intestinal fluid; FeSSGF—fed state simulated gastric fluid; FeSSIF—fed state simulated intestinal fluid; SFM—standardized food matrix; HTS—hydroxytyrosol; TS—tyrosol. Data belonging to the same group are marked with the same letter belong to the same statistical group ($p \leq 0.05$).

As mentioned before, the effect of food on bioaccessibility of particular compounds can be assessed by several methodological approaches, including biorelevant dissolution testing. Food effects on drug absorption are generally better predicted when using biorelevant media containing bile salts and lecithin as compared to the traditional media (SGF and SIF). Namely, the presence of food in gastrointestinal tract changes the conditions in the stomach and intestines, in terms of pH, osmolarity, and composition of digestion fluids, which can significantly influence the pharmacokinetic properties and bioaccessibility of particular compounds [27]. Mentioned differences of the fed/fasted gastric/intestinal state, can be mimicked by fed–fasted state biorelevant media with sufficient accuracy,

and although usually used in biorelevant dissolution testing of drugs, they can be applied in the field of nutraceutical formulation and testing. Food effects on bioaccessibility of OPE polyphenols, HTS and TS can be assessed by comparing the fasted state simulated gastric fluid (FaSSGF)/ fasted state simulated intestinal fluid (FaSSIF) and FeSSGF/FaSSIF data presented in Figure 1. Presented data clearly show that changes of pH and digestion fluid composition induced by presence of food in gastrointestinal tract do not influence the bioaccessibility of OPE polyphenols, HTS and TS. Moreover, there were no differences between results obtained by simulating digestion with SSF/SGF/SIF and biorelevant fasted state media. Considering the physicochemical characteristics of hydroxytyrosol and tyrosol, obtained results were partially expected. Namely, significant food effect can be anticipated based on both solubility and permeability of particular compound as described by the Biopharmaceutics Classification System (BCS) [28,29]. Taking into account high water solubility of HTS and TS (17.4 g/L and 25.3 g/L, respectively) and low apparent permeability assessed by predicted logP values (0.85–1.19 and 0.13–0.89, respectively) HTS and TS could be provisionally classified into class 3, according to BCS classification. BCS Class III (high solubility, low permeability) drugs tend to have negative food effects, but they can only be observed by investigating the interactions of food/food compounds with drug absorption and not biorelevant media bioaccessibility testing [30]. As mentioned before, introduction of SFM can be considered as a step forward in *in vitro* investigation of food effects on bioaccessibility of bioactive compounds because it takes into account wider spectra of possible bioactive compound–food matrix interactions that might affect pharmacokinetic properties of the particular compound [19]. In comparison to other applied methods, simulation of gastrointestinal digestion with SFM significantly increased bioaccessibility of OPE polyphenols in general but had no effect on HTS and TS bioaccessible fractions. This was partially expected, given the high water solubility and previously established gastrointestinal stability of HTS and TS [8]. Namely, positive food effects are usually associated with nonpolar polyphenols because the presence of fat in the food matrix enhances the formation of micelles during the intestinal digestion phase and increases polyphenol content in the soluble (bioaccessible) digest fraction [10]. Conversely to our results, absorption of tyrosol and hydroxytyrosol was positively influenced by the lipid-rich matrix (olive oil), resulting in 25% greater absorption compared to aqueous solution in the rat model [31]. However, the effect could have been achieved, not due to the lipid matrix, but the formation of HTS during gastrointestinal digestion (due to cleavage of more complex antioxidants (such as oleuropein)) and the protection of HTS and TS by other antioxidants present in the olive oil. Positive effects of food matrix on polyphenol bioavailability can also be attributed to the presence of fermentable fiber (such as pectin) that increase colonic bioavailability of particular polyphenols due to their prebiotic properties. These effects could not be monitored in our study. Therefore, observed slight, but statistically significant positive effects of SFM on phenolic bioaccessibility can likely be attributed to the impact of fat on micellization of nonpolar polyphenols present in OPE.

3.2. Gastrointestinal Interactions of HTS and TS with Particular Food

Bioavailability of olive polyphenols from food matrices other than olive oil has rarely been studied. Radić and co-workers [8] investigated the influence of olive pomace matrix and cyclodextrin encapsulation on bioavailability of olive polyphenols, and showed high bioaccessibility, but relatively low permeability, of hydroxytyrosol and tyrosol, which were both negatively affected by olive pomace matrix. Their results confirmed the previous findings of Malapert and co-workers [32], who showed that bioavailability of hydroxytyrosol was higher when it was applied as a pure compound than from Alperujo powder. They also showed that presence of foods (investigated by co-digestion with the test meal) significantly decreased hydroxytyrosol bioaccessibility and intestinal permeability (–20% and –10%, respectively). They supposed that negative food effects arise from the fact that polyphenols form complex bonds with particular food components, and that the investigation of the influence of the food matrix on the bioaccessibility of dietary plant phenols, such as HT, requires including real meal components into *in vitro* digestion studies [32]. This is consistent with our results (Figure 1),

where the comparison of results obtained in fed/fasted biorelevant media showed that changes in pH, ionic strength, or the amount of bile during gastrointestinal digestion are not determining factors for HTS and TS bioaccessibility. However, data presented in Table S5 and Figure 2 clearly show that both bioaccessibility and relative bioaccessibility of HTS and TS can be significantly changed during co-digestion with different foods, and that the extent of observed changes depends on chemical/nutritive composition of food matrices (Table S4). Depending on the food matrix investigated, bioaccessibility of OPE polyphenols was either not affected or it was significantly reduced. The most significant impact was observed after co-digestion of OPE with soy flakes, fresh low-fat cheese, and milk formula (relative bioaccessibility of TP was 51.8%, 60.6%, and 71.9% respectively), foods with relatively high protein content (52%, 12.4%, 10.5%) Bioaccessibility of HTS and TS was also significantly reduced by some of the investigated high-protein foods: soy flakes, sauce Bolognese, breakfast cereals, and whole-grain bread, but the effect was less pronounced than in the case of TP. Observed effects were, at least partially influenced, by polyphenol–protein interactions, well described in scientific literature and reviewed recently in the work of Jakobek [14]. Polyphenols (especially polyhydroxy polyphenols) form non-covalent hydrophobic interactions with proteins, which may subsequently be stabilized by hydrogen bonding [33], and can, among other effects, change the bioavailability of polyphenols. Different authors observed negative effects of milk proteins on bioavailability of polyphenols of different origin [34–38]. The majority of observed negative interactions in literature were observed for casein, which is consistent with our results showing the stronger negative effects of foods with casein content in comparison to other high-protein foods (although total protein content was relatively low). To our knowledge, impact of proteins on bioaccessibility of hydroxytyrosol and tyrosol has not been previously investigated, but it has been shown that hydroxytyrosol can form adducts with food proteins [39]. Obtained results suggest the negative interactions between proteins and polyphenols, but additional effects of matrix components, such as lipids or carbohydrates, must also be considered. For example, despite high protein content, canned tuna or milk formula did not negatively influence bioaccessibility of hydroxytyrosol, maybe partially due to high fat content (15.5 and 27.4%, respectively). Namely, although investigations suggest that interactions between lipids and polyphenols have only a small influence on the polyphenol accessibility for absorption [14], some studies show that when lipids interact with polyphenols, they can “capture” polyphenols and protect them from degradation or forming insoluble complexes in their passage through the gastrointestinal tract [40], or increase their solubility and activity in gastrointestinal tract by forming micelles and promoting emulsification [41]. In our study, such effects were not particularly pronounced, since OPE polyphenols have been proven stable during gastrointestinal digestion and are generally soluble in water [8].

Interactions between polyphenols and available carbohydrates have been described in scientific literature because polyphenols can reduce starch digestion, possibly via amylase inhibition, and inhibit glucose uptake transporters [42]. Far fewer studies have examined the impact of carbohydrates on polyphenol bioavailability and were mostly focused on polyphenol uptake. In our investigation, corn starch and honey (100% and 80% of available carbohydrates) were selected as model foods for investigation of available carbohydrate impact on olive polyphenols bioaccessibility. Relative bioaccessibility of TF, HTS, and TS after co-digestion with corn starch/honey was 94.5/113.6%; 94.9/89.5%, and 88.7/97.8%, and observed differences were not statistically significant (Figure 2).

While the negative impacts of indigestible carbohydrates (dietary fiber) on digestibility of fat and bioaccessibility of some minerals and trace elements is well known, their influence on the bioaccessibility of polyphenolic compounds has not been thoroughly investigated. The main direct mechanisms of negative impacts of dietary fiber on polyphenol intestinal availability are an incomplete release of polyphenols from fiber rich fruit and vegetable matrices (i); presence of polyphenols bound to polysaccharides requiring enzymatic hydrolysis to be absorbed (ii); and entrapment of polyphenols by dietary fiber during digestion in the upper intestine (iii) [43]. The possibility of the formation of insoluble polyphenol–dietary fiber complexes during gastrointestinal digestion is particularly important for

this investigation since it focused on the bioaccessibility of polyphenols previously isolated from its natural complex matrix. As shown in Figure 2, bioaccessibility of TP was not significantly reduced in the presence dietary fiber containing food (Table S4) except for soy flakes that contained high content of both proteins and fiber (52% and 16%, respectively). On the other hand, a negative impact of fiber was more noticeable in the case of TS and HTS. Bioaccessibility of TS was significantly reduced in the presence of breakfast cereals, wholegrain bread, apple and silver beat, ranging from 67% to 89% in relation to bioaccessibility of OPE consumed with no food. The negative effect of dietary fiber was even more pronounced in the case of HTS. Its relative bioaccessibility was significantly reduced in the presence of all fiber containing foods and ranged from 54.8% up to 84.2%. Pronounced negative effects on hydroxytyrosol bioaccessibility might be explained by the additional hydroxyl group in HTS structure since dietary fiber–polyphenol interactions were observed to appear through non-covalent bonds as electrostatic forces and hydrogen bonds as Van der Waals forces between hydroxyl groups of phenolic compounds and various components of dietary fibers [44]. The negative impact on HTS/TS bioaccessibility was not proportional to the content of dietary fiber in the food matrix probably because it is the consequence of complex interactions of all matrix components and also the chemical composition of the fiber. In order to investigate the gastrointestinal polyphenol–dietary fiber interactions more thoroughly, we additionally simulated gastrointestinal digestion of OPE in the presence of cellulose, pectin and inulin and assessed the impact on the relative bioaccessibility of OPE antioxidants (Figure 3). Presented data show that the impact of dietary fiber on polyphenol bioaccessibility depends on both types of dietary fiber and chemical characteristics of particular polyphenols. All investigated fiber significantly reduced relative bioaccessibility of TP from OPE (69.8–79%), and cellulose showed the strongest negative impact. Relative bioaccessibility of HTS was reduced to 88.1% by cellulose and to 93.2% by pectin and was not influenced by the presence of inulin in the reaction mixture. TS relative bioaccessibility was reduced to 87.4% by cellulose and was not affected by the presence of other dietary fiber. The comparison of fiber-in-food-matrix–polyphenol interactions to pure fiber–polyphenol interactions (Figures 2 and 3, respectively) indicate the significant effect of the food matrix on the type and extent of polyphenol–dietary fiber interactions. Therefore, data on the effect of fiber-rich food on polyphenol bioaccessibility cannot be extrapolated from the data obtained by *in vitro* digestion of polyphenols with pure dietary fiber. Therefore, in order to guide the design of functional foods enriched with phenolic substances, we should significantly increase the number of *in vitro* and *in vivo* studies on the bioavailability of particular phenolic substances in contact with particular foods. This is consistent with the conclusions of Pinarli and co-workers [45], stating that, due to complexity of interactions within the food matrix, every food material and component should be evaluated individually with respect to its interacting phenolic compound. Although HTS/TS–dietary fiber interactions have hardly been investigated, our observations are consistent with the recent findings of Bermúdez-Oria and co-authors [46], who confirmed the formation of predominant hydrogen–bonding interactions between HTS and strawberry dietary fiber. Tomas and co-authors [47] noticed modulation of polyphenols profile of blackberry purees by soluble dietary fiber (inulin or pectin), during a simulated *in vitro* gastrointestinal digestion and large intestine fermentation process, and explained it by the interactions of dietary fiber and polyphenols.

3.3. Impact of Food on Intestinal Permeability of TP, HTS, and TS

The influence of MIX and investigated food components on the viability of human epithelial colorectal adenocarcinoma cells Caco-2 was determined by using the MTT assay (Figure 4a) and by measuring TEER values, before and after the experiment (Figure 4b), as previously described. The results presented in Figure 4 show that cell viability was significantly decreased when cells were treated with the MIX and HPB, which is consistent with previously obtained data [48]. Although significantly decreased in relation to positive control (CTR+), viability of monolayers treated with MIX and HPB was above 80% (81.4 and 80.3%, respectively) and, therefore, suitable for further analysis. Moreover, it is evident from Figure 4b that, even though tested food matrices do not produce

significant cytotoxic effects, they can significantly affect the intestinal barrier, and may increase bilayer permeability or promote barrier integrity. Those effects are achieved through different mechanisms: affecting mucus permeation, paracellular permeation, bilayer permeability, or active transport and efflux transport [12,49]. As shown previously, food matrix can significantly affect intestinal permeability of polyphenols through formation of soluble or insoluble complexes, resulting with positive or negative effects on bioaccessibility. Therefore, the actual intestinal permeability of a particular compound is the result of all of the above-mentioned interactions and, because of that, it is very hard to predict.

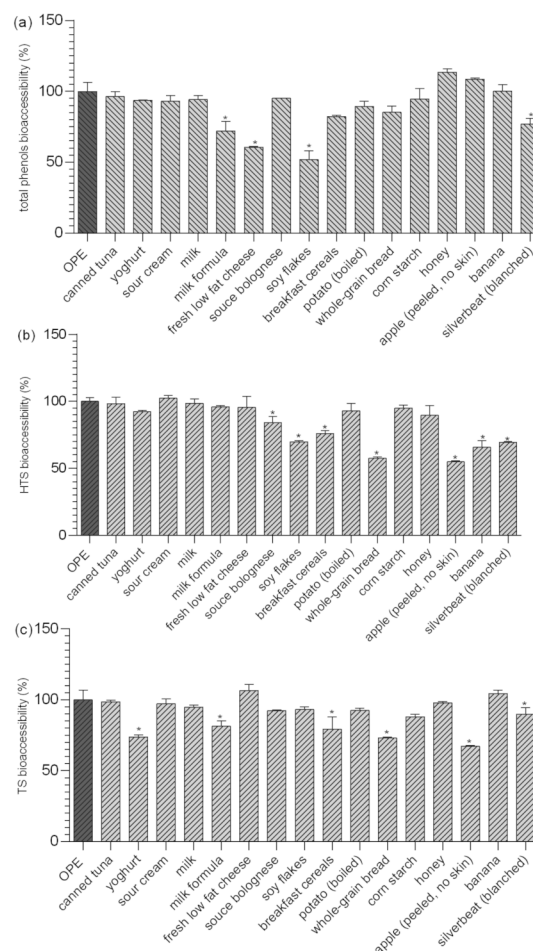


Figure 2. Impact of different foods on relative bioaccessibility* of total polyphenols (a), HTS (b) and TS (c) from OPE. * Relative bioaccessibility was calculated in relation to in vitro bioaccessibility of total phenols, HTS, and TS from OPE obtained after simulation of gastrointestinal digestion using FaSSIF/FaSSGF. Columns marked with * differ significantly from OPE ($p \leq 0.05$). OPE—olive pomace extract; FaSSGF—fasted state simulated gastric fluid; FaSSIF—fasted state simulated intestinal fluid; HTS—hydroxytyrosol; TS—tyrosol.

At the moment, available data on the impact of food matrix/nutrients on intestinal permeability of polyphenols are scarce. Recently, Nogueira Mendes and co-authors [50] showed that intestinal permeability of guarana catechins and procyanidins was not significantly affected by macronutrients (casein, starch and oil). On the other hand, transepithelial transport of indicaxanthin and betanin was negatively affected by particular food matrix components while intestinal transport of green tea catechins was positively affected by milk proteins, probably due to impact of milk on tight junction permeability [51,52]. The mentioned results point out the significance of chemical characteristics of targeted molecules and the exact composition of the food matrix in predicting food matrix effects, as well as the necessity of focused and tailored research.

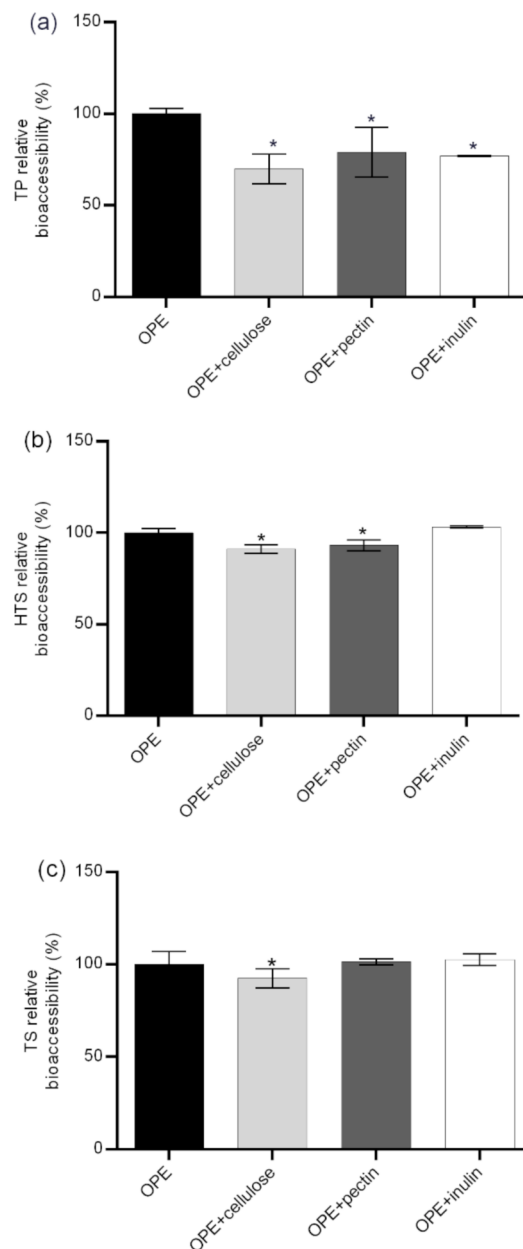


Figure 3. Impact of cellulose, pectin and inulin on relative bioaccessibility* of TP, HTS, and TS from OPE. * Relative bioaccessibility was calculated in relation to in vitro bioaccessibility of TP, HTS, and TS from OPE obtained after simulation of gastrointestinal digestion using FaSSIF/FeSSIF. Columns marked with * differ significantly from OPE ($p \leq 0.05$). OPE—olive pomace extract; FaSSGF—fasted state simulated gastric fluid; FaSSIF—fasted state simulated intestinal fluid; TP—total phenols; HTS—hydroxytyrosol; TS—tyrosol.

In order to investigate the impact of glucose, amino acids, dietary fiber (inulin and apple fiber), and HPB (excipient often used in nutraceutical production) on transepithelial permeability of HTS and TS, they were applied to Caco-2 monolayers in combination, and their permeability was compared to permeability of HTS and TS from pure mixture (MIX). As presented in Figure 5, permeability of HTS and TS was mostly unaffected by tested compounds, with few exceptions. First, glucose significantly improved permeability of both HTS and TS. The mechanisms of such interaction are unknown and beyond the scope of this investigation, but certain assumptions can be made based on available literature data. DeSouza and co-authors [53] investigated Caco-2 cell permeability was evaluated in isotonic media containing high (25 mM) or physiological (5.5 mM) glucose concentrations, and concluded

that high extracellular glucose concentration in isotonic media significantly alters physical barrier properties of Caco-2 cell monolayers, which predominantly affects transepithelial transport of solutes permeating the cell barrier by paracellular and transcellular passive diffusion through decreasing TEER of cell monolayers, increasing membrane fluidity. Their results are in consistence with data presented in Figure 4B showing that glucose significantly decreased TERR of Caco-2cell monolayers (20%).

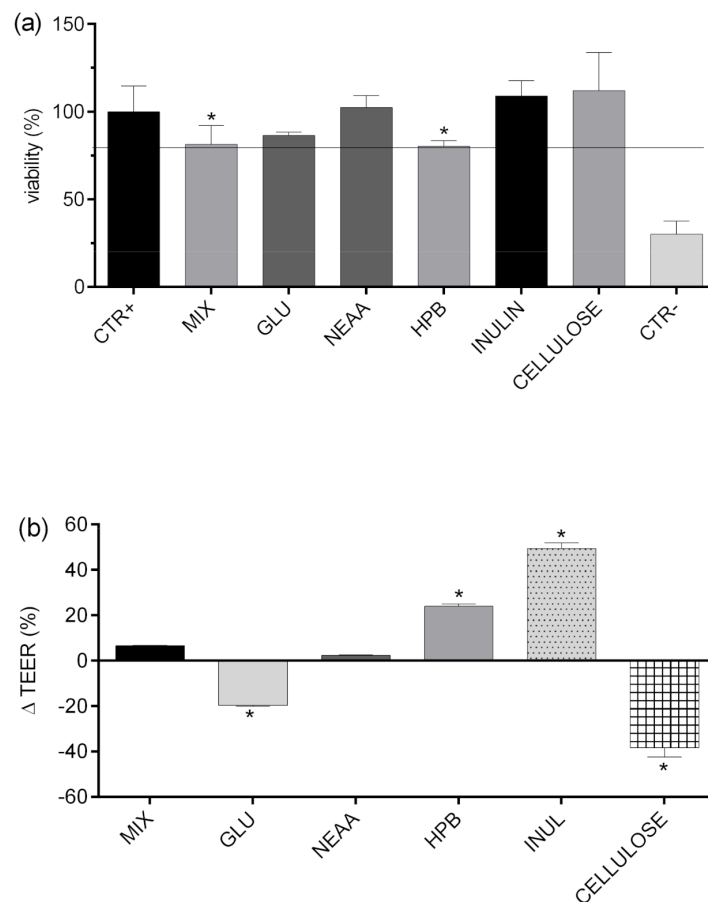


Figure 4. Viability (%) of Caco-2 cell monolayer determined by MTT test after exposure to analyzed food fractions (a). Relative change (%) of TEER values during the experiment (b). positive control (CTR+)—HBSS; MIX—TS + HTS (40 mg/L); EDTA (10 μM); GLU—glucose (10 mM); NEAA—non-essential amino acids (0.25%); HPB—hydroxypropyl beta-cyclodextrin (2.4 g/L); CELLULOSE—cellulose (1%); INULIN—inulin (1%); CTR—tBOOH (350 μM); TEER—transepithelial electrical resistance. Columns marked with * differ significantly from the reference column: (CTR+ (a); MIX (b)).

The TEER of cell monolayers was also negatively affected by cellulose (40%, but it did not result in increased permeability of HTS/TS. This is probably due to formation of insoluble complexes with HTS/TS, as shown in Figure 3, where cellulose decreased bioaccessibility of both investigated phenolic compounds. These observations are consistent with data obtained by Bermudez-Oria and co-authors [46], who showed that olive polyphenols form insoluble complexes with dietary fibers. On the other hand, inulin had the opposite effects on HTS and TS intestinal permeability. As shown in Figure 4B, it significantly increased TEER (40%). This observation is consistent with available literature data showing that fructooligosaccharides can improve intestinal barrier function through short fatty acid formation [54,55], but such effects could not have been noticed in our investigation (that did not include the step of microbial fermentation of fermentable fiber). However, our data show that inulin can have additional, direct impacts on Caco-2 cell permeability, and it resulted in a significant reduction of HTS transepithelial permeability.

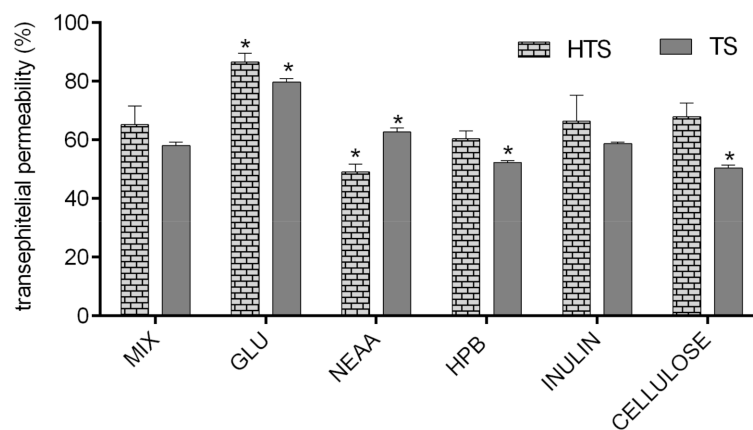


Figure 5. Permeability (%) of HTS and TS in Caco-2 cell monolayer (the amount of TS/HTS in basolateral compartment was expressed as the percentage of the amount applied to Caco-2 cell monolayer). MIX—TS + HTS (40 mg/L); GLU—glucose (10 mM); NEAA—non-essential amino acids (0.25%); HPB—hydroxypropyl beta-cyclodextrin (2.4 g/L); APPLE FIBRE—apple fiber (1%); INULIN (1%). Columns belonging to the same series marked with * differ significantly ($p \leq 0.05$) from the reference column (MIX).

4. Conclusions

Changes in pH, ionic strength, enzyme activity, or the amount of bile due to presence of food in digestive system, are not determining factors for HTS and TS bioaccessibility, but can slightly improve bioaccessibility of other OPE polyphenols. Soy, milk formula, cheese, and breakfast cereals exerted negative effects on HTS and TS bioaccessibility, probably due to negative HTS/TS-casein and HTS/TS-dietary fiber interactions. Particular nutrients significantly influenced the permeability of Caco-2 cell monolayer (without exerting cytotoxic effects); it was increased by glucose and cellulose while inulin improved monolayer barrier function. Those effects significantly affected transepithelial transport of HTS and TS. Our results provide new insights into factors affecting bioaccessibility and transepithelial permeability of HTS and TS and point out the complexity of polyphenol interactions within the food matrix. Therefore, in the process of formulating functional foods enriched with phenolic substances, every food matrix should be evaluated individually, with respect to its interacting phenolic compounds.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2304-8158/9/12/1831/s1>. Table S1. Composition of simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF); Table S2. Composition of standardized food model (SFM); Table S3. Chemical composition of OPE; Table S4. Macronutritive composition of food matrices used for investigations of gastrointestinal interactions with hydroxytyrosol and tyrosol; Table S5. Impact of different foods and dietary fibre on relative bioaccessibility* of HTS, TS and total polyphenols from OPE.

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Supplementary material

Table S1. Composition of simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

	Volume of stock solution needed for preparation of 200 mL of SSF/SGF/SIF		
	SSF	SGF	SIF
	pH 7	pH 3	pH 7
Stock solution	V/mL	V/mL	V/mL
KCl	7.55	3.455	3.4
KH₂PO₄	1.85	0.45	0.4
NaHCO₃	3.4	6.25	21.25
NaCl	-	5.9	4.8
MgCl₂(H₂O)₆	0.25	0.2	0.55
(NH₄)₂CO₃	0.03	0.25	-
Adjustment of pH	V/mL	V/mL	V/mL
1 M NaOH	-	-	-
1 M HCl	0.045	0.65	0.35

Table S2. Composition of standardized food model (SFM)

Macronutrient	Ingredient	g/100g of SFM
Protein	sodium caseinate	3.44
Available carbohydrate	sucrose	4.57
	starch	5.15
Dietary fibre	apple pectin	0.7
Fat	sunflower oil	3.42
Minerals	sodium chloride	0.53

Table S3. Chemical composition of OPE

Chemical analysis	g/100g OPE
Moisture[*]	4.1
Ash[*]	10.2
Protein[*]	22.7
Fat[*]	0.9
Total carbohydrates^{**}	55.6
Total polyphenols^{***}	6.5

**determined by standard AOAC methods;*

determined by difference;determine by Folin-Ciocalteu method and expressed as gallic acid equivalent*

Table S4. Macronutritive composition of food matrices used for investigations of gastrointestinal interactions with hydroxytyrosol and tyrosol

	protein	fat	available carbohydrates	total fibre	soluble fibre	insoluble fibre
	<i>g/100g of eddible part</i>					
canned tuna[*]	21.5	15.5	0.0	0.0	0.0	0.0
yoghurt[*]	4.3	2.8	5.1	0.0	0.0	0.0
sour cream[*]	3.0	12.0	4.4	0.0	0.0	0.0
milk[*]	3.3	3.2	4.6	0.0	0.0	0.0
milk formula[*]	10.5	27.4	62.1	0.0	0.0	0.0
fresh low fat cheese[*]	12.4	1.0	2.7	0.0	0.0	0.0
souce Bolognese[*]	6.2	5.3	7.1	1.8	1.2	0.4
soy flakes[*]	52.0	6.0	4.0	16.0	0.0	16.0
breakfast cereals[*]	12.8	2.3	68.9	11.2	1.1	10.1
potato (boiled)^{**}	0.9	0.2	17.6	2.8	1.6	1.2
whole grain bread[*]	8.0	2.0	46.0	2.7	2.5	0.2
corn starch	0.0	0.0	100.0	0.0	0.0	0.0
honey^{**}	0.4	0.0	80.0	0.0	0.0	0.0
apple (peeled, no skin)^{**}	0.3	0.1	12.6	1.4	1.1	0.3
banana^{**}	1.1	0.3	19.2	2.8	0.7	2.1
silverbeat (blanched)^{**}	1.3	0.1	1.5	1.2	0.3	0.9
cellulose fiber	-	-	-	100	-	100
pectin	-	-	-	100	100	-
inulin	-	-	-	100	100	-

** nutrition data tables of the product provided by the manufacturer/supplier; ** data taken from nutrition data tables (Kaić Rak i Antonić, 1990)*

Table S5. Impact of different foods and dietary fibre on relative bioaccessibility* of HTS, TS and total polyphenols from OPE.

	HTS	TS	total phenols
	relative bioaccessibility (%)		
OPE	100.0 ± 2.86	99.9 ± 6.75	99.9 ± 6.34
canned tuna	98.3 ± 4.92	98.5 ± 1.12	96.4 ± 3.48
yoghurt	92.5 ± 0.82	73.8 ± 1.42	93.7 ± 0.36
sour cream	102.3 ± 2.18	97.4 ± 3.34	93.1 ± 3.89
milk	98.6 ± 3.11	94.6 ± 1.63	94.1 ± 2.76
milk formula	95.9 ± 0.96	81.3 ± 3.95	71.9 ± 7.01
fresh low fat cheese	95.5 ± 8.16	106.4 ± 4.35	60.6 ± 0.63
souce bolognese	84.2 ± 4.61	92.4 ± 0.46	95.1 ± 0.14
soy flakes	69.8 ± 1.06	93.0 ± 1.93	51.8 ± 6.36
breakfast cereals	76.0 ± 2.30	79.3 ± 8.70	82.2 ± 0.95
potato (boiled)	92.8 ± 5.88	92.4 ± 1.64	89.5 ± 3.44
whole-grain bread	57.4 ± 0.97	73.0 ± 0.66	85.2 ± 4.40
corn starch	94.9 ± 2.19	88.1 ± 1.66	94.5 ± 7.45
honey	89.5 ± 7.26	97.8 ± 0.91	113.6 ± 2.30
apple (peeled, no skin)	54.8 ± 0.72	67.2 ± 0.55	108.6 ± 0.87
banana	65.8 ± 5.05	104.5 ± 2.15	100.2 ± 4.48
silverbeat (blanched)	69.3 ± 0.79	89.9 ± 4.59	76.8 ± 3.99
cellulose	108.2 ± 2.24	107.4 ± 7.05	69.8 ± 8.06
pectin	93.2 ± 3.04	101.5 ± 1.64	79.0 ± 13.56
inulin	103.3 ± 0.6	102.6 ± 3.17	76.8 ± 0.24

*relative bioaccessibility was calculated in relation to *in vitro* bioaccessibility of total phenols, HTS and TS from OPE obtained after simulation of gastrointestinal digestion using FaSSIF/FeSSIF. OPE-olive pomace extract; HTS-hydroxytyrosol; TS-tyrosol.

**4. Cellular Antioxidant Activity of Olive
Pomace Extracts: Impact of
Gastrointestinal Digestion and
Cyclodextrin Encapsulation**

Article

Cellular Antioxidant Activity of Olive Pomace Extracts: Impact of Gastrointestinal Digestion and Cyclodextrin Encapsulation

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Abstract: Olive pomace is a valuable secondary raw material rich in polyphenols, left behind after the production of olive oil. The present study investigated the protective effect of a polyphenolic extract from olive pomace (OPE) on cell viability and antioxidant defense of cultured human HepG2 cells submitted to oxidative stress induced by *tert*-butylhydroperoxide (tBOOH). The investigation considered possible matrix effects, impact of gastrointestinal digestion and cyclodextrin (CD) encapsulation. Pre-treatment of cells with OPE prevented cell damage and increased intracellular glutathione but did not affect the activity of glutathione peroxidase and superoxide dismutase. OPE matrix significantly enhanced cell protective effects of major antioxidants, such as hydroxytyrosol (HTS), while cyclodextrin encapsulation enhanced activity of OPE against intracellular reactive oxygen species (ROS) accumulation. The obtained results show that OPE is more potent antioxidant in comparison to equivalent dose of main polyphenols (HTS and TS) and that increasing solubility of OPE polyphenols by CD encapsulation or digestion enhances their potential to act as intracellular antioxidants. Antioxidative protection of cells by OPE was primarily achieved through direct radical-scavenging/reducing actions rather than activation of endogenous defense systems in the cell.

Keywords: olive pomace extract; cyclodextrin; in vitro gastrointestinal digestion; antioxidant

1. Introduction

Olive pomace is highly accessible, valuable secondary raw material, left behind after the production of olive oil. Its polyphenolic composition is similar to that of olive oil and characterized by a high content of the phenolic alcohol hydroxytyrosol (HTS) and its derivatives tyrosol (TS) and oleuropein (OLE), as the main antioxidative compounds. Their biological activity is strongly correlated to their antioxidant and anti-inflammatory properties since they are able to reduce the pool of reactive oxygen species (ROS) by acting as radical scavengers and metal chelators, as well as to counteract the inflammatory processes associated with the onset and progression of several pathological conditions [1]. The potential of olive polyphenols to protect low density lipoprotein (LDL) from oxidative damage has been well described in scientific literature and is confirmed by the European Food Safety Authority (EFSA) health claim [2]. Although proven to be valuable and low-cost source of HTS and its derivatives, olive pomace represents a highly complex matrix that makes an effective extraction procedure a challenging task. Several green and sustainable techniques have been developed and optimized in the last decade resulting in the enhancement of polyphenols' extraction yields [3,4].

However, raw dry olive pomace extracts (OPEs) are characterized by poor technological properties, such as marked hygroscopicity, intense odor and instability and therefore its usage is still limited. Our previous research suggests that the mentioned shortfalls can be successfully remedied by combining efficient green extraction techniques with the process of cyclodextrin (CD) encapsulation leading to improved extraction yields and achievement of desired OPE characteristics [5,6]. CDs are cyclic compounds, composed of at least six D-(+)-glucopyranoside units that are linked to each other with 1,4-glycosidic bonds. They possess a relatively hydrophobic cavity and a hydrophilic external surface, which enables them to form inclusion complexes with structurally varied compounds. Therefore, they have been increasingly used as an eco-friendly means of recovering polyphenolic substances from complex matrices [7]. Available research suggests that, in addition to modifying extraction yields and physio-chemical properties of dry OPE, application of CD in OPE formulation can have significant impact on functional properties of obtained extracts, particularly on their antioxidant activities. We already demonstrated CDs-mediated enhancement of the OPEs antioxidant activity in different food model systems by increasing significantly their polyphenolic content and probably the stability of antioxidants under oxidative conditions. The tested CDs significantly differed regarding their ability to improve the functionality of OPEs; encapsulation with hydroxypropyl- β -CD (hpbCD) and randomly methylated β -CD provided comparable and the most significant benefits [8]. Similar findings have been reported by other authors who observed that positive effects on antioxidant activity were usually associated with improved solubility of formed inclusion complexes [7,9,10].

Nutraceutical properties of polyphenol rich plant extracts can be additionally modified during gastrointestinal digestion through different mechanisms: chemical degradation, liberation from complex matrix or formation of metabolites. These modifications significantly depend on the polyphenolic profile of the plant extract and the conditions in gastrointestinal tract, which bias detailed prediction of such processes. Previous studies showed that HTS and OLE concentrations remain stable during OPE digestion, while the amount of bioaccessible TS increases significantly in comparison to native, undigested sample [11]. Other authors have also shown that polyphenolic composition of food- or plant-derived extracts and consequently their biological activity is significantly affected by gastrointestinal digestion [12–14]. The presence of CDs in the formulation also affected the bioaccessibility of polyphenolic antioxidants by increasing bioaccessibility of TS, probably by formation of inclusion complexes and prevention of TS adhesion to bile salts or other macromolecules. Particular CDs can also directly affect transepithelial permeability of OPE polyphenols, probably through direct effects on Caco-2 cell monolayer [11].

Antioxidant activity of polyphenols is usually attributed to their ability to scavenge free radicals, chelate metals and generally by their redox activity. However, newer experimental data indicate that polyphenols may also offer an indirect protection through activation of endogenous defense systems in the cell. The latest studies strongly suggest that dietary polyphenols can stimulate antioxidant transcription and detoxification defense systems through antioxidant responsive elements inducible by oxidative and chemical stress [15]. To our knowledge, antioxidant activity of OPE polyphenols has not been investigated in cellular model. Even though HTS and TS are believed to be the strongest antioxidants in OPE, there is also no study comparing their activity in the complex OPE matrix to that of pure compounds, in terms of the impact on their activity and the cumulative effect of other antioxidants in OPE.

Therefore, the aim of the present study was to assess the activity of OPE polyphenols against oxidative cell damage induced by *tert*-butyl hydroperoxide (tBOOH) by taking into account the impact of complex OPE matrix and possible polyphenol interactions, encapsulation with hpbCD, and changes in polyphenolic composition induced by gastrointestinal digestion.

2. Results and Discussion

In this study, the impact of olive pomace matrix and encapsulation with CD on olive polyphenols' antioxidative activity was examined in cellular model of oxidative stress. HepG2 cells constitute

a validated model to evaluate cellular antioxidative defense system that has been used in many nutritional studies to reveal the mechanisms of polyphenols' antioxidant activity. Samples used for cell treatment were: native sample (nat) and its bioaccessible fraction (nat_bf); hpbCD sample that contained hydroxypropyl β cyclodextrin and its digest (hpbCD_bf); mix that contained HTS and TS in the same concentration as in native sample; HTS and TS as one-compound samples.

2.1. Cell Viability

The cytotoxic effects of OPE and pure polyphenols on HepG2 cells were evaluated by a 3-[4-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. The results, shown in Figure S1, reveal no impact on cell viability compared to positive control cells. Therefore, the analyzed extracts and pure compounds were used at the noted concentrations (Table 1) for the examination of the impact on cell's viability and antioxidative defense system. Similar samples containing olive products' extracts and pure polyphenols were already reported as non-toxic by several authors in different cell lines [16–18].

Table 1. Final concentrations of the samples in wells.

	γ_{OPE} (mg/mL)	γ_{HTS} (μM)	γ_{TS} (μM)
nat	0.8	-	-
nat_bf	0.8	-	-
hpbCD	2.5	-	-
hpbCD_bf	2.5	-	-
mix	-	3.7	1.2
TS	-	-	20
HTS	-	20	-

OPE (olive pomace extract), nat (native OP), hpbCD (OP + hydroxypropyl β CD), bf (bioaccessible fraction), TS (tyrosol), HTS (hydroxytyrosol), mix (HTS + TS).

As shown in Figure 1, the treatment of HepG2 cells with 350 μM tBOOH significantly reduced cell viability clearly showing the toxic effect of peroxide. A significantly lower percentage of viable cells compared to the positive control indicated that pretreatment with analyzed extracts did not eliminate the prooxidative effect of tBOOH completely. This was probably due to the high concentration of tBOOH that was used to fulfil the requirement for clear statistical difference between positive and negative control ($0 < z' < 0.5$). However, a significant increase in viability was observed in cells treated with nat, hpb, nat_bf, and hpb_bf samples compared to negative control (Figure 1), while no difference between nat and hpb samples showed that cyclodextrin encapsulation did not affect the antioxidant activity of the analyzed OPEs. On the other hand, mix, TS and HTS samples did not exert protective effects on cell viability at the applied concentrations. Given the fact that mix, HTS and TS were applied in concentrations equivalent to those of nat, observed results indicate that the compounds other than TS and HTS in OPE, such as oleuropein, demethyloleuropein and ligstroside, possess strong antioxidative activity and significantly contribute to antioxidant activity of OPE. To our knowledge, this is the first study comparing antioxidative activity of extracts obtained from olive waste products to antioxidant activity of its main constituents, known as powerful antioxidants, such as HTS and TS. Obtained results also confirm the ability of polyphenols to exert their activity when present in the complex matrix. There was no difference in cell protective effects between digested and undigested OPEs indicating unaltered antioxidative potential during digestion, which is consistent with the results of our previous study where the changes of antioxidant activity of OPE during gastrointestinal digestion were monitored using non-cell based Trolox equivalent antioxidative assay [11]. Also, even though it is known that the additional hydroxyl group at position 3 on the phenol ring provides to HTS stronger antioxidative activity [19], there was no clear difference in TS and HTS effects of on cell viability. That was also probably due to the high concentration of prooxidant.

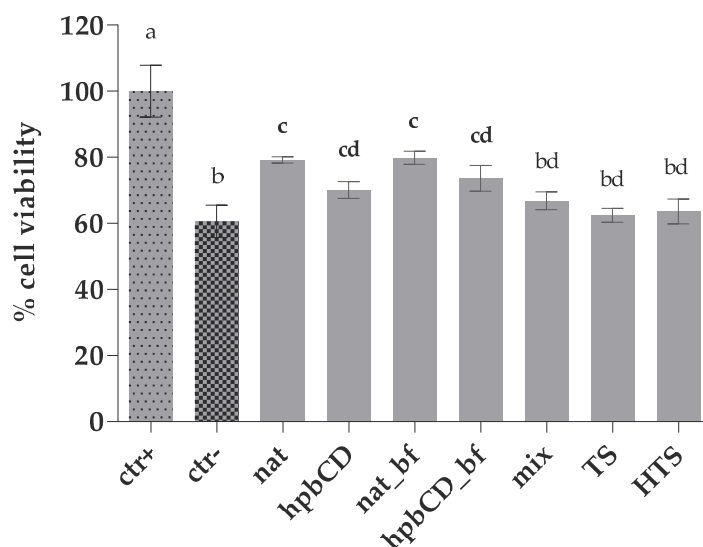


Figure 1. Protective effect of OPEs and pure polyphenols against oxidative stress on HepG2 viability. Data are presented as mean \pm standard deviation ($n = 3$) of the percentage calculated according to Equation (1); Positive control cells (ctr⁺) were treated with PBS while negative control cells (ctr⁻) were treated with tBOOH. All the other cells were treated both with samples and tBOOH. The final concentrations of the samples in wells are noted in Table 1. Different letters (a, b, c, d) indicate statistically significant differences compared to ctr⁺ ($p < 0.05$). Native olive pomace extract (nat), olive pomace extract with hydroxypropyl β -cyclodextrin (hpbCD); bioaccessible fraction (bf), tyrosol (TS), hydroxytyrosol (HTS), TS and HTS in the same concentration as in native sample (mix).

2.2. Reactive Oxygen Species (ROS) Determination

The treatment of HepG2 cells with 50 μ M tBOOH significantly increased intracellular ROS, clearly showing the toxic effect of peroxide. ROS are known to play a central role in mediating various diseases-originating processes, such as in cancer and coronary heart disease. Therefore, the elimination of the excessive ROS production and its scavenging is an essential strategy in the prevention of many illnesses. The ability of investigated samples to scavenge ROS was investigated by 2',7'-dichlorofluorescein diacetate (DCFDA) assay and expressed as relative fluorescence (RFU) (Figure 2). Obtained results showed significantly higher RFU values of samples compared to the positive control indicating that they did not eliminate the prooxidative effect of tBOOH completely. Again, this was also probably due to the high concentration of tBOOH that was used to fulfil the requirement for clear statistical difference between positive and negative control ($0 < z' < 0.5$). A significant decrease of RFU compared to negative control was observed at cells treated with hpb, nat_bf and HTS samples (Figure 2). The nat sample was the only OPE that did not decrease intracellular ROS but increased cellular viability (Figure 1). This unexpected result could be explained by methodological differences between the applied methods. In this method, contrary to the cell viability and intracellular GSH level determination (see below), the samples were removed from the wells before adding the prooxidant. Therefore, the measured antioxidative effect can be attributed only to the compounds that entered the cell during incubation period. It could be concluded that the ability of antioxidants to cross the cell membrane and to exert their activity intracellularly was the lowest in undigested native OPE. This is in consistence with our previous results that showed positive impact of gastrointestinal digestion on bioaccessibility of OPE polyphenols, and positive impact of cyclodextrin encapsulation of transcellular passage of OPE antioxidants [11]. Observed effects can be explained by the fact that OPE polyphenols, including HTS, are often found as part of larger complex molecules, such as oleuropein or hydroxytyrosol 4- β -D-glucoside which also have strong antioxidative properties [20]. However, due to their sizes and polarities, the cellular uptake of these compounds is usually limited by glucose transporters and is lower than the uptake of their aglycone [21]. The mix also did not show

protective effect on ROS production confirming that TS and HTS are not able to annul an intracellular deleterious effect of tBOOH in concentrations that are found in OPE. Interestingly, the hpb-containing sample showed antioxidative effect contrary to its bioaccessible fraction. Since hpb was already reported as small molecule extraction enhancer from OPE matrix [8,22], it probably increased the yield of small bioactive molecules during extraction. It seems that those smaller compounds were more susceptible to degradation during the digestion process as the antioxidative potential decreased in hpb_bf. According to Wojtunik-Kulesza [23], small polyphenols are more prone to pro-oxidation during digestion than large-molecular-weight compounds. Processes that can lead to pro-oxidation of polyphenols include the presence of metal ions, oxygen molecules, as most of all alkali pH. The latter was already reported as the main culprit for decreased bioaccessibility of small polyphenols including caffeic and rosmarinic acid [24]. Digestion of OPE resulted in unaltered concentrations of HTS and an increase of TS concentration (Figure S2) indicating that observed degradation may be linked to other small antioxidants from OPE such as ferulic and vanillic acid [25], which should be confirmed by future, targeted investigation.

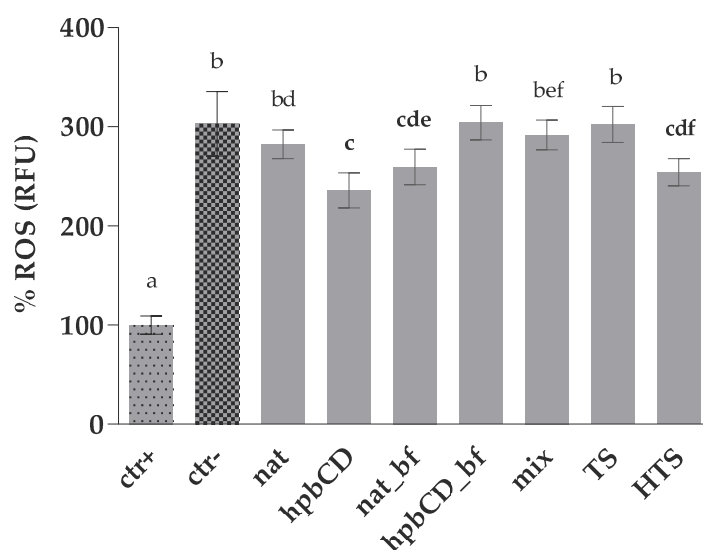


Figure 2. Protective effect of OPEs and pure polyphenols against oxidative stress on HepG2 ROS generation. Data are presented as mean \pm standard deviation ($n = 3$) of the relative fluorescence units (RFU) calculated according to Equation (2); Positive control cells (ctr+) were treated with PBS while negative control cells (ctr⁻) were treated with tBOOH. All the other cells were treated both with samples and tBOOH. The final concentrations of the samples in wells are noted in Table 1. Different letters (a, b, c, d, e, f) indicate statistically significant differences compared to ctr+ ($p < 0.05$). Native olive pomace extract (nat), olive pomace extract with hydroxypropyl β -cyclodextrin (hpbCD); bioaccessible fraction (bf), tyrosol (TS), hydroxytyrosol (HTS), TS and HTS in the same concentration as in native sample (mix).

The TS on the other hand was reported as an effective cellular antioxidant, probably due to its intracellular accumulation [19]. However, we were unable to confirm that postulate not even with the increased concentration of TS, observed during digestion. Unlike in the case of the cell viability, HTS sample (in a concentration approximately five times higher than in mix sample) decreased the ROS production while TS did not. It seems that the presence of HTS in concentration of 20 μ M could eliminate the intracellular effect of 50 μ M tBOOH as was obvious from the ROS measurement. Treatment with 350 μ M tBOOH limited the utility of cell viability assay to observe the protective effects of polyphenols in lower concentrations. These results confirm the importance of using multi-method approach to examine the antioxidative properties [26,27]. The TS in concentration of 20 μ M (approximately 17 times higher concentration than in OPE) failed to protect the cells from prooxidative effect of 50 μ M tBOOH in this method. In the already mentioned work from Di Benedetto [19], TS showed intracellular

accumulation and scavenging of ROS, but at a concentration of 500 μM , i.e., 25 times higher than the one we used in our assay. Therefore, the antioxidative effect of TS seems to be a dose- and incubation time-dependent.

2.3. GSH Determination

GSH is the main intracellular antioxidant and works as the substrate in GP-catalyzed neutralization of organic peroxides. Our results obtained by performing monochlorobimane (mBCl) assay showed that the treatment of HepG2 cells with 50 μM tBOOH significantly decreased intracellular GSH level clearly showing the toxic effect of peroxide while all tested samples succeeded to annul this effect as visible in an increase of RFU compared to the negative control (Figure 3). Interestingly, both nat and mix sample showed protective effect on GSH preservation, contrary to the increase in ROS level as observed by DCFDA assay. The difference could be explained by the fact that the samples were co-incubated with prooxidant in this method; so, both intra- and extra-cellular antioxidative activities against 50 μM tBOOH were measured. That observation confirms an ability of TS and HTS to scavenge the prooxidant in concentrations found in OPE and to maintain GSH in reduced form, which is in line with several previous *in vitro*- and *in vivo* studies. A study on healthy volunteers by Visoli [28] showed that polyphenols from olive mill waste waters increased total plasma glutathione. Pereira-Caro [29] on the other hand proved the ability of HTS in concentration 0.5 μM (approximately seven times lower concentration than the one found in OPE) to annul the deleterious effect of tBOOH at concentration of 400 μM , what is an eight times higher concentration than the concentration used in our assay. Therefore, the presence of HTS in OPE along with other antioxidants seem to be enough to exert their antioxidative activity against high concentrations of prooxidants. Cyclodextrin encapsulation did not affect the extent of their potential in decreasing oxidative stress as there was no difference between nat and hpb samples. The protective effect of HTS on GSH content was stronger than the effect of TS, as well as in ROS determination assay. However, the difference in GSH assay was not statistically significant.

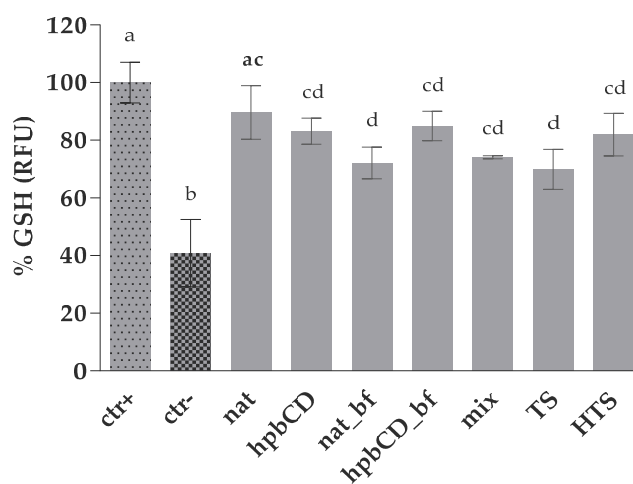


Figure 3. Protective effect of OPEs and pure polyphenols against oxidative stress on HepG2 GSH content. Data are presented as mean \pm standard deviation ($n = 3$) of the relative fluorescence units (RFU) calculated according to Equation (2); Positive control cells (ctr+) were treated with PBS while negative control cells (ctr⁻) were treated with tBOOH. All the other cells were treated both with samples and tBOOH. The final concentrations of the samples in wells are noted in Table 1. Different letters (a, b, c, d) indicate statistically significant differences compared to ctr+ ($p < 0.05$). Native olive pomace extract (nat), olive pomace extract with hydroxypropyl β -cyclodextrin (hpbCD); bioaccessible fraction (bf), tyrosol (TS), hydroxytyrosol (HTS), TS and HTS in the same concentration as in native sample (mix).

2.4. GPx and SOD Activity Determination

GPx and SOD play the crucial role in the defense against oxidative stress. SOD destroys highly reactive superoxide to hydrogen peroxide and oxygen. Hydrogen peroxide is further neutralized by GPx into oxygen and water by utilizing GSH as substrate.

In this study, cells pretreated with OPEs showed significantly lower GPx activity in comparison to positive control (treated only with PBS). All analyzed samples caused similar decrease in enzyme activity, with exception of bioaccessible fraction of hpb, which showed the strongest inhibition (Figure 4a). SOD activity was generally unaffected by tested OPEs and it was significantly reduced only in cells pretreated nat, hpb_bf, and HTS. Again, hpb_bf had the highest impact in lowering enzyme's activity (Figure 4b).

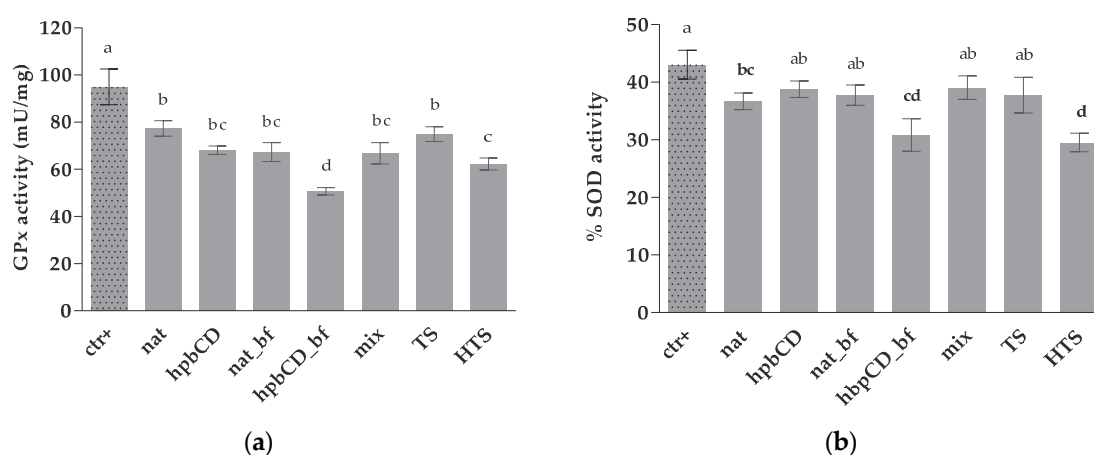


Figure 4. Effect of OPEs and pure polyphenols on enzyme activity. Data are presented as mean \pm standard deviation ($n = 3$) of the (a) GPx activity calculated according to Equation (3) and (b) SOD activity calculated according to Equation (4); Positive control cells (ctr+) were treated with PBS. The final concentrations of the samples in wells are noted in Table 1. Different letters (a, b, c, d) indicate statistically significant differences compared to ctr+ ($p < 0.05$). Native olive pomace extract (nat), olive pomace extract with hydroxypropyl β -cyclodextrin (hpbCD); bioaccessible fraction (bf), tyrosol (TS), hydroxytyrosol (HTS), TS and HTS in the same concentration as in native sample (mix).

Activity of SOD and GPx changes depending on the redox state of the cell. In vivo, their activity generally increases in response to oxidative stress, but if the stress continues, it is possible that the cell mechanisms will stop coping with persisting stress state, and in those late stages, the activity of enzymes will start to decrease. For example, extremely high concentrations of superoxide radicals in the cell will cause depletion of SOD and it will become unable to fight against free radicals. Therefore, polyphenols can affect the activity of mentioned enzymes indirectly, by affecting the redox state of the cell.

However, dietary polyphenols can also exert direct effects on the expression of genes regulating transcription of enzymes involved in antioxidant defense of the cell, acting similarly to numerous endogenous transcriptional factors [30]. For example, t-butylhydroquinone, has been identified as xenobiotic that can activate particular promoter regions called antioxidant responsive elements (ARE) and induce transcription of particular detoxifying, metal-binding or antioxidative enzymes. ARE activating effects have been proven for different fruit and vegetable extracts rich in polyphenols and carotenoids, and in those studies their ARE activating ability didn't correlate with direct radical scavenging or reducing potential [31,32].

The impact of OPE on the activity of antioxidative enzymes has not been investigated so far. However, Marinić and co-authors [33] showed that preexposure to olive oil polyphenols affected endogenous cellular defense mechanisms via the stress response gene-profiles associated with

hepatoprotection in the model of liver regeneration induced by one-third hepatectomy. Leskovec and co-workers [34] investigated the effect of olive leaf extract on the activity of GPx and SOD in animal model and found no changes in blood SOD activity while observed effects on GPx activity varied depending on the applied diet matrix. The results of the mentioned studies indicate different complex mechanism of involvement of olive polyphenols into regulation of oxidative response in vivo and emphasize the importance of the length of exposure to extracts, dosage and the existence of specie- and tissue- specific effects probably as the consequence of differences in bioavailability and metabolism pathways. Therefore, it is impossible to discuss data obtained in vitro in relation to available results of animal studies. The inhibitory effect of OPEs on the activity of GPx and SOD in cell lines tested here could be explained by their direct radical scavenging activities causing the decrease of intracellular ROS.

In conclusion, this study showed that a pretreatment of cells with OPE increased intracellular GSH and prevented radical-induced cell damage. In comparison to HTS, TS or their mix, OPE showed significantly higher ability to protect viability of cell subjected to treatment with free radicals, indicating significant contribution of other compounds in OPE (oleuropein, demethyloleuropein and ligstroside) to its antioxidative potential. The hpb, nat_bf and HTS showed the highest intracellular radical scavenging activity; however, measured antioxidative effect of polyphenols was dose- and incubation time-dependent and can vary significantly depending on the applied experimental conditions. Cells pretreated with OPEs showed significantly lower GPx activity, while SOD activity was generally unaffected indicating that OPE polyphenols do not exert direct effects on tested enzymes, but can change the redox state of the cell through multiple mechanisms (reductive potential, radical scavenging), resulting in consequential change of SOD/GPx activity.

3. Materials and Methods

3.1. Samples

Olive pomace (OP) was collected from several two-phase mills in Croatia during autumn 2018. OP was kept at $-20\text{ }^{\circ}\text{C}$ (Beko CN161220X, Istanbul, Turkey) in polypropylene bags until use. Pre-treatment included drying at $60\text{ }^{\circ}\text{C}$ for 24 h in an incubator (INKO, Zagreb, Croatia), shredding and sieving on φ 0.8 mm sieve (Prüfsieb DIN 4188, Kassel, Germany), defatting (~ 5 g of the sample was defatting for 2 h with petrol ether) using the Soxhlet apparatus (INKO SK6ESS, Zagreb, Croatia). Polyphenols were extracted with 20% ethanol from defatted OP (20 g/L) without (native sample) or with the addition of 8 g/L of hydroxypropyl β -cyclodextrin (hpbCD) according to previously optimized procedure [6]. The extraction was performed on 700 W of microwave power in high performance microwave digestion unit (Milestone 1200 mega, Sorisole, Italy) for 10 min. The extracts were cooled on ice and filtered to remove the crude parts. hpbCD was chosen according to the number of successful applications for olive polyphenols encapsulation in literature data. Obtained extracts were freeze dried for 48 h in a lyophilizer (Alpha 1–4 LOC-1, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). In addition, polyphenols specific for OP (hydroxytyrosol (HTS) and tyrosol (TS)) were included in this study mixed (in concentrations that are found in OPE) and as one-compound samples (220 μM). Stock solutions of pure compounds were prepared in DMSO (2 mg/mL) and diluted with PBS.

3.2. Reagents

Petrol ether was purchased from Carlo Erba Reagents (Barcelona, Spain) while ethanol and dimethyl sulfoxide (DMSO) were from Gram Mol (Zagreb, Croatia). Hydroxypropyl β -cyclodextrin was purchased from Wacker–Chemie GmbH (Burghausen, Germany). Methanol ($\geq 99.9\%$) and sodium acetate used for chromatographic analysis were from Sigma–Aldrich (St. Louis, MO, USA) while acetonitrile ($\geq 99.9\%$) was from Honeywell (Charlotte, NC, USA), and acetic acid from Kemika (Zagreb, Croatia). Reference standards of phenolic compounds 3-hydroxytyrosol (HTS) and tyrosol

(TS) were of analytical grade ($\geq 98\%$) and purchased from Sigma–Aldrich (St. Louis, MO, USA) as well as *tert*-butyl hydroperoxide (tBOOH). Dulbecco’s Phosphate Buffered Saline (PBS liquid, sterile filtered, without calcium, without magnesium, suitable for cell culture) was from Lonza (Basel, Switzerland). Bile salts, pancreatin from porcine pancreas ($4 \times$ USP), and Minimum Essential Eagle’s Medium (MEM with Earle’s salts, L-glutamine and sodium bicarbonate, sterile filtered, suitable for cell culture) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rabbit gastric extract (RGE > 25 U/mg) was from Lipolytech (Marseille, France). Heat inactivated fetal bovine serum (FBS), nonessential amino acids (NEAA), and trypsin were purchased from Capricorn Scientific (Ebsdorfergrund, Germany). 3-[4,5-Dimethyl-thiazole-2-yl]-2,5 diphenyltetrazolium bromide (MTT) was from Panreac AppliChem (Darmstadt, Germany). Fluorescent dyes 2',7'-dichlorofluorescein diacetate (DCFDA) and monochlorobimane (mBCL) were from Sigma–Aldrich (St. Louis, MO, USA). Glutathione Peroxidase Assay Kit (GPx) was from Cayman Chemical (Ann Arbor, MI, USA) while superoxide dismutase (SOD) assay kit, bicinchoninic acid (BCA), and bovine serum albumin (BSA) were from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water ($18\text{ M}\Omega$) was obtained from SG Reinstwassersystem Ultra Clear UV Plus coupled with SG Wasservollentsalzer-Patrone SG-2800 (Günzburg, Germany). Acqua pro injectione was obtained from the Croatian Institute of Transfusion Medicine (Zagreb, Croatia).

Cells were lysed with a lysis buffer that was made as follows: 25 mL of Solution 1, 250 μL of Solution 2 and 1 tablet of the Complete Protease Inhibitor (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Solution 1 was composed of 50 mM Tris, pH 8.0 (VWR International, Radnor, PA, USA), 137 mM NaCl (Kemig, Zagreb, Croatia), 1% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA), and 10% glycerol (Gram Mol, Zagreb, Croatia). Solution 2 was composed of 100 mM sodium orthovanadate (Sigma–Aldrich, St. Louis, MO, USA) and hydrogen peroxide (Gram Mol) in a final concentration of 3.6%.

All the solvents needed for chromatographic separation were degassed before analysis with Branson 1210 Ultrasonic Cleaner (Danbury, CT, USA). Acetate buffer was prepared by mixing sodium acetate 0.1 M: acetic acid 0.1 M (2:1 *v/v*) and adjusting the pH to 5 with pH meter (702 SM Titrimo, Metrohm, Herisau, Switzerland).

3.3. Quantification of Phenolic Components by HPLC-FLD

HTS and TS were identified and quantified by an Alliance 2695 HPLC system (Waters, Milford, MA, USA) coupled with a 2475 Multi λ detector (FLD) with Xenon lamp, according to modified method of Tzabopoulos and co-workers [35]. Samples were prepared by filtration through 0.45 μm PES syringe filters (Macherey–Nagel, Düren, Germany). Chromatographic separation was conducted by injecting 20 μL of sample on a reversed phase column ($250 \times 4.6\text{ mm}$, 5 μm) (Agilent Zorbax Eclipse Plus C18, Santa Clara, CA, USA). Mobile phases were 0.05 M sodium acetate buffer pH 5 and acetonitrile with the flow rate of 1 mL/min. Elution was conducted over 20 min at 25 $^{\circ}\text{C}$. Identification was performed with FLD set at the excitation wavelength of 280 nm, and emission wavelength of 316 nm. Polyphenols were identified by comparing the retention times of the eluting peaks with those of the standards. Peaks were quantified by using the Empower2 software (Waters, Milford, MA, USA) and compared to external standard calibration. Standard stock solutions were prepared by dissolving reference compounds in DMSO. Aliquots of these solutions were further diluted with ultrapure water to obtain calibration curve (1–81 mg/L).

3.4. In Vitro Simulated Gastrointestinal Digestion

Bioaccessible fractions (bf) of the OP polyphenols were obtained by in vitro static simulation of gastrointestinal digestion in the upper tract following the standardized protocol described by Brodkorb and co-workers [36]. The procedure was consisted of two sequential incubations; initially in simulated gastric fluid (SGF)/pepsin/gastric lipase to simulated gastric conditions followed by simulated intestinal fluid (SIF)/bile salts/pancreatin to simulate duodenal digestion. Briefly, 500 mg of

OPE were dissolved in 2 mL of ultrapure water and mixed with SGF. The samples were incubated at 37 °C for 2 h in a water bath (Büchi B-490, Flawil, Switzerland) with uniform shake at 110 rpm. Then SIF was added and incubated under the same conditions for 2 h. Total volume of the reaction mixture was approximately 18 mL, so the final concentration of extracts was 28 mg/mL. Enzyme solutions were prepared just before use. Samples were put on ice for 10 min and centrifuged (Heraeus Biofuge Stratos, Hanau, Germany) for 20 min at 4 °C and 4100 rpm in order to remove the crude parts of the sample. Supernatants were collected and the enzyme inactivation was done by a heat shock for 5 min at 100 °C in Thermomixer R (Eppendorf, Hamburg, Germany). Then, the samples were cooled in an ice bath for 10 min and centrifuged again under the same conditions. Supernatants, that represent bioaccessible fraction (bf) of the samples, were collected and stored at −80 °C until analyses.

3.5. Cell Culture

For investigation of antioxidative effect human liver cancer cell line (HepG2) was used. HepG2 cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in MEM supplemented with 10% heat-inactivated FBS and 1% A/A. Cell cultures were maintained at 37 °C, in a humidity saturated atmosphere consisted of 5% CO₂ (Sanyo MCO-20AIC CO₂ Incubator, Osaka, Japan). Medium was changed every 2–3 days. Cells were passaged at 80–90% confluence.

Samples used for cell treatment were prepared as follows: native sample (nat) and its bioaccessible fraction (nat_bf) contained 9 mg/mL of OPE without added CD; hpbCD sample and its digest (hpbCD_bf) contained OPE with CD in concentration of 28 mg/mL; mix contained HTS and TS in the same concentration as in native sample, i.e., 41 µM and 13 µM respectively (data obtained with HPLC analysis (nat OPE chromatograph is shown in Figure S3)); HTS and TS were one-compound samples and contained 220 µM of the analyte. A concentration of OPE in samples was chosen by taking into the account the total phenolic content obtained by analyzing samples with standard Folin-Ciocalteu method in order to annul the dilution of the OP by CD (Figure S4). The analysis revealed that the native sample had three times higher total phenolic content comparing to the sample with CD. Therefore, the native sample was diluted three times for the assays. Undigested OPE (dissolved in PBS) and OPEs' bioaccessible fractions were filtered over sterile syringe filter (PES membrane, φ 13 mm, pore size 0.22 µm) while stock solutions of pure polyphenols were diluted with PBS. The stock solutions were added directly to the cell culture medium so the final concentrations of OPE, mix (TS + HTS), TS and HTS in wells are noted in Table 1. Oxidative processes were induced with tBOOH in final concentrations noted in the description of each method [37].

3.6. Preparations of Cells for Cell Viability, ROS and GSH Determination

The first part of the cell viability, ROS, and GSH determination assays was similar. Also, all the photometric and fluorometric readings were done on a multilabel plate reader (Victor X3, Perkin Elmer, Waltham, MA, USA). The only difference was the type of the cell culture plates since for the MTT assay the transparent 96-well plates (Thermo Fisher Scientific 130188, Rochester, NY, USA) were used while for the ROS and GSH assays black 96-well plates (Thermo Fisher Scientific 137101, Rochester, NY, USA) were needed. Briefly, 2×10^4 cells/well were seeded in 100 µL of medium in 96-well plates and grown until reaching confluence (approximately 48 h) at 37 °C in a humidity saturated atmosphere consisted of 5% CO₂. Stock solutions of samples were added directly to the culture medium in volume of 10 µL. Cells were incubated for 20 h with samples or PBS for controls.

Cell viability was determined using the MTT [38] assay to check the toxicity of samples but also to determine the protective effect of samples after induction of oxidative stress with tBOOH. The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. That cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH. The formazan crystals formed are solubilized and the resulting colored solution is quantified spectrophotometrically at 570 nm. After the treatment with samples (as described previously), additional 3-h incubation with 15 µL of 350 µM tBOOH (final concentration in wells) followed only

in the assay that determined protective effect (in which positive control wells received 15 μL of PBS instead of tBOOH). The culture medium was then warily aspirated (Gilson Safe Aspiration Station, Middleton, WI, USA), and cells were washed with 250 μL PBS/well. Cell viability was assessed by the addition of 40 μL of MTT 0.5 mg/mL (diluted in PBS) and incubation for 3 h at 37 $^{\circ}\text{C}$, followed by dissolution of the formazan crystals in 170 μL of DMSO. Absorbance (A) was measured at 490 nm and cell viability was expressed as percentage (%) of the positive control according to Equation (1). Blank absorbance was measured in wells containing MTT without cells:

$$\% \text{ cell viability} = \frac{(A - A_b)}{(A_{\text{ctr}+} - A_b)} \times 100 \quad (1)$$

Quantitative measurement of the activity of hydroxyl, peroxy and other reactive oxygen species (ROS) was performed using 2',7'-dichlorofluorescein diacetate (DCFDA). DCFDA is a fluorescent dye that has the ability to diffuse into a cell where it is deacetylated by cell esterase to form a non-fluorescent compound [39]. Oxidation of this compound with ROS produces highly fluorescent compound 2',7'-dichlorofluorescein (DCF). DCF can be detected by a fluorescence spectrometer with a maximum excitation/emission spectrum at 495/529 nm. To assess antioxidative activity, the culture medium was warily aspirated (after the 20-h incubation with samples as described previously) and cells were washed with 100 μL of PBS. Then, cells were incubated with 100 μL of 25 μM DCFDA for 40 min. The dye was aspirated, and the cells were washed once again with 100 μL of PBS. Oxidative stress was induced by adding 100 μL of 50 μM tBOOH (diluted in PBS) for 1 h. Positive control wells received 15 μL of PBS instead of tBOOH. Fluorescence (F) was measured at 485/535 nm and % ROS was expressed as relative fluorescence (RFU) of the positive control according to Equation (2). Blank absorbance was measured in wells containing DCFDA without cells.

$$\text{RFU} = \frac{(F - F_b)}{(F_{\text{ctr}+} - F_b)} \times 100 \quad (2)$$

The intracellular concentration of reduced GSH was estimated by fluorometric assay that utilizes monochlorobimane (mBCL). Briefly, GSH reacts with mBCL in an enzyme reaction catalyzed with intracellular glutathione S-transferases. Adducts that form in that reaction can be measured fluorometrically at an excitation wavelength of 380 nm and an emission wavelength of 470 nm using a microplate reader [40]. After the 20-h incubation with samples (as described previously), 3-h incubation with prooxidant was done by adding 15 μL of 50 μM tBOOH (final concentration in wells). Positive control wells received 15 μL of PBS instead of tBOOH. The culture medium was then warily aspirated, and cells were washed with 100 μL of PBS after which 40-min incubation with 100 μL of 40 μM mBCL followed. Fluorescence (F) was measured at 355/460 nm and % GSH was expressed as relative fluorescence (RFU) of the positive control according to Equation (2). Blank absorbance was measured in wells containing mBCL without cells.

To assess the effect of olive pomace polyphenols on GPx and SOD activity, 10^6 cells were seeded in each well in 5 mL of culture medium in 6-well Falcon plate (Corning Inc., Corning, NY, USA). Cells were grown until reaching confluence (approximately 48 h) at 37 $^{\circ}\text{C}$ in a humidity saturated atmosphere consisted of 5% CO_2 . Samples were added directly to the culture medium in volume of 500 μL . Cells were incubated for 20 h with samples or PBS for control. Then the cells were washed with 1 mL of PBS and lysed with 200 μL of the lysis buffer in 20 min placed on ice. Cells were scraped, placed in Eppendorf tubes and centrifugated at 15000 G for 20 min at 4 $^{\circ}\text{C}$. Supernatant were collected and used for protein quantification and enzyme activity determination.

Protein concentration was determined by BCA method which is based on complex formation between protein and Cu^{2+} in alkali conditions. In those conditions, Cu^{2+} is reduced to Cu^+ that forms a purple complex with BCA that can be detected by measuring absorbance at 570 nm. The assay was done in 96-well plates by adding 4 μL of cell lysate, 21 μL of water and 100 μL of working reagent.

The plates were shaken slowly for 1 min in a multiplate reader and incubated at 37 °C, 30 min in total, after which the absorbance was measured at 490 nm. Blank contained 25 µL of water instead of samples and the obtained value was deducted from all the samples' values. The protein concentration was determined by comparing the result with bovine serum albumin (BSA) using the calibration curve made in the same conditions. The concentration range of BSA was 0.1–1 mg/mL prepared in aqua pro injectione.

GPx activity was measured indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in A₃₄₀ is directly proportional to the GPx activity in the sample [41]. One unit was defined as the amount of enzyme that will cause the oxidation of 1 nmol of NADPH to NADP⁺ per minute at 25 °C. Briefly, 20 µL of cell lysate was mixed with 50 µL of Assay Buffer, 50 µL of Co-Substrate Mixture, and 50 µL of NADPH after which the reaction was initiated by adding 20 µL of Cumene Hydroperoxide. The plate was slowly mixed for 5 s in a multiplate reader at room temperature and the absorbance was measured every minute for 10 min. The absorbance decreased linearly over time, so the specific enzyme activity was calculated as noted in Equation (3):

$$\text{GPx activity (nmol/min/mg)} = \frac{|\text{slope (min}^{-1})| \times V_t \text{ (mL)}}{\varepsilon \text{ (}\mu\text{M}^{-1}) \times V \text{ (mL)}} \quad (3)$$

where slope is from the A₃₄₀ decrease over time, V_t is the total volume in the well, V is the volume of the sample, ε is the NADPH extinction coefficient at 340 nm of 0.00373 µM⁻¹ (the value is adjusted for the pathlength of 0.6 cm of solution in the well).

SOD activity was measured by indirect method using Dojindo's tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). WST-1 produces a water-soluble formazan dye upon reduction with a superoxide anion that absorbs light at 440 nm. Since the absorbance is proportional to the amount of superoxide anion, the SOD activity as an inhibition rate can be quantified by measuring the decrease in the color development at 440 nm [42]. Briefly, 20 µL of cell lysate was mixed with 200 µL of WST Working Solution and 20 µL of Enzyme Working Solution in 96-well plate. The plate was incubated at 37 °C in a multilabel plate reader for 20 min and the absorbance was read at 450 nm. The control wells received water instead of sample. The SOD activity expressed as inhibition rate was calculated according to the Equation (4).

$$\text{SOD activity (inhibition rate (\%))} = \frac{(A_{b20} - A_{b0}) - (A_{20} - A_0)}{(A_{b20} - A_{b0})} \times 100 \quad (4)$$

where b represents the blank well in which water was added instead of cell lysate, 0 and 20 are times of measurement.

Supplementary Materials: The following are available online, Figure S1: Effect of a 20-hour exposure of olive pomace extracts and pure polyphenols on HepG2 cell viability, Figure S2: Bioaccessibility of hydroxytyrosol (HTS) and tyrosol (TS) from olive pomace extracts, Figure S3: Chromatograph of olive pomace extract, Figure S4: Total phenolic content determined by Folin-Ciocalteu method.

Author Contributions: Conceptualization, D.V.Č., I.V.V. and K.R.; methodology, I.V.V., I.P. and K.R.; validation, I.V.V. and I.P.; formal analysis, K.R. and D.V.Č.; investigation, K.R. and D.V.Č.; resources, D.V.Č. and I.V.V.; writing—original draft preparation, K.R.; writing—review and editing, D.V.Č.; visualization, K.R. and D.V.Č.; supervision, I.V.V. and D.V.Č.; funding acquisition, D.V.Č. and I.V.V. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples of the compounds are not available from the authors.

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Supplementary material

Figure S1: Effect of a 20-hour exposure of olive pomace extracts and pure polyphenols on HepG2 cell viability.

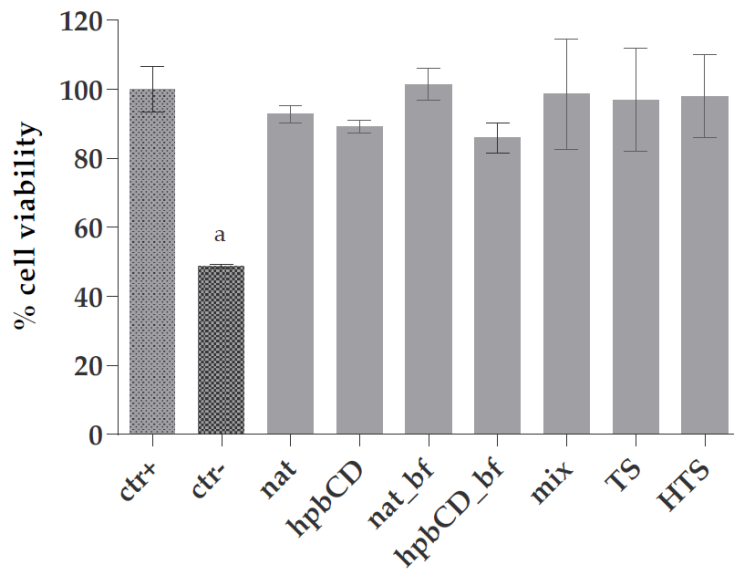


Figure S2: Bioaccessibility of hydroxytyrosol (HTS) and tyrosol (TS) from olive pomace extracts.

Examined parameters	nat	hpbCD
HTS ($\mu\text{g}/100\text{ mg}$)		
undigested extract	71.7	36.0
bioaccessible fraction	67.8	34.4
TS ($\mu\text{g}/100\text{ mg}$)		
undigested extract	23.5	8.9
bioaccessible fraction	30.4	15.0

Figure S3: Chromatograph of olive pomace extract.

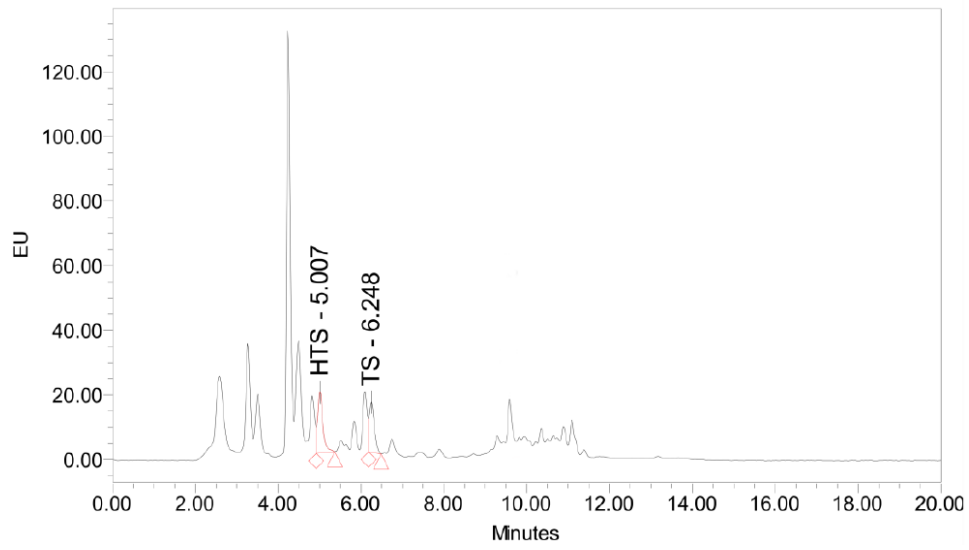
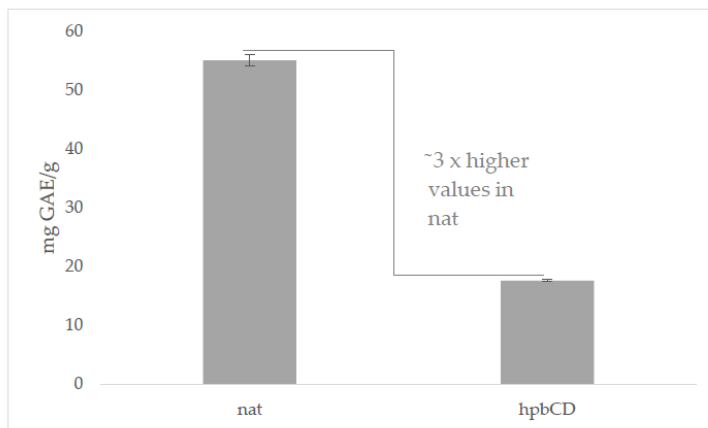


Figure S4: Total phenolic content determined by Folin-Ciocalteu method.



5. RASPRAVA

Komina masline sastoji se od lignina, celuloze, hemiceluloza, ostataka maslinovog ulja, proteina, minerala i složene smjese fenolnih sastavnica koja uključuje: fenolne alkohole (hidroksitirosol (HTS), tirosol (TS)); fenolne kiseline (kumarinska, cimetna, kofeinska, ferulična, galna, vanilinska, elenoična, sinapska, klorogenska, protokatehuidna, siringinska); flavonoide (apigenin, hesperidin, cijanidin flavon, antocijan, kvercetin, luteolin); lignane (acetoksipinoresinol, pinoresinol) [52]; sekoiridoide (oleuropein (OLE), oleokantal, oleacin, demetiloleuropein, oleuropein aglikon, ligstrozid) [53]. Navedeni spojevi, koje u nešto manjim količinama nalazimo i u maslinovom ulju, pokazuju širok spektar pozitivnih bioloških aktivnosti te tako doprinose održavanju zdravlja i prevenciji bolesti. Epidemiološkim studijama dokazana je povezanost unosa fenola masline (uglavnom putem konzumacije maslinovog ulja) i smanjene pojavnosti kardiovaskularnih bolesti i metaboličkog sindroma [54]; karcinoma [55] te različitih degenerativnih bolesti i stanja [56,57]. Navedeni učinci tumače se, između ostalog, antioksidacijskim [58], imunomodulatornim [59] i antikancerogenim svojstvima [60,61] fenolnih sastavnica.

S obzirom na vrlo sličan fenolni sastav maslinovog ulja i komine masline, visoku zastupljenost fenola u komini te dostupnost komine masline kao sekundarne sirovine, ona je u posljednjih dvadesetak godina prepoznata kao bogat i dobro iskoristiv izvor nutraceutika fenolnog tipa. Do danas je razvijen niz učinkovitih postupaka ekstrakcije biološki aktivnih fenola iz komine masline pri čemu se recentna istraživanja sve više usmjeravaju na „zelene“ i održive postupke koji koriste „zelena“ otapala te troše manje energije u odnosu na klasične ekstrakcije otapalom. Navedeni postupci najčešće uključuju primjenu ultrazvuka, mikrovalova te superkritičnih otapala [12,62–65].

Usprkos zadovoljavajućoj iskoristivosti postojećih postupaka ekstrakcije, razvoj suhih formulacija kompleksnih biljnih ekstrakata, kao što je ekstrakt komine masline (EKM), često je zahtjevan jer su sirovi ekstrakti izrazito higroskopni i ljepljivi, loše stabilnosti i slabe topljivosti te prodornog mirisa i neugodnog okusa, što značajno ograničava njihovu praktičnu primjenu [66]. U okviru naših preliminarnih istraživanja utvrdili smo izrazito loša fizikalno-kemijska svojstva sirovog EKM-a te pokazali da se primjenom nekoliko različitih vrsta ciklodekstrina (CD) tijekom procesa ekstrakcije i sušenja EKM-a značajno poboljšavaju prinosi ekstrakcijskog postupka, organoleptička svojstva suhog ekstrakta, njegova stabilnost te antioksidacijska učinkovitost u različitim model-sustavima hrane [12,64,67].

S obzirom na visok sadržaj biološki aktivnih fenola karakterističnih za maslinovo ulje, EKM možemo smatrati visokovrijednim nutraceutikom te pretpostaviti spektar bioloških učinaka sličan onima maslinovog ulja: izraženu antioksidacijsku aktivnost, imunomodulatorno djelovanje te kemoprotektivne i antitumorske učinke [58–61]. Međutim, potencijalni biološki učinci EKM-a bit će dodatno uvjetovani bioraspoloživošću svih aktivnih sastavnica. Iako bioraspoloživost primarno ovisi o fizikalno-kemijskim svojstvima biološki aktivnih sastavnica EKM-a, na nju značajno utječu i:

- matriks namirnice / ekstrakta
- korišteni ekscipijensi
- prisustvo hrane u gastrointestinalnom traktu (GIT)

koji mogu bitno promijeniti biodostupnost, ali i intestinalnu permeabilnost pojedinih aktivnih sastavnica EKM-a. Zbog toga, uzevši u obzir velike razlike u fizikalno-kemijskim svojstvima maslinovog ulja i suhog EKM-a, nije moguće, samo na temelju sličnog fizikalno-kemijskog profila bioaktivne komponente, pretpostaviti istu bioraspoloživost odnosno biološke učinke EKM-a i maslinovog ulja.

Stoga je osnovni cilj ovog istraživanja bio funkcionalna karakterizacija EKM-a i to u kontekstu istraživanja biodostupnosti i intestinalne permeabilnosti karakterističnih aktivnih sastavnica EKM-a (HTS-a, TS-a i OLE-a) te lokalnog antioksidacijskog učinka neprobavljive frakcije EKM-a u crijevu. Sistemski učinci fenola EKM-a bit će uvjetovani onom frakcijom fenola koja je biodostupna u tankom crijevu gdje se i apsorbira, njihovim metabolitima I II faze metabolizma te eventualno biološki aktivnim metabolitima neapsorbirane fenolne frakcije koji će nastati fermentacijom u debelom crijevu. Nositelji lokalnih učinaka na stijenku crijeva bit će neprobavljiva frakcija fenola EKM-a te metaboliti nastali djelovanjem crijevne mikrobiote. Biodostupnost i intestinalna permeabilnost posebno su istraženi u kontekstu utjecaja matriksa EKM-a i CD-a (koji su korišteni kao funkcionalni nosači) te prisustva hrane/obroka u GIT-u.

Intestinalna biodostupnost HTS-a, TS-a i OLE-a iz EKM-a istražena je primjenom standardiziranog statičkog *in vitro* modela probave [37]. Utjecaj matriksa EKM-a na biodostupnost HTS-a, TS-a i OLE-a istražen je usporedbom biodostupnosti smjese čistih sastavnica (HTS+TS+OLE) s biodostupnošću HTS-a, TS-a i OLE-a iz EKM-a, dok je utjecaj CD-a na biodostupnost HTS-a, TS-a i OLE-a istražen usporedbom biodostupnosti HTS-a, TS-a i OLE-a iz sirovog EKM-a s biodostupnošću iz EKM-a pripremljenih korištenjem β ciklodekstrina (bCD), hidroksipropil β ciklodekstrina (hpbCD), nasumično metiliranog β

ciklodekstrina (α CD) i γ ciklodekstrina (γ CD) [68]. Utjecaj hrane na biodostupnost istražen je korištenjem biorelevantnih medija te kodigestijom s pojedinim namirnicama / sastavnicama namirnica [69].

Udio HTS-a, TS-a i OLE-a je u svim analiziranim uzorcima EKM-a bio viši od 70 mg / 100g (HTS); 20 mg / 100g (TS) i 25 mg / 100g (OLE), izraženo na masu čistog ekstrakta, što je ili usporedivo ili značajno više od dostupnih literaturnih vrijednosti za maslinovo ulje [17,70]. U tom kontekstu, EKM predstavlja bolji nutritivni izvor biološki aktivnih spojeva fenolnog tipa od maslinovog ulja, međutim navedene podatke nužno je sagledati u kontekstu bioraspoloživosti, tj. biodostupnosti i intestinalne permeabilnosti navedenih spojeva iz EKM-a. Bioraspoloživost, obzirom na velike razlike u kemijskom sastavu, može biti bitno drugačija u usporedbi s maslinovim uljem.

Biodostupnost fenola iz EKM-a je uvjetovana stupnjem njihova oslobađanja iz matriksa ekstrakta (npr. iz kompleksa s prehrambenim vlaknima) ili stupnjem razgradnje kompleksnijih spojeva (oslobađanje HTS-a iz OLE-a; hidroliza glikozida); topljivošću u gastrointestinalnim tekućinama; interakcijama s probavnim enzimima i žučnim kiselinama i solima te drugim sastavnicama kompleksnog matriksa; te u konačnici kemijskom ili enzimskom razgradnjom u uvjetima GIT-a [20]. Osim toga prisutnost CD-a u ekstraktu [25,26] može bitno utjecati na njihovu biodostupnost u prvom redu zbog modificiranja stabilnosti u uvjetima probave i to na nekoliko načina: smanjenjem stupnja hidrolize, oksidacije, steričkih promjena, racemizacije i enzimske razgradnje; modificiranjem interakcija s drugim sastavnicama matriksa, druge hrane ili probavnih enzima / žučnih soli u lumenu GIT-a.

Rezultatima istraživanja utvrđeno je da HTS, TS i OLE kao i ostalih antioksidansi EKM-a pokazuju dobru gastrointestinalnu stabilnost te ostaju aktivni u uvjetima GIT-a. Količina HTS-a kao i udio ukupnih fenola EKM-a ostali su nepromijenjeni tijekom simulacije procesa probave ili je primijećen blagi porast u biodostupnoj frakciji. Nasuprot tome, u svim analiziranim uzorcima EKM-a primijećen je značajan porast koncentracije TS-a (129 % – 178 % početne količine TS-a) što je u skladu sa zapažanjima Corone i suradnika [21] vezanim uz dvosatnu inkubaciju maslinovog ulja u HCl-u. Zanimljivo je da isti autori izvještavaju također o povećanju koncentracije HTS-a, što nije u skladu s rezultatima naše studije. Navedene razlike ukazuju na važnost primjene standardiziranih postupaka simulacije probave te na specifičan učinak matriksa kao „nosača“ fenolnog spoja, što je u skladu s dostupnim literaturnim podacima [71]. U tom kontekstu porast koncentracije TS-a tijekom probave možemo pojasniti

formiranjem fenolnih alkohola kao produkata raspada složenijih fenolnih spojeva (sekoiridoida ili verbaskozida) u kiselim uvjetima [21]. Na konačnu količinu biodostupnog TS-a bitno je utjecala i prisutnost bCD-a i hpbCD-a u EKM-u. Naime, količina biodostupnog TS-a u navedenim uzorcima bila je značajno veća u usporedbi sa sirovim EKM-om. Uočene promjene mogu se objasniti snažnim kapacitetom vezanja žučnih soli s fenolnom hidroksilnom skupinom vodikovim/ionskim vezama [26]. Stvaranjem inkluzijskih kompleksa ili agregata TS-a s bCD-om i hpbCD-om [72] može se spriječiti vezanje sa žučnim solima što rezultira povećanom biodostupnošću. Udio glavnog sekoiridoida masline OLE-a nakon simulacije probave je bio nepromijenjena u odnosu na količine utvrđene u EKM-u prije probave. Dobiveni podaci u skladu su s dostupnim podacima iz literature [73] i činjenicom da glikozilirani sekoiridoidi ne podliježu kiselinskoj hidrolizi u želucu [32].

U usporedbi s podacima dobivenim za maslinovo ulje [17,70] biodostupnost HTS-a, TS-a i OLE-a iz EKM-a u GIT-u usporediva je ili veća, što potvrđuje primjenjivost EKM-a kao potencijalnog alternativnog nutritivnog izvora HTS-a, TS-a i OLE-a.

Utjecaj hrane na biodostupnost fenola EKM-a istražen je primjenom dva *in vitro* pristupa: ispitivanjem oslobađanja HTS-a, TS-a i OLE-a iz matriksa EKM-a u biorelevantnim medijima te istraživanjem konkretnih EKM-hrana interakcija kodigestijom s pojedinim namirnicama / komponentama namirnica.

Simulacija stanja natašte odnosno stanja sitosti korištenjem biorelevantnih medija pokazala je da promjene pH, ionske snage, aktivnosti enzima ili prisutnosti/količine žučnih soli u GIT-u ne utječu bitno na biodostupnost promatranih fenolnih sastavnica (ukupnih fenola, HTS-a, TS-a i OLE-a). Uzimajući u obzir fizikalno-kemijske karakteristike HTS-a i TS-a, dobiveni rezultati djelomično su očekivani. Naime, promjene u GIT-u koje su simulirane primjenom biorelevantnih medija uglavnom mogu imati pozitivan utjecaj na biodostupnost spojeva slabo topljivih u vodi sukladno biofarmaceutskoj klasifikaciji (engl. *Biopharmaceutics Classification System* (BCS)) djelatnih tvari / lijekova [74,75]. U tom kontekstu, s obzirom na dobru topljivost HTS-a i TS-a u vodi (17,4 g / L; i 25,3 g/L) te dobru permeabilnost procijenjenu na temelju $\log P$ vrijednosti (0,85 – 1,19 i 0,13 – 0,89) HTS i TS mogli bi se okvirno svrstati u BCS klasu I (dobra topljivost, dobra permeabilnost) (Tablica 1). Stoga se očekivani negativan utjecaj hrane na njihovu bioraspoloživost može istraživati jedino preko direktnih učinaka hrane na biodostupnost, a ne ispitivanjem biodostupnosti korištenjem biorelevantnih medija [76].

Tablica 1. Fizikalno-kemijske karakteristike fenolnih spojeva masline

Fenolni spoj	Topljivost u vodi (g / L)	logP	pKa (najjača kiselina)	pKa (najjača baza)	Mr (g / mol)
Hidroksitirosol	17,4	0,13 – 0,89	9,4	-2,4	154,2
Tirosol	25,3	0,85 – 1,19	10,2	-2,4	138,2
Oleuropein	0,73	0,11 – 0,63	9,3	-3,0	540,2

Interakcije EKM-a s hranom i posljedični utjecaj na biodostupnost ukupnih fenola, HTS-a i TS-a istražena je kodigestijom EKM-a s različitim namirnicama i komponentama namirnica, primjenom standardiziranog statičkog *in vitro* modela probave [37]. U jedinom dostupnom sličnom istraživanju, autori su utvrdili da testni obrok negativno utječe na bioraspoloživost HTS-a iz komine masline, smanjujući biodostupnost za otprilike 20 % [25]. Zaključci navedene studije bili su da negativni učinci hrane vjerojatno potječu od stvaranja kompleksnih veza HTS-a s određenim komponentama hrane te da je za realnu procjenu značajnosti interakcija EKM-hrana potrebno u istraživanje uključiti što širi spektar stvarnih namirnica. Nastavno na njihove zaključke, u okviru našeg istraživanja, istražene su interakcije fenola EKM-a sa 16 različitih vrsta namirnica, kategoriziranih s obzirom na udio masti, proteina, raspoloživih ugljikohidrata i prehrambenih vlakana. Ovisno o tipu namirnice, biodostupnost fenola EKM-a ili se nije značajno promijenila ili je značajno smanjena. Izrazito negativan učinak na biodostupnost imale su namirnice s relativno visokim udjelom proteina (52 %, 12,4 %, 10,5 %), koje su smanjile biodostupnost fenola EKM-a za 51,8 %, 60,6 %, odnosno 71,9 %. Promatrani učinci vjerojatno su barem dijelom posljedica interakcija fenolnih spojeva i proteina opisanih u radu Jakobeka [44] gdje se navodi kako fenoli (posebno polihidroksifenoli) tvore nekovalentne hidrofobne interakcije s proteinima, koje se mogu naknadno stabilizirati vodikovom vezom [77] i mogu, između ostalih učinaka, promijeniti bioraspoloživost fenola.

Na biodostupnost HTS-a i TS-a negativno su utjecala i prehrambena vlakna te je u namirnicama s visokim udjelom prehrambenih vlakana relativna biodostupnost TS-a smanjena za 67 – 89 %, dok je biodostupnost HTS-a smanjena za 54,8 – 84,2 %. Negativan utjecaj prehrambenih vlakana na biodostupnost HTS-a i TS-a nije bio proporcionalan sadržaju prehrambenih vlakana u namirnici vjerojatno zato što je isti posljedica složenih interakcija svih komponenata namirnice, a također je bitno uvjetovan kemijskim karakteristikama prehrambenog vlakna. Na temelju dobivenih rezultata zaključeno je da se utjecaj prehrambenih vlakana na biodostupnost fenola ne može ekstrapolirati iz podataka dobivenih *in vitro* simulacijom digestije fenola s izoliranim prehrambenim vlaknima već isključivo kodigestijom s kompletnom namirnicom, a

zbog značajnog utjecaja ostalih sastavnica namirnice. Navedeno je u skladu sa zaključcima studije Pinarlija i suradnika [47] koji također navode da se zbog složenosti interakcija unutar matrice hrane, svaka interakcija fenola s hranom treba istraživati posebno.

Intestinalna permeabilnost i mehanizmi intestinalnog transporta HTS-a, TS-a i OLE-a nisu dobro istraženi. Dostupni podaci ukazuju na pasivnu apsorpciju HTS-a i TS-a [35,78,79], a vrijednosti prividnih koeficijenata permeabilnosti dobivene u okviru našeg istraživanja u skladu su s vrijednostima dostupnim u literaturi ($1 \times 10^{-5} \text{ cm s}^{-1}$ (za HTS) i $3 \times 10^{-5} \text{ cm s}^{-1}$ (za TS)). Prividni koeficijent permeabilnosti OLE-a bio je vrlo nizak ($1 \times 10^{-7} \text{ cm s}^{-1}$) što upućuje na lošu permeabilnost odnosno vrlo intenzivan metabolizam OLE-a u Caco-2 stanicama. Dobiveni rezultati u skladu su sa zaključcima studije Edgecombea i suradnika [80] koji navode da je OLE slabo bioraspoloživ te naglašavaju potencijalnu važnost paracelularnog transporta OLE-a u crijevu, uz pretpostavku da su biološki učinci OLE-a posljedica djelovanja njegovih bioaktivnih metabolita.

Kako bismo istražili mogućnost postojanja interakcija na razini apsorpcije između HTS-a, TS-a i OLE-a, određeni su prividni koeficijenti permeabilnosti za svaki od navedenih spojeva kada su primijenjeni na monosloj Caco-2 stanica kao smjesa spojeva. S obzirom na to da nisu utvrđene značajne razlike u odnosu na koeficijente permeabilnosti dobivene ispitivanjem svakog pojedinačnog spoja zaključujemo da oni međusobno ne interferiraju značajno što je važan preduvjet za daljnji razvoj kompleksnih nutraceutika iz komine masline. Istraživanjem antioksidacijskog potencijala u apikalnom i bazolateralnom odjeljku Caco-2 staničnog modela, primijećeno je da tijekom transporta smjese dolazi do porasta antioksidacijske aktivnosti koja je u bazolateralnom odjeljku 2,8 puta veća nego u apikalnom. Pretpostavljamo da je navedeno posljedica enzimske hidrolize OLE-a posredovane glikozidazama te apsorpcije i daljnjeg metabolizma oleuropein aglikona, čiji metaboliti zadržavaju antioksidacijsku aktivnost. To je u skladu s dobivenim podacima o niskoj permeabilnosti i značajnom metabolizmu OLE-a, a može se objasniti opažanjima Németha i suradnika [81] o doprinosu membranskih i citosolnih glikozidaza u tankom crijevu apsorpciji i metabolizmu fenolnih sastavnica glikozidnog tipa.

Utjecaj matriksa EKM-a na permeabilnost ukupnih fenola, HTS-a, TS-a i OLE-a istražen je usporedbom rezultata dobivenih ispitivanjem permeabilnosti iz smjese navedenih fenola te iz kompleksne matrice EKM-a. Dobiveni rezultati pokazuju da su antioksidansi iz EKM-a permeabilni, ali podložni intenzivnom metabolizmu te da matriks EKM-a ima snažan negativan utjecaj na njihovu permeabilnost. Primjerice, aplikacijom EKM-a na Caco-2 stanični monosloj,

izostaje učinak porasta antioksidacijskog potencijala u bazolateralnom odjeljku, vjerojatno zbog zasićenja membranskih i citosolnih glikozidaza drugim sastavnicama prisutnim u kompleksnom matriksu EKM-a. Matriks EKM-a negativno je utjecao i na intestinalnu permeabilnosti HTS-a, TS-a i OLE-a. Navedeni negativni utjecaj bi se mogao objasniti nekovalentnim interakcijama HTS-a, TS-a i OLE-a s makromolekulama prisutnim u EKM-u koje su velikim dijelom posljedica slabih asocijacija (kombinacija vodikovih veza i hidrofobnih interakcija) i može umanjiti mogućnost pasivne difuzije kroz crijevni epitel (stanični monosloj), glavnog načina transporta HTS-a i TS-a [42].

Utjecaj CD-a na permeabilnost fenola je bio generalno negativan (ali u puno manjoj mjeri u usporedbi s utjecajem matriksa EKM-a), iako su opaženi učinci CD-a specifični s obzirom na vrstu CD-a i promatranu fenolnu sastavnicu. Prisutnost CD-a u formulaciji negativno je utjecala na permeabilnost TS-a i OLE-a, međutim bCD i ramCD značajno su povećali transmembranski transport HTS-a (s 5 % na 17 % odnosno 12 %). Opaženi učinci vjerojatno su posljedica navedenih CD-a da uklanjanjem kolesterola iz membrane povećavaju permeabilnost Caco-2 staničnog monosloja [82]. Isti učinak, s druge strane, nije bio opažen kod TS-a vjerojatno zbog prethodno primijećenog stvaranja inkluzijskih kompleksa TS-a i ciklodekstrina.

Hrana može značajno utjecati na permeabilnost intestinalne barijere i to različitim mehanizmima: utjecajem na produkciju sluzi, utjecajem na paracelularnu propusnost, utjecajem na propusnost dvosloja ili utjecajem na aktivni transport [41,83]. Ako uz navedeno uzmemo u obzir prethodno prikazane učinke pojedinih namirnica na biodostupnost fenola EKM-a, jasno je da se utjecaj obroka odnosno pojedinih namirnica na intestinalnu permeabilnost fenola teško može procijeniti.

Malo je literaturnih podataka o utjecaju namirnica ili pojedinih nutrijenata na intestinalnu permeabilnost fenola. Nedavno su Mendes i suradnici [84] pokazali da hrana (kazein, škrob i mast) ne utječu značajno na intestinalnu permeabilnost katehina i procijanidina guarane. S druge strane, transepitelni transport indikaksantina i betanina iz zelenog čaja bio je manji u prisutnosti hrane, dok je kazein pozitivno utjecao na intestinalnu permeabilnost katehina vjerojatno zbog utjecaja mlijeka na propusnost čvrstih spojeva [85,86]. Iz dostupnih podataka vidljivo je da su učinci visoko specifični za pojedine kombinacije namirnica/nutrijenta i fenola te da je u okviru funkcionalne karakterizacije svakog nutraceutika potrebno posebno provesti i analizu mogućih interakcija s hranom s obzirom na to da za sad nije moguće donositi zaključke ekstrapolacijom opažanja dobivenih analizom sličnih sustava.

U okviru naših istraživanja istražen je utjecaj glukoze, aminokiselina, prehrambenih vlakana (inulin i jabučna vlakna) i hpbCD (vrsta CD-a koja se pokazala najprimjenjivija za formulaciju EKM-a) na intestinalnu permeabilnost HTS-a i TS-a. Glukoza je pokazala statistički značajan pozitivan učinak na intestinalnu permeabilnost HTS-a i TS-a. Mogući mehanizmi opažene interakcije mogli bi se objasniti rezultatima D'Souze i suradnika [87] koji su istraživali permeabilnost Caco-2 staničnog monosloja u prisutnosti medija s visokom (25 mM) ili fiziološkom (5,5 mM) koncentracijom glukoze. Njihova studija pokazala je da visoka koncentracija izvanstanične glukoze utječe na svojstva Caco-2 staničnog sloja tako da smanjuje transepitelni električni otpor (TEER) i povećava fluidnost membrana čime se povećava mogućnost paracelularnog i transcelularnog pasivnog transporta. Navedeno je u skladu s rezultatima mjerenja TEER-a staničnih dvosloja, gdje je inkubacija s otopinom glukoze dovela do 20 %-tnog smanjenja TEER-a.

Celuloza je također imala značajan negativan učinak na TEER Caco-2 staničnih monoslojeva, međutim navedeno nije rezultiralo povećanom permeabilnošću HTS-a i TS-a. To je vjerojatno posljedica stvaranja netopljivih kompleksa između HTS-a/TS-a i celuloze što je u skladu s našim rezultatima istraživanja utjecaja prehrambenih vlakana na biodostupnost HTS-a i TS-a, ali i s rezultatima Bermúdez-Oria i suradnika [88] koji su pokazali da fenoli masline tvore netopljive komplekse s prehrambenim vlaknima.

Iz do sada prezentiranih rezultata proizlazi da su fenoli EKM-a biodostupni i stabilni tijekom probave, a zbog ograničene apsorpcije u tankom crijevu imaju tendenciju koncentriranja u GIT-u. Stoga imaju potencijal za prevenciju i/ili liječenje brojnih poremećaja GIT-a.

Za procjenu biološke aktivnosti nutraceutika u smislu istraživanja njihovog lokalnog antioksidacijskog učinka vrlo često se koristi kombinacija *in vitro* modela digestije sa staničnim modelima epitela crijeva [49]. Smanjenje oksidacijskog stresa u GIT-u je osobito važno s obzirom na to da su brojne studije pokazale da je oksidacijski stres izravno povezan s razvojem bolesti GIT-a kao što su upalna bolest crijeva i karcinom kolona koji je trenutno drugi najsmrtonosniji oblik karcinoma [50]. Dobiveni rezultati pokazuju da je EKM učinkovito zaštitio Caco-2 stanice od oksidacijskog stresa, a na antioksidacijsku aktivnost pozitivno je utjecala prisutnost bCD-a, hpbCD-a i ramCD-a u formulaciji. Pretpostavljeni mehanizam opaženog učinka CD-a je njihova sposobnost da povećaju biodostupnost fenola EKM-a u tankom crijevu, što je potkrijepljeno prethodno prikazanim rezultatima.

Mehanizmi kojim fenolni spojevi masline ostvaruju protektivne antioksidacijske učinke do sada nisu u potpunosti razjašnjeni, ali generalno mogu uključivati izravni antioksidacijski učinak, utjecaj na staničnu signalizaciju i utjecaj na stanični ciklus [89].

S obzirom na to da je za konačnu potvrdu učinka i razumijevanje mehanizma antioksidacijskog djelovanja potrebno provesti istraživanje na nekoliko staničnih linija i koristiti nekoliko različitih testova, dodatne analize provedene su na stanicama humanog karcinoma jetre (HepG2). HepG2 stanična linija se često koristi kao *in vitro* model sustav za ispitivanje mehanizama brojnih učinaka nutraceutika zbog relativno jednostavnog, brzog i jeftinog uzgoja uz istovremeno adekvatno predstavljanje složenosti bioloških sustava [51]. Direktna antioksidacijska aktivnost fenola posljedica je njihove sposobnosti gašenja slobodnih radikala, kompleksacije kelirajućih metala i općenito njihove redukcijske aktivnosti. Međutim, noviji literaturni podaci pokazuju da fenoli također aktivno sudjeluju u antioksidacijskoj obrani organizma putem neizravnih mehanizama djelovanja, primarno aktivacijom endogenih obrambenih sustava u stanici (stimulacija transkripcije glavnih antioksidacijskih i detoksikacijskih enzimskih obrambenih sustava) [48].

Rezultati dobiveni u okviru naših istraživanja pokazuju da EKM dovodi do porasta razine unutarstaničnog glutaciona, najvažnijeg neenzimskog antioksidansa, ali ne utječe na aktivnost antioksidacijskih enzima glutation peroksidaze i superoksid dismutaze. Naime, stanice u kojima je izazivan oksidacijski stres i koje su koinkubirane s EKM-om sadržavale su istu količinu glutaciona kao i stanice u kojima stres nije izazivan dok su aktivnosti antioksidacijskih enzima ostale iste prije i poslije inkubacije s EKM-om. Stoga se može zaključiti da je antioksidacijska zaštita stanica EKM-om primarno postignuta izravnim uklanjanjem radikala, a ne aktiviranjem sustava endogene obrane u stanici. EKM se pokazao potentnijim antioksidansom u usporedbi s ekvivalentnim količinama HTS-a i TS-a (12 % veća vijabilnost stanica tretiranih s EKM-om), potvrđujući prisutnost brojnih drugih sastavnica s antioksidacijskim potencijalom u EKM-u. hpbCD je iskazao pozitivan utjecaj na antioksidacijski potencijal EKM-a i to 16 %-tnim smanjenjem akumulacije unutarstaničnih reaktivnih kisikovih spojeva (ROS). Kao i u studiji na Caco-2 staničnom modelu, pretpostavljeni mehanizam kojim hpbCD utječe na antioksidacijski potencijal je poboljšavanje topljivosti svih antioksidanasa prisutnih u EKM-u u gastrointestinalnim tekućinama. No, obzirom na primijećeni učinak hpbCD-a povećavanja biodostupnosti TS-a, uklanjanje ROS bi moglo biti i posljedica akumulacije upravo tog snažnog unutarstaničnog antioksidansa.

6. ZAKLJUČAK

- Rezultati dobiveni u okviru istraživanja pokazuju da je ekstrakt komine masline (EKM) bogat izvor biološki aktivnih fenola hidroksitirosola (HTS), tirosola (TS) i oleuropeina (OLE) te da je njihov sadržaj u EKM-u usporediv ili značajno veći nego u maslinovom ulju.
- Tijekom procesa probave, zbog razgradnje kompleksnijih molekula, dolazi do dodatnog oslobađanja HTS-a i TS-a iz EKM-a, a njihova biodostupnost usporediva je ili bolja od one u maslinovom ulju. Prisutnost beta ciklodekstrina (bCD) i hidroksipropil beta ciklodekstrina (hpbCD) u EKM-u značajno je poboljšala biodostupnost TS-a, vjerojatno putem stvaranja inkluzijskih kompleksa i posljedičnom prevencijom interakcija TS-a sa žučnim solima koje negativno utječu na njegovu biodostupnost.
- Utjecaj hrane na biodostupnost istraživanih spojeva je varijabilan i značajno se mijenja ovisno o nutritivnom sastavu obroka/namirnice. Izrazito negativan učinak na biodostupnost imale su namirnice s relativno visokim udjelom proteina koje su smanjile biodostupnost fenola EKM-a za 51,8 %, 60,6 %, odnosno 71,9 %. Na biodostupnost HTS-a i TS-a negativno su utjecala i prehrambena vlakna smanjujući relativnu biodostupnost TS-a za 67 – 89 %, a biodostupnost HTS-a za 54,8 – 84,2 %. Negativan utjecaj prehrambenih vlakana na biodostupnost HTS-a i TS-a nije bio proporcionalan sadržaju prehrambenih vlakana u namirnici te je značajno uvjetovan kemijskim karakteristikama prehrambenog vlakna.
- Istraživanje intestinalne permeabilnosti je pokazalo da su HTS i TS dobro permeabilni (prividan koeficijent permeabilnosti $1 \times 10^{-5} \text{ cm s}^{-1}$, odnosno $3 \times 10^{-5} \text{ cm s}^{-1}$) dok je OLE slabo permeabilan (prividan koeficijent permeabilnosti $1 \times 10^{-7} \text{ cm s}^{-1}$); da se značajno metaboliziraju u stanicama tankog crijeva i da je pasivna difuzija glavni mehanizam transmembranskog transporta HTS-a i TS-a. HTS, TS i OLE ne interferiraju značajno na razini transmembranskog transporta što je važan preduvjet za daljnji razvoj kompleksnih nutraceutika iz komine masline.
- Matriks EKM-a pokazao je značajan negativan utjecaj na permeabilnost navedenih spojeva dok je utjecaj CD-a bio slabiji i specifičan s obzirom na vrstu primijenjenog CD-a i promatrane fenolne sastavnice. Prisutnost CD-a u formulaciji negativno je utjecala na permeabilnost TS-a i OLE-a, međutim bCD i ramCD značajno su povećali transmembranski transport HTS-a (s 5 % na 17 odnosno 12 %). Opaženi učinci vjerojatno su dijelom uvjetovani sposobnošću navedenih CD-a da uklanjanjem kolesterola iz membrane povećavaju permeabilnost Caco-2 staničnog monosloja.

- Istraživanjem direktnih utjecaja hrane/nutrijenata na intestinalnu permeabilnost HTS-a, TS-a i OLE-a utvrđeno je da su učinci visoko specifični s obzirom na promatrani nutrijent te su uglavnom posljedica utjecaja na permeabilnost čvrstih spojeva. Glukoza je pokazala statistički značajan pozitivan učinak na intestinalnu permeabilnost HTS-a i TS-a, što je posljedica smanjenja transepitelnog električnog otpora (TEER) i povećanja fluidnosti membrana. Celuloza je također imala značajan negativan učinak na TEER Caco-2 staničnih monoslojeva, međutim navedeno nije rezultiralo povećanom permeabilnošću HTS-a i TS-a. Navedeno je vjerojatno posljedica stvaranja netopljivih kompleksa između HTS-a/TS-a i celuloze što je u skladu s rezultatima istraživanja utjecaja prehrambenih vlakana na biodostupnost HTS-a i TS-a.
- EKM je učinkovito zaštitio Caco-2 stanice od oksidacijskog stresa, a na antioksidacijsku aktivnost pozitivno je utjecala prisutnost bCD-a, hpbCD-a i nasumično metiliranog beta ciklodekstrina (ramCD) u formulaciji. Istraživanjem mehanizama opaženih učinaka utvrđeno je da njihovoj aktivnosti najviše doprinose direktni antiradikalni učinak fenolnih spojeva te utjecaj na porast unutarstaničnih koncentracija glutationa.
- Dobiveni rezultati pokazuju da je EKM visokovrijedan izvor biološki aktivnih spojeva masline i s obzirom na kemijski sastav i biodostupnost promatranih spojeva, usporediv s maslinovim uljem. Dodatno, EKM pruža učinkovitu antioksidativnu zaštitu stanicama tankog crijeva. Rezultati studije ukazuju na imperativ sveobuhvatne karakterizacije u procesu oblikovanja visoko vrijednih nutraceutika komine masline te mogućnosti jednostavnih intervencija tijekom formulacije koje rezultiraju poboljšanom funkcionalnošću završnog proizvoda.
- U širem smislu, rezultati ovog doktorskog rada doprinose valorizaciji komine masline kao sekundarne sirovine u formulaciji visokovrijednih proizvoda dodane vrijednosti te indirektno, smanjenju ekološkog otiska proizvodnje maslinovog ulja i stvaranja otpada od hrane.

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Doktorski rad

BIODOSTUPNOST, PERMEABILNOST I ANTIOKSIDACIJSKA AKTIVNOST NUTRACEUTIKA IZ KOMINE MASLINE

Kristina Radić

SAŽETAK

Tijekom proizvodnje maslinovog ulja iz ploda masline (*Olea europaea* L.) nastaju velike količine otpada u obliku otpadnih voda i komine (krutog ostatka). Njihov kemijski sastav karakterizira značajan udio fenolnih spojeva koje nalazimo i u maslinovom ulju. Osnovni cilj ovog istraživanja bio je funkcionalna karakterizacija ekstrakta komine masline (EKM) i to u kontekstu istraživanja biodostupnosti i intestinalne permeabilnosti karakterističnih aktivnih sastavnica EKM-a: hidroksitirosola (HTS), tirosola (TS) i oleuropeina (OLE) te istraživanje lokalnog antioksidacijskog učinka neprobavljive frakcije EKM-a na stanice crijevnog epitela. Zaključak ove studije je da je EKM bogat izvor biološki aktivnih i biodostupnih fenola HTS-a, TS-a i OLE-a, a njihov sadržaj i biodostupnost usporedivi su ili bolji u odnosu na maslinovo ulje. Također, zaključeno je da navedeni fenoli međusobno ne interferiraju na razini permeabilnosti što je važan preduvjet za daljnji razvoj kompleksnih nutraceutika iz komine masline. EKM ostvaruje značajne antioksidacijske učinke na stanice crijevnog epitela koji su posljedica direktnog antiradikalnog učinka, ali i učinaka na produkciju endogenih antioksidansa stanice. Matriks EKM-a, prisutnost i vrsta ciklodekstrina u formulaciji te prisutnost hrane u probavnom sustavu značajno utječu na sve promatrane parametre. Stoga rezultati studije ukazuju na važnost usmjerene, ali sveobuhvatne funkcionalne karakterizacije u procesu oblikovanja visoko vrijednih nutraceutika komine masline te naglašavaju mogućnosti uvođenja jednostavnih intervencija tijekom formulacije koje će rezultirati poboljšanom funkcionalnošću završnog proizvoda.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Rad sadrži: 96 stranica, 29 grafičkih prikaza, 12 tablica i 90 literaturnih navoda. Izvornik je na hrvatskom jeziku.

Ključne riječi: fenoli komine masline; antioksidans; bioraspoloživost; interakcije fenol-hrana

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Doctoral thesis

BIOACCESSIBILITY, PERMEABILITY AND ANTIOXIDATIVE ACTIVITY OF OLIVE POMACE-BASED NUTRACEUTICALS

Kristina Radić

SUMMARY

During the production of olive oil from the olive fruit (*Olea europaea* L.) large amounts of waste is generated in the form of wastewater and pomace (solid residue). Their chemical composition is characterized by a significant proportion of phenolic compounds that are also found in olive oil. The main goal of this study was a functional characterization of olive pomace's extract (OPE), namely, the bioavailability and intestinal permeability study of characteristic active components of OPE: hydroxytyrosol (HTS), tyrosol (TS) and oleuropein (OLE) and local antioxidative effect of indigestible OPE fraction on intestinal epithelial cells. The conclusion of this study is that OPE is a rich source of biologically active and bioavailable phenols HTS, TS and OLE, and their content and bioavailability are comparable or better comparing to olive oil. Also, it was concluded that these phenols do not interfere with each other at the level of permeability, which is an important prerequisite for the further development of complex nutraceuticals from olive pomace. OPE achieves significant antioxidative effects on intestinal epithelial cells as a result of direct antiradical effect, but also of the endogenous cells' antioxidants production. The OPE matrix, the presence and type of cyclodextrin in the formulation and the presence of food in the digestive system significantly affect all the observed parameters. Therefore, the results of the study indicate the importance of targeted but comprehensive functional characterization in the process of formulating high-value olive pomace nutraceuticals and emphasize the possibility of introducing simple interventions during formulation that will result in improved final product functionality.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 96 pages, 29 figures, 12 tables and 90 references. Original is in Croatian language.

Keywords: olive pomace phenols; antioxidant; bioavailability; food-phenol interaction

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